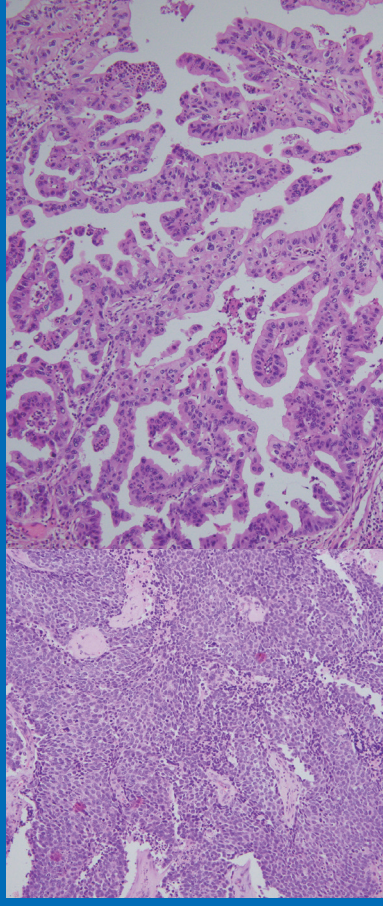
pISSN 1598-2998
eISSN 2005-9256

https://www.e-crt.org

CANCER RESEARCH AND TREATMENT



VOLUME 54
NUMBER 4
OCTOBER 2022

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Long-term Exposure to PM_{10} Increases Lung Cancer Risks: A Cohort Analysis

The Korean Cancer Association

Korean Society of Medical Oncology

Korean Association for Lung Cancer

CANCER RESEARCH *AND* TREATMENT

pISSN 1598-2998
eISSN 2005-9256
https://www.e-crt.org

It is indexed in Science Citation Index Expanded SCIE, PubMed, PubMed Central, Medline, Scopus, KoreaMed, KoreaMed Synapse, KoMCI, WPRIM, Worldwidescience.org, DOI/CrossRef and Google Scholar.

It has been published since 1966 with a title of The Journal of Korean Cancer Association.
Its title was changed from the year of 2001 as Cancer Research and Treatment.

Aims and Scope

Cancer Research and Treatment is a peer-reviewed open access publication of the Korean Cancer Association, Korean Society of Medical Oncology, and Korean Association for Lung Cancer. It is published quarterly. Abbreviated title is *Cancer Res Treat*. It accepts manuscripts relevant to experimental and clinical cancer research. Subjects include carcinogenesis, tumor biology, molecular oncology, cancer genetics, tumor immunology, epidemiology, predictive markers and cancer prevention, pathology, cancer diagnosis, screening and therapies including chemotherapy, surgery, radiation therapy, immunotherapy, gene therapy, multimodality treatment and palliative care.

Volume 54, No. 4
October 2022

Printed on October 10, 2022
Published on October 15, 2022

Published by Korean Cancer Association
Room 1824, Gwanghwamun Officia,
92 Saemunan-ro, Jongno-gu,
Seoul 03186, Korea
Tel: 82-2-792-1486
Fax: 82-2-792-1410
E-mail: cancer@kams.or.kr
Homepage: http://www.cancer.or.kr

Edited by Korean Cancer Association
E-mail: journal@cancer.or.kr

Printed by ONBEST Inc.
17, World Cup-ro 12-gil, Mapo-gu,
Seoul 04004, Korea
Tel: 82-2-322-4020
E-mail: onbest2@gmail.com

This journal was supported by the Korean Federation of Science and Technology Societies Grant funded by the Korean Government (Ministry of Education).

CANCER
RESEARCH *AND*
TREATMENT

Volume 54, No. 4, October 2022

pISSN 1598-2998
eISSN 2005-9256
https://www.e-crt.org

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pISSN 1598-2998
eISSN 2005-9256
https://www.e-crt.org

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2022.1326
Cancer Res Treat. 2022;54(4):951-952

Editorial

Treating Oligometastses, Prelude or Just Hassles of Systemic Treatment

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The concept of "oligometastatic disease" was first proposed by Hellman and Weichselbaum as a distinct clinical state between locally confined and systemically metastasized disease in 1995 [1]. The thought that treating metastasis-directed therapy (MDT) for oligometastatic disease has the potential for cure or long-term disease control has been very appealing like two old hypotheses of Paget's "seed and soil" and Ewing's "mechanical mechanism" [2-4]. The hypotheses could explain clinical situations that resection of limited metastatic tumors in the brain, lung or liver resulted in very good outcomes and even cure. Since the concept of oligometastatic disease has become more generally accepted, MDT, such as surgical resection or radiotherapy, is often offered with curative rather than palliative intent. In addition, owing to rapid advancement in technologies and radiotherapy techniques, stereotactic body radiotherapy (SBRT) has become more affordable recently. The number of publications of MDT has skyrocketed and more data has accumulated [5]. But, there is still some hesitancy or reluctance due to the lack of confirmative results by phase III studies on the efficacy or (cost-) effectiveness. What is worse, a disappointing result was recently reported from NRG-BR002 study which is one of the ongoing phase III studies evaluating the role of MDT when added to systemic therapy for predefined oligometastatic disease (Table 1) [6]. Based on NRG-BR002 study, the addition of MDT did not translate into better survival outcomes, suggesting that it might be of no or little use in the context of currently effective systemic treat-

Table 1. Currently ongoing prospective randomized phase III studies for oligometastatic disease

Trial	Histology	Treatment	No. of patients	Inclusion criteria	Primary endpoint
NRG-BR002 (NCT02364557)	Breast cancer	Systemic treatment±metastasis-directedtreatment (SBRT or surgery or both)	402	≤ 4 mets (maximum diameter ≤ 5 cm) Controlled primary tumor ECOG status ≤ 2	Overall survival
NRG-LU002 (NCT03137771)	NSCLC	Systemic chemotherapy±localized treatment (SBRT to metastases & SBRT or hypofractionated RT to primary tumor)	300	≤ 3 mets without progression after first-line systemic treatment ECOG status ≤ 2	Overall survival
SABR-COMET 3 (NCT03862911)	Any cancer	SOC treatment (chemotherapy, immunotherapy, hormones, or observation, at the discretion of the treating oncologist)±SBRT	297	1-3 mets (maximum diameter ≤ 5 cm) Controlled primary tumor Karnofsky performance status > 60 Life-expectancy > 6 mo	Overall survival
SABR-COMET 10 (NCT03721341)	Any cancer	SOC treatment (chemotherapy, immunotherapy, hormones, or observation, at the discretion of the treating oncologist)±SBRT	159	4-10 mets (maximum diameter ≤ 5 cm) Controlled primary tumor Karnofsky performance status > 60 Life-expectancy > 6 mo	Overall survival
CORE (NCT02759783)	Breast, prostate, or NSCLC	Systemic treatment (c/s palliative radiotherapy)±localized treatment	245	≤ 3 mets (maximum diameter < 5 cm in lung, < 6 cm in all other tissues) Controlled primary tumor ECOG status ≤ 2 Life-expectancy > 6 mo	Progression-free survival

c/s, with/without; ECOG, Eastern Cooperative Oncology Group; mets, metastasis; NSCLC, non–small cell lung cancer; RT, radiotherapy; SBRT, stereotactic body radiotherapy; SOC, standard of care.

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Received September 30, 2022 **Accepted** September 30, 2022 **Published Online** October 4, 2022

ment. We cannot draw a conclusion, however, that MDT plays no or little role in all tumor types or all clinical settings. There are many tumor types with different tumor biology, and furthermore, oligometastatic disease should encompass quite diverse clinical situations, such as *de novo* oligometastasis, oligo-recurrence, oligo-progression, and oligo-persistence [7]. In this regard, a meta-analysis reported by Rim et al is very helpful to gain an insight and clinical guidance in treating oligometastatic disease [8]. Nevertheless, one question occurs inevitably to us. What if systemic therapy improves enough to eradicate cancer cells or metastatic disease? This is actually happening! Advance and repeating success of immune-oncology has rapidly changed the landscape of systemic treatment so that the indications have expanded rapidly from clinically over metastatic disease to micrometastatic disease in adjuvant and neo-adjuvant settings with improving the chances for cure. Therefore, subsequent research following Rim et al.'s meta-analysis is needed to focus on MDT in the context of systemic therapy in very diverse clinical situations and could define its role more specifically. The devil is in the detail, and God is also in the detail likewise.

Unfortunately, Rim et al.'s meta-analysis did not deal with biological aspects of oligometastatic disease. Basically, the biologic concept of "oligometastatic disease" is challengeable like that "seed and soil" or "mechanical mechanism" hypothesis was challenged by others such as "tumor self-seeding" hypothesis [9,10]. There is cumulative evidence that both mechanical mechanism, such as hemodynamic and anatomical factors, and fruitful soil play complementary roles in tumor dissemination. There is also contradictory evidence, however, that intrinsic metastatic traits of seeds or cancer cells play a critical role. Research for identifying the biomarkers representing seed, soil or mechanical factors will be needed. On the other hand, "oligometastatic disease" might be a certain point in the temporospatial continuum of cancer rather than the binary or all-or-nothing state, leading to the postulation that a low-volume metastatic disease may still be curable with definitive MDT. Research for defining "oligometastatic disease" or "oligometastatic state" could be more important than that for identifying the biomarkers representing each factor. In this regard, the development of analyzing technology of circulating tumor cells or tumor DNA is noteworthy as a good method for defining the oligometastatic disease or state. Up to a certain point, we can give curative MDT without additional or concurrent systemic therapy. The point might change according to the state and the nature of tumors but MDT can be played like a prelude, which is played as an introduction to a larger musical piece, but also is played as a brief and self-contained one.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2022.329
Cancer Res Treat. 2022;54(4):953-969

Original Article

Role of Local Treatment for Oligometastasis: A Comparability-Based Meta-Analysis

Chai Hong Rim[1], **Won Kyung Cho**[2], **Jong Hoon Lee**[3], **Young Seok Kim**[4], **Yang-Gun Suh**[5], **Kyung Hwan Kim**[6], **Eui Kyu Chie**[7], **Yong Chan Ahn**[2], **The Oligometastasis Working Group, Korea Cancer Association**

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Purpose We intend to investigate the oncological efficacy and feasibility of local consolidative therapy (LCT) through a meta-analysis method.

Materials and Methods Four databases including PubMed, MEDLINE, Embase, and Cochrane library were searched. Target studies are controlled trials comparing outcomes of LCT versus a control group. Primary endpoints are overall survival (OS) and progression-free survival (PFS).

Results A total of 54 studies involving 7,242 patients were included. Pooled analyses showed that the LCT arm could achieve improved OS with pooled odds ratio of 2.896 (95% confidence interval [CI], 2.377 to 3.528; p < 0.001). Regarding PFS, pooled analyses showed pooled odds ratio of 3.045 (95% CI, 2.356 to 3.937; p < 0.001) in favor of the LCT arm. In the subgroup analyses including the studies with reliable comparability (e.g. randomized studies or intentionally matched studies without significant favorable prognosticator in LCT arms), pooled odds ratio was 2.548 (95% CI, 1.808 to 3.591; p < 0.001) favoring the LCT arm regarding OS. Regarding PFS, pooled OR was 2.656 (95% CI, 1.713 to 4.120; p < 0.001) which also favored the LCT arm. Subgroup analyses limited to the randomized controlled trials (RCT) were also performed and pooled odds ratios on OS and PFS were 1.535 (95% CI, 1.082 to 2.177; p=0.016) and 1.668 (95% CI, 1.187 to 2.344; p=0.003). The rates of grade ≥ 3 complications related to LCT was mostly low (< 10%) and not significantly higher compared to the control arm.

Conclusion Pooled analyses results of all included studies, selected studies with reliable comparability, and RCT's demonstrated the survival benefit of LCT. These consistent results suggest that LCT was beneficial to the patients with oligometastasis.

Key words Oligometastasis, Local therapy, Radiotherapy, Surgery, Meta-analysis

Introduction

With the classic general oncologic concept, the role of aggressive local treatment in the patients with systemic metastatic lesions used to be limited in a few clinical situations. Nevertheless, the benefit of locally ablative therapy for metastatic lesion(s) and/or primary disease has recently been proposed in the patients with "oligometastasis", which has been defined as the disease status with only a few, but not disseminated, metastatic foci [1]. Resection of limited metastatic lesions involving the lung or liver, for example, enabled favorable long-term survival outcome in significant portion of the colorectal cancer patients [2,3]. Given the advances in radiation therapy (RT) techniques capable of high-dose delivery with precise targeting, the utilization of local consolidative therapy (LCT) in oligometastatic setting has become dramatically and increasingly popular over the recent past years [4,5].

Many recently published studies have shown that improved long-term survival outcomes were achieved, by applying LCT to oligometastasis, when compared to the historic controls. Majority of these studies, however, were single arm studies or small phase 2 comparative series [6-10]. Furthermore, there was no preclinical evidence on the role of local treatment in the process of tumors undergoing the events of metastatic cascade. Therefore, it is unclear whether the

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Received May 24, 2022 **Accepted** August 13, 2022
Published Online August 16, 2022

improved outcomes following LCT were by virtue of the provided local therapy per se, or the bias of selecting out more favorable patients' subgroup having better clinical conditions and indolent disease nature. In clinical practice, there is still insufficient consensus in regards to the role of additional LCT to systemic therapy or supportive care in oligometastatic setting.

In this meta-analysis, the oncologic benefit of LCT in oligometastic disease was investigated by analyzing the literatures that explored the role of LCT in terms of survival outcomes as the endpoints with their comparative groups. In particular, the clinical balancing between the LCT and control arms was taken into account in further detail.

Materials and Methods

1. Study design and eligibility criteria

Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines [11] were strictly observed. The population, intervention, comparison, and outcome (PICO) question of the hypothesis was as follows: "Did LCT confer an oncologic benefit (regarding overall survival [OS] and progression-free survival [PFS]) in managing the patients with oligometastasis?" The following inclusion criteria were used to include the eligible studies: (1) controlled trial involving the patients with oligometastasis that compared the outcomes of those who underwent LCT versus a control group; (2) 10 or more patients in each arm; (3) at least one primary endpoint provided; and (4) oligometastasis defined as five or fewer metastases or as the metastatic lesions that could definitely be encompassed and treated by the provided LCT.

2. Protocol registration

This study is registered in PROSPERO (protocol No. CRD42022316613).

3. Information sources and search strategy

Four databases including PubMed, MEDLINE, Embase, and Cochrane library were systematically searched, as recommended by Cochrane handbook [12], and the last date of the search was the 14th of March, 2022. Detailed searching strategy including the search terms are as shown in the Supplement Data 1. The conference abstracts and in-press studies were also searched and included if they met the inclusion criteria. No language limitation was applied. For the studies possibly having the overlapping patients' cohort, those with the larger number of patients or those published more recently, if the number of patients are similar between competing studies, were chosen. Searching process was performed independently by two investigators (CH Rim, WK Cho) and any disagreements were resolved by discussion or re-evaluation of the databases in question.

4. Data items and collection process

The primary endpoints were OS and PFS. The incidences and types of grade 3 or higher adverse events were collected and subjectively reviewed. A pre-designed data sheet included the followings.

(1) General information including the author, affiliation, year of publication, patient recruitment, type of study, target disease, and definition of oligometastasis.

(2) Clinical data including the number of patients in each arm (LCT arm vs. control arm), target sites for LCT (e.g., metastatic or primary site), number of oligometastasis, treatment modality employed, OS, PFS, and adverse events of grade 3 or higher.

The survival data were acquired from the descriptive graphs if the numerical data were not provided in the articles. Data collection processes were also performed by two independent investigators (CH Rim, WK Cho) and any disagreements were resolved by re-evaluation of the literature.

5. Risk of bias and subgroup analyses

Although the current study intended to investigate on the studies that had the control arms (LCT vs. control), only few were in randomized study design, whereas majority were in retrospective ones. Possible confounders were carefully analyzed following the guidelines provided by the Cochrane group [13]. Reliable comparability was defined as either randomized controlled trials (RCT) or the studies with clinical balancing effort (e.g., propensity score matching) without any major prognosticators skewed in favor of any arm. Major prognosticators include the number of metastases, patients' age, performance status, and TNM stage, respectively, which were common and important clinical factors across various cancer primaries. The studies were regarded as having non-reliable comparability, if any of the above prognosticators or disease-specific factors were regarded important at the authors' discretion (e.g., prostate specific antigen in prostate cancer and or α-fetoprotein in hepatocellular carcinoma, respectively) and had favorable slant toward the LCT arm (e.g., statistically significant or > 20% difference). After the pooled analyses of all included studies, subgroup analyses were serially performed for the studies with reliable comparability and RCT's, and RCT's only, respectively.

Since the included studies dealt with heterogenous primary sites, subgroup analyses per primary were also subsequently performed. Subgroup analyses were performed according to hierarchical comparability and study designs, as suggested by Shin and Rim [14].

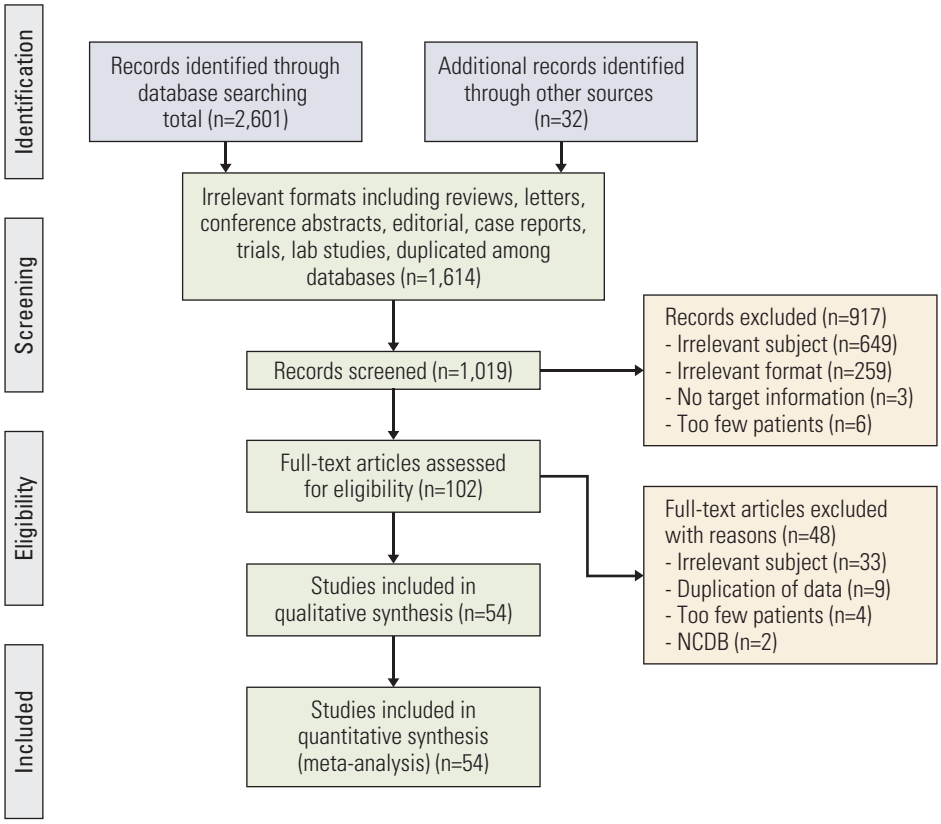


Fig. 1. Study inclusion plot. NCDB, National Cancer Database.

6. Quality assessment

Considering that most eligible studies were non-randomized, Newcastle-Ottawa scales of the included studies were used for the quantitative quality analyses [15]. The studies having quality scale of high (8 or 9 points) and moderate (6 or 7 points) were included in the pooled analysis, but not those with low score (5 or lower points) [13].

7. Statistics

The effect measures of primary endpoints (OS and PFS) were assessed as the odds ratio (OR) in comparison to percentile OS or PFS rates at 2-years between the LCT and control arms. 1- or 5-year rates were evaluated considering the natural courses of different primaries and histology (e.g., OS or PFS nearly nil at 2 years in small cell lung cancer [SCLC] studies; minimal OS or PFS changes within 2 years in prostate cancer studies). For pooled analyses of OR's, the random effects model was used based on the possible heterogeneity in clinical setting and study designs, referencing the Cochrane handbook [13]. In subgroup analyses that included RCT's only, fixed-effect model was applied if heterogeneity among the studies were regarded insignificant ($p < 0.1$ and $I^2 \leq 50\%$). In addition, pooled analyses of temporal OS percentile were performed according to the primary sites, using the random effects model.

In pooled analyses, heterogeneity was assessed using the Cochrane Q test [16] and I^2 statistics [17]. Studies with an I^2 statistic of 25%, 50%, and 75% were regarded to have low, moderate, and high heterogeneity, respectively. Publication bias was assessed in pooled analyses including 10 or more studies, using visual funnel plot evaluation and quantitative Egger's test [18]. If 2-tailed p-value was < 0.1 in Egger's test and asymmetry was noted in funnel plot, Duval and Tweedie's trim and fill methods were performed for the sensitivity analyses [19]. All the statistical analyses were performed using the Comprehensive Meta-Analysis ver. 3 (Biostat Inc., Englewood, NJ).

Results

1. Study selection and characteristics

The selection process is illustrated in Fig. 1. At the initial search across the databases, a total of 2,601 studies were identified. Thirty-two studies were added from the reference lists of the searched studies. After filtering of 1,614 studies

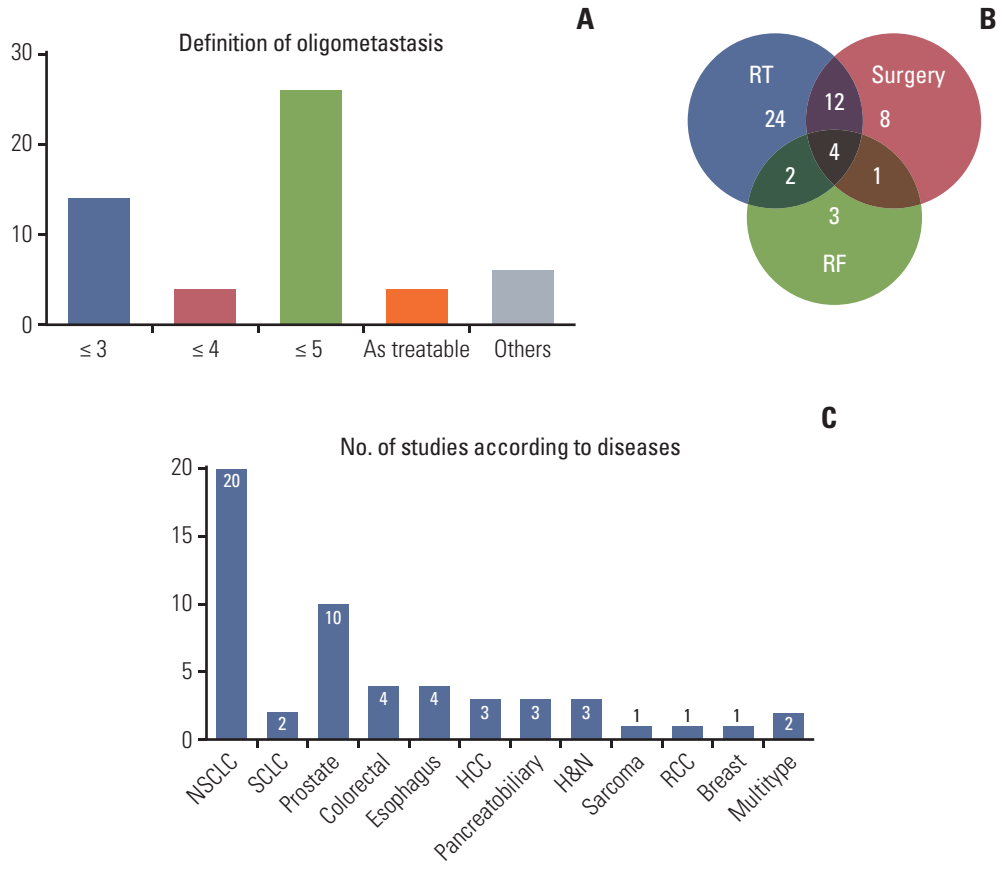


Fig. 2. Descriptive summary of definition of oligometastases among included studies (A), modality of local consolidative therapy used in included studies (B), and number of studies included according to site of origin (C). HCC, hepatocellular carcinoma; H&N, head and neck; NSCLC, non–small cell lung cancer; RCC, renal cell carcinoma; SCLC, small cell lung cancer.

with irrelevant format or duplicates among databases, abstracts of 1,019 studies were screened. Full-text evaluation was performed for 102 studies, and 54 studies finally fulfilled the inclusion criteria, which comprised as the final cohort of the current study [6,7,20-71].

Regarding the study design, eight studies were prospective RCTs, whereas the remainders were retrospective series. Fourteen studies did clinical balancing effort (e.g., propensity score matching) between the LCT and control arms. Among all 54 selected studies, 26 studies (48.1%) defined oligometastases as having metastatic foci of 5 or less, four studies (7.4%) as having 4 or less, and 14 studies (25.9%) as having 3 or less, respectively. Remaining studies used various individualized clinical definitions such as "resectable" "controllable with surgery", "within RT portal", or "confined to a single organ", respectively (Fig. 2A). Regarding the LCT modality, RT was performed in 42 studies (77.8%), surgery in 25 studies (46.3%), and radiofrequency ablation in 10 studies (18.5%), respectively (Fig. 2B). Twenty-two studies investigated oligometastasis of lung primary (40.7%, 20 on non–small cell lung cancer (NSCLC) plus 2 on SCLC, 10 of prostate primary (18.5%), four of colorectal primary, four of esophagus primary, three of liver primary, three of pancreatobiliary primary, and three of head and neck primary, respectively. There were three studies that focused only on single disease site: soft tissue sarcoma; renal cell carcinoma; and breast cancer, respectively. Two studies included various primaries (Fig. 2C). Further information on the included studies are summarized in S1 and S2 Tables.

2. Quality assessment

As for the selection category of Newcastle-Ottawa scale, all included studies acquired 4 points. All included studies had high representativeness as investigating a specific disease condition (oligometastases of cancers), adequate selection of non-exposed cohort (drawn from the same community), ascertainment of exposure (all studies acquired data from the secure medical records), and demonstrated the outcomes of interest (e.g., death or recurrence) not present at the initiation of study, respectively. Regarding the outcome category,

Table 1. Pooled analyses of studies

	No. of studies	No. of patients	Heterogeneity p-value	I^2 (%)	Heterogeneity	Pooled OR (95% CI)	p-value favoring LCT
Overall survival							
All studies	48	6,759	< 0.001	50.6	Moderate	2.896 (2.337-3.528)	< 0.001
Reliable comparability	15	2,690	0.007	53.4	Moderate	2.548 (1.808-3.591)	< 0.001
RCTs only	5	1,172	0.346	10.5	Low	1.535 (1.082-2.177)	0.016
NSCLC	17	1,525	0.06	37.5	Low to moderate	2.928 (2.151-3.985)	< 0.001
SCLC	2	130	0.184	43.2	Moderate	1.043 (0.336-3.240)	0.942
Prostate	6	2,055	0.2	31.4	Low to moderate	1.941 (1.282-2.938)	0.002
Colorectal	4	914	0.016	70.9	Moderate to high	4.453 (2.103-9.429)	< 0.001
HCC	3	218	0.541	~0	Very low	4.436 (2.439-8.069)	< 0.001
Esophagus	4	777	0.556	~0	Very low	2.092 (1.485-2.947)	< 0.001
Progression-free survival							
All studies	39	5,021	< 0.001	62.1	Moderate to high	3.045 (2.356-3.937)	< 0.001
Reliable comparability	16	2,109	0.001	60.3	Moderate to high	2.656 (1.713-4.120)	< 0.001
RCTs only	8	1,317	0.282	18.0	Low	1.668 (1.187-2.344)	0.003
NSCLC	13	1,277	0.049	43.0	Moderate	3.993 (2.262-5.087)	< 0.001
SCLC	2	130	0.276	15.8	Low	1.654 (0.544-5.034)	0.376
Prostate	10	1,726	0.003	63.6	Moderate to high	2.278 (1.463-3.546)	< 0.001
Colorectal	3	684	0.031	71.3	Moderate to high	4.911 (2.212-10.903)	< 0.001
HCC	2	126	0.854	~0	Very low	7.974 (2.081-30.547)	0.002
Esophagus	2	675	0.016	82.8	High	2.895 (0.524-15.984)	0.223

CI, confidence interval; HCC, hepatocellular carcinoma; LCT, local consolidative therapy; NSCLC, non–small cell lung cancer; OR, odds ratio; RCT, randomized controlled trial; SCLC, small cell lung cancer.

majority of studies acquired 3 points as they acquired data based on the medical records and few or negligible proportion of follow-up loss. Several studies, however, were regarded as having 2 points if the duration of follow-up was less than one year. Since RCTs and studies with matched control compared at least two known clinical prognosticators, they acquired 2 points in comparability category, whereas others acquired 0 points. The resulting quality points of the selected studies were at least 6 (S3 Table), which met the pre-defined cut-off value, and all were included in the pooled analyses.

3. Synthesized results

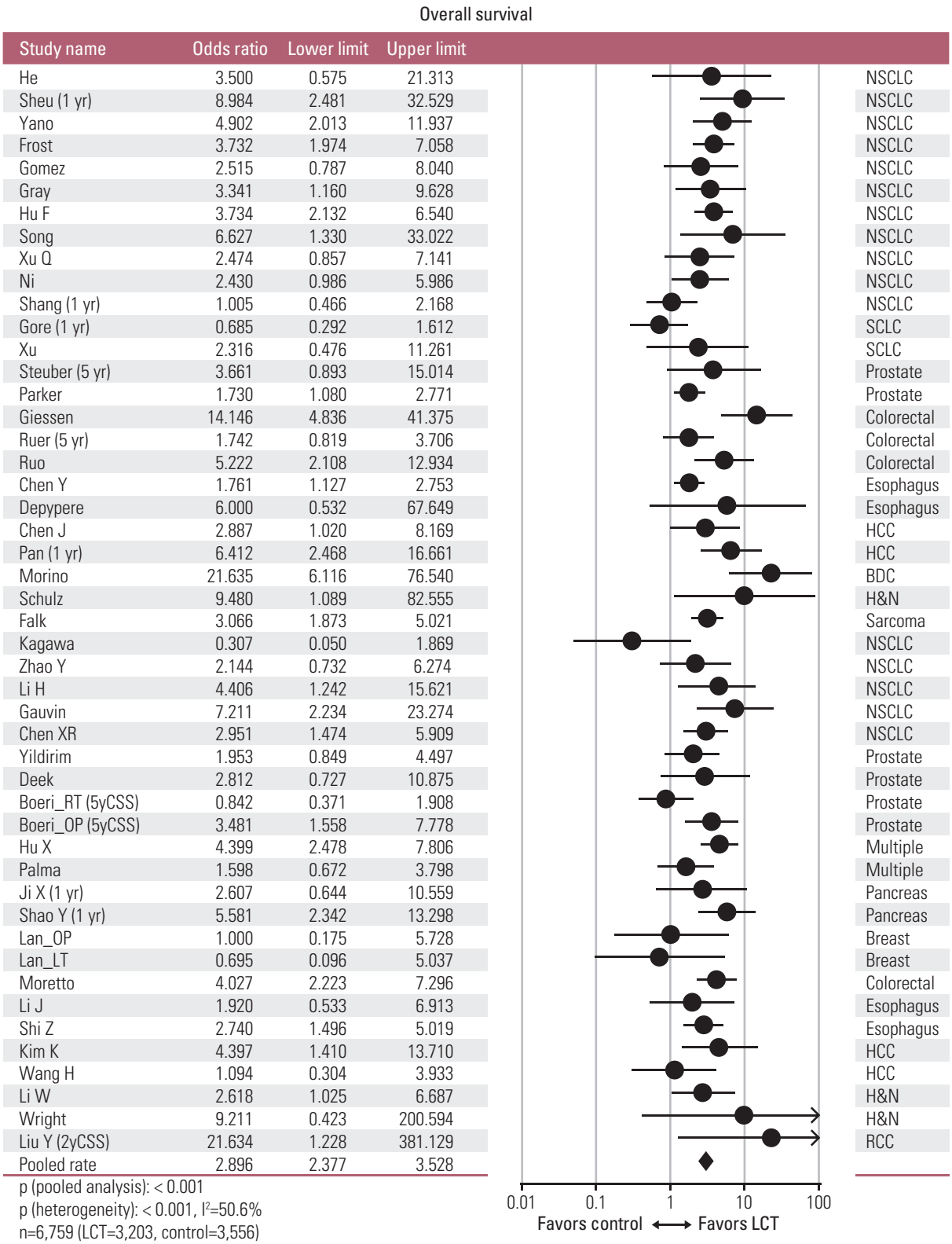
Pooled analyses of all included studies showed that the patients in the LCT arm could achieve improved OS with pooled OR of 2.896 (95% confidence interval [CI], 2.377 to 3.528; $p < 0.001$), with moderate heterogeneity ($p < 0.001$, I^2=50.6%) (Table 1, Fig. 3A). Regarding PFS, pooled analyses showed pooled OR of 3.045 (95% CI, 2.356 to 3.937; $p < 0.001$), with moderate heterogeneity ($p < 0.001$, I^2=62.1%) in favor of the LCT arm (Table 1, Fig. 4A).

In the subgroup analyses including the studies with reliable comparability (RCTs and intentional matched studies without known favorable prognosticator in the LCT arms), pooled OR was 2.548 (95% CI, 1.808 to 3.591; $p < 0.001$) favoring the LCT arm regarding OS, with moderate heterogeneity (p=0.007, I^2=53.4%) (Table 1, Fig. 3B). Regarding PFS, pooled OR was 2.656 (95% CI, 1.713 to 4.120; $p < 0.001$) which also favored the LCT arm, with moderate to high heterogeneity among studies (p=0.001, I^2=60.3%) (Table 1, Fig. 4B). Subgroup analyses limited to the RCT's only were also performed and all favored the LCT arm: pooled ORs on OS and PFS were 1.535 (95% CI, 1.082 to 2.177; p=0.016) with low heterogeneity (p=0.346, I^2=10.5%) (Fig. 3C) and 1.668 (95% CI, 1.187 to 2.344; p=0.003) with low heterogeneity (p=0.282, I^2=18.0%) (Table 1, Fig. 4C), respectively.

4. Pooled survival according to the primary site

Subgroup pooled analyses were performed according to the primary sites (Table 1). Pooled OR's for OS in NSCLC, SCLC, prostate cancer, colorectal cancer, liver cancer, and esophageal cancer were 2.928 (95% CI, 2.151 to 3.985; $p < 0.001$), 1.043 (95% CI, 0.336 to 3.240; p=0.942), 1.941 (95% CI, 1.282 to 2.938; p=0.002), 4.453 (95% CI, 2.103 to 9.429; $p < 0.001$), 4.436 (95% CI, 2.439 to 8.069; $p < 0.001$), and 2.092 (95% CI, 1.485 to 2.947; $p < 0.001$), respectively. Improved OS was achievable in the LCT arm in all disease sites except in SCLC. For PFS, pooled OR's for NSCLC, SCLC, prostate cancer, colorectal cancer, liver cancer, and esophageal can-

A



p (pooled analysis): < 0.001
p (heterogeneity): < 0.001, I^2=50.6%
n=6,759 (LCT=3,203, control=3,556)

Fig. 3. Forest plots of pooled analyses regarding overall survival, including all studies (A), studies with reliable comparability (B), and randomized controlled trials (C) [7,8,21-31,34,35,37-45,47-53,55-66,68-71,73]. BDC, biliary duct cancer; HCC, hepatocellular carcinoma; H&N, head and neck; LCT, local consolidative therapy; NSCLC, non–small cell lung cancer; RCC, renal cell carcinoma; SCLC, small cell lung cancer; 2yCSS, 2-year cancer-specific survival; 5yCSS, 5-year cancer-specific survival. *(Continued to the next page)*

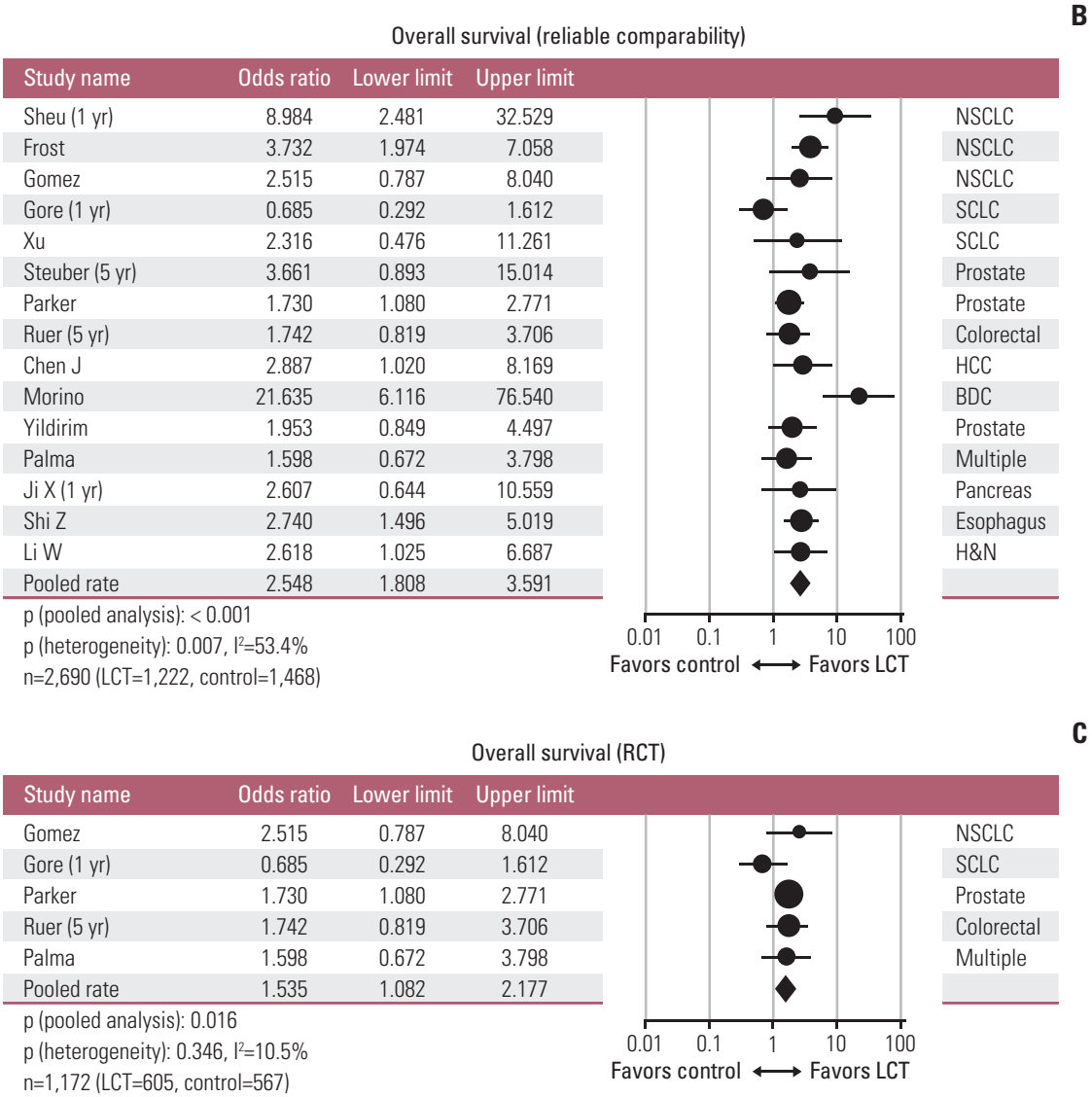


Fig. 3. *(Continued from the previous page)*

cer were 3.993 (95% CI, 2.262 to 5.087; p < 0.001), 1.654 (95% CI, 0.554 to 5.034; p=0.376), 2.278 (95% CI, 1.463 to 3.546; p < 0.001), 4.911 (95% CI, 2.212 to 10.903), 7.974 (95% CI, 2.081 to 30.547; p=0.002), and 2.895 (95% CI, 0.524 to 15.984; p=0.223), respectively. Again, improved PFS was achievable in the LCT arm in all disease sites except SCLC. The percentile rates of OS and PFS by pooled analyses according to the primary sites are illustrated and summarized in Table 2 and Fig. 5.

5. Publication bias

Regarding OS, no significant publication bias was noted (Egger's p=0.234). However, publication bias was highly suggested in the pooled analysis regarding PFS (Egger's p < 0.001). The trimmed OR using Duval and Tweedie's method was 2.278 (95% CI, 1.753 to 2.961). Funnel plots and results of quantitative Egger's test are shown in S4 Fig.

6. Adverse events

Twenty studies (seven on lung cancer; five on prostate cancer; two on pancreas cancer; two on esophageal cancer; one on colorectal cancer; one on liver cancer; and two on various cancers, respectively) involving 2,963 patients (1,487 in the LCT arm, 1,476 in the control arm) provided comparative information on the incidences and grade of adverse events. Regarding lung cancer studies, LCT-related adverse events were relatively more frequent when compared to other primaries, with grade 3 or higher rates ranging from 8% to 28.6%. Three studies reported the possibility of excessive

A

Progression-free survival

Study name	Odds ratio	Lower limit	Upper limit	
Iyengar (1 yr)	3.619	0.570	22.979	NSCLC
Sheu (1 yr)	3.938	0.924	16.775	NSCLC
Frost	11.178	4.849	25.768	NSCLC
Gomez	2.722	0.612	12.101	NSCLC
Hu F	1.887	0.853	4.175	NSCLC
Xu Q (1 yr)	2.636	0.919	7.562	NSCLC
Ni (1 yr)	4.679	1.435	15.261	NSCLC
Shang (1 yr)	1.630	0.781	3.404	NSCLC
Gore (1 yr)	1.218	0.440	3.375	SCLC
Xu	4.743	0.514	43.809	SCLC
Bouman-Wammes (1 yr)	8.850	2.556	30.640	Prostate
Lan	2.731	1.194	6.245	Prostate
Ost	1.729	0.615	4.857	Prostate
Parker	1.169	0.864	1.583	Prostate
Tsumura	2.917	0.538	15.828	Prostate
Giessen	10.615	4.347	25.923	Colorectal
Ruer	2.114	0.925	4.833	Colorectal
Chen Y (1 yr)	1.359	0.886	2.085	Esophagus
Chen J (1 yr)	10.211	0.528	197.396	HCC
Hsu KH	8.376	2.173	32.279	NSCLC
Zhao Y (1 yr)	5.127	1.515	17.354	NSCLC
Li H	2.796	0.564	13.854	NSCLC
Gauvin	6.544	1.509	28.374	NSCLC
Wang	1.693	0.865	3.317	NSCLC
Yildirim	1.332	0.312	5.679	Prostate
Phillips (1 yr)	2.672	0.835	8.543	Prostate
Deek	1.629	0.487	5.449	Prostate
Boeri_RT (5ySSFS)	1.715	0.870	3.378	Prostate
Boeri_OP (5ySSFS)	4.220	2.393	7.443	Prostate
Palma	3.920	1.244	12.353	Multiple
Ji X (6 mo)	1.605	0.416	6.199	Pancreas
Lan_OP	7.429	1.778	31.040	Breast
Lan_LT	1.714	0.301	9.773	Breast
Moretto	5.375	3.174	9.101	Colorectal
Shi Z	7.911	2.016	31.043	Esophagus
Kim K	7.479	1.657	33.754	HCC
Li W	2.070	0.630	6.794	H&N
Wright (1 yr)	58.586	4.253	806.971	H&N
Liu Y	2.108	0.901	4.931	RCC
Pooled rate	3.045	2.356	3.937	

p (pooled analysis): < 0.001
p (heterogeneity): < 0.001, I^2=62.1%
n=5,021 (LCT=2,454, control=2,567)

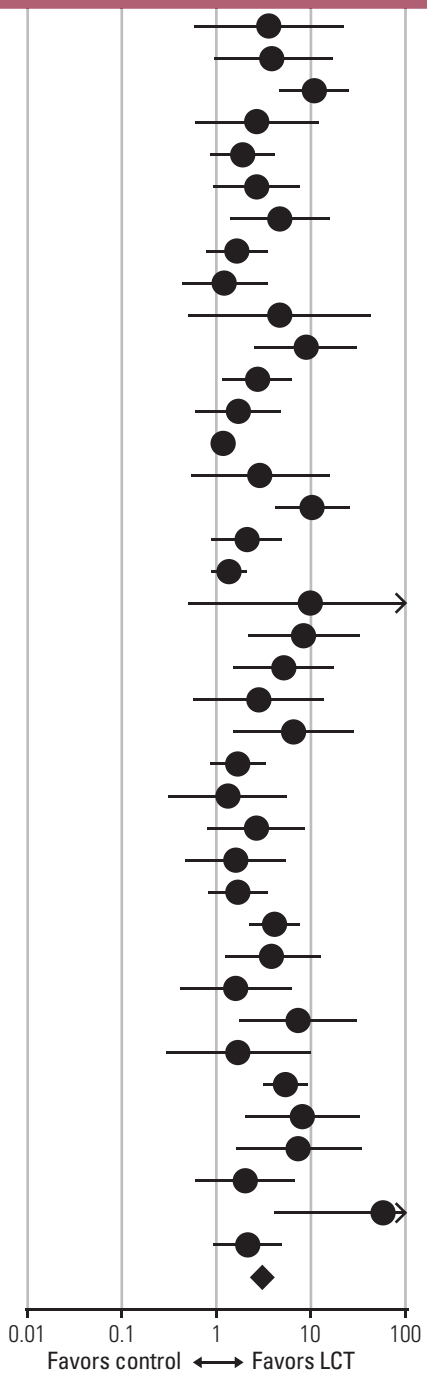


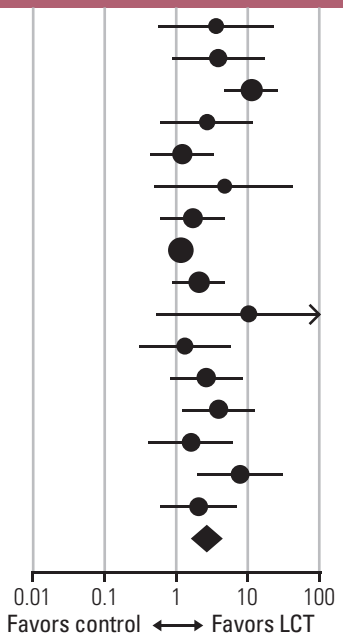
Fig. 4. Forest plots of pooled analyses regarding progression-free survival, including all studies (A), studies with reliable comparability (B), and randomized controlled trials (C) [6-8,20-22,25-28,31-33,35,36,38,39,42,43,46-48,50,52-54,56,58-60,62-64,66,67,69-71,73]. HCC, hepatocellular carcinoma; H&N, head and neck; LCT, local consolidative therapy; NSCLC, non–small cell lung cancer; RCC, renal cell carcinoma; SCLC, small cell lung cancer; 5ySSFS, 5-year second-line systemic therapy free survival. *(Continued to the next page)*

B

Progression-free survival (reliable comparability)

Study name	Odds ratio	Lower limit	Upper limit	
Iyengar (1 yr)	3.619	0.570	22.929	NSCLC
Sheu (1 yr)	3.938	0.924	16.775	NSCLC
Frost	11.178	4.849	25.768	NSCLC
Gomez	2.722	0.612	12.101	NSCLC
Gore (1 yr)	1.218	0.440	3.375	SCLC
Xu	4.743	0.514	43.809	SCLC
Ost	1.729	0.615	4.857	Prostate
Parker	1.169	0.864	1.583	Prostate
Ruer	2.114	0.925	4.833	Colorectal
Chen J (1 yr)	10.211	0.528	197.396	HCC
Yildirim	1.332	0.312	5.679	Prostate
Phillips (1 yr)	2.672	0.835	8.543	Prostate
Palma	3.920	1.244	12.353	Multiple
Ji X (6 mo)	1.605	0.416	6.199	Pancreas
Shi Z	7.911	2.016	31.043	Esophagus
Li W	2.070	0.630	6.794	H&N
Pooled rate	2.656	1.713	4.120	

p (pooled analysis): < 0.001
p (heterogeneity): 0.001, I^2=60.3%
n=2,109 (LCT=1,105, control=1,004)



C

Progression-free survival (RCT only)

Study name	Odds ratio	Lower limit	Upper limit	
Iyengar (1 yr)	3.619	0.570	22.979	NSCLC
Gomez	2.722	0.612	12.101	NSCLC
Gore (1 yr)	1.218	0.440	3.375	SCLC
Ost	1.729	0.615	4.857	Prostate
Parker	1.169	0.864	1.583	Prostate
Ruer	2.114	0.925	4.833	Colorectal
Philips (1 yr)	2.672	0.835	8.543	Prostate
Palma	3.920	1.244	12.353	Multiple
Pooled rate	1.668	1.187	2.344	

p (pooled analysis): 0.003
p (heterogeneity): 0.288, I^2=18.0%
n=1,317 (LCT=686, control=631)

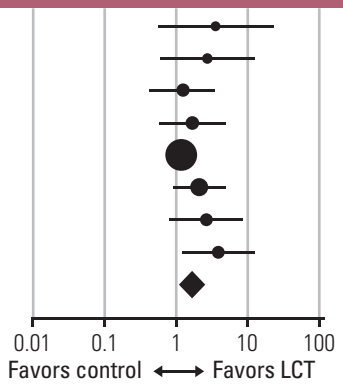


Fig. 4. *(Continued from the previous page)*

grade 3 or higher adverse events related to LCT [28,35,67]. Grade 5 adverse event, potentially related to the LCT, occurred in three cases among seven lung cancer studies (3 of 281, 1.07%). Regarding prostate cancer studies, grade 3 or higher adverse events related to the LCT was quite rare, and no studies reported significantly excessive adverse events related to the LCT, and grade 5 case, however, was also not reported. Palma et al. [7] reported three grade 5 adverse events among 66 patients (4.5%) following stereotactic body radiotherapy (SBRT) (radiation pneumonitis, pulmonary abscess, gastric ulcer). Ruo et al. [40] reported two out of 127 patients (1.6%) with postoperative death, and significant postoperative morbidity incidence of 20.5%. The types and rates of adverse events varied in other studies. The rates of grade 3 or higher adverse events related to LCT was mostly low (< 10%) and not significantly excessive when compared to the control arm (Table 3).

Table 2. Pooled survival rates according to disease

	No. of studies	No. of patients	LCT	Control	p-value
Overall survival					
NSCLC					
1-Year OS	17	1,539	84.1 (77.0-89.3)	66.0 (54.0-76.2)	0.004
2-Year OS	16	1,387	60.5 (52.5-68.0)	35.1 (26.3-45.0)	< 0.001
SCLC					
1-Year OS	2	130	60.7 (38.1-79.4)	42.8 (14.7-76.4)	0.411
Prostate					
3-Year OS	6	1,980	86.6 (65.0-95.7)	77.3 (44.6-93.5)	0.512
Colorectal					
1-Year OS	4	914	92.3 (67.9-98.6)	73.2 (48.1-89.0)	0.157
2-Year OS	4	914	72.5 (33.7-93.2)	40.5 (19.3-65.9)	0.173
Esophagus					
1-Year OS	4	777	72.8 (68.0-77.2)	59.0 (46.6-70.3)	0.026
2-Year OS	4	777	31.5 (22.6-42.0)	18.0 (14.6-22.0)	0.005
Pancreas					
1-Year OS	2	146	30.6 (21.1-42.1)	6.9 (8-40.2)	0.122
HCC					
1-Year OS	3	218	72.1 (51.8-86.1)	36.7 (16.0-63.8)	0.039
2-Year OS	3	218	38.8 (13.1-72.7)	18.4 (6.3-43.2)	0.282
H&N					
1-Year OS	3	145	83.7 (58.9-94.8)	67.3 (20.4-94.3)	0.463
2-Year OS	3	145	61.9 (41.1-79.1)	40.8 (13.8-74.9)	0.321
Progression-free survival					
NSCLC					
1-Year PFS	13	1,291	60.3 (51.0-68.9)	34.7 (26.2-44.3)	< 0.001
2-Year PFS	10	1,036	32.1 (22.2-43.9)	10.6 (5.7-19.0)	0.001
SCLC					
1-Year PFS	2	130	30.9 (17.2-49.2)	16.6 (8.0-31.3)	0.159
Prostate					
1-Year PFS	8	1,324	71.7 (51.4-85.9)	56.5 (30.7-79.2)	0.344
2-Year PFS	7	1,270	46.8 (26.0-68.7)	30.3 (13.4-54.9)	0.316
Colorectal					
1-Year PFS	3	684	68.1 (52.3-80.6)	34.6 (19.7-53.3)	0.007
2-Year PFS	3	684	41.8 (31.5-53.0)	12.2 (5.7-24.4)	0.001
Esophagus					
1-Year PFS	2	675	33.7 (22.0-47.8)	23.2 (19.2-27.8)	0.108
2-Year PFS	2	675	8.9 (2.6-26.4)	1.4 (0.6-3.6)	0.021
H&N					
1-Year PFS	2	98	69.6 (50.6-83.6)	25.4 (3.1-78.1)	0.133
2-Year PFS	2	98	37.5 (14.4-68.1)	12.4 (5.6-25.5)	0.068

HCC, hepatocellular carcinoma; H&N, head and neck; LCT, local consolidative therapy; NSCLC, non–small cell lung cancer; OS, overall survival; PFS, progression-free survival; SCLC, small cell lung cancer.

Discussion

There is little disagreement that the patients with a lower metastatic burden have a far better prognosis, when compared to those with higher metastatic burden. There exist controversies, however, whether aggressive local treatment directed to oligometastasis may derive oncological benefits either by delaying disease progression or hindering metastatic cascade [5,72,73]. In addition to the several previous prospective studies which reported their conclusive results, the current analysis could provide a support on the role of LCT in managing the patients with oligometastatic disease.

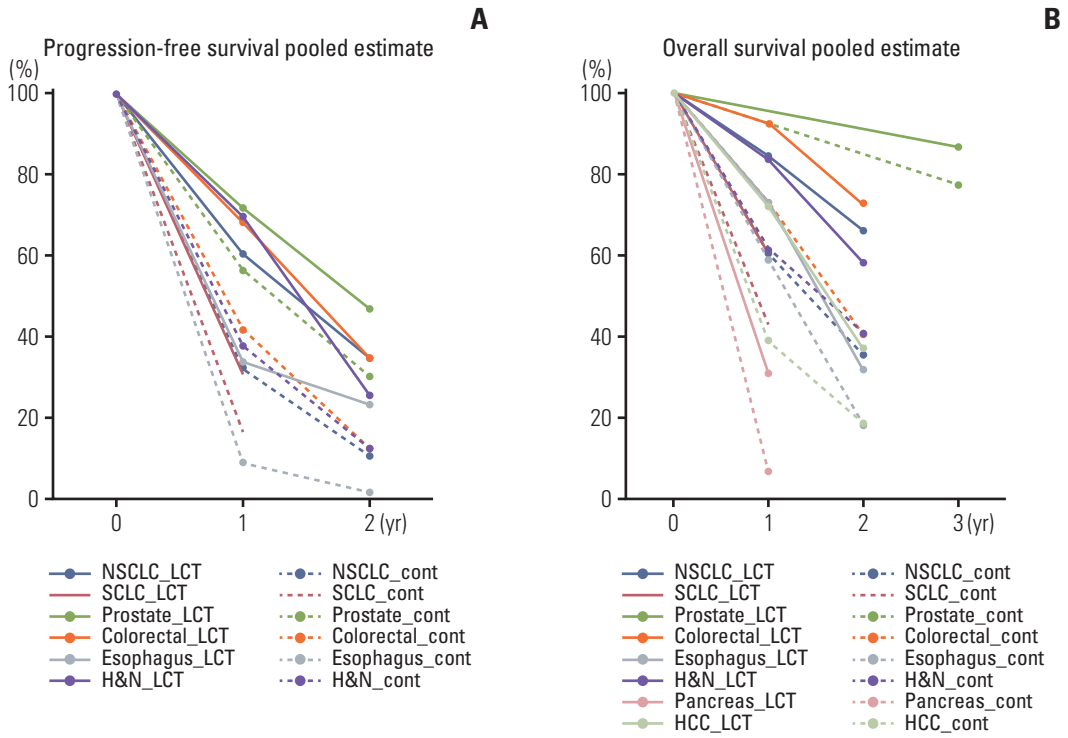


Fig. 5. Pooled survival percentile of overall survival (A) and progression-free survival (B) according to site of origin. HCC, hepatocellular carcinoma; H&N, head and neck; LCT, local consolidative therapy; NSCLC, non–small cell lung cancer; SCLC, small cell lung cancer.

In the present study, application of LCT resulted in significant benefit in terms of OS or PFS through the analyses for all included studies. In a subsequent subgroup analysis confined to the studies with reliable comparability, the effect size and the degree of hetererogeneity were similar to those from all studies. Finally, in an analysis limited to randomized controlled studies, the benefit of LCT remained significant in favor of the LCT, and the heterogeneity between studies was small. In another subgroup analyses on the various primary sites, the effect sizes related to the benefit of LCT varied and the degree of heterogeneity generally tended to decrease, when compared to those on all studies. As the benefit of LCT was consistently significant across all the analyses, we would speculate that the current study results strongly support the role of LCT in oligometastatic disease. The benefit of LCT in subgroup of RCTs with low heterogeneity suggested significant clinical advantages of LCT in oligometastatic patients. In addition, the effect size was different for primary sites, which suggests the needs for disease-specific approach.

The key question that remains is whether LCT can alter the biologic course of the oligometastatic patients. Several researchers recently suggested their hypotheses based on their own observations. Gomez et al. [8,27] investigated the role of LCT in the oligoprogression following the first-line chemotherapy for NSCLC, and reported that PFS benefit from LCT might have led to long-term OS benefit. Moreover, they also suggested that LCT could have removed the treatment-resistant cancer clones, or at least, could have slowed down the progression of metastatic spread by reduction of residual disease burden [27]. In another recent study, Phillips et al. [6] reported that total ablation of disease detectable by prostate-specific membrane antigen positron emission tomography–computed tomography using SBRT could reduce the development of new metastases. Based on these results, they suggested that the application of SBRT to metastatic lesions would not only delay the time for reemergence of detectable metastases, but also could prevent the progression of remaining micrometastases. Although the initial report by Palma et al. [7], with follow-up of approximately 2 years, failed to demonstrate the survival benefit following LCT, they later proved significant OS benefit in favor of LCT arm in the latest update with 51-months' follow-up (median OS difference of 22 months). As such, majority of clinical studies suggested the oncologic benefit by LCT and relevant hypotheses. However, preclinical studies are insufficient to provide biologic rationale and underlying mechanism. Phillips et al. [6] found that the circulating tumor DNA (ctDNA) was lower in the oligometastatic patients, when compared to

Table 3. Complication assessment

Author, target disease	Modality of LCT	No.	Control	No.	Grade ≥ 3 toxicity
Gomez, NSCLC [8]	RT or surgery & standard maintenance	25	Standard maintenance	24	2 cases G3 esophagitis in LCT; 1 G3 fatigue and 1 G3 anemia cases in control
Ni, NSCLC [35]	TKI & MWA	34	TKI	52	4 (9.3%) of MWA group needed chest tube drainage No G ≥ 3 toxicity related to TKI
Shang, NSCLC [42] (postop)	RT or RFA and/or CTx	105	CTx or BSC	47	Overall: 24.8% vs. 21.2% (LCT vs. control) (most common complication was myelosuppression) 1 case (0.9%) G5 infection in LCT arm
Wang, NSCLC [67]	RT (one site only) & ICI	59	ICI	93	9 of 59 (15%); mostly pneumonia or BM toxicity; 1 G5 mortality case due to severe pneumonia in LCT arm
Iyengar, NSCLC [32]	SBRT & CTx	14	CTx	15	Total 4 (28.6%) and 3 (20%) cases at LCT and control; no G5 toxicity
Wang, NSCLC [68]	^{125}I brachy	25	CTx	28	≥ G3 complication is lower in LCT arm (8%, pneumothorax vs. 25%, hematologic & nausea/vomiting)
Gore, SCLC [28]	PCI and cRT (45 Gy/15 F)	44	PCI	42	Overall: 25% vs. 9.5% 1 case of G5 pneumonitis in LCT arm
Bouman-Wammes, prostate [20]	SBRT (mostly 30 Gy/3 F or 35 Gy/7 F)	43	Active surveillance	20	No SBRT related toxicity
Ost, prostate [36]	SBRT (81%) or resection	31	Active surveillance	31	No grade 2 or higher toxicity in LCT arm
Parker, prostate [38]	RT and ADT	410	ADT	409	No data in low metastatic burden subgroup; 4% vs. 1% for whole population
Tsumura, prostate [46]	RT to metastases, prostate brachy & HTx	22	Prostate brachy & HTx	18	No difference in grade ≥ 2 toxicity
Phillips, prostate [6]	SBRT 24-48 Gy/3-5 F	36	Observation (allow CTx or ADT after 6 mo)	18	No G3 or higher adverse event in both arms
Ruo, colorectal [40]	Bowel surgery and CTx	127	CTx (83.5%)	103	Grade 5 cases (2 postoperative cases, 1.6%) postop OP morbidity (20.5%)
Ji, pancreas [56]	SBRT (m 41 Gy/5-7 F)+CTx	23	CTx	23	1 case of G3 duodenal bleeding in LCT arm
Shao, pancreas [65]	Liver and pancreas surgery+CTx	50	Palliative surgery + CTx	50	Longer hospital stay (21 vs. 13 days, $p < 0.001$), more transfusion and OP time in LCT arm
Chen, esophagus [22]	CCRT (IMRT, 50 Gy/25 F to primary; 45 Gy/15 F to metastases; cisplatin/paclitaxel)	196	CTx	265	No significant difference in both arms
Li J, esophagus [61]	IMRT (60 Gy) and/or CTx	55	CTx (90%), BSC (10%)	27	G3 complication: 7.3% (LCT) vs. 11.1% (control) No G4 or 5 toxicity in both arms
Kim K, HCC [58]	Surgery, RT and/or CTx	36	CTx	22	1 case of G3 pneumonitis after surgery
Palma, multiple [7]	SBRT and/or standard CTx	66	CTx	33	More in LCT (10.6% vs. 3%) grade 5 cases due to SBRT

(Continued to the next page)

Table 3. Continued

Author, target disease	Modality of LCT	No.	Control	No.	Grade ≥ 3 toxicity
Hu, multiple [55]	RT (SBRT, WBRT) & CTx or HTx	86	CTx or HTx	156	G3 pneumonia 3 cases (3.5%) and G3 leukopenia 1 case (1.2%) in LCT arm

ADT, androgen deprivation therapy; BM, bone marrow; BSC, best supportive care; CCRT, concurrent chemoradiation; cRT, chest radiotherapy; CTx, chemotherapy; HCC, hepatocellular carcinoma; HTx, hormone therapy; ICI, immune-checkpoint inhibitor; IMRT, intensity modulated radiation therapy; LCT, local consolidation therapy; MWA, microwave ablation; NSCLC, non-small cell lung cancer; OP, operation; PCI, prophylactic cranial irradiation; RFA, radiofrequency ablation; RT, radiotherapy; SBRT, stereotactic body radiotherapy; SCLC, small cell lung cancer; TKI, tyrosine kinase inhibitor.

that in polymetastaic patients, but failed to elucidate the relationship between ctDNA and oncologic outcomes [6]. Ongoing studies by Palma et al. [7], namely SABR-COMET 3 and SABR-COMET 10, intend to elucidate the biologic characteristics of oligometastasis by collecting ctDNA and circulating tumor cells, whose results are highly awaited.

Current study is not free from a few limitations. Relative significance of meta-analysis including the observational studies could be debated, because the uncontrolled confounders and possible heterogeneity could have affected pooled analyses [13]. However, the clinical decisions in oncology field could not be based solely on the level-1 evidence drawn from multiple well-designed prospective randomized clinical trials [74]. Furthermore, with the increasing evidence in favor of the role of LCT, it might be quite difficult to initiate a large-scale prospective trials in this clinical setting. On the other hand, there are suggestions that well-designed observational studies may provide high level of evidence similar to those from the prospective randomized trials [75]. In addition, our study comprehensively analyzed various cancer types, which is a rather unfamiliar method in oncology studies. Such approach can yield the heterogeneity of the pooled analysis. However, because the existence of oligometastic status having potential benefit of local treatment is still controversial, an integrated study is needed for overall clinical decisions [76]. To overcome the heterogeneity of pooled analysis, we performed hierarchical analysis, disease-specific subgroup analyses, and quantitative heterogeneity analyses. However, through the comprehensive analysis, the authors' could demonstrate, more or less, consistent and reliable results in favor of LCT based on various primaries, which may be shared in the clinical setting of the intermediate metastatic cascade called oligometsatasis. Another weakness of the current study included the fact that the innate mechanism of LCT could not be verified, as the speculations of the current study are based on external integration of the published clinical series. Based on these perspectives, we would strongly believe that the current analyses would be fruitful in our routine clinical practice, through our comparability-based formal meta-analyses, to support the necessity of applying LCT to the oligometastatic patients, and also in promoting the relevant basic biologic researches on oncologic mechanism of LCT, respectively.

The result of the current analyses suggested that LCT application be beneficial to the oligometastatic patients, based on the consistent findings by pooled analyses among (1) all included studies, (2) selected studies with reliable comparability, and (3) RCT's, respectively. LCTs might have different magnitude of oncologic benefits according to the primary sites, since the pooled survival percentiles varied among different primaries. Additional adverse events relat-

ed to LCT, however, need to be considered in the treatment decision process, especially for optimizing the potency of LCT when treating the lesions adjacent to the critical organs at risk.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Author Contributions

Conceived and designed the analysis: Ahn YC, Chie EK, Lee JH, Kim YS, Suh YG, Kim KH, Rim CH, Cho WK.
Collected the data: Rim CH, Cho WK.
Contributed data or analysis tools: Ahn YC, Chie EK, Lee JH, Kim YS, Suh YG, Kim KH, Rim CH, Cho WK.
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Conflicts of Interest

Yong Chan Ahn, the editor-in-chief of the Cancer Research and Treatment, was not involved in the editorial evaluation or decision to publish this article. All remaining authors have declared no conflicts of interest.

Acknowledgments

This work has been approved and assisted by the board of directors of Korean Cancer Association.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1078
Cancer Res Treat. 2022;54(4):970-984

Original Article

Reclassification of Five *BRCA1/2* Variants with Unknown Significance Using Complex Functional Study

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Purpose While *BRCA1/2* genes are commonly investigated, variants of unknown significance (VUS) and variants with potential splice effect are still being detected and they represent a substantial challenge in genetic counseling and therapy.

Materials and Methods Out of genetically tested 3,568 hereditary breast and ovarian cancer probands five, functionally not investigated variants with potential splice-modifying effect were subjected to functional characterization. Transcript-level analysis on peripheral blood-derived RNA of the carriers was performed to test aberrant splicing. The completeness of the aberrant splicing event was also studied, existence and extent of nonsense-mediated decay was even addressed. Clinical and phenotype data, pedigree and co-segregation analyses were also done. Locus-specific loss of heterozygosity (LOH) in tumor tissues was additionally tested.

Results In case of the *BRCA1*:c.4484+4dupA and the *BRCA1*:c.5407-10G>A variants functional results allowed us to reclassify them from VUS into likely pathogenic category. *BRCA1*:c.4358-31A>C, by producing incomplete aberrant splicing, was highlighted as strong VUS, but in lack of other supporting evidence, re-categorization was not possible. The likely pathogenic assertion of previously not reported *BRCA2*:c.8487G>T was reinforced based on its spliceogenic property and tumor LOH, while *BRCA2*:c.793G>A failed to present aberrant splicing in spite of suggestive predictions, which altered its original VUS evaluation into likely benign class.

Conclusion We presented molecular and clinical evidence for reclassification of four out of five *BRCA1/2* variants. Both up- and down-classification harbour important clinical significance. Patients carrying re-classified pathogenic variants in the future will not be dropped out from medical surveillance, preventive measures, treatment and predictive family screening in relatives at risk.

Key words *BRCA1*, *BRCA2*, Breast neoplasms, Reclassification, Variants of unknown significance, Splicing

Introduction

As high-throughput next-generation sequencing (NGS) is becoming more and more recognized in routine diagnostics, there are an increasing number of novel rare variants, which are either not registered in locus-specific databases or clinically not interpreted. These variants with uncertain significance (VUS) pose challenge to genetic counseling and clinical managements [1,2]. Regarding *BRCA1/2* genes, it is recommended to report VUS in the clinical genetics test records by the European consensus statement and expert recommendations [3]. However, VUS should not be used for medical decisions (surveillance, treatment, or preventive measures) or for predictive testing in relatives at risk; therefore, patients harboring such genetic alterations cannot benefit from the mutation-based therapies. This explains the strong demand to assert these variants into definite pathogenic or benign clinical categories aided by various gene-based functional studies. The American College of Medical Genetics and Genomics (ACMG) elaborated the standards and guidelines for the interpretation of sequence variants through the synthesis of categorical evidence [4]. According to these guidelines, well-established functional studies, for example, mRNA-level tests examining these variants' possible adverse effects on splicing may promote their pathological assertion. In the further assessment of the clinical relevance of VUS, variant-phenotype co-segregation in the family by clinical geneticists, potential loss-of-heterozygosity testing or functional *in vitro* assays represent important landmarks [2,4].

Germline pathogenic variants of the *BRCA1* and *BRCA2* genes account for 15%-20% of hereditary breast and ovarian cancer (HBOC) cases and represent the main genetic cause of hereditary familiar tumors of these types [5]. The Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) Consortium [6] registers and curates *BRCA1/2* variants in the BRCA Exchange database [7]. The consortium assembles genetic and clinical information originating from international expert laboratories in order to categorize these variants based on gene-specifically calibrated criteria (ver. 2.5.1, 29 June 2017). Another valuable repository

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Received September 29, 2021 **Accepted** February 2, 2022 **Published Online** February 8, 2022

for annotated *BRCA1/2* variants is the LOVD (Leyden Open Variation Database, https://grenada.lumc.nl/LSDB_list/lsdbs), especially that, curated by HCI/Tavtigian (http://hci-exlovd.hci.utah.edu/home.php). Still, a substantial amount of *BRCA1/2* variants fall into the VUS category, approximately 5%-10% of patients who undergo genetic testing of *BRCA1* and *BRCA2* receive a result reporting a VUS [8]. The most VUS are extremely rare or not even registered in population databases. Our department has performed routine genetic testing of HBOC families for germline mutations of *BRCA1/2* genes with NGS techniques for over 6 years. During this period, we tested 3,568 probands, whose personal and/or family history of tumors conformed to the criteria of the relevant National Comprehensive Cancer Network (NCCN) guidelines for genetic testing [9]. In the course of the genetic diagnostic workflow, we regularly detected variants, which were either clinically not reviewed in the proper locus-specific databases, or their functional assessment was conflicting, so we regarded them as VUS. Besides VUS, variant classification is a dynamic process, and previously classified variants sometimes need periodic reevaluation. The knowledge base for variant classification is continuously increasing with the expanding data in both public and in-house databases, publications reporting functional studies, as well as improvements in computational algorithms for predicting pathogenicity and genotype-phenotype association [10]. Therefore, following current recommendations, our laboratory periodically reviews previously identified genetic test results and performs variant-level reassessment.

The relevance of splicing in the *BRCA* genes, whether alternative or aberrant, was reported in various studies in connection with functionality [11,12]. Since any type of genetic variation (missense, nonsense, synonymous as well as intronic) may influence correct splicing, we systematically subjected these variants to diverse *in silico* splice predictions, and those that were predicted to be potentially spliceogenic, were further analyzed. We selected five VUS with potential splice effect and we studied them at transcriptional level, using blood RNA samples of the proband. Additional clinical evidence, as clinico-pathological features of carriers, pedigree analysis during clinical genetic counseling, presence of potential locus-specific loss of heterozygosity (LOH) in the corresponding tumor tissues and co-segregation of the variant with the disease were involved in the establishment of the plausible clinical relevance.

We present transcript-level genetical evidence as well as phenotype rationales for reclassification of five *BRCA1/2* variants.

Materials and Methods

1. Patients, clinical genetic counseling, and *BRCA1/2* genotyping

In this study, we analyzed 3,568 Hungarian HBOC patients by NGS method within the frame of routine genetic testing for *BRCA1* and *BRCA2* genes at the Department of Molecular Genetics of the National Institute of Oncology, Budapest, Hungary between 2015-2020. In Hungary, a national guideline was published in 2020 by the Board of Clinical Geneticists about the criteria for germline testing of patients with breast cancer (http://www.hbcs.hu/uploads/jogszabaly/3278/fajlok/2020_EuK_20_szam_EMMI_szakmai_iranyelv_2.pdf). In brief, breast/ovarian cancer under the age of 50, triple-negative breast cancer or ovarian cancer or male breast cancer at any age, breast cancer at any age with two or more first-degree relatives ($1 \leq 50$) or at least one ovarian first-degree cancer relatives [13]. Prior to molecular genetic testing, clinical genetic counseling was performed in each case according to the Hungarian legal and ethical regulations, where personal and familial tumor history was registered. All participants gave written informed consent for the genetic testing. Genotyping was carried out for all coding exons and exon-intron boundaries of *BRCA1* and *BRCA2* genes with Multiplicom amplicon-based enrichment *BRCA* MASTR Dx or *BRCA* MASTR Plus Dx library preparation kit (Agilent Technologies, Santa Clara, CA) and sequenced on MiSeq Illumina platform (Illumina, San Diego, CA). Bioinformatics analysis was done with the MASTR Reporter software v.1.1 (Agilent Technologies). When a VUS was identified, the significance of this result was extensively explained and discussed with the patients during the post-test counseling. Then, if the patient agreed to participate in research studies (including *in vitro* characterization and family screening for studying segregation), a second sampling was performed and these samples were further used for functional characterization. Upon conclusive result, the patients were re-counseled in the light of the new result. The availability of genetic testing was offered for all at-risk relatives of the variant carriers' families.

2. *In silico* predictions and variant selection

Splice alteration predictions for splice consensus regions (–3 to +8 at the 5′ splice site and –12 to +2 at the 3′ splice site) were taken from ADA_score and RF_score, arising from adaptive boosting [14] and random forest ensemble [15] learning methods integrated into the annotations of dbNSFP v4.0 [16]. Cutoff scores > 0.9 for ADA and > 0.7 for RF were considered. Possible splice effects of intronic variants outside of the consensus splice regions were queried by varSEAK, an online public access program (JSI Medical Systems, Etten-

Table 1. Five variants selected for transcript analysis according to *in silico* predictions

Gene	HGVS name	Exon	rs ID	GnomAD frequency[a]	Carrier number in our cohort	Splice predictions			
						ADA	RF	VarSeak	LaBranchoR, RNABP
BRCA1	LRG_292t1:c.4484+4dupA	Ex-14	NA	NA	2/3,568	NA	NA	5[b]	NA
BRCA1	LRG_292t1:c.5407-10G>A	Ex-23	rs273901767	4×10^{-6}	2/3,568	0.999[b]	0.912[b]	5[b]	NA
BRCA1	LRG_292t1:c.4358-31A>C	Ex-14	rs764503776	NA	1/3,568	NA	NA	1	High, 35%[b]
BRCA2	LRG_293t1:c.8487G>T	Ex-19	NA	NA	4/3,568	0.999[b]	0.998[b]	5[b]	NA
BRCA2	LRG_293t1:c.793G>A	Ex-09	rs1403242422	NA	1/3,568	0.067	0.398	4[b]	NA

ADA, adaptive boosting, ensemble machine learning (scoring interval 0-1); HGVS, Human Genome Variation Society; LaBranchoR, branchpoint position site predictor; RNABP, branchpoint site predictor (probability: 0-100%); NA, not applicable; RF, random Forest, ensemble machine learning (scoring interval 0-1); VarSeak, combined score for splice prediction (scoring interval 1-5). [a]v2.1.1, [b]High scores, that are predictive for aberrant splicing.

Table 2. Baseline characteristics of index patients from each family

Family No.	Variant (HGVS)	Age (at tumor onset)	Tumor type	Histology	No. of family members[a] with HBOC related cancer (see details on the pedigrees)
1	LRG_292t1:c.4484+4dupA	29	Breast	Invasive ductal carcinoma; ER+; PR-; HER2–; Ki67: 20%	1
2	LRG_292t1:c.4484+4dupA	44	Ovarian	Ovarian serous papillary carcinoma, high grade; multiplex metastases	2
3	LRG_292t1:c.4358-31A>C	49	Breast	Ductal carcinoma *in situ*; ER+; PR+; HER2+; Ki67: 10%	5
4	LRG_292t1:c.5407-10G>A	48	Breast	Invasive ductal carcinoma; ER+; PR–; HER2–; Ki67: 30%	None
5	LRG_292t1:c.5407-10G>A	37	Breast	Invasive ductal carcinoma; ER+; PR+; HER2–; Ki67: 80%	None
6	LRG_293t1:c.8487G>T p.(Gln2829His)	41	Breast	Invasive ductal carcinoma; ER+; PR-; HER2–; Ki67: 30%	3
7	LRG_293t1:c.8487G>T p.(Gln2829His)	36	Breast	Invasive ductal carcinoma; ER+; PR+; HER2–; Ki67: 15%	3
8	LRG_293t1:c.8487G>T p.(Gln2829His)	45	Breast	Invasive lobular carcinoma; ER+; PR+; HER2–; Ki67: na	3
9	LRG_293t1:c.8487G>T p.(Gln2829His)	50	Breast	Invasive ductal carcinoma; ER+; PR+; HER2–; Ki67: 15%	None
10	LRG_293t1:c.793G>A p.(Gly265Arg)	27	Breast	Invasive ductal carcinoma; ER–; PR–; HER2–; Ki67: 60%	1

Reference sequences: *BRCA1*: LRG_292t1; *BRCA2*: LRG_293t1. ER, estrogen receptor; HBOC, hereditary breast and ovarian cancer; HER2, human epidermal growth factor receptor 2; HGVS, Human Genome Variation Society; PR, progesterone receptor. [a]First, second, and third degree.

heim, Germany), based on mainly MaxEntScan calculations. Scores ≥ 4 were taken as a cutoff for plausible splice impact. RNABP (http://nsclbio.jbnu.ac.kr/tools/RNABP) [17] and LaBranchoR [18] predictors were applied for determining 3′ splice branchpoint positions. LaBranchoR defines the most probable position of the active adenine and RNABP predicts the odds for a nucleotide being a potential branchpoint site. Variants were selected for cDNA-level study if any of these predictions were suggestive of possible aberrant splicing and variant frequency was extremely low (< 0.001) in various populations. Variants, elected for transcript-level analysis are listed in Table 1. Phenotype and family characteristics of the probands carrying these variants are listed in Table 2 and S1 Fig.

3. cDNA qualitative analyses

RNA was isolated using the Tempus Spin RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) from peripheral blood taken in Tempus Blood RNA tubes (Thermo Fisher Scientific) according to the manufacturer's recommendations. First-strand reverse transcription was carried out by SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific). Reverse transcription PCRs (RT-PCRs) amplifying the variant-containing exons along with at least two adjacent exons were designed individually (list of cDNA primers is given in S2 Table). Amplification products were visualized on 1% agarose gel next to Hyper Ladder 1 kb DNA sizing standard (Bioline, London, UK) and subsequently sequenced by conventional Sanger sequencing method on ABI3130 Genetic Analyzer using the BigDye v.1.1 Kit (Thermo Fisher Scientific). Sequencing was done for the whole RT-PCR product without separation of the respective bands to compare peak intensities of normal and aberrantly spliced products. Where it was necessary to remove the interfering predominant normal alternative splice product, fragments of different sizes were cut out and cleaned from the gel by Monarch Gel Extraction Kit (New England Biolabs, Ipswich, MA) and the purified product was sequenced as above.

4. cDNA semi-quantitative measurements

Relative quantitation of the normal and aberrantly spliced isoforms was assessed by two analytical methods: quantitative real-time PCR (qPCR), as well as quantitative multiplex PCR of short fluorescent fragments (QMPSF), and the averaged results of the two methods were considered for the subsequent calculations. Selective amplification of the two types of transcripts was performed with specific primer pairs engineered to discriminate between the two splice forms. At least one primer of the pairs was designed so that it should span exon borders, to amplify only from the cDNA (exact primer sequences are listed in S2 Table). qPCR was run on QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) in relative quantification mode using SYBRGreen chemistry (Xceed HRM 2× Mix, Institute of Applied Biotechnologies, Prague, Czech Republic). Since both types of transcripts were amplified from the same template cDNA, no calibrator sample was needed, the expressions of the normal and aberrantly spliced products were directly comparable. Each test was performed in technical triplicates and three independent measurements were done. Means of the measurements with standard deviations were calculated. For QMPSF, one of the primer pairs of the respective amplicons was labeled with FAM fluorescence. PCR was conducted using Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) for 24 cycles at 62°C annealing temperature. The resulted products were subjected to capillary electrophoresis on an ABI3130 Genetic Analyzer (Thermo Fisher Scientific) in Microsatellite analysis mode. Peaks were visualized using the Peak Scanner Software 2.0 provided for the instrument. Peak ratios were calculated based on the area under the curve (AUC). Three biological measurement replicates were done.

Relative allelic expressions for allelic imbalance tests were measured on cDNA calculating the AUC ratios of exonic heterozygote positions in sequencing electropherograms [19]. The peak ratios defined on cDNA were normalized to the ratios of the same positions measured on gDNA.

5. LOH tests

Loss of the normal allele was tested in the tumor DNA of the probands, where it was available. DNA was extracted from the tumor using the Maxwell RSC DNA FFPE kit (Promega Corporation, Madison, WI). The PCR-amplicon of the variant-containing region was subjected to sequencing and allelic AUC ratio of the electropherogram peaks at the variant position was determined. LOH was calculated by normalizing the AUC ratios of the variant position of the tumor to that of the gDNA, and the tumor content was also taken into account by using the formula below:

$R=(AUC_{\text{reference in tumor}}AUC_{\text{variant in tumor}})\times(AUC_{\text{variant in germline}}/AUC_{\text{reference in germline}})\times$Proportion of tumor content $R < 0.5$ was considered as LOH.

6. Complex evaluation of pathogenicity

We employed the VarSome software's built-in pathogenicity calculator [20], corresponding with the statements of Goldgar et al. (2004) [21], for allocating variants into the 5-tier categories along with current ACMG guidelines [4]. As additional supporting evidence, at some variants we took into consideration co-segregation, LOH, family history, and proband phenotype characteristics to underpin their clinical relevance.

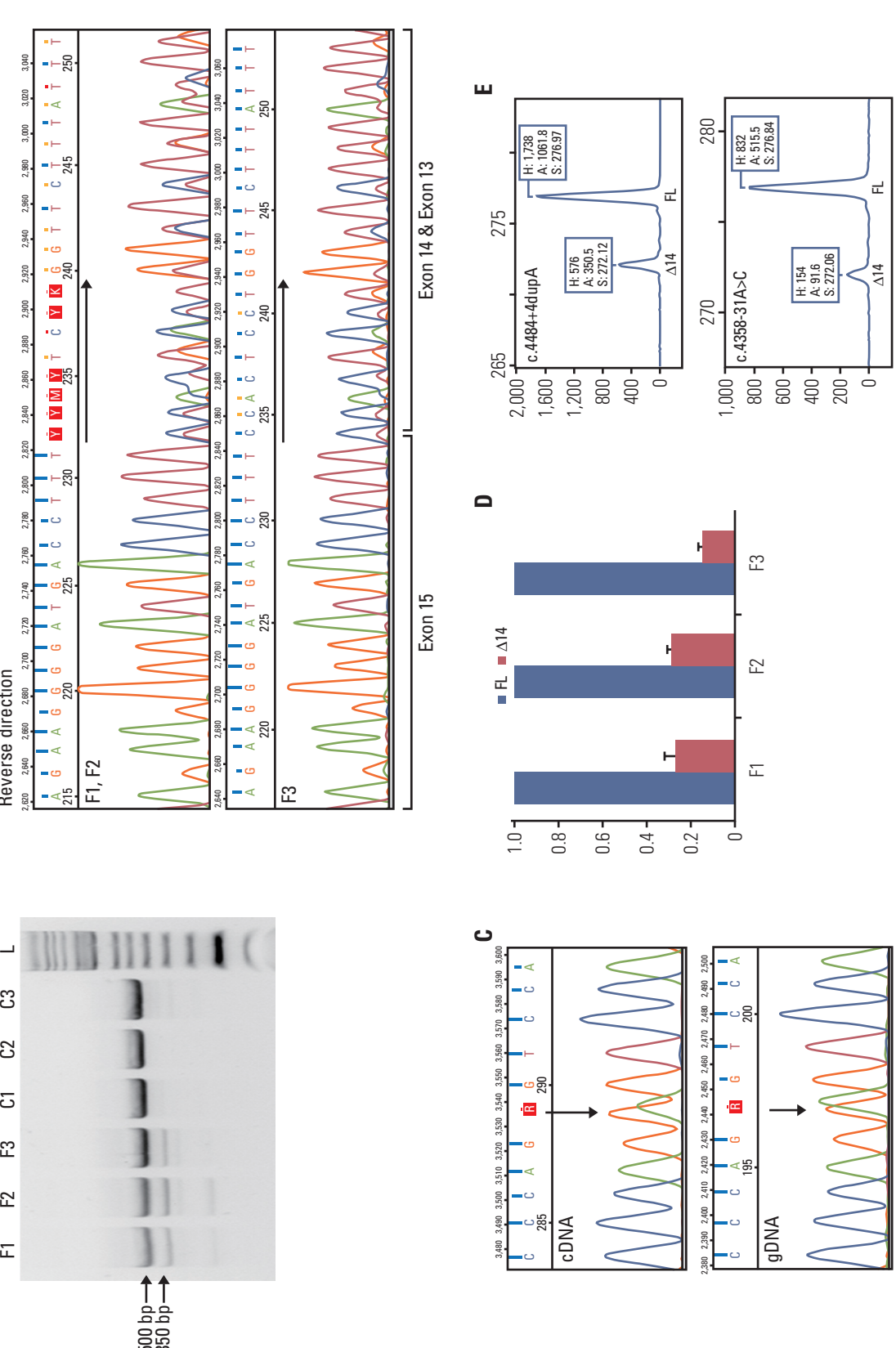

Fig. 1. *BRCA1* c.4484+4dupA and *BRCA1* c.4358-31A>C variants both cause *BRCA1* exon 14 skipping (A-F). (A) RT-PCR results of probands of F1 and F2 (*BRCA1* 4484+4dupA carriers) and F3 (*BRCA1* c.4358-31A>C carrier) yielded an additional 150 bp shorter product on agarose gel, not present in controls. (B) Sanger sequencing of the RT-PCR products of the variant carriers showed whole exon 14 skipping in all probands, with a lower extent in the case of F3. The sequencing shows the reverse direction. (C) Allelic imbalance test of a nearby heterozygote exonic variant *BRCA1* c.4837A>G in F1 shows a 1:2 ratios for the A:G alleles on cDNA relative to gDNA. (D) Relative abundance of the FL (blue bars) and Δ14 (red bars) transcripts measured by qPCR in F1, F2, and F3. Error bars represent standard deviation of three measurements. (E) Relative abundance of the FL and Δ14 transcripts measured by QMPSF in *BRCA1* 4484+4dupA carrier (F1) and *BRCA1* c.4358-31A>C carrier (F3). *(Continued to the next page)*

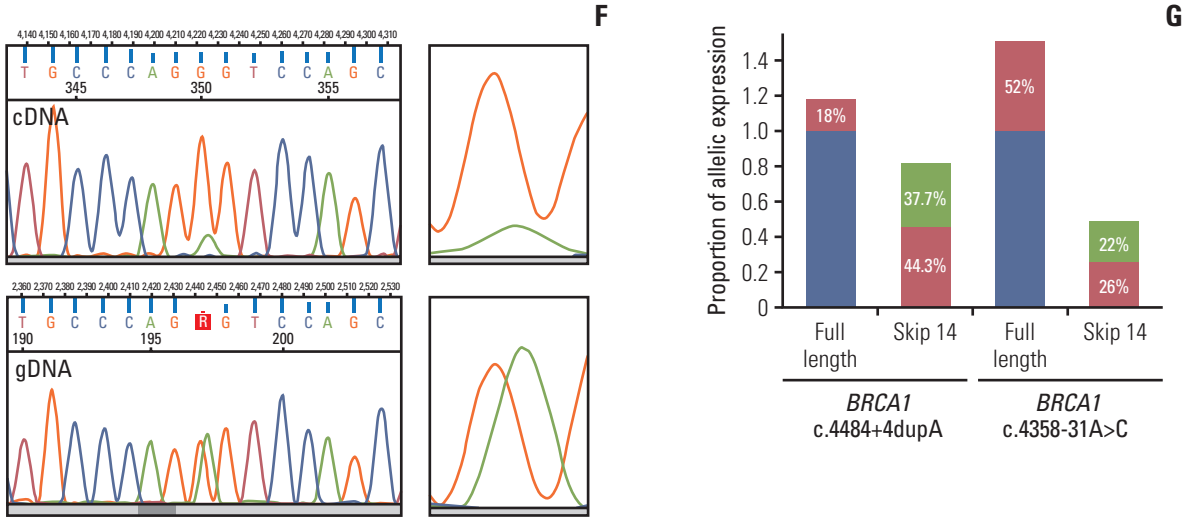

Fig. 1. *(Continued from the previous page)* (F) Detection of incomplete aberrant splicing on RT-PCR product amplified exclusively from the FL transcript by sequencing tagging polymorphism *BRCA1* c.4837A>G. The electropherogram of the variant position is enlarged on the right side. (G) The composition of FL and Δ14 transcripts from the wild type (blue) and variant carrier (red) alleles in *BRCA1* 4484+4dupA carrier (F1) and *BRCA1* c.4358-31A>C carrier (F3). The green segment represents the NMD-degraded fraction. The extent of FL transcript originating from the *BRCA1* c.4358-31A>C carrier allele is only imputed. FL, full length; F1, family 1; F2, family 2; F3, family 3; C1-3, wild type controls; L, 1 kb ladder (Promega); NMD, nonsense-mediated mRNA decay; RT-PCR, reverse transcription polymerase chain reaction; QMPSF, quantitative multiplex PCR of short fluorescent fragments; qPCR, quantitative real-time polymerase chain reaction.

Results

1. Selection of VUS potentially affecting splicing

In the course of our routine *BRCA1/2* diagnostic NGS-sequencing, we tested altogether 3,568 probands whom clinical presentation fulfilled the HBOC criteria for genetic testing (S3 Fig.) [9]. As a result of the comprehensive exon and exon-intron boundary sequencing of both genes, we detected 560 different variants, 130 of which were VUS according to the relevant ACMG criteria or not registered in *BRCA1/2* locus-specific databases. The majority of them were extremely rare or absent in various population databases. We subjected these variants to diverse *in silico* splice prediction algorithms defining canonical splice disruptions or creation of novel splice sites (see "Materials and Methods"). Seven of the variants were suggestive for having spliceogenic effect by at least one of the *in silico* tools and for five variants out of them (with pan-population frequencies < 0.01 each), RNA samples were available for transcript-level analysis (Table 1). The study involved 10 nonrelated families altogether, carrying any of these five variants (Table 2, S1 Fig.).

2. *BRCA1* intronic variants causing partial exon 14 skipping

Two probands of nonrelated breast cancer families (family 1 and family 2) carried a *BRCA1* c.4484+4dupA variant, which was an insertion of an additional adenine nucleotide after the 4th basis of the *BRCA1* intron 14, close to the canonical splice donor site. VarSeak prediction gave a high score for splice alteration (Table 1). Another proband in a different family was a c.4358-31A>C variant carrier (family 3). Branchpoint predictors anticipated that this latter variant affects the active adenine upstream the splice acceptor site of exon 14 (Table 1, S4 Fig.). RT-PCR amplification from the cDNA of the variant carriers with primers flanking exon 14 yielded a smaller-sized extra band in all three cases (Fig. 1A), which was sequenced and identified as an aberrant splice product with whole exon 14 skipping (Δ14) (Fig. 1B, S5 Fig.).

Semi-quantitative real-time PCR, as well as QMPSF analyses designed for specific amplification of the normal and aberrantly spliced RNA products showed that the Δ14 transcript is present in a lower quantity than the normal, full-length transcript (Fig. 1D and E). The average proportions were 0.32 (approx. ratio 1:3) for c.4484+4dupA carriers and 0.17 (approx. ratio 1:5.5) for c.4358-31A>C carrier calculated based on both detection methods. In addition, one c.4484+4dupA carrier proband carried numerous exonic heterozygote variants that allowed testing of allelic imbalance. Interestingly, AUC calculations of a heterozygote position in exon 16 (c.4837A>G) showed a 1:2 ratio of the two nucleotides in the electropherogram superposition (Fig. 1C). To resolve the discrepancy between the two ratios (1:3 and 1:2), we raised the possibility that incomplete aberrant splicing

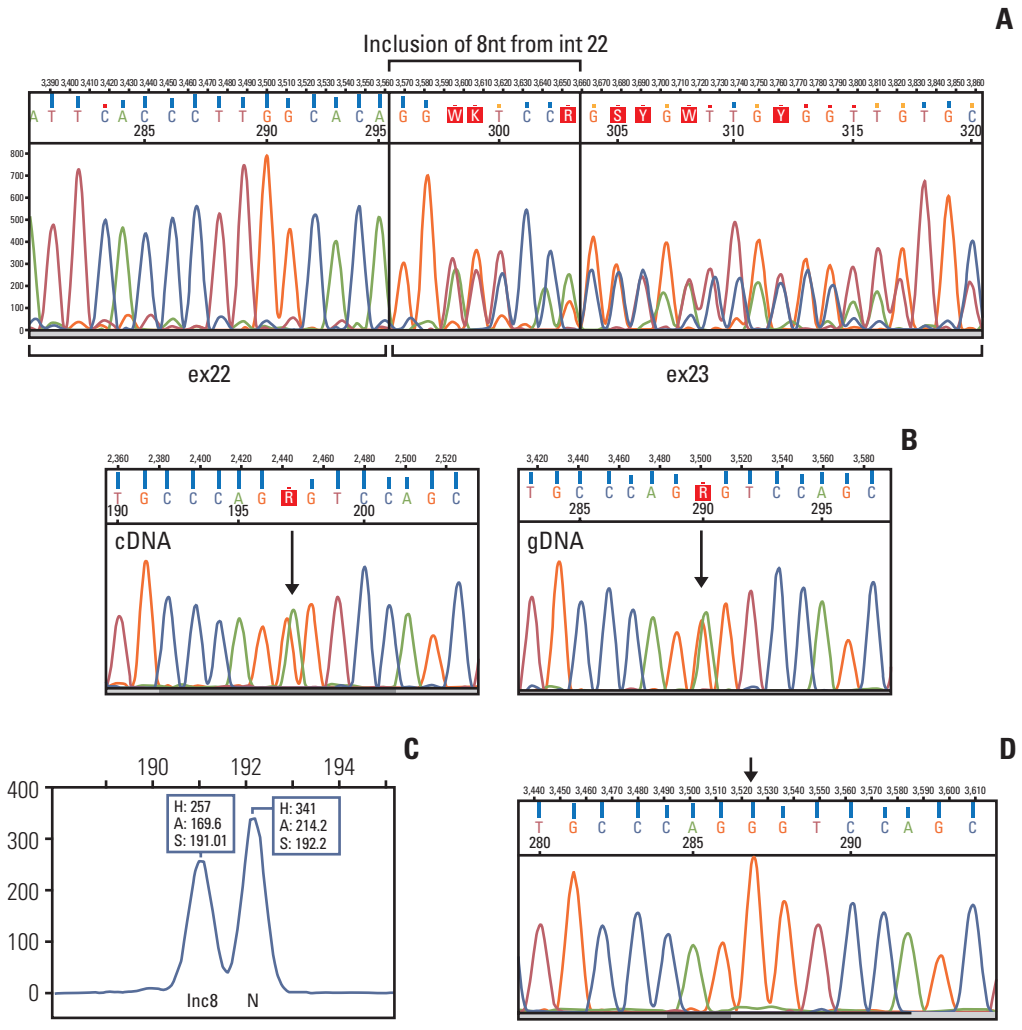

Fig. 2. cDNA analysis of *BRCA1* c.5407-10G>A variant. (A) Sanger sequencing result of the RT-PCR product of the *BRCA1* c.5407-10G>A variant carrier proband of F5. Aberrant transcript revealed the inclusion of eight nucleotides of intron 22 into exon 23 generating a frameshift from this position. The peak intensities of the normal and aberrant sequences are equal. (B) Allelic imbalance test harnessing a heterozygote position *BRCA1* c.4837A>G outside of the aberrantly spliced exon shows a 1:1 allelic ratio compared to the same position in gDNA. This confirms that the aberrant transcript is not degraded by NMD. (C) Relative abundance of the normal (N) and aberrant (inc8) transcripts measured by QMPSF. The slight difference may arise from suboptimal specificity of the discriminative primers. (D) Detection of complete aberrant splicing on RT-PCR product amplified exclusively from the normal transcript by sequencing tagging polymorphism *BRCA1* c.4837A>G. Arrow points to the variant position, which represents only G, corresponding to the wild type allele. *(Continued to the next page)*

may interfere with the allele expression ratios measured. We tested this hypothesis using the c.4837A>G variant as a tagging polymorphism: we performed allele-specific RT-PCR with primers amplifying only the full-length, exon 14-containing transcript encompassing the c.4837A>G variant position. Concomitant Sanger sequencing of the resulted PCR product and measurement of the AUC of the electropherogram peaks in the tagging position yielded that the ratio was A:G=1:5.5 (Fig. 1F). Regarding the fact that Δ14-containing allele-specific PCR resulted exclusively in allele A, we could state that allele A is in "cis" position with 4484+4dupA variant, therefore the presence of allele A in the full-length transcript with 1:5.5 ratio markers that ~20% of the 4484+4dupA variant-containing allele also produced normally spliced RNA product. A remarkable portion of the remaining ~80% aberrantly spliced product may be partially degraded by nonsense-mediated decay (NMD), leaving only ~40% of the allelic expression as aberrant transcript (Fig. 1G). This

indirectly suggests that NMD degrades approximately half of the *BRCA1* Δ14 transcript. Unfortunately, the two other probands did not carry any exonic alterations in heterozygote form. Consequently, the calculation of the measure of incomplete aberrant splicing was feasible only indirectly in the case of the c.4358-31A>C carrier. Calculating with the same extent of NMD (~50% degradation of the *BRCA1* Δ14 transcript), we yielded that nearly half of the variant-carrier allele might be normally spliced. Therefore, most probably, only half of the amount of aberrant splice product was detectable at c.4358-31A>C carrier relative to c.4484+4dupA carrier (Fig. 1G), which may be attributable to the larger incompleteness of this variant's aberrant splicing effect.

The pedigrees of the three families are depicted in S1 Fig. All showed characteristic personal and familial HBOC features (Table 2). Family members in family 1 were available for genetic testing allowing genotype-phenotype co-segregation analysis. The proband's mother, who was nonaffected turned out to be a non-carrier. The paternal grandmother, who had breast cancer at the age of 54, carried the variant. The proband's father turned out to be also a variant carrier, but without clinical symptoms.

3. *BRCA1* c.5407-10G>A causes partial intron inclusion

The *BRCA1* c.5407-10G>A variant was detected in two independent probands in our tested cohort (family 4 and family 5) and both harbored characteristic personal and familial HBOC features (Table 2, S1 Fig.). The variant changes a G nucleotide to an A in intron 22, ten nucleotides upstream of the exon 23, which was predicted *in silico* to disturb canonical splicing (Table 1). RNA sample was available from the proband of family 4. RT-PCR amplification yielded a fragment, which was indistinguishable from the wild type in length. Nonetheless, sequencing analysis of the fragment revealed aberrant splicing with retention of eight nucleotides of intron 22 upstream of the *BRCA1* exon 23 (Fig. 2A). The nucleotide change introduced a novel AG acceptor dinucleotide within the AG exclusion zone [22], which acted as a novel strong acceptor site. Since the mutant transcript generated stop codon only in the last exon (exon 24), the resulting aberrant transcript was not subject to NMD. This was verified on the cDNA by a heterozygote exonic position, which actually did not show allelic imbalance (Fig. 2B). Therefore, the relative abundance of the normal and alternative transcripts reflects reliably the original ratio of the two splicing events. This was also confirmed by QMPSF technique (Fig. 2C). The completeness of the aberrant splicing was also studied applying a tagging variant c.4837A>G in exon 16, which was present in heterozygote form in one of the carriers. The tagging variant was co-amplified in a specific PCR reaction, which was designed for selective amplification of the normal, wild-type transcript. Sequencing electropherogram of the tagging variant position yielded only the G allele, no traces of the A allele (which was "in cis" with c.5407-10G>A) was detectable (Fig. 2D). This result ascertained that all the transcripts generated from the c.5407-10G>A variant-carrier allele were aberrant, so the aberrant splicing induced by this variant was complete. Furthermore, LOH test of the breast tumor tissues of index cases was available both in family 4 and family 5. Loss of the normal allele was demonstrated in both cases with R=0.26 and R=0.3 scores, respectively (Fig. 2E).

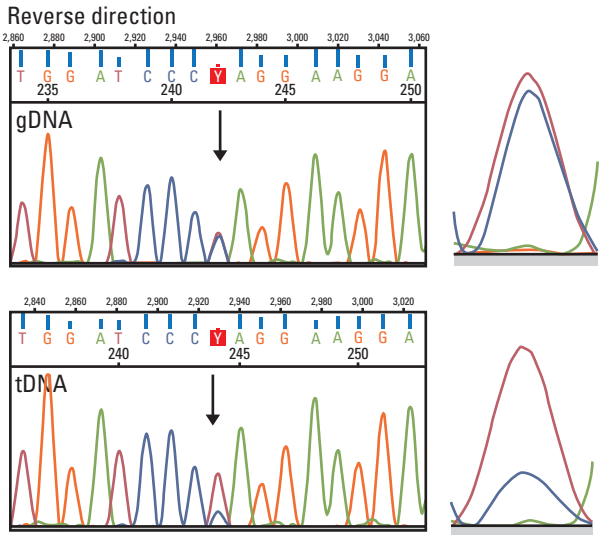

Fig. 2. *(Continued from the previous page)* (E) Representative example for LOH in family 4. The electropherogram of the variant position is enlarged on the right side. F5, family 5; gDNA, genomic DNA; inc8, aberrant transcript with 8 nucleotide inclusion from intron 22; LOH, loss of heterozygosity; N, normal transcript; NMD, nonsense-mediated mRNA decay; QMPSF, quantitative multiplex PCR of short fluorescent fragments; RT-PCR, reverse transcription polymerase chain reaction; tDNA, tumor DNA.

4. Transcript-level study of *BRCA2* putatively spliceogenic exonic variants

Based on *in silico* predictions, we selected two different *BRCA2* variants for cDNA analysis, for which it was anticipated that the canonical splice sites might be affected. Of these, *BRCA2* c.8487G>T, positioned in the last nucleotide of the *BRCA2* exon 19, occurred in four unrelated probands in our cohort 4/3,568 (0.11%). Blood RNA sample was available from only one proband (family 6). RT-PCR–amplification of the region flanking the variant carrier exon yielded two products: one corresponded to the full-length transcript, while the

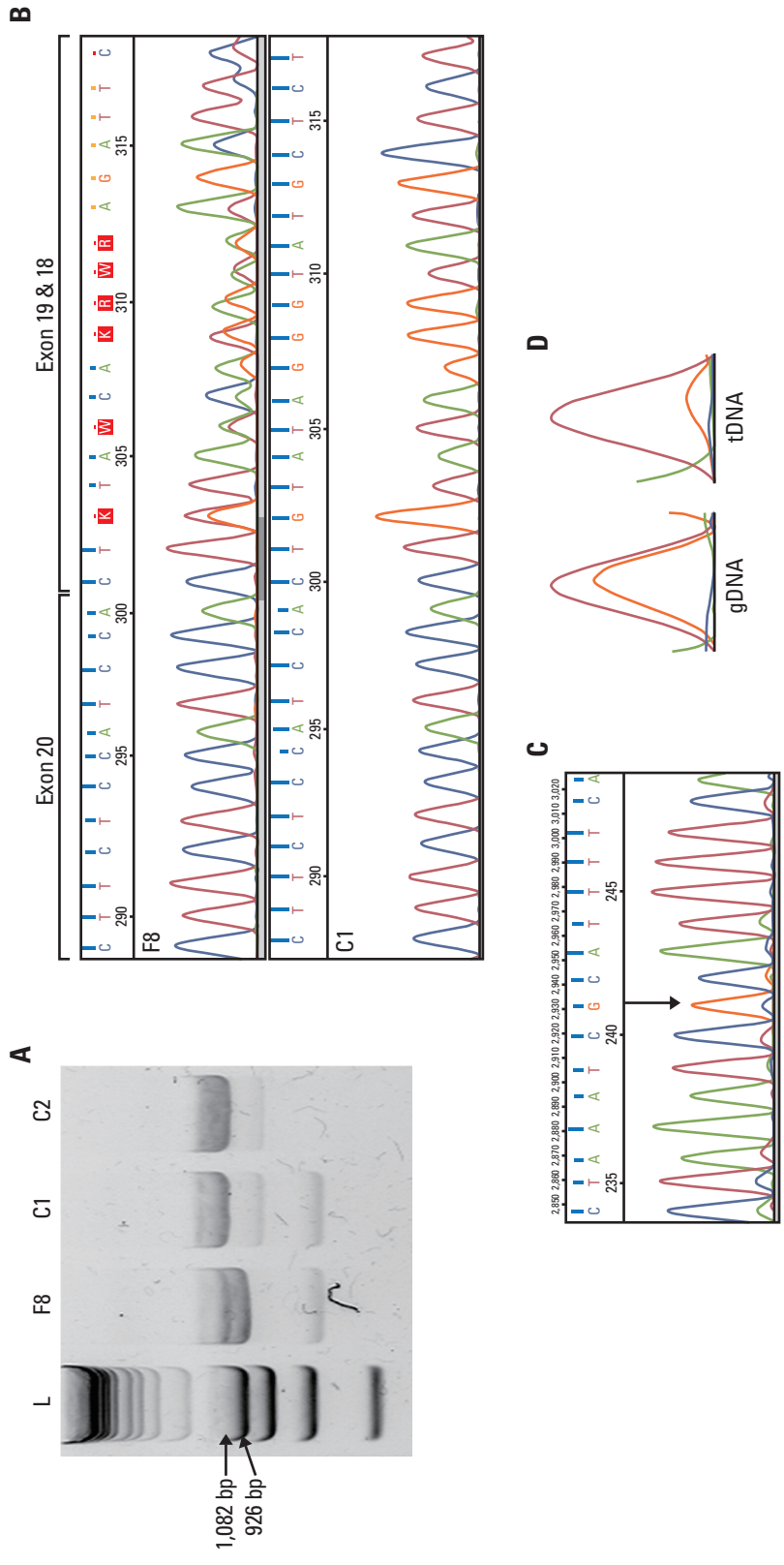

Fig. 3. cDNA analysis of *BRCA2* exonic variants. Panels A-D show the results of the *BRCA2* c.8487G>T variant. (A) RT-PCR gel electrophoresis results of proband of F8 (*BRCA2* c.8487G>T carrier) yielded an additional 120 bp shorter product on agarose gel, not present in controls. (B) Sanger sequencing of the RT-PCR products of the variant carrier showed whole exon 19 skipping. Reverse direction sequencing is depicted. (C) Demonstration of complete aberrant splicing on RT-PCR product amplified exclusively from the FL transcript. At the tagging *BRCA2* c.7242A>G variant position only G allele was detected and not any A (arrow points it out). (D) Representative example for LOH in family 7 highlighting the heterozygote superposition of the electropherogram in gDNA versus tDNA. *(Continued to the next page)*

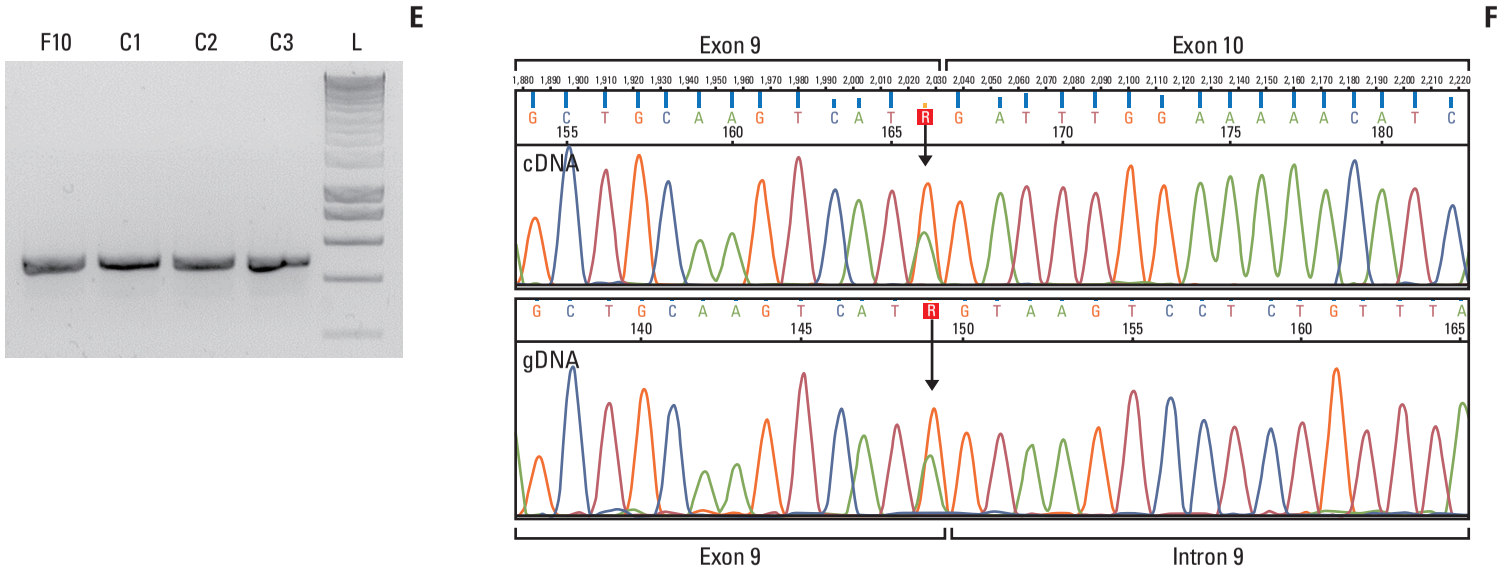

Fig. 3. *(Continued from the previous page)* (E, F) The results of the *BRCA2* c.793G>A variant. (E) RT-PCR gel electrophoresis results of proband of F10 (*BRCA2* c.793G>A carrier). No additional band different from the wild type is present. (F) Aligned sequencing electropherogram of cDNA and gDNA of the same region. Arrow points the variant c.793G>A. Neither an aberrant transcript, nor allelic imbalance is experienced. C1-3, wild type controls; F8, family 8; F10, family 10; gDNA, genomic DNA; L, 1 kb ladder (Promega); LOH, loss of heterozygosity; RT-PCR, reverse transcription polymerase chain reaction; tDNA, tumor DNA.

Table 3. Variant classification

Variant	ACMG class	ACMG criteria	BRCA exchange	ClinVar	ClinVar Review status	Reclassification	ACMG criteria with our evidence	Further supporting evidence
BRCA1: c.4484+4dupA	VUS	PM2, PP3, PP4	No variant listed	No data	NA	LP	PM2, PP3, PP4, PS3[a)]	
BRCA1: c.5407-10G>A	VUS	PM2, PP4	Variant not interpreted	VUS	[b)]	LP	PM2, PP4, PS3[a)]	LOH in tumor, Saturation mutagenesis Findlay et al. [23]
BRCA1: c.4358-31A>C	VUS	PM2, PP4, BP7	Variant not interpreted	No data	NA	Strong VUS	PM2, PP4, BP7, PS3[a)]	
BRCA2: c.8487G>T (p.Gln2829His)	LP	PVS1, PM2, PP3, PP4, BP1	No variant listed	NA	NA	P	PVS1, PM2, PP3, PP4, BP1, PS3[a)]	LOH in tumor, Aberrant splicing was formerly detected by Houdayer et al. [24].
BRCA2: c.793G>A (p.Gly265Arg)	VUS	PVS1, PM2, PP4, BP1, BP4	Not interpreted	Conflicting interpretations of pathogenicity: VUS (3) – LB (1)	[b)]	LB	PM2, PP4, BP1, BP4	

PVS1: Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease; PM2: Variant not found in gnomAD exomes; PP3: Pathogenic computational verdict based on 1 pathogenic prediction from GERP vs. no benign predictions; PP4: Patient's phenotype or family history is highly specific for a disease with a single genetic etiology; PS3: Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product; BP1: Missense variant in a gene for which primarily truncating variants are known to cause disease; BP4: Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.); BP6: Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation; BP7: A synonymous (silent) variant for which splicing prediction algorithms predict no impact on the splice consensus sequence nor the creation of a new splice site and the nucleotide is not highly conserved. ACMG, American College of Medical Genetics and Genomics; LB, likely benign; LOH, loss of heterozygosity; LP, likely pathogenic; NA, not applicable; P, pathogenic; VUS, variant of unknown significance. [a)]Supporting pathogenic evidences arising from our tests, [b)]Reviewed by expert panel.

other, shorter fragment proved to be an aberrant transcript with whole exon 19 skipping (Fig. 3A). The peak intensities of the sequencing electropherogram at the superposition of the normal and aberrant transcript sequences were equal (Fig. 3B); therefore, we suspected that the aberrant splicing was complete. Indeed, a tagging exonic variant position (c.7242A>G), which was also present in the proband in heterozygote state, we detected only the allele G, when amplifying the normal transcript selectively (Fig. 3C). No traces of allele A was present, implying that no full-length transcript was generated from the c.8487G>T variant carrier allele. Tumor sample DNA was available from three probands (family 6, 7, 9). LOH was demonstrated in all three samples with a mean Z score=0.25 (standard deviation, 0.03) (Fig. 3D).

BRCA2 c.793G>A affected the last nucleotide of *BRCA2* exon 9 and was prognosticated as potentially spliceogenic variant affecting canonical splice donor site by varSEAK program (score 4). Opposed to this prediction, we observed neither aberrant transcript nor allelic imbalance when tested cDNA of the variant carrier (Fig. 3E and F).

Discussion

This study based on 15 randomized controlled trials including 2,867 patients and aCorrect splicing regulation is indispensable for generating functional transcripts, so adequate evaluation of genetic variants' role in aberrant splicing is of paramount clinical relevance. We analyzed five rare *BRCA1/2* variants on cDNA-level, which were suggested a priori as potentially splice-altering changes according to various *in silico* splice predictions. Although RNA expression data arose from peripheral blood rather than tumor tissue samples of the carriers, data are authentical, since surveys give evidence that *BRCA1* alternative splicing is similar in blood and breast tissue, a finding supporting the clinical relevance of blood-based *in vitro* splicing assays [25]. Besides the presence of aberrant splicing, the extent of that is also an issue in determining pathogenicity, since surveys argue that incomplete aberrant splicing may yield normal transcript in sufficient quantity for physiological function [26]. Since the analyzed RNAs were collected in Tempus Blood RNA tubes, we could not perform NMD-inhibition prior to cDNA analyses of the samples, but we were able to calculate its extent indirectly in most of the cases, where it was applicable. Utilizing tagging polymorphism is an acknowledged way of determining if the lower extent of alternative allele expression is the result of incomplete aberrant splicing or nonsense-mediated decay [27]. Additionally, as further steps, we investigated locus-specific LOH in breast cancer tumor tissues and also performed co-segration analysis of these variants with clinical phenotype.

Multiple lines of evidence were synthesized to prove pathogenicity using the standardized variant interpretation recommendations of the ACMG (Table 3). Of the variants studied, *BRCA1* c.4484+4dupA and *BRCA1* c.5407-10G>A had enough supportive evidence for reclassification from VUS into likely pathogenic (Tier 2) category. These main arguments are (1) multiple lines of computational evidence support a deleterious effect on the gene or gene product (PP3 evidence), (2) the variants were found in patients with disease phenotype (PP4), since the probands were young age at onset with personal disease phenotypes characteristic of *BRCA1* mutation-carriers, in addition, *BRCA1* c.4484+4dupA variant carrier families had several relatives having various tumors in the syndromic spectrum, (3) the variant alleles were absent in diverse variant databases such as Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org) or the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org) (PM2) and (4) finally, our results fulfilled the PS3 category: 'well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product are strong evidence for pathogenicity' [4]. Our study provided transcript-level evidence for pathogenicity (pathogenic splice product, elicited unequivocally by the variant position), which was eligible for the assertion. Furthermore, CRISPR-based saturation genome editing surveys performed by Findlay et al. (2018) [23] also pointed out the possible functional relevance of *BRCA1* c.5407-10G>A, with an intermediate functional score of –0.95. An additional layer of a posteriori evidence for pathogenicity was also provided by LOH test of the variant *BRCA1* c.5407-10G>A, where the loss of the normal allele with $R < 0.5$ was demonstrated in the tumor tissues removed from both probands. Although the tumor sample of the proband carrying *BRCA1* c.4484+4dupA did not show LOH, it is not a strong proof against pathogenicity, since the ACMG scoring system does not make use of the somatic results as independent evidence for clinical assertion [28]. Even the bona fide pathogenic *BRCA1* mutations do not always accompanied by LOH. As much as 10% of the *BRCA1* germline mutation-associated breast tumors did not show locus-specific LOH [29]. Co-segregation analysis, however, was achievable in a c.4484+4dupA variant carrier family, where the variant segregated with the phenotype in additional family members, corroborating its pathogenic nature.

cDNA-level analysis of the variant *BRCA1* c.4358-31A>C clearly showed the presence of aberrant splicing, which was also whole exon 14 skipping. This is most probably the consequence of the disturbance of the active branchpoint adenine in the splicing intermediate lariat formation. Nonetheless, indirect calculations based on the inferred extent of

the NMD showed that the aberrant splicing is only partial, it totals up to only half of the transcripts of the variant-carrier allele. Functional surveys by de la Hoya et al. [26] revealed that *BRCA*-associated cancer risk is not markedly increased for individuals who carry a *BRCA1* allele, which permits 20%-30% of tumor suppressor function. In contrast, Bonnet et al. (2008) [30] judged *BRCA2* c.9501+3A-T variant with partial exon 25 skipping as a biologically significant mutation with reduced penetrance, although a significant portion of the variant-carrier allele produced normal transcript. Similarly, Zhang et al. (2009) [27] published that *BRCA2* IVS4-12del5 is a mutation, though this variant causes only partial deletion of exon 5 as a result of inefficient aberrant splicing. In our case, the proband's family (family 3) harbored strong characteristics of HBOC with five affected relatives, each fitting in the disease spectrum. Unfortunately, samples from other members of the family were not available for co-segregation analysis, similarly LOH test of the tumor was not feasible. In summary, this variant, although deserves attention, still requires further analysis to be equivocally asserted into the likely pathogenic category.

Transcript of the *BRCA2* c.8487G>T variant allele showed complete exon 19 skipping in our study. The *BRCA2* Δ19 is a minor naturally occurring alternative in-frame isoform but it is proved to be non-functional in complementation assays [31]. Spliceogenic capacity of this variant was formerly witnessed by Houdayer et al. [24] but they did not determine the amount of the aberrant splicing, which was assessed as 100% in our study. The other novelty provided by our experiments was the demonstration of LOH in several variant-carrier tumor samples, which is also corroborative for its pathogenic nature [28]. The variant is not registered in the dbSNP or ClinVar databases, but occurred relatively frequently in our familiar breast cancer cohort (4/3, 568). Phenotypes of the probands, as well as family tumor history, were characteristic for the pathology of *BRCA1* carriers. ACMG scoring by VarSome ver. 2021 predicts this variant as likely pathogenic. Indeed, by the combined supportive evidence, we can reinforce this assertion.

As for the variant *BRCA2* c.793G>A, as opposed to its spliceogenic prediction by varSEAK, our studies yielded neither aberrant transcript nor allelic imbalance at cDNA-level. The results provided sufficient evidence for this variant to alter the VUS ACMG verdict to likely benign.

Poor participant rate in family (cascade) screening is considered a limitation of the current study. While genetic testing was offered to all first-degree, asymptomatic and second-degree affected relatives during genetic counseling, in the 10 families only 14.8% (4/27) check-in rate was observed. Referred reasons from probands were elderly parents, living in different city or countryside and loose family bonds. Therefore, interpretation of co-segregation data has not represented strong relevance in our study.

As a summary, out of the five investigated variants, we were able to reclassify two VUS (*BRCA1*:c.4484+4dupA; *BRCA1*:c.5407-10G>A) into likely pathogenic class; one likely pathogenic variant (*BRCA2*:c.8487G>T, p.(Gln2829His)) into pathogenic category and one VUS (*BRCA2*:c.793G>A, p.(Gly265Arg)) into likely benign class.

With the spread of the high-throughput NGS in the routine molecular genetic diagnostics of hereditary cancer predisposition, there are emerging numbers of rare variants with unknown significance. The presence of VUS represents a significant challenge for the clinical geneticist, for the managing clinicians and for the patients as well. According to the current guidelines, VUS of the *BRCA1/2* genes are reportable, however should not be used for medical decisions, which can result in considerable stress for the proband and the proband's family. All of these emphasize the need for the molecular and clinical characterization of VUS. Both up- and down-classification harbor important clinical significance. Patients carrying re-classified pathogenic variants (previously known as VUS) in the future will not be dropped out from medical surveillance, preventive measures, treatment, and predictive family screening in relatives at risk. In the current study, we presented molecular and clinical evidence as a basis of reclassification and clinical evaluation of five *BRCA1/2* variants that can be used in the interpretation of molecular genetic reports.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study was approved by the Institutional Ethical Board and the Research and Ethics Committee of the Hungarian Health Science Council (ETT-TUKEB 53720-7/2019/EÜIG) and performed in accordance with the principles of the Declaration of Helsinki. After genetic counseling, written informed consents were obtained from all patients.

Author Contributions

Conceived and designed the analysis: Bozsik A, Papp J, Grolmusz VK, Patócs A, Oláh E, Butz H.

Collected the data: Bozsik A, Papp J, Grolmusz VK, Patócs A, Oláh E, Butz H.

Contributed data or analysis tools: Bozsik A, Papp J, Grolmusz VK, Patócs A, Oláh E, Butz H.

Performed the analysis: Bozsik A.

Wrote the paper: Bozsik A, Papp J, Butz H.

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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

Acknowledgments
This work was supported by TKP2020-NKA-26 to A.P. and NKFI FK 135065 to H.B.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.857
Cancer Res Treat. 2022;54(4):985-995

Original Article

Assessment of Anti-tumor Efficacy of Osimertinib in Non–Small Cell Lung Cancer Patients by Liquid Biopsy Using Bronchoalveolar Lavage Fluid, Plasma, or Pleural Effusion

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Purpose This study was to evaluate anti-tumor efficacy of osimertinib in patients positive for acquired epidermal growth factor receptor (*EGFR*) T790M mutation in liquid biopsy using plasma, bronchoalveolar lavage fluid (BALF) or bronchial washing fluid (BWF), and pleural effusion.

Materials and Methods Among patients benefited from previous EGFR–tyrosine kinase inhibitor (TKI) treatment followed by treatment failure, patients in whom T790M mutations are detected in at least one of the samples including tumor tissues, BALF/BWF, plasma, and pleural effusion were enrolled. T790M mutation was detected by extracting cell free DNA from liquid biopsy samples, using PANA Mutyper. Objective response rate (ORR) and progression-free survival (PFS) with osimertinib treatment were evaluated.

Results Between January 2018 and December 2019, 63 patients were enrolled and received osimertinib. Mean age was 63 years, and 38 (60.3%) were female. Twenty-six patients had T790M mutation in both liquid and tissue samples (group A), 19 patients had only in tissue biopsy samples (group B), and 18 patients had T790M mutation only in liquid biopsy samples (group C). ORR in overall population was 63.5%, and was 61.5% in group A, 68.4% in group B, and 61.1% in group C, respectively. Median PFS in overall patients was 15.6 months (95% confidence interval, 10.7 to 24.2). There was no significant difference in ORR or PFS between groups.

Conclusion Osimertinib showed favorable efficacy in lung cancer patients with acquired resistance to prior EGFR-TKI therapies, who screened positive for harboring T790M mutation detected from cell free DNA extracted from plasma, BALF/BWF, and pleural effusion.

Key words Non-small cell lung carcinoma, Osimertinib, T790M, Liquid biopsies

Introduction

First-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have conferred significant clinical benefits in patients with advanced *EGFR* mutant non–small cell lung cancer (NSCLC), thus being the standard first-line treatment options. However, majority of patients ultimately develop disease progression after 12-24 months of treatment, most commonly due to acquisition of Thr790Met (T790M) EGFR-TKI resistance mutation [1,2].

Osimertinib is a novel drug that potently inhibits signaling pathways and cellular growth in both *EGFR* mutation–positive and *EGFR*/T790M mutation–positive cell lines. Based on the results of the prior AURA phase III study demonstrating an efficacy of the drug with objective response rate (ORR) of 71% and the median progression-free survival (PFS) of 10.1 months [3] and phase III FLAURA trial confirming ORR of 80% and PFS of 18.9 months [4], osimertinib is approved for first-line treatment of patients with metastatic NSCLC harboring EGFR-sensitizing and T790M resistant mutations [5].

In South Korea, positivity of T790M mutation is pre-requisite for reimbursement of the drug for the second-line treatment in EGFR-positive progressive or metastatic NSCLC patients [6]. Accordingly, to diagnose T790M mutation positivity, repeated tumor biopsies should be performed in patients with acquired resistance when those patients develop disease progression following prior therapy with EGFR-TKI. However, such tissue biopsies are invasive methods accompanying discomfort and risk of procedure-associated complications and may not always supply enough tumor tissues for genetic profiling.

To overcome these limitations regarding tissue biopsies, new technologies called 'liquid biopsy' using circulating tumor DNA (ctDNA) in plasma have emerged [7-9]. The Korea National Health Insurance Service (NHIS) has covered ctDNA tests for *EGFR* mutations in advanced NSCLC since 2018, but only plasma or pleural fluid sample is indicated for reimbursement of EGFR-TKIs due to the limited diagnos-

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Received July 28, 2021 **Accepted** January 14, 2022 **Published Online** January 17, 2022

tic efficacy of other types of body fluid [6]. Therefore, efforts to improve diagnostic efficacy and clinical utility of liquid biopsy should be done by diversifying body fluid specimens including especially bronchoalveolar lavage fluid (BALF) or bronchial washing fluid (BWF). Recent studies reported BALF/BWF based EGFR genotyping have superior diagnostic performance to plasma [10,11], but specific detection for T790M mutation was not conducted in these studies and feasibility of liquid biopsy along with clinical response to osimertinib was not confirmed.

Therefore, this study was to evaluate diagnostic performance of liquid biopsy along with anti-tumor efficacy of osimertinib in patients who test positive for T790M mutations in liquid biopsy using at least one of the samples such as plasma, BALF/BWF and pleural effusion (especially focusing on liquid biopsy using BALF/BWF vs. other types of biopsy).

Materials and Methods

1. Study population and patient selection

This was a phase II, open-label, single-arm, single-center study to evaluate anti-tumor efficacy of osimertinib in NSCLC in whom T790M mutations are detected by liquid biopsy using at least one of the samples such as plasma, BALF/BWF, and pleural effusion. Among patients diagnosed and treated for NSCLC at Asan Medical Center between January 2018 and December 2019, we prospectively enrolled 63 patients who met following inclusion criteria: (1) patients who are aged ≥ 20 years and histologically or cytologically diagnosed as inoperable stage IIIB or IV NSCLC according to the 7th edition of the TNM staging system by the international association for the study of lung cancer, and patients who understand information about the trial and voluntarily agree to participate in the trial; (2) patients with EGFR sensitizing mutation (E19Del, L858R, L861Q, G719X) positive, who had shown clinical benefits (complete responders [CR] or partial response [PR] and stable disease ≥ 6 months) from EGFR-TKIs and had developed progressive disease; (3) patients in whom T790 mutations are detected in at least one of the samples including tumor tissues, BALF/BWF (cell-free DNA), plasma (cell-free DNA), and pleural effusion (cell-free DNA). Patients who received drugs targeting T790M mutations prior to enrolment, who have coexisting malignancies, severe or unstable medical conditions, who previously received other treatments including chemotherapy, radiotherapy, or surgery with less than 2 weeks of time interval at the time of starting study treatment were excluded.

2. Liquid biopsy and tissue sample preparation

All patients willing to be enrolled for the study underwent bronchoscopy with or without endobronchial ultrasound–guided transbronchial needle aspiration (EBUS-TBNA) for obtaining tumor tissues and BALF/BWF. At least 20 mL of BALF/BWF was taken by instilling 100 mL of sterile 0.9% saline by wedging the bronchoscope at the lung cancer site. If the obtained BALF/BWF specimen was less than 5 mL, an additional specimen was obtained by bronchial washing. Fifteen to twenty milliliters of blood sample was also obtained in heparin bottle from subjects at the time of screening for eligibility. Twenty milliliters of pleural fluid was also obtained in the patients with pleural effusion.

For liquid biopsy samples, centrifugation of samples was performed immediately after collection of the liquid specimens and 1 mL of supernatant was used for ctDNA extraction. ctDNA was purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) [12]. The purity and concentration of DNA was measured using a NanoDrop machine (Thermo Fisher Scientific, Waltham, MA). *EGFR* mutation analysis were conducted using PANA Mutyper (Panagene, Daejeon, Korea) with the peptide nucleic acid–mediated PCR clamping method [13] according to the instructions from manufacturers.

DNA of tumor tissue was extracted from paraffin sections, by deparaffinizing sections with xylene and alcohol.

3. Therapeutic methods

Osimertinib was administered as 80 mg once daily, and dose reduction to 40 mg once daily was permitted under physician's judgement based on individual safety and tolerability. A cycle of study treatment was defined as 28 days, day 1 of next cycle being 29 day of previous cycle, and the time window for each visit being ±7 days. Each cycle was scheduled as D29±7 (cycle 2), D57±7 (cycle 3), D85±7 (cycle 4), D113±7 (cycle 5) from cycle 1 day 1, and then every 8 weeks. Response evaluation was performed every 8 weeks (±7 days) from day 1 of first cycle. Each subject was recommended to continue the study drug until disease progression or manifestation of unacceptable toxicity.

4. Study variables and endpoints

Baseline demographic and clinical characteristics such as age, sex, smoking history, histologic subtype, *EGFR* mutation status, and the presence or absence of previous surgery or irradiation were extracted from each patient's medical record.

ORR was defined as the proportion of patients achieving a best clinical response to osimertinib of either CR or PR, as recorded in the patient's medical record, based on Response Evaluation Criteria in Solid Tumors ver. 1.1. PFS was defined

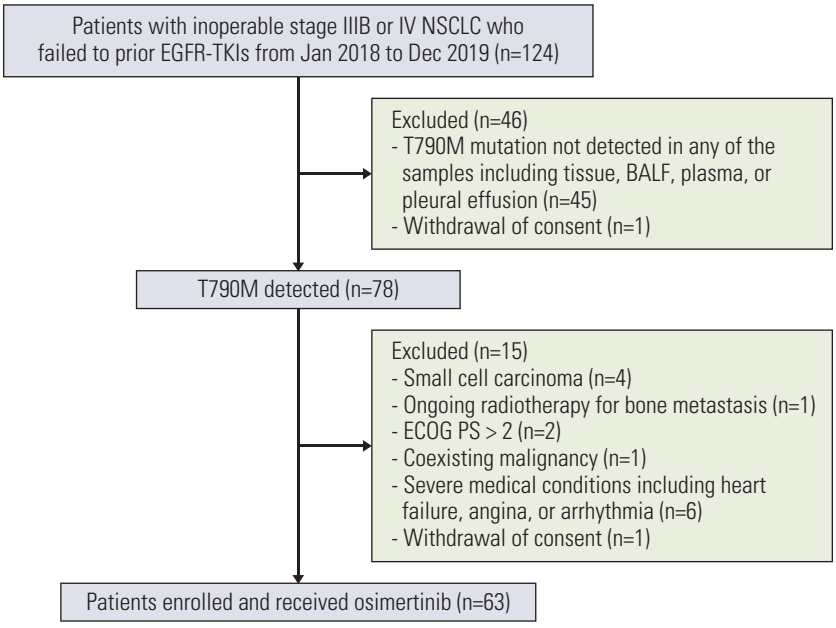

Fig. 1. Patient flowchart. One hundred twenty-four patients who previously benefited from EGFR-TKI treatment and eventually experienced disease progression were enrolled. From 78 T790M detected in either tissue or liquid biopsy specimens, 63 patients were enrolled and received osimertinib. BALF, bronchoalveolar lavage fluid; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; NSCLC, non–small cell lung cancer; T790M, (c.2369C>T; p.Thr790Met); TKI, tyrosine kinase inhibitor.

as the time (in months) from the first date of Osimertinib treatment until the date of objective disease progression or death, whichever comes first.

Adverse events (AEs) related to osimertinib treatment were reported according to the Common Terminology Criteria for Adverse Events (CTCAE), ver. 4.03. If a patient experienced a CTCAE of grade 3 or higher and/or unacceptable toxicity (any grade) that was associated with osimertinib, drug interruption was permitted for up to 3 weeks. If the toxicity resolved or reverted to CTCAE grade ≤ 2 within 3 weeks of onset, osimertinib could be restarted at the same dose (80 mg, daily) or a lower dose (40 mg, daily), excluding cases with any grade of pulmonary toxicity, symptomatic corrected QT interval prolongation, or corneal ulceration. Once a dose had been reduced, it was not re-escalated at future cycles.

5. Statistical Analysis

Subject number was calculated using z-test based on the non-inferiority test. We assumed the null hypothesis as ORR 35% and alternative hypothesis as ORR 60%, adopted from the AURA phase I study [14]. We intended to prove the alternative hypothesis that the difference between ORRs would be lower than 0.25 versus the null hypothesis that the difference between ORRs would be higher than 0.25 using the level of significance of 2.5%. When ORR difference is lower than 0.25, 56 subjects were estimated to be needed to have the power of the test of 80% for rejecting the null hypothesis. However, considering a halfway dropout-rate of 10%, a total of 63 subjects were thought to be needed. Among them, given that the likelihood of detecting T790M mutation in TKI-acquired-resistant patients is around 60%, about 105 patients are expected to be tested for T790M mutation status and 63 patients would be administered osimertinib. The diagnostic performance of each method for detecting mutations in plasma or BALF/BWF samples was expressed in terms of the sensitivity, specificity, and accuracy, with the mutation status determined in tissue sample as the reference standard. Analysis variables were summarized and were stratified by the type of biopsy samples which were detected to harbor T790M mutation (tissue or liquid biopsy). We grouped the subjects as they harbor T790M mutation detected in both tissue and liquid biopsy samples (group A), only in tissue sample (group B), or only in liquid biopsy samples (group C). Significant differences in descriptive variables between these groups were assessed with the chi-squared or Fisher exact tests for qualitative variables and Student's t test for quantitative variables. $p < 0.05$ was considered statistically significant for all tests. All analyses were conducted using the IBM SPSS ver. 25.0 (IBM Corp., Armonk, NY) or the R statistical package ver. 3.5.3 (Institute for Statistics and Mathematics, Vienna, Austria; http://www.R-project.org).

Table 1. Baseline characteristics

	Total	Group A	Group B	Group C
No.	63	26	19	18
Age (yr)	63 (45-84)	60.3 (47-74)	63.7 (45-84)	66.9 (54-81)
Female sex	38 (60.3)	15 (57.7)	11 (57.9)	15 (66.7)
ECOG				
0-1	59 (93.7)	25 (96.2)	17 (89.5)	17 (94.4)
2-3	4 (6.3)	1 (3.8)	2 (10.5)	1 (5.6)
Previous surgery	12 (19.0)	3 (11.5)	2 (10.5)	7 (38.9)
Previous RTx	21 (33.3)	10 (38.5)	7 (36.8)	4 (22.2)
Extrathoracic metastasis	37 (58.7)	15 (57.7)	12 (63.2)	10 (55.6)
Brain	18 (28.6)	9 (34.6)	4 (21.1)	5 (27.8)
Extrathoracic visceral metastases	28 (44.4)	11 (42.3)	10 (52.6)	7 (38.9)
Coexisting *EGFR* mutation				
E19del	45 (71.4)	23 (88.5)	13 (68.4)	9 (50.0)
L858R	16 (25.4)	3 (11.5)	5 (26.3)	8 (44.4)
G719X	2 (3.2)	1 (3.8)	1 (5.3)	0
Other (L861Q, S768I)	1 (1.6)	0	0	1 (5.6)
T790M positivity				
Plasma	18	13	0	5
BALF/BWF	32	19	0	13
Pleural effusion	8	4	0	4
Tissue	45	26	19	0
Reason for absence of *EGFR* mutation test in tissue sample				
Unable to conduct tissue biopsy	-	-	-	7 (38.9)
Inadequate amount of sample	-	-	-	3 (16.7)
No malignant cells	-	-	-	4 (22.2)

Values are presented as number (%) or median (range). *EGFR* mutation: T790M, (c.2369C>T; p.Thr790Met); E19del, (c.2235del15; p.E746_A750del); L858R, (c.2573T>G; p.Leu858Arg); G719X, (c.2155G>A; p.Gly719Ser); L861Q, (c.2582T>A; p.Leu861Gln); S768I, (c.2303G>T; p.Ser768Ile). Group A, patients who have T790M mutation detected in both tissue and liquid biopsy samples; Group B, patients who have T790M mutation detected only in tissue; Group C, patients who have T790M mutation detected only in liquid biopsy samples. BALF, bronchoalveolar lavage fluid; BWF, bronchial washing fluid; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; RTx, radiotherapy.

Results

1. Clinical characteristics of study population

One hundred twenty-four patients with acquired resistance after treatment with EGFR-TKIs were screened for the T790M resistance mutation in any of samples including tissue, BALF/BWF, plasma, or pleural effusion from January 2018 to December 2019. After screening procedure, 63 patients were finally enrolled and received osimertinib treatment (Fig. 1). Median age was 63 years old (range, 45 to 84 years) and 38 (60.3%) were female. From the enrolled subjects, 56 tissue samples were obtained via bronchoscopy or EBUS-TBNA at the time of screening procedure. Among them, three samples had inadequate amount to perform *EGFR* mutation test and four specimens showed no malignant cells, being unable to undergo mutation test. Therefore, 45 cases out of 49 showed T790M mutation detected from tissue sample. In terms of liquid biopsy samples, 32 BALF/BWF, 18 plasma, and eight pleural fluid samples had T790M positivity (Table 1).

Among the enrolees, 26 patients had T790M mutation detected in both tissue and liquid biopsy samples (group A), 19 only in tissue sample (group B), and 18 only in liquid biopsy samples (group C) (Fig. 2). Subjects in group C seemed to be older, and had more frequent history of previous surgery and L858R mutation as coexisting *EGFR* mutation along with T790M mutation compared with group A and B, but there was no statistically significant difference (p-value for age difference=0.356, previous surgery=0.063, and L858R coexistence=0.064) (Table 1).

2. Diagnostic performance of liquid biopsy specimen for detection of *EGFR* mutation

We compared the diagnostic yields of BALF/BWF and

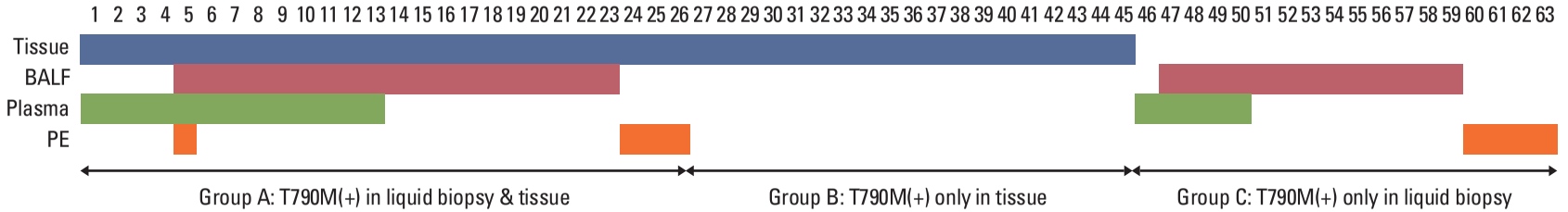

Fig. 2. Grouping of the subjects according to T790M mutation status in tissue or liquid biopsy samples. T790M positivity by the type of biopsy sample in overall population are shown as a diagram. Group A, patients who have T790M mutation detected in both tissue and liquid biopsy samples; Group B, patients who have T790M mutation detected only in tissue; Group C, patients who have T790M mutation detected only in liquid biopsy samples. BALF, bronchoalveolar lavage fluid; PE, pleural effusion; T790M, (c.2369C>T; p.Thr790Met).

Table 2. Diagnostic performance of liquid biopsy according to *EGFR* mutation

	T790M			E19del			L858R			All *EGFR* mutations		
	Plasma	BALF/BWF	p-value	Plasma	BALF/BWF	p-value	Plasma	BALF/BWF	p-value	Plasma	BALF/BWF	p-value
Sensitivity (95% CI)	28.9 (16.4-44.3)	42.2 (27.7-57.9)	0.083	52.8 (35.5-69.6)	80.6 (64.0-91.8)	0.026	40.0 (12.2-73.8)	80.0 (44.4-97.5)	0.381	54.2 (39.2-68.6)	81.3 (67.4-91.1)	0.012
Specificity (95% CI)	75.0 (19.4-99.4)	25.0 (0.63-80.6)		92.3 (64.0-99.8)	92.3 (64.0-99.8)		97.4 (86.5-99.9)	100.0 (91.0-100.0)		0.0 (0.0-97.5)	0.0 (0.0-97.5)	
Accuracy, n (%)	16/49 (32.7)	20/49 (40.8)		63.3 (31/49)	83.7 (41/49)		42/49 (85.7)	47/49 (95.9)		26/49 (53.1)	39/49 (79.6)	
TP	13	19		19	29		4	8		26	39	
TN	3	1		12	12		38	39		0	0	
FP	1	3		1	1		1	0		1	1	
FN	32	26		17	7		6	2		22	9	

EGFR mutation: T790M, (c.2369C>T; p.Thr790Met); E19del, (c.2235del15; p.E746_A750del); L858R, (c.2573T>G; p.Leu858Arg). BALF, bronchoalveolar lavage fluid; BWF, bronchial washing fluid; CI, confidence interval; EGFR, epidermal growth factor receptor; FN, false negative; FP, false positive; TN, true negative; TP, true positive.

Table 3. Clinical efficacy of osimertinib treatment by T790M positivity status in tissue or liquid biopsy samples

	Total	Group A	Group B	Group C
No.	63	26	19	18
Type of response				
CR	0	0	0	0
PR	40	16	13	11
SD	21	10	6	5
PD	2	0	0	2
Response rate (CR+PR) (95% CI, %)	63.5 (51.3-75.7)	61.5 (42.4-80.6)	68.4 (46.9-89.9)	61.1 (37.9-84.3)
PFS (95% CI, mo)	15.6 (10.7-24.2)	10.7 (7.2-16.7)	NR	20.3 (11.1-24.4)

Group A, patients who have T790M mutation detected in both tissue and liquid biopsy samples; Group B, patients who have T790M mutation detected only in tissue; Group C, patients who have T790M mutation detected only in liquid biopsy samples. CI, confidence interval; CR, complete response; NR, not reached to median; PD, progression of disease; PFS, progression-free survival; PR, partial response; SD, stable disease; T790M, (c.2369C>T; p.Thr790Met).

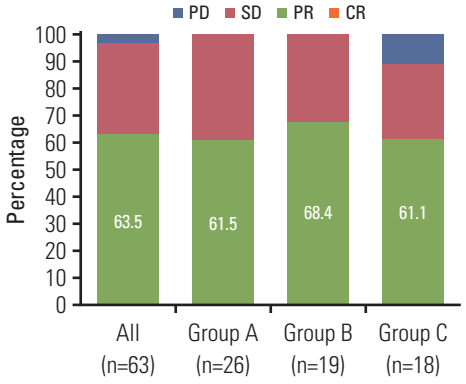

Fig. 3. Objective response rates by T790M positivity status in tissue or liquid biopsy samples. Objective response rates according to Response Evaluation Criteria in Solid Tumors in the response evaluable population are shown by T790M positivity in tissue or liquid biopsy samples. Group A, patients who have T790M mutation detected in both tissue and liquid biopsy samples; Group B, patients who have T790M mutation detected only in tissue; Group C, patients who have T790M mutation detected only in liquid biopsy samples. CR, complete response; PD, progression of disease; PR, partial response; SD, stable disease; T790M, (c.2369C>T; p.Thr790Met).

plasma samples for detecting *EGFR* mutations in 49 cases with adequate tissue samples. Sensitivity for predicting tissue T790M mutation using BALF/BWF was 42.2%, higher compared to that of plasma (28.9%), but was not significantly better (p=0.077). Similar results were shown in detecting tissue L858R (80.0% vs. 40.0%, p=0.381). Sensitivity for E19del and overall *EGFR* mutations was significantly superior using BALF/BWF compared to plasma. Specificity, however, was lower in BALF/BWF (25.0%) for detecting T790M mutation than in plasma (75%). There was no difference in specificity for the diagnosis of E19del or L858R, evaluated by plasma and BALF/BWF (Table 2). When combining the results of BALF/BWF and plasma ctDNA tests, sensitivity for detection of T790M was 51.1%, specificity was 25.0%, and accuracy was 49.0%. The sensitivity for predicting T790M mutation using both BALF/BWF and plasma was significantly higher than using plasma ($p < 0.001$), but was not significant compared to using BALF/BWF (p=0.125). Similar results were observed in detection of E19del and overall *EGFR* mutations (S1 Table). Ten out of 63 patients underwent additional bronchial washing, and exclusion of these cases did not result any significant difference in diagnosis rate for T790M in BALF/BWF (sensitivity 43.8% [95% confidence interval (CI), 26.4 to 62.3], specificity 25% [95% CI, 0.63 to 80.6], accuracy 41.7%, p=0.830).

3. Clinical efficacy of osimertinib according to T790M positivity status in tissue or liquid biopsy

The response to osimertinib was evaluable in all 63 enrolled patients at the time of data analysis. In the overall population, CR was not observed, PR was observed in 40 patients (ORR, 63.5%) (Table 3, Fig. 3). Subjects with group A (n=26) had ORR of 61.5%, while group B (n=19) and C (n=18) showed ORR of 68.4% and 61.1%, respectively. Although patients who harbor T790M mutation only in tissue have shown the highest ORR among the three groups, the intergroup difference was not significant (p=0.631 comparing A and B, p=0.970 comparing A and C, p=0.642 comparing B and C) (Figs. 3 and 4).

Response to osimertinib in patients of group C was not significantly different according to the type of liquid biopsy samples. Patients with T790M detected in both BALF/BWF and plasma, ORR was 100%; for BALF/BWF only, 44.4%; plasma only, 100%; pleural effusion only, 50% ($p > 0.05$) (S2 Table, S3 and S4 Figs.). ORRs by coexisting *EGFR* mutation status (E19del and L858R) along with T790M mutation was

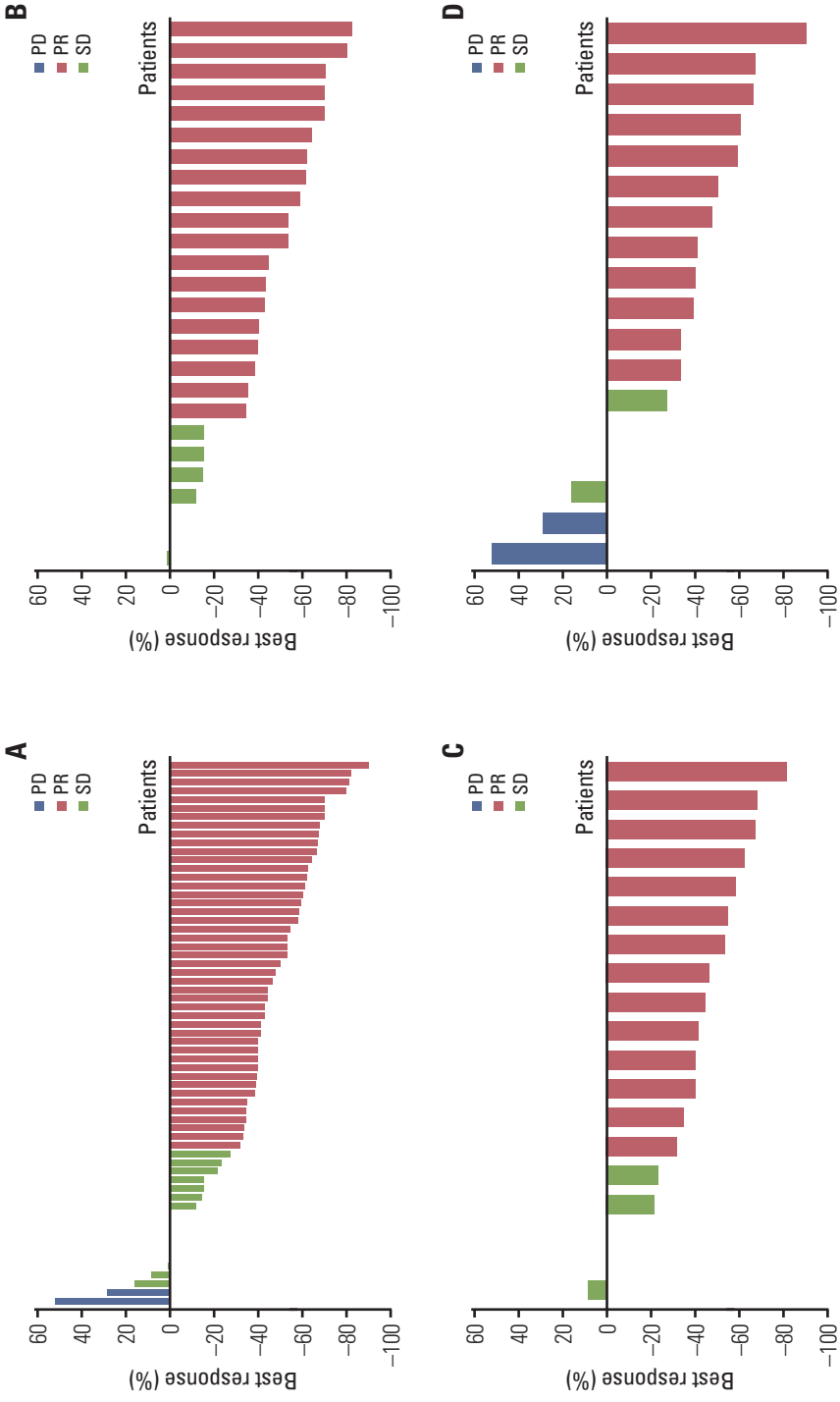

Fig. 4. Tumor response to osimertinib by T790M positivity status in tissue or liquid biopsy samples. Waterfall plot according to Response Evaluation Criteria in Solid Tumors in the response evaluable population are shown by T790M positivity in tissue or liquid biopsy samples. (A) Overall patients (n=63). (B) Group A (n=26): patients who have T790M mutation detected in both tissue and liquid biopsy samples. (C) Group B (n=19): patients who have T790M mutation detected only in tissue. (D) Group C (n=18): patients who have T790M mutation detected only in liquid biopsy samples. PD (blue bar), progression of disease; PR (red bar), partial response; SD (green bar), stable disease; T790M, (c.2369C>T; p.Thr790Met).

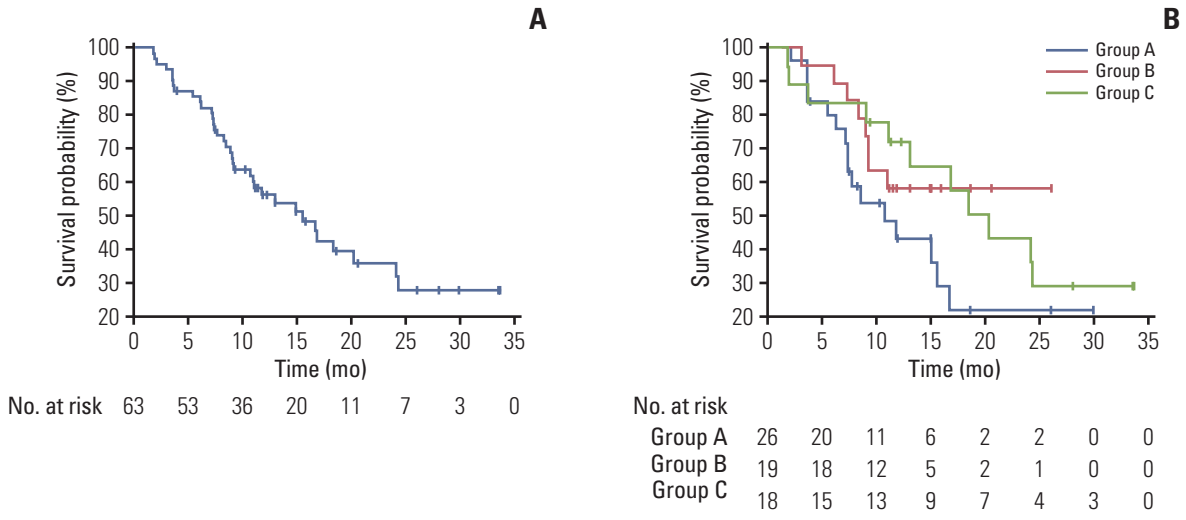

Fig. 5. Progression-free survival after osimertinib treatment. Progression-free survival after treatment with osimertinib in overall patients (n=63) (A) and by T790M positivity (B) in tissue or liquid biopsy samples are shown. Group A (n=26), patients who have T790M mutation detected in both tissue and liquid biopsy samples; Group B (n=19), patients who have T790M mutation detected only in tissue; Group C (n=18), patients who have T790M mutation detected only in liquid biopsy samples; T790M, (c.2369C>T; p.Thr790Met).

Table 4. Adverse events with CTCAE grade ≥ 3 related to osimertinib treatment

Adverse events	No. (%)	Grade	Action taken	Outcome
Hyponatremia	1 (1.6)	3	Drug interrupted	Resolved
Neutropenia	2 (3.2)	3	Drug interrupted	Resolved
QTc prolongation	1 (1.6)	3	Drug withdrawn	Resolved
Pneumonia	2 (3.2)	5	Drug withdrawn	Death

CTCAE, Common Terminology Criteria for Adverse Events.

not significantly different (S5 Table).

The final analysis of PFS was performed on the data cut-off date of December 3, 2020 and the median follow-up duration was 20.6 months (95% CI, 17.2 to 24.0). The median PFS in overall population was 15.6 months (95% CI, 10.7 to 24.2) (Fig. 5A). PFS according to T790M mutation status of biopsy samples was as follows: group A, 10.7 months (95% CI, 7.2 to 16.7); group B, not reached to median; group C, 20.3 months (95% CI, 11.1 to 24.4). Although patients in group B and C seemed to have numerically better PFS than group A, there were no statistical difference in PFS between groups (p=0.137) (Fig. 5B).

4. Safety assessment of osimertinib

AEs of grade 3 to 5 related to osimertinib treatment developed in six patients (9.5%). Two patients experienced grade 3 neutropenia and one patient experienced grade 3 hyponatremia, which resulted in drug interruption for 2 weeks, and AEs were resolved. One patient had prolongation of QTc interval related to osimertinib, which resolved with permanent drug withdrawal. Pneumonia developed in two patients treated with osimertinib, and worsened despite of drug withdrawal, resulting in death (Table 4).

Discussion

This was the novel prospective trial evaluating the clinical efficacy of osimertinib as 3rd generation EGFR–TKI in patients with NSCLC who harbor *EGFR* T790M mutation detected from either tissue or liquid biopsy samples, especially in BALF/BWF. In the present study, there is indication that osimertinib may have favorable efficacy in patients who had T790M mutation only detected in liquid biopsy samples, which is not inferior compared to other group of the patients.

After acquiring resistance to EGFR-TKIs, demonstration of T790M mutation is mandatory to use osimertinib, which requires re-biopsy usually based on tumor genotyping. However, obtaining adequate tissue through re-biopsy is clearly limited in clinical practice, due to inaccessible tumor

site, poor performance status of patients, and potential complications related to procedure [15-17]. Liquid biopsies using ctDNA in blood, which are less invasive and more convenient than conventional tissue biopsy therefore had been approved for the alternative tests [18,19]. In this prospective study, although the number of patients was only five, ORR in patients with T790M-positive plasma and T790M-negative tumor sample was 100%, supporting the promising role of plasma T790M detection as a feasible biomarker to osimertinib treatment outcome. However, some limitations remain regarding feasibility of liquid biopsy. Proportion of ctDNA in blood samples is generally low, and half-life of ctDNA is short, casting challenges with respect to low sensitivity and high false-negative rates. For example, detecting mutations with plasma ctDNA has widely ranged sensitivity, with 39%-86% sensitivity for *EGFR* mutations and 27%-75% sensitivity for T790M mutation [20-23]. *A post hoc* analysis of AURA phase III trial demonstrated detection rate of T790M as 51 to 66% (51% by cobas plasma, 58% by droplet digital polymerase chain reaction (ddPCR), and 66% by next-generation sequencing) [24]. In this prospective study, sensitivity of T790M mutation by cobas plasma test (51%) was lower than the values for E19del (82%) and L858R (68%). In the current study, we used PANAMutyper probe only and sensitivity of plasma ctDNA for detecting T790M mutations and overall *EGFR* mutation was 28.9% and 54.2%, respectively. Detection rate of plasma T790M mutation in our study is noticeably low when compared to the study of Park et al. [25], which reported same detection rate of plasma T790M mutation by either PANAMutyper or cobas test as 45.9% (17/37 patients). We assume that relatively lower disease burden in our study population could explain the low sensitivity of plasma ctDNA test. As shown in Table 1, our patients seem to have lower proportion of extrathoracic metastasis (28.6% of brain metastasis and 44.4% of extrathoracic visceral metastasis) than that of AURA3 (33% of brain metastasis and 52% of extrathoracic visceral metastasis) [3] or study of Park et al. (52.4% of brain metastasis) [25]. In addition to tumor burden, DNA instability while processing plasma or difference in analytic methods could be related to the low sensitivity of plasma T790M detection in our study. Nevertheless, varied sensitivity in detecting T790M mutation from plasma requires further utilization of other types of liquid biopsy along with blood sample.

BALF/BWF plays a supporting role in the diagnosis of lung cancer and the detection of *EGFR* mutations. Diagnostic yield of BALF/BWF identifying malignant cells in adenocarcinoma was 77% in previous study [26]. Park et al. [27] suggested that BALF/BWF might be effective for determining the *EGFR* genotype, with high concordance rate (91.7%) between BALF/BWF and tissue using PANA Mutyper in 20 patients. Hur et al. [28] reported BALF/BWF extracellular vesicle (EV)–based *EGFR* genotyping had average sensitivity and specificity of 76% and 87%, respectively. Lee et al. [11] compared diagnostic yields of plasma and BWF for detecting EGFR-TKI sensitizing mutations (E19del and L858R) by ddPCR, reporting superior diagnostic performance of BWF (sensitivity and specificity being 68.42% and 98.15% for E19del, 89.47 and 96.30 for L858R) compared to plasma (sensitivity and specificity being 31.58% and 94.44% for E19del, 47.37% and 98.15% for L858R). In our study, similar superior sensitivity of BALF/BWF compared to plasma was observed in detecting E19del and overall *EGFR* mutations, but not significant in T790M. Also, sensitivity in detection of T790M mutation of BALF/BWF (42.2%) was considerably low compared to previous studies which used BALF/BWF or plasma ctDNA [11,24-28], which suggest role of ctDNA in BALF/BWF may not fully substitute for tissue biopsy. This relatively low diagnostic performance might be associated to DNA instability in the BALF/BWF, difference in the detection method, location of targeted tumor lesion, or spatial heterogeneity of tumor. Still, sensitivity for T790M detection has been significantly improved from 28.9% to 51.1% when we combined the results of plasma ctDNA and BALF/BWF tests, which reveals the additive effect of BALF on plasma ctDNA test. Indeed, the cost and risks for complication of bronchoscopic procedures must be considered. However, we suggest active measurement of ctDNA from BALF/BWF would enable more patients to be detected as harboring T790M mutation, thus, to be benefited for osimertinib.

Kiura et al. [29] assessed ORR as 75% in Japanese cohort of AURA Phase I study, which contained 12 subjects who had positive T790M result from BALF/BWF samples. In the current study, patients who showed T790M positivity only in BALF/BWF demonstrated ORR of 44.4%, and when combined with patients who harbor T790M mutation in both plasma and BALF/BWF, patients demonstrated ORR of 61.5%. Furthermore, ORR in five patients with T790M-positive plasma and T790M-negative tumor sample was 100%. The number of each patient for T790M positivity in various liquid biopsy samples was too small to draw any firm conclusion, we carefully assume that this relatively low ORR despite of high sensitivity of BALF/BWF was not related to coexisting *EGFR* activating mutation status according to the data on S2 Table. Rather it could be related to tumor burden, which was not fully evaluated in this study.

Malignant effusion was also under consideration of our study, but number of patients who harbor T790M mutation in pleural fluid was small, limiting exact assessment of diagnostic performance and ORR. *EGFR* genotyping using both EV DNAs (DNA inside the EV shed by tumor cells, protected

by dual lipid membranous coating) and ctDNAs from supernatant of pleural effusion resulted in 100% agreement with tissue *EGFR* genotyping in both EGFR-TKI naive patients and patients who had acquired resistance to EGFR-TKI in a recent study [30], suggesting pleural effusion is also a useful liquid biopsy sample.

Safety profile of osimertinib in the current study was consistent with previous reports of AURA trials [3,31]. Osimertinib was well tolerated, and no dose reductions were needed related to AEs in current study. However, interruptions and discontinuation of the drug did occur, and two mortality cases (3.2% of overall population) developed. The incidence of pneumonia in patients with disease progression in the central nervous system was 3%-5% in previous trial, which is consistent with our data.

This study has several limitations. We only used ctDNA, which is known to have relatively low sensitivity compared to EV-derived liquid biopsy tests. But ctDNA is simple and cost-effective, and our study showed permissive sensitivity and specificity in detecting T790M mutation using diverse liquid biopsy samples. In addition, due to the study maturation was not fully achieved, the median overall survival of this study was not evaluated. However, this is the first study prospectively evaluating efficacy of osimertinib in patients who harbor T790M mutations in liquid biopsy samples, reflecting real world practice setting.

In conclusion, osimertinib had favorable efficacy in patients with NSCLC harboring T790M mutation detected in liquid biopsy samples, which is non-inferior to those detected in tissue, supporting feasibility of liquid biopsy as another tool for re-biopsy for identifying T790M mutation. BALF/BWF have non-inferior diagnostic performance in detecting T790M mutation compared to plasma.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study protocol was approved by the Institutional Review Board of Asan Medical Center (approval number: 2017-0295) and written informed consent was obtained from all patients.

Author Contributions

Conceived and designed the analysis: Ji WJ, Lee JC, Choi CM.
Collected the data: Ji WJ, Lee JC, Choi CM.
Contributed data or analysis tools: Ji WJ, Lee JC, Chun SM, Choi CM.
Performed the analysis: Kim YJ, Chun SM, Choi CM.
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Conflicts of Interest

This study was funded by Astrazeneca, Inc. (study sponsor). The authors have no conflicts of interest to declare otherwise.

Acknowledgments

This work was supported by the Korea Medical Device Development Fund grant funded by the Korea Government (the Ministry of Science and ICT, the Ministry of Trade, Industry and Energy, The Ministry of Health & Welfare, Republic of Korea, the Ministry of Food and Drug Safety) (Project Number: 202011B13).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.902
Cancer Res Treat. 2022;54(4):996-1004

Original Article

The Value of the Illness-Death Model for Predicting Outcomes in Patients with Non–Small Cell Lung Cancer

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Purpose The illness-death model (IDM) is a comprehensive approach to evaluate the relationship between relapse and death. This study aimed to illustrate the value of the IDM for identifying risk factors and evaluating predictive probabilities for relapse and death in patients with non–small cell lung cancer (NSCLC) in comparison with the disease-free survival (DFS) model.

Materials and Methods We retrospectively analyzed 612 NSCLC patients who underwent a curative operation. Using the IDM, the risk factors and predictive probabilities for relapse, death without relapse, and death after relapse were simultaneously evaluated and compared to those obtained from a DFS model.

Results The IDM provided more detailed risk factors according to the patient's disease course, including relapse, death without relapse, and death after relapse, in patients with resected lung cancer. In the IDM, history of malignancy (other than lung cancer) was related to relapse and smoking history was associated with death without relapse; both were indistinguishable in the DFS model. In addition, the IDM was able to evaluate the predictive probability and risk factors for death after relapse; this information could not be obtained from the DFS model.

Conclusion Compared to the DFS model, we found that the IDM provides more comprehensive information on transitions between states and disease stages and provides deeper insights with respect to understanding the disease process among lung cancer patients.

Key words Non–small cell lung carcinoma, Prognosis, Disease-free survival, Risk factors

Introduction

Lung cancer is one of the most common cancers in the world, and its mortality of 25% is the highest among all cancers [1]. Non–small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases and surgery is the mainstay treatment for patients with early-stage NSCLC [2]. Surgery is generally performed in patients with stage I to IIIA NSCLC, when the tumor is resectable in patients who are suitable for surgery [3]. The 5-year overall survival (OS) rate for NSCLC is approximately 50% for resected lung cancers [1] and approximately 30%-55% of patients with resected NSCLC eventually show relapse during follow-up [4]. There are several risk factors affecting OS or relapse-free survival (RFS) in patients with resected NSCLC. Tumor-related factors, including tumor-node-metastasis (TNM) staging, cell types, and host-related factors (i.e., sex, age, smoking history) are predictive of OS in patients with NSCLC [5]. In addition, TNM staging, tumor markers, and performance status are reported as significant factors in predicting RFS after surgical resection in patients with NSCLC [5].

In patients who have undergone NSCLC surgery, events such as relapse or metastasis are called intermediate events because they are not fatal, whereas death is called a terminal event. Studies have typically focused on whether or not a patient survives without experiencing relapse or death when analyzing cancer-related survival data. This concept is called disease-free survival (DFS). In DFS studies, the intermediate event and terminal event are considered to have a competing relationship, survival time is defined as the time of occurrence of the first of the two events, and censoring is defined as whether either of the two events has occurred or not. On the other hand, the illness-death model (IDM) [6-8] considers the intermediate event and terminal event to have a semi-competing relationship, so it has the advantage of observing the survival time from the occurrence of the intermediate event to the occurrence of the terminal event, which cannot be observed in DFS.

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Received August 9, 2021 **Accepted** November 18, 2021 **Published Online** November 19, 2021
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The purpose of this study was to identify risk factors affecting relapse and death via the IDM among NSCLC patients and evaluate personalized predictive probabilities of disease outcomes to help in establishing follow-up plans or treatment strategies.

Materials and Methods

1. Study population

A total of 917 patients who underwent curative surgery for primary lung cancer from 2010 to 2018 were initially included in this study. Exclusion criteria were as follows: (1) sublobar resection, (2) the presence of synchronous or metachronous lung cancer, (3) an unknown origin of cancer recurrence in patients with double primary cancer, (4) the presence of distant metastases, (5) other than adenocarcinoma or squamous cell carcinoma, and (6) pre-invasive cancers. A total of 612 consecutive patients with resected lung adenocarcinoma or squamous cell carcinoma were ultimately included in the study (Fig. 1).

2. Data processing

Patient demographics, history of malignancy other than lung cancer, family history of lung cancer, smoking history (never smokers vs. previous or current smokers), cancer location (upper lobes, including the middle lobe vs. lower lobes), cancer type (adenocarcinoma vs. squamous cell carcinoma), TNM stage, operation method (lobectomy vs. bi-lobectomy or pneumonectomy), treatment method, and the presence of relapse were recorded from electronic medical records and radiology reports. TNM stage was determined according to the eighth edition of the American Joint Commission on Cancer staging system for lung cancer [9]. The T category was determined based on pathologic reports following lobectomy, and the N category was determined by lymph node dissection or endobronchial ultrasound-guided biopsy.

Time to relapse was defined as the time from the date of operation to the date of the first recorded evidence of intrathoracic or distant metastasis as confirmed by imaging or histology. Loss to follow-up was defined as a case where imaging follow-up was not performed after December 2018 (1 year before the end of the study). The time of censoring was determined as the date of the last imaging evaluation. Date of death and cause of death data were obtained via linkage to the Korean Statistical Information Service.

3. Statistical analysis

For analyses of the DFS model and the IDM, proportional hazard models were used to examine the associations of covariates (age, sex, smoking history, family history of lung cancer, history of other malignancies, nodule location, operation method, cancer type, treatment method, interaction of cancer types and treatment methods, TNM stage) with relapse or death. For the analysis of DFS, the primary endpoint was defined as the time of relapse or death. The IDM was fitted to estimate the intensities in transitions from surgery to relapse (0→1), from surgery to death (0→2), and from relapse

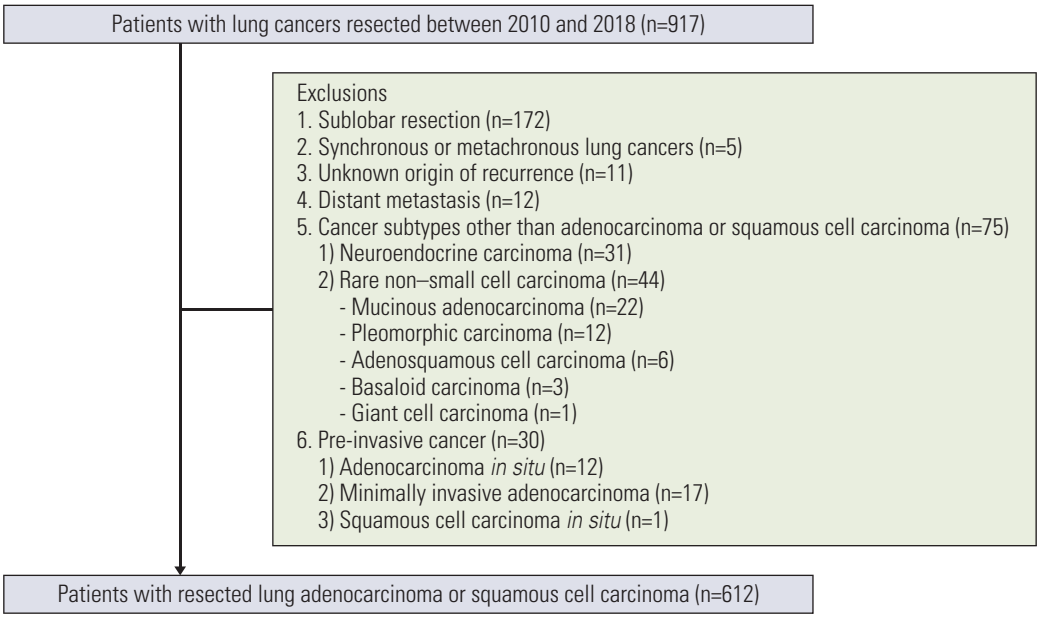
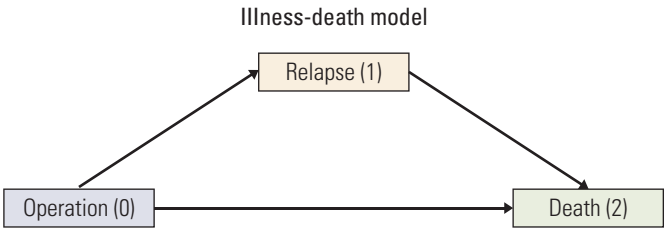
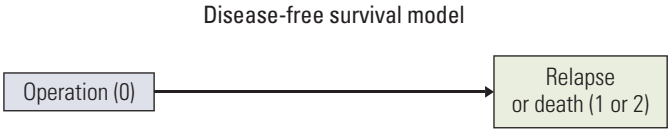


Fig. 1. Flow diagram of patient inclusion and exclusion.



Progression path	No. of patients
Alive without relapse (0→0)	392
Alive after relapse (0→1)	65
Dead without relapse (0→2)	74
Dead after relapse (0→1→2)	81



Progression path	No. of patients
Disease free (0→0)	392
Relapse or death (0→1 or 2)	220

Fig. 2. Comparison of disease-free survival and illness-death models and their progression paths and counts for lung cancer patients.

to death (1→2), as illustrated in Fig. 2. These three transitions could occur at any time until the end of follow-up. The most widely used R package for analyzing DFS models is survival, but this package can only handle right-censored data. Therefore, the icensReg [10] R package was used instead of survival because the event of interest, such as relapse, was interval censored in the NSCLC data. In addition, the most widely used R package to analyze multi-state models is msm, but this package can only analyze right-censored data with intermediate events. Therefore, the SmoothHazard [11] R package was used to deal with interval-censored intermediate events.

In both the DFS and ID models, interval censoring was considered as the assessment of relapse was not continuous; the exact date of relapse could have occurred in the interval between two consecutive clinical examinations. As for baseline transition intensities, a Weibull distribution was assumed in both the DFS and ID models. Multivariable analyses in both the DFS and ID models were conducted to select the most parsimonious model that would reduce overfitting. Risk factors included in initial multivariable analyses were selected based on a p-value < 0.2 in TNM-adjusted univariable analyses because TNM stage is considered a primary risk factor. The Akaike information criterion (AIC) was used to decide on final models (i.e., models with the smallest AIC value), thus indicating the best model through a backward elimination strategy.

The IDM can discern four different predictive probabilities for patients who underwent surgery, while the DFS model includes only two predictions. For the former, the probabilities of being alive after relapse, being dead after relapse, being dead without relapse, and being alive without relapse are predictable at each elapsed time since surgery; for the latter, we calculated the probabilities of being relapsed or dead and being alive without experiencing any event. As an illustration, we examined a patient with the following risk factors: age, 65 years; cancer type, adenocarcinoma; sex, male; treatment method, operation only; history of malignancy other than lung cancer, no; smoking history, previous or current smoker; nodule location, upper lobes, and middle lobes; and operation method, lobectomy. Those values correspond to the sample median or mode of each risk factor. We estimated the predictive probabilities of survivorship and relapse by TNM stage (1A, 2A, or 3A) in order to investigate characteristics within each stage and to compare changes in trends according to TNM stage.

Results

1. Patient characteristics

Among the 612 included patients with resected lung adeno-carcinoma or squamous cell carcinoma, 69.3% (424/612) were men (mean age±standard deviation, 66.3±9.6 years) and 30.7% (188/612) were women (mean age±standard deviation, 65.1±8.9 years). Sixteen percent (99/612) had a history of malignancy other than lung cancer, 4% (26/612) had a family history of lung cancer, and 56% (345/612) were previous or current smokers. Forty percent (246/612) of the lung tumors were located in the lower lobes. With respect to TNM staging, 48% (294/612) of cases were 1A, 12.3% (75/612) were 1B, 5.9% (36/612) were 2A, 15.8% (97/612) were 2B, 14.4% (88/612) were 3A, 3.4% (21/612) were 3B, and 0.2% (1/612) were 3C. A combined TNM stage (3B and 3C) was used in the analysis because there was only one patient with stage 3C cancer. Sixty-two percent (382/612) of the patients had adenocarcinoma, and 38% (230/612) had squamous cell carcinoma. Fifty-three percent (323/612) underwent surgery without additional treatment modalities, and 47% (289/612)

Table 1. Patient demographic and medical characteristics

Variable	Included patients (n=612)
Sex	
Female	188 (30.7)
Male	424 (69.3)
Age (yr)	66.0±9.4
History of malignancy other than lung cancer	
No	513 (83.8)
Yes	99 (16.2)
Family history of lung cancer	
No	586 (95.8)
Yes	26 (4.2)
Smoking history	
Never smoker	267 (43.6)
Former or current smoker	345 (56.4)
Nodule location	
Upper and middle lobes	366 (59.8)
Lower lobes	246 (40.2)
TNM stage[a]	
1A	294 (48.0)
1B	75 (12.3)
2A	36 (5.9)
2B	97 (15.8)
3A	88 (14.4)
3B	21 (3.4)
3C	1 (0.2)
Pathologic diagnosis	
Squamous cell carcinoma	230 (37.6)
Adenocarcinoma	382 (62.4)
Operation method	
Lobectomy	559 (91.3)
Bilobectomy or pneumonectomy	53 (8.7)
Treatment	
Operation only	323 (52.8)
Adjuvant chemo- or radiation therapy	289 (47.2)
Relapse	
No	415 (67.8)
Yes	146 (23.9)
Loss to follow-up	51 (8.3)
Death	
Alive	426 (69.6)
Death (lung cancer)	155 (25.3)
Death (with a cause other than lung cancer)	31 (5.1)[b]

Values are presented as number (%) or mean±standard deviation. [a]The pathologic T categorization was based on the eighth edition staging system for lung cancer, [b]Patients were classified as censored at the time of death.

received chemotherapy or radiation therapy in addition to surgery. With respect to relapse, 51 of the 612 patients (8.3%) were lost to follow-up, 68% (415/612) did not experience relapse, and 24% did experience relapse (146/612). Seventy percent (426/612) were alive, 25% (155/612) of the included patients died from lung cancer, and 5% (31/612) died from causes other than lung cancer during the course of the study. Detailed results are described in Table 1.

2. Comparative risks: DFS and ID models

In univariable analyses of the DFS and ID models adjusted for TNM stage, the p-values of all variables except family history of lung cancer were < 0.20. Those variables were considered for multivariable analyses. S1 Table shows the detailed results of univariable analyses.

The DFS model accounting for age, smoking history, history of other malignancies, operation method, TNM stage, cancer type, treatment method, and the interaction of cancer type and treatment method had the lowest AIC value (AIC, 980.4) (Table 2).

The IDM (AIC, 1,868.3) included age, smoking history, history of other malignancies, nodule location, operation method, TNM stage, cancer type, treatment method, and the interaction of cancer type and treatment method (Table 3). Pneumonectomy against lobectomy had a statistically significantly higher risk within all three transitions (relapse, 0→1: hazard ratio [HR], 1.97; p=0.007; death without relapse, 0→2: HR, 2.58; p=0.009; death following relapse, 1→2: HR, 1.84; p=0.039). Risks for relapse and death without relapse increased substantially with an increase in TNM stage. Smoking history was a statistically significant predictor of death without relapse, with an HR of 4.05 (p < 0.001), though there was no statistically significant association with relapse or death after relapse. History of other malignancies was observed as a statistically significant predictor of relapse, though not of other transitions. There was a statistically significant interaction effect between cancer type and treatment method for relapse (p < 0.001) and death without relapse (p < 0.001), as in the multivariable DFS analysis (p < 0.001). For death following relapse, adenocarcinoma showed substantially less risk compared to squamous cell carcinoma (HR, 0.52; p=0.006). Both the DFS and ID models indicated that family history of lung cancer and sex were not statistically significant predictors of disease outcomes. The IDM revealed a statistically significant relative risk of nodule location on disease outcomes that was not observed in the DFS model.

3. Predictions from the DFS and ID models

Fig. 3 displays predictive probabilities by TNM stage, as estimated from both the ID and DFS models. For the IDM, the

Table 2. Multivariable analysis for the disease-free survival model based on the Akaike information criterion in the patients with lung cancer

Variable	Disease-free survival model	
	HR (95% CI)	p-value
Age (yr)	1.03 (1.01-1.05)	0.001
Former or current smoker	1.68 (1.22-2.32)	0.001
History of malignancy other than lung cancer	1.44 (1.00-2.06)	0.049
Pneumonectomy (reference: lobectomy)	2.09 (1.41-3.11)	< 0.001
TNM stage (reference: 1A)		
1B	1.62 (0.96-2.71)	0.070
2A	3.17 (1.66-6.05)	< 0.001
2B	3.94 (2.36-6.59)	< 0.001
3A	7.38 (4.29-12.70)	< 0.001
3B or 3C	15.41 (8.08-29.4)	< 0.001
Interaction of cancer type and treatment (reference: SqCC and operation only)		
SqCC and adjuvant therapy	0.26 (0.16-0.44)	< 0.001
ADC and operation only	0.39 (0.24-0.65)	< 0.001
ADC and adjuvant therapy	0.72 (0.45-1.15)	0.170

ADC, adenocarcinoma; Adjuvant therapy, adjuvant chemo- or radiation therapy; CI, confidence interval; HR, hazard ratio; SqCC, squamous cell carcinoma.

Table 3. Multivariable analysis for the illness-death model based on the Akaike information criterion in patients with lung cancer

Variable	Relapse		Death without relapse		Death after relapse	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (yr)	1.02 (1.00-1.04)	0.115	1.07 (1.03-1.11)	< 0.001	-	-
Former or current smoker	-	-	4.05 (1.95-8.39)	< 0.001	-	-
History of malignancy other than lung cancer	1.67 (1.07-2.6)	0.023	-	-	-	-
Location at a lower lobe	0.63 (0.44-0.89)	0.009	1.97 (1.17-3.32)	0.011	-	-
Pneumonectomy (reference: lobectomy)	1.97 (1.20-3.23)	0.007	2.58 (1.26-5.28)	0.009	1.84 (1.03-3.28)	0.039
TNM stage (reference: 1A)						
1B	1.19 (0.58-2.42)	0.632	2.42 (1.1-5.32)	0.027	-	-
2A	2.98 (1.32-6.73)	0.009	2.30 (0.71-7.44)	0.164	-	-
2B	3.55 (1.80-7.01)	< 0.001	3.16 (1.38-7.23)	0.007	-	-
3A	5.26 (2.61-10.59)	< 0.001	11.20 (4.61-27.21)	< 0.001	-	-
3B or 3C	12.06 (5.35-27.22)	< 0.001	16.35 (5.47-48.91)	< 0.001	-	-
Interaction of cancer type and treatment (reference: SqCC and operation only)						
SqCC and adjuvant therapy	0.62 (0.29-1.32)	0.214	0.10 (0.05-0.23)	< 0.001	-	-
ADC and operation only	0.44 (0.21-0.92)	0.029	0.44 (0.22-0.89)	0.022	0.52 (0.33-0.83)[a]	0.006
ADC and adjuvant therapy	1.65 (0.83-3.28)	0.157	0.17 (0.07-0.40)	< 0.001		

ADC, adenocarcinoma; Adjuvant therapy, adjuvant chemo- or radiation therapy; CI, confidence interval; HR, hazard ratio, SqCC, squamous cell carcinoma. [a]HR of ADC relative to SqCC, ignoring treatment.

height of the sum of the two bottom regions represents the probability of relapse, and the sum of the two middle regions represents the probability of death. Moreover, the height of the sum of the colored regions in the IDM corresponds to the probability of death or relapse in the DFS model.

As expected, a patient diagnosed with a TNM stage of 1A had the lowest estimated probability of being alive after relapse (5-year probability, 5.1%) as well as of death after

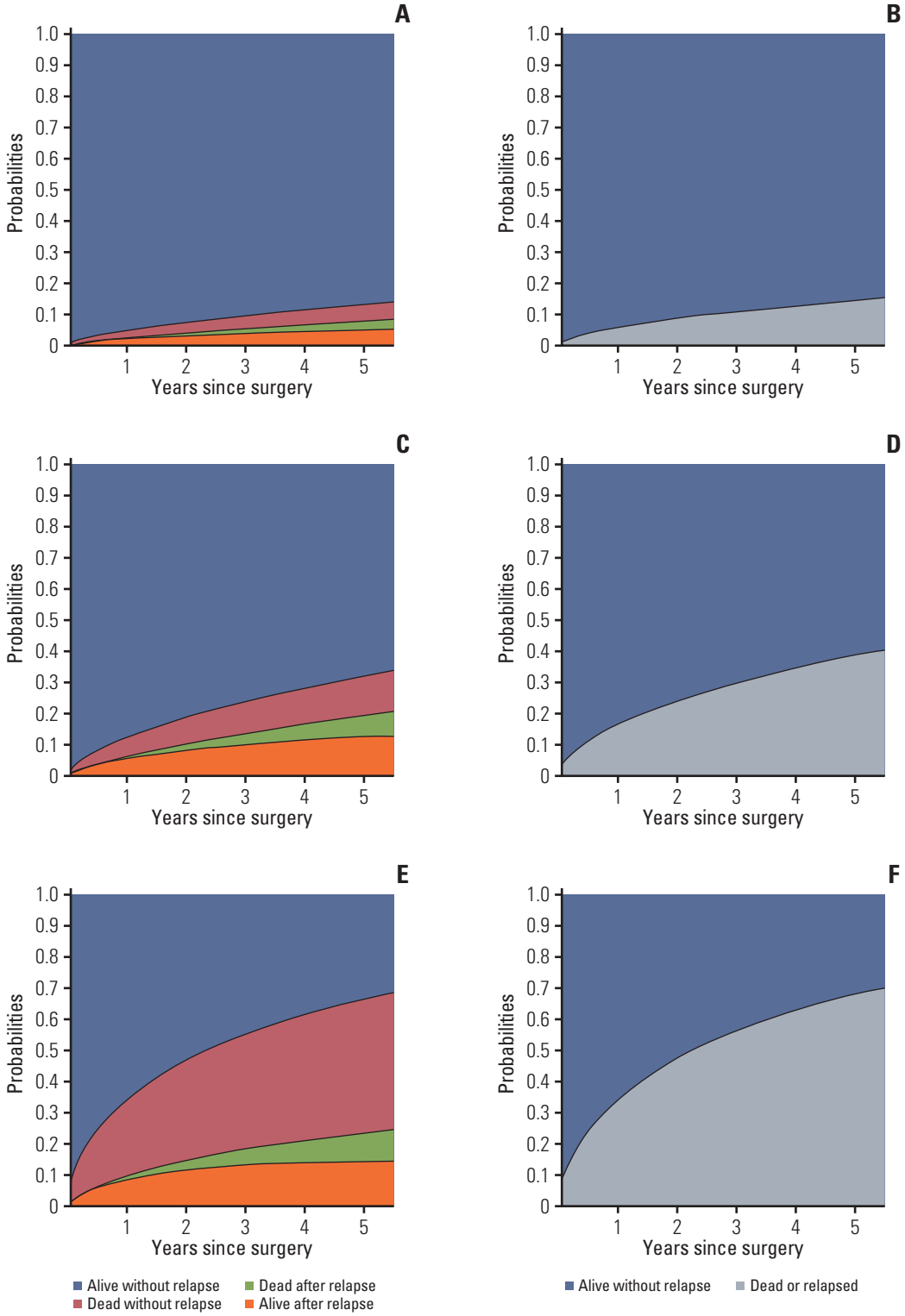


Fig. 3. Predictive probabilities for an illustrative patient[a)] with the sample median or mode for each risk factor presented according to TNM stage (1A [A, B], 2A [C, D], 3A [E, F]), as estimated from the illness-death model (left) and the disease-free survival model (right). [a)]This patient's risk factors were set to age=65, cancer type=adenocarcinoma, sex=male, treatment method=operation only, history of malignancy other than lung cancer=no, smoking history=previous or current smoker, nodule location=upper lobes and middle lobes, and operation method=lobectomy.

relapse (2.7%) and death without relapse (5.5%). Probabilities were highest for patients with a TNM stage of 3A (with 5-year probabilities of 14.4%, 9.1%, and 42.8%, respectively). Moreover, the sum of three 5-year probabilities in the IDM gave similar results for the probability of death or relapse as compared to the DFS model (1A, 13.3 vs. 14.2%; 2A, 32.1 vs. 38.5%; 3A, 66.3 vs. 67.8%).

Discussion

In our study, we demonstrated that it is possible to evaluate detailed predictive probabilities of disease outcomes via the IDM in NSCLC patients who received surgical resection. The main reason for this finding is that the IDM evaluates the occurrences of relapse and death simultaneously as well as in conjunction with interactions between these outcomes. As compared to the DFS model, the IDM can identify statistically significant risk factors for disease outcomes according to the patient's disease course, including relapse, death without relapse, and death after relapse. To our knowledge, this is the first study to apply the IDM for an epidemiological investigation of lung cancer. Our findings facilitate an understanding of multi-state processes in the post-surgery follow-up period for lung cancer patients.

The IDM proposed in our study showed several strengths as compared to the DFS model. First, history of malignancy other than lung cancer (HR, 1.44; p=0.049) and smoking history (HR, 1.68; p=0.001) were statistically significant risk factors related to decreased DFS; the IDM demonstrated that a history of malignancy other than lung cancer was related to relapse (HR, 1.67; p=0.023) and smoking history was related to death without relapse (HR, 4.05; $p < 0.001$). Second, the location of the lung tumor was not a statistically significant risk factor in the DFS model; however, lower lobe location was positively associated with death without relapse (HR, 1.97; p=0.011) and negatively associated with relapse (HR, 0.63; p=0.009) according to the IDM. This finding suggests that the DFS model offsets these effects. Third, we observed a statistically significant interaction between cancer type and treatment methods in both the DFS and the IDM. However, the IDM could additionally differentiate the magnitude of interaction between cancer type and treatment methods in death without relapse from death after relapse.

Various events can occur in the disease course of a patient who has undergone surgery for NSCLC. Usually, researchers perform separate analyses for each event setting as a primary endpoint, considering the other endpoints as censoring. For example, studies of OS aim to estimate the probability of death by considering relapse as censoring. Conversely, evaluating RFS aims to estimate the probability of relapse by considering death as censoring. However, those separate analyses do not provide completely satisfying results due to a failure to reveal associations between relapse and death [12,13]. In contrast, the IDM simultaneously deals with endpoints focusing on transitioning from one state to another. Moreover, the IDM provides predictions of patient clinical prognoses at specific points in their relapse or death process. Several medical articles have estimated event-related predictive probabilities via the IDM in patients with breast cancer [12,14,15], ovarian cancer [16], or colon cancer [13]. However, to our knowledge, the IDM has not been implemented in patients with lung cancer.

As mentioned above, the IDM allowed us to explore the effects of risk factors on relapse leading to death. Regarding the NSCLC patients who experienced relapse in our study, we found that pneumonectomy and squamous cell carcinoma cancer subtype were unfavorably associated with death after relapse. Sekihara et al. [17] reported that female gender, adenocarcinoma histology, and absence of distant metastasis were favorably associated with post-recurrence survival (PRS), and Shimada et al. [18] revealed that adenocarcinoma showed a favorable PRS; however, an unfavorable PRS was observed among patients with pneumonectomy as well as adjuvant therapy. Our study identified risk factors from the IDM that were similar to previous results obtained with the PRS model, though these two models have different starting points. Specifically, the PRS model examines survival probability only among patients who experienced relapse, and the survival rate is calculated from the time of relapse [19]. In contrast, the IDM evaluates the probability of death after relapse starting from the time of lung cancer surgery. We believe that the IDM shows a more comprehensive view of risk factors for mortality as well as other patient outcomes experienced during lung cancer follow-up.

As shown in Fig. 3, the predictive probability of relapse or death obtained from the DFS model can be decomposed into three parts in the IDM (i.e., the predictive probabilities of being alive after relapse and of having died with or without relapse). This decomposition enables us to investigate the evolving process of events such as relapse and death [20-22]. For example, for a patient diagnosed with lung cancer with a TNM stage of 3A, there is a 67.8% probability of cancer relapse or death within five years of a lung cancer operation based on the results of the DFS model. We report the following separate five-year probabilities for survival after relapse, death after relapse, and death without relapse via the IDM: 14.4%, 9.1%, and 42.8%, respectively. Moreover, the sum of these probabilities corresponds to the predictive probability of relapse (23.5%) and the two mortality probabilities (51.9%) within 5 years following the operation.

Our study has several limitations. First, this was a retro-

spective single-center study and our results were evaluated within a small number of included patients. Our primary focus was to introduce the IDM and to compare it with the typical DFS model among patients with surgically resected NSCLC. As a further step, implementing the IDM within a larger multi-institutional cohort will help us to better understand the association between relapse and death among NSCLC patients. Second, our analysis was limited to patients with resected adenocarcinoma and squamous cell carcinoma, which are two of the most common histologic NSCLC types. Further studies including other cancer subtypes and advanced-stage lung cancers are warranted to gain a comprehensive understanding of this issue. Third, loss to follow-up was defined as a case where imaging follow-up was not performed as of a year before the end of the study, and patients who died of causes other than lung cancer were regarded as being censored at the time of death. Fourth, because the follow-up interval is longer in patients undergoing surgery at an earlier stage, there is a possibility that the date of relapse may not be accurate for early-stage patients, which had a total follow-up period of 8 years. Fifth, although treatment methods change before and after relapse in lung cancer patients, we assumed models in which the effects of treatment methods do not change during follow-up. However, if there are risk factors that may show altered effects before and after relapse, such as treatment methods, a model that considers the effects of these risk factors as time-varying would be more appropriate.

In conclusion, our results demonstrate the IDM can be used as a complementary tool to simultaneously evaluate the predictive probabilities of relapse and death during the post-operation follow-up of NSCLC patients. Furthermore, the IDM may help in establishing follow-up plans or treatment strategies according to individual risk prediction. We believe that the IDM provides a more comprehensive picture of risk factors as compared to the DFS model and may facilitate an improved understanding of the multi-state disease processes occurring among lung cancer patients.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This retrospective preliminary study was approved (approval number: CUH-2020-11-004) by the institutional review board of the Chonbuk National University Hospital (Jeonju, Korea). The requirement for informed consent was waived due to the retrospective nature of this study. This research was conducted in accordance with the principles of the Declaration of Helsinki and its later amendments.

Author Contributions

Conceived and designed the analysis: Chae KJ, Choi H, Jeong WG, Kim J.
Collected the data: Chae KJ.
Contributed data or analysis tools: Choi H, Kim J.
Performed the analysis: Choi H, Kim J.
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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2020R1F1A1A01048397).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.986
Cancer Res Treat. 2022;54(4):1005-1016

Original Article

A Phase I/IIa Randomized Trial Evaluating the Safety and Efficacy of SNK01 Plus Pembrolizumab in Patients with Stage IV Non–Small Cell Lung Cancer

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Purpose The aim of this study is to evaluate the safety and efficacy of *ex vivo* activated and expanded natural killer (NK) cell therapy (SNK01) plus pembrolizumab in a randomized phase I/IIa clinical trial.

Materials and Methods Overall, 18 patients with advanced non–small cell lung cancer (NSCLC) and a programmed death ligand 1 tumor proportion score of 1% or greater who had a history of failed frontline platinum-based therapy were randomized (2:1) to receive pembrolizumab every 3 weeks +/− 6 weekly infusions of SNK01 at either 2×10^9 or 4×10^9 cells per infusion (pembrolizumab monotherapy vs. SNK01 combination). The primary endpoint was safety, whereas the secondary endpoints were the objective response rate (ORR), progression-free survival (PFS), overall survival, and quality of life.

Results Since no dose-limiting toxicity was observed, the maximum tolerated dose was determined as SNK01 4×10^9 cells/dose. The safety data did not show any new safety signals when SNK01 was combined with pembrolizumab. The ORR and the 1-year survival rate in the NK combination group were higher than those in patients who underwent pembrolizumab monotherapy (ORR, 41.7% vs. 0%; 1-year survival rate, 66.7% vs. 50.0%). Furthermore, the median PFS was higher in the SNK01 combination group (6.2 months vs. 1.6 months, p=0.001).

Conclusion Based on the findings of this study, the NK cell combination therapy may consider as a safe treatment method for stage IV NSCLC patients who had a history of failed platinum-based therapy without an increase in adverse events.

Key words Non-small cell lung carcinoma, NK cell, Pembrolizumab, Combination therapy

Introduction

The incidence of non–small cell lung cancer (NSCLC), consisting approximately 80% of lung cancers, has drastically increased, and NSCLC remains to be one of the leading causes of cancer-related death worldwide [1]. Although platinum-based chemotherapies, such as cisplatin with gemcitabine (GP therapy) or pemetrexed (PP therapy), have been used as the first-line treatment for NSCLC patients, the clinical benefits from these therapies are restricted to only a small portion of patients accompanied with a plateau [1-3]. Recently, the new development of immune checkpoint inhibitors (ICIs) has moved into the breakthrough advances in NSCLC treatment [4]. Pembrolizumab has replaced chemotherapy as the first-line treatment for patients with a programmed death-ligand 1 (PD-L1) tumor proportion score (TPS) of at least 50% [5,6]. However, the low response rates of ICIs for NSCLC patients is still a problem encountered in current immunotherapies.

Many studies aim to improve the efficacy of ICIs. These studies were focused on searching predictive biomarkers to tumor response and novel combination approaches for ICIs. Among the most widely known predictive biomarkers for ICIs are PD-L1, microsatellite instability/defective mismatch repair (MSI/dMMR), and tumor mutational burden [7,8]. Although MSI/dMMR is approved for clinical use in all types of solid tumors and PD-L1 is approved only for clinical use in specific cancer types (e.g., for predicting the response

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Received September 3, 2021 **Accepted** November 29, 2021
Published Online December 3, 2021

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to first-line pembrolizumab monotherapy in NSCLC) [9,10], many researchers suggest that any single biomarker cannot effectively identify the benefit populations. Thus, they think that the specificity and efficacy of prediction will be greatly improved through the combination of multiple factors. Aside from the predictive biomarkers, many studies have been using combinatorial approaches to improve the efficacy of ICIs. Indeed, chemotherapy, radiation therapy, molecularly targeted therapy, and cell therapy are considered as combination regimens [11,12]. However, an effective way to incorporate the molecularly targeted and immune targeted therapies into combination regimens is yet to be determined. Moreover, many problems, including side effects and efficacy, must be taken into consideration when designing the ICI-based combination regimens because the other types of therapy may have significant influence on host immunity or tumor microenvironment.

Natural killer (NK) cells, which are innate lymphocytes, account for 5%-15% of human peripheral blood leukocytes and are considered as a major type of immune cells that can kill foreign target cells [13,14]. NK cells are also an essential population for tumor immunosurveillance by orchestrating the innate immunity in the heterogeneous microenvironment [15,16]. NK cells participate in the immune response against solid and hematopoietic cancer cells by their capacity to recognize the molecular patterns characteristic of stressed cells. Indeed, higher cancer susceptibility and tumor progression to metastasis are significantly associated in NSCLC patients with a higher NK cell count [13,17]. Moreover, unlike T cells, the NK cells can recognize and attack cancer cells without neo-antigen in high mutation loaded patients and loss of MHC expression which often occurs in human cancer [16,18,19]. The NK cells are activated by ligands that are often upregulated in the condition with oncogenic stress [16]. Therefore, the development of NK cell-mediated immunotherapies would be an ideal strategy to increase the efficacy of current T cell–mediated immunotherapy and increase the response rate of current T cell–mediated immunotherapy.

In this study, we generated the non-genetically modified and autologous super NK cells (SNK01) by using the NK cell activation condition. We also investigated the safety and tolerability as well as the preliminary antitumor activity of SNK01 when administered in combination with pembrolizumab in patients with NSCLC.

Materials and Methods

1. Study design

The aim of this randomized, open-label, single-center study is to evaluate the safety, tolerability, and anti tumor activity of SNK01 in combination with pembrolizumab in patients with advanced or metastatic NSCLC (PD-L1 TPS ≥ 1%) who had a history of failed frontline platinum-based therapy. The primary endpoint is safety, and the secondary endpoint is efficacy, represented by objective response rate (ORR), progression-free survival (PFS), overall survival, time to progression, and quality of life (QoL).

2. Patients

Eligible patients were recruited at Asan Medical Center (Seoul, South Korea) between February 2019 and March 2020. In the phase I study, patients with advanced and/or metastatic NSCLC were sequentially enrolled in cohorts of 3-6 subjects. The eligible subjects received study drugs that begin on cycle 1 day 1 and continued in 3-week cycles until the occurrence of the unequivocal radiographic disease progression using Response Evaluation Criteria in Solid Tumor (RECIST) ver. 1.1 as assessed by the investigator, unacceptable toxicity, or other reasons for discontinuation.

Eighteen patients with advanced NSCLC with a PD-L1+ TPS of 1% or greater who had a history of failed frontline platinum-based therapy were randomized (2:1) to pembrolizumab every 3 weeks +/– 6 weekly infusions of SNK01 at either 2×10^9 or 4×10^9 cells per infusion (pembrolizumab monotherapy [cohort 0] vs. SNK combination [cohort 1, 2, respectively]).

3. NK cell isolation and expansion

All the manufacturing and testing procedures used to produce *ex vivo* expanded NK cells (SNK01) were performed under good manufacturing practice conditions (NKMAX Co., Ltd., Seongnam, Korea). Peripheral blood mononuclear cells (PBMCs) were collected from the leukapheresis products of enrolled patients in the treatment group and then used for NK cell expansions as described previously with some modification [20]. The detailed method for NK cell expansion is described in the Supplementary Methods.

4. Characterization of the NK cells

The phenotype of culture-expanded NK cells was determined via flow cytometric analysis. For assessing the NK cell activity, cytotoxicity and degranulation assays were performed. The detailed method of these assays is described in the Supplementary Methods.

5. Treatments

Dose escalation was evaluated in a phase I study of SNK01, which was administered in combination with pembrolizumab. The purpose of the dose escalation phase was to gather preliminary safety and tolerability data for SNK01 in combination with pembrolizumab, as well as SNK01 in com-

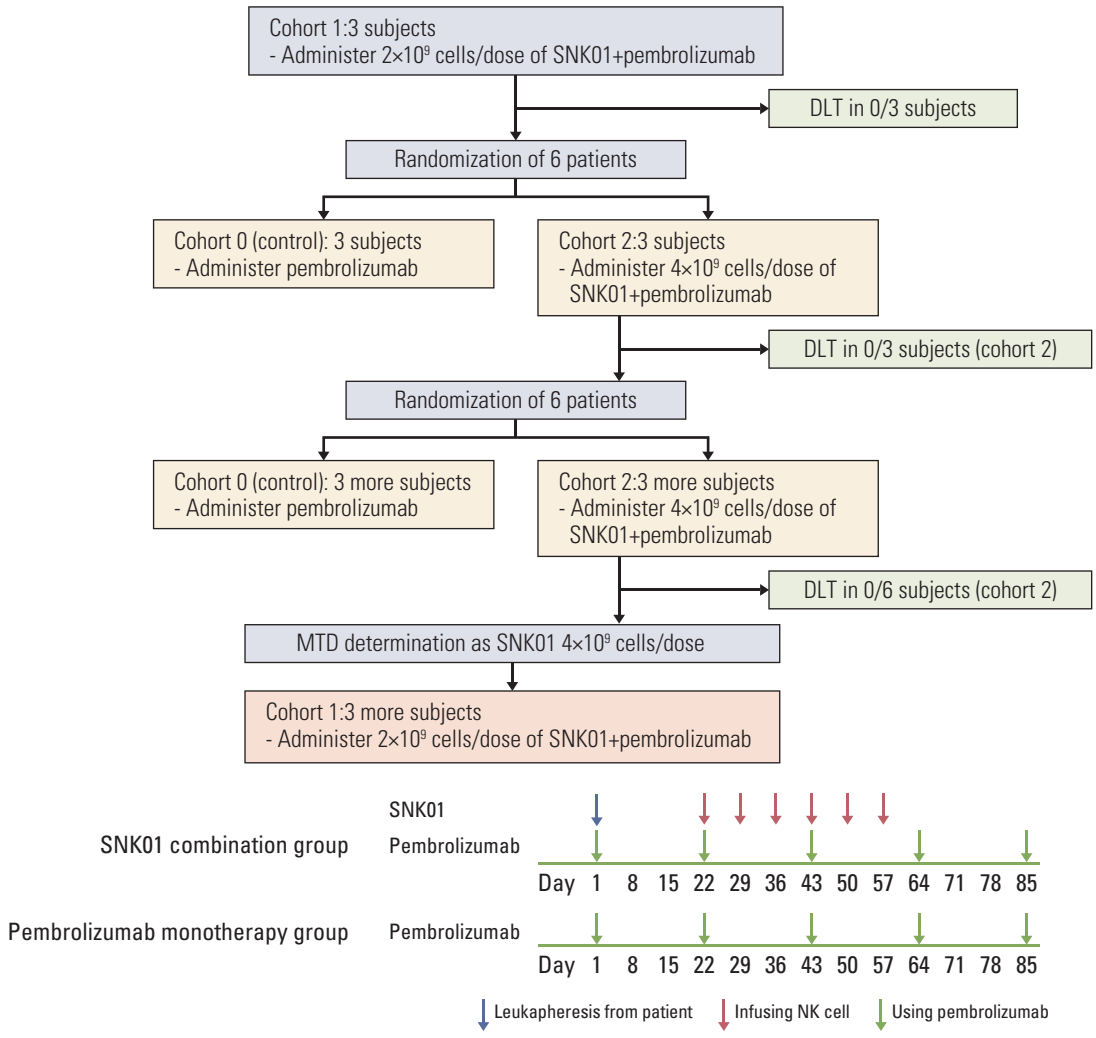

Fig. 1. Clinical trial profile. In total, 20 patients were enrolled to the trial. Except for the first three and the last three patients (cohort 1), the remaining patients were randomly assigned to cohort 0 or 2. The pembrolizumab monotherapy group (cohort 0) received regular therapy with intravenous injection of pembrolizumab (200 mg) on the indicated time. The pembrolizumab plus SNK01 group (cohort 1 or 2) received pembrolizumab plus a total of 6 SNK01 infusions in 42 days, i.e., weekly infusion for 6 weeks. DLT, dose-limiting toxicity; MTD, maximum tolerated dose; NK, natural killer.

bination with pembrolizumab, to determine the maximum tolerated dose (MTD)/recommended phase 2 dose (RP2D) for each combination regimen for the phase IIa portion of the study.

The dose escalation followed the standard oncology phase I "3+3" dose escalation design in cohort 1 and cohort 2 (Fig. 1). After cohort 1, The patients were randomly allocated at 1:1 ratio to receive pembrolizumab only (200 mg every 3 weeks) or pembrolizumab in combination with either 2×10^9or 4×10^9 cells/dose of SNK01 (weekly infusion for 6 weeks). cohort 0 served as the control group for cohort 1 and cohort 2. Three eligible subjects were initially enrolled into cohort 1. The subjects were administered with 2.0×10^9 SNK01 in combination with pembrolizumab. If no dose-limiting toxicities (DLTs) were observed during the DLT observation period, three eligible subjects were enrolled into cohort 0 and cohort 2 and received pembrolizumab alone or 4.0×10^9 SNK01 in combination with pembrolizumab, respectively. Again, if no DLTs occur during the DLT observation period, three more eligible subjects were enrolled into cohort 2 to confirm the MTD/RP2D for SNK01 in combination with pembrolizumab. Following the completion of the DLT observation period in cohorts 1 and 3, additional subjects were allowed.

The expansion of up to three subjects was allowed if 0 of three subjects has a DLT to further examine the preliminary efficacy, while assessing the RP2D, which is consistent with

backfilling a cohort. If one subject develops a DLT at a specific dose during the DLT observation period, additional three subjects are enrolled into that same dose cohort. The development of DLTs in more than one of six subjects in a specific dose cohort suggests that the MTD has been exceeded and further dose escalation was not pursued.

A DLT was defined as a Common Terminology Criteria for Adverse Events (CTCAE) grade of ≥ 3 for any adverse event related or at least possibly related to the administration of SNK01 occurring within the DLT observation period. The subjects were eligible for DLT evaluation if they experience a DLT after at least one dose of study drug or do not experience a DLT having taken a minimum of 75% of the doses expected during the DLT observation period. The subjects who did not fulfill these requirements and who discontinued their study participation prior to completing the DLT observation period were replaced for DLT evaluation but remained in the overall safety and efficacy analyses.

6. Follow-up and adverse events

On-study imaging for tumor assessments was performed with the use of RECIST ver. 1.1, every 6 weeks (±7 days) after the first dose of the study treatment and should follow calendar days and not be adjusted for delays in cycle starts. The same imaging technique should be used in a subject throughout the study. Safety was monitored via laboratory assessments, physical examinations, and vital signs. It was graded by physicians in accordance with the U.S. National Cancer Institute's (NCI) CTCAE ver. 5.0.

Subjects who discontinued the study treatment for a reason other than disease progression moved into the long-term follow-up phase and should be assessed every 6 weeks (±7 days) via radiologic imaging to monitor the disease status. Every effort should be made to collect information with regard to the disease status until the start of a new therapy, during a disease progression, at death, or until the end of the study. If a subject prematurely withdraws from the study, all evaluations described under the End of Study Visit were performed. Additionally, once a subject has presented with a confirmed disease progression or starts a new anticancer therapy, the subject moved into the survival follow-up phase and should be contacted through telephone or clinical visit every 12 weeks to assess for survival status until death, withdrawal of consent, or the end of the study, whichever occurs first.

7. Patient-reported outcomes

The patient-reported outcomes (PROs) were collected to evaluate the disease-related symptoms and health-related quality of life (HRQoL) to support the finding of a survival benefit. Moreover, the PROs were collected upon screening: 3rd visit (day of the 2nd pembrolizumab administration), 6th visit (day of the 3rd pembrolizumab administration), 9th to 13th visit (day of the 4th, 6th, 8th, 12th, and 16th pembrolizumab administration), and 14th visit (end-of-treatment visit) for patients who completed one baseline and one post-baseline PRO assessment. The European Organization for the Research and Treatment of Cancer (EORTC) QoL questionnaire and lung cancer module was used to assess the PROs. The PROs reflecting the lung cancer symptoms, commonly reported treatment-related symptoms, functioning in daily life, and HRQoL were collected using two self-administered questionnaires that have been routinely used in lung cancer studies: the EORTC quality-of-life questionnaire (QLQ-C30) and its lung cancer module (QLQ-LC13).

8. Statistical analysis

Descriptive statistics were used for the baseline characteristics of the patients. Pearson's chi-square test and Fisher exact test were used for data comparison, and the Mann-Whitney U test for the comparison of the nonparametric variables. The survival was estimated using the Kaplan-Meier method, and the log-rank test was used to determine the significance of any differences in the survival curves. All tests were two-sided, and a p-value of < 0.05 was considered statistically significant. The SPSS ver. 25.0 (IBM Corp., Armonk, NY) and SAS ver. 9.4 (SAS Institute Inc., Cary, NC) were used for the analyses.

Results

1. Characteristics of the NK cell products

To manufacture the *ex vivo* expanded NK cell products, the $CD56^+$ cells were isolated from the patients' PBMCs and expanded as described previously [20]. In freshly isolated $CD56^+$ cells from the leukapheresis products of enrolled patients in the treatment group, the proportion of NK cells ($CD56^+CD3^-$) varied among donors (66.47%±18.67%). However, as stated previously [20], after 17-18 days of culture with γ-irradiated KL-1 and LCL feeders in the presence of interleukin (IL)-2 and IL-21, the proportion of NK cells ($CD56^+CD3^-$) was significantly increased (99.10%±0.87%) in the NK cell products from all donors with a minimal contamination of the $CD3^+$ T cells (0.76%±0.83%), $CD20^+$ B cells (0.17%±0.14%), and $CD14^+$ monocytes (0.11%±0.13%) (Fig. 2A, S1 Table). In the expansion culture, the NK cells were efficiently expanded (2,858±1,774-fold; median, 1,964; range, 1,171 to 5,867) with high viability (97%±0.94%) (S1 Table), which were sufficient for multiple injections in all donors. As the cytotoxicity of the NK cells is finely regulated by the net balance of signals from activating and

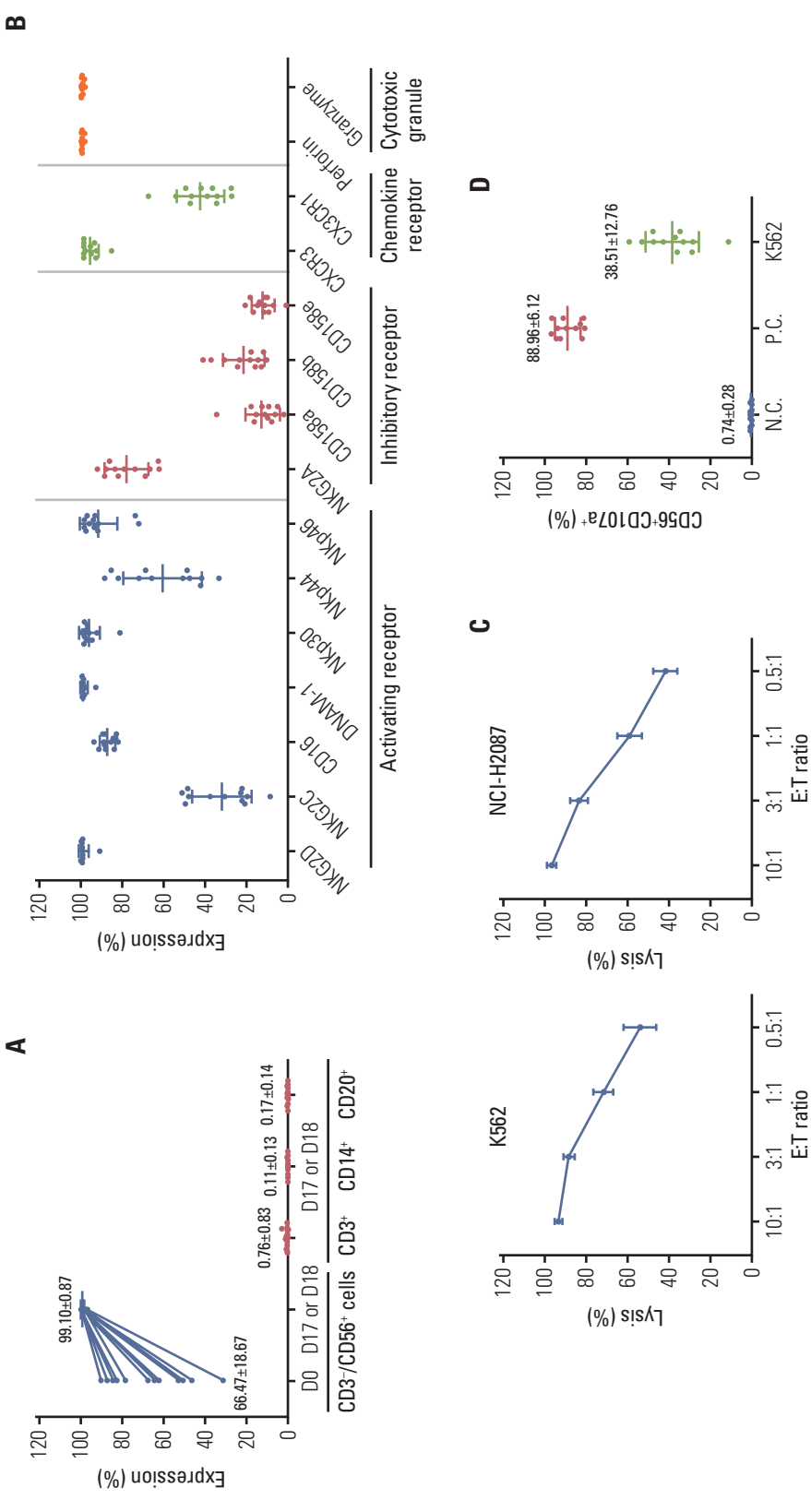

Fig. 2. Characteristics of expanded NK cells. (A) The percentages of $CD3^-CD56^+$ NK cells, $CD3^+$ T cells, $CD20^+$ B cells, and $CD14^+$ monocytes were analyzed flow cytometrically on freshly isolated positive cells using CliniMACS CD56 microbeads (D0; before expansion) and expanded NK cells for 17-18 days of culture (D17-18). (B) The fold expansion of the total cell population after 17-18 days of culture (D17-18). The expression levels of activating receptors, inhibitory receptors, chemokine receptors, perforin, and granzyme B were analyzed flow cytometrically among $CD56^+$ gated cells after 17-18 days of culture. (C) The cytotoxic activity of expanded NK cells against the K562 and NCI-H2087 cell lines was measured via calcein-release assay at E:T ratios of 10:1 to 0.5:1 in triplicate. (D) The NK cell degranulation activity was measured flow cytometrically with % of $CD56^+CD107a^+$ in coincubation with K562 cells (E:T ratio=1:1), with phorbol 12-myristate 13-acetate/ionomycin treatment (as positive control, P.C.), or without treatment (negative control, N.C.). Dots represent the mean value of each patient from 5 to 6 cultures for clinical trial. Horizontal bars indicate the mean value, and each point represents mean±standard deviation. NK, natural killer.

Table 1. Baseline clinical characteristics of study patients (n=20)

Characteristic	Pembrolizumab monotherapy (n=6)	SNK combination (n=14)	p-value
Age (yr)			
Median (range)	56.5 (49-70)	62 (49-73)	
≥ 65	1 (16.7)	6 (42.9)	0.35
Sex			
Male	2 (33.3)	11 (78.6)	0.12
Female	4 (66.7)	3 (21.4)	
Smoking status			
Current smoker	0	0	0.12
Ex-smoker	4 (66.7)	11 (78.6)	
Never smoker	2 (33.3)	3 (21.4)	
ECOG performance status			
0	0	0	
1	6 (100)	14 (100)	
Histology			
Adenocarcinoma	6 (100)	13 (92.9)	0.99
Squamous cell carcinoma	0	0	
Pleomorphic carcinoma	0	1 (7.1)	
PD-L1 22c3 TPS			
Median (range, %)	1 (1-15)	25 (1-100)	
≥ 50%	0	6 (42.9)	0.12
EGFR status			
Wild type	1 (16.7)	11 (78.6)	0.02
Mutant	5 (83.3)	3 (21.4)	
ALK translocation			
No	6 (100)	14 (100)	
Yes	0	0	
Previous lines of chemotherapy			
1	0	10 (71.4)	0.01
2	2 (33.3)	2 (14.3)	
≥ 3	4 (66.7)	2 (14.3)	
NK cell activity (pg/mL)			
Median (range)	972.4 (102.8-1,639.0)	1570.5 (145.0-3,563.2)	

Values are presented as number (%) unless otherwise indicated. ALK, anaplastic lymphoma kinase; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; NK, natural killer; PD-L1, programmed death-ligand 1; TPS, tumor proportion score.

inhibitory receptors on their surface, the expression levels of activating and inhibitory receptors were analyzed. The culture-expanded NK cells from all donors highly expressed activating receptors, including NKG2D (98.87%±2.43%), CD16 (87.37%±3.56%), DNAM-1 (98.63%±1.84%), NKp30 (96.05%±5.05%), NKp46 (91.59%±9.01%), inhibitory receptor NKG2A (78.01%±10.60%), and chemokine receptor CXCR3 (95.65%±4.12%), whereas the expression level of inhibitory receptors, CD158a (KIR2DL1; 12.53%±8.36%), CD158b (KIR2DL2/L3; 21.63%±10.08%), and CD158e (KIR2DL1; 12.23%±5.35%), was relatively low. Moreover, the culture-expanded NK cells highly expressed cytotoxic granules of perforin (99.44%±0.62%) and granzyme B (99.47%±0.61%) in all NK cells (Fig. 2B, S2 Table). The cytotoxic activity of culture-expanded NK cells was examined 1 day before injection day (16-17 days of culture) against the standard K562 cells which are a NK-sensitive target and the NCI-H2087 lung adenocarcinoma cells. As expected from high expression levels of several activating receptors and cytotoxic granules, the expanded NK cells from all patients exerted a strong cytotoxic activity against both K562 and NCI-H2087 cells even at a low E:T ratio of 0.5:1 (54.2%±7.9% and 42.2%±5.9% of the K562 and NCI-H2087 targets, respectively) (Fig. 2C, S3 Table). In untreated NK cells (0.74%±0.28%), NK cell degran-

Table 2. AEs reported in study patients (n=20)

Type of adverse event	Pembrolizumab monotherapy (n=8)		SNK combination (n=12)	
	Any grade	Grade 3-5	Any grade	Grade 3-5
All AEs	8 (100)	2 (25.0)	12 (100)	1 (8.3)
Treatment-related AEs				
Pembrolizumab-related AEs	6 (75.0)	1 (12.5)	11 (91.7)	0
SNK01-related AEs	0	0	0	0
Common AEs occurring in ≥ 2 patients				
Anorexia	3 (37.5)	0	4 (33.3)	0
Myalgia	2 (25.0)	1 (12.5)	1 (8.3)	0
Arthralgia	1 (12.5)	1 (12.5)	2 (16.7)	0
Pneumonia	0	0	3 (25.0)	0
Back pain	1 (12.5)	0	2 (16.7)	0
Hyperkalemia	0	0	3 (25.0)	0
Fatigue	2 (25.0)	0	0	0
Insomnia	0	0	2 (16.7)	0
Urticaria	0	0	2 (16.7)	0
Headache	0	0	2 (16.7)	0
Immune-related AEs	0	0	5 (41.7)	0
Hyperthyroidism	0	0	3 (25.0)	0
Hypothyroidism	0	0	3 (25.0)	0
Pneumonitis	0	0	1 (8.3)	0

Values are presented as number (%). AE, adverse event.

ulation activity was upregulated when cocultured with K562 cells (38.51%±12.76%) or treated with phorbol 12-myristate 13-acetate/ionomycin (88.96%±6.12%) (Fig. 2D, S4 Table). Collectively, we could produce a large number of clinical-grade NK cells (SNK01) with minimal contamination of other immune cells and high cytotoxic activity against cancer cells for multiple injections via *ex vivo* expansion using two feeder cells and cytokines.

2. Patients and treatments

Between February 2019 and March 2020, a total of 20 patients (13 male and 7 female) were enrolled. Table 1 summarizes the baseline characteristics and NK cell activities. The median age was 61 years (range, 31 to 77 years), and the most common histologic type of tumor was adenocarcinoma, except for one pleomorphic carcinoma. The baseline characteristics including the PD-L1 expression status were balanced between the two groups.

As shown in Fig. 1, a total of 18 patients were scheduled to be enrolled and to be randomized for pembrolizumab monotherapy group (6 patients) or pembrolizumab plus SNK01 (SNK combination) group (12 patients). However, two patients in the SNK combination group (one patient in cohort 1 and the other in cohort 2) received a single dose of pembrolizumab and then were dropped out due to serious adverse event (SAE) before initiating SNK01 administration. Therefore, two additional patients were enrolled to the SNK combination group. Thus, the final number of enrolled patients in the study was 20: six were assigned to receive pembrolizumab monotherapy, and 14 to receive SNK combination. Every six patients completed therapy with pembrolizumab alone, pembrolizumab plus 2×10^9 SNK01, or pembrolizumab plus 4×10^9 SNK01.

3. MTD determination

Nine patients were involved in the dose escalation part of the study and received pembrolizumab plus SNK01. SNK01 was administered intravenously for 6 consecutive weeks (2×10^9 cells/dose, n=3; 4×10^9 cells/dose, n=6), except for three patients who were administered with five doses of SNK01 due to a progressive disease. Because no DLT was observed, MTD was determined as SNK01 4×10^9 cells/dose.

4. Safety

Twenty patients were included for safety analysis. The treatment was well tolerated throughout the trial. Moreover, no adverse events related to SNK01, as well as any new safety signals in the SNK combination group, were observed.

Table 2 summarizes the adverse events. Immune-related hyperthyroidism (n=3), hypothyroidism (n=3), and pneumonitis occurred in the SNK combination group. No grade 3-5 immune-related adverse events (AEs) were observed.

Table 3. Comparison of efficacy outcomes between two treatment groups (n=18)

	Pembrolizumab monotherapy (n=6)	SNK combination (n=12[a])	p-value[b]	Cohort 1 SNK01 2×10^9 (n=6)	Cohort 2 SNK01 4×10^9 (n=6)
ORR	0/6 (0)	5/12 (41.7)	0.11	2/6 (33.3)	3/6 (50.0)
DCR	1/6 (16.7)	8/12 (66.7)	0.13	4/6 (66.7)	4/6 (66.7)
PR	0/6	5/12		2/6	3/6
SD	1/6	3/12		2/6	1/6
PD	5/6	4/12		2/6	2/6
Median PFS (95% CI, mo)	1.6 (0.6-4.7)	6.2 (1.4)	0.001	4.8	9.4
1-Year survival rate (%)	50.0	66.7	0.39	50.0	83.3

Values are presented as number (%) unless otherwise indicated. CI, confidence interval; DCR, disease control rate; ORR, objective response rate; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease. [a]The efficacy outcomes were not evaluated in 2 patients who were scheduled to receive natural killer (NK) combination but received pembrolizumab monotherapy due to adverse event, [b]p-value for pembrolizumab monotherapy-administered patients vs. NK combination patients.

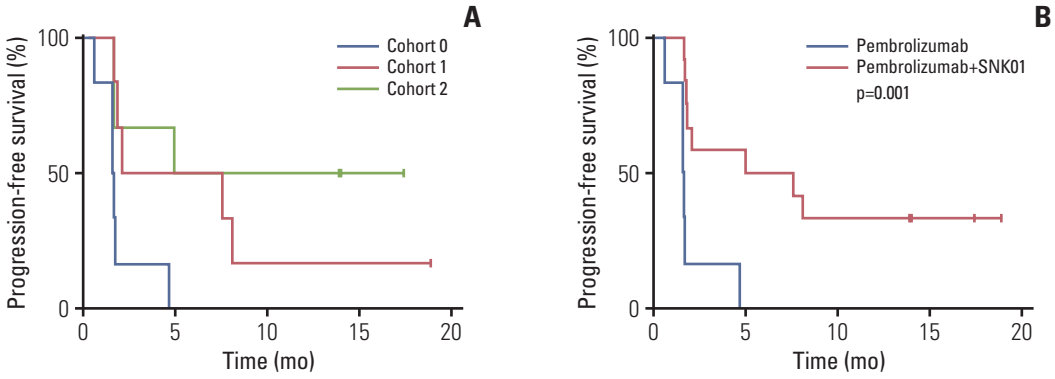

Fig. 3. Progression-free survival analysis. Kaplan-Meier analysis was used to estimate the progression-free survival of each cohort (A) or group patients who received natural killer cell infusion (B).

The median time to the occurrence of immune-related AEs was 2.7 months after the first pembrolizumab administration (range, 0.7 to 7.4). One immune-related AE occurred before the SNK01 administration due to pembrolizumab, and the other six occurred median 2.2 months after the first SNK01 administration (range, 0.2 to 6.7). The patients receiving pembrolizumab plus SNK01 tended to experience more immune-related AEs than those receiving pembrolizumab alone (35.7% vs. 0%, p=0.26), but it was not statistically significant.

The safety data analyzed by putting the two patients who discontinued the scheduled treatment before the initiation of SNK01 due to SAEs in the pembrolizumab monotherapy group (n=8 for pembrolizumab monotherapy, and n=12 for SNK combination) were similar to that with intention to treat (ITT) population (S5 Table).

5. Efficacy

Eighteen patients were included for the analysis of efficacy outcomes per protocol, excluding two patients who discontinued the scheduled treatment before the initiation of SNK01 due to SAEs. Table 3 shows the ORR, median PFS, and 1-year survival rate evaluated in the pembrolizumab monotherapy and SNK combination groups. The ORR for the total population was 27.8% (5/18), whereas the ORR for the SNK combination group (41.7%) was superior to that for the pembrolizumab monotherapy group (0%), but the differences were not statistically significant (p=0.11).

At the time of the data cutoff, 14 of 18 patients presented with disease progression, including eight of 12 patients (66.7%) who underwent SNK combination treatment and six of six patients (100.0%) who underwent pembrolizumab monotherapy. Fig. 3A shows the PFS curve in each cohort. The median PFS was significantly longer in patients who underwent SNK combination treatment than in those who underwent pembrolizumab monotherapy (6.2 months vs.

1.6 months, p=0.001, with a median follow-up duration of 17.5 months) (Table 3, Fig. 3B). Seven patients died, including four of 12 patients (33.3%) who underwent SNK combination treatment and three of six patients (50.0%) who underwent pembrolizumab monotherapy. One-year survival rate tended to be higher for the SNK combination treatment than the pembrolizumab monotherapy (66.7% vs. 50.0%, p=0.39). The result of analyses with ITT population (n=6 for pembrolizumab monotherapy, and n=14 for SNK combination) were similar to that with per protocol population (data not shown).

The efficacy outcomes tended to be higher for patients administered with 4×10^9 cells/dose of SNK01 than those administered with 2×10^9 cells/dose of SNK01, but the differences were not statistically significant (ORR: 33.3% vs. 50.0%, p=0.19; median PFS: 9.4 vs. 4.8 months, p=0.45; 1-year survival rate: 50.5% vs. 83.3%, p=0.19).

The ORR, PFS, and 1-year survival rate were significantly higher in the patients with the immune-related AE compared to those without AE (ORR: 80.0% vs. 7.7%, p=0.008; median PFS: not reached vs. 1.7 months, p=0.005; 1-year survival rate: 100% vs. 44.9%, $p < 0.001$).

6. Patient-reported outcomes

The baseline PRO scores during the screening period were similar between the pembrolizumab monotherapy group and SNK01 combination group for all PRO scales. The patients in both groups (pembrolizumab monotherapy vs. SNK01 combination) reported a moderate-to-high HRQoL at baseline. No statistically significant difference was observed in HRQoL between patients receiving pembrolizumab alone and those receiving pembrolizumab plus SNK01 (p=0.15). The SNK combination group showed a longer time to deterioration in physical function and role function than the pembrolizumab monotherapy group did (physical function: hazard ratio [HR], 0.29, p=0.058; role function: HR, 0.18, p=0.036).

The analyses of the mean change from baseline throughout each visit showed a modest trend favorably toward the SNK01 combination group relative to pembrolizumab monotherapy in HRQoL, physical function, role function, and dyspnea and chest pain symptom scales, which was represented by higher values of mean change in functional scales and lower values in symptom scales.

Discussion

ICI monotherapy, including pembrolizumab (KEYTRUDA, Merck), has been Food and Drug Administration–approved as the first-line treatment of choice for NSCLC with a PD-L1 TPS of 50% or greater in patients who are not eligible for or who have failed tyrosine-kinase inhibitor treatment [21]. However, restricted populations with NSCLC have a PD-L1 TPS of over 50%, and the clinical benefits of pembrolizumab monotherapy are limited to only a small proportion of NSCLC patients [22]. The median PFS of pembrolizumab monotherapy in previously treated and PD-L1–positive NSCLC patients is known as approximately 5.0 months with the percentage of grade 3-5 treatment-related adverse events being 13% [23]. Recently, to overcome these limitations of current immunotherapies, the combinations of various other therapies with ICIs have been intensively investigated [22,24]. In this study, we evaluated the clinical safety and efficacy of the autologous NK cell and pembrolizumab combination and also investigated the role of immune cells in this combination therapy with both enrolled patients and mouse model.

We observed that T cells as well as NK cells are also participated in the therapeutic process of programmed death-1 (PD-1)/PD-L1 axis blockade, as shown by our xenograft tumors which derived from NSCLC cells depleting T cells, NK cells, and both cells, respectively (S6 Fig.), consistent with previous study [16], indicating that NK cells may play roles in helping to recruit a T cell response and/or by killing tumor cells directly. Given our *in vivo* immune cell depletion experiment results showing the participation of the NK cells in the therapeutic effects of the PD-1 blockade and the cytotoxic effects of the NK cells on MHC- and neoantigen-deficient cancer cells [14,25], we determined the therapeutic effects of the combination therapy of NK cell and PD-1 blockade for treating NSCLC. In this study, to optimize the cytotoxic ability of autologous NK cells, we established the super NK cells (SNK01) modulating the culture conditions, which resulted in the activation of the NK cells. We also showed an enhanced cytotoxic ability of SNK01 compared with their corresponding NK cells. PD-1 blockade therapy with SNK01 resulted in the enhanced tumor growth inhibition of xenografted tumors by using the NSCLC cells, regardless of the genetically modified PD-L1 overexpression or knockout (S7 Fig.), suggesting that the combination therapy would be also effective in PD-L1–negative NSCLC patients. Moreover, further confirmation of the PD-L1 independency in therapeutic effects of these combinations would widen the clinical benefits of NSCLC patients, avoiding the unnecessary restriction of the patient cohorts.

Next, we aimed to investigate that the clinical usage of pembrolizumab treatment with autologous SNK cells and evaluate the possible usage of combination therapy for NSCLC as the therapy of choice after platinum-based therapy. Our clinical trial data showed that the ORR and PFS were higher in the SNK01 and pembrolizumab combination group

(ORR, 41.7%; PFS, 6.2 months) compared with pembrolizumab alone (ORR, 0%; PFS, 1.6 months). The therapeutic effects of combination therapy were also superior to those of pembrolizumab monotherapy (PFS; 5.0 months) from previous KEYNOTE-010 trial [23]. Moreover, four patients in the SNK01 combination group have not presented with disease progression and been continuing with the treatment. Although the baseline characteristics including epidermal growth factor receptor (*EGFR*) mutational status and the number of previous lines of chemotherapy were not balanced, these results suggest that the SNK01 and pembrolizumab combination treatment has potential to exhibit more clinical benefit for treating NSCLC patients without severe AEs.

A recent study has shown that the combination therapy of allogenic NK cells and pembrolizumab have survival benefits in PD-L1+, advanced NSCLC patients. We also showed the clinical efficacy of combination treatment with autologous NK cells and pembrolizumab in NSCLC patients for the first time. Autologous NK cell infusion alone has shown limited efficacy, possibly because inhibitory receptors on autologous NK cells matches self MHC I presented on tumor cells and this 'self' recognition signals subsequently inhibit activation of NK cells, and autologous NK cells derived from cancer patients are actually in immune-suppression state with impaired functions, making these cells difficult to exhibit antitumor capability [26-28]. However, in the present study, autologous NK cell infusion in combination with pembrolizumab showed potential to improve clinical efficacy of pembrolizumab monotherapy. This could be explained by several reasons that pembrolizumab augments NK cells by expressing PD-L1, or interferon-γ secreted by NK cells expresses PD-L1 in cancer cells [29]. Furthermore, autologous NK cell infusion has an advantage of no need to find donors with human leukocyte antigen typing, compared to allogeneic infusion.

From the viewpoint of safety, patients receiving SNK combination experienced more immune-related AEs than those who receiving pembrolizumab monotherapy (35.7% vs. 0%, p=0.26); however, the difference was not statistically significant. There is a possibility that such a numeric difference occurred due to the small number of study patients. In addition, the SNK combination group had a longer PFS than the pembrolizumab monotherapy group, therefore more pembrolizumab was administered (mean, 5.4 times vs 3.5 times; p=0.12), and the follow-up period was also longer (median, 14.6 months vs. 11.0 months; p=0.24). Of note, unlike cyto-toxic chemotherapy, immune-related AEs are known to occur mainly several months after beginning pembrolizumab treatment [30]. Thus, they may explain why immune-related AEs tended to be detected more frequently in SNK combination group. In addition, there are reports that the occurrence of immune-related AEs is a predictor of the efficacy of immunotherapy [31]. Since the response rate tended to be higher in SNK combination group in the present study, it is also possible that more frequent immune-related AEs in SNK combination group was associated with the relatively favorable response. Moreover, in this study, the efficacy outcomes including ORR, PFS, and 1-year survival rate were significantly higher in the patients with the immune-related AE compared to those without AE. Whether SNK01 combination shows better efficacy than immunotherapy alone without increasing immune-related AEs need be confirmed through additional large-scale studies. Until now, there has been no clear discussion on how to prevent immune-related AEs, but close monitoring of the occurrence of the AEs and active and personalized managements for them are necessary [32].

Herein, we conducted phase 1 to evaluate toxicity and a pilot phase 2a study to determine the appropriate dose for future studies. It was difficult to determine the therapeutic dose of SNK01 in a preclinical model because there was no animal model unlike other anticancer drugs. Moreover, for cell therapy, the dose is not always proportional to the toxic/therapeutic effect, and DLT does not easily occur [33]. In the case of autologous NK cells, few adverse events were reported in previous clinical studies since the patient's own cells were proliferated [34]. In the present study design, it was planned to increase the SNK01 dose up to 4×10^9 cells/dose, because DLT was less likely to be observed even if the dose was continuously increased. In addition, continuously increasing the number of cells had various limitations such as cell production capacity and the time required for administration. According to the protocol, the maximum dose administered during clinical trials, 4×10^9 cells/dose, was determined as the MTD.

There are several limitations in this study. The first limitatin is the small number of patients. This study was a phase I/IIa clinical trial of the new treatment option, which will require a further large-scale randomized study based on these results. The second limitation was that the baseline tumor characteristics including *EGFR* mutation status and the number of previous lines of chemotherapy were not balanced between SNK01 combination and pembrolizumab monotherapy group because of the small number of patients. ORR of pembrolizumab alone was 0%, less than 18% of the previous study [23]. Considering that EGFR-positive patients have shown a poor response to ICI in the previous study [35], the higher *EGFR* mutation rate and the higher number of previous lines of chemotherapy in the pembrolizumab monotherapy group might explain its poor ORR and PFS in the present study. In addition, although not statisti-

cally significant, PD-L1 expression was also lower in the pembrolizumab monotherapy group, which may also have contributed to poor response and PFS of the pembrolizumab monotherapy group. Moreover, only seven out of 18 patients died and 4 patients did not present with disease progression due to the short study period. Further follow-up and long-term studies could help confirm these findings.

In conclusion, given our randomized phase I/IIa clinical trials showing a promising ORR and PFS without severe AEs after SNK01 and pembrolizumab combination therapy compared with pembrolizumab alone in NSCLC patients, combination therapy with pembrolizumab and autologous NK cell therapy would potentially improve the therapeutic effects of pembrolizumab. This study also provides the basis for performing large-scale phase III clinical trials.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This prospective clinical trial was approved by the institutional review board (IRB, 2018-1479) of Asan Medical Center (Seoul, South Korea) and registered at the Clinical Research Information Service (CRIS, KCT0003463). Informed consent was obtained from all participants prior to enrollment. The trial was designed and conducted in accordance with the Helsinki Declaration and the Ethical Guidelines for Clinical Studies.

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Contributed data or analysis tools: Kim EJ, Cho YH, Kim YM, Jung JS, Kang Y, Rho JK, Choi CM.
Performed the analysis: Kim EJ, Cho YH, Kim DH, Ko DH, Do EJ, Kim SY, Ji W, Choi MG, Lee JC, Rho JK, Choi CM.
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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1007
Cancer Res Treat. 2022;54(4):1017-1029

Original Article

The Role of Neutrophil-to-Lymphocyte Ratio in Predicting Pathological Response for Resectable Non–Small Cell Lung Cancer Treated with Neoadjuvant Chemotherapy Combined with PD-1 Checkpoint Inhibitors

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Purpose The aim of our study was to investigate the value of baseline and preoperative neutrophil-to-lymphocyte ratio (NLR) in predicting the pathological response and disease-free survival (DFS) of neoadjuvant chemotherapy alone or combined with programmed cell death-1 (PD-1) checkpoint inhibitors in patients with resectable non–small cell lung cancer (NSCLC).

Materials and Methods Resectable NSCLC patients who underwent neoadjuvant chemotherapy alone or combined with PD-1 checkpoint inhibitors between January 2018 and January 2020 were included. Peripheral venous blood samples of the patients were collected within 3 days prior to the first neoadjuvant treatment and within 3 days prior to surgery.

Results A total of 79 patients in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group and 89 patients in neoadjuvant chemotherapy alone group were included. Thirty-five point four percent of the patients achieved pathological complete response (pCR) in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group, whereas only 9.0% reached pCR in the group of neoadjuvant chemotherapy. High NLR level were correlated with poor pathological response and DFS in neoadjuvant chemotherapy or combined with PD-1 checkpoint inhibitors group. Multivariate analysis revealed that baseline NLR could independently predict pathological response and DFS in the neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group.

Conclusion High NLR level were correlated with poor pathological response and shorter DFS in patients with NSCLC undergoing neoadjuvant chemotherapy or combined with PD-1 checkpoint inhibitors. Meanwhile, baseline NLR could independently predict response to pathological response and DFS, revealing its potential as a screening tool in NSCLC patients who received neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors.

Key words Non–small cell lung neoplasm, Neutrophil-lymphocyte ratio, PD-1 checkpoint inhibitors, Neoadjuvant chemotherapy, Pathological response

Introduction

Global cancer statistics showed that lung cancer is the leading cause of cancer death [1]. Non–small cell lung cancer (NSCLC) accounts for 85% of all lung cancer, mainly consisting of adenocarcinoma and squamous cell carcinoma [2]. The emergence of immune checkpoint blockade targeting programmed cell death-1/programmed cell death ligand-1 (PD-1/PD-L1) has revolutionized the treatment of NSCLC. These drugs unleash antitumor immunity, resulting in tumor regression and improved survival in some patients with advanced NSCLC [3,4]. Combining anti–PD-1/PD-L1 with chemotherapy in metastatic NSCLC has also shown a survival advantage over chemotherapy alone, regardless of the level of PD-L1 expression or tumor mutation burden [5,6]. It has been hypothesized that neoadjuvant immunotherapy for early-stage NSCLC has the advantage of maximizing T-cell activation using the primary tumor as an antigen source, thereby systemically eliminating micro-metastases [7]. Using immune checkpoint inhibitors as neoadjuvant treatment could be superior to using them as adjuvant treatment since they could release neoantigens from dying tumor cells and stimulate the priming and expansion of neoantigen-specific T cells in the tumor before surgical resection [8]. A trial of neoadjuvant ipilimumab combined with chemotherapy showed that 58% of patients with NSCLC had an objective response [9].

There is a pressing need to find easy-to-use, reliable, and inexpensive biomarkers to identify NSCLC patients who may respond to neoadjuvant anti–PD-1 antibody. Cancer-related

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Received September 8, 2021 **Accepted** November 18, 2021 **Published Online** November 23, 2021
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inflammation is a critical determinant of disease progression and survival in most cancers [10]. Hematologic parameters neutrophil-to-lymphocyte ratio (NLR) that may reflect the balance between inflammation and immune response, has been shown to be useful for predicting the prognosis of patients with advanced NSCLC after immunotherapy [11,12].

The predictive value of NLR has not been evaluated in patients with NSCLC after neoadjuvant chemotherapy combined with immunotherapy, the aim of our study was to investigate the baseline and preoperative hematologic parameter NLR to predict the pathological response and disease-free survival (DFS) of NSCLC patients receiving neoadjuvant chemotherapy combined with immunotherapy compared with neoadjuvant chemotherapy alone.

Materials and Methods

1. Patients

A total of 79 resectable NSCLC patients (II and IIIA stage) receiving surgery after neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors and 89 patients receiving surgery after neoadjuvant chemotherapy alone were enrolled in Tianjin cancer Hospital (Tianjin, China) from January 2018 to January 2020. The inclusion criteria were as follows: (1) patients with NSCLC confirmed by bronchoscopy biopsy or computed tomography (CT) guided puncture biopsy; (2) the preoperative staging was done with contrast-enhanced CT or positron emission tomography; (3) neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors (nivolumab, camrelizumab, or tislelizumab) or neoadjuvant chemotherapy alone were applied for at least two cycles before surgery; (4) peripheral blood was collected before neoadjuvant therapy initiation and surgery, respectively. The following data were collected from the medical records: age, sex, smoking history, histology, neoadjuvant therapy number of cycles, tumor size at baseline, and pathological response. The rate of major pathologic response (MPR; residual viable tumor in NSCLC ≤ 10%), pathological complete response (pCR; absence of any viable invasive tumor in the lung tissue and lymph node) and DFS which was defined by the symptom-, metastasis-, and recurrence-free survival time of patients after treatment were well calculated. The cutoff date was June 31, 2021.

2. Specimen collection

Peripheral venous blood samples of the patients were collected within 3 days prior to the first neoadjuvant treatment and within 3 days prior to surgery. Total white blood cell count (WBC), absolute neutrophil count (ANC), platelet count (PLT), absolute lymphocyte count (ALC), and tumor markers (carcinoembryonic antigen [CEA], squamous cell carcinoma antigen [SCC], and total prostate specific antigen [TPSA]) were collected. NLR was defined as the ratio of ANC to ALC. The upper limit of normal value is 5 μg/L for CEA, 1.5 μg/L for SCC, and 80 U/L for TPSA, respectively. Based on the reference range, the baseline levels of serum tumor markers CEA, SCC, and TPSA were categorized into normal and high.

3. Statistical analysis

Categorical variables were summarized as frequencies and percentages and analyzed by using the chi-square test or Fisher's exact test. Continuous variables were analyzed by using Mann-Whitney U test for skewed distributed variables. The receiver operating characteristic (ROC) curve was plotted to determine the optimal cutoff values of baseline and preoperative NLR. The univariate and multivariate logistic regression analyses were performed to identify the independent predictors for pathological responses of neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors and neoadjuvant chemotherapy alone by using a forward stepwise procedure. The Kaplan-Meier method was used to estimate the probability of DFS, and the log-rank test was used to investigate the significance of differences between different NLR groups. The prognostic values of each variable were evaluated with univariate cox proportional hazard regression analyses. Multivariate analysis for DFS was performed using the variables that were significant on univariate analysis. The p-value less than 0.05 was considered statistically significant. SPSS ver. 24 (IBM Corp., Armonk, NY) was used for statistical analysis.

Results

1. Baseline clinical characteristics of patients

The main clinical characteristics of participants were presented in Table 1. The neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group had 79 NSCLC patients (79.7% were smokers or ex-smokers), whose median age was 61 years old (range, 40 to 77 years), including 63 men (79.7%) and 16 women (20.3%). The squamous cell carcinomas, adenocarcinoma, and large cell carcinoma accounted for 55.7%, 26.6%, and 17.7% of these patients, respectively. The postoperative pathological results that there were 21 patients (26.6%) who had lymph node metastasis. Before surgery, all patients had received two or more cycles of neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors treatment: 39 (49.4%) cases had received two cycles of neoadjuvant treatment, 30 (38.0%) had received three cycles, and 10 (12.6%) had received four cycles.

Table 1. The correlation between pathological response and clinical-pathological variables in the two different treatment groups

Characteristic	Neoadjuvant chemotherapy combined with immunotherapy group				Neoadjuvant chemotherapy group			
	pCR (n=28)	Non-pCR (n=51)	Total	p-value	pCR (n=8)	Non-pCR (n=81)	Total	p-value
Sex								
Male	25	38	63 (79.7)	0.118	6	67	73 (82.0)	0.593
Female	3	13	16 (20.3)		2	14	16 (18.0)	
Age (yr)								
< 61	9	22	31 (39.2)	0.338	4	40	44 (49.4)	0.973
≥ 61	19	29	48 (60.8)		4	41	45 (50.6)	
Smoking history								
Smoker or ex-smoker	23	40	63 (79.7)	0.695	8	69	77 (86.5)	0.262
Never smoker	5	11	16 (20.3)		0	11	11 (12.4)	
Histology								
Squamous cell carcinomas	19	25	44 (55.7)	0.061	5	49	54 (60.7)	0.462
Adenocarcinoma	3	18	21 (26.6)		2	28	30 (33.7)	
Large cell carcinoma	6	8	14 (17.7)		1	4	5 (5.6)	
Neoadjuvant therapy number of cycles								
2	11	28	39 (49.4)	0.095	6	52	58 (65.2)	0.857
3	15	15	30 (38.0)		1	19	20 (22.5)	
4	2	8	10 (12.6)		1	10	11 (12.4)	
N stage								
N0	27	31	58 (73.4)	0.003	7	44	51 (57.3)	0.070
N1-2	1	20	5 (26.6)		1	37	38 (42.7)	
Baseline CEA								
Normal	18	23	41 (56.9)	0.029	5	48	53 (59.6)	0.859
High	6	25	31 (43.1)		3	33	36 (40.4)	
Baseline SCC								
Normal	13	32	45 (62.5)	0.302	5	52	57 (64.0)	0.920
High	11	16	27 (37.5)		3	29	32 (36.0)	
Baseline TPSA								
Normal	13	26	39 (59.1)	0.539	5	44	49 (55.1)	0.657
High	11	16	27 (40.9)		3	37	40 (44.9)	

CEA, carcinoembryonic antigen; pCR, pathological complete response; SCC, squamous cell carcinoma antigen; TPSA, total prostate specific antigen.

The neoadjuvant chemotherapy alone group had 89 NSCLC patients (86.5% were smokers or ex-smokers), including 73 men (82.0%) and 16 women (18.0%). The squamous cell carcinomas, adenocarcinoma, and large cell carcinoma accounted for 60.7%, 33.7%, and 5.6% of these patients, respectively. There were 38 patients (42.7%) who had pathological lymph node metastasis.

2. Cutoff determination of baseline and preoperative NLR

The optimum cutoff values for baseline and preoperative NLR were determined by ROC analysis, respectively. In neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group, the cutoff point of baseline and preoperative NLR for predicting pCR were 1.96 (area under the curve [AUC], 0.679; sensitivity, 0.860; specificity, 0.500) and 1.89 (AUC, 0.657; sensitivity, 0.600; specificity, 0.750). The cutoff point of baseline NLR for predicting MPR was 2.05 (AUC, 0.664; sensitivity, 0.829; specificity, 0.500) and preoperative NLR for predicting MPR was 1.93 (AUC, 0.594; sensitivity, 0.575, specificity, 0.684). In neoadjuvant chemotherapy alone group, the cutoff point of baseline and preoperative NLR for predicting pCR were 1.01 (AUC, 0.433; sensitivity, 0.97; specificity, 0.25) and 1.43 (AUC, 0.516; sensitivity, 0.61; specificity, 0.63). The cutoff point of baseline NLR for predicting MPR was 1.01 (AUC, 0.422; sensitivity, 0.968; specificity, 0.154) and preoperative NLR for predicting MPR was 1.43 (AUC, 0.615;

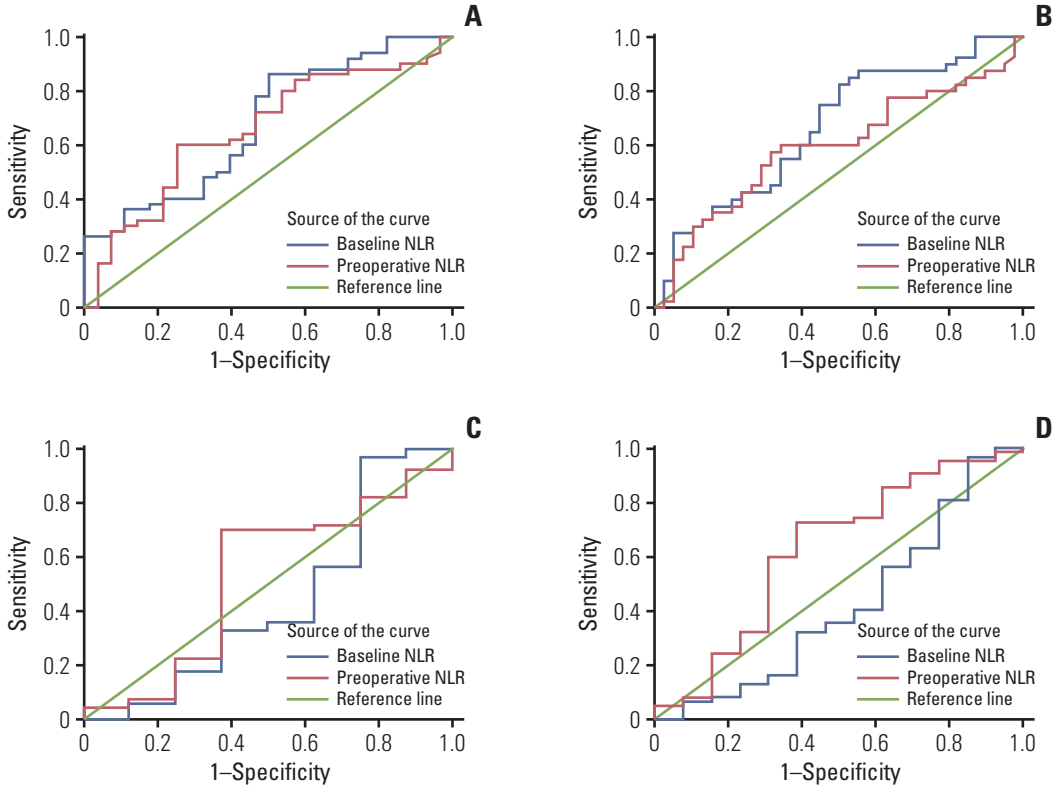

Fig. 1. Receiver operating characteristic curve identified the cutoff point of neutrophil-to-lymphocyte ratio (NLR). (A) Baseline and preoperative NLR for predicting pathological complete response (pCR) were 1.96 (area under the curve [AUC], 0.679; sensitivity, 0.86; specificity, 0.50] and 1.89 (AUC, 0.657; sensitivity, 0.60; specificity, 0.75) in neoadjuvant chemotherapy combined with immunotherapy group. (B) Baseline and preoperative NLR for predicting major pathologic response (MPR) were 2.05 (AUC, 0.664; sensitivity, 0.829; specificity, 0.500) and 1.93 (AUC, 0.594; sensitivity, 0.575; specificity, 0.684) in neoadjuvant chemotherapy combined with immunotherapy group. (C) Baseline and preoperative NLR for predicting pCR were 1.01 (AUC, 0.433; sensitivity, 0.97; specificity, 0.25) and 1.43 (AUC, 0.516; sensitivity, 0.61; specificity, 0.63) in neoadjuvant chemotherapy alone group. (D) Preoperative NLR for predicting MPR were 1.01 (AUC, 0.422; sensitivity, 0.968; specificity, 0.154) and 1.43 (AUC, 0.615; sensitivity, 0.694; specificity, 0.615) in neoadjuvant chemotherapy alone group.

sensitivity, 0.694; specificity, 0.615) (Fig. 1). Patients were then divided into low and high NLR groups according to the cutoff values.

3. Correlations between baseline and preoperative hematological parameters and pathological response of neoadjuvant treatment

In neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group, 48.1% (n=38) and 35.4% (n=28) of the patients reached MPR and pCR. While in the group of neoadjuvant chemotherapy alone group, the MPR and pCR were only 14.6% and 9.0%, respectively. The patients were divided into two groups: the group that achieved pCR after neoadjuvant therapy and the group that did not achieve pCR. The clinicopathological characteristics and hematological parameters between patients showing pCR and those not showing pCR were compared in Tables 1 and 2, respectively.

In neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group, higher baseline and preoperative WBC, neutrophils, NLR, and higher preoperative PLT were observed in the non-pCR group than pCR group. However, in neoadjuvant chemotherapy alone group, there were no significant differences in these hematological parameters between the non-pCR and pCR group (Table 2).

Then we assessed the correlations between NLR and MPR and found that higher baseline NLR was observed in the non-MPR groups in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group. What's more, there were no significant differences between NLR and MPR in neoadjuvant chemotherapy alone group (Fig. 2). Fig. 2 showed the correlations between NLR and pathological responses.

Univariate logistic regression analysis (Table 3) showed that histology, lymph node metastasis or not, baseline CEA,

Table 2. The correlation between pathological response and hematologic parameters in the two different neoadjuvant treatment groups

Characteristic	Neoadjuvant chemotherapy combined with immunotherapy group				Neoadjuvant chemotherapy group			
	pCR (n=28)	Non-pCR (n=51)	Total	p-value	pCR (n=8)	Non-pCR (n=81)	Total	p-value
Baseline WBC (×10⁹/L)	6.69 (4.99-12.18)	8.12 (3.35-23.56)	7.42 (3.35-23.56)	0.009	7.19 (4.95-12.47)	7.05 (3.23-18.99)	7.06 (3.23-18.99)	0.605
Baseline neutrophils (×10⁹/L)	3.93 (2.34-9.01)	5.60 (1.73-19.24)	4.80 (1.73-19.24)	0.001	4.70 (1.94-10.85)	4.32 (1.5-12.53)	4.38 (1.50-12.53)	0.789
Baseline monocytes (×10⁹/L)	0.51 (0.11-1.03)	0.56 (0.06-5.90)	0.53 (0.06-5.90)	0.166	0.52 (0.27-0.84)	0.53 (0.09-1.13)	0.53 (0.09-1.13)	0.746
Baseline lymphocyte (×10⁹/L)	2.10 (1.16-3.75)	1.84 (0.78-3.99)	1.90 (0.78-3.99)	0.335	1.92 (0.73-3.52)	1.88 (0.82-5.07)	1.89 (0.73-5.07)	0.817
Baseline platelet (×10⁹/L)	248.50 (103.00-387.00)	286.00 (146.00-541.00)	267.00 (103.00-541.00)	0.026	272.00 (145.00-317.00)	277.00 (160.00-563.00)	273.00 (145.00-563.00)	0.733
Baseline NLR	2.00 (0.85-4.76)	2.88 (1.20-13.23)	2.61 (0.85-13.23)	0.009	2.74 (0.77-14.86)	2.25 (0.86-6.28)	2.31 (0.77-14.86)	0.537
Preoperative WBC (×10⁹/L)	5.30 (3.80-15.36)	6.47 (2.43-12.66)	5.86 (2.43-15.36)	0.035	5.28 (4.39-7.29)	6.14 (3.06-15.70)	5.99 (3.06-15.70)	0.128
Preoperative neutrophils (×10⁹/L)	2.76 (1.21-13.65)	3.31 (1.04-9.34)	3.19 (1.04-13.65)	0.013	2.86 (2.27-3.58)	3.37 (0.58-12.63)	3.32 (0.58-12.63)	0.127
Preoperative monocytes (×10⁹/L)	0.56 (0.30-1.08)	0.60 (0.18-1.35)	0.58 (0.18-1.35)	0.059	0.48 (0.33-0.93)	0.57 (0.24-1.44)	0.56 (0.24-1.44)	0.134
Preoperative lymphocyte (×10⁹/L)	2.06 (0.63-2.79)	1.73 (0.79-3.09)	1.83 (0.63-3.09)	0.632	1.78 (0.84-3.30)	2.03 (0.47-3.83)	2.02 (0.47-3.83)	0.851
Preoperative platelet (×109/L)	222.00 (93.00-690.00)	237.50 (147.00-384.00)	233.50 (93.00-690.00)	0.186	215.50 (166.00-260.00)	230.00 (117.00-509.00)	224.00 (117.00-509.00)	0.489
Preoperative NLR	1.63 (0.53-21.67)	2.02 (0.79-7.52)	1.83 (0.53-21.67)	0.022	1.38 (0.85-6.20)	1.83 (0.21-17.68)	1.69 (0.21-17.68)	0.880

Values are presented as median (range). NLR, neutrophil-to-lymphocyte ratio; pCR, pathological complete response; WBC, white blood cell.

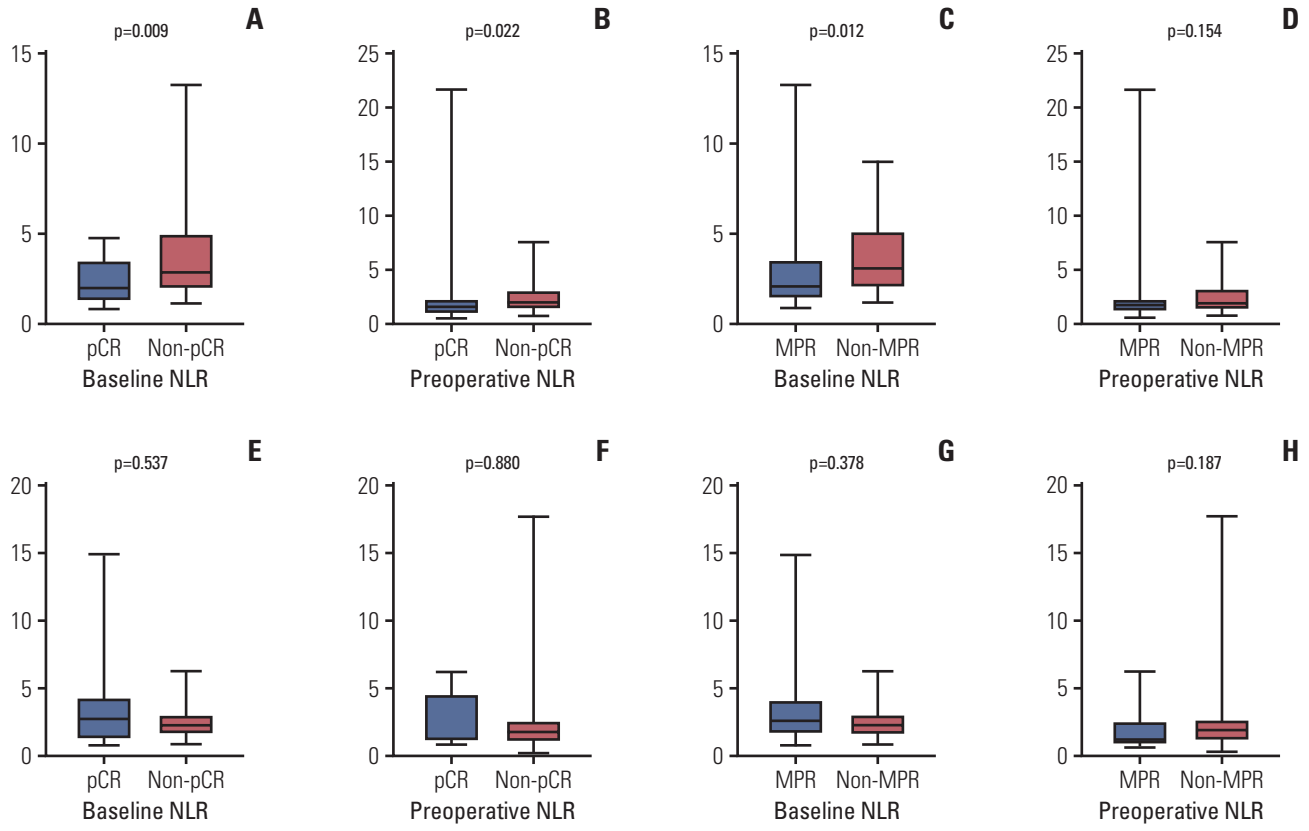

Fig. 2. The correlations between baseline and preoperative neutrophil-to-lymphocyte ratio (NLR) and pathological responses. (A-D) Neoadjuvant chemotherapy combined with programmed death-1 (PD-1) checkpoint inhibitors group. (A) The difference of baseline NLR between pathological complete response (pCR; mean±standard deviation, 2.39±1.19) and non-pCR (3.60±2.27) patients (p=0.009). (B) The difference of preoperative NLR between pCR (2.33±3.84) and non-pCR (2.32±1.30) patients (p=0.022). (C) The difference of baseline NLR between major pathologic response (MPR; 2.76±2.15) and non-MPR (3.55±1.86) patients (p=0.012). (D) The difference of preoperative NLR between MPR (2.35±3.33) and non-MPR (2.30±1.33) patients (p=0.154). (E-H) Neoadjuvant chemotherapy alone group. (E) The difference of baseline NLR between pCR (3.98±4.56) and non-pCR (2.48±1.11) patients (p=0.537). (F) the difference of preoperative NLR between pCR (2.50±2.04) and non-pCR (2.29±2.21) patients (p=0.880). (G) The difference of baseline NLR between MPR (3.52±3.60) and non-MPR (2.45±1.12) patients (p=0.378). (H) The difference of preoperative NLR between MPR (2.03±1.72) and non-MPR (2.36±2.26) patients (p=0.187).

baseline and preoperative NLR were significantly correlated with pCR and MPR in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group. Lymph node metastasis, baseline and preoperative NLR were significantly correlated with pCR and MPR in neoadjuvant chemotherapy alone group. Multivariate logistic regression analysis (Table 4) revealed that lymph node metastasis or not (pCR: p=0.033; hazard ratio [HR], 11.741; 95% confidence interval [CI], 1.212 to 11.371; MPR: p=0.013; HR, 26.385; 95% CI, 1.980 to 35.163), and baseline NLR (pCR: p=0.030; HR, 5.407; 95% CI, 1.178 to 24.825; MPR: p=0.015; HR, 10.549; 95% CI, 1.562 to 72.924) could independently predict pCR and MPR after neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors in patients with NSCLC. However, the baseline and preoperative NLR were not independently predictive factor for pCR or MPR in neoadjuvant chemotherapy alone group.

4. Correlations between baseline and preoperative NLR and DFS

We further analyzed the correlations between NLR and DFS in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group. After a median follow-up of 18 months for the entire cohort, 26 patients had disease relapse or progression. The optimum cutoff values of baseline and preoperative NLR for predicting DFS were 2.43 and 1.48 determined by ROC analysis as mentioned above. Low baseline and preoperative NLR group had better DFS than high baseline and preoperative NLR group (Fig. 3). The univari-

Table 3. Univariate analysis of pCR and MPR for neoadjuvant chemotherapy or combined with immunotherapy group

Characteristic	Univariate for pCR and MPR in neoadjuvant chemotherapy combined with immunotherapy group				Univariate for pCR and MPR in neoadjuvant chemotherapy group			
	p-value for pCR	Odds ratio (95% CI)	p-value for MPR	Odds ratio (95% CI)	p-value for pCR	Odds ratio (95% CI)	p-value for MPR	Odds ratio (95% CI)
Sex								
Male or female	0.129	2.851 (0.737-11.031)	0.102	3.517 (1.023-12.097)	0.590	-	0.793	0.805 (0.160-4.046)
Age (yr)								
< 61 or ≥ 61	0.340	0.624 (0.237-1.643)	0.388	0.534 (0.922-5.930)	0.973	0.976 (0.228-4.171)	0.349	0.563 (0.169-1.876)
Smoking history (smoker, ex-smoker or never smoker)	0.695	1.265 (0.391-4.096)	0.138	2.420 (0.753-7.774)	0.860	1.217 (0.137-10.803)	0.456	2.250 (0.267-18.955)
Histology	0.082		0.014		0.644		0.349	
Squamous cell carcinomas	0.983	0.987 (0.293-3.326)	0.897	0.923 (0.273-3.118)	0.460	0.408 (0.038-4.395)	0.935	0.909 (0.091-9.035)
Adenocarcinoma	0.068	4.500 (0.893-22.668)	0.025	5.777 (1.241-25.878)	0.349	0.286 (0.021-3.921)	0.349	0.286 (0.021-3.921)
Others	1		1		1		1	
Neoadjuvant therapy number of cycles (≤ 2 or >2)	0.187	0.532 (0.208-1.358)	0.429	0.699 (0.288-1.696)	0.544	1.673 (0.317-8.830)	0.342	1.944 (0.493-7.666)
N (N0 or N1-2)	0.007	17.419 (2.190-138.540)	0.001	9.130 (2.413-34.546)	0.095	0.162 (0.019-1.374)	0.039	0.192 (0.040-0.923)
Baseline CEA (normal or high)	0.033	3.261 (1.103-9.637)	0.018	3.281 (1.231-8.745)	0.859	1.146 (0.256-5.127)	0.874	1.102 (0.330-3.687)
Preoperative CEA (normal or high)	0.150	3.266 (0.653-16.338)	0.296	2.000 (0.545-7.337)	0.657	0.710 (0.156-3.221)	0.988	0.990 (0.275-3.561)
Baseline SCC (normal or high)	0.304	0.591 (0.217-1.610)	0.164	0.502 (0.191-1.324)	0.924	0.929 (0.207-4.173)	0.410	0.607 (0.185-1.992)
Preoperative SCC (normal or high)	0.564	0.684 (0.189-2.478)	0.135	2.640 (0.738-9.439)	0.409	0.540 (0.125-2.332)	0.643	0.741 (0.208-2.640)
Baseline TPSA (normal or high)	0.539	0.727 (0.263-2.009)	0.964	0.977 (0.366-2.609)	0.658	1.403 (0.314-6.260)	0.612	1.366 (0.409-4.557)
Preoperative TPSA (normal or high)	0.173	2.631 (0.654-10.590)	0.101	3.136 (0.878-10.209)	0.628	1.429 (0.260-7.854)	0.643	1.343 (0.386-4.668)
Baseline NLR (normal or high)	0.001	6.286 (2.117-18.668)	0.005	4.371 (1.556-12.282)	0.020	12.000 (1.498-11.28)	0.090	6.000 (0.756-47.63)
Preoperative NLR (normal or high)	0.004	4.500 (1.614-12.549)	0.023	2.931 (1.159-7.413)	0.032	6.240 (1.175-33.144)	0.039	3.636 (1.069-12.375)

CEA, carcinoembryonic antigen; CI, confidence interval; MPR, major pathologic response; NLR, neutrophil-to-lymphocyte ratio; pCR, pathological complete response; SCC, squamous cell carcinoma antigen; TPSA, total prostate specific antigen.

Table 4. Multivariate analysis of pCR and MPR for neoadjuvant chemotherapy or combined with immunotherapy group

Characteristic	Multivariate for pCR and MPR in neoadjuvant chemotherapy combined with immunotherapy group				Multivariate for pCR and MPR in neoadjuvant chemotherapy group			
	p-value for pCR	Odds ratio (95% CI)	p-value for MPR	Odds ratio (95% CI)	p-value for pCR	Odds ratio (95% CI)	p-value for MPR	Odds ratio (95% CI)
Histology	0.654	-	0.313	-	-	-	-	-
Squamous cell carcinomas	0.820	0.839 (0.185-3.809)	0.334	0.375 (0.051-2.744)	-	-	-	-
Adenocarcinoma	0.531	1.851 (0.270-12.697)	0.710	1.522 (0.166-13.960)	-	-	-	-
Others	1		1					
N (N0 or N1-2)	0.033	11.741 (1.212-11.371)	0.013	26.385 (1.980-35.163)	0.278	0.275 (0.027-2.830)	0.129	0.271 (0.050-1.460)
Baseline CEA (normal or high)	0.333	1.908 (0.516-7.052)	0.834	1.172 (0.267-5.144)	-	-	-	-
Baseline NLR (normal or high)	0.030	5.407 (1.178-24.825)	0.015	10.549 (1.562-72.924)	0.095	7.774 (0.700-8.628)	0.318	3.284 (0.319-33.795)
Preoperative NLR (normal or high)	0.546	1.520 (0.391-5.916)	0.876	1.130 (0.190-4.119)	0.264	2.979 (0.439-20.192)	0.109	3.223 (0.770-13.480)

CEA, carcinoembryonic antigen; CI, confidence interval; MPR, major pathologic response; NLR, neutrophil-to-lymphocyte ratio; pCR, pathological complete response.

ate cox analysis results revealed that the baseline NLR and preoperative NLR were significantly correlated with DFS, as well as lymph node metastasis or not and baseline CEA. Multivariate cox regression analysis determined that baseline NLR and lymph node metastasis or not were independent predictors of DFS in patients with NSCLC in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group (Table 5).

In neoadjuvant chemotherapy alone group, after a median follow-up of 24 months for the entire cohort, 33 patients had disease relapse or progression. The optimum cutoff values of baseline and preoperative NLR for predicting DFS were 4.78 and 1.43, respectively. Low baseline and preoperative NLR group had better DFS than high baseline and preoperative NLR group (Fig. 3). However, the baseline and preoperative NLR were not independently predictive factor for DFS in neoadjuvant chemotherapy alone group (Table 5).

Discussion

To the best of our knowledge, this study is the first time to evaluate the predictive values of baseline and preoperative inflammatory factor NLR for pathological response and DFS of patients with resectable NSCLC receiving neoadjuvant chemotherapy or combined with PD-1 checkpoint inhibitors. The findings suggested that high baseline and preoperative NLR level were correlated with poor pathological response and DFS in NSCLC patients undergoing neoadjuvant chemotherapy alone or combined with PD-1 checkpoint inhibitors. In addition, NLR level significantly declined from baseline to preoperative after neoadjuvant therapy. Furthermore, baseline NLR could independently predict pCR, MPR, and DFS of NSCLC patients undergoing neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors.

It is very well known that patients achieving pCR or MPR after neoadjuvant immunotherapy usually had longer DFS and overall survival (OS) compared with neoadjuvant therapy. For this reason, primary endpoint in recent neoadjuvant therapy studies are pCR and MPR to predict the DFS and OS [13]. Previous studies showed that only 5%-8% of patients had pCR for neoadjuvant chemotherapy alone in patients with stage IIIA NSCLC [14]. Neoadjuvant chemotherapy combined with PD-1/PD-L1 inhibitors has changed the treatment landscape of metastatic NSCLC, which guided us to explore the effectiveness of this strategy in the neoadjuvant therapy. Our study showed that 48.1% of patients had MPR and 35.4% of patients achieved pCR in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group, whereas the MPR and pCR were only 14.6% and 9.0% in neoadjuvant chemotherapy alone group, respectively.

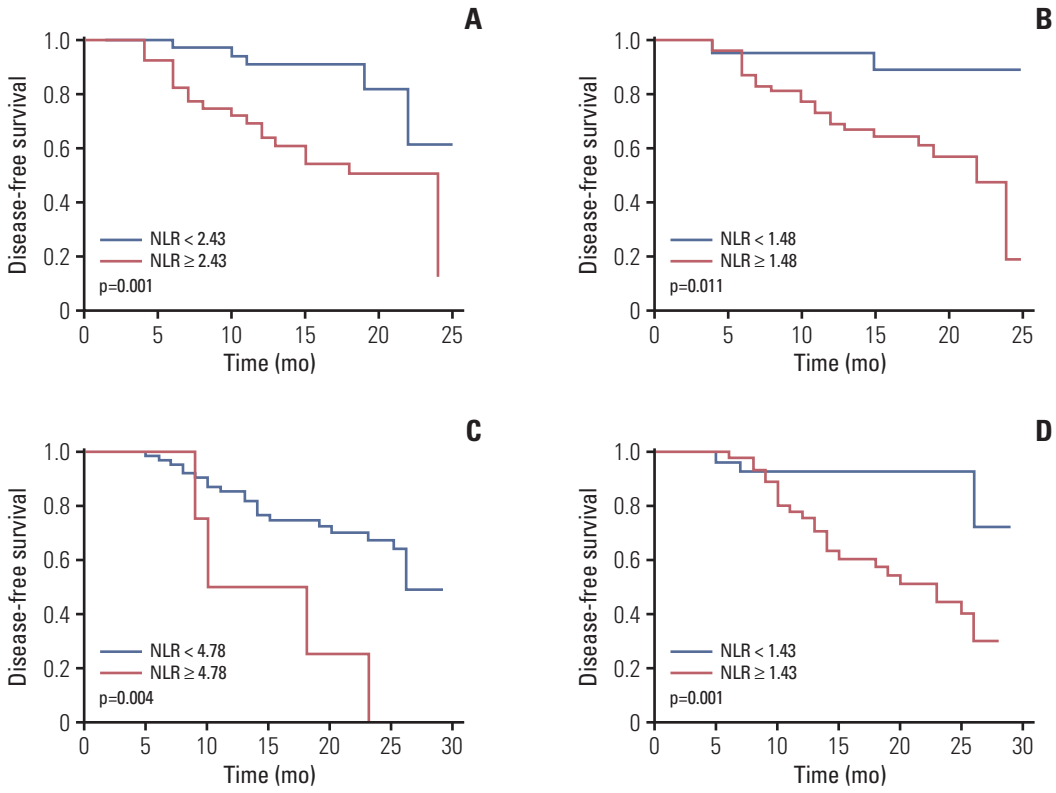

Fig. 3. Kaplan-Meier analysis of disease-free survival in relation to neutrophil-to-lymphocyte ratio (NLR). Kaplan-Meier curves for disease-free survival (DFS). (A) DFS curve of patients with baseline NLR in neoadjuvant chemotherapy combined with programmed death-1 (PD-1) checkpoint inhibitors group (p=0.001). (B) DFS curve of patients with preoperative NLR in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group (p=0.011). (C) DFS curve of patients with baseline NLR in neoadjuvant chemotherapy alone group (p=0.004). (D) DFS curve of patients with preoperative NLR in neoadjuvant chemotherapy alone group (p=0.001).

Inflammation can stimulate angiogenesis and affect immune surveillance as well as treatment response [15]. Tumorigenesis and progress are driven by the production of inflammatory cytokines, which could recruit inflammatory cells like neutrophils and platelet counts. Lymphopenia has a negative effect on cell-mediated immunity that initiate tumor cell death, and there has been growing evidence supporting the relationship between lymphopenia during neoadjuvant therapy and pathological response in patients with cancer [16,17]. Fang et al. [18] found that a higher lymphocyte level during neoadjuvant therapy was associated with a higher rate of pCR in patients with esophageal adenocarcinoma. In this study, baseline and preoperative WBC, neutrophils, and preoperative platelet counts were significantly higher in the non-PCR group than in the pCR group in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group. Thus, by assessing the status of these tumor-associated inflammatory responses, this might be evidence for early clinical evaluation of antitumor activity and potential therapeutic effects. This could be further explored to help differentiate between those who are not achieved pCR responding to treatment versus those achieved pCR.

Recent study reported that pretreatment derived neutrophil-to-lymphocyte ratio was found to be a predictive factor for pCR in patients with breast cancer treated with neoadjuvant chemotherapy [19]. While high NLR was found to be associated with poor survival in NSCLC patients receiving neoadjuvant chemotherapy [20], its relationship with pathological response is uncertain. Our results indicated that high baseline NLR was related to poor pCR and MPR in patients with NSCLC receiving neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors, as well as baseline CEA and lymph node metastasis or not. CEA has been mainly investigated as prognostic or predictive markers in NSCLC patients treated with chemotherapy. Recent study reported that CEA may serve as a reliable marker of efficacy in NSCLC patients treated with nivolumab when considering the determination of the markers at baseline [21]. Lymph node stations is a more accurate prognostic indicator in patients with completely resected non-small cell lung cancer

Table 5. Univariate and multivariate analysis of DFS in neoadjuvant chemotherapy or combined with immunotherapy group

Characteristic	Univariate for DFS in neoadjuvant chemotherapy combined with immunotherapy group		Multivariate for DFS in neoadjuvant chemotherapy combined with immunotherapy group		Univariate for DFS in neoadjuvant chemotherapy group		Multivariate for DFS in neoadjuvant chemotherapy group	
	p-value	Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value	Odds ratio (95% CI)
Sex (male or female)	0.284	1.579 (0.685-3.639)	-	-	0.500	0.755 (0.334-1.708)	-	-
Age (< 61 yr or ≥ 61 yr)	0.524	0.756 (0.320-1.787)	-	-	0.319	0.682 (0.322-1.448)	-	-
Smoking history (smoker, ex-smoker or never smoker)	0.336	1.532 (0.642-3.657)	-	-	0.245	1.626 (0.717-3.689)	-	-
Histology	0.120	-	-	-	0.284	-	-	-
Squamous cell carcinomas	0.247	2.413 (0.543-10.721)			0.945	1.074 (0.140-8.219)		
Adenocarcinoma	0.061	4.232 (0.933-19.187)	-	-	0.526	1.928 (0.254-14.638)	-	-
Others	1				1			
Neoadjuvant therapy number of cycles (≤ 2 or > 2)	0.587	0.807 (0.373-1.749)	-	-	0.849	1.076 (0.507-2.283)	-	-
N (N0 or N1-2)	0.001	4.067 (1.739-9.512)	0.038	1.645 (1.028-2.633)	0.002	0.265 (0.113-0.621)	0.212	0.549 (0.214-1.410)
Baseline CEA (normal or high)	0.010	3.108 (1.310-7.375)	0.077	2.277 (0.915-5.663)	0.336	1.433 (0.688-2.982)	-	-
Preoperative CEA (normal or high)	0.922	1.056 (0.355-3.141)	-	-	0.374	0.714 (0.340-1.500)	-	-
Baseline SCC (normal or high)	0.143	0.496 (0.194-1.268)	-	-	0.335	0.658 (0.281-1.542)	-	-
Preoperative SCC (normal or high)	0.808	0.882 (0.321-2.423)	-	-	0.343	0.695 (0.327-1.476)	-	-
Baseline TPSA (normal or high)	0.491	0.711 (0.269-1.877)	-	-	0.122	1.789 (0.856-3.741)	-	-
Preoperative TPSA (normal or high)	0.302	1.625 (0.647-4.082)	-	-	0.425	1.396 (0.615-3.172)	-	-
Baseline NLR (normal or high)	0.004	4.264 (1.605-11.328)	0.019	3.848 (1.252-11.827)	0.008	4.337 (1.460-12.886)	0.109	2.517 (0.813-7.791)
Preoperative NLR (normal or high)	0.026	5.131 (1.212-21.727)	0.151	3.034 (0.668-13.776)	0.004	4.741 (1.644-13.669)	0.067	2.904 (0.926-9.102)

CEA, carcinoembryonic antigen; CI, confidence interval; DFS, disease-free survival; NLR, neutrophil-to-lymphocyte ratio; SCC, squamous cell carcinoma antigen; TPSA, total prostate specific antigen.

[22]. But their effect on pathological responses and prognosis in neoadjuvant therapy has rarely been reported and needs further investigation. However, there were no significant differences in neoadjuvant chemotherapy alone group and it might be due to the low number of patients reaching pCR or MPR. We will increase the sample size for further verification in the subsequent study.

Hematologic parameter NLR value has been reported as a prognostic biomarker in patients with solid tumor [23,24]. Recent studies have showed that NLR was significantly associated with prognosis in patients with NSCLC and other metastatic solid tumors treated with immunotherapy [25,26]. It was reported that high pretreatment NLR (≥ 5) was independently related to poorer OS and PFS in advanced NSCLC patients treated with nivolumab [11,26]. In operable NSCLC patient, previous studies showed that a high preoperative NLR was an independent negative prognostic indicator [27]. Our results indicate that a high degree of NLR was corresponded to a poor DFS in patients with NSCLC and to the best of our knowledge is the first to report the prognostic value of inflammatory factor NLR in NSCLC patients receiving neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors.

In this study, baseline NLR showed an independent predictive ability for pathological response and DFS after neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors. However, the preoperative NLR did not show an independent predictive ability for pCR or MPR in multivariate analysis, which may be related to the inherent correlation between baseline and preoperative NLR. This phenomenon has been described in some studies [28], and it still needs to be further explored.

Because of readily available, non-invasive, and economic advantages, NLR can be used as a predictor of efficacy of neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors. We are aware of the limitations of our study. The study is a retrospective study and cannot control the influence of confounding factors on the results. In addition, the sample size is small and samples only come from single center and single-race that will not be generalized. No significant difference in NLR between the different pathological response groups especially in neoadjuvant chemotherapy alone group might due to the limited sample size. We will further expand the sample size for verification in the future. Therefore, whether it can be widely used in clinical practice to help evaluate the pathological response and prognosis of NSCLC patients treated with neoadjuvant therapy will require further study.

Our study proposed that a high baseline and preoperative NLR level were correlated with poor pathological response and DFS in NSCLC patients undergoing neoadjuvant chemotherapy alone or combined with PD-1 checkpoint inhibitors. Moreover, baseline NLR could independently predict pCR, MPR and DFS in neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors group. We believe that the inflammatory factor NLR, which can be detected in a simple, quick, cheap, and practical manner, may be used as an auxiliary clinical indicator to increase the predictability of pathological response and DFS in NSCLC patients undergoing neoadjuvant chemotherapy combined with PD-1 checkpoint inhibitors.

Ethical Statement

The study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Individual written informed consent for this study was acquired (approval number: bc2021187).

Author Contributions

Conceived and designed the analysis: Sun X, Feng Y, Zhao X, Zhang B.
Collected the data: Sun X, Feng Y.
Contributed data or analysis tools: Sun X, Feng Y, Huang W, Zhao X, Zhang H.
Performed the analysis: Sun X, Feng Y, Huang W.
Wrote the paper: Sun X, Feng Y.
Corrected the manuscript critically for important intellectual content: Zhang B, Yue D, Wang C.

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

This work was supported by National Natural Science Foundation of China (grant number 81772484); Tianjin Cancer Hospital Clinical Trial Project (grant number: C1705). We thank all the participants who contributed to the article.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1030
Cancer Res Treat. 2022;54(4):1030-1037

Original Article

Long-term Exposure to PM_{10} Increases Lung Cancer Risks: A Cohort Analysis

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Purpose Although lung cancer incidences in female never-smokers have increased, few studies focus on explicit investigation. We aimed to investigate the relationship between long-term exposure to ambient particulate matter sized 10 μm or less in diameter (PM_{10}) and the incidence of lung cancer within different genders and smoking status populations.

Materials and Methods We included Seoul metropolitan residents, aged between 20 and 65 years, who underwent a national health screening examination from 2005-2007 and were followed up until 2015. Individual-level long-term exposure to PM_{10} was assessed based on subject home addresses. To assess the relationship between PM_{10} and lung cancer, we estimated hazard ratios (HRs) for increased lung cancer incidence from a 10 $\mu g/m^3$ increase in PM_{10}.

Results Among 5,831,039 individuals, 36,225 (0.6%) developed lung cancer within the 7 years observed. In females, the majority (94.4%) of lung cancer development was found in never-smokers. In adjusted analyses, a significant relationship between lung cancer development and PM_{10} was observed in males, regardless of smoking status (never-smoker: HR, 1.14 [95% confidence interval (CI), 1.13 to 1.15]; ex-smoker: HR, 1.16 [95% CI, 1.14 to 1.17]; current smoker: HR, 1.18 [95% CI, 1.17 to 1.19]). We also found significant associations in female never- or ex-smokers with smaller HRs (never-smoker: HR, 1.06 [95% CI, 1.05 to 1.07]; ex-smoker: HR, 1.13 [95% CI, 1.02 to 1.23]; current smoker: HR, 1.04 [95% CI, 0.99 to 1.10]).

Conclusion Our findings suggest that long-term exposure to PM_{10} is associated with lung cancer development. A novel approach to lung cancer screening needs to be considered depending on the exposed PM_{10} level.

Key words Particulate matter, Lung neoplasms, Incidence, Women, Men, Non-smokers, Smokers

Introduction

Lung cancer is one of the most prevalent malignant neoplasms in many countries [1]. Increasing knowledge has enlightened the complex and intertwined effects of demographic, environmental, and genetic susceptibility on lung cancer development [2]. In general, lung cancer is more prevalent in cigarette smokers and men [3]. Recently though, incidence pattern of lung cancer according to sex and smoking status has changed [4]. As the incidence of lung cancer in females has increased in recent years, the difference in lung cancer prevalence between men and women is remarkably decreasing [5]. Although smoking has been well studied as an important carcinogen in the development of lung cancer [6], recent evidences have presented that lung cancer incidence is increasing in never-smokers [7].

Particulate matter (PM) has been considered as one of the environmental lung carcinogens. Globally, it has been continuously reported that an increasing concentration of PM 10 μm or less in diameter (PM_{10}) is related with an increasing risk of newly developed lung cancer [8]. A systematic review and meta-analysis showed a significantly positive association between PM_{10} and lung adenocarcinoma [9]. Based on accumulating evidence, the International Agency for Research on Cancer declared that outdoor air pollution is a carcinogen for lung cancer [10]. Given the relationship between ambient PM_{10} and lung cancer incidence, recent interests have focused on the role of PM_{10} in lung cancer pathogenesis in the patients naïve to cigarette smoking. Epidemiologic evidence has suggested that the development of lung cancer in never-

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Received September 14, 2021 **Accepted** January 14, 2022
Published Online January 17, 2022

smokers can be contributed to by an increased concentration of indoor or outdoor air pollution [11]. However, no study has clearly identified the relationship between long-term exposure to ambient PM_{10} and the incidence of lung cancer within different genders and smoking status, especially in female never-smokers.

The present cohort study aimed to clarify the long-term impact of exposure to PM_{10} on lung cancer incidence according to different gender and smoking status in a large portion of Seoul metropolitan residents, using the universally covered national health insurance database and annually-updated address information.

Materials and Methods

This study complied with STROBE guidelines.

1. Study design and eligibility criteria

We screened all Seoul residents under coverage of the national health insurance who received a health screening examination from January 2005 to December 2007, including all adults aged 20 to 65 years. We excluded subjects previously diagnosed with lung cancer before examinations or within 1 year since the baseline (January 2008). We observed all included subjects from January 2008 to December 2015. Follow-up observation was discontinued if with; diagnosis of lung cancer, death, or transfer of residence outside of the Seoul metropolitan area. The study subjects without address information were excluded from analysis.

2. Individual characteristics and lung cancer

Individual characteristics such as sociodemographic, behaviors, and medical information were extracted from the Korean National Health Insurance Service (NHIS) claim database [12]. We acquired anthropometric assessments and standardized questionnaires regarding social and medical history for all subjects. Never-smokers were defined as subjects who smoked < 100 cigarettes in their lifetime. Ex-smokers were defined as subjects who smoked > 100 cigarettes in their lifetime, but quit smoking at least 30 days before their screening examination. We created five groups based on radiologist readings; normal, suspicious active pulmonary tuberculosis, suspicious inactive pulmonary tuberculosis, suspicious lung disease other than pulmonary tuberculosis, and suspicious cardiovascular disease. Lung cancer incidence was determined based on initial diagnosis dates of lung cancer from the anonymized database of the National Health Insurance Review and Assessment Service (HIRA). Lung cancer was defined using the International Classification of Diseases-10 codes C33 and C34.

3. Assessment of individual-level long-term exposure to PM_{10}

We used annual average PM_{10} concentrations predicted within subject home addresses for 2002-2006 from a validated exposure prediction model to assess individual long-term exposure to PM_{10}. This model was constructed in a universal kriging framework, which is composed of a few summary predictors estimated from hundreds of geographic variables and a spatial correlation structure modeled based on regulatory monitoring data [13]. For residential address data, we obtained annually-updated home addresses for all subjects on a 100-m grid produced by the National Geographic Information Institute. Lastly, we computed 5-year averages of annual average PM_{10} concentrations across all addresses of each subject from 2002-2006.

4. Statistical analyses

Demographic characteristics, clinical features, and PM_{10} concentrations were descriptively analyzed. Cox regression analyses, stratified by sex and smoking status, were performed to estimate hazard ratios (HRs) and 95% confidential intervals (95% CIs) for lung cancer incidence per 10 $\mu g/m^3$ increase of PM_{10}. Confounders included in the adjusted models were: age, body mass index, income, previous malignancy history, and chest X-ray abnormality. Survival time was calculated from January 2008 to lung cancer diagnosis or censoring dates. Study subjects who died, transferred outside of the Seoul metropolitan area during the observation period, or survived by the end of study period (December 31, 2015) were classified as censored. Age-stratified analysis was conducted as a subgroup analysis. We used SAS ver. 9.4 software (SAS Institute Inc., Cary, NC) for all statistical analyses.

Results

1. Baseline characteristics and clinical features

The Seoul metropolitan health screening cohort contained 5,831,039 individuals, from whom a total of 2,622,914 (45.0%) were identified as women (Table 1). Approximately, 54% never moved and 91% resided within the metropolitan area during the follow-up period. The most commonly included age group was 30-39 years old (31.4%) for men and 40-49 years old (31.5%) for women. Median body mass index was calculated as 23.4. Generally, female individuals engaged in less risky health-related behaviors than male. In male individuals, 43.1% were current smokers and 38.2% were never-smokers, while 88.8% of female individuals were never-smokers. The lowest percentage of male individuals was seen in the lowest income group, indicating limited participation

Table 1. Sociodemographic characteristics of the included patients who received national health screening during 2005-2007

	Total patients (n=5,831,039)	Male (n=3,208,125)	Female (n=2,622,914)
Age (yr)			
≥ 20 and < 30	1,028,358 (17.6)	499,383 (15.6)	528,975 (20.2)
≥ 30 and < 40	1,411,323 (24.2)	1,007,648 (31.4)	403,675 (15.4)
≥ 40 and < 50	1,710,016 (29.3)	884,570 (27.6)	825,446 (31.5)
≥ 50 and < 60	1,203,381 (20.6)	586,348 (18.3)	617,033 (23.5)
≥ 60 and < 65	477,961 (8.2)	230,176 (7.2)	247,785 (9.4)
BMI	23.4 (21.3-25.6)	24.1 (22.1-26.0)	22.4 (20.4-24.7)
Screening year			
2005	1,608,929 (27.6)	869,492 (27.1)	739,437 (28.2)
2006	2,709,626 (46.5)	1,551,651 (48.4)	1,157,975 (44.1)
2007	1,512,484 (25.9)	786,982 (24.5)	725,502 (27.7)
Smoking history			
Never-smoker	3,553,833 (60.9)	1,225,457 (38.2)	2,328,376 (88.8)
Ex-smoker	512,534 (8.8)	468,370 (14.6)	44,164 (1.7)
Current smoker	1,477,450 (25.3)	1,384,209 (43.1)	93,241 (3.6)
Not recorded	287,222 (4.9)	130,089 (4.1)	157,133 (6.0)
Income			
1st (0%-25%)	1,131,845 (19.4)	469,056 (14.6)	662,789 (25.3)
2nd (25%-50%)	1,357,739 (23.3)	721,011 (22.5)	636,728 (24.3)
3rd (50%-75%)	1,583,787 (27.2)	955,146 (29.8)	628,641 (24.0)
4th (75%-100%)	1,655,162 (28.4)	983,715 (30.7)	671,447 (25.6)
Not recorded	102,506 (1.8)	79,197 (2.5)	23,309 (0.9)
Drinking behavior			
< 3/wk	5,108,203 (87.6)	2,663,189 (83.0)	2,445,014 (93.2)
≥ 3/wk	490,980 (8.4)	433,887 (13.5)	57,093 (2.2)
Not recorded	231,856 (4.0)	111,049 (3.5)	120,807 (4.6)
Exercise behavior			
< 3/wk	4,539,320 (77.8)	2,499,046 (77.9)	2,040,274 (77.8)
≥ 3/wk	1,013,490 (17.4)	570,131 (17.8)	443,359 (16.9)
Not recorded	278,229 (4.8)	138,948 (4.3)	139,281 (5.3)
Underlying chronic disease			
Hypertension	390,603 (6.7)	200,085 (6.2)	190,518 (7.3)
Diabetes mellitus	130,321 (2.2)	76,698 (2.4)	53,623 (2.0)
Cardiovascular disease	35,808 (0.6)	19,129 (0.6)	16,679 (0.6)
Stroke	17,064 (0.3)	10,048 (0.3)	7,016 (0.3)
Liver disease	77,767 (1.3)	53,628 (1.7)	24,139 (0.9)
Previous malignancy	24,205 (0.4)	8,230 (0.3)	15,975 (0.6)
Blood chemistry			
Hemoglobin (g/dL)	14.1 (13.0-15.3)	15.1 (14.4-15.8)	13.0 (12.3-13.6)
Fasting glucose (mg/dL)	90 (83-99)	92 (83-101)	89 (82-97)
Total cholesterol (mg/dL)	190 (167-215)	191 (169-216)	188 (165-213)
Chest radiography			
Normal	5,189,969 (89.0)	2,853,769 (89.0)	2,336,200 (89.1)
Suspicious active pulmonary tuberculosis	242,56 (0.4)	16,505 (0.5)	7,751 (0.3)
Suspicious inactive pulmonary tuberculosis	301,362 (5.2)	192,083 (6.0)	109,279 (4.2)
Suspicious lung disease other than pulmonary tuberculosis	117,372 (2.0)	67,782 (2.1)	49,590 (1.9)
Suspicious cardiovascular disease	85,068 (1.5)	33,142 (1.0)	51,926 (2.0)
Not recorded	113,012 (1.9)	44,844 (1.4)	68,168 (2.6)

Values are presented as number (%) or median (IQR). BMI, body mass index; IQR, interquartile range.

Table 2. Exposed mean PM_{10} levels in Seoul metropolitan area each grid of one hectare cell unit, weighted by individual residence, from 2002 to 2006

Year	Annually measured PM_{10} ($\mu g/m^3$)						
	Mean	Standard deviation	5th percentile	25th percentile	Median	75th percentile	95th percentile
2002	63.3	11.8	43.1	56.1	64.0	70.8	81.5
2003	57.9	6.2	47.2	53.6	58.2	62.3	67.3
2004	58.0	7.7	45.4	52.9	58.3	63.3	69.9
2005	58.9	5.6	50.4	55.4	58.9	62.4	67.2
2006	60.6	6.0	51.6	56.6	60.3	64.3	70.8

PM_{10}, particulate matter 10 μm or less in diameter.

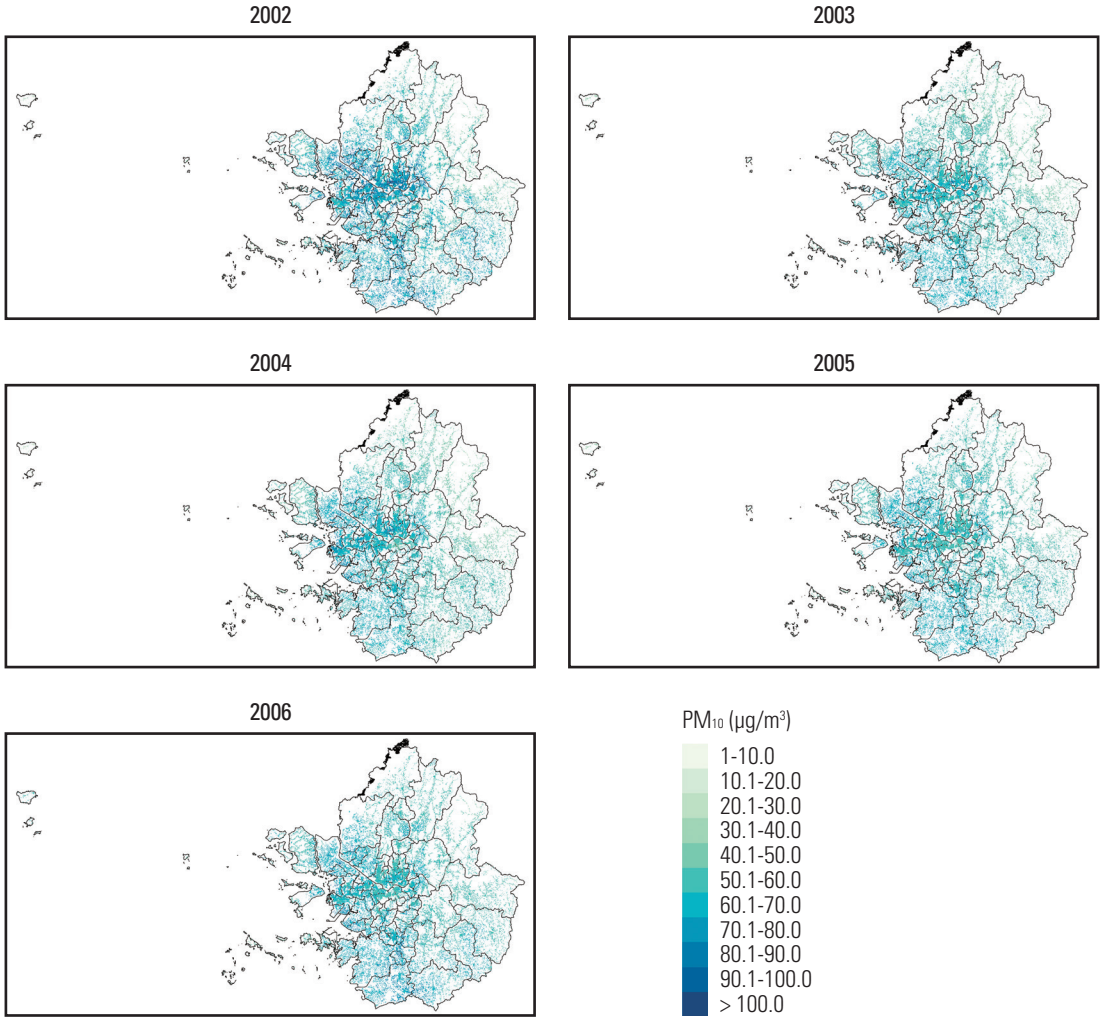

Fig. 1. Maps of mean PM_{10} concentrations in the Seoul metropolitan area of South Korea. PM_{10}, particulate matter 10 μm or less in diameter.

of male individuals with low income in the health screening examination, while female individuals showed similar participation rates among different income groups. In addition, more frequent alcoholic use (≥ 3/wk) was found in men.

At baseline, underlying chronic comorbidities were identified, including hypertension (6.7%), diabetes mellitus (2.2%),

cardiovascular disease (0.6%), stroke (0.3%), liver disease (1.3%), and previous malignancy (0.4%). In baseline blood tests, median hemoglobin was lower in women, but within the normal range. In chest radiography, most included subjects had normal features (89.0%).

Among a total of 5,831,039 individuals, 36,225 (0.6%) newly diagnosed cases of lung cancer were identified from 2008 to 2015 (S1 Table). A majority of incident lung cancer was found in individuals ≥ 40 years old who were never-smokers. The incidence of lung cancer was 0.76% in male patients and 0.45% in female patients. In the male patients with lung cancer, current, ex-, and never-smokers comprised 52.2%, 15.8%, and 32.0%, respectively. In contrast, these proportions were 4.3%, 1.3%, and 94.4%, respectively, in the female patients with lung cancer. The proportion of never-smokers in females was 30.7% among all lung cancer patients, and 58.8% among never-smoker patients with lung cancer. Among the total lung cancer patients, 2.5% had previous malignancy history and only 70.7% had normal features on initial chest radiography.

2. Air pollutant exposure and lung cancer incidence

Annual average PM_{10} concentrations, predicted within 100-m of patient home addresses, were summarized in Table 2. The mean PM_{10} concentration was 59.7 μg/m^3 (standard deviation, 7.5). Temporal and spatial distribution of annual mean PM_{10} concentrations are depicted in Fig. 1. Among the entire Seoul metropolitan areas, PM_{10} concentrations were higher in the central urban areas than in surrounding rural areas and this pattern was consistent over time.

The association between long-term exposure to PM_{10} and incident lung cancer is presented in Table 3. The incidence rate of lung cancer was found increased in the following populations: never-smokers, ex-smokers, and current smokers in both sexes. Compared to never-smokers, incidence rate ratio (IRR) of lung cancer per 10 μg/m^3 increase in PM_{10} was significantly higher in current smokers (male: IRR, 1.400 [95% CI, 1.342 to 1.460]; female: IRR, 1.400 [95% CI, 1.323 to 1.479]) and ex-smokers (male: IRR, 1.223 [95% CI, 1.154 to 1.295]; female: IRR, 1.222 [95% CI, 1.144 to 1.303]). In males, regardless of smoking status, we observed positive association between lung cancer and PM_{10} after adjustments were made (never-smoker: HR, 1.138 [95% CI, 1.127 to 1.149]; ex-smoker: HR, 1.155 [95% CI, 1.138 to 1.172]; current smoker: HR, 1.180 [95% CI, 1.165 to 1.194]). In females, we found a positive association between lung cancer and PM_{10} in never-smokers (HR, 1.062 [95% CI, 1.050 to 1.072]) and ex-smokers (HR, 1.127 [95% CI, 1.021 to 1.234]), but a marginal association in current smokers (HR, 1.043 [95% CI, 0.985 to 1.101]). In addition, when stratified by age, young (age 20-44) female never-smokers showed an association between PM_{10} expo-

Table 3. Risk of lung cancer development according to PM_{10} level within different sex and smoking statuses

	Male					Female				
	Incidence rate[a] (95% CI)	Unadjusted analysis		Adjusted analysis[b]		Incidence rate[a] (95% CI)	Unadjusted analysis		Adjusted analysis[b]	
		Hazard ratio	95% CI	Hazard ratio	95% CI		Hazard ratio	95% CI	Hazard ratio	95% CI
Never-smoker	50.2 (49.1-51.4)	1.144	1.127-1.161	1.138	1.127-1.149	36.0 (35.3-36.8)	1.062	1.051-1.072	1.061	1.050-1.072
Ex-smoker	61.4 (59.3-63.6)	1.161	1.138-1.184	1.155	1.138-1.172	44.0 (42.1-46.0)	1.136	1.030-1.242	1.127	1.021-1.234
Current smoker	70.3 (69.0-71.7)	1.184	1.172-1.195	1.180	1.165-1.194	50.4 (48.7-52.2)	1.048	0.990-1.105	1.043	0.985-1.101

Hazard ratio was estimated based on per 10 μg/m^3 change. CI, confidence interval; PM_{10}, particulate matter 10 μm or less in diameter. [a]Incidence rate was expressed as 100,000 person-year, [b]Covariables including age, body mass index, income, previous malignancy history, and chest X-ray abnormality were adjusted.

sure and lung cancer whereas none was found in older (age 45-64) female never-smokers (S2 Table).

Discussion

Our large population-based cohort study investigated the impact of long-term PM_{10} exposure on the incidence of lung cancer, especially in male and female never-smokers, using spatially-resolved PM_{10} predictions. In females, the majority (94.4%) of lung cancer development was found in never-smokers. The HR for lung cancer was higher in men, in which positive association with PM_{10} was identified regardless of smoking status. Considering that the HR for lung cancer development from long-term exposure to PM_{10} was rising in multiple population subsets in the order of never-smokers, ex-smokers, and current smokers, there may be a synergetic mechanism between cigarette smoking and PM_{10} on the lung carcinogenesis in males. We found that IRR of lung cancer per $\mu g/m^3$ increase in PM_{10} was higher in current smokers and ex-smokers compared to never-smokers. However, this synergetic mechanism may also not exist because of the low HR and weak association between PM_{10} and lung cancer found in female current smokers. In females, a significant relationship between PM_{10} exposure and lung cancer development was found in never-smokers or ex-smokers. Our result indicates that even women who never smoked can be at a higher risk for lung cancer development if they reside in a region with higher PM_{10} concentrations compared to those living in a low pollution area.

Lung cancer occurring in never-smoker females is considered as a distinct entity with different epidemiologic, biologic, and genetic features compared to lung cancer associated with cigarette smoking [14]. Globally, about 25% of lung cancer patients are considered never-smokers and the incidence of lung cancer in never-smoker females is increasing [15]. Never-smoker females have a higher rate of lung adenocarcinoma and targetable genetic mutations. The proportion of lung cancer development in never-smoker females was higher compared to never-smoker males [16]. One important review reported that the percentage of female never-smokers among lung cancer patients was about 43%-94% in Asia [17]. In a previous study including 9,685 Korean patients diagnosed with lung cancer in 2005, about 24% of lung cancer patients were found to be female and about 80% of female patients with lung cancer were never-smokers [18]. Our study included 36,225 patients diagnosed with lung cancer from 2008 to 2015 and showed a higher proportion of female lung cancer patients (32.6%) and never-smokers among female lung cancer patients (94.4%). Considering that female lung cancer rates are increasing [19], this epidemiologic difference can be explained by the different observation period.

Little evidence has been established regarding the etiology of lung cancer development in female never-smokers. Although numerous etiologic factors for lung cancer in never-smokers, including environmental, genetic, hormonal, and viral factors have been evaluated [16], currently established factors remain ambient toxic chemicals mainly related with occupational exposure [20]. A prospective study reported that occupational carcinogens were significantly related with lung cancer in male never-smokers [14]. Currently, PM exposure has been considered as another potential etiology of lung cancer. The European Study of Cohorts for Air Pollution Effects (ESCAPE) study prospectively identified that ambient PM_{10} was associated with a higher risk of lung cancer, especially lung adenocarcinoma [21]. Several European studies have shown a significant impact of long-term PM_{10} exposure on lung cancer in ever-smokers or male populations [22]. The relationship between long-term PM_{10} exposure and female never-smokers with lung cancer has not been well elucidated. Our study showed that long-term exposure to PM_{10} was related with a higher incidence of lung cancer in both sexes. Importantly, long-term exposure to a higher level of PM_{10} was significantly related with an increased risk of lung cancer in female never-smokers.

Although recent epidemiological evidence showed $PM_{2.5}$ as a stronger risk factor than PM_{10} for lung cancer, our study did not include $PM_{2.5}$ because $PM_{2.5}$ data for the Seoul metropolitan area has only been available since 2015. However, our findings focusing on PM_{10} can still provide important implication in the association with lung cancer particularly under high-dose PM exposure. Until recently, spatially and temporally extensive $PM_{2.5}$ data have been available mostly in countries with low-dose PM exposure. In the ESCAPE study, where mean PM_{10} was 21.3 $\mu g/m^3$, adjusted HR for lung cancer incidence was 1.22 (95% CI, 1.03 to 1.45) per 10 $\mu g/m^3$ increase of PM_{10} [21] and 1.28 (95% CI, 1.10 to 1.51) per 10 $\mu g/m^3$ increase of $PM_{2.5}$ [23]. In United States, where mean PM_{10} was 21.6 $\mu g/m^3$, adjusted HR for lung cancer incidence in female was 1.04 (95% CI, 0.95 to 1.14) per 10 $\mu g/m^3$ increase of PM_{10} and 1.06 (95% CI, 0.91 to 1.25) per 10 $\mu g/m^3$ increase of $PM_{2.5}$ [24]. $PM_{2.5}$ has been reported a higher effect estimates for lung cancer development. In a similar condition, the association of $PM_{2.5}$ with lung cancer could be stronger in Korea given our findings of the relationship between PM_{10} exposure and lung cancer incidence.

In fact, two similar studies were published before we finish the present study [25,26]. Yang et al. [25] analyzed 489 cases of lung cancer in 83,478 individuals and found that a higher level of PM was related with lung cancer development in heavy smokers or those with family history of cancer. The

relationship between PM and lung cancer in never-smoker or female population was insignificant in this study. Yang et al. [25] reported limitations of small number of lung cancer cases and less detailed geographic information. Moon et al. [26] overcame these limitations by using a larger cohort database including 6,567,909 individuals and district-specific home addresses, which is a similar methodology with our study. They showed a significantly elevated risk of lung adenocarcinoma development in male smokers, but not in female or never-smokers. In our consideration, they might fail to find a significant relationship between lung cancer development and PM_{10} level in female or never-smoker because the elderly were included. In fact, our study set a washout period for 1 year before enrollment and excluded the elderly (≥ 65 years old) that was considered as a uncontrollable confounding factor. Compared to former two studies, our study emphasized hazardous effects of PM on lung cancer development in female never-smokers.

The present study had several strengths. First, we performed our investigation on individuals who were under the coverage of a national health insurance and received a national health screening examination. Our study evaluated a total of 5,831,039 individuals, which is a large sample size compared to previous studies [9]. This approach can reduce biases attributed to medical inequality on lung cancer detection in smaller cohort samples. Second, our exposure assessment relied on annually-updated addresses on the 100-m grid. These spatially-resolved and mobility-incorporated exposures helped accurate assessment of the association with lung cancer incidence [27].

There were several limitations in our study. First, the PM_{10} level estimated by our kriging model using geographic information and regulatory monitoring data is not exactly same with actual PM_{10} exposure at individual level. Discrepancy between indoor and outdoor air pollution level and distant movement while awake needs to be considered but relevant information was not available. In spite of this major limitation on interpreting results, well-designed studies on environmental epidemiology have used geographic information system-based spatiotemporal exposure model, because any alternative measure on air pollution exposure at the individual basis was not available. In fact, most epidemiologic studies evaluate the impact of air pollution on human health with an effect estimation model using continuously measured PM_{10} like ours [28]. In the United States, a prospective study used a prediction model for spatiotemporal exposure to PM_{10} based on a 100-m grid geographic information [24]. In Europe, a multicentre prospective study assessed PM_{10} exposure by land-use regression models [21]. In addition, even if individual exposure measurement is possible, many new problems arise due to errors between measuring devices. Second, data on ambient PM_{10} levels before 2002 were not available in South Korea. Third, our study used a relatively short period of exposure before lung cancer incidence. There should be a long latency period before the detection of lung cancer as a result of past exposure to PM_{10}. However, the optimal lag time to evaluate the risk for lung cancer after PM_{10} exposure has not been evaluated yet [27]. Fourth, other ambient exposures were not considered in this analysis. Occupational or environmental exposure is related with a higher risk of lung cancer in never-smokers. In addition, second-hand smoke exposure can increase the risk of lung cancer among never-smokers, especially in female [29]. Nevertheless, we could not adjust these confounders because of lack of information. Fifth, histological subtype data of lung cancer was not available in our cohort dataset. Considering that female lung cancer has been increasing and a majority of lung cancers in female were adenocarcinoma, lung adenocarcinoma incidence may specifically be at a risk of increasing alongside higher exposure levels of PM_{10} in females [7]. In a previous systematic review and meta-analysis, adenocarcinoma was reportedly associated with outdoor PM_{10} [9].

In conclusion, our study suggests that long-term exposure to PM is associated with lung cancer development. An extended indication of lung cancer screening examination needs to be considered to include never-smokers depending on the degree of population exposure to PM.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

Our study protocol was authorized by the institutional review board committee in Seoul National University Bundang Hospital. Informed consent was waived (IRB No. X-2101-661-904).

Author Contributions

Conceived and designed the analysis: Lee HW, Kim SY, Cho YJ, Hwang S.
Collected the data: Kang SC.
Contributed data or analysis tools: Lee HW, Kang SC, Cho YJ, Hwang S.
Performed the analysis: Lee HW, Kang SC.
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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

Acknowledgments
This research was supported by the Seoul National University Bundang Hospital Research Fund (No. 13-2018-014) and the National Cancer Center, Korea (No. NCC-2110570).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.698
Cancer Res Treat. 2022;54(4):1038-1052

Original Article

Clinical Evidence of Chemotherapy or Endocrine Therapy Maintenance in Patients with Metastatic Breast Cancer: Meta-Analysis of Randomized Clinical Trials and Propensity Score Matching of Multicenter Cohort Study

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Purpose This study aims to comprehensively evaluate the clinical efficacy of chemotherapy or endocrine therapy maintenance in metastatic breast cancer (MBC) patients.

Materials and Methods The meta-analysis of randomized clinical trials (RCTs) and propensity score matching of multicenter cohort study evaluated MBC patients who underwent first-line chemotherapy or endocrine therapy maintenance. This study is registered with PROSPERO: CRD42017071858 and ClinicalTrials.gov: NCT04258163.

Results A total of 2,867 patients from 15 RCTs and 760 patients from multicenter cohort were included. The results from meta-analysis showed that chemotherapy maintenance improved progression-free survival (PFS) (hazard ratio [HR], 0.63; 95% confidence interval [CI], 0.54 to 0.73; p < 0.001; moderate-quality evidence) and overall survival (OS) (HR, 0.87; 95% CI, 0.78 to 0.97; p=0.016; high-quality evidence) than observation. In the cohort study, for hormone receptor–positive MBC patients, chemotherapy maintenance improved PFS (HR, 0.67; 95% CI, 0.52 to 0.85; p < 0.001) and OS (HR, 0.55; 95% CI, 0.42 to 0.73; p < 0.001) compared with observation, and endocrine therapy maintenance also improved PFS (HR, 0.65; 95% CI, 0.53 to 0.80; p < 0.001) and OS (HR, 0.55; 95% CI, 0.44 to 0.69; p < 0.001). There were no differences between chemotherapy and endocrine therapy maintenance in PFS and OS (all p > 0.05). Regardless of the continuum or switch maintenance therapy, showed prolonged survival in MBC patients who were response to first-line treatment.

Conclusion This study provided evidences for survival benefits of chemotherapy and endocrine therapy maintenance in MBC patients, and there was no difference efficacy between chemotherapy and endocrine therapy maintenance for hormone receptor–positive patients.

Key words Metastatic breast neoplasms, Chemotherapy, Endocrine therapy, Overall survival, Progression-free survival

Introduction

The primary goals of treatment for metastatic breast cancer (MBC) are to reduce symptoms, maintain quality of life, slow tumor progression, and extend survival. After first-line chemotherapy, further treatment always determined by the patient's response, individual tolerance, and physician preferences. However, there are several options for MBC patients who are responding to chemotherapy, to continue treatment with a fix number of cycles or until disease progression, stop chemotherapy, take a watch and wait strategy, and the optimal maintenance treatment has not been determined [1-4].

For hormone receptor–positive MBC patients, switch endocrine therapy maintenance is also a common option following first-line chemotherapy. A GINECO group study, phase III trial of taxane plus bevacizumab compared with exemestane plus bevacizumab duration in estrogen receptor–positive, human epidermal growth factor receptor 2 (HER2)–negative MBC patients after first-line taxane and bevacizumab indicated that maintenance therapy with exemestane plus

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Received June 10, 2021 **Accepted** February 2, 2022 **Published Online** February 4, 2022
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bevacizumab did not achieve longer progression-free survival (PFS) [5]. The current clinical practice has established neither a clear clinical benefit of chemotherapy over endocrine therapy maintenance for hormone receptor–positive MBC patients.

Overall, high-quality studies are warranted to further clarify the association between chemotherapy or endocrine therapy maintenance and clinical benefit in patients with MBC after first-line chemotherapy, specifically hormone receptor–positive MBC patients. This study aimed to perform a comprehensive meta-analysis of randomized controlled trials (RCTs) and machine learning propensity score matched analysis of multicenter cohort data to evaluate the efficacy of chemotherapy or endocrine therapy maintenance in MBC patients after first-line chemotherapy.

Materials and Methods

1. Meta-analysis of randomized clinical trials

The meta-analysis was conducted according to the Cochrane Collaboration recommendations and PRISMA statement [6]. We searched PubMed, EMBASE, the Cochrane Central Register of Controlled Trials, and ClinicalTrials.gov for RCTs up to December 30, 2019 using the following terms: "chemotherapy" or "endocrine therapy", "breast cancer" and "randomized clinical trials." The proceedings of American Society of Clinical Oncology, European Society for Medical Oncology and American Society for Therapeutic Radiology and Oncology, and the references in the included RCTs and relevant meta-analysis were also reviewed manually.

Trials with any of the following study designs were included: trials comparing a fixed number of cycles of with a longer cycle, regardless of whether the longer cycle is a few more cycles or until the disease progresses, it also doesn't matter whether maintenance therapy is the original regimen or alternative, chemotherapy or endocrine therapy. We have excluded studies whose abstracts or full texts were no English, and studies that do not have available data. Three investigators (Y.Y., Q.G. and D.L.) screened the eligibility of the studies. The risk of bias was assessed based on the recommendation of the Cochrane Collaboration Handbook [7].

2. Propensity score matched analysis of multicenter cohort study

The multicenter cohort study was reported according to the CONSORT and STROBE guideline. hormone receptor–positive MBC patients who underwent chemotherapy, endocrine therapy maintenance, or observation were retrospectively collected from three hospitals in China between January 2003 and September 2017. A total of 760 patients were recruited from at the Sun Yat-sen Memorial Hospital of Sun Yat-sen University (Guangzhou, China), the Sun Yat-sen University Cancer Center (Guangzhou, China), and the Foshan Afflicted Hospital of Sun Yat-sen University (Foshan, China).

Patient selection was performed according to the following inclusion criteria: (1) primary diagnosed as hormone receptor–positive breast cancer, which was defined as immunohistochemical staining showed that at least 1% of the nuclei were positive for either estrogen receptor or progesterone receptor. (2) Patients with measurable disease, who have response to first-line chemotherapy, including the patients were evaluated as complete response, partial response, or stable disease according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria, ver. 1.1 [8]. (3) After the last cycle of first-line chemotherapy, it was still in a state of no progress for at least 4 weeks, otherwise it was considered to be a failure of first-line chemotherapy. The key exclusion criteria were as follows: (1) the presence of immeasurable disease, and (2) the endocrine therapy was administered before first-line chemotherapy. The data were censored on April 30, 2018. The follow-up was performed according to the recommendation of the National Comprehensive Cancer Network guidelines.

3. Endpoint definition

The primary endpoints were PFS and overall survival (OS). The PFS was defined as the time from therapy to the first assessed progression, or death. The OS was defined as the time from the date of the histologically documented diagnosis to the date of death or final follow-up.

4. Statistical analysis

For the meta-analysis, we pooled the data from different studies using a DerSimonian-Laird random effects model weighted by the sample size in each trial [9]. Then, to incorporate the indirect comparison with the direct comparison, we conducted a random effects Bayesian network meta-analysis. The treatment effect on the time-to-event outcome was estimated by the hazard ratio (HR) with 95% confidence interval (CI). Weighted averages of treatment effects were calculated by pooling log HRs for PFS and OS across the studies, by inverse variance weighting. The I^2 statistic was used to assess the heterogeneity across the trials. $I^2 \geq 50\%$ was considered substantial heterogeneity [10]. The Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology was used to assess the quality of evidence as high, moderate, low, or very low [11].

For the individual patient-level analysis, the exact chi-square test was used to compare the patient characteristics. Propensity score matching was used to reduce baseline bias

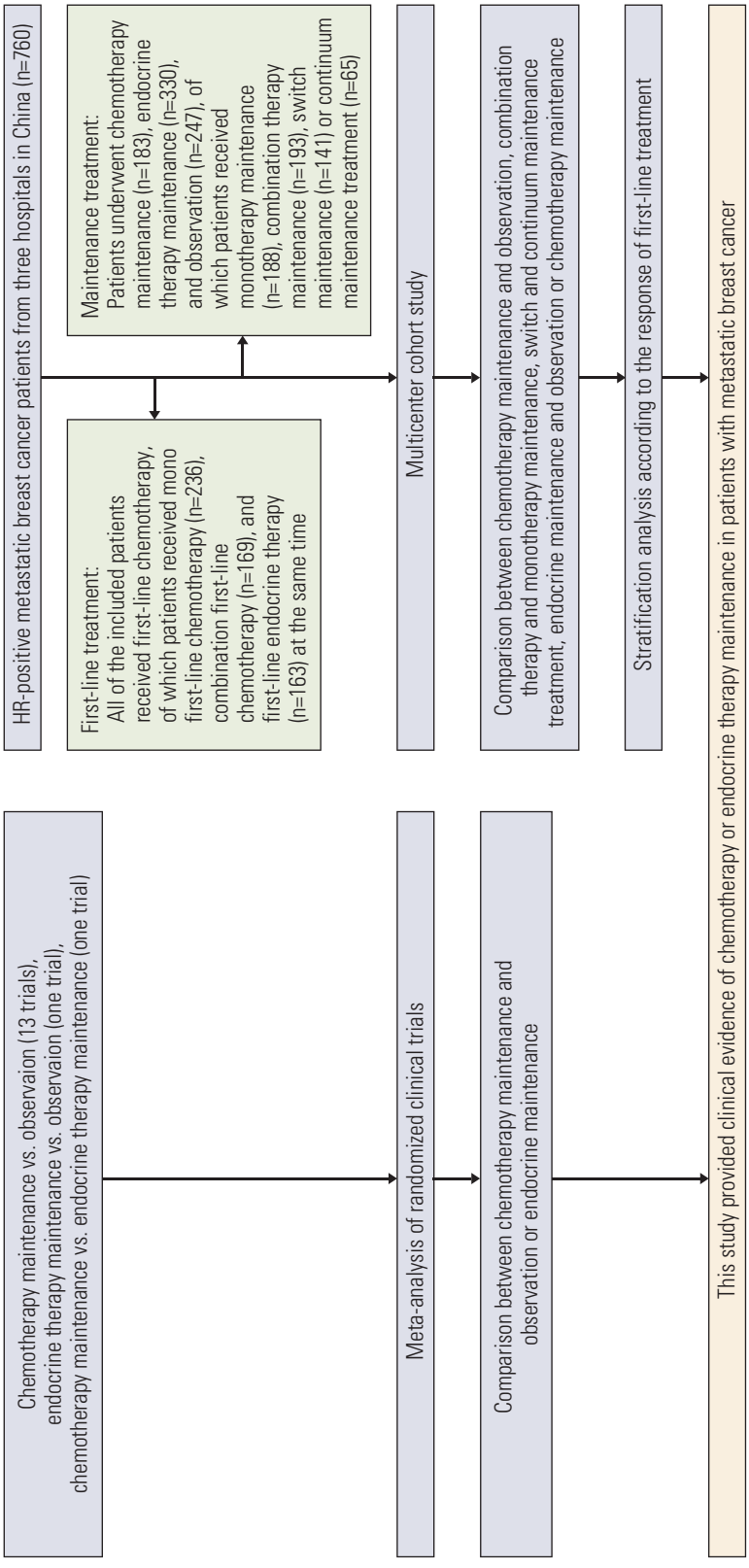


Fig. 1. Study design and patient recruitment. HR, hormone receptor.

Table 1. Characteristics of trials included in the meta-analysis

Study	Year	Chemotherapy regimen	Maintenance duration	Regimen of maintenance	Switch agent therapy	Time of random
			Until PD or additional a fixed No. of cycles	Combination or single-agent therapy		Before/ After first-line CT
Coates et al. [14]	1987	AC or CMF×3 vs. AC or CMF until PD	Until PD	Combination agent	No	Before first-line CT
Harris et al. [19]	1990	Mitox×4 then Mitox until PD vs. control	Until PD	Single-agent therapy	No	After first-line CT
Muss et al. [2]	1991	FAC×6 then CMF×12 vs. control	Additional a fixed No. of cycles	Combination agent	Yes	After first-line CT
Ejlertsen et al. [4]	1993	FEC×8 vs. FEC×24	Additional a fixed No. of cycles	Combination agent	No	Before first-line CT
Gregory et al. [1]	1997	VAC/VEC/MMM×6 then VAC/VEC/ MMM×6 vs. control	Additional a fixed No. of cycles	Combination agent	No	After first-line CT
Falkson et al. [15]	1998	Doxorubicin×6 then CMFPTH until PD vs. control	Until PD	Combination agent	Yes	After first-line CT
Kloke et al. [21]	1999	IE×6 then MPA until PD vs. control	Until PD	Single-agent therapy	Yes	After first-line CT
French Epirubicin Study Group [16]	2000	FEC×4 vs. FEC×11/12	Additional a fixed	Combination agent	No	Before first-line CT
Nooij et al. [22]	2003	CMF×6 then CMF until PD vs. control	Until PD	Combination agent	No	After first-line CT
Gennari et al. [17]	2006	AT/ET×6/8 then TXL ×8 vs. control	Additional a fixed No. of cycles	Combination agent	Yes	After first-line CT
Mayordomo et al. [20]	2009	E×3→TXL×3 then w TXL until PD vs. control	Until PD	Combination agent	Yes	Before first-line CT
Alba et al. [13]	2010	AT×6 then PLD until PD vs. control	Until PD	Combination agent	Yes	After first-line CT
Park et al. [3]	2013	PG×6 then PG until PD vs. control	Until PD	Combination agent	No	After first-line CT
Gligorov et al. [18]	2014	Bev+Doc×3/6 then Bev+X vs. Bev until PD	Until PD	Combination agent	Yes	After first-line CT
Tredan et al. [5]	2016	T+Bev×4/6 then T+Bev vs. E+Bev until PD	Until PD	Combination agent	Yes	After first-line CT

AC, doxorubicin, cyclophosphamide; AT, doxorubicin, paclitaxel; Bev, bevacizumab; Bev+Doc, bevacizumab, docetaxel; Bev+X, bevacizumab and capecitabine; CMF, cyclophosphamide, methotrexate, fluorouracil; CMFPTH, cyclophosphamide, methotrexate, fluorouracil, prednisone, tamoxifen and halotestin; CT, chemotherapy; E, epirubicin; E+Bev, exemestane, bevacizumab; ET, epirubicin, paclitaxel; FAC, fluorouracil, doxorubicin, cyclophosphamide; FEC, fluorouracil, epirubicin, cyclophosphamide; IE, ifosfamide, epirubicin; Mitox, mitoxantrone; MMM, mitoxantrone, methotrexate, mytomicin; MPA, medroxyprogesterone acetate; PD, progressive disease; PG, paclitaxel, gemcitabine; PLD, pegylated liposomal doxorubicin; T+Bev, taxane, bevacizumab; TXL, paclitaxel; VAC, vincristine, doxorubicin, cyclophosphamide; VEC, vincristine, epirubicin, cyclophosphamide; w TXL, weekly paclitaxel.

based on neural network machine learning [12]. The PFS and OS were calculated using the Kaplan-Meier method and the log-rank test. Univariate and multivariable Cox regression models were applied to determine the independent prediction factors. Furthermore, we developed a model to predict the OS and evaluate the suitability of chemotherapy or endocrine therapy maintenance. The optimal cutoff values were used to separate patients into low-risk and high-risk groups were generated using the "survminer" package in R (R Foundation for Statistical Computing, Vienna, Austria).

Table 2. Clinical characteristics among patients with chemotherapy maintenance versus observation before and after propensity score matching in multicenter cohort

Characteristic	No. of patients (%)[a]		p-value	No. of patients (%)[b]		p-value
	Chemotherapy	Observation		Chemotherapy	Observation	
Total	183 (42.6)	247 (57.4)		176 (50.0)	176 (50.0)	
Age (yr)						
Median (95% CI)	49.5 (47.8-51.3)	48.8 (47.2-50.4)	0.548	49.5 (47.7-51.3)	49.0 (47.2-50.8)	0.670
< 50	89 (48.6)	136 (55.1)	0.222	86 (48.9)	94 (53.4)	0.455
≥ 50	94 (51.4)	111 (44.9)		90 (51.1)	82 (46.6)	
Follow-up, median (95% CI, mo)	27.2 (24.0-30.5)	21.1 (17.9-24.3)	0.010	27.6 (24.3-30.9)	20.5 (16.7-24.3)	0.006
ECOG PS						
0-1	169 (92.3)	223 (90.3)	0.566	163 (92.6)	157 (89.2)	0.354
≥ 2	14 (7.7)	24 (9.7)		13 (7.4)	19 (10.8)	
First diagnosis						
Yes	0	2 (0.8)	0.615	0	2 (1.1)	0.478
No	183 (100)	245 (99.2)		176 (100)	174 (98.9)	
Ki-67 status						
< 14	31 (21.5)	22 (14.3)	0.138	29 (20.9)	19 (16.7)	0.493
≥ 14	113 (78.5)	132 (85.7)		110 (79.1)	95 (83.3)	
Bone metastasis						
Yes	108 (59.0)	107 (43.3)	0.002	101 (57.4)	101 (57.4)	> 0.99
No	75 (41.0)	140 (56.7)		75 (42.6)	75 (42.6)	
Liver metastasis						
Yes	61 (33.3)	70 (28.3)	0.314	57 (32.4)	49 (27.8)	0.416
No	122 (66.7)	177 (71.7)		119 (67.6)	127 (72.2)	
Pulmonary metastasis						
Yes	60 (32.8)	77 (31.2)	0.802	55 (31.2)	62 (35.2)	0.497
No	123 (67.2)	170 (68.8)		121 (68.8)	114 (64.8)	
Brain metastases						
Yes	6 (3.3)	18 (7.3)	0.115	6 (3.4)	14 (8.0)	0.107
No	177 (96.7)	229 (92.7)		170 (96.6)	162 (92.0)	
Soft tissue metastasis						
Yes	11 (13.4)	9 (10.7)	0.767	10 (12.7)	6 (9.1)	0.677
No	71 (86.6)	75 (89.3)		69 (87.3)	60 (90.9)	
Lymph node metastasis						
Yes	73 (39.9)	92 (37.2)	0.648	67 (38.1)	68 (38.6)	> 0.99
No	110 (60.1)	155 (62.8)		109 (61.9)	108 (61.4)	
Menopausal status						
Premenopausal	104 (56.8)	138 (55.9)	0.920	101 (57.4)	96 (54.5)	0.668
Postmenopausal	79 (43.2)	109 (44.1)		75 (42.6)	80 (45.5)	
No. of metastatic sites						
1-2	131 (71.6)	198 (80.2)	0.050	131 (74.4)	131 (74.4)	> 0.99
≥ 3	52 (28.4)	49 (19.8)		45 (25.6)	45 (25.6)	
Response to first-line chemotherapy						
CR+PR	82 (44.8)	128 (51.8)	0.180	82 (46.6)	94 (53.4)	0.241
SD	101 (55.2)	119 (48.2)		94 (53.4)	82 (46.6)	

CI, confidence interval; CR, complete response; ECOG, Eastern Cooperative Oncology Group; PR, partial response; PS, performance status; SD, stable disease. [a]Study patients before propensity score matching, [b]Study patients after propensity score matching.

Table 3. Clinical characteristics among patients with endocrine therapy maintenance versus observation before and after propensity score matching in multicenter cohort

Characteristic	No. of patients (%)[a]		p-value	No. of patients (%)[b]		p-value
	Endocrine therapy	Observation		Endocrine therapy	Observation	
Total	330 (57.2)	247 (42.8)		221 (50.0)	221 (50.0)	
Age (yr)						
Median (95% CI)	49.1 (47.8-50.4)	48.8 (47.2-50.4)	0.753	48.4 (46.9-50.0)	49.2 (47.6-50.8)	0.509
< 50	168 (50.6)	136 (55.1)	0.366	119 (53.8)	120 (54.3)	> 0.99
≥ 50	162 (49.4)	111 (44.9)		102 (46.2)	101 (45.7)	
Follow-up, median (95% CI, mo)	35.3 (32.6-38.1)	21.1 (17.9-24.3)	< 0.001	35.0 (31.5-38.5)	19.6 (16.4-22.7)	< 0.001
ECOG PS						
0-1	302 (91.5)	223 (90.3)	0.716	201 (91.0)	197 (89.1)	0.634
≥ 2	28 (8.5)	24 (9.7)		20 (9.0)	24 (10.9)	
First diagnosis						
Yes	1 (0.3)	2 (0.8)	0.801	1 (0.5)	2 (0.9)	> 0.99
No	329 (99.7)	245 (99.2)		220 (99.5)	219 (99.1)	
Ki-67 status						
< 14	56 (22.1)	22 (14.3)	0.069	27 (16.7)	20 (13.9)	0.607
≥ 14	197 (77.9)	132 (85.7)		135 (83.3)	124 (86.1)	
Bone metastasis						
Yes	216 (65.5)	107 (43.3)	< 0.001	107 (48.4)	107 (48.4)	> 0.99
No	114 (34.5)	140 (56.7)		114 (51.6)	114 (51.6)	
Liver metastasis						
Yes	63 (19.1)	70 (28.3)	0.012	51 (23.1)	66 (29.9)	0.131
No	267 (80.9)	177 (71.7)		170 (76.9)	155 (70.1)	
Pulmonary metastasis						
Yes	87 (26.4)	77 (31.2)	0.240	65 (29.4)	70 (31.7)	0.680
No	243 (73.6)	170 (68.8)		156 (70.6)	151 (68.3)	
Brain metastases						
Yes	10 (3.0)	18 (7.3)	0.031	9 (4.1)	17 (7.7)	0.157
No	320 (97.0)	229 (92.7)		212 (95.9)	204 (92.3)	
Soft tissue metastasis						
Yes	13 (8.8)	9 (10.7)	0.803	10 (10.4)	9 (11.0)	> 0.99
No	135 (91.2)	75 (89.3)		86 (89.6)	73 (89.0)	
Lymph node metastasis						
Yes	88 (26.7)	92 (37.2)	0.009	64 (29.0)	84 (38.0)	0.055
No	242 (73.3)	155 (62.8)		157 (71.0)	137 (62.0)	
Menopausal status						
Premenopausal	175 (53.0)	138 (55.9)	0.553	120 (54.3)	122 (55.2)	0.924
Postmenopausal	155 (47.0)	109 (44.1)		101 (45.7)	99 (44.8)	
No. of metastatic sites						
1-2	273 (82.7)	198 (80.2)	0.497	180 (81.4)	172 (77.8)	0.408
≥ 3	57 (17.3)	49 (19.8)		41 (18.6)	49 (22.2)	
Response to first-line chemotherapy						
CR+PR	155 (47.0)	128 (51.8)	0.285	111 (50.2)	120 (54.3)	0.446
SD	175 (53.0)	119 (48.2)		110 (49.8)	101 (45.7)	

CI, confidence interval; CR, complete response; ECOG, Eastern Cooperative Oncology Group; PR, partial response; PS, performance status; SD, stable disease. [a]Study patients before propensity score matching, [b]Study patients after propensity score matching.

Table 4. Clinical characteristics among patients with endocrine therapy versus chemotherapy maintenance before and after propensity score matching in multicenter cohort

Characteristic	No. of patients (%)[a]		p-value	No. of patients (%)[b]		p-value
	Endocrine therapy	Chemotherapy		Endocrine therapy	Chemotherapy	
Total	330 (64.3)	183 (35.7)		176 (50.0)	176 (50.0)	
Age (yr)						
Median (95% CI)	49.1 (47.8-50.4)	49.5 (47.8-51.3)	0.712	49.7 (47.9-51.4)	49.6 (47.8-51.4)	0.943
< 50	168 (50.6)	89 (48.6)	0.688	88 (50.0)	85 (48.3)	0.831
≥ 50	162 (49.4)	94 (51.4)		88 (50.0)	91 (51.7)	
Follow-up, median (95% CI, mo)	35.3 (32.6-38.1)	27.2 (24.0-30.5)	< 0.001	31.3 (28.3-34.4)	27.7 (24.3-31.0)	0.111
ECOG PS						
0-1	302 (91.5)	169 (92.3)	0.871	161 (91.5)	162 (92.0)	> 0.99
≥ 2	28 (8.5)	14 (7.7)		15 (8.5)	14 (8.0)	
First diagnosis						
Yes	1 (0.3)	0	> 0.99	1 (0.6)	0	> 0.99
No	329 (99.7)	183 (100)		175 (99.4)	176 (100)	
Ki67 status						
< 14	56 (22.1)	31 (21.5)	0.989	24 (17.9)	30 (21.9)	0.503
≥ 14	197 (77.9)	113 (78.5)		110 (82.1)	107 (78.1)	
Bone metastasis						
Yes	216 (65.5)	108 (59.0)	0.176	112 (63.6)	104 (59.1)	0.444
No	114 (34.5)	75 (41.0)		64 (36.4)	72 (40.9)	
Liver metastasis						
Yes	63 (19.1)	61 (33.3)	< 0.001	54 (30.7)	54 (30.7)	> 0.99
No	267 (80.9)	122 (66.7)		122 (69.3)	122 (69.3)	
Pulmonary metastasis						
Yes	87 (26.4)	60 (32.8)	0.150	53 (30.1)	60 (34.1)	0.493
No	243 (73.6)	123 (67.2)		123 (69.9)	116 (65.9)	
Brain metastases						
Yes	10 (3.0)	6 (3.3)	> 0.99	8 (4.5)	6 (3.4)	0.785
No	320 (97.0)	177 (96.7)		168 (95.5)	170 (96.6)	
Soft tissue metastasis						
Yes	13 (8.8)	11 (13.4)	0.381	9 (10.5)	11 (14.5)	0.593
No	135 (91.2)	71 (86.6)		77 (89.5)	65 (85.5)	
Lymph node metastasis						
Yes	88 (26.7)	73 (39.9)	0.003	66 (37.5)	66 (37.5)	> 0.99
No	242 (73.3)	110 (60.1)		110 (62.5)	110 (62.5)	
Menopausal status						
Premenopausal	175 (53.0)	104 (56.8)	0.462	92 (52.3)	101 (57.4)	0.392
Postmenopausal	155 (47.0)	79 (43.2)		84 (47.7)	75 (42.6)	
No. of metastatic sites						
1-2	273 (82.7)	131 (71.6)	0.004	133 (75.6)	128 (72.7)	0.626
≥ 3	57 (17.3)	52 (28.4)		43 (24.4)	48 (27.3)	
Response to first-line chemotherapy						
CR+PR	155 (47.0)	82 (44.8)	0.706	81 (46.0)	81 (46.0)	> 0.99
SD	175 (53.0)	101 (55.2)		95 (54.0)	95 (54.0)	

CI, confidence interval; CR, complete response; ECOG, Eastern Cooperative Oncology Group; PR, partial response; PS, performance status; SD, stable disease. [a]Study patients before propensity score matching, [b]Study patients after propensity score matching.

Additionally, to evaluate the potential correlation between the different outcomes, a matrix correlation analysis was first conducted, and a weighted linear regression model was further applied to quantify any existing correlations. An F-statistical significance test of the regression coefficient (β) was performed to confirm the validity of this model. Pearson correlation coefficients (ρ) and the coefficient of determination (R^2) with its 95% CI were used to estimate the strength of the correlation. All statistical tests were two-sided, and p-values less than 0.05 were considered statistically significant. The statistical analyses were performed using R ver. 3.4.3.

This study combined a meta-analysis of RCTs that was registered on PROSPERO (Identifier: CRD42017071858), and a retrospectively, machine learning propensity score matched analysis of multicenter cohort study that has been registered at ClinicalTrials.gov (Identifier: NCT04258163).

Results

1. Trials and patients' characteristics

The study design and patient recruitment shows in Fig. 1. The characteristics of RCTs are summarized in Table 1. This included 15 trials including 2,867 patients, 13 trials [1-4,13-22] compared chemotherapy maintenance and observation, one trial [5] compared endocrine therapy maintenance and observation, and one trial [21] compared chemotherapy and endocrine therapy maintenance. Most trials had a low risk of bias (S1 Fig.).

The multicenter cohort recruited 760 patients, including 183 patients (24.1%) who underwent chemotherapy maintenance, 330 patients (43.4%) received endocrine therapy maintenance, and 247 patients (32.5%) were observation after first-line chemotherapy. All of the included patients received first-line chemotherapy, of which 236 patients (31.1%) received mono first-line chemotherapy, 169 patients (22.2%) received combination first-line chemotherapy, and 163 patients (21.4%) received first-line endocrine therapy at the same time. As for the maintenance treatment, 188 patients (24.7%) were treated with monotherapy of cytotoxic chemotherapy (n=28) or endocrine therapy (n=160), 193 (25.4%) patients received combination therapy of cytotoxic chemotherapy (n=74) or endocrine therapy (n=119), 141 (18.6%) patients underwent switch chemotherapy maintenance (n=42) or endocrine therapy (n=99), and 65 (8.6%) patients received continuum maintenance treatment of cytotoxic chemotherapy (n=20) or endocrine therapy (n=45). Via machine learning-based propensity score matching, there were 176 patients in each group for the comparison between chemotherapy maintenance and observation, 221 patients in each group for the comparison between endocrine therapy maintenance and observation, and 176 patients in each group for the comparison between chemotherapy and endocrine therapy maintenance. The demographic features are detailed in Tables 2, 3, and 4, the baseline bias was reduced after matching.

2. Chemotherapy maintenance with better clinical benefit than observation in RCTs

In the meta-analysis of RCTs, comparing with observation, chemotherapy maintenance significantly improved PFS (HR, 0.63; 95% CI, 0.54 to 0.73; $p < 0.001$; moderate-quality evidence) (Fig. 2A) and OS (HR, 0.87; 95% CI, 0.78 to 0.97; p=0.016; high-quality evidence) (Fig. 2B). Similar survival benefits were also recorded in subgroups defined by timing of random assignment, duration of maintenance chemotherapy in the study arm, combined agent or single-agent chemotherapy maintenance, and switch agent therapy or not (S2 and S3 Tables). The GRADE evidence ranged from moderate to high quality.

3. No difference between chemotherapy and endocrine therapy maintenance in RCTs

In the meta-analysis of RCTs, patients who received endocrine therapy showed similar PFS than chemotherapy maintenance (HR, 1.00; 95% CI, 0.70 to 1.50; p=0.998) (S4A Fig.). Only one trial comparing endocrine therapy maintenance and observation, which found that endocrine therapy maintenance could extend the time to progression (p=0.020), but no improved survival (p=0.390) [21]. The overall network meta-analysis comparison between chemotherapy and endocrine therapy maintenance showed similar PFS (HR, 1.00; 95% CI, 0.73 to 1.37; $p > 0.99$) (S4B Fig.). Patients who received treatment with chemotherapy or endocrine therapy maintenance had similar OS (HR, 1.15; 95% CI, 0.59 to 2.22; p=0.679) (S4C Fig.). The HR of OS for the overall network meta-analysis comparison between chemotherapy and endocrine therapy maintenance was 1.02 (95% CI, 0.68 to 1.53; p=0.920) (S4D Fig.).

4. Chemotherapy maintenance with better clinical benefit than observation in cohort study

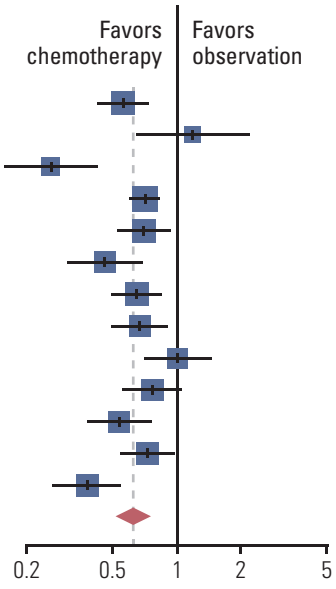
In the multicenter cohort study, before matching, chemotherapy maintenance was associated with a significant improvement in PFS (HR, 0.66; 95% CI, 0.53 to 0.83; $p < 0.001$) (Fig. 3A) and OS (HR, 0.56; 95% CI, 0.43 to 0.73; $p < 0.001$) (Fig. 3B) compared with observation. After matched, chemotherapy maintenance also significantly improved PFS (HR, 0.67; 95% CI, 0.52 to 0.85; $p < 0.001$) (Fig. 3C) and OS (HR, 0.55; 95% CI, 0.42 to 0.73; $p < 0.001$) (Fig. 3D) compared with observation. The majority of subgroups showed the OS advantage of chemotherapy maintenance compared with

A

Progression-free survival

Study	Chemotherapy (total)	Observation (total)	HR (95% CI)	Weight (%)
Coates 1987	154	151	0.56 (0.43-0.73)	8.8
Harris 1990	22	21	1.18 (0.65-2.15)	4.3
Muss 1991	71	74	0.26 (0.16-0.43)	5.4
Ejlertsen 1993	157	161	0.71 (0.61-0.83)	10.5
Gregory 1997	48	52	0.70 (0.53-0.92)	8.6
Falkson 1998	99	96	0.46 (0.31-0.68)	6.7
FESG 2000	266	126	0.65 (0.50-0.84)	8.8
Nooij 2003	96	100	0.67 (0.50-0.90)	8.3
Gennari 2006	109	106	1.01 (0.71-1.43)	7.4
Mayordomo 2009	90	90	0.77 (0.57-1.05)	8.1
Alba 2010	78	77	0.54 (0.39-0.75)	7.6
Park 2013	116	115	0.73 (0.55-0.97)	8.4
Gligorov 2014	91	94	0.38 (0.27-0.54)	7.3
Total	1,397	1,263	0.63 (0.54-0.73)	100.0

Heterogeneity: I^2=70%, T^2=0.0539, p < 0.001
Test for overall effect: Z=–5.92 (p < 0.001)



B

Overall survival

Study	Chemotherapy (total)	Observation (total)	HR (95% CI)	Weight (%)
Coates 1987	154	151	0.77 (0.60-0.98)	11.0
Harris 1990	22	21	1.06 (0.57-1.97)	2.8
Muss 1991	71	74	1.11 (0.74-1.67)	5.7
Ejlertsen 1993	157	161	0.78 (0.63-0.97)	12.5
Gregory 1997	48	52	0.81 (0.54-1.21)	5.8
Falkson 1998	99	96	0.94 (0.69-1.28)	8.4
FESG 2000	266	126	0.96 (0.78-1.18)	12.9
Nooij 2003	96	100	1.03 (0.83-1.27)	12.6
Gennari 2006	109	106	1.12 (0.73-1.72)	5.3
Mayordomo 2009	90	90	0.94 (0.67-1.32)	7.4
Alba 2010	78	77	0.86 (0.58-1.27)	6.0
Park 2013	116	115	0.65 (0.42-1.00)	5.3
Gligorov 2014	91	94	0.43 (0.26-0.70)	4.3
Total	1,397	1,263	0.87 (0.78-0.97)	100.0

Heterogeneity: I^2=36%, T^2=0.0140, p=0.100
Test for overall effect: Z=–2.41 (p=0.016)

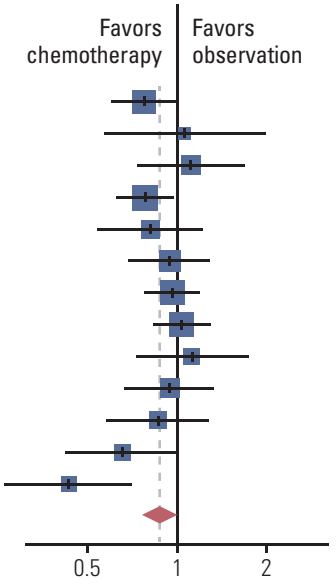


Fig. 2. Pooled HRs for progression-free survival (A) and overall survival (B) with chemotherapy maintenance versus observation [1-4,13-20,22]. CI, confidence interval; FESG, French Epirubicin Study Group; HR, hazard ratio.

the observation (S5 Fig.). Furthermore, comparing with combination therapy of cytotoxic chemotherapy, monotherapy of cytotoxic chemotherapy after first-line treatment in MBC showed similar PFS (HR, 0.94; 95% CI, 0.56 to 1.59; p=0.832) and OS (HR, 0.71; 95% CI, 0.40 to 1.27; p=0.241). The comparison between the switch and continuum chemotherapy maintenance treatment was also performed. Results showed that there were no differences between switch and continuum chemotherapy maintenance in PFS (HR, 0.67; 95% CI, 0.32 to 1.42; p=0.285) and OS (HR, 0.54; 95% CI, 0.19 to 1.58; p=0.254) (S6 Fig.).

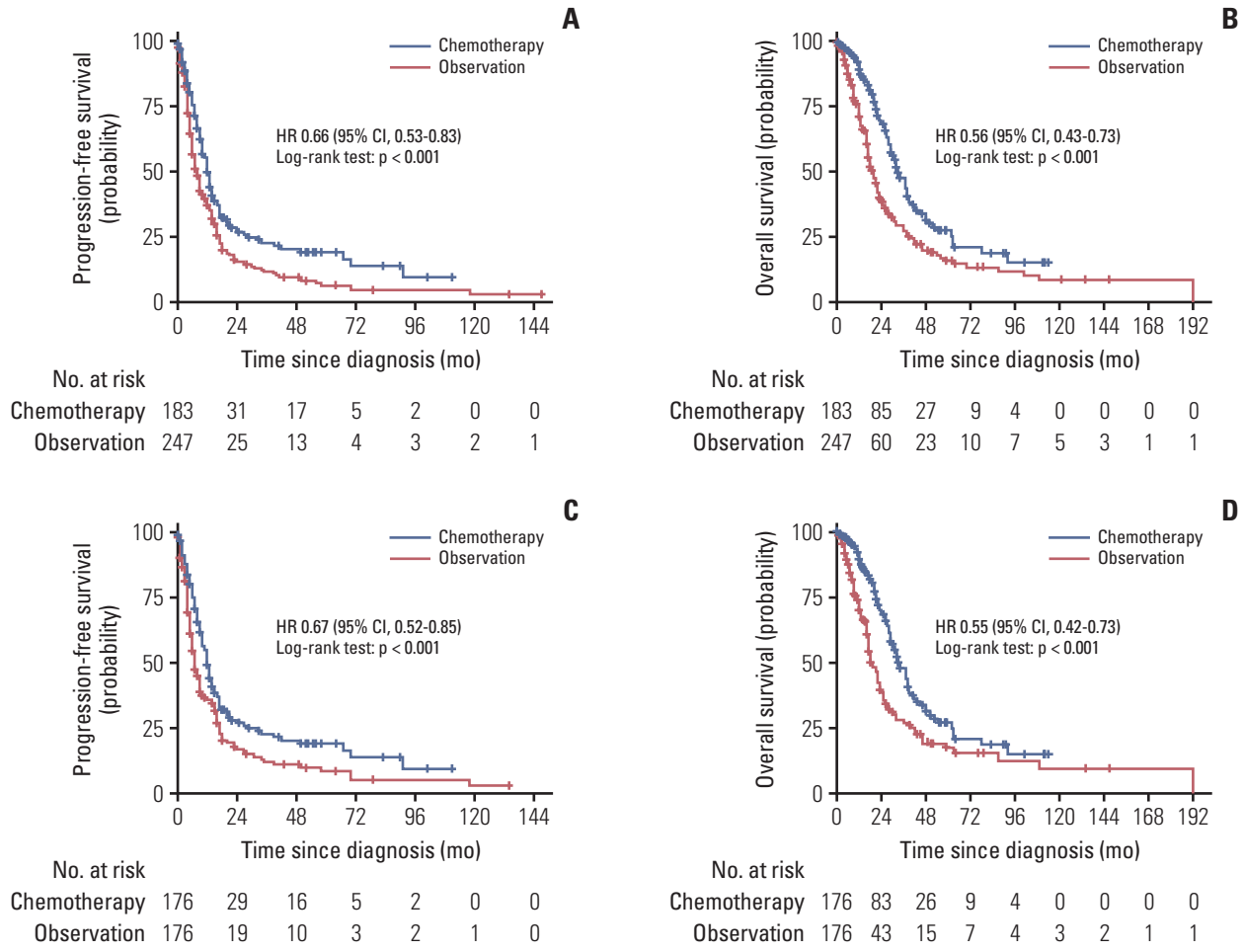


Fig. 3. Progression-free survival and overall survival among patients with chemotherapy maintenance versus observation before and after matching in multicenter cohort: progression-free survival before matching (A), overall survival before matching (B), progression-free survival after matching (C), and overall survival after matching (D). CI, confidence interval; HR, hazard ratio.

5. Endocrine therapy maintenance with better clinical benefit than observation in cohort study

In the cohort study, for hormone receptor–positive MBC patients, before matching, compared with the observation group, the endocrine therapy maintenance group significantly prolonged the PFS (HR, 0.63; 95% CI, 0.53 to 0.76; p < 0.001) (Fig. 4A) and OS (HR, 0.56; 95% CI, 0.45 to 0.69; p < 0.001) (Fig. 4B). After matching, endocrine therapy maintenance also improved PFS (HR, 0.65; 95% CI, 0.53 to 0.80; p < 0.001) (Fig. 4C) and OS (HR, 0.55; 95% CI, 0.44 to 0.69; p < 0.001) (Fig. 4D). Compared with observation group, the endocrine therapy maintenance group showed OS superiority in most subgroups, more results are shown in S7 Fig. It was observed that there were no differences between combination therapy and monotherapy of endocrine therapy maintenance in PFS (HR, 1.12; 95% CI, 0.87 to 1.44; p=0.397) and OS (HR, 1.11; 95% CI, 0.82 to 1.49; p=0.497). Results of the comparison between switch and continuum endocrine therapy maintenance indicated that continuum endocrine therapy maintenance significantly improved PFS (HR, 0.63; 95% CI, 0.43 to 0.92; p=0.017), whereas no differences were presented in OS (HR, 0.99; 95% CI, 0.64 to 1.52; p=0.948) (S8 Fig.).

6. No difference between chemotherapy and endocrine therapy maintenance in cohort study

In the cohort study, for hormone receptor–positive MBC patients, before matching, there were no differences between chemotherapy and endocrine therapy maintenance in PFS (HR, 1.03; 95% CI, 0.84 to 1.27; p=0.760) (Fig. 5A) and OS (HR, 1.06; 95% CI, 0.83 to 1.35; p=0.660) (Fig. 5B). After matching, the chemotherapy was also similar to the endocrine therapy maintenance in PFS (HR, 0.96; 95% CI, 0.76 to 1.21; p=0.726) (Fig. 5C) and OS (HR, 0.85; 95% CI, 0.65 to 1.11;

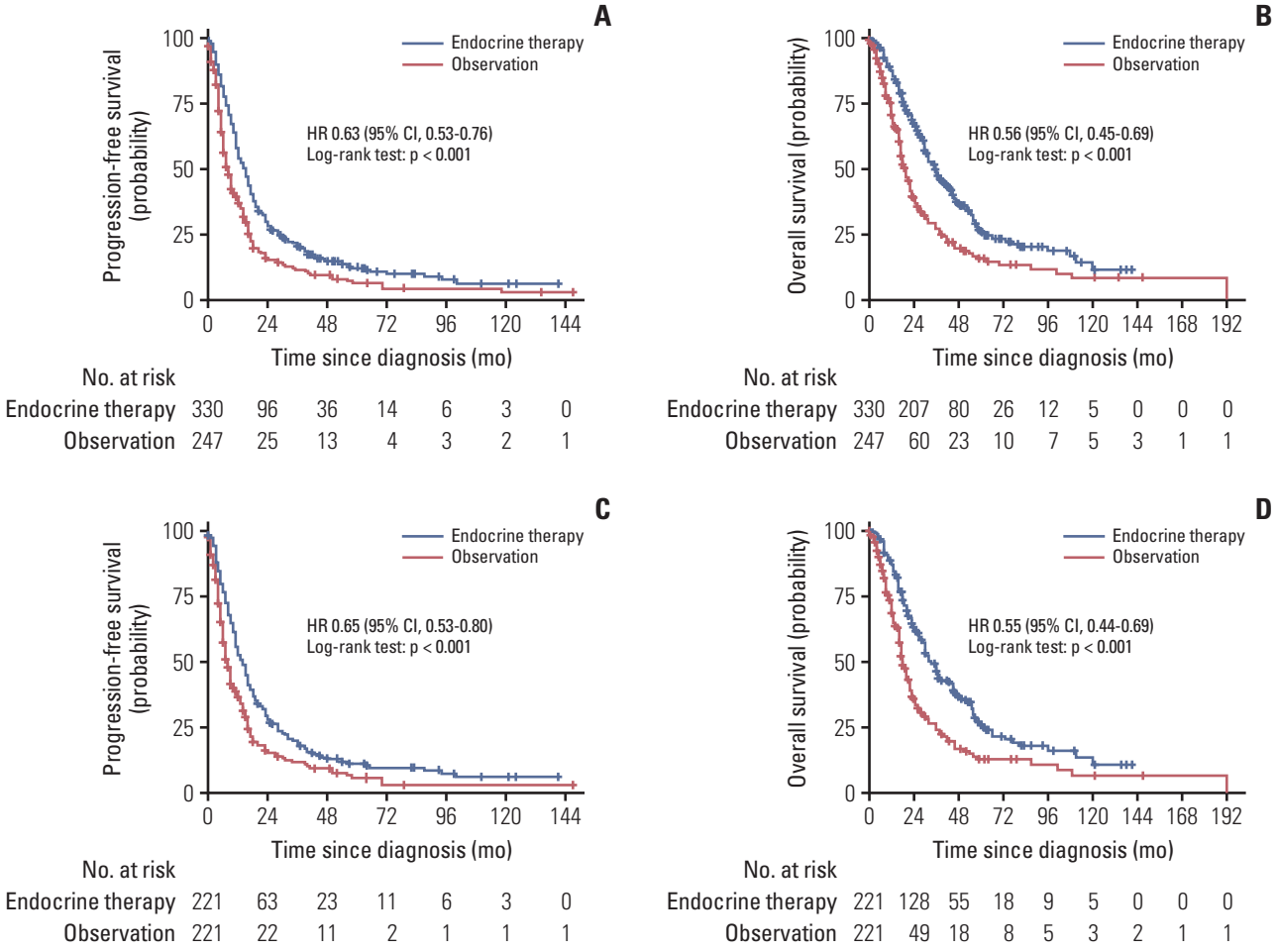


Fig. 4. Progression-free survival and overall survival among patients with endocrine therapy maintenance versus observation before and after matching in multicenter cohort: progression-free survival before matching (A), overall survival before matching (B), progression-free survival after matching (C), and overall survival after matching (D). CI, confidence interval; HR, hazard ratio.

p=0.219) (Fig. 5D). Chemotherapy and endocrine therapy maintenance have comparable OS in most subgroups, more results are shown in S9 Fig.

Furthermore, for all 513 patients who received chemotherapy or endocrine therapy maintenance, we built a prediction model incorporating factors with the response to first-line chemotherapy (HR, 1.83; 95% CI, 1.45 to 2.30), liver metastasis or not (HR, 2.05; 95% CI, 1.61 to 2.62), pulmonary metastasis or not (HR, 1.61; 95% CI, 1.27 to 2.03), soft tissue metastasis or not (HR, 1.20; 95% CI, 1.06 to 1.35), lymph node metastasis or not (HR, 1.90; 95% CI, 1.50 to 2.40), and Ki-67 ≥ 14% or < 14% (HR, 1.35; 95% CI, 1.17 to 1.55), and then a risk score of death was calculated for each patient using a formula derived from the levels of these predictive variables weighted by their corresponding regression coefficients as follows: Risk score= (0.49359×level of best response to chemotherapy)+(0.64785× level of liver metastasis)+(0.33138×level of pulmonary metastasis)+(0.28485×level of soft tissue metastasis)+(0.62951× level of lymph node metastasis)+(0.36584×level of ki67 expression) to categorize patients into high-risk or low-risk group according to OS. After obtaining the risk scores of deaths from the prediction model, the patients were separated into low-risk and high-risk groups (HR for PFS, 0.44; 95% CI, 0.35 to 0.55; p < 0.001; HR for OS, 0.30; 95% CI, 0.23 to 0.40; p < 0.001) (S10 and S11 Figs.). There was no significant difference in PFS or OS between chemotherapy or endocrine therapy maintenance in either high-risk (HR for PFS, 0.88; 95% CI, 0.70 to 1.12; p=0.296; HR for OS, 0.81; 95% CI, 0.62 to 1.06; p=0.122) (S12A and S12B Fig.) or low-risk groups (HR for PFS, 1.01; 95% CI, 0.63 to 1.61; p=0.971; HR for OS, 1.31; 95% CI, 0.74 to 2.35; p=0.358) (S12C and S12D Fig.).

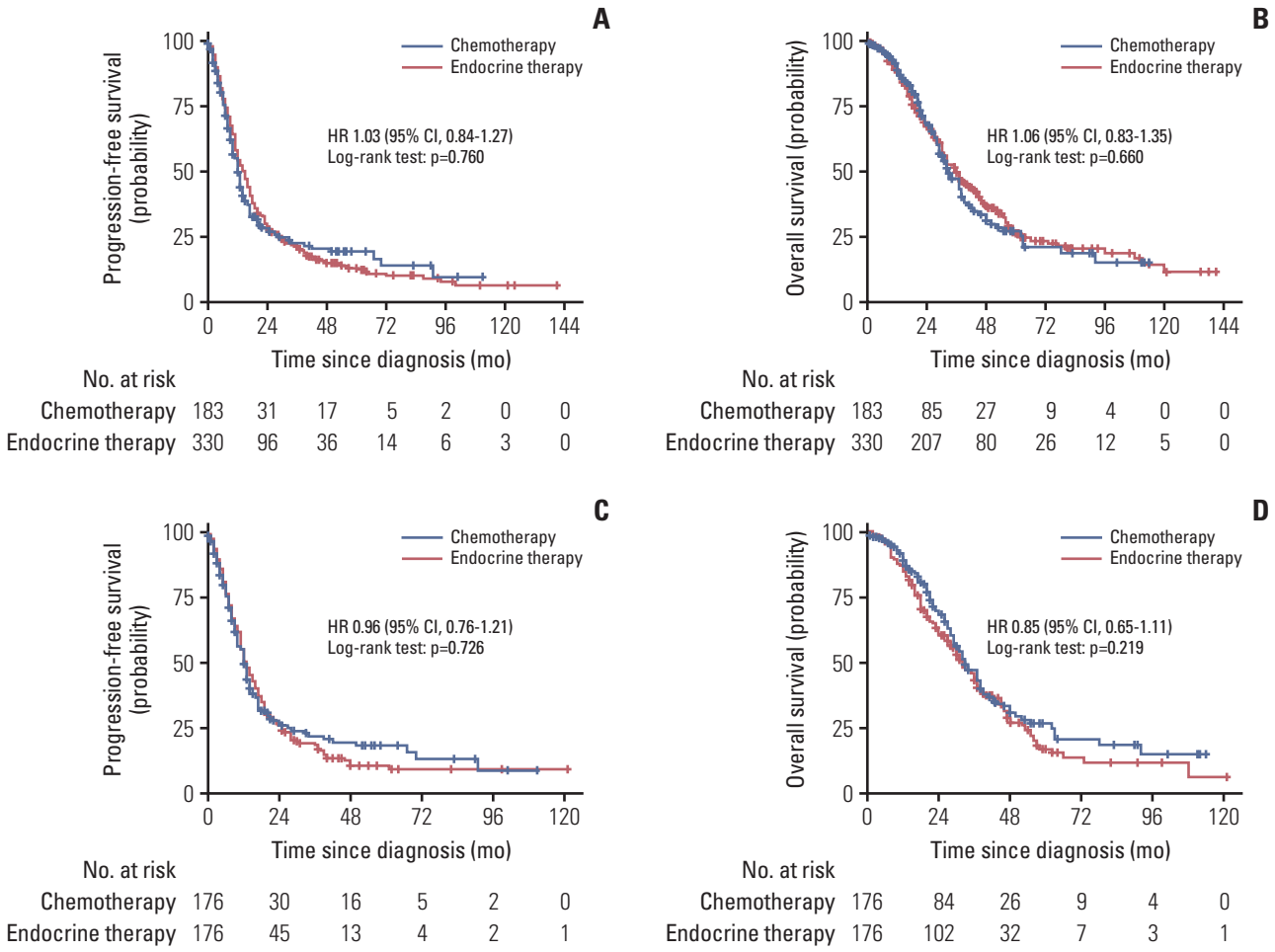


Fig. 5. Progression-free survival and overall survival among patients with chemotherapy versus endocrine therapy maintenance before and after matching in multicenter cohort: progression-free survival before matching (A), overall survival before matching (B), progression-free survival after matching (C), and overall survival after matching (D). CI, confidence interval; HR, hazard ratio.

7. Stratification analysis according to the response of first-line treatment in cohort study

In the cohort study, for the complete response patients after first-line treatment, patients received endocrine therapy showed similar PFS (HR, 0.39; 95% CI, 0.14 to 1.11; p=0.064) (S13A Fig.) and OS (HR, 1.17; 95% CI, 0.14 to 9.49; p=0.867) (S13B Fig.) than chemotherapy maintenance. Similar PFS (HR, 0.80; 95% CI, 0.31 to 2.07; p=0.622) (S13C Fig.) and OS (HR, 0.97; 95% CI, 0.24 to 3.93; p=0.969) (S13D Fig.) benefits were observed in patients received monotherapy and combination therapy. Patients who received switch or continuum maintenance treatment also had similar PFS (HR, 0.82; 95% CI, 0.22 to 2.98; p=0.718) (S13E Fig.) and OS (p=0.073) (S13F Fig.). For the patients who were partial response to the first-line treatment, there were no differences between chemotherapy and endocrine maintenance therapy in PFS (HR, 0.83; 95% CI, 0.56 to 1.23; p=0.343) (S14A Fig.) and OS (HR, 0.83; 95% CI, 0.52 to 1.34; p=0.444) (S14B Fig.). Comparing with combination therapy, monotherapy maintenance significantly improved PFS (HR, 1.48; 95% CI, 1.00 to 2.20; p=0.047) (S14C Fig.), whereas no differences were observed in OS (HR, 1.37; 95% CI, 0.86 to 2.18; p=0.177) (S14D Fig.). Moreover, the switch maintenance was similar to the continuum maintenance treatment in PFS (HR, 0.56; 95% CI, 0.31 to 1.02; p=0.058) (S14E Fig.) and OS (HR, 0.89; 95% CI, 0.46 to 1.70; p=0.701) (S14F Fig.). As for patients with stable disease after the first-line treatment, the chemotherapy was similar to the endocrine maintenance therapy in PFS (HR, 0.86; 95% CI, 0.60 to 1.23; p=0.409) (S15A Fig.) and OS (HR, 0.75; 95% CI, 0.50 to 1.14; p=0.176) (S15B Fig.). Monotherapy maintenance showed similar PFS (HR, 1.26; 95% CI, 0.88 to 1.80; p=0.197) (S15C Fig.) and OS (HR, 1.44; 95% CI, 0.94 to 2.21; p=0.087) (S15D Fig.) compared with combination therapy. Patients who received switch or continuum maintenance

treatment had similar PFS (HR, 0.66; 95% CI, 0.39 to 1.14; p=0.138) (S15E Fig.) and OS (HR, 0.94; 95% CI, 0.49 to 1.79; p=0.854) (S15F Fig.).

8. PFS as surrogate for OS

In order to further explore the potential surrogate value of PFS for OS in maintenance treatment in MBC patients. Differences were greater with PFS than OS for trials of chemotherapy maintenance compared with observation (HR, 0.72; 95% CI, 0.59 to 0.80; p < 0.001) (S16 Fig.), and the correlation coefficient R^2 between treatment effects on PFS and on OS was 12% (95% CI, 8% to 16%) when all trials were considered to 40% (95% CI, 30% to 54%) after exclusion of one highly influential trial3 by sensitivity analysis (S17 Fig.). Additionally, in the cohort study, among patients (n=513) who were treated with chemotherapy or endocrine therapy maintenance, the association between PFS and OS was R^2=0.609 (p < 0.001) (S18 Fig.).

Discussion

This study based on 15 RCTs including 2,867 patients and a multicenter cohort recruited 760 patients quantitatively evaluated the clinical benefits of chemotherapy or endocrine therapy maintenance after first-line chemotherapy for MBC, which indicated that maintenance treatment has clinically benefits for both PFS and OS than observation in MBC patients, and there were no difference efficacy between chemotherapy and endocrine therapy maintenance for hormone receptor–positive MBC patients. Additionally, treatment effect sizes were greater for OS than for PFS, and a moderate correlation between PFS and OS was identified for determining the effectiveness of maintenance treatment.

Results of this study were consistent with a meta-analysis of the duration of chemotherapy for MBC [23], which showed prolonged chemotherapy had a statistically significant survival advantage, thus support policies to extend treatment until the disease progresses without unacceptable toxicity. We included several new trials [3,5,18,21] that were not included in the previous study [23], in particular studies that included new antitumor drugs including gemcitabine and capecitabine. A meta-analysis [24] included four RCTs with 1,044 participants and found that the combination of doublet chemotherapy with trastuzumab compared with single-agent chemotherapy as first-line therapy for HER2-positive MBC is associated with longer PFS and OS, and recommended that doublet chemotherapy appears to be an appropriate regimen for good performance status patients. This meta-analysis with multicenter cohort study findings support PFS and OS benefit of chemotherapy than observation after first-line chemotherapy in MBC. Furthermore, it was observed that comparing with combination therapy, monotherapy maintenance significantly improved PFS for the patients who were partial response to the first-line treatment. Overall, we recommend the use of sequential monotherapy chemotherapy maintenance for MBC patients, and combination of doublet chemotherapy recommend for patients who with rapid disease progression, or life-threatening visceral metastases occurs, or the need for rapid symptom or disease control is present.

Previous meta-analysis study included eight RCTs with 4,580 participants indicated that cyclin-dependent kinases 4 and 6 inhibitors combined with endocrine therapy can significantly prolong PFS, OS and improve the objective response rate, clinical benefit response in patients with hormone receptor–positive, HER2-negative advanced breast cancer [25]. But even in the first-line treatment of hormone receptor–positive MBC patients, more than half of the patients receive chemotherapy as the first-line treatment, for the reasons that patients with high tumor load and visceral crisis, clinicians who consider that some patients need a fast response, and the efficacy of chemotherapy is higher than that of endocrine therapy [26]. It is worth noting that there are few studies provide high evidence for endocrine therapy maintenance after disease control by previous chemotherapy in hormone receptor–positive MBC patients.

Moreover, in previous clinical practice, using endocrine therapy maintenance before disease progression might not be recommended after first-line chemotherapy, patients who received endocrine therapy before disease progression may lose the opportunity to receive other endocrine therapy after disease progression [27]. Due to the limited trials comparing endocrine therapy maintenance and observation in hormone receptor–positive MBC patients, our multicenter cohort data analysis provides evidence that the endocrine therapy maintenance plays an important role in hormone receptor–positive MBC patients.

This study further confirmed the benefit of maintenance endocrine therapy in hormone receptor–positive MBC patients, which could provide clinical evidence for further clinical trials. Although the results revealed that chemotherapy and endocrine therapy maintenance have similar effects on PFS and OS, further validation in prospective clinical trials was needed. Independent biomarkers, such as circulating tumor cell, long noncoding RNAs and tumor immune-microenvironment were adequately predicting therapeutic response and identifying patients who could derive the greatest therapeutic benefit in breast cancer [28-30]. But there were no clear evidences that tumor immune-microenvironment biomarkers can guide maintenance therapy. Therefore, in order to further validate the results of this study, over-

come within-tumor microenvironments heterogeneity, and explore the mechanism of maintenance therapy efficacy, we conducted a phase 3 randomized trial comparing the efficacy of fulvestrant versus capecitabine as maintenance therapy after first-line combination chemotherapy in patients with hormone receptor+/HER2– MBC, and the trial recruitment is ongoing (NCT04263298).

There are some limitations in this study. The heterogeneity of molecular subtype of MBC, and the schedule of chemotherapy that some of the regimens used in the study are outdated from the current point of view. Although prolonged chemotherapy maintenance has significant clinical benefits, which can reduce symptoms and improve quality of life by delaying disease progression, however, only two studies included in the meta-analysis focused on the quality of life. Due to the retrospective nature of the multicenter cohort study and the meta-analytic approach taken in this study, not all of the included patients have available data for us to further analyze. Additionally, due to a lack of available tumor microenvironment-based variables, we were unable to further consider the potential mechanisms driving the interaction between clinical benefit and tumor microenvironment, which warrants further investigation to better guide chemotherapy or endocrine therapy maintenance precisely.

In conclusion, this study provided evidences for PFS and OS benefits of chemotherapy or endocrine therapy maintenance over observation after first-line chemotherapy in MBC, and there was no difference efficacy between chemotherapy and endocrine therapy maintenance for hormone receptor–positive MBC patients. Additionally, treatment effect sizes were greater for OS than for PFS, and a moderate correlation between PFS and OS was identified and suggested that both PFS and OS should be evaluated to determine the effectiveness of maintenance therapy in future clinical trials.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study protocol was approved by the ethics committee (SYSEC-KY-KS-2019-171-001) of the Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Author Contributions

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Contributed data or analysis tools: Ren W, Yu Y, Hong H, Wang Y, Gao Q, Chen Y, Yao H.
Performed the analysis: Ren W, Yu Y, Hong H, Wang Y, Gao Q, Chen Y, Yao H.
Wrote the paper: Ren W, Yu Y, Hong H, Wang Y, Gao Q, Chen Y, Chen P, Zhao J, Ou Q, Lin D, Fu T, Tan Y, Li C, Xie X, Ye G, Tang J, Yao H.
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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

We thank the patients who participated in this study and all investigators contributed to these trials. This study was supported by grants 81972471 and 82073408 from the National Natural Science Foundation of China, grants 202206010078 and 202201020574 from the Guangzhou Science and Technology Project, grant 2018007 from the Sun Yat-Sen University Clinical Research 5010 Program, grant SYS-C-201801 from the Sun Yat-Sen Clinical Research Cultivating Program, grant A2020558 from the Guangdong Medical Science and Technology Program, grant 7670020025 from Tencent Charity Foundation, grant YXQH202209 from the Scientific Research Launch Project of Sun Yat-Sen Memorial Hospital.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.748
Cancer Res Treat. 2022;54(4):1053-1064

Original Article

Clinical Significance of Major Angiogenesis-Related Effectors in Patients with Metastatic Breast Cancer Treated with Trastuzumab-Based Regimens

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Purpose Angiogenesis is a crucial phenomenon in the development and progression of breast cancer (BC), but the clinical significance of angiogenesis-related proteins in metastatic BC remains unknown. This study investigates the prognostic value of vascular endothelial growth factor receptors 1, 2, 3 (VEGFR1, VEGFR2, VEGFR3) as well as vascular endothelial growth factors A and C (VEGFA and VEGFC) in metastatic BC patients treated with trastuzumab-based regimens.

Materials and Methods Two hundred female patients were included. Protein and mRNA expression of the studied angiogenesis-related factors were evaluated by immunohistochemistry and quantitative polymerase chain reaction, respectively.

Results High expression of VEGFA, VEGFC, VEGFR1, VEGFR2, and VEGFR3 in the tumor cells was observed in 43.5%, 24.2%, 36%, 29.5%, and 43%, respectively. Stromal elements expressed high levels of VEGFA, VEGFC, VEGFR1, VEGFR2, and VEGFR3 in 78.9%, 93.3%, 90.7%, 90.2%, and 74.8% of tumors with available data. High tumor cell expression of VEGFR1 was a favorable prognosticator for survival among patients with human epidermal growth factor receptor 2 (HER2)–positive tumors (hazard ratio [HR], 0.55; p=0.013). A trend towards longer progression-free survival was detected univariately for patients with HER2-negative tumors and high expression of VEGFR2 (HR, 0.60; p=0.059).

Conclusion VEGFR1 and VEGFR2 seem to have significant prognostic value in BC patients with metastatic disease treated with trastuzumab-based regimens.

Key words Breast neoplasms, HER2, Vascular endothelial growth factor A, VEGFR, Angiogenesis, Prognosis

Introduction

Breast cancer (BC) remains the most frequent cancer type worldwide and one of the leading cancer-related causes of death in women [1]. Locally limited disease is curable in 70%-80% of patients, while distant metastases result in poor prognosis despite the vast variety of recent emerging therapeutic options [2].

Human epidermal growth factor receptor 2 (HER2) targeting, one of the most successful achievements in the treatment of BC during the last 30 years, originated from the identification of HER2, a surface tyrosine kinase receptor with pivotal role in the initiation and development of HER2-enriched BC [3]. Slamon et al. [4] showed that the combination of the anti-HER2 monoclonal antibody trastuzumab with chemotherapy in metastatic BC patients, yields significant improvements in objective responses and duration of responses, and prolongs time to disease progression and overall survival (OS), reforming the treatment landscape of HER2-positive disease.

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Received June 23, 2021 **Accepted** November 15, 2021 **Published Online** November 17, 2021
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Although the mechanism of function and clinical aspects of trastuzumab in the treatment of BC have been extensively studied, the role of angiogenesis in the management of HER2-positive BC is still unclear. Angiogenesis is a crucial factor in the initiation and development of BC and has been recently reviewed elsewhere [5].

Preclinical studies have outlined the cross-talk pathways between HER2 signaling and angiogenesis in animal and human cell lines, linking HER2 activation with vascular endothelial growth factor (VEGF) mRNA expression, hypoxia-inducible factor-α (HIF-α) synthesis and microvessel counts (reviewed by Alameddine et al. [6]). In clinical studies, BC tissue samples were found to display HER2 expression in significant association with VEGF and the proangiogenic cyclooxygenase-2 (COX-2) (reviewed by Alameddine et al. [6]). The mechanisms linking HER2 signaling with angiogenesis involve primarily direct induction of COX-2 transcription, activation of mitogen-activated protein kinases (MAPKs) and resultant VEGF transcription and activation of phosphoinositide 3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) and MEK-ERK-mTOR with subsequent HIF-α expression (reviewed by Alameddine et al. [6]). Clinical studies of HER2-positive BC, at various phases (0-III), have investigated the use of anti-angiogenic agents along with HER2 targeting, namely employing the anti-VEGF monoclonal antibody bevacizumab, in the neoadjuvant or adjuvant setting, anti–vascular endothelial growth factor receptor tyrosine kinase inhibitors, mTOR inhibitors, COX-2 inhibitors or the antiangiogenic effect of metronomic administration of chemotherapeutic regimens. Frequent obstacles in the establishment of such regimens involve toxicity or modest results (reviewed by Alameddine et al. [6]), both accentuated by the still elusive reliable predictive factors.

In the present study, in view of the link between HER2 signaling and angiogenesis, we hypothesized that the expression of VEGFA and VEGFC and VEGF receptors 1, 2, 3 (VEGFR1, VEGFR2, VEGFR3), the pivotal angiogenesis effectors, may have clinical prognostic significance for female BC patients with metastatic disease treated with trastuzumab.

Materials and Methods

1. Study design, population, and data collection

The study was conducted following the Helsinki Declaration on ethical guidelines (2013) [7] was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine and was performed by the Hellenic Cooperative Oncology Group (HeCOG).

It is a retrospective translational research study of patients with histologically confirmed, metastatic BC, treated with trastuzumab-based regimens. The medical records of all patients were retrospectively reviewed. Adequacy of clinical, pathological and treatment data on patients' medical records and availability of adequate tumor tissue were also included in the eligibility criteria. Initial HER2 expression was centrally reassessed due to known issues with inter-laboratory discordances. All experiments were designed and performed in the Laboratory of Molecular Oncology, Aristotle University of Thessaloniki.

2. Immunohistochemical analysis

Formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples were retrospectively collected from the enrolled patients. Representative hematoxylin-eosin-stained tumor sections were reviewed by a pathologist (M.B.) and representative tumor areas were selected for the construction of the tissue microarray (TMA) blocks, as previously described [8]. Each case was represented by two tissue cores, 1.5 mm in diameter. Various neoplastic, non-neoplastic, and reactive tissues were also included in each TMA block to assist in the orientation and provide internal assay controls. Cases inadequately represented on the TMA sections were re-cut from the original blocks and whole tissue sections were used for immunohistochemical analysis.

Serial 2.5-μm-thick thick tissue sections from the TMA or the original blocks were used for the immunohistochemical staining, performed in the Laboratory of Molecular Oncology of the Hellenic Foundation of Cancer Research / Aristotle University of Thessaloniki, using a Bond MaxTM autostainer machine (Leica Microsystems, Wetzlar, Germany). The primary antibodies employed, retrieval conditions, dilution, and incubation time are presented in Table 1, as previously described [9].

3. Evaluation of immunohistochemistry

All immunohistochemical stains were assessed by one pathologist (E.K.) blinded to the case and patient characteristics. The angiogenesis-related proteins VEGFA, VEGFC VEGFR1, VEGFR2, and VEGFR3 were variably expressed and evaluated in the neoplastic cells, stromal lymphocytes, plasma cells, and the vascular endothelium. The non-neoplastic elements served as internal positive control in each tissue core. Scoring of immunostaining was performed based on the Allred score (values 0-8), calculated as the sum of points for the percentage of stained neoplastic cells (points 0-5) and the staining intensity (points 0-3), as follows: points 0-5 correspond to the following percentages of positive tumor cells 0%, < 1%, 1%-10%, 11%-33%, 34%-66%, and 67%-100%, respectively; points 0-3 for intensity were assigned for negative, weak, moderate or intense staining intensity, respectively.

Table 1. Primary antibodies and staining conditions

Antibody	Clone/Source	Dilution	Antigen retrieval	Incubation time
VEGF-A (m)	VG1/Dako, Glostrup, Denmark	1:75	20/EDTA	60 min
VEGF-C (r, PL)	Z-CVC7/Zymed, Invitrogen, Carlsbad, CA	1:250	20/CA	Overnight
VEGFR1 (r)	RB-1527/Thermo Fisher Scientific, Fremont, CA	1:450	15/CA	Overnight
VEGFR2 (r)	55B11/Signaling Technology, Beverly, MA	1:450	20/EDTA	Overnight
VEGFR3 (m)	KLT9/Novocastra, Leica Biosystems, Newcastle Upon Tyne, UK	1:50	15/CA	Overnight

CA, citric acid, pH 6.0; m, mouse; PL, polyclonal; r, rabbit.

4. Gene expression analysis

The protein expression and mRNA levels of VEGFA, VEGFC, VEGFR1, VEGFR2, and VEGFR3, were evaluated in FFPE tumor tissues. FFPE tissue blocks were processed for RNA extraction, following histological examination to estimate tumor tissue abundance and mark areas with highest tumor cell density. Manual macrodissection was performed in cases with < 50% tumor cell content. RNA extraction from whole or macrodissected 10-μm paraffin sections was carried out with a fully automated nucleic acid isolation method, based on silica-coated magnetic beads (Versant Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) in combination with a liquid handling robot, as before [10,11]. Finally, to ensure the presence of pure RNA, DNase I was added to each nucleic acid extract to remove DNA.

Subsequent cDNA synthesis was performed with random primers and SuperScript III reverse transcriptase (cat. No. 48190011 and 18080044, Invitrogen, Carlsbad, CA), according to standard procedures. cDNAs were assessed in duplicate 10 μL reactions with quantitative polymerase chain reaction in an ABI7900HT system for 45 cycles of amplification using premade exon-spanning Taqman-MGB assays (Applied Biosystems/Life Technologies, Fisher Scientific, Foster City, CA) for the following transcripts (data in parentheses refer to assay ID and amplicon size): *FLT1* (VEGFR1) exons 17-18 (Hs00176573_m1; 55 bp), *KDR* (VEGFR2) exons 15-16 (Hs00176676_m1; 84 bp), *VEGFC* exons 4-5 (Hs00153458_m1; 126 bp), *VEGFA* exons 1-2 (Hs00173626_m1; 77 bp), *HIF1A* exons 4-5 (Hs00153153_m1; 76 bp), *NRP1* exons 7-8 (Hs00826128_m1; 90 bp), *TEK* exons 11-12 (Hs00176096_m1; 82 bp), *VCAM1* exons 7-8 & 8-9, (Hs00365486_m1; 122 bp), *VHL* (Von Hippel-Lindau tumor suppressor) exons 2-3 (Hs00184451_m1; 72 bp).

For the detection of VEGF xa and xb transcript variants, custom assays were designed spanning exons 7b-8a for VEGFA165a, 189a, 206a, and exons 7b-8b for VEGF165b, 189b, 206b (sense primer, antisense primer, and, Taqman MGB probe, all sequences 5′-3′): for xa, AAACACAGACTCGCGTTGCA, AGAGATCTGGTTCCCGAAACC, and CGAGGCAGCTTGAG; for xb, AGGCGAGGCAGCTTGAGTTA, ACGTTCTGTCGATGGTGATGGT, and CGAACGTACTTGCAGATC. The size of xa transcripts was 129 bp and for xb 122 bp, respectively.

A Taqman-MGB expression assay targeting β-glucuronidase (*GUSB*) exons 8-9 (Hs00939627_m1; 96 bp) was used as the endogenous reference for relative quantification. The commercially available TaqMan Control Total RNA (cat. No. 4307281, Applied Biosystems) was applied as a positive control for inter-run evaluation of polymerase chain reaction assay efficiency, alongside no-template controls. Finally, to obtain linear relative quantification values, relative expression was assessed as (40-dCT), as described before [10]. Samples were considered eligible for GUSB CT < 36 and deltaRQ for each duplicate pair (intra-run variation) of < 1. Based on the above criteria, RNA samples for 60 patients yielded informative results for all aforementioned targets and were considered eligible for relative mRNA expression analysis.

The biomarkers were associated with patient characteristics and other markers of interest, related to HER2 resistance and available in the HeCOG database, including PTEN (phosphatase and tensin homolog), phosphorylated forms of HER2 ($pHER2^{Tyr\ 877}$ $pHER2^{Tyr\ 1221/1222}$) and HER3 protein expression, *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) status as well as mRNA expression of *HER2*, *HER3*, *HER4*, *Src* (SRC proto-oncogene, non-receptor tyrosine kinase), *CDKN1B* (cyclin dependent kinase inhibitor 1B), and *JAK2* (Janus kinase 2).

5. Statistical analysis

Descriptive statistics including counts with the corresponding percentages (for categorical variables) and medians with range (for continuous variables) were used to summarize patient characteristics and the distribution of examined markers for the entire cohort and by central HER2 status (according to central assessment of HER2). The median value of the Allred scores was used as a cut-off point for VEGFR1, VEGFR2, VEGFR3, VEGFA, and VEGFC to categorize tumors into low or high-expressing. The associations of the

expression of examined markers with patient characteristics and other markers of interest were assessed in the entire cohort using the chi-square or the Fisher exact test if appropriate (for categorical variables) and the Wilcoxon rank-sum test (for continuous variables). Spearman correlations were used to evaluate the associations between the mRNA levels of the markers of interest.

Progression-free survival (PFS) was defined as the time from the initiation of trastuzumab treatment for metastatic BC (with or without concurrent chemotherapy/hormonal therapy) to the date of the first documented disease progression, death from any cause or last contact (whichever occurred first). Survival was measured from the initiation of trastuzumab treatment to the date of death (from any cause), with patients alive and those lost to follow-up being censored at the date last known to be alive. Survival curves were estimated with the Kaplan-Meier method and compared between groups with the log-rank test.

The prognostic value of the tumoral and stromal expression of the examined markers was evaluated with respect to PFS and survival separately in patients with HER2-positive and HER2-negative tumors, only for those markers with adequate number of patients and events of interest (PFS or survival events) in each group. The prognostic value of the mRNA expression of examined markers was not assessed due to the small number of patients with available data. All parameters were tested for proportionality using time-dependent covariates. The associations between the stromal and tumoral expression of the examined markers and progression/mortality rates were assessed with hazard ratios (HR) and 95% confidence intervals (CI) estimated by univariate Cox proportional hazard regression models. Multivariate Cox regression analyses with a backwards selection criterion of $p < 0.10$ were also performed, including: menopausal status (premenopausal, postmenopausal), performance status (0, 1-2), estrogen receptor (ER)/progesterone receptor (PR) status (negative, positive), number of metastatic sites (1-2, 3), as well as each marker that was found to be a significant prognosticator or revealed a trend towards significance in the univariate analyses ($p < 0.10$).

Follow-up information for all patients was updated in October 2019. All tests were two-sided at an alpha 5% level of significance. Analyses were conducted using the SAS ver. 9.3 software (SAS Institute Inc., Cary, NC).

Results

1. Patient population

A total of 200 female patients, with available data for at least one of the markers of interest, were included in the current study. Trastuzumab was administered for HER2-positive disease according to the original HER2 assessment in the local institution. Nevertheless, upon central re-evaluation of HER2 status, 78 patients (39%) were found to have in fact HER2-negative disease. Selected patient and tumor characteristics according to central assessment of HER2 status are depicted in Table 2. The median age at the time of trastuzumab initiation was 57 years (range, 28 to 95 years), with patients carrying HER2-negative tumors being older (Wilcoxon rank-sum p=0.041). Most patients had relapsed metastatic BC (R-MBC), while 31.5% had *de novo* metastatic disease.

2. Trastuzumab exposure

In total, 86% of the study cohort received trastuzumab as first-line treatment (59 HER2-negative and 113 HER2-positive), while 12.5% of patients were treated with second line trastuzumab (16 HER2-negative, 9 HER2-positive). Additionally, in three patients with HER2-negative tumors, trastuzumab was administered as a third-, fourth-, and seventh-line treatment, respectively. In 90% of patients, trastuzumab was given with concurrent chemotherapy (66 HER2-negative and 114 HER2-positve), while 16 patients (8%) received trastuzumab in combination with hormone therapy and in four patients (2%) the drug was administered as monotherapy.

3. Distribution of examined markers

The expression of VEGFA, VEGFC, VEGFR1, VEGFR2, and VEGFR3 in the neoplastic cells was cytoplasmic. No membranous staining was observed. Nuclear expression was observed in 42.6% (20 of 47 informative cases) for VEGFA, 35% (62 of 177 informative cases) for VEGFC, 42.5% (82 of 193 informative cases) for VEGFR1, 64.6% (122 of 189 informative cases) for VEGFR2, and 17.9% (19 of 106 informative cases) for VEGFR3. For all immunohistochemically examined parameters, except VEGFC, the Allred score values ranged from 0-8, the median Allred score value was 6, and the mean ranged from 5.75-6.02. For VEGFC the Allred score values ranged from 3-8, the median value was 7 and the mean 6.84. Using the median value as a cutoff, high expression (greater than the median) of VEGFA, VEGFC, VEGFR1, VEGFR2, and VEGFR3 in tumor cells was observed in 43.5%, 24.2%, 36%, 29.5%, and 43%, respectively. Stromal elements, namely lymphocytes, plasma cells and endothelial cells, displayed expression of VEGFA, VEGFC, VEGFR1, VEGFR2, and VEGFR3 in 78.9%, 93.3%, 90.7%, 90.2%, and 74.8% of patients with available data, respectively. The frequency distribution of tumor and stromal expression was similar in patients with HER2-positive and HER2-negative disease (S1 Table). mRNA expression of the examined markers was available for tumors of 60 patients (30%) and did not differ between patients with HER2-positive and HER2-negative disease.

Table 2. Selected patient and tumor characteristics

	Total (n=200)	HER2-negative (n=78)	HER2-positive (n=122)
Age (yr)[a]			
Median (min-max)	57.2 (28.4-95.0)	59.3 (31.8-78.8)	55.1 (28.4-95.0)
Menopausal status[a]			
Postmenopausal	152 (76.0)	62 (79.5)	90 (73.8)
Premenopausal	47 (23.5)	16 (20.5)	31 (25.4)
Unknown	1 (0.5)	0	1 (0.8)
PS[a]			
0	142 (71.0)	56 (71.8)	86 (70.5)
1	48 (24.0)	18 (23.1)	30 (24.6)
2	9 (4.5)	3 (3.8)	6 (4.9)
Unknown	1 (0.5)	1 (1.3)	0
Histological grade			
I	7 (3.5)	3 (3.8)	4 (3.3)
II	77 (38.5)	31 (39.7)	46 (37.7)
III	103 (51.5)	39 (50.0)	64 (52.5)
Unknown	13 (6.5)	5 (6.4)	8 (6.6)
ER/PR status			
Negative	50 (25.0)	11 (14.1)	39 (32.0)
Positive	150 (75.0)	67 (85.9)	83 (68.0)
Subtypes			
HER2-enriched	39 (19.5)	0	39 (32.0)
Luminal A	15 (7.5)	15 (19.2)	0
Luminal B	50 (25.0)	50 (64.1)	0
Luminal HER2	83 (41.5)	0	83 (68.0)
TNBC	11 (5.5)	11 (14.1)	0
Unknown	2 (1.0)	2 (2.6)	0
No. of metastatic sites[a]			
1-2	184 (92.0)	71 (91.0)	113 (92.6)
≥ 3	15 (7.5)	6 (7.7)	9 (7.4)
Unknown	1 (0.5)	1 (1.3)	0
Visceral metastasis[a]			
No	65 (32.5)	28 (35.9)	37 (30.3)
Yes	133 (66.5)	48 (61.5)	85 (69.7)
Unknown	2 (1.0)	2 (2.6)	0
R-MBC	137 (68.5)	53 (67.9)	84 (68.9)
History of adjuvant CT[b]	113 (82.5)	45 (84.9)	68 (81.0)
History of adjuvant RT[b]	72 (52.6)	28 (52.8)	44 (52.4)
History of adjuvant HT[b]	98 (71.5)	39 (73.6)	59 (70.2)
***De novo* MBC**	63 (31.5)	25 (32.1)	38 (31.1)

Values are presented as number (%) unless otherwise indicated. CT, chemotherapy; ER, estrogen receptor; HER2, human epidermal growth factor receptor; HT, hormonal therapy; MBC, metastatic breast cancer; PR, progesterone receptor; PS, performance status; R-MBC, relapsed metastatic breast cancer; RT, radiotherapy; TNBC, triple-negative breast cancer. [a]At the time of trastuzumab initiation, [b]Only for patients with R-MBC.

Tumors from patients with *de novo* metastatic BC had higher VEGFxa mRNA expression (Wilcoxon rank-sum p=0.008), as compared to those with R-MBC, and expressed less frequently tumor VEGFC and stromal VEGFR1 (chi-square p < 0.001 and p=0.019, respectively) (S2 Table). No differences in the distribution of the examined markers were detected between patients with ER/PR-positive and negative tumors (data not shown).

Table 3. Significant associations of examined markers with patient characteristics and other markers of interest

	Total	Low	High	p-value
Tumor VEGFA				
pHER2$^{Tyr\ 1221/1222}$ protein expression	155			0.019[a]
Negative	105 (67.7)	61 (76.3)	44 (58.7)	
Positive	50 (32.3)	19 (23.8)	31 (41.3)	
HER3 protein expression	135			0.004[a]
Negative	40 (29.6)	28 (40.6)	12 (18.2)	
Positive	95 (70.4)	41 (59.4)	54 (81.8)	
PTEN status	161			0.027[a]
Loss	91 (56.5)	55 (64.7)	36 (47.4)	
No loss	70 (43.5)	30 (35.3)	40 (52.6)	
Tumor VEGFC				
Menopausal status	185			0.001[a]
Postmenopausal	140 (75.7)	114 (81.4)	26 (57.8)	
Premenopausal	45 (24.3)	26 (18.6)	19 (42.2)	
Age	186			0.031[b]
Median (min-max)	57.1 (28.4-95.0)	57.7 (32.1-95.0)	53.6 (28.4-85.9)	
Tumor VEGFR1				
Visceral metastases	185			0.008[a]
No	58 (31.4)	45 (38.1)	13 (19.4)	
Yes	127 (68.6)	73 (61.9)	54 (80.6)	
Tumor VEGFR2				
Histological grade	168			0.033[a]
I-II	73 (43.5)	45 (38.1)	28 (56.0)	
III	95 (56.5)	73 (61.9)	22 (44.0)	
JAK2 mRNA expression	117			0.016[b]
Median (min-max)	36.9 (27.9-41.0)	37.3 (30.5-41.0)	36.6 (27.9-38.9)	
PTEN status	155			0.033[a]
Loss	88 (56.8)	70 (61.9)	18 (42.9)	
No loss	67 (43.2)	43 (38.1)	24 (57.1)	
Tumor VEGFR3				
pHER2$^{Tyr\ 877}$ protein expression	153			0.029[a]
Negative	125 (81.7)	73 (88.0)	52 (74.3)	
Positive	28 (18.3)	10 (12.0)	18 (25.7)	
PTEN status	160			0.004[a]
Loss	92 (57.5)	59 (67.8)	33 (45.2)	
No loss	68 (42.5)	28 (32.2)	40 (54.8)	
Stromal VEGFR3				
JAK2 mRNA expression	86			0.007[b]
Median (min-max)	37.1 (28.7-41.0)	37.8 (30.2-39.4)	36.7 (28.7-41.0)	
HER2 mRNA expression	69			0.005[b]
Median (min-max)	39.3 (27.0-42.9)	37.8 (27.0-42.5)	39.9 (28.7-42.9)	

Values presented as median (min-max) or number (%). HER, human epidermal growth factor receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor. [a]Pearson's chi-square/Fisher's exact test, [b]Wilcoxon rank-sum test.

4. Association with clinicopathological characteristics and other markers of interest

Patients with VEGFR1-high tumors had more frequently visceral metastases in comparison to the VEGFR1-low BCs (p=0.008). VEGFR2-low tumors were more frequently of higher grade, with loss of PTEN and higher *JAK2* mRNA expression compared to VEGFR2-high tumors (p=0.033, p=0.033, and p=0.016, respectively). VEGFR3-high expression was associated with pHER2^{Tyr877} protein expression and preservation of PTEN (p=0.029 and p=0.004, respectively).

Patients carrying tumors with high tumor VEGFC expression compared to those with low VEGFC expression were of younger age at the time of trastuzumab administration and less frequently of postmenopausal status (p=0.031 and p=0.001, respectively). Tumors with high tumor VEGFA expression had more frequently positive pHER2$^{Tyr1221/1222}$ (p=0.019) and HER3 protein expression (p=0.004) and PTEN preservation (p=0.027). Tumors with stromal VEGFR3 high expression had lower mRNA levels of *JAK2* and higher levels of *HER2* (p=0.007 and p=0.005, respectively) (Table 3). In addition, *VEGFA* mRNA expression was positively, marginally correlated with *Src* (rho=0.27, p=0.045) and *VEGFR1* mRNA expression was positively correlated with *HER2* mRNA expression (rho=0.33, p=0.015). Positive correlations were also observed between *VHL* mRNA expression and *Src* (rho=0.42, p=0.012), *CDKN1B* (rho=0.37, p=0.006), *JAK2* (rho=0.28, p=0.044), *HER2* (rho=0.38, p=0.005), *HER3* (rho=0.44, p < 0.001), and *HER4* mRNA (rho=0.33, p=0.016). However, none of these correlations were strong. No further significant associations were detected between the expressions of examined markers and patient characteristics or other markers of interest.

We further examined potential associations between expression of the examined markers in the neoplastic cells. Tumors with high VEGFR1 expression had less frequently low tumor expression of VEGFR2 (p=0.014), VEGFR3 (p=0.003), and VEGFA (p=0.043) as compared to tumors with low VEGFR1 expression. In addition, tumors with low expression of VEGFR3, as compared to those with high expression, had more frequently low expression of VEGFR1 (p=0.003), VEGFR2 (p=0.012), VEGFC (p=0.006), and VEGFA (p < 0.001) (Table 4). Co-expression of tumor VEGFR1 and VEGFR2 was noticed in 26 tumors (15.1%), VEGFR1 and VEGFA in 35 tumors (19.3%), VEGFR2 and VEGFA in 26 tumors (15.3%), and VEGFR3 and VEGFA in 45 tumors (25.1%).

5. Association of markers with clinical outcome

Within a median follow-up of 13.3 years (95% CI, 12.8 to 14.4), a total of 180 events of progression or death (PFS events) were reported and 168 patients (84%) died. The median PFS was 11.5 months (95% CI, 9.6 to 14.0). Patients with HER2-positive tumors had longer PFS compared to those with HER2-negative disease (median PFS, 14.0 months [95% CI, 11.0 to 19.6] vs. 8.9 months [95% CI, 7.8 to 10.7]; log-rank p=0.018). The median survival was 3.4 years (95% CI, 2.9 to 3.9) and was significantly longer for patients with HER2-positive compared to those with HER2-negative tumors (median survival, 4.0 years [95% CI, 3.1 to 5.0] vs. 2.9 years [95% CI, 2.3 to 3.4]; log-rank p=0.010).

Among patients with HER2-positive BC, none of the exa-

Table 4. Associations between the neoplastic cell expression of examined markers

	VEGFR1			VEGFR2			VEGFR3			VEGFA		
	Low	High	p-value	Low	High	p-value	Low	High	p-value	Low	High	p-value
VEGFR1												
Low	-	-	-	83 (68.0)	24 (48.0)	0.014	76 (73.1)	40 (51.9)	0.003	72 (69.2)	42 (54.5)	0.043
High	-	-		39 (32.0)	26 (52.0)		28 (26.9)	37 (48.1)		32 (30.8)	35 (45.5)	
VEGFR2												
Low	83 (77.6)	39 (60.0)	0.014	-	-	-	76 (78.4)	45 (60.8)	0.012	70 (73.7)	49 (65.3)	0.24
High	24 (22.4)	26 (40.0)		-	-		21 (21.6)	29 (39.2)		25 (26.3)	26 (34.7)	
VEGFR3												
Low	76 (65.5)	28 (43.1)	0.003	76 (62.8)	21 (42.0)	0.012	-	-	-	70 (68.0)	31 (40.8)	< 0.001
High	40 (34.5)	37 (56.9)		45 (37.2)	29 (58.0)		-	-		33 (32.0)	45 (59.2)	
VEGFC												
Low	90 (78.9)	45 (69.2)	0.15	90 (74.4)	39 (76.5)	0.77	84 (83.2)	51 (65.4)	0.006	77 (77.0)	57 (72.2)	0.46
High	24 (21.1)	20 (30.8)		31 (25.6)	12 (23.5)		17 (16.8)	27 (34.6)		23 (23.0)	22 (27.8)	
VEGFA												
Low	72 (63.2)	32 (47.8)	0.043	70 (58.8)	25 (49.0)	0.24	70 (69.3)	33 (42.3)	< 0.001	-	-	-
High	42 (36.8)	35 (52.2)		49 (41.2)	26 (51.0)		31 (30.7)	45 (57.7)		-	-	

Values are presented as number (%). VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

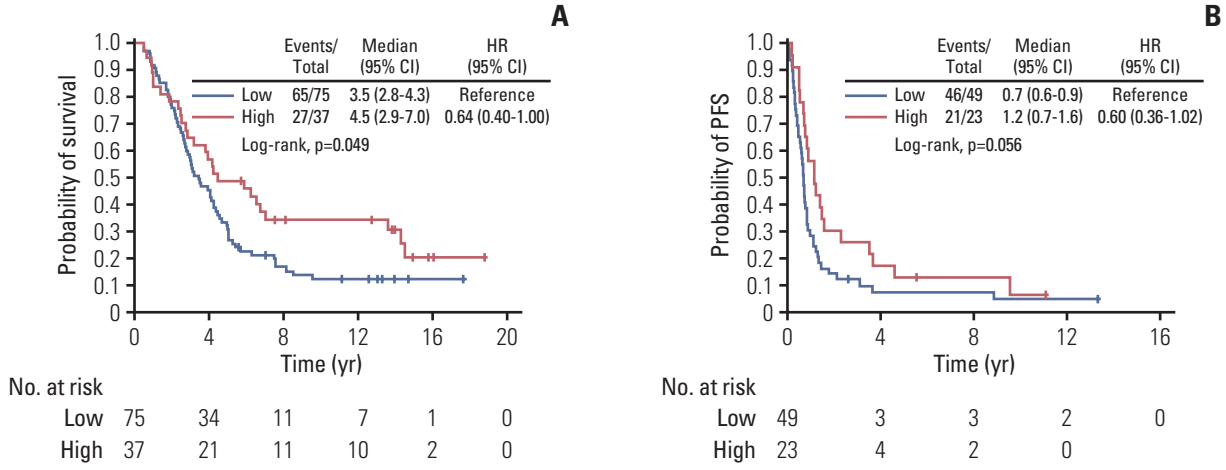

Fig. 1. Kaplan-Meier curves with respect to survival based on tumor cell expression of VEGFR1 in patients with HER2-positive tumors (A) and PFS based on tumor cell VEGFR2 expression in patients with HER2-negative disease (B). CI, confidence interval; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; PFS, progression-free survival; VEGFR, vascular endothelial growth factor receptor.

mined markers showed prognostic significance for PFS univariately, whereas high VEGFR1 expression in the tumor cells was marginally associated with longer survival (HR, 0.64; 95% CI, 0.40 to 1.00; Wald's p=0.051) (Fig. 1A). In multivariate analysis, high expression of VEGFR1 remained a favorable prognostic factor for survival among patients with HER2-positive tumors (HR, 0.55; 95% CI, 0.34 to 0.88; p=0.013) along with 1-2 PS (HR, 0.58; 95% CI, 0.37 to 0.93; p=0.025), while menopausal status, ER/PR status and the number of metastatic sites were removed from the final model (p=0.23, p=0.51, and p=0.16, respectively).

A trend towards longer PFS was detected in univariate analysis for patients with HER2-negative tumors and high tumor expression of VEGFR2 (HR, 0.60; 95% CI, 0.36 to 1.02; p=0.059) (Table 5, Fig. 1B). However, high expression of VEGFR2 was not found to be prognostic for PFS upon adjustment for selected clinicopathological parameters (p=0.19).

Tumor co-expression of VEGF1/VEGFR2, VEGFR1/VEGFA, VEGFR2/VEGFA, and VEGFR3/VEGFA did not show prognostic significance either among patients with HER2-positive tumors or among those with HER2-negative disease (Table 5).

Discussion

Over the last decades, the clinical course of metastatic BC has been transformed most remarkably via HER2 targeting. Although many aspects of HER2-positive BC and trastuzumab effects are illuminated, the clinical significance of angiogenesis in patients treated with trastuzumab-based regimens is still unclear. In this study, we assessed the protein and gene expression of pivotal angiogenesis-related molecules with regards to their interrelations and prognostic value in metastatic BC patients, treated with trastuzumab.

A major finding of this study was the correlation of VEGFR1 immunohistochemical expression by tumor cells with prolonged survival, in HER2-positive patients. Similarly, Lebok et al. [12] have reported that reduced or lost membranous expression of VEGFR1 in BC patients is associated with poor prognosis, and a previous study from our group found that VEGFR1 mRNA expression had prognostic value, depending on HER2 status, in patients with high-risk early BC treated in the adjuvant setting [8]. On the contrary, Ghosh et al. [13], using data from a cohort of 642 cases with primary breast carcinomas treated with a combination of surgical excision with or without local irradiation and/or hormonal therapy, have reported that high levels of VEGFR1 were significantly associated with decreased OS. In addition, Kosaka et al. [14] have shown that increased VEGFR1 mRNA levels in peripheral blood of BC patients with stage 0 to III disease and positive or negative ER/PR/HER2 status, who underwent surgery, are associated with larger tumor size, lymph node (LN) infiltration, advanced clinical stage as well as with poor survival outcome. Along these lines, a previous report from our group of 124 metastatic HER2-negative BC patients found that high expression of VEGFR1 was associated with poor survival in multivariate analysis [9]. The above findings highlight the biological heterogeneity of BC and suggest that in the various intrinsic subtypes of BC, importantly based on HER2 status the effect of angiogenesis-related factor may differ.

Another interesting finding was the significant association of VEGFR1 tumor expression with visceral metastases. The

Table 5. Expression of the studied molecules and hazard ratios estimated by univariate Cox regression analysis

	HER2-positive						HER2-negative					
	PFS			Survival			PFS			Survival		
	Events/ Total	HR (95% CI)	p-value	Events/ Total	HR (95% CI)	p-value	Events/ Total	HR (95% CI)	p-value	Events/ Total	HR (95% CI)	p-value
Tumor VEGFR1												
Low	68/75	Reference	-	65/75	Reference	-	40/44	Reference	-	38/44	Reference	-
High	32/37	0.80 (0.52-1.22)	0.30	27/37	0.64 (0.40-1.00)	0.051	29/30	0.98 (0.61-1.59)	0.94	28/30	1.12 (0.69-1.83)	0.64
Tumor VEGFR2												
Low	66/75	Reference	-	59/75	Reference	-	46/49	Reference	-	45/49	Reference	-
High	27/29	1.15 (0.74-1.81)	0.53	25/29	1.16 (0.73-1.85)	0.53	21/23	0.60 (0.36-1.02)	0.059	19/23	0.67 (0.39-1.15)	0.15
Tumor VEGFR3												
Low	53/58	Reference	-	48/58	Reference	-	43/48	Reference	-	41/48	Reference	-
High	44/52	0.85 (0.57-1.27)	0.43	41/52	0.87 (0.57-1.32)	0.50	28/28	0.98 (0.61-1.58)	0.94	27/28	1.17 (0.72-1.91)	0.53
Tumor VEGFC												
Low	78/86	Reference	-	71/86	Reference	-	50/55	Reference	-	49/55	Reference	-
High	24/28	0.70 (0.44-1.11)	0.13	22/28	0.82 (0.51-1.32)	0.42	17/17	1.27 (0.73-2.21)	0.41	15/17	1.07 (0.60-1.92)	0.81
Tumor VEGFA												
Low	52/58	Reference	-	47/58	Reference	-	43/46	Reference	-	41/46	Reference	-
High	47/53	1.05 (0.71-1.56)	0.81	43/53	1.06 (0.70-1.60)	0.79	25/27	1.00 (0.61-1.64)	0.99	24/27	1.19 (0.72-1.98)	0.50
Tumor VEGFR1/VEGFR2												
Low	77/85	Reference	-	70/85	Reference	-	57/61	Reference	-	54/61	Reference	-
High	14/16	0.84 (0.48-1.49)	0.56	13/16	0.87 (0.48-1.57)	0.63	9/10	0.53 (0.26-1.08)	0.079	9/10	0.77 (0.38-1.56)	0.46
Tumor VEGFR1/VEGFA												
Other	80/90	Reference	-	73/90	Reference	-	52/56	Reference	-	50/56	Reference	-
Both high	17/19	0.95 (0.56-1.61)	0.85	16/19	0.90 (0.52-1.55)	0.69	15/16	0.86 (0.48-1.53)	0.60	14/16	1.06 (0.58-1.92)	0.85
Tumor VEGFR2/VEGFA												
Other	73/83	Reference	-	65/83	Reference	-	57/61	Reference	-	54/61	Reference	-
Both high	17/17	1.32 (0.78-2.24)	0.30	16/17	1.18 (0.68-2.05)	0.55	8/9	0.62 (0.29-1.30)	0.21	8/9	0.69 (0.32-1.46)	0.33
Tumor VEGFR3/VEGFA												
Other	67/75	Reference	-	62/75	Reference	-	54/59	Reference	-	51/59	Reference	-
Both high	27/31	0.89 (0.57-1.40)	0.61	24/31	0.87 (0.54-1.39)	0.55	14/14	1.18 (0.65-2.13)	0.58	14/14	1.53 (0.84-2.78)	0.17
Stromal VEGFR3												
Negative	15/17	Reference	-	13/17	Reference	-	17/19	Reference	-	16/19	Reference	-
Positive	63/70	0.81 (0.46-1.42)	0.45	58/70	1.00 (0.55-1.83)	> 0.99	34/37	0.84 (0.47-1.50)	0.55	33/37	1.20 (0.66-2.19)	0.56
Stromal VEGFA												
Negative	17/18	Reference	-	15/18	Reference	-	14/16	Reference	-	14/16	Reference	-
Positive	71/79	0.72 (0.42-1.22)	0.22	64/79	0.81 (0.46-1.41)	0.45	45/48	1.41 (0.77-2.58)	0.26	42/48	1.08 (0.59-1.98)	0.80

CI, confidence interval; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; PFS, progression-free survival; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

role of VEGF/VEGFR axis in the development of metastases is supported by many studies. Interestingly, Ning et al. [15], some years ago, showed that the activation of VEGFR1 can induce epithelial-mesenchymal transition (EMT), promoting migration and invasion of BC cells. Previously, it had been documented that VEGF and its receptors VEGFR1 and 2 exert an osteolytic role in bone metastases from BC [16]. Furthermore, Kaplan et al. [17], using a mouse model, have shown that VEGFR1-positive bone marrow-derived hematopoietic progenitor cells participate in the formation of tumor-specific pre-metastatic sites. More recently, Sadremomtaz et al. [18] published that dual blockade of VEGFR1 and VEGFR2 by a novel peptide can stop VEGF-driven metastasis via manipulation of PI3K/AKT and MAPK/ERK1/2 signaling pathways. Although the association of VEGFR1 with increased visceral metastases seems contradictory to the associated increased survival, it may not be so in the setting of HER2-positive tumors treated with trastuzumab, further referring to the association of HER2 pathways and angiogenesis blockade, namely via the PI3K/AKT and MAPK/ERK1/2 pathways. In a study of angiogenesis in early BC, VEGFR1 was associated with better disease-free survival in ER/PR-positive BC but worse disease-free survival in triple-negative BC but no statisitical significance was observed to HER2-positive BC [19]. The varying results compared to the present work, introduce the question of heterogeneity of angiogenesis-related parameters and their significance in early versus advanced/metastatic BCs stemming from the associated clonal evolution of tumors. These findings point to the following hypothesis: the expression of angiogenesis-related parameters and their significance may vary among both the various intrinsic subtypes and the stage of BC. The question may be addressed in larger cohorts of early or metastatic BC, along with the immune profile of tumors, in light of the combinations of treatment modalities, particularly in metastatic BC [20].

Also interesting was the marginally significant association of tumor VEGFR2 expression with improved PFS in patients with HER2-negative tumors. This is the first report according to our knowledge, which implicates VEGR2 in the clinical outcome of HER2-negative patients. In preclinical mouse models of *HER2*-amplified BC brain metastasis, combined targeting of HER2 (trastuzumab and lapatinib) with an anti-VEGFR2 antibody significantly reduced tumor growth prolonging median OS 5-fold [21]. Yan et al. [22] have reported that VEGFR2 expression in BC was positively correlated with LN metastasis and negatively with OS. In addition, in the same study, higher VEGFR2 expression was associated EMT markers (such as Twist1 and Vimentin), implicating VEGFR2 in EMT of BC. Thus, the prognostic value of VEGFR2 in trastuzumab-treated BC remains to be further clarified in larger studies.

Discordance between local and central evaluation of HER2, as observed herein, have been previously reported from our group [10,23] and others [24-26]. Central assessment of the first 104 cases enrolled in the National Surgical Adjuvant Breast and Bowel Project (NSABP) Protocol B-31 (NSABP B-31) did not confirm the community-based initial evaluation in 18% of cases [24]. Similarly, in the N9831 trial of 119 patients, there was poor concordance (74%) between local and central testing for HER2 [25]. More recently, Griggs et al. [26], on a cohort of 367 patients, reported that HER2 discordance by IHC between local and central laboratories was 26%. Interestingly, 4% of patients initially characterized as negative were finally positive upon retesting [27]. These observations support the necessity of centralization of HER2 testing in order to maximize the number of patients appropriately treated with trastuzumab, who will experience clinical benefit, minimizing concurrently those who will face side effects without any benefit.

Despite the promising results of the current study, we have to acknowledge some limitations. A weakness of our study is that it has been performed based on the analysis of retrospectively collected data, and in a relatively small cohort. Another weakness relates to the heterogeneity of the cohort with regards to trastuzumab exposure. Moreover, a two-phase design should also be used to validate the value of the studied biomarkers.

To conclude, our findings suggest that angiogenesis-related factors may have significant prognostic value for BC patients with metastatic disease treated with transtuzumab-based regimens. It seems that VEGFR1 and VEGFR2 are the most promising markers, however, more studies are needed to further define their potential clinical value.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study was conducted following the Helsinki Declaration on ethical guidelines (2013) was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (Protocol #4283; January 14, 2008) under the general title "Investigation of major mechanisms of resistance to treatment with trastuzumab in patients with metastatic breast cancer" and was performed by the Hellenic Cooperative Oncology Group (HeCOG). All patients had provided written informed consent forms, before receiving any treatment, with the exception of patients treated before 2005, for whom a waiver of consent was granted by the Bioethics Committee.

Author Contributions

Conceived and designed the analysis: Kourea HP, Kotoula V, Fountzilas G.
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Performed the analysis: Koliou GA.
Wrote the paper: Kourea HP, Dimitrakopoulos FI, Koliou GA, Papadopoulou K.
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Conflicts of Interest

H.P.K received Honoraria by Roche. G.P. received Research Funding by Roche and had Advisory Role. D.P. had Advisory Role and received Honoraria by Roche. P.P. had Advisory Role and received Honoraria by Roche. A.K. had advisory role, Roche. G.F. participated in Advisory Board organized by Roche. The rest of the authors declare no conflict of interest.

Acknowledgments

The study was supported by a research grant from F. Hoffmann-La Roche and by an internal HeCOG research grant (HE TRANS_BR). The authors are indebted to the patients and their families for their trust and participation in the present registry.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.835
Cancer Res Treat. 2022;54(4):1065-1073

Original Article

Fear of Cancer Recurrence and Its Negative Impact on Health-Related Quality of Life in Long-term Breast Cancer Survivors

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Purpose Fear of cancer recurrence (FCR) is a common psychological issue in breast cancer (BC) survivors during early survivorship but whether the same is true among long-term survivors has yet to be empirically evaluated. This study investigated FCR level, its associated factors, and impact on quality of life (QoL) in long-term BC survivors.

Materials and Methods Participants included women diagnosed with BC between 2004 and 2010 at two tertiary hospitals. Survey was conducted in 2020. The study measured FCR with the Fear of Cancer Recurrence Inventory and other patient-reported outcomes, including depression and cancer-related QoL. Logistic regression was used to identify factors associated with FCR, and structural equation modeling was conducted to explore the impact of FCR on other outcomes.

Results Of 333 participants, the mean age at diagnosis was 45.5, and 46% experienced FCR. Age at diagnosis ≤ 45 (adjusted odds ratio [aOR], 2.64; 95% confidence interval [CI], 1.51 to 4.60), shorter time since diagnosis (aOR, 1.75, 95% CI, 1.08 to 2.89), and having a history of recurrence (aOR, 2.56; 95% CI, 1.16 to 5.65) was associated with more FCR. FCR was significantly associated with an increased risk of depression (β=0.471, p < 0.001) and negatively impacted emotional functioning (β=–0.531, p < 0.001). In addition, a higher FCR level may impair overall health-related QoL in long-term BC survivors (β=–0.108, p=0.021).

Conclusion Ten years after diagnosis, long-term BC survivors still experienced a high level of FCR. Further, the negative impact of FCR on QoL and increased depression risk require an FCR screening and appropriate interventions to enhance long-term BC survivors' QoL.

Key words Fear of cancer recurrence, Depression, Quality of life, Breast cancer survivor, Young breast cancer

Introduction

Survival after breast cancer (BC) has improved over the past decades, thanks to advances in diagnosis and targeted treatments. Ninety percent of female BC patients in Korea survive up to 5 years, and more than 80% can survive up to 10 years. This contributed to more than two hundred thousand BC survivors living in Korea at the end of 2017 [1]. The cumulative increase in incident cases and improvement in survival outcomes have contributed to continuous growth in the number of BC survivors. Though most cancer survivors improve their health status and return to everyday life after cancer, evidence suggests that some cancer survivors experience psychological problems such as depression, distress, or anxiety attributable to their fear that cancer might come back. The ongoing fear or concern might negatively impact the patients' overall health status and physical and social functioning.

In general, fear of cancer recurrence (FCR) is defined as worry that cancer will return or progress in the same or another part of the body [2]. It is one of the most common psychological effects in all cancers. FCR is thought to persist long after the termination of cancer treatment [3]. Also, previous studies have documented that 42%-70% of BC survi-

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Received July 22, 2021 **Accepted** December 8, 2021
Published Online December 8, 2021

vors experienced a high FCR level [4], and 21%-40% of them needed help dealing with FCR [5]. However, in most studies, participants were surveyed in the early years of survivorship, during which time the concerns of diagnosis and treatment are probably most intense. Generally, long-term survivors are defined as those who have survived more than 5 years or more since the time of diagnosis [5,6], and their experience of FCR might be differ with those whose treatment are ongoing. However, the prevalence of FCR and other mood disorders in long-term cancer survivors has been much less extensively investigated.

Research on FCR has been predominantly conducted in Western populations, with few studies that have been conducted with Korean cancer survivors [7,8]. Furthermore, Korean long-term BC survivors' characteristics might differ from those of BC survivors from other countries due to diagnosis at an early age and peaks in the 40- and 50-year age group [9]. Therefore, this study's findings may help enhance the knowledge of FCR and health-related quality of life (HRQoL) in long-term BC survivors diagnosed at an early age. In this study, we targeted long-term BC survivors who have survived on an average of 10 years postdiagnosis, and aimed to assess their FCR level, investigate which factors are associated with FCR, and finally, how FCR impacts other patient-reported outcomes (PROs).

Materials and Methods

1. Participants and data collection

The study's target population was BC survivors who participated in a cohort study at two tertiary hospitals in Korea: the National Cancer Center (NCC) and Samsung Medical Center (SMC), which started between 2004-2010. Details of baseline recruitment were described elsewhere [10,11]. In 2020, we conducted a long-term follow-up survey to assess the current status of survivors, including FCR, HRQoL, and other PROs.

Nurses at the hospitals contacted potential participants via a phone call and explained the study's purpose. Next, participants were asked if they agreed to participate in the survey. A few days after phone contact, we sent the participant a greeting letter with a questionnaire and a stamped and addressed return envelope for those who agreed to participate. The participants were asked to complete the questionnaire and return it within 2 weeks.

2. Measures

1) Fear of cancer recurrence

The Fear of Cancer Recurrence Inventory (FCRI) was developed and validated in 2009 by Simard and Savard [12]. It has been utilized in many studies to measure the prevalence of FCR and its associated factors. The questionnaire was translated and validated into the Korean language in 2017 and is one of the most common long-form instruments to assess FCR available in the Korean language [8]. The FCRI comprises seven subscales: triggers, severity, psychological distress, coping strategies, functional impairment, insight, and reassurance. Items are responded to using a Likert scale ranging from zero ("*not at all*" or "*never*") to four ("*a great deal*" or "*all the time*"). A total score is calculated for each subscale and the total questionnaire by summing the items' responses. The total score on the FCRI ranges from 0 to 168, where a higher score indicates a higher FCR level. The 9-item Severity subscale of the FCRI can be used to assess the prevalence of FCR and its severity in clinical settings [12]. In this study, a "Severity subscale" score of 13 or higher indicated having FCR based on evidence from previous research [13,14]. The Korean version of the FCRI is considered a reliable instrument to assess FCR in cancer survivors with a reported Cronbach's alpha of 0.85 [8].

2) Depression

The Patient Health Questionnaire (PHQ-9) with nine items was used to assess depression. The total score is calculated by summing the responses to all items and ranges from 0 to 27.

3) Cancer-related HRQoL

Cancer-related quality of life (QoL) was measured by the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire-Core 30 (QLQ-C30). This study focused on the Global Health Status subscale and five subscales that assess physical, role, cognitive, emotional, and social functioning. Raw scores are transformed to a linear scale ranging from 0 to 100, with a higher score representing a better QoL and higher functioning level. A Korean version of the EORTC QLQ-C30 was translated and validated in 2004 [15].

4) Overall HRQoL

The overall HRQoL was measured using the EuroQol 5-Dimension Questionnaire (EQ-5D). The five dimensions are measured by five items that ask about a participant's overall health status in the areas of mobility, self-care, usual activity, pain/discomfort, and depression/anxiety. The EQ-5D was adapted and validated for use with the Korean population. We applied the weighted quality values for Koreans to calculate the EQ-5D utility index and used it in the final analysis. The EQ-5D utility index score ranges from 0 to 1, and a higher score indicates a better overall HRQoL [16].

Table 1. Sociodemographic and clinical characteristics of the study population (n=333)

Characteristic	Total (n=333)	By FCR status		
		No (n=180, 54%)	Yes (n=153, 46%)	p-value
Sociodemographic				
Age at diagnosis (yr)				
Mean±SD	45.5±8.1	47.3±8.0	43.5±0.7	< 0.001[a]
≤ 45	166 (49.9)	70 (38.9)	96 (62.8)	< 0.001[b]
> 45	167 (50.1)	110 (61.1)	57 (37.2)	
Age at survey (yr)				
Mean±SD	57.2±8.4	59.1±8.3	54.9±7.9	< 0.001[a]
≤ 65	282 (84.7)	144 (80.0)	138 (90.2)	0.010[b]
> 65	51 (15.3)	36 (20.0)	15 (9.8)	
Household income				
< 3 mil KRW	135 (40.8)	70 (39.3)	65 (42.5)	0.632[b]
3 mil to < 5 mil KRW	95 (28.7)	55 (30.9)	40 (26.1)	
≥ 5 mil KRW	101 (30.5)	53 (29.8)	48 (31.4)	
Employment status				
Housewife/Unemployed	158 (47.4)	86 (47.8)	72 (47.1)	0.896[b]
Employed	175 (52.6)	94 (52.2)	81 (52.9)	
Education level				
High school graduate or lower	196 (59.0)	109 (60.9)	87 (56.9)	0.457[b]
University graduate or higher	136 (41.0)	70 (39.1)	66 (43.1)	
Marital status				
Married and living with a spouse	243 (72.9)	136 (75.6)	107 (69.9)	0.250[b]
Other	90 (27.1)	44 (24.4)	46 (30.1)	
Obesity status: yes	67 (20.1)	35 (19.4)	32 (20.9)	0.739[b]
Comorbidity status: yes	79 (23.8)	49 (27.2)	30 (19.7)	0.111[b]
Menopausal status: yes	281 (84.4)	160 (88.9)	121 (79.1)	0.014[b]
Pregnancy history: yes	301 (90.9)	164 (91.1)	137 (90.7)	0.904[b]
Clinical				
Time since diagnosis (yr)				
≤ 10	209 (62.8)	104 (57.8)	105 (68.6)	0.041[b]
> 10	124 (37.2)	76 (42.2)	48 (31.4)	
Stage at diagnosis				
Stage 0, I, II	300 (90.1)	164 (91.1)	136 (88.9)	0.499[b]
Stage III, IV	33 (9.9)	16 (8.9)	17 (11.1)	
Recurrence: yes	32 (9.6)	11 (6.1)	21 (13.7)	0.019[b]
Surgery: lumpectomy	286 (85.9)	155 (86.1)	131 (85.6)	0.898[b]
Chemotherapy: yes	239 (72.2)	124 (69.3)	115 (75.7)	0.196[b]
Radiotherapy: yes	287 (86.7)	154 (86.0)	133 (87.5)	0.695[b]
Hormone therapy: yes	268 (80.9)	143 (79.9)	125 (82.2)	0.588[b]
BC molecular subtype				
Luminal A	238 (71.9)	129 (72.5)	109 (71.2)	0.522[b]
Luminal B	31 (9.3)	14 (7.9)	17 (11.1)	
HER2 positive	23 (6.9)	15 (8.4)	8 (5.2)	
Triple-negative	39 (11.8)	20 (11.2)	19 (12.4)	
Histological subtypes: IDC	291 (92.4)	157 (93.5)	134 (91.2)	0.444[b]

Values are presented as number (%) unless otherwise indicated. BC, breast cancer; FCR, fear of cancer recurrence; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; KRW, Korean Won; SD, standard deviation. [a]p-value obtained from the t test, [b] Chi-square test.

5) Sociodemographic and clinical factors

The sociodemographic factors assessed included age, marital status, household income, education level, and employment status. The health-related factors included body mass index, comorbidity status, menopausal status, and pregnancy history. Information on stage at diagnosis, treatment modalities, histological subtype, BC molecular subtype, and cancer recurrence history was obtained from the hospital's electronic medical records. The diagnosis stage was re-categorized into two levels: stage 0 to II, and stage III to IV. Time since diagnosis ranged from 9 to 16 years and was divided into two groups: > 10 years versus ≤ 10 years.

3. Statistical analysis

Demographic characteristics and clinical factors were calculated using frequencies and percentages for categorical variables and standard deviations for continuous variables. Statistical significance of differences was tested by Fisher exact test or t test where appropriate. Logistic regression was fitted to identify the factors associated with having FCR (yes/no) status. The univariate and multivariate logistic regression with stepwise selection was performed to identify the final factors associated with FCR after adjustment for other covariates. All measured sociodemographic and clinical factors were considered as covariates in the analysis.

A pathway analysis using the structural equation model (SEM) was performed to evaluate the impact of FCR on HRQoL. The final SEM model included demographic and clinical factors that were statistically significantly with high FCR based on multivariate logistic regression. Bivariate analyses were also conducted to examine the correlation coefficients between FCR and other PROs. The path analysis was then performed by fitting the SEM using the "*lavaan*" package in R software [17]. The R package "*lavaan*" was developed in 2011 to provide fully open-source structural equation modeling. The model fit indices were calculated and are reported as recommended [18].

All analyses were conducted using SAS software, ver. 9.4 (SAS Institute Inc., Cary, NC) and R software version R ver. 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria), with a two-sided type I error and an alpha of 0.05.

Results

1. Characteristics of the study population

Of the 333 participants in this study, the mean age at diagnosis was 45.5±8.1, and the mean age at the survey was 57.2±8.4 (Table 1). Half of the participants were diagnosed with BC at age 45 or earlier, and 85% of them were age 65 or younger at the survey time. Sixty-three percent of participants had a time since diagnosis ranging from 9 to 10 years, and 37% had a time since diagnosis of more than 10 years. Ninety percent of participants were diagnosed at early stages (0 to II).

Among 333 long-term BC survivors, 153 women (46%) reported having FCR. Those who experienced FCR had a significantly lower age at diagnosis (47.3 vs. 43.5), a lower proportion of having menopause (79% vs. 89%), and a higher proportion having a time since diagnosis of 10 years or less (69% vs. 58%). No difference in other demographic characteristics, including household income level, employment status, education level, and marital status, was observed between the two groups. Among those who experience FCR, 21 participants (14%) had a history of recurrence, which was significantly higher than that among those without experience of FCR (6%). No difference was observed in stage at diagnosis, treatment modalities, and histological subtype between the two groups.

2. FCR scores and other outcomes

Overall, the total FCRI scores ranged from 0 to 142 (over a maximum of 168), with an average of 54.4±25.7 (S1 Table). The mean Trigger and Severity subscales were 12±7 over a maximum of 32 scores. Among the EQ-5D dimensions, 44% of participants reported having problems with pain/discomfort, and 45% reported having problems with anxiety/depression, resulting in an overall EQ-5D index score of 0.918. Regarding depression measures using PHQ-9, 17 (5%) and 53 (16%) participants reported having moderate/severe and mild depression, respectively. All functioning scales assessed by the EORTC QLQ-C30 were approximately 80 or higher.

A significantly lower EQ-5D summary index, higher depression score, and lower functioning scores were reported in those with FCR (Table 2). The overall EQ-5D index score of those with FCR was 0.887±0.088, which was lower compared with 0.945±0.074 reported by those without FCR. Furthermore, a significant difference was observed in the functioning scores between the two groups, with more inferior results in those with FCR: 14.4 scores on the emotional functioning scale, 10.3 scores on the social functioning scale, and 10.1 scores on the role functioning scale ($p < 0.001$).

3. Factors associated factors with FCR

Results from both univariate (S2 Table) and multivariate logistic analysis (Table 3) showed that younger age at diagnosis (adjusted odds ratio [aOR], 2.64; 95% confidence interval [CI], 1.51 to 4.60), shorter time since diagnosis (aOR, 1.75; 95% CI, 1.08 to 2.89), and having a history of cancer recurrence (aOR, 2.56; 95% CI, 1.16 to 5.65) was significantly associated with having FCR. None of the other sociodemograph-

Table 2. HRQoL and depression score by FCR status (n=333)

HRQoL and depression	Range	Mean±SD		Difference	p-value
		FCR: No (n=180)	FCR: Yes (n=153)		
EQ-5D index	0-1	0.945±0.074	0.887±0.088	0.057	< 0.001
PHQ-9: total depression score	0-27	1.4±2.1	4.1±4.5	2.8	< 0.001
EORTC QLQ-C30 functions					
Physical Functioning	0-100	89.2±9.2	83.6±13.9	5.6	< 0.001
Role Functioning	0-100	91.8±14.0	81.7±23.2	10.1	< 0.001
Emotional Functioning	0-100	86.7±15.0	72.3±20.9	14.4	< 0.001
Cognitive Functioning	0-100	82.9±16.3	75.1±17.7	7.8	< 0.001
Social Functioning	0-100	93.0±14.0	82.7±22.3	10.3	< 0.001

EORTC QLQ-C30, European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Core 30; EQ-5D, EuroQoL; FCR, fear of cancer recurrence; HRQoL, health-related quality of life; PHQ-9, Patient Health Questionnaire-9; SD, standard deviation.

Table 3. Multivariate logistic regression on factors associated with FCR (n=333)

Factor	FCR		
	aOR[a)]	95% CI	p-value
Age at diagnosis (yr)			
> 45	1.00		
≤ 45	2.64	1.51-4.60	0.001
Age at survey (yr)			
> 65	1.00		
≤ 65	1.11	0.53-2.33	0.775
Menopausal status			
Yes	1.00		
No	1.20	0.60-2.39	0.613
Time since diagnosis (yr)			
> 10	1.00		
≤ 10	1.75	1.08-2.89	0.028
Stage at diagnosis			
Stage 0, I, II	1.00		
Stage III, IV	1.17	0.55-2.49	0.785
Recurrence			
No	1.00		
Yes	2.56	1.16-5.65	0.020

aOR, adjusted odds ratio; CI, confidence interval; FCR, fear of cancer recurrence. [a)]Adjusted for age at survey, menopausal status, age at diagnosis, time since diagnosis, stage at diagnosis, and recurrence history.

ic and clinical variables were associated with having FCR.

4. Impact of FCR on other PROs from path analysis

Both the total FCR score and the severity score were significantly correlated with the scores of other PROs (Fig. 1). The total FCRI score had significant negative correlations with overall QoL status (the EQ-5D index), all EORTC QLQ-C30 functioning subscale scores, and a significant positive correlation with depression by PHQ-9 scores.

The path analysis showed consistent results that younger age was associated with a higher FCR level (standardized β=–0.224, p < 0.001) (Fig. 2, S3 Table). A higher FCR level had a negative association with emotional function (β=–0.531, p < 0.001) and a positive association with depression (β=0.471, p < 0.001). Furthermore, a higher FCRI score was significantly associated with a lower EQ-5D index score (β=–0.108, p=0.021) which suggests that FCR might impair overall HRQoL in cancer survivors. Depression and emotional functioning were also significantly associated with overall HRQoL (β=–0.334, p < 0.001 and β=0.366, p < 0.001, respectively). The path analysis model had a decent fit with the following indices: Goodness of Fit Index=0.976, Adjusted Goodness of Fit Index=0.926, Normed-Fit Index=0.970, Comparative Fit Index=0.986, root mean square error of approximation=0.049, and standardized root mean square residual=0.034 (S4 Table).

Discussion

The current study targeted long-term young BC survivors with a time since diagnosis ranging from 9 to 16 years, and the study population were those diagnosed with BC at an early age (an average of 45 years). Even though FCR persists long after treatment completion [3], most previous studies assessed FCR in the early course of treatment or survivorship [4,5], and few have focused on FCR among long-term survivors, especially those 10 years or more postdiagnosis. Cancer survivors who have survived more than 5 years or more since the time of diagnosis are considered as long-term survivors [5,6]. In this study, the mean and median survival time of the participants were 11.6 years and 10 years respectively, and

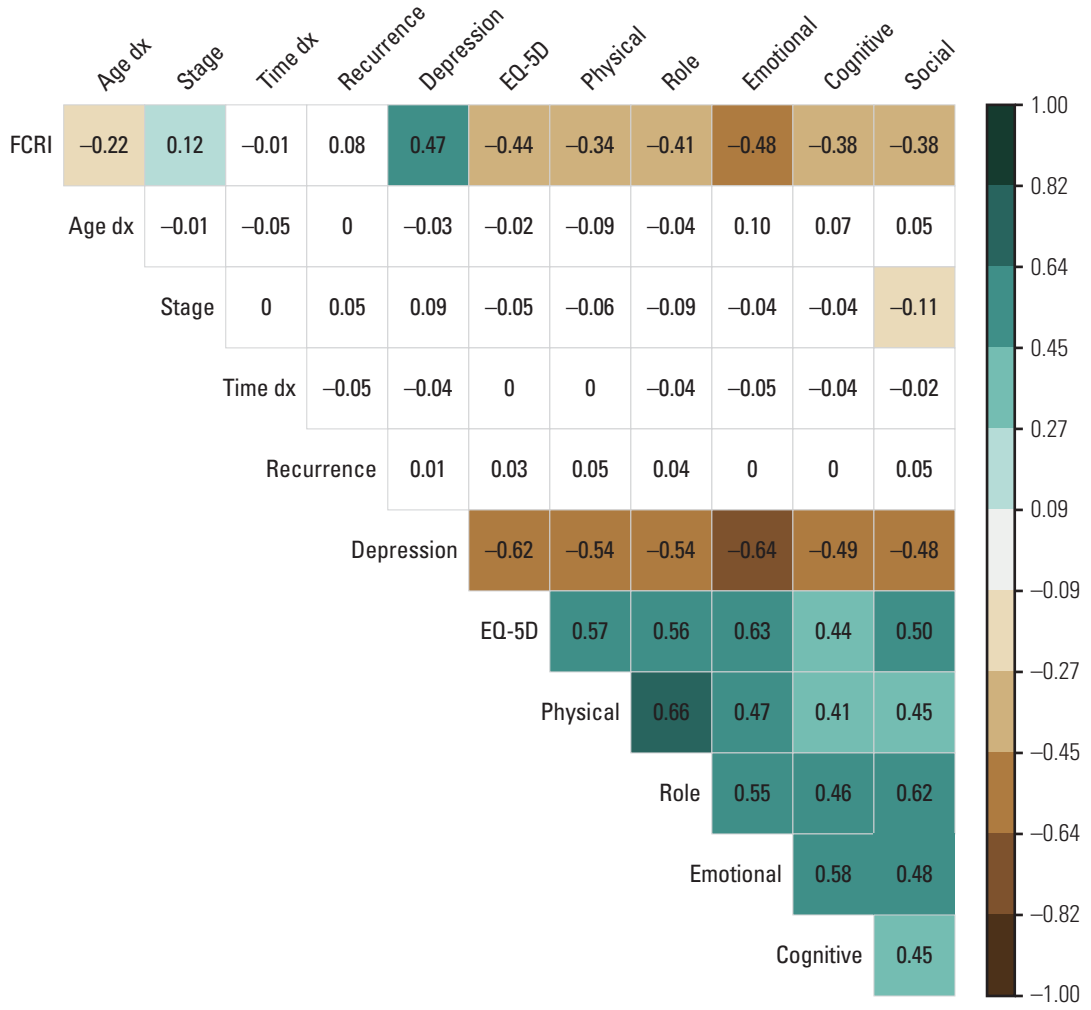

Fig. 1. Correlations between FCRI and other factors. The brown color indicates the statistically significant negative associations. The green color indicates the statistically significant positive associations. The white color indicates non-significant associations. A darker color indicates stronger correlations. EQ-5D, EuroQol 5-Dimension Questionnaire Index; FCRI, Fear of Cancer Inventory; stage, stage at diagnosis; Time dx, time since diagnosis (in year).

thus, they can be considered long-term BC survivors. These are the unique characteristics that differentiate our findings from previous work. Given the increasing number of long-term BC survivors and the limited knowledge on psychological issues in young BC survivors, findings from the current study might help provide the information needed to develop support programs for this particular population.

A low FCR level can be considered a normal reaction of survivors to cancer progression, encouraging survivors to adopt better health behaviors. However, when fear becomes clinically significant or problematic, survivors have chronic intrusive thoughts about a possible recurrence, which might increase depression risk and deteriorate their QoL. Even though 10 years had passed since the initial diagnosis, our results prove that BC survivors still had concerns and worries about their cancer disease. Approximately half of the participants had FCR, consistent with previous studies that reported an FCR prevalence of 40% to 60% [19-21]. In addition, the FCRI subscale scores and total FCRI score reported by our participants were relatively similar to previous studies despite the difference in time since cancer diagnosis [7,14]. In this study, 10% of the participants had a history of recurrence, and these women had a 2.5-fold higher risk of experiencing FCR than those without recurrence. Thus, our findings suggest that long-term BC survivors with a history of FCR might need support to reduce their FCR, and future research on a larger sample of this particular population is needed to provide more comprehensive findings.

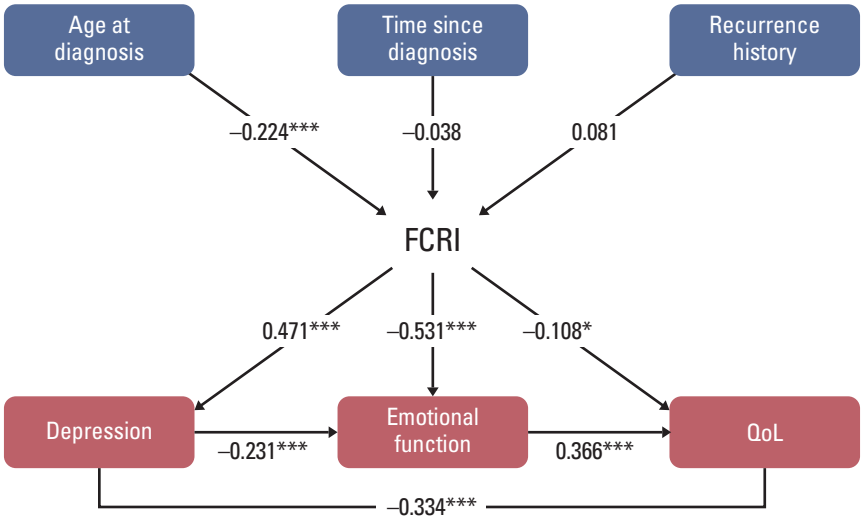

Fig. 2. Path diagram of the SEM model for the associations between fear of cancer recurrence and other patient-reported outcomes. The structural model presents the effects of age at diagnosis, time since diagnosis, history of recurrence, fear of cancer recurrence, and other patient-reported outcomes. FCRI, Fear of Cancer Recurrence Inventory; QoL, quality of life; SEM, structural equation model. *$p < 0.05$, ***$p < 0.001$.

Consistent with our research, other studies [4,22,23] found that younger cancer survivors experienced more FCR. For example, a study reported that more than 60% of young cancer survivors had clinical FCR [24]. A more aggressive tumor subtype, and worse prognosis [25] may explain why BC patients diagnosed at a younger age had more concerns and more fears about their disease's progress. Thus, accumulated evidence supports that early age at diagnosis is a robust predictor for higher FCR. In terms of clinical factors, previous studies found a shorter time since diagnosis [22,24] and worse disease prognosis, such as having a previous recurrence or advanced stage, were associated with higher FCR levels [24,26]. Again, these findings were consistent with our study. However, it should be noted that the time since diagnosis was longer in the present study, with a minimum of 9 years, whereas other studies were commonly conducted among survivors with less than 5 years since diagnosis.

Cancer survivors are at an elevated risk for psychological issues such as FCR, distress, anxiety, and depression that might persist years after receiving treatment. For example, according to previous studies, a higher FCR level was strongly associated with higher levels of depression [2,12,27], emotional distress [7,22,24], and anxiety [27,28]. Consequently, having FCR might worsen cancer survivors' overall QoL [14,28], which was consistent with our findings.

When fear or concern about cancer recurrence becomes problematic, screening and management for FCR are crucial. Cancer survivors with a high FCR level might benefit from psychoeducation programs or other psychological interventions such as attention training, metacognitive therapy, acceptance, or mindfulness [29,30]. The increasing number of clinical trials to develop therapies to reduce FCR in recent years indicates that FCR is one of the common unmet needs among cancer survivors, and healthcare providers are trying to help survivors with these psychological issues. However, most of the interventions were conducted in Western countries. Thus far, no research on interventions to reduce FCR among Korean cancer survivors and especially long-term cancer survivors has been conducted.

Our study had several limitations. First, due to the nature of cross-sectional data, we could not assess variation in FCR over time and likewise cannot determine causal relationships between FCR and other PROs measured in our research. Second, our results should be interpreted with caution because 90% of the study participants were in BC stage 0 to II, with nine years as the minimum time since diagnosis. In addition, this study might have selection bias due to the fact that only participants agreed to participate were included in the analysis. Thus, they would generally have better HRQoL than advanced stage-patients or those who rejected to participate in the survey.

After 5 years of adjuvant endocrine therapy, BC survivors remain at risk for cancer recurrence. The most recent National Comprehensive Cancer Network Survivorship Guidelines recommend that psychological issues, including FCR, are among the eight most common issues that should be managed in cancer survivors [31]. Long-term BC survivors in our study, especially those diagnosed at a younger age and

had recurrence history, still expressed fear or concerns about cancer, which deteriorates their overall QoL and increases depression risk. Thus, long-term BC survivors who experience these psychological issues should be identified and supported.

Though BC survivors often experience worries and uncertainties about their disease, not many directly express these feelings to healthcare providers. Therefore, doctors and other healthcare providers should identify emotional distress in BC survivors and provide appropriate intervention. We believe that our research findings are of interest to healthcare providers and BC survivors because it provides a better understanding of FCR and its impact on long-term BC survivors. Future research should focus on developing screening and psychological interventions to reduce FCR for this growing survivor population, especially in Asian BC survivors.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was approved by the National Cancer Center institutional review board (IRB approval number: NCC2019-0281). All participants gave their consent to participate by signing an informed consent form.

Author Contributions

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

This work was supported by the National Cancer Center of Korea (grant numbers NCC-04101502 and NCC-1911271). The funder had no role in the study design, in the collection, analysis, and interpretation of data, in the writing of the report; or in the decision to submit the article for publication.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.864
Cancer Res Treat. 2022;54(4):1074-1080

Original Article

Lobular Carcinoma *In Situ* during Preoperative Biopsy and the Rate of Upgrade

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Purpose There is a potential risk that lobular carcinoma *in situ* (LCIS) on preoperative biopsy might be diagnosed as ductal carcinoma *in situ* (DCIS) or invasive carcinoma in the final pathology. This study aimed to evaluate the rate of upgrade of LCIS on preoperative biopsy to DCIS or invasive carcinoma.

Materials and Methods Data of 55 patients with LCIS on preoperative biopsy were analyzed. All patients underwent surgery between 1991 and 2016 at Severance Hospital in Seoul, Korea. We analyzed the rate of upgrade of preoperative LCIS to DCIS or invasive cancer in the final pathology. The clinicopathologic features related to the upgrade were evaluated.

Results The rate of upgrade of LCIS to DCIS or invasive carcinoma was 16.4% (9/55). In multivariate analysis, microcalcification and progesterone receptor expression were significantly associated with the upgrade of LCIS (p=0.023 and p=0.044, respectively).

Conclusion The current study showed a relatively high rate of upgrade of LCIS on preoperative biopsy to DCIS or invasive cancer. The presence of microcalcification and progesterone receptor expression may be potential predictors of upgradation of LCIS on preoperative biopsy. Surgical excision of the LCIS during preoperative biopsy could be a management option to identify the concealed malignancy.

Key words Breast carcinoma *in situ*, Breast neoplasms, Core needle biopsy, Lobular carcinoma *in situ*, Surgical diagnostic technics

Introduction

Traditionally, an excision is recommended for patients with lobular carcinoma *in situ* (LCIS) diagnosed on core needle biopsy [1]. However, the management of LCIS has been controversial, and some authors advocate observation rather than surgical excision [2,3].

LCIS was excluded from the malignant category in the 8th American Joint Committee on Cancer (AJCC) staging system for breast cancer [4]. The National Comprehensive Cancer Network (NCCN) guidelines recommend active surveillance, surgical excision, and/or other interventions such as counseling for lifestyle modification, medication, or surgery for reducing the risk of breast cancer in patients with LCIS diagnosed on core needle biopsy [1]. Surgical excision should be considered only in patients with pleomorphic LCIS or lesions that are non-concordant with imaging findings [1]. Patients with classic LCIS and those with lesions that are concordant with imaging findings can be followed up with close observation [1].

Across the literature, the upgrade rates of LCIS on core needle biopsy to invasive carcinoma or ductal carcinoma *in situ* (DCIS) at surgical excision have been reported from 0% to 50% [5]. Previous studies have shown that the upgrade rate varies, and there is still no consensus about surgical treatment or observation in cases of LCIS [5].

In this study, we analyzed the upgrade rate and risk factors associated with the upgrade of LCIS diagnosed at preoperative biopsy and performed surgical excision in a single institution.

Materials and Methods

We reviewed electronic medical records (EMR) and data from the Breast Cancer Registry database of Severance Hospital, Yonsei University Health System, and conducted a retrospective study. The computerized medical database included information about the clinical, radiological, and pathological characteristics of patients; treatment methods; preoperative and postoperative pathologic findings; preoperative findings on physical examination, mammography, and ultrasonography; recurrence and mortality; and follow-up data, as previously described [6].

We reviewed the data of 80 patients who underwent breast surgery for LCIS at Severance Hospital between January 1991 and December 2016 using EMRs and data from the database. We excluded patients diagnosed with invasive

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Received July 30, 2021 **Accepted** December 20, 2021 **Published Online** December 21, 2021
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cancer at preoperative biopsy (n=6) and non-LCIS at preoperative biopsy (n=18); we also excluded one case involving unavailable data. Finally, 55 cases of LCIS diagnosed at preoperative biopsy were enrolled in the study (Fig. 1).

The patients underwent breast-conserving surgery or mastectomy, according to the patients' and surgeons' preferences based on the tumor size, location, and multiplicity of tumors. After surgery, some patients who underwent breast-conserving surgery received adjuvant radiotherapy according to the multidisciplinary team approach.

Patient characteristics such as age, clinical findings, preoperative biopsy methods, pathological findings, and treatment methods were reviewed. A preoperative physical examination was performed by experienced surgeons, and a palpable mass was described in the medical database with or without information about the location or size of the lesion. Preoperative imaging evaluations including mammography, ultrasonography, and magnetic resonance imaging (MRI) were performed. The initial reports of preoperative imaging studies were reviewed for their correlation with the final pathology.

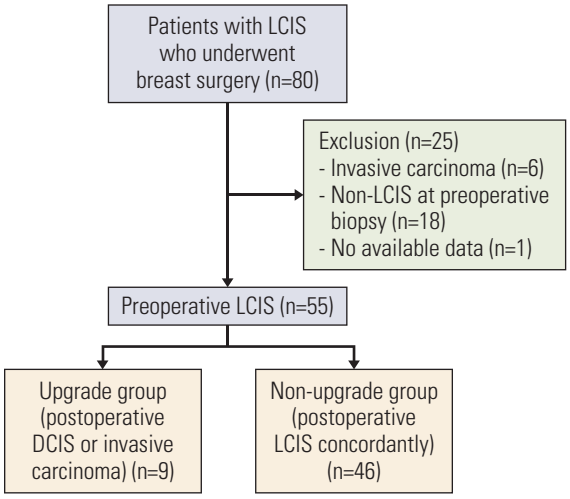

Fig. 1. Schema of the study design to analyze the upgrade rate of preoperative LCIS. DCIS, ductal carcinoma *in situ*; LCIS, lobular carcinoma *in situ*.

Final pathology records were reviewed to analyze histopathological variables including tumor size, hormone receptor status, E-cadherin expression, pleomorphism, and comedo necrosis. Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2)/neu expression were evaluated on formalin-fixed, paraffin-embedded whole sections of the surgically resected breast specimens using immunohistochemistry (IHC). The cutoff value for ER and PR positivity was > 1% staining on IHC. Pleomorphism and comedo necrosis were reviewed by an experienced breast pathologist (J.S.K.) and categorized as either absent or present.

Categorical variables were analyzed using the chi-square test or Fisher exact test. Continuous variables were analyzed using the Student's t test or Mann-Whitney U test. Univariate and multivariate analyses for calculating the odds ratios of significant risk factors for the upgrade of preoperative LCIS were performed using binary logistic regression. Multivariate analysis was adjusted for age, microcalcification on mammography, and PR status as covariates. Risk factors were selected using backward stepwise regression based on the probability of the likelihood ratio. A $p < 0.05$ was considered significant; all tests were two-sided. Statistical analyses were conducted using a commercially available statistical software SPSS Statistics ver. 25 (IBM Corp., Armonk, NY).

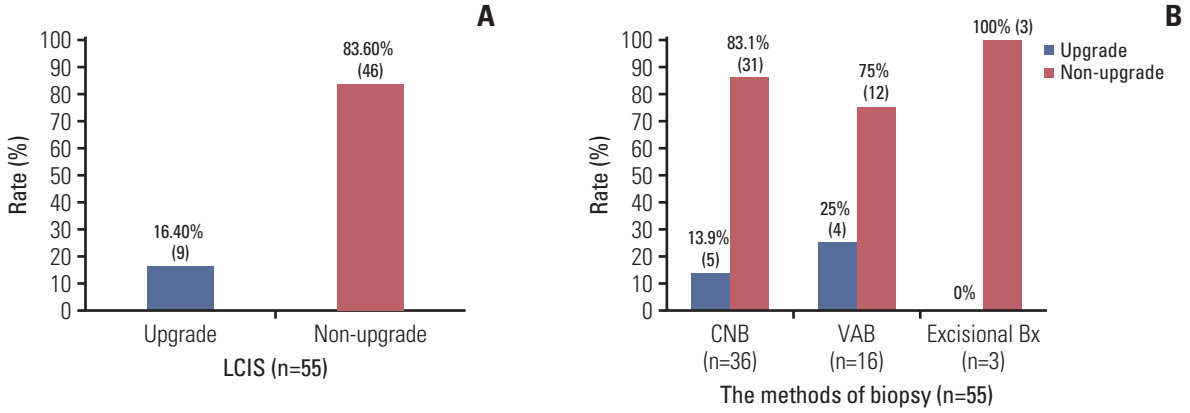

Fig. 2. The rate of upgrade of preoperative LCIS. (n=55). (A) The rate of upgrade of preoperative LCIS. (B) The rate of upgrade of preoperative LCIS according to the methods of preoperative biopsy. Bx, biopsy; CNB, core needle biopsy; LCIS, lobular carcinoma *in situ*; VAB, vacuum assisted biopsy.

Table 1. Comparison of clinicopathologic features between the upgrade group and non-upgrade group

Characteristic	Preoperative LCIS (n=55)		p-value
	Upgrade group (n=9, 16.4%)	Non-upgrade group (n=46, 83.6%)	
Age (yr)			
≤ 50	4 (44.4)	28 (60.9)	0.467
> 50	5 (55.6)	18 (39.1)	
Physical exam			
Non-palpable	7 (77.8)	36 (78.3)	> 0.999
Palpable	2 (22.2)	10 (21.7)	
Microcalcification on mammography			
Negative	1 (11.1)	24 (52.2)	0.050
Positive	7 (77.8)	19 (41.3)	
Unknown	1 (11.1)	3 (6.5)	
USG mass			
Negative	6 (66.7)	28 (63.6)	> 0.999
Positive	3 (33.3)	16 (36.4)	
BI-RADS category			
Category 4	8 (88.9)	34 (73.9)	0.767
Category 5	0	2 (4.3)	
Others (category 2, 3, 6)	1 (11.1)	10 (21.7)	
MRI enhancement			
Negative	1 (16.7)	12 (32.4)	0.649
Positive	5 (83.3)	25 (67.6)	
Biopsy methods			
Core needle biopsy	5 (55.6)	31 (67.4)	> 0.999
Vacuum assisted biopsy	4 (44.4)	12 (26.1)	
Excisional biopsy	0	3 (6.5)	
Surgery type			
Partial mastectomy	7 (77.8)	36 (78.3)	> 0.999
Total mastectomy	2 (22.2)	10 (21.7)	
Tumor site			
Left	6 (66.7)	23 (50.0)	0.475
Right	3 (33.3)	23 (50.0)	
Tumor size (cm)			
≤ 2	7 (77.8)	30 (75.0)	> 0.999
> 2	2 (22.2)	10 (25.0)	
ER			
Negative	0	1 (2.3)	> 0.999
Positive	9 (100)	42 (97.7)	
PR			
Negative	1 (11.1)	16 (38.1)	0.241
Positive	8 (88.9)	26 (61.9)	
HER-2			
0 to 1+	5 (55.6)	21 (45.7)	0.796
2+	2 (22.2)	17 (37.0)	
3+	2 (22.2)	4 (8.7)	
E-cadherin expression			
Negative	9 (100)	38 (82.6)	0.327
Positive[a)]	0	1 (2.2)	
Not done	0	7 (15.2)	

(Continued to the next page)

Table 1. Continued

Characteristic	Preoperative LCIS (n=55)		p-value
	Upgrade group (n=9, 16.4%)	Non-upgrade group(n=46, 83.6%)	
Pleomorphic type[b]			
No	8 (100)	25 (75.8)	0.318
Yes	0	8 (24.2)	
Comedo necrosis[b]			
No	8 (100)	26 (78.8)	0.310
Yes	0	7 (21.2)	

Values are presented as number (%). BI-RADS, Breast Imaging-Reporting and Data System; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; LCIS, lobular carcinoma *in situ*; MRI, magnetic resonance imaging; PR, progesterone receptor; USG, ultrasonography. [a]Weak positive, [b]Missing data were excluded from the analysis (pleomorphic n=14, comedo necrosis n=14).

Table 2. Univariate and multivariate analysis for the upgradation of preoperative LCIS (n=55)

Clinicopathologic factor	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Age (≤ 50 yr vs. > 50 yr)	1.944 (0.460-8.223)	0.366	-	-
Microcalcification on MMG (negative vs. positive)	8.842 (1.000-78.221)	0.050	14.155 (1.448-138.394)	0.023
PR (negative vs. positive)	4.923 (0.562-43.123)	0.150	10.621 (1.069-105.559)	0.044

CI, confidence interval; LCIS, lobular carcinoma *in situ*; MMG, mammography; OR, odds ratio; PR, progesterone receptor.

Results

Overall, 55 cases of preoperative LCIS were identified. They were classified into two different groups based on the final pathology: nine cases of postoperative DCIS or invasive carcinoma in the upgrade group and 46 cases of postoperative LCIS in the non-upgrade group (Fig. 1). The upgrade rate of preoperative LCIS to DCIS or invasive carcinoma was 16.4% (9/55) (Fig. 2A).

Clinicopathologic features were compared between the upgrade group and the non-upgrade group (Table 1). There was no significant difference between the two groups in terms of age; physical examination findings; ultrasonographic findings; Breast Imaging-Reporting and Data System (BI-RADS) category; MRI findings; biopsy methods; surgical methods; tumor site; tumor size; and the status of ER, PR, HER-2, and E-cadherin expression at baseline. Microcalcification on mammography was seen in 41.3% (19/46) of patients in the non-upgrade group and in 77.8% (7/9) of patients in the upgrade group, which indicated a marginally significant difference (p=0.05). In the multivariate analysis, microcalcification on mammography and PR positivity were significantly associated with the risk of upgrade of preoperative LCIS (odds ratio [OR], 14.155; p=0.023 and OR, 10.621; p=0.044) (Table 2).

Pleomorphism of LCIS was analyzed by preoperative biopsy (Table 1). The rates of pleomorphic LCIS and comedo necrosis were 19.5% and 17.1%, respectively. Based on preoperative biopsy findings, there were no cases of pleomorphic LCIS in the upgrade group, whereas there were eight cases of pleomorphic LCIS in the non-upgrade group. There were only seven cases of comedo necrosis in the non-upgrade group. There were no significant differences between the two groups in terms of pleomorphism and comedo necrosis (p=0.318 and p=0.310, respectively).

Discussion

The current study demonstrated that the upgrade rate of preoperative LCIS was 16.4%. A relatively significant proportion of the patients with preoperative LCIS had hidden invasive cancer that might be missed if only core needle biopsy is used as a definitive diagnostic tool. A previous study reported an 8.4%-9.3% rate of upgrade for LCIS, which was considerably higher than the acceptable target for surveillance, and the authors suggested excision of preoperative LCIS confirmed by a core needle biopsy [5]. Li et al. [7] suggested that LCIS might be a precursor of invasive carcinoma, and localized treatment for LCIS is warranted. Cheng et al. [8] also suggested that lumpectomy is the most appropriate management for LCIS. In this study, all upgrade groups were

Table 3. Upgrade rates of lobular neoplasia or LCIS during the recent 5 years

Study	Year	Pathology	No.	Upgrade rate (%)	Feature
Calhoun and Collins [12]	2016	LN	76	13	Included pLCIS as an upgraded pathology
Khoury et al. [13]	2016	LN LCIS	63 34	24 32 67 (pLCIS)	MRI-guided core biopsy
Schmidt et al. [9]	2018	LN	115	11 (all LN) 4 (except pLCIS and discordant lesions)	Observation vs. excision
Desai et al. [14]	2018	pLCIS	15	20	
Genco et al. [15]	2019	LN cLCIS	287 115	3.8 7	Classic LN diagnosed on breast core needle biopsy
Holbrook et al. [16]	2019	LN	66	7.6	
Nakhlis et al. [17]	2019	NC-LCIS	76	36	Supporting routine excision

cLCIS, classic lobular carcinoma *in situ*; LCIS, lobular carcinoma *in situ*; LN, lobular neoplasia; NC-LCIS, non-classic lobular carcinoma *in situ*; pLCIS, pleomorphic lobular carcinoma *in situ*.

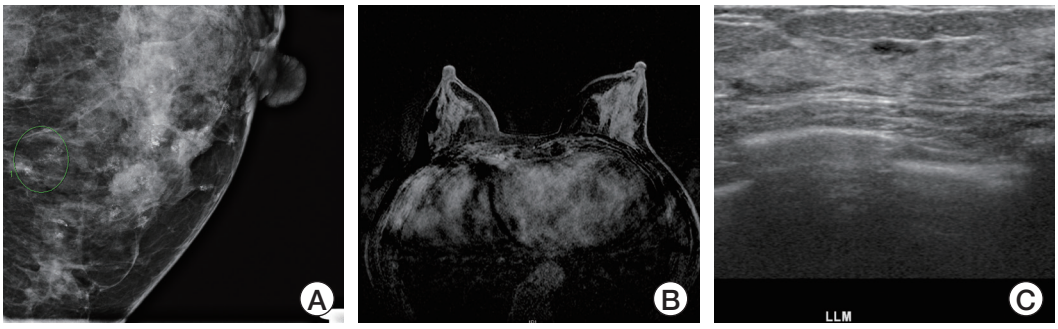

Fig. 3. A case of the upgrade group with definite microcalcification on mammography without USG and MRI findings and the PR positivity. (A) Increased extent and amount of grouped microcalcification in lower central portion of the left breast on mammography. (B) Multiple probable benign enhancement without localized suspicious enhancements in both breasts on MRI. (C) Increased benign looking lesions in the both breasts on USG. MRI, magnetic resonance imaging; PR, progesterone receptor; USG, ultrasonography.

diagnosed by core needle or vacuum assisted biopsy, not by excisional biopsy (Fig. 2B). This result suggested that excision could be considered in case of LCIS for reliable tissue confirmation.

Nevertheless, there is controversy about the treatment of LCIS diagnosed on core needle biopsy, because of the varying upgrade rates mentioned in the literature (Table 3). Schmidt et al. [9] reported that the true upgrade rate of LCIS was 4% on excluding pleomorphic LCIS and image-discordant lesions. They suggested that surgery for classic LCIS is unnecessary, and careful radio-pathological correlation during initial biopsy is critical. Wen and Brogi [10] suggested that classic LCIS on core needle biopsy with concordant imaging does not require surgical resection. However, our study showed a relatively high rate of upgrade of LCIS, regardless of the presence of pleomorphism. There was no association between the upgrade rate and presence of pleomorphic LCIS or comedo necrosis in this study. Even though the pathologic slides of all cases of LCIS were reviewed by a specialized pathologist, no pleomorphism or comedo necrosis was detected in the upgrade group. Pleomorphic LCIS is considered to be an aggressive type of LCIS associated with high-grade DCIS and invasive cancer [11,12]. In the previous studies, the upgrade rates of pleomorphic LCIS were relatively high, at 20%-100% [9,13,14]. Hence, the NCCN guidelines recommend surgical excision for pleomorphic LCIS [1]. The difference in the upgrade rate of pleomorphic LCIS between the previous and current study might be due to the small sample size of each study and inter-observer variability in the pathological evaluations.

The definition of concordant images and pathologic results is variable [18]. Youk et al. [18] summarized five categories of radio-pathological correlation in a sonography-guided core needle biopsy of a breast lesion: concordant malignancy, discordant malignancy, concordant benign, discordant benign, and borderline or high-risk. Before the AJCC system 8th edition was published, LCIS was considered a malignant lesion; thus, when core needle biopsy for a BI-RADS category 4a lesion reveals classic LCIS, it could be considered either as discordant benign or borderline. When the radio-pathological correlation is borderline, a multidisciplinary approach or surgical excision to identify the hidden malignancy could be adopted. However, after the AJCC system 8th edition was published, when core needle biopsy for a category 4a lesion revealed classic LCIS, it was considered as concordant benign and not borderline or high-risk. In such cases, surgical excision should not be routinely recommended according to the NCCN guidelines. Since most previous studies used data obtained before the AJCC system 8th edition was published, the definition of the radio-pathological correlation for classic LCIS was considered to be either discordant benign or borderline. When we reviewed a previous study, the exact definition of the radio-pathological correlation was not specified [19,20]. Since the general recommendation of close follow-up for classic LCIS is based on ambiguous or arbitrary definition of the radio-pathological correlation for classic LCIS, it is difficult to routinely follow the revised guidelines for classic LCIS. A more detailed definition of the radio-pathological correlation for LCIS should be developed to avoid miscommunication among physicians, radiologists, pathologists, and surgeons.

The current study found that microcalcification on mammography and the expression of PR were significant in predicting the likelihood of upgrade of LCIS. A previous study reported a similar association between the upgrade rate of LCIS and mammographic calcification [5]. This result was similar to that observed in our study. Therefore, when cases of LCIS diagnosed on preoperative biopsy involve mammographic microcalcification and PR positivity, hidden invasive cancer or DCIS might be discovered after surgical excision (Fig. 3).

There were some limitations to this study. First, we analyzed a small number of cases. Few cases of preoperative LCIS were evaluated for calculating the upgrade rate. Second, when we reviewed the pathologic data, including data on pleomorphism, there were some missing data that might have affected the accuracy of the analysis. In the future, multi-center, large-scale, and long-term research on LCIS is necessary. Furthermore, IHC for core needle biopsy at preoperative pathologic evaluations is not always available in many institutions; thus, there is a limitation of generalization of the result of the current study. A multidisciplinary approach for evaluation of upgrading LCIS would be valuable for patients with LCIS in preoperative diagnosis. Finally, our research has a limitation of retrospective studies, selection bias. Nevertheless, the current study has valuable implications for clinical practice. Our study focused only on LCIS and not on lobular neoplasia or atypical lobular hyperplasia. We found that the ambiguity in the definition of radio-pathological correlation in the previous studies might have weakened the evidence of the current guidelines. In the current study, multivariate analysis found two significant predictors of the upgrade of preoperative LCIS.

This study showed a relatively high rate of upgrade to DCIS or invasive cancer in cases of preoperative LCIS. The presence of microcalcification on mammography and PRs can be potential predictors of upgrade. Surgical excision of LCIS during core needle biopsy could be considered as a management option to identify a hidden malignancy.

Ethical Statement

This study was approved by the Institutional Review Board (IRB) of Severance Hospital, Yonsei University Health System (4-2020-0716). The informed consent was waived by IRB because of the retrospective design of the study.

Author Contributions

Conceived and designed the analysis: Park HS.
Collected the data: Lee J, Ku GY, Lee H, Park HS, Ku JS, Kim JY, Park S, Park BW.
Contributed data or analysis tools: Lee J, Ku GY, Lee H, Park HS, Ku JS, Kim JY, Park S, Park BW.
Performed the analysis: Lee J, Ku GY, Lee H, Park HS.
Wrote the paper: Lee J, Ku GY, Lee H, Park HS.
Revised and approved the submitted version of the manuscript: all authors.

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.890
Cancer Res Treat. 2022;54(4):1081-1090

Original Article

Effect of Estrogen Receptor Expression Level and Hormonal Therapy on Prognosis of Early Breast Cancer

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Purpose Estrogen receptor (ER) expression in breast cancer plays an essential role in carcinogenesis and disease progression. Recently, tumors with low level (1%-10%) of ER expression have been separately defined as ER low positive (ER^{low}). It is suggested that ER^{low} tumors might be morphologically and behaviorally different from tumors with high ER expression (ER^{high}).

Materials and Methods Retrospective analysis of a prospective cohort database was performed. Patients who underwent curative surgery for early breast cancer and had available medical records were included for analysis. Difference in clinicopathological characteristics, endocrine responsiveness and five-year recurrence-free survival was evaluated between different ER subgroups (ER^{high}, ER^{low}, and ER-negative [ER^{-}]).

Results A total of 2,162 breast cancer patients were included in the analysis, Tis and T1 stage. Among them, 1,654 (76.5%) were ER^{high}, 54 (2.5%) were ER^{low}, and 454 (21.0%) were ER^{-} patients. ER^{low} cases were associated with smaller size, higher histologic grade, positive human epidermal growth factor receptor 2, negative progesterone receptor, and higher Ki-67 expression. Recurrence rate was highest in ER^{-} tumors and was inversely proportional to ER expression. Recurrence-free survival was not affected by hormonal therapy in the ER^{low} group (p=0.418).

Conclusion ER^{low} breast cancer showed distinct clinicopathological features. ER^{low} tumors seemed to have higher recurrence rates compared to ER^{high} tumors, and they showed no significant benefit from hormonal therapy. Future large scale prospective studies are necessary to validate the treatment options for ER^{low} breast cancer.

Key words Breast neoplasms, Hormone receptor, Estrogen receptor, Hormonal therapy

Introduction

Breast cancer, the most common malignancy in women worldwide, is considered a heterogeneous disease with high degree of diversity [1]. Risk stratification for recurrence after surgery depends on various clinicopathological factors including patient age, tumor size, lymph node involvement, and hormone receptor expression [2]. Since the discovery of hormone receptors in the 1960s, estrogen receptor (ER) and progesterone receptor (PR) expression has remained essential in the decision-making algorithm for breast cancer treatment [3].

ER positivity is closely associated with major hormonal risk factors of breast cancer [4]. At the same time, ER-positive (ER^{+}) disease exhibits distinct clinicopathological features such as older age, smaller size, lower grade, and most importantly, favorable prognosis [5,6]. Yet the hallmark of ER expression is its predictive role in hormonal therapy response; adjuvant tamoxifen therapy for ER^{+} breast cancer has led to a significant decrease in recurrence and mortality [7].

It is undebatable that ER-negative (ER^{-}) patients do not benefit from hormonal therapy; however, defining ER positivity with a clear cutoff point remains challenging [8]. The traditional cutoff value for ER^{+} disease was over 10% of cells staining, which was later lowered to 1%; however, a recent update in the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guideline recommends defining samples with low level (1%-10%) of ER expression separately as ER low positive (ER^{low}) [9]. Recent reports in the literature suggest that ER^{low} tumors might be morphologically and behaviorally different from tumors with high ER expression (ER^{high}) [10-12]. In the present study, we aim to compare ER^{high}, ER^{low}, and ER^{-} subtypes of early breast cancer in terms of clinicopathological characteristics, endocrine responsiveness, and prognosis.

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Received August 7, 2021 **Accepted** November 15, 2021 **Published Online** November 17, 2021
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Materials and Methods

1. Study population

Retrospective analysis was performed on a prospective cohort of 2,411 patients who underwent curative surgery for early stage breast cancer between January 2005 and December 2015 at Seoul National University Bundang Hospital. The inclusion criteria for the current study were as follows: (1) histologically confirmed stage 0 of ductal carcinoma *in situ* (DCIS) or stage I of invasive ductal carcinoma (IDC), (2) available surgical records and pathology reports, and (3) available immunohistochemistry (IHC) staining results on ER, PR, human epidermal growth factor receptor 2 (HER2) and Ki-67. Patients with contralateral advanced stage breast cancer were excluded from the study. A total of 2,162 patients were included for analysis.

2. Data collection

Demographic information of study participants was obtained through review of medical records. Surgical records were reviewed for operation date, method, and extent of axillary dissection. Information on tumor size, histological type, histological grade, lymphovascular invasion, lymph node metastasis, and pathological stage was retrieved from pathology reports. IHC staining was routinely performed for ER, PR, HER2, and Ki-67. Follow-up data was collected until each patient's last visit to the hospital and included adjuvant therapy (radiation therapy, hormonal therapy, chemotherapy), recurrence status (date of recurrence, initial recurrence site, additional treatment), and survival status (date and cause of death). 5-Year recurrence-free survival (RFS) was analyzed by censoring events at 5 years.

3. Immunohistochemistry staining

Hormone receptor status was determined by our pathologists who are fully dedicated to breast cancer pathology. Patients were separated into three groups based on IHC result of ER staining: (1) ER^{high}, when ≥ 10% of tumor cell nuclei were immunoreactive, (2) ER^{low}, with 1%-9% of cells staining, and (3) ER^{-}, if less than 1% of tumor cells showed IHC staining for ER.

4. Statistical analysis

All statistical analyses were performed using SPSS ver. 23.0 (IBM Corp., Armonk, NY). Continuous variables were compared using Student's t test; categorical variables were compared using chi-square test or Fisher exact test. Survival analysis was conducted using Kaplan-Meier method and log-rank test. Hazard ratio for recurrence was obtained through Cox regression analysis. Subgroup analysis was performed for DCIS and IDC patients separately. All p-values were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Among the 2,162 patients included in the study, 1,654 (76.5%) were ER^{high}, 54 (2.5%) were ER^{low}, and 454 (21.0%) were ER^{-}. Clinicopathological characteristics of the study participants are summarized in Table 1. When compared to ER^{high} cases, ER^{low} patients were associated with higher grade, negative PR, positive HER2, and higher Ki-67 expression. When compared to ER^{-} cases, ER^{low} patients were associated with younger age, lower grade, positive PR, positive HER2, and lower Ki-67 expression. ER^{low} breast cancer was smaller in size than both ER^{high} and ER^{-} groups ($p < 0.001$ and $p=0.010$, respectively).

Postoperative treatment data was available for all cases. Eighty seven point one percentage (1,441/1,654) of ER^{high} patients, 68.5% (37/54) of ER^{low} patients, and 4.4% (20/454) of ER^{-} patients received hormonal therapy ($p < 0.001$ between all groups). Hormonal therapy included selective ER modulators and aromatase inhibitors. 22.6% (373/1,654) of ER^{high} patients, 38.9% (21/54) of ER^{low} patients, and 53.3% (242/454) of ER^{-} patients received adjuvant chemotherapy ($p < 0.001$ between all groups).

Follow-up information was available for 2,161 patients (mean follow-up of 6.59 years; range, 0.01 to 15.79 years). Five-year recurrence rate was 5.1% (84/1,654), 7.4% (4/54), and 9.7% (44/454) in ER^{high}, ER^{low}, and ER^{-} groups, respectively ($p < 0.001$). Recurrence data included local recurrence, regional recurrence, and systemic recurrence. When two groups were compared to each other independently, RFS was significantly worse in ER^{-} cases compared to ER^{high} cases ($p < 0.001$), but there was no statistically significant difference between ER^{low} and ER^{high} cases ($p=0.597$) or ER^{low} and ER^{-} cases ($p=0.400$) (Fig. 1). Similar results were found in subgroup analysis of IDC patients; only ER^{-} patients showed worse RFS compared to ER^{high} patients ($p < 0.001$), and no significant difference in recurrence was observed between ER^{low} and ER^{high} patients ($p=0.613$) or ER^{low} and ER^{-} patients ($p=0.385$) (Fig. 2).

To evaluate endocrine responsiveness of ER^{high} and ER^{low} patients, 5-year RFS was compared between patients with our without hormonal therapy (Fig. 3). ER^{-} patients were excluded from this analysis as hormonal therapy was routinely not included in their treatment plan. ER^{high} patients showed significantly worse prognosis when hormonal therapy was omitted ($p=0.020$). This difference was not observed in ER^{low} cases; there was no difference in recurrence between-patients who received hormonal therapy and those who did not receive the treatment ($p=0.418$).

Table 1. Clinicopathological characteristics according to ER expression in early breast cancer patients

	ER				p-value		
	ER^{-} (n=454)	ER^{low} (n=54)	ER^{high} (n=1,654)	Total (n=2,162)	Total	ER^{low} vs. ER^{-}	ER^{low} vs. ER^{high}
Age (yr)							
Mean±SD	54.0±11.1	48.9±10.5	51.2±11.0	51.7±11.1	< 0.001	0.001	0.570
Median (range)	54 (25-85)	49 (29-72)	49 (25-88)	50 (25-88)			
Sex							
Female	454 (100)	54 (100)	1,644 (99.4)	2,152 (99.5)	0.326	-	> 0.99
Male	0	0	10 (0.6)	10 (0.5)			
Operation							
Breast conserving surgery	281 (61.9)	30 (55.6)	1,217 (73.6)	1,528 (70.7)	< 0.001	0.366	0.003
Total mastectomy	173 (38.1)	24 (44.4)	437 (26.4)	634 (29.3)			
Axillary dissection							
Not done	40 (8.8)	9 (16.7)	299 (18.1)	348 (16.1)	< 0.001	0.130	0.482
Sentinel lymph node biopsy	399 (87.9)	43 (79.6)	1,324 (80.0)	1,766 (81.7)			
Axillary lymph node dissection	15 (3.3)	2 (3.7)	31 (1.9)	48 (2.2)			
Size (cm)							
Mean±SD	1.0±0.7	0.8±0.6	1.2±0.6	1.1±0.6	< 0.001	0.010	< 0.001
Median (range)	1.1 (0.1-2.0)	0.6 (0.1-2.0)	1.2 (0.0-2.0)	1.1 (0.0-2.0)			
Type							
Ductal carcinoma *in situ*	86 (18.9)	13 (24.1)	457 (27.6)	556 (25.7)	0.001	0.404	0.870
Invasive ductal carcinoma	357 (78.6)	39 (72.2)	1,131 (68.4)	1,527 (70.6)			
Others	11 (2.4)	2 (3.7)	66 (4.0)	79 (3.7)			
T category							
Tis	86 (18.9)	13 (24.1)	457 (27.6)	556 (25.7)	< 0.001	0.270	< 0.001
T1mic	81 (17.8)	13 (24.1)	81 (4.9)	175 (8.1)			
T1a	41 (9.0)	6 (11.1)	117 (7.1)	164 (7.6)			
T1b	56 (12.3)	8 (14.8)	325 (19.6)	389 (18.0)			
T1c	190 (41.9)	14 (25.9)	647 (40.7)	878 (40.6)			
N category							
Nx	41 (9.0)	8 (14.8)	297 (18.0)	346 (16.0)	< 0.001	0.249	0.904
N0	408 (89.9)	45 (83.3)	1,311 (79.3)	1,764 (81.6)			
N1mic	5 (1.1)	1 (1.9)	46 (2.8)	52 (2.4)			
Stage							
0	86 (18.9)	13 (24.1)	457 (27.6)	556 (25.7)	< 0.001	0.579	0.877
IA	363 (80.0)	40 (74.1)	1,152 (69.6)	1,555 (71.9)			
IB	5 (1.1)	1 (1.9)	45 (2.7)	51 (2.4)			

(Continued to the next page)

Table 1. Continued

	ER				p-value		
	ER⁻ (n=454)	ER^{low} (n=54)	ER^{high} (n=1,654)	Total (n=2,162)	Total	ER^{low} vs. ER⁻	ER^{low} vs. ER^{high}
Grade							
G1	3 (0.7)	5 (9.3)	418 (25.3)	426 (19.7)	< 0.001	< 0.001	0.017
G2	88 (19.4)	17 (31.5)	513 (31.0)	618 (28.6)			
G3	221 (48.7)	12 (22.2)	200 (12.1)	433 (20.0)			
Unknown	142 (31.3)	20 (37.0)	523 (31.6)	685 (31.7)			
Lymphovascular invasion							
Present	41 (9.0)	1 (1.9)	184 (11.1)	226 (10.5)	0.055	0.057	0.056
Absent	284 (62.6)	31 (57.4)	959 (58.0)	1,274 (58.9)			
Unknown	129 (28.4)	22 (40.7)	511 (30.9)	662 (30.6)			
Progesterone receptor							
Positive	16 (3.5)	22 (40.7)	1,491 (90.1)	1,529 (70.7)	< 0.001	< 0.001	< 0.001
Negative	438 (96.5)	32 (59.3)	163 (9.9)	633 (29.3)			
HER2							
Negative	102 (22.5)	9 (16.7)	584 (35.3)	695 (32.1)	< 0.001	0.269	< 0.001
Equivocal	0	0	4 (0.2)	4 (0.2)			
Positive	66 (14.5)	12 (22.2)	71 (4.3)	149 (6.9)			
Not done	286 (63.0)	33 (61.1)	995 (60.2)	1,314 (60.8)			
Ki-67 (%)							
Mean±SD	26.7±19.0	18.1±13.9	9.1±9.0	13.0±13.9	< 0.001	< 0.001	< 0.001
Median (range)	20 (0-90)	15 (5-60)	5 (0-70)	7 (0-90)			
Radiotherapy							
Done	263 (57.9)	27 (50.0)	1,138 (68.8)	1,428 (66.0)	< 0.001	0.508	0.009
Not done	186 (38.8)	25 (46.3)	488 (29.5)	689 (31.9)			
Unknown	15 (3.3)	2 (3.7)	28 (1.7)	45 (2.1)			
Hormonal therapy							
Done	20 (4.4)	37 (68.5)	1,441 (87.1)	1,498 (69.3)	< 0.001	< 0.001	< 0.001
Not done	434 (95.6)	17 (31.5)	213 (12.9)	664 (30.7)			
Chemotherapy							
Done	242 (53.3)	21 (38.9)	373 (22.6)	636 (29.4)	< 0.001	0.045	0.005
Not done	212 (46.7)	33 (61.1)	1,281 (77.4)	1,526 (70.6)			
Recurrence							
Yes	44 (9.7)	4 (7.4)	84 (5.1)	132 (6.1)	0.001	0.587	0.357
Local	16 (3.5)	2 (3.7)	34 (2.1)	52 (2.4)			
Regional	7 (1.5)	0	5 (0.3)	12 (0.6)			
Systemic	13 (2.9)	1 (1.9)	21 (1.3)	35 (1.6)			

Values are presented as number (%) unless otherwise indicated. ER, estrogen receptor; ER⁻, estrogen receptor negative; ER^{high}, estrogen receptor high positive; ER^{low}, estrogen receptor low positive; HER2, human epidermal growth factor receptor 2; SD, standard deviation.

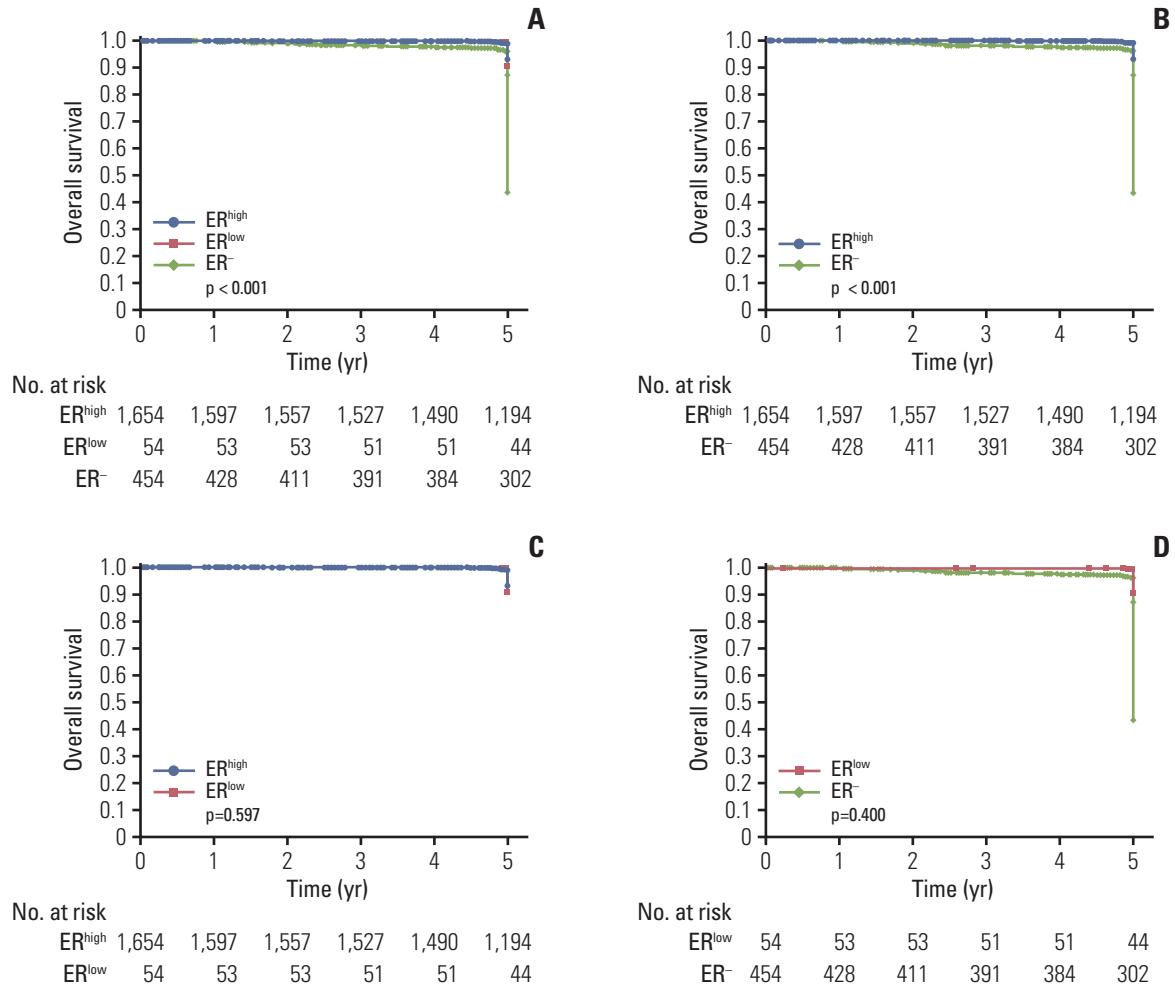

Fig. 1. Survival analysis between different estrogen receptor (ER) subgroups in early breast cancer patients. Difference in 5-year recurrence-free survival between ER^{high}/ER^{low}/ER^- (A), ER^{high}/ER^- (B), ER^{high}/ER^{low} (C), and ER^{low}/ER^- (D) patients. ER^-, estrogen receptor negative; ER^{high}, estrogen receptor high positive; ER^{low}, estrogen receptor low positive.

Risk factors for recurrence in the study population were analyzed by Cox proportional regression (Table 2). In univariate analysis, younger age, higher grade, ER^- status, higher Ki-67 expression, and omission of hormonal therapy were associated with increased risk of recurrence. In multivariate analysis, all factors except ER^- status and Ki-67 expression remained statistically significant. Subgroup analysis was performed for DCIS and IDC patients. In the DCIS group, only age was associated with recurrence (p=0.007). In the IDC group, univariate analysis revealed that younger age, higher grade, ER^- status, lower PR expression, higher Ki-67 expression, and omission of hormonal therapy were associated with higher recurrence rate. In multivariate analysis, only age and hormonal therapy remained statistically significant.

Discussion

ER plays an important role in the signaling pathway for breast cancer carcinogenesis and disease expression [13]. Hormonal therapy targeting ER including selective ER modulators, aromatase inhibitors, ER down-regulators, and ovarian suppression has led to significant improvement in the clinical outcome of breast cancer treatment [7]. ER^+ tumors show excellent response to hormonal therapy, and therapeutic effect depends on the proportion of ER expression [14,15]. In contrast, ER^- tumors show no response to hormonal therapy; however, these tumors respond relatively better to chemotherapy compared to ER^+ tumors [16]. Therefore, it is critical to set an optimal cutoff point for ER positivity to properly select patients eligible for individualized treatment options [17].

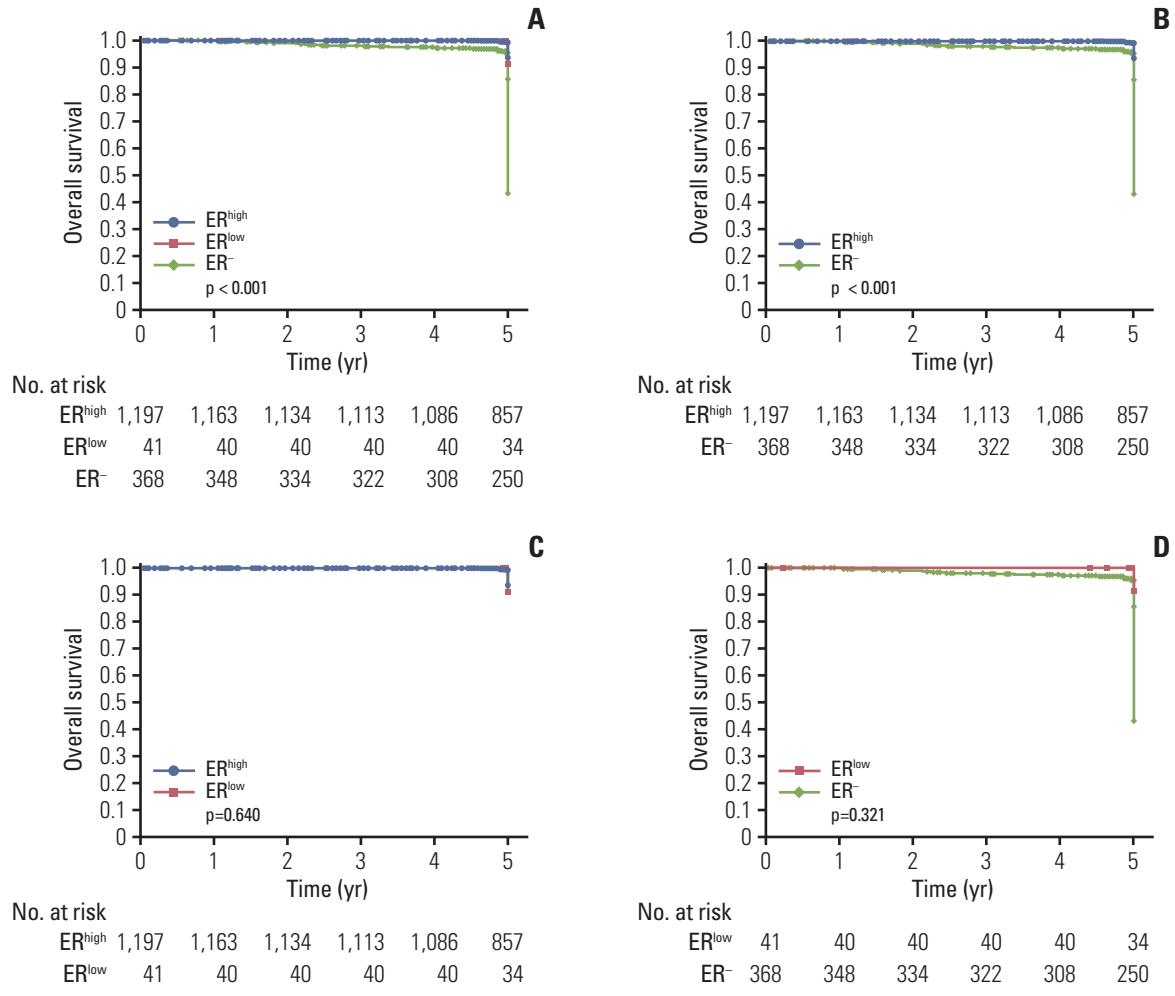

Fig. 2. Survival analysis between different estrogen receptor (ER) subgroups in early stage invasive ductal carcinoma patients. Difference in 5-year recurrence-free survival between ER^{high}/ER^{low}/ER^{-} (A), ER^{high}/ER^{-} (B), ER^{high}/ER^{low} (C), and ER^{low}/ER^{-} (D) patients. ER^{-}, estrogen receptor negative; ER^{high}, estrogen receptor high positive; ER^{low}, estrogen receptor low positive.

In 2010, the cutoff value for ER positivity was lowered to 1% from 10% by the ASCO/CAP guideline update [18]. Although the currently accepted cutoff is 1%, multiple studies have since reported that ER^{low} tumors with ER expression less than 10% show characteristics closer to ER^{-} tumors, including questionable response to hormonal therapy [10-12]. The latest recommendation of the ASCO/CAP guideline to report these tumors separately as ER low positive reflects this concern. If ER^{low} breast cancer is indeed a distinct disease subtype closer to ER^{-}, ER^{low} patients currently classified as ER^{+} will not only receive unnecessary hormonal treatment with potential side effects, but they might also fail to receive chemotherapy that is needed [17].

Several studies have addressed the clinicopathological features of ER^{low} tumors. Compared to ER^{high}, ER^{low} breast cancer is associated with younger age, advanced stage, larger tumor size, higher HER2 expression, and lower PR expression [19,20]. When morphologically analyzed, ER^{low} tumors exhibit features previously described for basal-like and triple-negative tumors, including higher grade, higher proliferation index, sheet-like growth pattern, intratumoral lymphocytic inflammatory infiltrate, and necrosis [12]. In our current study, we focused specifically on early stage breast cancer, a novel approach not presented in previous literature. ER^{low} tumors showed higher grade, positive HER2, negative PR, and higher proliferation index compared to ER^{high} tumors, which was consistent with previous studies. Age at diagnosis showed no statistically significant difference between ER^{low} and ER^{high} groups, and tumor size was smallest in the ER^{low} group compared to both ER^{high} and ER^{-} patients. Detailed morphological analysis was not performed in this study. Patients with ER^{high} tumors were more likely to

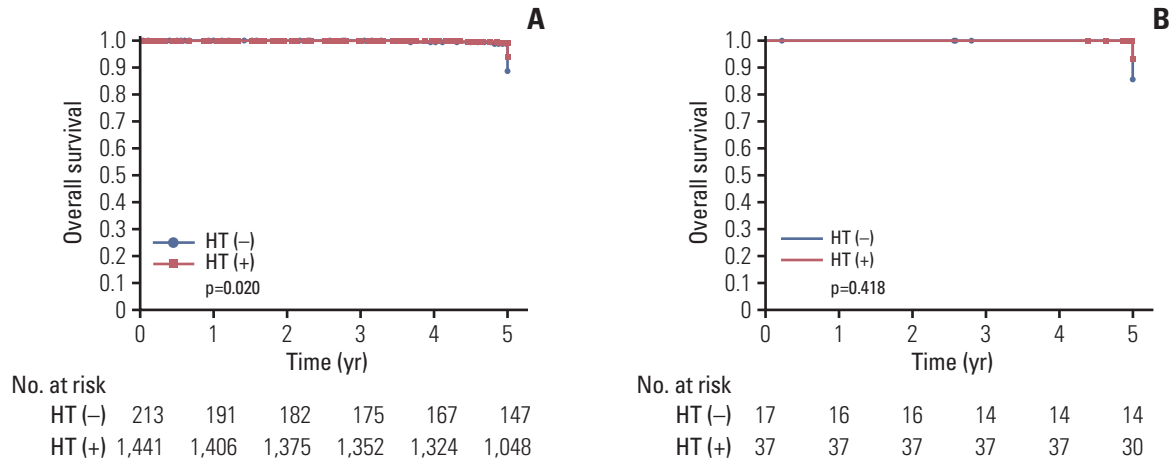

Fig. 3. Effect of estrogen receptor (ER) expression level on hormonal therapy (HT) response. (A) Difference in 5-year recurrence-free survival in ER^{high} patients. (B) Difference in 5-year recurrence-free survival in ER^{low} patients. ER^{high}, estrogen receptor high positive; ER^{low}, estrogen receptor low positive.

receive hormonal therapy compared to ER^{low} and ER^- groups; in contrast, a significantly small proportion of ER^{high} patients received chemotherapy in comparison to their ER^{low} or ER^- counterparts. This result was in concordance with previous literature [17,19,20].

Although limited data is available on the survival outcome of ER^{low} breast cancer, a few previous studies showed that ER^{low} patients exhibit significantly worse disease-free and overall survival rates compared to ER^{high} patients, but similar to those who are ER^- [11,21,22]. In the current study, the ER^{low} group had a slight, but not statistically significant, survival benefit over the ER^- group. At the same time, ER^{low} tumors showed worse prognosis compared to ER^{high} tumors, yet also with no statistical significance. Recurrence rate showed a proportional decrease with ER expression level. In multivariate regression analysis, we failed to prove the effect of ER expression level on recurrence. This study was confined to DCIS and stage I IDC, and the overall recurrence rate was low. It is possible that the low proportion of recurrent cases hindered to show a clear difference between ER subgroups. Future prospective studies with larger cohorts might validate the difference in survival outcome between ER^{low} and ER^{high} groups.

Most breast cancers exhibit either strong ER expression or its complete absence, and the number of patients in the ER^{low} subgroup is limited [23]. Therefore, prospective data on the endocrine responsiveness of ER^{low} tumors is scarce [19]. Yet many retrospective studies have suggested that primary breast cancer patients with low ER expression might not benefit significantly from hormonal therapy [17]. Viale et al. [21] compared disease-free and overall survival of ER^{low} and ER^- groups and reported that hormonal therapy had no effect on survival outcomes. In HER2-negative stage II/III breast cancer, ER^{low} tumors showed limited benefit from hormonal therapy and better response to neoadjuvant chemotherapy [24]. In our current study, we found that hormonal therapy had no effect on recurrence in ER^{low} patients; on the contrary, ER^{high} patients showed clear endocrine responsiveness. This suggests that hormonal therapy might have limited apparent benefit in early stage ER^{low} breast cancer.

ER^+ tumors have been subjected to multigene assays to identify more aggressive types that are expected to benefit from additional chemotherapy [12]. Our study sheds light on the possibility that early stage ER^{low} breast cancer might be a high risk subtype and potential candidate for chemotherapy. It is suggested that treatment options for ER^- tumors may be appropriate for some ER^{low} tumors; however, endocrine responsiveness of primary breast cancer patients with low ER expression needs to be further explored in prospective studies [20].

This study has certain limitations. First, the study was limited by its retrospective design, and treatment options were not assigned in a randomized manner. Second, although the current study was performed on a large cohort, the sample size of the ER^{low} group was relatively small. It is known that majority of breast cancers show either completely absent or strongly positive ER staining, and tumors with low ER expression are rare. Future studies with larger study populations could possibly overcome this limitation and provide more information on ER^{low} tumors.

In conclusion, ER^{low} breast cancer shows distinct clinicopathological features compared to ER^{high} and ER^- types. ER^{low} tumors seem to have higher recurrence rates compared to ER^{high} tumors, although future large scale prospective stud-

Table 2. Cox regression model for risk factors of recurrence in early breast cancer

	Total				DCIS				IDC			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (yr)												
< 50	Reference		Reference		Reference		Reference		Reference		Reference	
≥ 50	0.51 (0.35-0.73)	< 0.001	0.46 (0.32-0.66)	< 0.001	0.29 (0.12-0.72)	0.007	0.29 (0.12-0.72)	0.007	0.57 (0.38-0.86)	0.007	0.53 (0.36-0.80)	0.002
Type												
DCIS	Reference											
IDC	1.20 (0.80-1.80)	0.390	-	-	-	-	-	-	-	-	-	-
Grade												
1	Reference		Reference						Reference		Reference	
2	2.06 (1.10-3.87)	0.025	1.90 (1.00-3.61)	0.050	-	-	-	-	2.06 (1.10-3.87)	0.025	1.89 (0.99-3.61)	0.054
3	3.07 (1.65-5.73)	< 0.001	2.18 (1.05-4.52)	0.036	-	-	-	-	3.08 (1.65-5.73)	< 0.001	2.12 (0.99-4.54)	0.052
LVI												
No	Reference								Reference			
Yes	1.46 (0.90-2.37)	0.131	-	-	-	-	-	-	1.46 (0.90-2.37)	0.130	-	-
ER												
ER^{high}	Reference		Reference		Reference				Reference		Reference	
ER^{low}	1.34 (0.49-3.65)	0.571	0.95 (0.50-1.82)	0.882	1.31 (0.18-9.63)	0.793	-	-	1.36 (0.43-4.33)	0.606	1.09 (0.29-4.14)	0.897
ER^{-}	1.96 (1.35-2.85)	< 0.001	0.94 (0.34-2.64)	0.907	0.74 (0.23-2.46)	0.626	-	-	2.29 (1.52-3.44)	< 0.001	1.27 (0.36-4.44)	0.707
PR												
Positive	Reference				Reference				Reference		Reference	
Negative	1.20 (1.00-1.43)	0.054	-	-	0.81 (0.48-1.37)	0.426	-	-	1.62 (1.09-2.42)	0.018	0.52 (0.21-1.33)	0.175
HER2												
Positive	Reference								Reference			
Negative	0.76 (0.27-2.19)	0.613	-	-	-	-	-	-	0.77 (0.38-1.57)	0.477	-	-
Ki-67												
< 14	Reference		Reference		Reference				Reference		Reference	
≥ 14	1.66 (1.18-2.34)	0.004	1.00 (0.64-1.57)	0.989	1.13 (0.46-2.76)	0.794	-	-	1.79 (1.21-2.64)	0.003	0.99 (0.60-1.65)	0.971
HT												
No	Reference		Reference		Reference				Reference		Reference	
Yes	0.50 (0.35-0.70)	< 0.001	0.45 (0.25-0.79)	0.006	0.75 (0.37-1.54)	0.754	-	-	0.40 (0.27-0.59)	< 0.001	0.30 (0.12-0.77)	0.012
CT												
No	Reference								Reference			
Yes	1.06 (0.73-1.52)	0.777	-	-	-	-	-	-	1.00 (0.67-1.50)	0.985	-	-

CI, confidence interval; CT, chemotherapy; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; ER^{-}, estrogen receptor negative; ER^{high}, estrogen receptor high positive; ER^{low}, estrogen receptor low positive; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; HT, hormonal therapy; IDC, invasive ductal carcinoma; LVI, lymphovascular invasion; PR, progesterone receptor.

ies are necessary. Similar to patients with ER- tumors, those with ER^{low} tumors do not appear to benefit from hormonal therapy. Treatment options for ER^{low} breast cancer should be reconsidered, including omission of hormonal therapy and addition of adjuvant chemotherapy.

Ethical Statement

This study was approved by the institutional review board of (blinded for review) (IRB No. B-2105-682-103). All procedures performed were in accordance with the ethical standards of the institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Author Contributions

Conceived and designed the analysis: Kang E, Kim EK, Shin HC.
Collected the data: Kim SM, Jang M, Yun BL, Park SY.
Contributed data or analysis tools: Yoon KH, Park Y.
Performed the analysis: Yoon KH, Park Y.
Wrote the paper: Yoon KH, Park Y.
Critical revision of the manuscript for important intellectual content: Kim JH, Kim SH, Suh KJ.
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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.901
Cancer Res Treat. 2022;54(4):1091-1098

Original Article

Real World Evidence of Neoadjuvant Docetaxel/Carboplatin/Trastuzumab/Pertuzumab (TCHP) in Patients with HER2-Positive Early or Locally Advanced Breast Cancer: A Single-Institutional Clinical Experience

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Purpose Docetaxel/carboplatin/trastuzumab/pertuzumab (TCHP) regimen is frequently used to treat early and locally advanced human epidermal growth factor receptor 2 (HER2)–positive breast cancer (BC) in neoadjuvant setting. However, large-scaled real-world evidence did not exist.

Materials and Methods We retrospectively reviewed medical records of patients with early or locally advanced HER2-positive BC who underwent neoadjuvant TCHP followed by curative surgery at Samsung Medical Center between January 2016 and August 2020.

Results Of 447 patients, 316 (70.7%) received breast-conserving surgery and 131 (29.3%) received total mastectomy. In terms of neoadjuvant chemotherapy response, pathologic complete response (pCR) and residual cancer burden (RCB) score were analyzed. The rate of pCR was 64% a class of RCB 0 was observed in 65% of cases, RCB class I in 12%, RCB class II in 14%, and RCB class III in 2%. The 3-year event-free survival rate was 90.6%, BC with pCR occurred in 92.8%, and BC with non-pCR in 86.3% (p=0.016). In terms of distant metastasis, the 3-year distant recurrence-free survival rate was 93.5%; BC with pCR occurred in 95.9% and BC with non-pCR in 89.2% (p=0.013). Mucositis (85.2%), pain (83.2%), and diarrhea (70.5%) were the most common non-hematologic adverse events. In terms of hematologic adverse events, anemia (89.9%) was the most commonly observed adverse events followed by thrombocytopenia (29.8%).

Conclusion Neoadjuvant TCHP therapy had a pCR rate of 64% and a 3-year event-free survival of 90% in real world experience. In terms of toxicity profile, anemia was frequently observed and adequate management including occasional transfusion was required.

Key words Neoadjuvant TCHP regimen, HER2-positive breast cancer, Real world evidence

Introduction

Anti–human epidermal growth factor receptor-2 (HER2) monoclonal antibody, trastuzumab improved survival of patients with early and advanced HER2-positive breast cancer (BC) [1,2]. In terms of neoadjuvant setting, adding trastuzumab in cytotoxic chemotherapy improved pathologic complete response (pCR) and event-free survival (EFS) [3].

The addition of pertuzumab to trastuzumab and cytotoxic chemotherapy significantly improved overall survival in HER2-positive metastatic BC [4,5]. Subsequently, many clinical trials have demonstrated successful outcomes with pertuzumab and trastuzumab combination in HER2-positive BCs regardless of treatment setting [4,6-8].

As neoadjuvant therapy, pertuzumab added to trastuzumab and cytotoxic chemotherapy improved pCR and patient survival [7,9]. Accordingly, treatment guidelines for HER2-positive early or locally advanced BC have been established with trastuzumab±pertuzumab and chemotherapy as the standard treatment strategy [10,11].

Among clinical trials with pertuzumab for early or locally advanced HER2-positive BC, the TRYPHAENA clinical trial was designed to evaluate the safety and efficacy of pertuzumab and trastuzumab in combination with anthracycline- or carboplatin-based neoadjuvant chemotherapy (NAC) in HER2-positive BC [12]. The reported safety profile in this study indicated that six cycles of docetaxel/carboplatin/trastuzumab/pertuzumab (TCHP) had severe adverse

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Received August 9, 2021 **Accepted** January 4, 2022
Published Online January 10, 2022

*Part of this work was presented at the San Antonio Breast Cancer Symposium in December 2020.

events (SAEs) in approximately 30% of cases. This represents the highest rate of SAEs compared with other treatment arms despite lowest cardiac toxicity [12]. Approximately 70% of patients experienced diarrhea, and grade 3/4 neutropenia was observed in about 50% of patients who received the neoadjuvant TCHP regimen [12]. In terms of efficacy, TCHP had a pCR of 64% and 90% 3-year disease-free survival (DFS) [12,13].

Recent advance of adjuvant treatment strategy suggested that trastuzumab emtansine (T-DM1) significantly improved 3-year DFS in HER2-positive BC which did not achieved pCR after NAC compared with trastuzumab treatment despite several toxicities [14]. Therefore, pCR achievement is the important factor to decide adjuvant treatment strategy as well as surrogate marker of survival. Now, TCHP regimen is frequently used for HER2-positive BC in neoadjuvant settings because of the best in terms of pCR.

Here, we report our clinical experience with BC patients treated with neoadjuvant TCHP followed by curative surgery. Comprehensive analysis of the efficacy and safety of the neoadjuvant TCHP regimen were performed in real world experience (RWE).

Materials and Methods

1. Patients

We retrospectively reviewed electronic medical records of patients with early or locally advanced HER2-positive BC who underwent neoadjuvant TCHP chemotherapy followed by curative surgery at Samsung Medical Center between January 2016 and August 2020. We included patients diagnosed with clinical stage II to IIIC BC by diagnostic examinations (breast ultrasonography and/or magnetic resonance imaging, chest and abdomino-pelvic computed tomography (CT) scan, bone scan, and/or positron emission tomography–CT scans, if indicated). Stage was based on American Joint Committee on Cancer (AJCC) 7th edition. Patients who received previous BC surgery due to local recurrence after curative surgery or who underwent palliative surgery were excluded. In the event of bilateral BC, one BC that required NAC was selected.

2. Neoadjuvant chemotherapy

Patients received six cycles of TCHP neoadjuvant therapy. The study drugs were administered intravenously once every 3 weeks. Details of administration method were described in previous article [12]. Prophylactic pegfilgrastim was administered at every treatment cycle.

3. BC pathology

Pathologists determined BC histology and receptor status (estrogen receptor [ER], progesterone receptor [PR], and HER2) according to hematoxylin and eosin and immunohistochemical (IHC) staining. ER and PR positivity were defined as Allred score in the range of 3-8 according to IHC staining with antibodies to ER (Immunotech, Marseille, France) and PR (Novocastra, Newcastle upon Tyne, UK), respectively. HER2 status was evaluated using the appropriate antibody (DAKO, Carpinteria, CA) and/or silver *in situ* hybridization (SISH). HER2 grades of 0 and 1 were defined as negative results, while grade 3 was identified as a positive result. *HER2* amplification was confirmed by SISH results of 2+. All HER2-positive BC were included regardless of ER and PR state.

After surgery, pathologic response to NAC was determined as pCR or residual cancer burden (RCB) [15]. We defined pCR as no residual invasive tumor in the primary tumor bed and ipsilateral axillary lymph nodes regardless of the presence of ductal carcinoma *in situ* (DCIS) (ypT0/is ypN0) [16].

4. Statistical analysis

EFS was defined as the elapsed time from date of curative surgery to detection of local or distant tumor recurrence. We also included contralateral or ipsilateral DCIS as an observed event. Distant recurrence-free survival (DRFS) was defined as the elapsed time from date of curative surgery to detection of distant metastasis. Overall survival (OS) was defined as the duration between curative surgery and death. DRFS and OS were analyzed using the Kaplan-Meier method. Cox proportional-hazard regression was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). Two-tailed p-values < 0.05 were considered statistically significant, and IBM SPSS Statistics ver. 21 (IBM Corp., Armonk, NY) was used for analysis of all data.

Results

1. Patient cohort

Between February 2016 and August 2019, 1,840 BC patients received NAC followed by curative surgery. Among these patients, those with HER2-positive BC numbered 539 (38.0%), and the TCHP regimen was used in 447 (24.3%) (S1 Fig.). Baseline characteristics of these 447 patients are described in Table 1. Hormone receptor positivity was observed in 48.3% of these patients, and 45.4% were stage III. Median age of patients was 49.

Table 1. Clinicopathological characteristics of patients

Characteristic	No. (%) (n=447)
Age, median (range, yr)	49 (19-80)
< 40	75 (16.8)
≥ 40 and < 50	166 (47.1)
≥ 50 and < 60	157 (35.1)
≥ 60	49 (11.0)
Female sex	447 (100)
Menopausal status	
Pre-menopause	258 (57.7)
Post-menopause	187 (41.8)
Unknown	2 (0.5)
Familiar history	
Yes	66 (14.8)
No	381 (85.2)
Clinical stage (AJCC 7th)	
2A	112 (25.1)
2B	132 (29.5)
3A	133 (29.8)
3B	5 (1.1)
3C	65 (14.5)
Type of surgery	
BCS with SLNB	279 (62.4)
BCS with ALND	43 (9.6)
TM with SLNB	83 (18.6)
TM with ALND	42 (9.4)
BRCA status (n=129)	
BRCA1 alteration	0
BRCA2 alteration	3 (2.3)
Histology	
IDC	402 (89.9)
IDC with ILC	11 (2.5)
Micropapillary	21 (4.7)
Others	13 (2.9)
Histologic grade	
1	3 (0.7)
2	216 (48.3)
3	225 (50.3)
Unknown	3 (0.7)
Nuclear grade	
1	0
2	189 (42.3)
3	257 (57.5)
Unknown	1 (0.2)
Subtype	
ER+/PR+	141 (31.5)
ER+/PR–	71 (15.9)
ER–/PR+	3 (0.7)
ER–/PR–	231 (51.7)
Unknown	1 (0.2)

AJCC, American Joint Committee on Cancer; ALND, axillary lymph node dissection; BCS, breast-conserving surgery; ER, estrogen receptor; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; PR, progesterone receptor; SLNB, sentinel lymph node biopsy; TM, total mastectomy including nipple sparing mastectomy.

2. Response to neoadjuvant TCHP regimen

Of 447 patients, 279 (62.4%) received breast-conserving surgery (BCS) with sentinel lymph node biopsy (SLNB), 43 (9.6%) received BCS with axillary lymph node dissection (ALND), 83 (18.6%) opted for total mastectomy (TM) with SLNB, and 42 (9.4%) underwent TM with ALND. When axillary lymph node metastasis was suspected, fine needle aspiration was performed at the time of BC diagnosis. We confirmed axillary lymph node metastases pathologically in 172 patients. Of these 172 patients, ALND was performed in 47 (27.3%). Twenty-eight patients (16.3%) had axillary lymph node metastasis at the time of curative surgery.

In terms of NAC response, we evaluated pathologic response at the time of curative surgery. This evaluation included pCR and RCB score. The rate of pCR was 64% and differed according to hormone receptor status ($p < 0.001$), clinical stage ($p=0.028$), and histologic grade ($p=0.010$) (Fig. 1). Other factors that affected NAC response are described in S2 Table. In this analysis, relative dose intensities (RDIs) of docetaxel and carboplatin did not affect NAC response ($p=0.187$ and 0.917, respectively). In terms of RCB score, a class of RCB 0 was observed in 65% of cases, RCB class I in 12%, RCB class II in 14%, and RCB class III in 2% (S3 Table). Four cases did not achieve pCR but in RCB class 0 because lymphovascular invasions remained in two cases, lymphatic emboli in one case and isolated tumor cell in lymph node in one case after NAC.

In multivariate analysis of the associations between baseline characteristics and pCR status, hormone receptor negativity was positively related to pCR (HR, 0.36; 95% CI, 0.24 to 0.56; $p=0.001$) whereas pre-menopausal status and advanced clinical stage were oppositely (HR, 1.91; 95% CI, 1.27 to 2.95; $p=0.004$ and HR, 2.62; 95% CI, 1.36 to 5.07; $p=0.004$, respectively) (Fig. 2A).

3. Survival analysis

We ceased data acquisition in December 2020, and the median follow-up duration was 36 months. During follow-up, 33 events have occurred, 24 cases of distant metastasis and nine of local recurrence. The 3-year EFS rate was 90.6%,

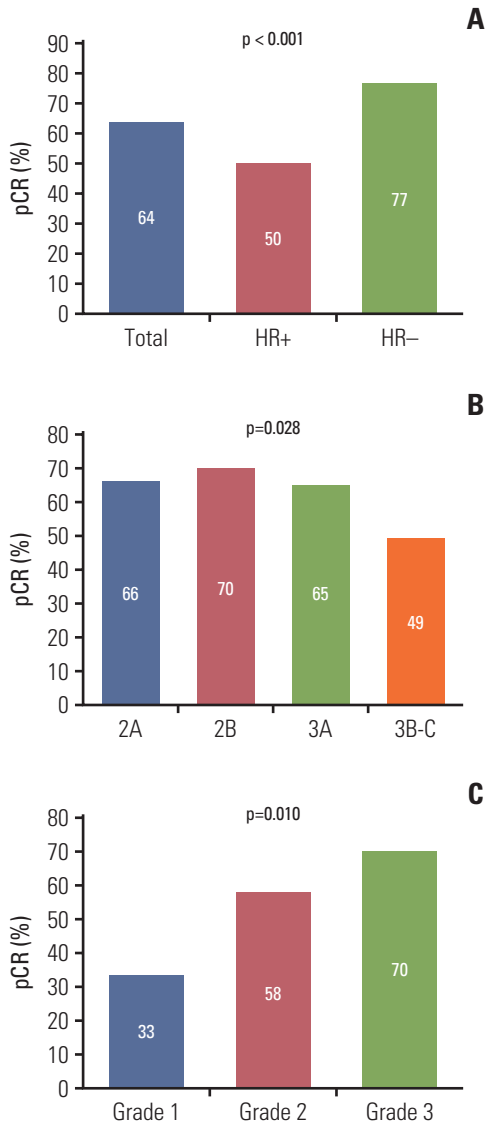

Fig. 1. Pathologic complete response (pCR) according to hormone receptor (HR) status (A), clinical stage (B), and histologic grade (C).

BC with pCR occurred in 92.8%, and BC with non-pCR in 86.3% (p=0.016) (Fig. 3). In terms of distant metastasis, the 3-year DRFS rate was 93.5% in the total patients, 95.9% in pCR patients, and 89.2% in non-pCR patients (p=0.013). There were two deaths, and the 3-year OS rate was 99.6%. Both deaths were in the non-pCR group. OS was also grouped according to pCR status, but significant difference was not observed (p=0.059).

We also analyzed the association between survival rate and RCB class (S4 Fig.). EFS was associated with red blood cell (RBC) class: the 3-year EFS of RCB classes 0, 1, 2, and 3 were 93.0%, 93.3%, 82.1%, and 62.5%, respectively, p < 0.001. DRFS and OS were also associated with RCB class. Three-year DRFS of RCB classes 0, 1, 2, and 3 were 96.0%, 93.3%, 88.2%, and 72.9%, respectively (p=0.014). OS was associated with RCB class (p < 0.001).

The effects of key characteristics on EFS were shown in Fig. 2B. Clinical stage IIIB or IIIC (HR, 8.83; 95% CI, 2.58 to 30.15; p=0.001) and pCR status (HR, 0.39; 95% CI, 0.18 to 0.86; p=0.019) demonstrated effects on EFS.

4. Safety and toxicity

Mucositis (85.2%), pain (83.2%), and diarrhea (70.5%) were the most common non-hematologic adverse events (Table 2). In terms of grade 3 or higher adverse events, anorexia (5.8%) and diarrhea (3.1%) were most commonly observed. Hematologic adverse events were also frequently observed (Table 2). Anemia (89.9%) was the most commonly observed adverse events followed by thrombocytopenia (29.8%). Grade 3 or higher anemia occurred in 7.2% of patients and 90 patients (20.1%) received RBC transfusion. Neutropenia (24.5%) was frequently observed even though prophylactic pegfilgrastim was used at every NAC cycle. Grade 3 or higher neutropenia occurred in 5.4% and febrile neutropenia in 2.0%.

Five patients (1.1%) did not complete six cycles of neoadjuvant TCHP chemotherapy. Two patients (0.4%) only received three cycles, four cycles in two patients (0.4%) and five cycles in one patient (0.2%). RDIs of docetaxel and carboplatin were 0.965 and 0.876, respectively (S5 Table). The RDIs of both decreased as cycles progressed.

In terms of cardiac toxicity, median left ventricle ejection fraction (LVEF) at baseline echocardiography was 65.6 (interquartile range, 62 to 69) and 64.0 (interquartile range, 60 to 67). We observed ejection fraction (EF) decrease in 257 patients during NAC and more than 10% decrease of EF was observed in 88 patients. However, no patients underwent significant declines in LVEF (≥ 10% points from baseline to < 50%) and symptomatic left ventricle systolic dysfunction (S6 Fig.).

5. Treatment after curative surgery

After curative surgery, patients received adjuvant targeted agents per the established protocol. In BC patients achieving pCR, 96.5% received adjuvant trastuzumab; 3.5% received the trastuzumab and pertuzumab combination. In BC patients without pCR, adjuvant trastuzumab was used in 88.8% of patients, the trastuzumab and pertuzumab combination in 7.5%, and T-DM1 in 3.7%.

Of 447 patients, 411 (91.9%) patients received adjuvant radiotherapy (RTx) after curative surgery and 258 patients (62.8%) of patients received adjuvant RTx were performed in our institute. We evaluated the relationship between the

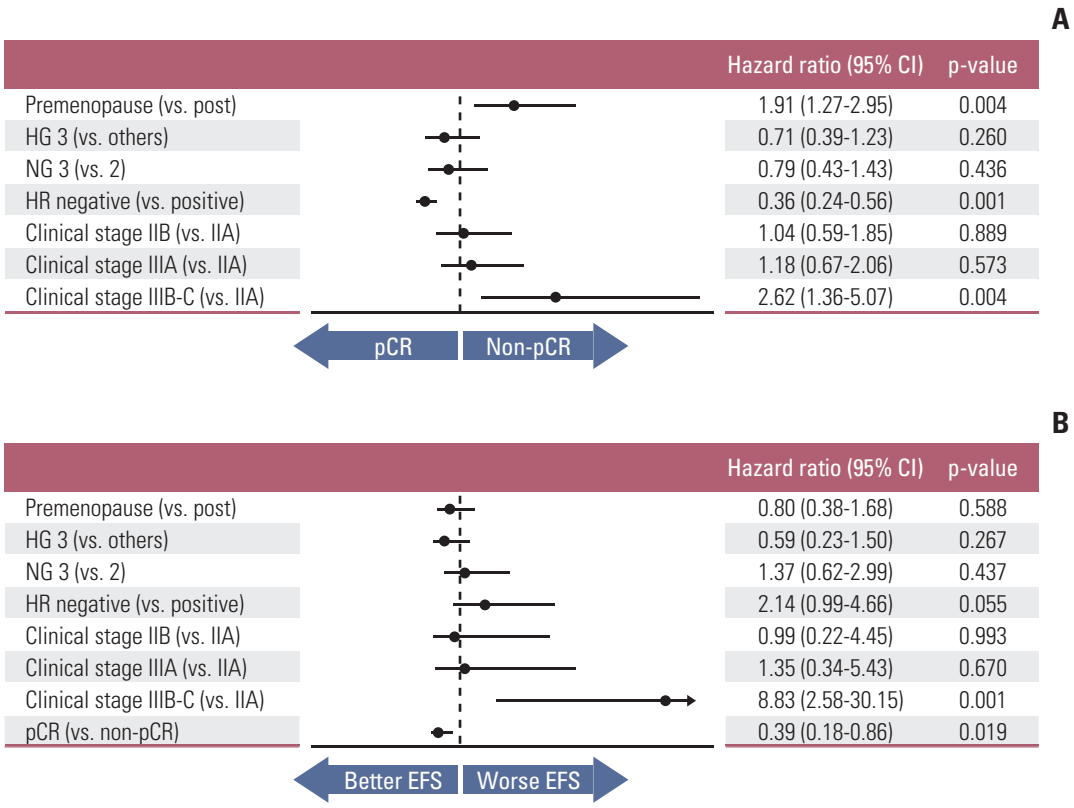

Fig. 2. Multivariate analysis of factors affecting to pathologic complete response (pCR) (A) and event-free survival (EFS) (B). CI, confidence interval; HG, histologic grade; NG, nuclear grade.

radiation dose and pCR status. In this analysis, patients who achieved pCR received less radiation dose compared with patients without pCR ($p < 0.001$) (S7 Table).

Discussion

We evaluated the efficacy and safety profile of neoadjuvant TCHP chemotherapy in real world practice. pCR rate was 64% and 3-year EFS was 90.6%. In terms of adverse events (AEs), approximately 90% of patients experienced anemia. Mucositis, pain, and diarrhea were the most frequently observed non-hematologic AEs.

This result was compatible with that of the TRYPHAENA clinical trial [12]. This previous clinical trial demonstrated a pCR rate of 66% and a 3-year DFS rate of 90% [13].

We analyzed the incidence of down-staging in terms of axillary lymph node status, one of the advantages of NAC. Approximately 72.7% of patients experienced down-staging in terms of axillary node evaluation. Moreover, we performed further subgroup analysis to find the factors associated with pCR and survival. Hormone receptor negativity, low clinical stage (stage IIA-IIIA), and post-menopausal status were favorable to pCR. In terms of survival, clinical stage and pCR affected the EFS. Interestingly, hormone receptor negativity increased pCR rate but negatively affected EFS although statistical significance was marginal (p=0.055). Previous studies have suggested that hormone receptor-negative, HER2-positive BC had higher pCR rate compared with hormone receptor-positive, HER2-positive BC, whereas progression-free survival (PFS) was longer in hormone receptor-positive, HER2-positive BC rather than hormone receptor-negative, HER2-positive BC according to the NeoSphere trial.

Moreover, clinical stage significantly affected survival regardless of pCR status. This result suggests that negative hormone receptor status with initially high clinical stage BC has increased disease recurrence even though pCR had been achieved. Longer-term follow-up is warranted to confirm our suggestion.

In terms of AEs, there are s differences between our clinical experience and what was observed in clinical trial [12]. We observed anemia of 89.9% and 20.1% of patients received RBC transfusion compared with anemia of 36.8% in the clinical trial. The incidence of neutropenia (24.2%) was lower than that of the clinical trial (48.7%); however, prophylactic pegfilgrastim was administered in real world practice.

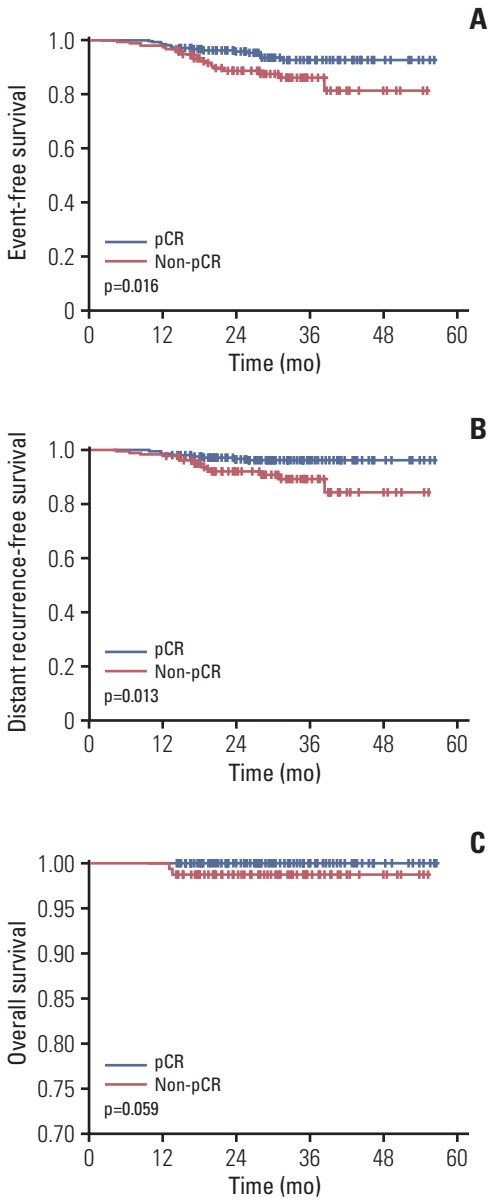

Fig. 3. Event-free survival (A), distant recurrence-free survival (B), and overall survival (C) according to pathologic complete response (pCR) or non-pCR.

Table 2. Adverse events for neoadjuvant TCHP chemotherapy

Adverse event	All grade	≥ Grade 3
Anorexia	302 (67.6)	26 (5.8)
Vomiting	129 (27.8)	13 (2.9)
Diarrhea	327 (70.5)	14 (3.1)
Constipation	115 (25.7)	1 (0.2)
Dermatologic adverse event	149 (33.3)	2 (0.4)
Rash	108 (24.2)	2 (0.4)
Pruritus	92 (20.6)	0
Acne like reaction	50 (11.2)	1 (0.2)
Liver function test		
Albumin	49 (11.0)	0
Bilirubin, total	29 (6.5)	0
AST	240 (53.7)	5 (1.1)
ALT	281 (62.9)	6 (1.3)
ALP	137 (30.6)	0
Mucositis	381 (85.2)	1 (0.2)
Fatigue	242 (54.1)	3 (0.7)
Peripheral neuropathy	270 (60.4)	3 (0.7)
Pain	372 (83.2)	1 (0.2)
Hematologic adverse event		
Anemia	402 (89.9)	32 (7.2)
RBC transfusion	90 (20.1)	0
Leukopenia	95 (21.3)	6 (1.3)
Neutropenia	108 (24.2)	24 (5.4)
Febrile neutropenia	0	9 (2.0)
Thrombocytopenia	133 (29.8)	5 (1.1)
PLT transfusion	6 (1.3)	0

Values are presented as number (%). ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PLT, platelet; RBC, red blood cell; TCHP, docetaxel/carboplatin/trastuzumab/pertuzumab.

We observed high incidence of all grade AEs in both non-hematologic and hematologic fields. But grade 3 or higher AEs were not frequently observed. In our data, median age of patients was 49 and 90% of patients were under 60 years of age. We suggested that young aged patients resulted low incidence of high-grade AEs.

RDI of docetaxel decreased during NAC. Especially patients over 60 years of age received less dose of docetaxel compared with whom under 60 years of age. This result suggested that elderly patient had high risk of serious AEs and dose reduction would be needed. Therefore, physicians should carefully examine elderly patients with underlying disease during NAC with TCHP regimen. In terms of carboplatin, we initially decreased the dosage of carboplatin in patients who had risk factors of renal impairment or emesis. Therefore, the dose intensity of carboplatin in the first cycle was low despite the high proportion of young patients.

The combination of docetaxel, pertuzumab, and trastuzumab is used for metastatic HER2-positive BC as first-line treatment; its efficacy was demonstrated in the CLEOPATRA clinical trial [4]. The *post hoc* analysis of duration of docetaxel administration showed that 14.2% of patients received fewer than six cycles because of AEs. Interestingly, these patients had shorter PFS and OS compared with those who received six cycles of docetaxel treatment [17]. Another post hoc analysis of the safety profile in Asian patients showed that these patients had more frequent docetaxel dose reduc-

tion than patients in other regions because Asian patients experienced more AEs. However, efficacy in terms of PFS and OS was similar in Asian patients to those from other regions [18].

Approximately 90% of patients in the TRYPAENA trial received the scheduled number of cycles of docetaxel and carboplatin [12]. In our study, only five (1.1%) of our patients failed to receive six cycles of treatment, and the docetaxel RDI was 0.965 and that of carboplatin was 0.876. We suggest that high RDIs of cytotoxic agents were possible due to prophylactic use of pegfilgrastim and relatively young aged patients, but high incidence of anemia and thrombocytopenia occurred because of high RDIs. In the analysis of the relationship between the RDIs of cytotoxic agents and pCR status, we did not observe the impact of RDI on pCR status. This result may be preliminary but considering toxicities of TCHP regimen, careful dose modification is necessary to maintain a balance between efficacy and safety of neoadjuvant TCHP treatment.

Although this study s retrospective analysis of neoadjuvant TCHP chemotherapy, the sample size is relatively large, and details of AEs are described. Neoadjuvant TCHP regimen is now popularly used for HER2-positive BC and therefore RWE of this regimen may be useful as treatment reference. In contrast with previous clinical trial, we focused on the factors affecting the efficacy of NAC and NAC-related AEs rather than cardiac toxicity. Relatively short follow-up duration is limitation of our study and long-term follow-up would be warranted.

In conclusion, neoadjuvant TCHP therapy had a pCR rate of 64% and a 3-year EFS of 90% in RWE. In terms of toxicity profile, anemia was frequently observed and adequate management including occasional transfusion was required.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was reviewed and approved by the Institutional Review Board (IRB) of Samsung Medical Center, Seoul, Korea (IRB No. 2019-04-021). The requirement for informed consent was waived due to the use of medical records with retrospective clinical data.

Author Contributions

Conceived and designed the analysis: Kim SW, Park YH.
Collected the data: Kim JY, Nam SJ, Lee JE, Yu J, Chae BJ, Lee SK, Ryu JM, Ahn JS, Im YH, Kim SW, Park YH.
Contributed data or analysis tools: Kim JY, Ahn JS, Im YH, Kim SW, Park YH.
Performed the analysis: Kim JY.
Wrote the paper: Kim JY, Park YH.

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Conflicts of Interest

YHP reports grants from Pfizer, AstraZeneca, Novartis, Merck, Roche, and Eisai. All other authors declare no competing interests.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.978
Cancer Res Treat. 2022;54(4):1099-1110

Original Article

Implication and Influence of Multigene Panel Testing with Genetic Counseling in Korean Patients with *BRCA1/2* Mutation–Negative Breast Cancer

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Purpose The aim of the study was to evaluate the clinical implication of multigene panel testing of beyond *BRCA* genes in Korean patients with *BRCA1/2* mutation-negative breast cancer.

Materials and Methods Between 2016 and 2019, a total of 700 *BRCA1/2* mutation-negative breast cancer patients received comprehensive multigene panel testing and genetic counseling. Among them, 347 patients completed a questionnaire about cancer worry, genetic knowledge, and preference for the method of genetic tests during pre- and post-genetic test counseling. The frequency of pathogenic and likely pathogenic variants (PV/LPV) were analyzed.

Results At least one PV/LPV of 26 genes was found in 76 out of 700 patients (10.9 %). The rate for PV/LPV was 3.4% for high-risk genes (17 *PALB2*, 6 *TP53*, and 1 *PTEN*). PV/LPVs of clinical actionable genes for breast cancer management, high-risk genes and other moderate-risk genes such as *ATM*, *BARD1*, *BRIP*, *CHEK2*, *NF1*, and *RAD51D*, were observed in 7.4%. Patients who completed the questionnaire showed decreased concerns about the risk of additional cancer development (average score, 4.21 to 3.94; $p < 0.001$), influence on mood (3.27 to 3.13; $p < 0.001$), influence on daily functioning (3.03 to 2.94; $p=0.006$); and increased knowledge about hereditary cancer syndrome (66.9 to 68.8; $p=0.025$) in post-test genetic counseling. High cancer worry scales (CWSs) were associated with age ≤ 40 years and the identification of PV/LPV. Low CWSs were related to the satisfaction of the counselee.

Conclusion Comprehensive multigene panel test with genetic counseling is clinically applicable. It should be based on interpretable genetic information, consideration of potential psychological consequences, and proper preventive strategies.

Key words Breast neoplasms, Genetic testing, Multigene panels, Beyond *BRCA*, Cancer worry

Introduction

With the discovery of breast cancer susceptibility genes and recognizing the significantly increased breast cancer risks in the carriers with pathogenic variant (PV) or likely pathogenic variant (LPV), clinical practice regarding hereditary or familial breast cancers has undergone considerable changes for several decades. The most well-known hereditary breast cancer susceptibility genes, *BRCA1* and *BRCA2* mutations result in cumulative risk of female breast cancer by the age of 80 of 57%-72% and 45%-69%, respectively [1-3]. Many current clinical guidelines recommend preventive strategies for carriers of *BRCA* mutations [4-7].

In addition, with the commercialization of multigene panel tests using next-generation sequencing, it has become more common to test germline mutations of other breast cancer susceptibility genes beyond *BRCA* using multigene panels. Despite the cost effectiveness and shortened turnaround time to test multiple genes, comprehensive multigene panel tests still have several limitations, including a high likelihood for detection of variants of unknown significance (VUS) or secondary findings, as well as limited information and preventive strategies especially for the carriers with low to moderate-penetrance cancer susceptibility genetic variants.

A recent study reported that multigene panel tests did not increase cancer worry in the patients with breast cancer, compared to those who underwent *BRCA1*/2-only testing [8]. However, because the results of multigene panel tests can provoke negative emotional effects exceeding potential preventive benefit for some patients, there is still the opinion that multigene panel tests should be carefully applied in accordance with phenotypical features of multiple hereditary cancer syndrome or limited to individuals without a known

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Received August 30, 2021 **Accepted** November 15, 2021 **Published Online** November 17, 2021

genetic mutation of a single syndrome [7,9]. Therefore, the use of comprehensive multigene panel testing requires discussion of clinical actionability and consideration of possible negative emotional effects.

In this study, we prospectively tested the germline genetic variants beyond *BRCA* in Korean *BRCA1/2* mutation-negative breast cancer patients with high risk of hereditary cancer syndrome using a comprehensive multigene panel. Subsequently, we evaluated the frequency of PV/LPV in clinically actionable genes for breast cancer, cancer worry, genetic knowledge, and preference for the sequence and methods of multigene panel testing among the patients. In this manner, we considered clinical actionability and emotional effect of comprehensive multigene panel testing.

Materials and Methods

1. Study population

We enrolled Korean *BRCA1/2* mutation-negative breast cancer patients with at least one high-risk factor for hereditary breast cancer syndrome. Risk factors of hereditary breast cancer were defined as follows: (1) at least one case of breast or ovarian cancer in first- or second-degree relatives; (2) a first diagnosis of breast cancer before age 40; (3) bilateral breast cancer; (4) male breast cancer and (5) co-diagnosis with breast and other cancers in the same patient. Between March 2016 and December 2019, 1,866 breast cancer patients with high-risk factors were tested for *BRCA1/2* germline mutations, and 76 carriers with *BRCA1* mutations and 119 carriers with *BRCA2* mutations (one patient had both *BRCA1* and *BRCA2* mutations) were identified in Yonsei Cancer Center, Yonsei University College of Medicine, Seoul, Republic of Korea. Out of the patients without *BRCA1/2* mutations, we conducted comprehensive multigene panel tests for 700 participants, and additionally evaluated cancer worry, genetic knowledge, and attitude toward the multigene panel tests for 374 participants who agreed to answer questionnaires before and after the genetic tests. A flowchart including study design and process is shown in S1 Fig.

2. Comprehensive multigene panel-based variant analysis

Genomic DNA was extracted from the patients' peripheral blood samples. We used a customized targeted capture sequencing panel which included all coding sequences and intron-exon boundaries of the coding exon from 65 cancer predisposition genes (*APC*, *ALK*, *ATM*, *AXIN1*, *AXIN2*, *BARD1*, *BLM*, *BMPR1A*, *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CDK4*, *CDKN2A*, *CHEK2*, *CTNNB1*, *EPCAM*, *EXO1*, *FANCM*, *FLCN*, *GALNT12*, *GPC3*, *GREM1*, *KIF1B*, *KRAS*, *LMO1*, *MEN1*, *MLH1*, *MLH3*, *MRE11A*, *MSH2*, *MSH6*, *MUTYH*, *NBN*, *NF1*, *NF2*, *NRAS*, *NTRK1*, *PALB2*, *PAX6*, *PHOX2B*, *PMS1*, *PMS2*, *POLD1*, *POLE*, *PPM1D*, *PRSS1*, *PTCH1*, *PTEN*, *RAD50*, *RAD51*, *RAD51C*, *RAD51D*, *RB1*, *RET*, *RUNX1*, *SDHA*, *SDHAF2*, *SDHB*, *SLX4*, *SMAD4*, *STK11*, *TP53*, *VHL*, and *WT1*). Products with each capture reaction were sequenced by 151 base pair paired-end reads on a NextSeq 550Dx instrument (Illumina, San Diego, CA). High-quality sequencing data with an average depth of 500-1,000 fold was obtained.

We identified all single base pair substitutions, insertion-deletions, and copy number variants (CNVs) in each gene. All likely deleterious variants were validated by Sanger sequencing. Split-read-based detection of large insertions and deletions was conducted using the Pindel and Manta algorithms. CNVs detected by ExomeDepth software [10] were further crosschecked with a base-level read depth normalization algorithm implemented in the DxSeq Analyzer (Dxome, Seoul, Korea). All possible large rearrangements were confirmed by the multiplex ligation-dependent probe amplification method. Genetic variants were classified using a five-tier system following guidelines from the American College of Medical Genetics and Genomics [11], and PV/LPV was considered to be a mutation in the current study [12].

3. Clinical data collection

Sociodemographic factors (sex, current age, age at first diagnosis of breast cancer, education level, marital status, and the number of children) were obtained during the baseline interview prior to pre-test counseling. The family history of cancer within the third-degree relatives was assessed by drawing a pedigree for each family during the pre-test counseling. The characteristics of breast cancer (pathological diagnosis, laterality, and subtype) and presence of other primary malignancy were obtained by review of medical records with permission from each participant.

4. Definition of the genes of interest

Among the genes tested using the comprehensive multigene panel, 14 genes were defined as clinically actionable genes [13] for risk-reduction of breast cancer using recommended strategies according to the NCCN, ASCO, or ESMO guidelines [4,6,7]: *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CHEK2*, *NF1*, *PALB2*, *PTEN*, *RAD51C*, *RAD51D*, *STK11*, and *TP53*. Considering the penetrance for hereditary breast cancer in the previous reports and guidelines, we defined *BRCA1*, *BRCA2*, *CDH1*, *PALB2*, *PTEN*, *STK11*, and *TP53* as high-risk genes; *ATM*, *BARD1*, *BRIP1*, *CHEK2*, *NF1*, *RAD51C*, and *RAD51D* as moderate-risk genes; and other genes as unknown-risk genes for breast cancer [7,14-16].

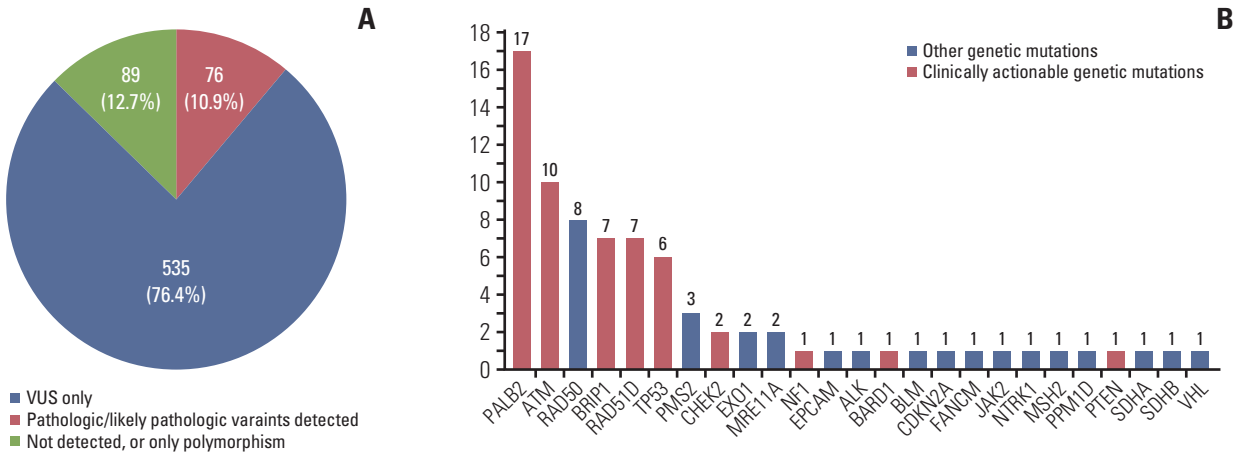

Fig. 1. Result of germline multigene panel tests in *BRCA* mutation-negative patients with high risk for hereditary breast cancer (n=700). (A) Proportion of the patients according to the results. (B) Frequency of likely pathogenic/pathogenic genetic variants (genes, n=26; patients, n=76)[a]. VUS, variants of unknown significance. [a]Three patients had two likely pathogenic/pathogenic variants simultaneously (*RAD50* and *PMS2*, *EPCAM* and *SDHA*, and *JAK2* and *NTRK1*).

5. Genetic counseling

For all the patients enrolled in this study, the researchers provided pre- and post-test counseling. Genetic counseling was conducted by a trained medical oncologist and two registered nurses. The three researchers had completed a genetic counseling program certified by the Korean Breast Cancer Society. The pre-test counseling included the significance and utility of genetic variants with information, possible discrimination in insurance and employment, and alternatives to genetic testing. Post-test counseling was regarding interpretation of the genetic tests results and recommendations based on the results. For the mutation carriers, we provided preventive strategies via a multidisciplinary clinic consisting of various cancer specialists. We also recommended familial disclosure of genetic test result, and provided familial genetic testing with counseling for the family members.

6. Questionnaires about cancer worry, genetic knowledge, and attitude to genetic tests

In this study, cancer worry and its influence on mood and daily functioning were measured using a five-point Likert scale from Lerman's Cancer Worry Scale (CWS) [17], which was modified under Korean translation [18,19], with a Cronbach's alpha of 0.853. Genetic knowledge was measured using a 12-item true-false scale test adapted from Erblich's Breast Cancer Genetic Counseling Knowledge Questionnaire (BGKQ) [20], which was translated and applied to previous studies [21,22], with a Cronbach's alpha of 0.817 in this study. Total score of the test was calculated on a scale of 100 points. After a genetic test and post-test counseling, we assessed the patient's satisfaction about the comprehensive multigene panel tests with counseling using the question, "How much were your questions regarding the possibility of hereditary breast cancer answered after the multigene panel test?" with answer choices using the five ordinal variables of "very satisfied," "satisfied," "neutral," "dissatisfied," and "very dissatisfied." The second question was asked to assess the patient preference for the sequence of genetic tests, "You did multigene panel tests after confirmation of negative for *BRCA1/2* mutation. If you can select the sequence for testing *BRCA1/2* genes and other genetic variants beyond *BRCA*, which of the method would you prefer?" with four choices, "concurrent tests using multigene panel," "multigene panel test only for *BRCA1/2* negative patients," "*BRCA1/2* mutation tests only," or "not sure."

7. Statistical analysis

Correlation between each risk factor and identified PV/LPV was analyzed using a chi-square test or Fisher's exact test if indicated. Differences between pre-test and post-test values of CWS and BGKQ were compared using paired t tests. Clinico-genetic factors associated with genetic test results and post-test cancer worry were analyzed using single and multiple linear logistic regression modeling. Multiple linear logistic regression modeling was conducted using the variables with a p-value < 0.2 in the simple linear logistic regression model. A p-value < 0.05 was designated as statistically significant and all tests were two-sided. All statistical analyses were performed using IBM SPSS ver. 25.0 (IBM Corp., Armonk, NY).

Table 1. Baseline characteristics of the patients according to the genetic results (n=700)

Clinicopathological variable	PV/LPV (n=76)	VUS or ND (n=624)	p-value
Age at first diagnosis of breast cancer (yr)	44 (25-82)	43 (17-83)	0.628
Sex			
Male	2 (2.6)	5 (0.8)	0.171[a)]
Female	74 (97.4)	619 (99.2)	
Breast cancer, laterality			
Unilateral	64 (84.2)	554 (88.8)	0.250[a)]
Bilateral (metachronous)	6 (7.9)	24 (3.8)	
Bilateral (synchronous)	6 (7.9)	46 (7.4)	
Pathology			
IDC	55 (72.4)	435 (69.7)	0.545[a)]
ILC	5 (6.6)	18 (2.9)	
DCIS	11 (14.5)	119 (19.1)	
LCIS	1 (1.3)	10 (1.6)	
Others	4 (5.2)	39 (6.3)	
Unknown	0	3 (0.5)	
Hormone receptor			
Positive	51 (67.1)	156 (25.0)	0.255[a)]
Negative	23 (30.3)	460 (73.7)	
Unknown	2 (2.6)	8 (1.3)	
TNBC			
TNBC	15 (19.7)	98 (15.7)	0.367
Others	61 (80.3)	526 (84.3)	
Education			
University/College graduate	43 (56.6)	375 (60.1)	0.301[a)]
High school graduate	24 (31.6)	161 (25.8)	
Middle graduate	1 (1.3)	26 (4.2)	
No/Elementary school graduate	3 (3.9)	9 (1.5)	
Unknown	5 (6.6)	53 (8.5)	
Family history of breast cancer			
Yes	30 (39.5)	288 (46.2)	0.269
No	46 (60.5)	336 (53.8)	
Family history of ovarian cancer			
Yes	7 (9.2)	26 (4.2)	0.076[a)]
No	69 (90.8)	598 (95.8)	

(Continued to the next page)

Results

1. Overview of clinical characteristics of the patients according to the results of genetic tests

This study included a total of 700 *BRCA1/2* mutation–negative breast cancer patients aged 18-83 years who had at least one high-risk factor for hereditary breast cancer syndrome. Among the patients, we identified at least one PV/LPV of 26 genes in 76 patients (10.9 %). The frequency and spectrum of genetic variants are shown in Fig. 1. Another 535 patients (76.4%) had at least one VUS of 63 genes. No mutation nor VUS was found in 89 patients (12.7%) in this study. The baseline characteristics of the patients according to the presence of PV/LPV are presented in Table 1.

Among the 76 patients with any PV/LPV, 24 patients (31.6% of the PV/LPV carriers, and 3.4% of the total participants) had PV/LPV in one of three high-risk genes: 17 in *PALB2*, six in *TP53*, and one in *PTEN*. Information on the genetic variants is shown in Table 2 with detailed clinicopathologic characteristics of the patients. PV/LPV in moderate-risk genes were identified in 28 patients (36.8% of the PV/LPV carriers, and 4% of the total participants) for six

Table 1. Continued

Clinicopathological variable	PV/LPV (n=76)	VUS or ND (n=624)	p-value
Second cancer history (multi-selection)			
Yes	19 (25.0)	86 (13.8)	0.010
Thyroid	9 (11.8)	40 (6.4)	0.080
Colorectal	4 (5.3)	10 (1.6)	0.055[a)]
Lung	2 (2.6)	4 (0.6)	0.131[a)]
Endometrium	1 (1.3)	6 (1.0)	0.554[a)]
Ovary	2 (2.6)	3 (0.6)	0.131[a)]
Pancreas	0	2 (0.3)	> 0.99[a)]
Sarcoma	0	2 (0.3)	> 0.99[a)]
Lymphoma	0	2 (0.3)	> 0.99[a)]
Leukemia	0	1 (0.2)	> 0.99[a)]
Kidney	0	4 (0.6)	> 0.99[a)]
Urothelial	0	1 (0.2)	> 0.99[a)]
Stomach	0	12 (1.9)	0.630[a)]
Small bowel	1 (1.3)	1 (0.2)	0.205[a)]
Paraganglioma	1 (1.3)	0	0.109[a)]
Liver	0	3 (0.5)	> 0.99[a)]
Uterine cervix	4 (5.3)	2 (0.3)	0.002[a)]
No	57 (75.0)	538 (86.2)	
Experience of full-term delivery			
Yes	63 (82.9)	461 (73.9)	0.087
No	13 (17.1)	163 (26.1)	

Values are presented as median (range) or number (%). DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma *in situ*; LPV, likely pathogenic variant; ND, not detected; PV, pathogenic variant; TNBC, triple negative breast cancer; VUS, variant of unknown significance. [a)]These values were analyzed using Fisher exact tests.

genes: 10 in ATM, seven in *BRIP1*, seven in *RAD51D*, two in *CHEK2*, one in *BARD1*, and one in *NF1* (S2 Table). PV/LPV in unknown-risk genes were found in 24 patients (31.6% of the PV/LPV carriers, and 3.4% of the total participants) for 16 genes: seven in *RAD50*, two in *PMS2*, two in *EXO1*, two in *MRE11A*, one in *ALK*, one in *BLM*, one in *CDKN2A*, one in *FANCM*, one in *MSH2*, one in *PPM1D*, one in SDHB, one in *VHL*, one in both *EPCAM* and *SDHA*, one in both *JAK2* and *NTRK1*, and one in both *PMS2* and *RAD50* (S3 Table).

Fifty-two patients with PV/LPV in clinically actionable genes beyond *BRCA* (68.4% of the PV/LPV carriers and 7.4% of the total participants) were more likely to have bilateral breast cancer compared to those without any PV/LPV and VUS (odds ratio, 5.619; 95% confidence interval, 1.623 to 19.455; p=0.006) (Table 3).

2. Cancer worry and genetic knowledge before and after multigene panel testing with genetic counseling

A total of 374 patients completed the questionnaires regarding cancer worry, its influence on mood and daily functioning, and genetic knowledge before and after genetic tests with counseling with a median time interval of 21 days between questionnaires (range, 14 to 85). After genetic tests with counseling about multigene panel, the patients showed decreased concern about the possibility of cancer in the future (average score of pre-test, 4.21±0.883 to post-test, 3.94±1.048; $p < 0.001$), decreased influence of cancer worry on mood (average score of pre-test, 3.27±0.645 to post-test, 3.13±0.694; $p < 0.001$), and decreased influence of cancer worry on daily functioning (average score of pre-test, 3.03±0.758 to post-test, 2.94±0.729; p=0.006). In addition, there was a slight but significant increase in the average score of knowledge about hereditary cancer (pre-test, 66.9±21.7 to post-test, 68.8±21.8; p=0.025) (Table 4).

3. Satisfaction and preference about comprehensive multigene panel tests beyond *BRCA*

Among the 374 patients who answered the survey about satisfaction after the comprehensive multigene panel tests with counseling, the answer about hereditary cancer risks were "very satisfied" for 173 patients (46.3%) and "satisfied" for 182 patients (48.7%). Another 11 patients (2.9%) were dis-

Table 2. Characteristics of patients with pathogenic or likely pathogenic variants in high-risk genes for breast cancer beyond *BRCA* (n=24)

Case	Sex	Age at first diagnosis of breast cancer (yr)	Breast cancer Side/Path (subtype)	Family history kind (degree*n)	Second cancer	Affected genes	Nucleotide change	Amino acid change	Effect	Mode of inheritance	Zygosity	dbSNP
P027	F	34	Rt/IDC (TNBC)	CRC (2*1)	-	*PALB2*	c.1381C>G	p.Gln461Glu	MS	AD	Hetero	-
P030	F	38	Lt/ILC (ER+/HER2–)	Breast (1*1), Lung (1*1)	-	*PALB2*	c.1426delA	p.Arg476Glufs	FS	AD	Hetero	-
P041	F	50	Lt/DCIS (ER+/HER2–)	Breast (1*1), Lung (1*1)	-	*PALB2*	c.1426delA	p.Arg476Glufs	FS	AD	Hetero	-
P032	F	38	Lt/LCIS (ER+/HER2–)	Breast (2*2)	-	*PALB2*	c.1516C>T	p.Gln506Ter	NS	AD	Hetero	-
P036	F	47	Rt/IDC (ER+/HER2–)	Breast (1*1), Stomach (1*2)	AoV	*PALB2*	c.2257C>T	p.Arg753Ter	NS	AD	Hetero	-
P042	F	54	Lt/IDC (ER+/HER2–)	Breast (1*1), Kidney (1*1), Esophagus (1*1)	-	*PALB2*	c.228_229delAT	p.Ile76Metfs	FS	AD	Hetero	-
P048	F	44	Rt/DCIS (ER+/HER2–)	Stomach (2*1)	Ovary	*PALB2*	c.355C>T	p.Gln119Ter	NS	AD	Hetero	-
P005	F	28	Rt/DCIS (ER+/HER2–)	Stomach (2*1), Pros (2*1), Liver (2*1)	-	*PALB2*	c.2406_2407delTG	p.Cys802Ter	NS	AD	Hetero	-
P040	F	52	Lt/IDC (ER+/HER2–)	Stomach (1*1), Skin (1*1)	Cervix	*PALB2*	c.2485C>T	p.Gln829Ter	NS	AD	Hetero	-
P014	F	25	Lt/Medullary (TNBC)	Breast (2*2), CRC (2*2)	-	*PALB2*	c.2748+1G>A	-	Splicing	AD	Hetero	rs753153576
P049	F	62	Lt/IDC (TNBC)	Breast (1*1), Stomach (1*2)	-	*PALB2*	c.2748+1G>A	-	Splicing	AD	Hetero	rs753153576
P063	F	45	Rt/ILC (ER+/HER2–)	Breast (2*1, 3*1)	-	*PALB2*	c.3256C>T	p.Arg1086Ter	NS	AD	Hetero	rs587776527
P101	F	60	Lt/IDC (ER+/HER2–)	Ovary (1*1)	-	*PALB2*	c.3350+5G>A	-	Splicing	AD	Hetero	rs587782566
P102	F	38	Bil(syn)/IDC (ER+/HER2+)	Ovary (1*1)	-	*PALB2*	c.3350+5G>A	-	Splicing	AD	Hetero	rs587782566
P010	F	50	Rt/IDC (ER+/HER2–)	Breast (1*1), Stomach (1*1), Panc (2*1), Sarcoma (1*1)	-	*PALB2*	Exon 1_11 deletion	-	Exon deletion	AD	Hetero	-
P011	F	47	Bil(met)/IDC (TNBC)	Breast (1*2)	-	*PALB2*	Exon 11 deletion	-	Exon deletion	AD	Hetero	-

(Continued to the next page)

Table 2. Continued

Case	Sex	Age at first diagnosis of breast cancer (yr)	Breast cancer Side/Path (subtype)	Family history kind (degree*n)	Second cancer	Affected genes	Nucleotide change	Amino acid change	Effect	Mode of inheritance	Zygosity	dbSNP
P059	F	47	Rt/IDC (TNBC)	Breast (2*2), CRC (1*1, 2*1), cervix	-	*PALB2*	Exon 8 deletion	-	Exon deletion	AD	Hetero	-
P070	F	47	Lt/mucinous (ER+/HER2–)	Breast (2*1), Lung (2*1), Leukemia (2*1)	-	*TP53*	c.542G>A	p.Arg181His	MS	AD	Hetero	rs397514495
P023	F	51	Lt/IDC (ER+/HER2–)	Breast (1*2), Ovary (1*1)	-	*TP53*	c.542G>A	p.Arg181His	MS	AD	Hetero	rs397514495
P006	F	41	Bil(met)/DCIS (ER–/HER2+)	0	Lung, melanoma, sarcoma	*TP53*	c.646G>A	p.Val216Met	MS	AD	Hetero	rs730882025
P034	F	32	Bil(syn)/IDC (ER–/HER2+)	Stomach (1*1), Panc (1*1)	-	*TP53*	c.733G>A	p.Gly245Ser	MS	AD	Hetero	rs28934575
P025	F	38	Rt/DCIS (unknown)	0	-	*TP53*	c.743G>A	p.Arg248Gln	MS	AD	Hetero	rs11540652
P021	F	30	Rt/IDC (ER+/HER2+)	Stomach (2*2), Lung (2*1), Liver (2*1), Lymphoma (2*1)	-	*TP53*	c.824G>A	p.Cys275Tyr	MS	AD	Hetero	-
P062	F	37	Bil(syn)/IDC (ER+/HER2–)	Thyroid (1*1)	-	*PTEN*	c.813_815delinsCC	p.His272Profs	FS	AD	Hetero	-

AD, autosomal dominant; AoV, ampulla of Vater; Bil(met), bilateral breast cancer, metachronous; Bil(syn), bilateral breast cancer, synchronous; CRC, colorectal cancer; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; F, female; FS, frameshift; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Lt, left; MS, missense; NS, nonsense; Panc, pancreas; Pros, prostate; Rt, right; TNBC, triple negative breast cancer.

Table 3. Risk factors with odds ratio related to the identification of clinically actionable[a] genetic mutations for breast cancer beyond *BRCA* (n=52)

Clinicopathological risk factor	Mutation (n=52)	Vs. not detected (n=89)					
		Simple regression			Multiple regression		
		OR	95% CI	p-value	OR	95% CI	p-value
Age at first diagnosis of breast cancer ≤ 40 yr	21	0.758	0.379-1.516	0.433	-	-	-
Male breast cancer	0	N/A	-	-	N/A	-	-
Bilateral breast cancer	10	5.060	1.498-17.087	0.009	5.619	1.623-19.455	0.006
TNBC	10	1.175	0.485-2.847	0.722	-	-	-
Family history of breast cancer	23	0.741	0.373-1.474	0.393	-	-	-
Family history of ovarian cancer	5	4.628	0.864-24.775	0.073	5.470	0.980-30.545	0.053
Presence of other primary cancer	10	2.116	0.798-5.610	0.132	2.665	0.977-7.267	0.055

CI, confidence interval; N/A, not applicable; OR, odds ratio; TNBC, triple negative breast cancer. [a]Genes with inheritance of increased breast cancer risk according to the National Comprehensive Cancer Network, American Society of Clinical Oncology, or European Society of Medical Oncology guidelines: number of patients with mutation in each gene; *ATM* (10), *BARD1* (1), *BRIP1* (7), *CHEK2* (2), *NF1* (1), *PALB2* (17), *PTEN* (1), *RAD51D* (7), *TP53* (6).

Table 4. Difference in cancer worry scores (5-point Likert scale) and genetic knowledge between pre- and post-test (n=374)

	Pre-test	Post-test	p-value
Concern about the possibility of cancer in the future	4.21±0.883	3.94±1.048	< 0.001
Influence on mood	3.27±0.645	3.13±0.694	< 0.001
Influence on daily functioning	3.03±0.758	2.94±0.729	0.006
Knowledge about hereditary cancer (max. point 100)	66.9±21.7	68.8±21.8	0.025

Values are presented as average±standard deviation.

satisfied, and eight patients (2.1%) were neutral about the genetic tests with counseling (Fig. 2A). When the answers were converted to a five-point Likert score (from "very dissatisfied" as point 1, to "very satisfied" as point 5), the median point for satisfaction was 4 (range 2 to 5). Meanwhile, in the simple regression and multiple regression models, a high CWSs were associated with young patients (aged ≤ 40 years) and the identification of PV/LPV, and low CWSs were related to higher satisfaction regarding genetic test with counseling (Tables 5 and 6).

For the sequence of genetic tests, 176 patients (47.1%) preferred to simultaneously test *BRCA1/2* and the genes beyond *BRCA* using comprehensive multigene panel, and 164 patients (43.9%) selected a sequential test including *BRCA1/2* mutation tests followed by multigene panel testing beyond *BRCA* for the *BRCA1/2* mutation-negative patients. Another three patients (0.8%) wanted to test *BRCA1/2* mutations only, and 31 patients (8.3%) had no preference for the sequence or method of genetic tests (Fig. 2B).

Discussion

The current study demonstrated that one out of ten patients with germline *BRCA1/2* mutation-negative breast cancer and risk factors for hereditary breast cancer had PV or LPV of cancer predisposition genes. Considering the general rule of 10 for threshold of certain testing, multigene panel tests can be justified and applicable in clinical practice for patients with germline *BRCA1/2* mutation-negative breast cancer and risk factors for hereditary breast cancer. For those with PV/LPV, clinical actionability and psychological influence should be considered in genetic counseling.

In this study, among the germline *BRCA1/2* mutation-negative breast cancer patients, PV/LPV were identified in 3.4% of the subjects with high-risk genes, and a total of 7.4% of the subjects with clinically actionable genes with recommendations in the current clinical guidelines for hereditary breast cancer, which was consistent with 4.9%-11.4% frequency of PV/LPV beyond *BRCA* in the previous results of multigene panel tests [23-27]. We provided intensive screening using mammography and breast magnetic resonance imaging to all of 52 patients with clinically actionable genetic mutations. No contralateral prophylactic mastectomy was conducted.

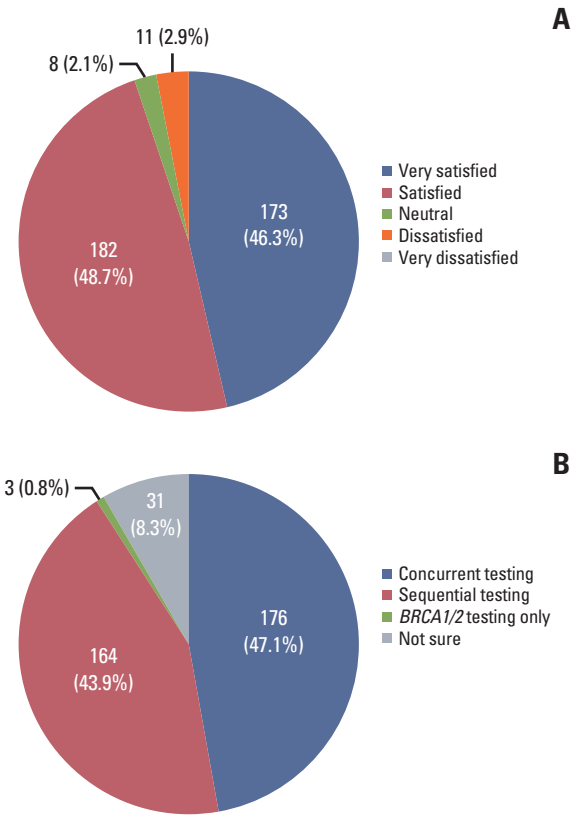

Fig. 2. Result of the survey about genetic counseling on multigene panel testing (n=374). (A) Satisfaction about the information gained by genetic tests with counseling. (B) Preference of the sequence and method of genetic testing for *BRCA1/2* mutation test and multigene test beyond *BRCA*.

Among the high-risk genes, PV/LPV were most frequently identified in *PALB2* gene (n=17). The carriers of *PALB2* PV/LPV were diagnosed with the primary breast cancer at median 47.1 years of age (range, 28.2 to 62.7), and 11 of 17 (54.7%) carriers had family history of breast cancer (Table 2). Zhou et al. [28] reported that *PALB2*-related breast cancer showed clinical characteristics including a family history of cancer, larger tumor, triple-negative breast cancer (TNBC), lymph nodal positivity, and bilateral breast cancer. Although proportion of TNBC (29.4%), frequency of family history of breast and/or cancer (76.5%), and proportion of bilateral breast cancer (11.8%) in this study were slightly higher than those in the report, clinical significance could not be shown due to small number of the participants and control group. The second most frequent high-risk PV/LPV was found in *TP53* (n=6). Among six carriers, five did not meet the criteria for the classic Li-Fraumeni syndrome (LFS) [29], or those of Birch et al. [30], Eeles [31], and Bougeard et al. [32]. All PV/LPVs found in this study were missense variants (Table 2). Bougeard et al. [32] previously suggested early-onset breast cancer diagnosed before age 31 years as a novel criterion for *TP53* genetic testing, based on the clinical findings of the carriers with missense variants in *TP53*, and tumor spectrum of the adult *TP53* PV/LPV carriers . However, most of the carriers in our study had neither personal /family history of LFS tumors nor early-onset breast cancer. In addition, eight kinds of missense VUS were also identified (S4 Table). It is necessary to further investigate the clinical penetrance and tumor spectrum of the carriers with *TP53* missense variants.

The present study additionally focused on the effect of

Table 5. Correlation between clinic-social factors and the cancer worry after multigene panel testing with counseling (simple regression analysis, n=374)

	Concern about the possibility of breast cancer in the future			Influence on mood			Influence on daily functioning		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Age ≤ 40 yr	0.255	0.042 to 0.467	0.019	0.150	0.009 to 0.291	0.037	0.173	0.025 to 0.321	0.022
Bilateral breast cancer	–0.391	–0.716 to –0.065	0.019	–0.146	–0.363 to 0.071	0.187	–0.079	–0.307 to 0.150	0.498
TNBC	0.283	–0.005 to 0.570	0.054	0.140	–0.050 to 0.331	0.149	0.214	0.014 to 0.414	0.036
Family history of breast or ovarian cancer	–0.052	–0.266 to 0.161	0.630	0.031	–0.110 to 0.172	0.666	–0.016	–0.164 to 0.133	0.837
Highly educated (above college graduates)	0.002	–0.002 to 0.006	0.388	0.0005	–0.002 to 0.003	0.724	0.0003	–0.002 to 0.003	0.816
PV/LPV detected	0.503	0.140 to 0.866	0.007	0.142	–0.100 to 0.384	0.250	0.102	–0.152 to 0.357	0.477
Counselee's satisfaction to genetic test with counseling	–0.176	–0.333 to –0.019	0.028	–0.125	–0.229 to –0.021	0.018	–0.134	–0.243 to –0.025	0.016
Stage IV breast cancer	0.396	–0.453 to 1.244	0.360	0.547	–0.013 to 1.107	0.055	0.235	–0.356 to 0.825	0.435
Genetic knowledge (post-test)	0.339	–0.150 to 0.829	0.174	0.049	–0.276 to 0.375	0.765	0.136	–0.206 to 0.477	0.435

B, beta regression coefficient value; CI, confidence interval; LPV, likely pathogenic variant; PV, pathogenic variant; TNBC, triple negative breast cancer.

Table 6. Correlation between clinic-social factors and the cancer worry after multigene panel testing with counseling (multiple regression analysis, n=374)

	Concern about the possibility of breast cancer in the future			Influence on mood			Influence on daily functioning		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Age ≤ 40 yr	0.201	–0.015 to 0.417	0.068	0.149	0.009 to 0.288	0.037	0.173	0.018 to 0.312	0.027
Bilateral breast cancer	–0.292	–0.624 to –0.040	0.085	-	-	-	-	-	-
TNBC	0.196	–0.090 to 0.481	0.179	-	-	-	0.181	–0.018 to 0.379	0.075
PV/LPV detected	0.427	0.057 to 0.797	0.024	-	-	-	-	-	-
Counselee's satisfaction to genetic test with counseling	–0.117	–0.276 to 0.043	0.152	–0.125	–0.228 to –0.022	0.018	–0.125	–0.234 to –0.017	0.024
Stage IV breast cancer	-	-	-	0.535	–0.019 to 1.089	0.058	-	-	-

B, beta regression coefficient value; CI, confidence interval; LPV, likely pathogenic variant; PV, pathogenic variant; TNBC, triple negative breast cancer.

genetic counseling after multigene panel tests. Among 374 patients who answered the questionnaire, 35 patients (9.4%) had PV/LPV in the genes beyond *BRCA* (S5 Table). Our results demonstrated that comprehensive multigene panel tests with genetic counseling can decrease the patients' cancer worry and increase the patients' knowledge about hereditary cancer syndrome. However, cancer worry of the PV/LPV carriers did not change after genetic tests with counseling (S6 Table), which was consistent with the previous study [33]. Decreased cancer worry was probably related to the psychologic relief of the patients with VUS or negative results. In a previous study regarding *BRCA1/2* mutation tests, Richter et al. [34] reported that 36% of the VUS carriers failed to recall the clinical significance of their result, and their cancer worry and cancer preventive strategies were similar to those for patients without mutation. Otherwise, in a meta-analysis study including the results of 13 multigene panel tests and two exome sequencing tests of hereditary syndromes, the patients with VUS had higher genetic test-specific concerns compared to those with negative results, and lower concerns compared to those with positive results [35]. Katz et al. [8] suggested that the impact of cancer worry was not different by genetic test type or test results, but is rather influenced by ethnic and educational factors. In addition to the debate about the correlation between genetic testing result and cancer worry, the impact of the multigene panel testing result and clinical factors on cancer worry of Asian breast cancer patients has not been fully evaluated, since most previous studies were conducted in Western countries [8,34,35].

Genetic counseling is defined as a communication process which deals with human problems associated with the occurrence, or risk of occurrence, of a genetic disorder in a family [36]. Considering that one of the goals of genetic counseling is to facilitate the ability to use genetic information under the cognitive interpretation [37], we assessed the satisfaction level using the counselees' subjective degree of interpretation of the genetic information to the possibility of hereditary breast cancer. Although the satisfaction of the counselee was distributed at lower scores in the carriers with PV/LPV (median, 4; range, 2 to 5) than in those with VUS (median, 4; range, 2 to 5; $p < 0.001$), or than in those with negative result (median, 5; range, 2 to 5; p=0.001), 85.7% of the patients answered that they were satisfied with the information gained by genetic testing with counseling, even among the carriers with PV/LPV (S7 Fig.).

Based on the results that clinically actionable PV/LPV were commonly identified in multigene panel tests and that cancer worry was decreased after multigene panel tests with genetic counseling, the authors suggest that multigene panel tests can be usefully applied in clinical practice. However, we are needed to embrace the potential discomfort of the patients who still prefer *BRCA1/2* mutation tests prior to multigene panel tests beyond *BRCA*. In this study, the patients who preferred concurrent multigene panel tests were younger (median years of age, 39.8 vs. 44.6; p=0.004) and more highly educated (proportion of college or university graduated, 74.2% vs. 62.7%; p=0.029) than the patients who preferred sequential tests. Given that comprehensive multigene panel includes complex genetic information about multiple disease penetrance and diverse kinds of malignancy, well-structured genetic counseling will help to support comprehension and clinical decisions of the patients who have difficulties in getting multigene tests.

There are several limitations in this study. First, considering the frequency of PV/LPV in moderate- or low-penetrance genes beyond *BRCA*, a larger number of patients is needed to analyze an accurate incidence rate of each variant and clinical features of the carriers. Second, clinical action-

ability was assessed only based on the detection of genetic variant described in current clinical guidelines. Whether the identification of genetic mutation with counseling can actually improve a long-term preventive strategy and the survival outcome of the carriers is still controversial. Third, cancer worry and satisfaction of the patients with VUS and negative results could be influenced by miscomprehension about VUS and uninformative results, respectively. Despite the limitations, to the best of our knowledge, this is the first study simultaneously analyzed the potential actionability and psychological influence of comprehensive multigene panel tests in hereditary breast cancer.

Despite several debates, multigene panel tests are rapidly replacing the traditional single-gene direct sequencing methods. It is important for clinicians to improve the comprehensive multigene panel tests with genetic counseling programs based on the interpretable genetic information, consideration of potential psychological consequences, and proper preventive strategies for the carrier.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The ethical principles for medical research established by the World Medical Association Declaration of Helsinki were followed throughout the study. The institutional review board at Severance Hospital, Seoul, Korea reviewed and approved this study (IRB approval number: 4-2015-0819 and 4-2018-0259). We obtained informed consent from all patients who participated in this study.

Author Contributions

Conceived and designed the analysis: Park JS, Park HS.
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Contributed data or analysis tools: Park JS, Shin S, Lee YJ, Lee ST, Nam EJ, Han JW, Lee SH, Kim TI, Park HS.
Performed the analysis: Park JS, Shin S, Lee YJ, Lee ST, Park HS.
Wrote the paper: Park JS, Park HS.
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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

Some of the results of this study were presented as a poster presentation at the 2019 ESMO ASIA held on November 22-24, 2019, in Singapore, and as an E-poster presentation at the 13th Annual Meeting of Korean Society of Medical Oncology & 2020 International Conference held on September 3-4, 2020, in Republic of Korea.

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NRF-2018R1C1B6009449).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1017
Cancer Res Treat. 2022;54(4):1111-1120

Original Article

The Association of Estrogen Receptor Activity, Interferon Signaling, and MHC Class I Expression in Breast Cancer

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Purpose The expression of major histocompatibility complex class I (MHC I) has previously been reported to be negatively associated with estrogen receptor (ER) expression. Furthermore, MHC I expression, level of tumor-infiltrating lymphocytes (TILs), and expression of interferon (IFN) mediator MxA are positively associated with one another in human breast cancers. This study aimed to investigate the mechanisms of association of MHC I with ER and IFN signaling.

Materials and Methods The human leukocyte antigen (HLA)-ABC protein expression was analyzed in breast cancer cell lines. The expressions of *HLA-A* and *MxA* mRNAs were analyzed in MCF-7 cells in Gene Expression Omnibus (GEO) data. ER and HLA-ABC expressions, Ki-67 labeling index and TIL levels in tumor tissue were also analyzed in ER+/human epidermal growth factor receptor 2– breast cancer patients who randomly received either neoadjuvant chemotherapy or estrogen modulator treatment followed by resection.

Results HLA-ABC protein expression was decreased after β-estradiol treatment or hESR-GFP transfection and increased after fulvestrant or IFN-γ treatment in cell lines. In GEO data, *HLA-A* and *MxA* expression was increased after *ESR1* shRNA transfection. In patients, ER Allred score was significantly lower and the HLA-ABC expression, TIL levels, and Ki-67 were significantly higher in the estrogen modulator treated group than the chemotherapy treated group.

Conclusion MHC I expression and TIL levels might be affected by ER pathway modulation and IFN treatment. Further studies elucidating the mechanism of MHC I regulation could suggest a way to boost TIL influx in cancer in a clinical setting.

Key words Breast neoplasms, Estrogens, Receptors, Interferons, Major histocompatibility complex, Tumor-infiltrating lymphocytes

Introduction

Tumor-infiltrating lymphocytes (TILs) have consistently been reported to play an important role in breast cancer [1-5]. TILs have a strong prognostic and predictive significance, particularly in triple-negative breast cancer (TNBC). CD8+ cytotoxic TILs are activated by the T cell receptor-recognition of a specific peptide, which is generally generated from endogenous proteins, and are presented by a major histocompatibility complex class I (MHC I) on the surface of tumor cells [6]. The recognition of these peptides by cytotoxic CD8+ TILs triggers a series of events that can result in tumor cell lysis. A better understanding of TILs and related features could facilitate the development of efficient immunotherapeutic approaches in breast cancer.

MHC I proteins are membrane proteins that are expressed on almost all nucleated cells and are encoded by human leukocyte antigen (HLA)-A, -B, and -C genes. The expression of HLAs varies from tissue to tissue and is largely stimulated by interferon (IFN) signaling. The downregulation of HLAs is frequently observed in tumors and is reported to be correlated with disease progression [7]. Aberrant HLA expression in tumor cells might be caused by alterations in *HLA* gene transcription, the translation of *HLA* mRNA, or post-translational modifications. Torigoe et al. [8] established a monoclonal anti-pan HLA class I antibody suitable for the immunostaining of formalin-fixed tissue and found a high rate (85%, 35 out of 41 cases) of HLA downregulation in breast cancer compared with other malignancies (20%-42%). Since HLA expression on tumor cells is important for the function of TILs, the downregulation of HLA might compromise the effective immune response in patients with breast cancer. Moreover, recent studies have reported increased IFN signaling in cancer cells and their association with a good

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Received September 11, 2021 **Accepted** December 20, 2021
Published Online December 21, 2021

response to anthracycline-based chemotherapy in breast cancer [9]. However, HLA expression, the level of IFN signaling activation, and their relationship in normal breast tissue and various subtypes of breast cancer have not been extensively studied.

We previously demonstrated the differential expression of HLA-ABC in breast cancer. HLA-ABC protein expression was negatively correlated with estrogen receptor (ER) protein expression but was not significantly correlated with human epidermal growth factor receptor 2 (HER2) protein expression [10,11]. HLA-ABC expression was higher in TNBC and hormone receptor (HR)–/HER2+ breast cancers than in HR+ breast cancers, was positively correlated with TILs, and was associated with better clinical outcomes in breast cancer patients [1,10,11]. In The Cancer Genome Atlas (TCGA) data analysis, *HLA-A* gene expression was positively correlated with *CD8B* gene expression but was not significantly correlated with the total number of mutations. Instead, *HLAs*, *CD3*, and *CD8* gene expression were positively correlated with IFN receptor genes and the IFN-inducible *MxA* gene [10]. Additionally, MxA protein expression was higher in TNBC than in other types of breast cancer, was positively correlated with TIL levels, and was associated with better clinical outcomes [12]. Therefore, it can be hypothesized that ER activity, IFN signaling, and MHC I expression regulate one another and influence TIL influx.

The current study aimed to clarify the mechanisms of the association of MHC I with estrogen and IFN signaling.

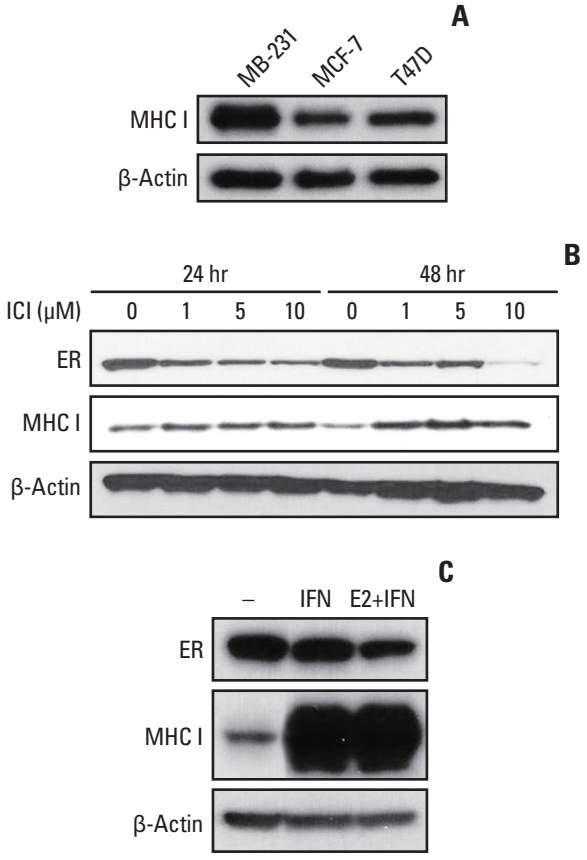

Fig. 1. The effect of estrogen signaling and interferon signaling on HLA-ABC expression. (A) The baseline HLA-ABC protein expression is higher in ER-negative MDA-MB-231 cell lines than in ER-positive MCF-7 and T47D cell lines. (B) After 24 or 48 hours of ICI treatment (1 to 10 μM), the ER protein expression decreased, and HLA-ABC protein increased in MCF-7 cells. (C) The HLA-ABC protein expression increased in MCF-7 cells 48 hours after 100 units/mL of IFN-γ treatment. ER, estrogen receptor; HLA, human leukocyte antigen; IFN, interferon; MHC I, major histocompatibility complex class I.

Materials and Methods

1. Cell lines, cultures, drug treatments, and plasmid transfections

This study used breast cancer cell lines obtained from ATCC, including ERα+ (MCF-7 and T47D) and ERα– (MDA-MB-231). The MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (cat No. 11995, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (cat No. 16000, Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (cat No. 15140, Invitrogen). The T47D and MDA-MB-231 cells were grown in Roswell Park Memorial Institute 1640 (Gibco, El Paso, TX) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were cultured at 37°C in the presence of 5% CO_2. The cells were starved for 24 hours and treated with ICI (1 to 10 μM, fulvestrant, Sigma-Aldrich, St. Louis, MO), IFN-γ (100 units/mL, R&D Systems, Minneapolis, MN), or β-estradiol (1 nM, Sigma-Aldrich) in 2 mL of medium for an appropriate time. The cells were then used in the protein expression assays.

For the *ESR1* plasmid transfection, ERα-cells were plated and cultured in a 6-well plate at 90% confluency and transfected with 2.5 μg of hESR-GFP (cat No. #28230, Addgene, Cambridge, MA) using 3.75 μL of Lipofectamine 3000 reagent (Life Technologies) and 5 μL of P3000 reagent (Life Technologies) per well according to the manufacturer's protocol.

2. Protein isolation and Western blotting

The cells were lysed with RIPA buffer, and the Pierce BCA Protein Assay Reagent Kit (cat No. 23225, Thermo Fisher, Waltham, MA) was used to measure the protein concentration. Approximately 10 μg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresi-

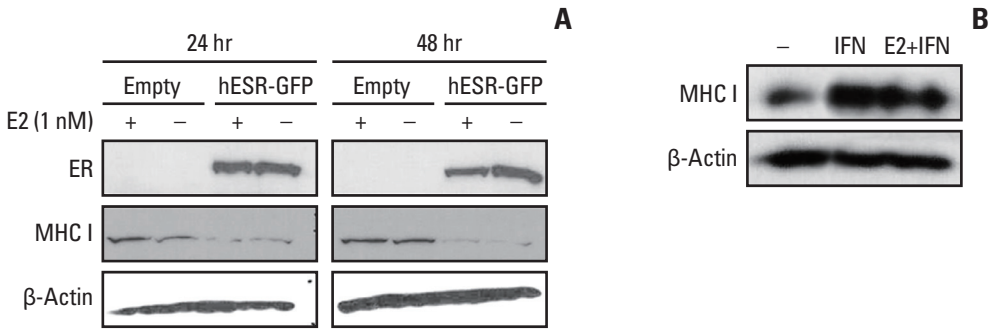

Fig. 2. HLA-ABC protein expression in the MDA-MB-231 cells. (A) Twenty-four or 48 hours after transfection with the hESR-GFP plasmid, MDA-MB-231 cells show ER expression and decreased HLA-ABC protein levels. (B) The HLA-ABC protein expression was markedly increased 48 hours after 100 units/mL of IFN-γ treatment in MDA-MB-231 cells. ER, estrogen receptor; HLA, human leukocyte antigen; IFN, interferon; MHC I, major histocompatibility complex class I.

sand transferred to a PVDF membrane (Millipore, Bedford, MA). S1 Table summarizes the antibodies used for protein detection. Equal loading of the protein samples was verified with an antibody to β-actin. Immunoreactive signals were detected with the Promega Western Blot Detection System (cat No. W1008, Promega, Madison, WI).

3. Gene Expression Omnibus data analysis

We analyzed one dataset from the Gene Expression Omnibus (GEO) database that included Affymetrix RNA microarray analysis data from MCF-7 breast cancer cells that were transfected with *ESR1* shRNA (5′-GCTTCAGGCTACCATTATGttcaagagacataATGGTAGCCTGAAGCttttttacgcgt-3′) (accession No. GDS4061) [13]. The fold changes of *ESR1*, *HLA-A*, and *MxA* mRNA expressions were calculated.

4. Patients and tissue specimens

A total of 126 patients who were diagnosed with ER+/HER2– invasive ductal carcinoma were randomized to receive either estrogen modulator treatment or chemotherapy for 24 weeks as their neoadjuvant systemic therapy [14]. We analyzed the clinicopathologic data of the patients and the HLA-ABC and ER protein expressions and TIL levels in the pre-neoadjuvant biopsy tissues and the post-neoadjuvant resected tissues.

5. Histological evaluation

The histologic type was defined based on the 2019 World Health Organization classification criteria, and the histologic grade was assessed using the modified Bloom-Richardson classification [15]. The hematoxylin and eosin–stained slides were histopathologically analyzed for TILs (defined as the percentage of the invasive carcinoma's stroma that was infiltrated by lymphocytes in 10% increments; if less than 10% of the stroma was infiltrated by TILs, 1% or 5% criteria were used; all available full sections were evaluated), histological subtype and grade, tumor size, pT category, pN category, and lymphovascular invasion [1,16]. The tumor response to neoadjuvant systemic therapy was evaluated based on the Miller-Payne grade (1, no change; 2, up to 30% reduction; 3, 30%-90% reduction; 4, more than 90% reduction; 5, no residual malignant cells) [17]. A pathologic complete response (pCR) was defined as the absence of residual invasive cancer cells in the breast and lymph nodes [18].

6. Tissue microarray construction and immunohistochemical evaluation

All 126 patients were checked for ER, progesterone receptor (PR) and HER2 expression and Ki-67 labeling index both in the pre-neoadjuvant biopsy tissues and the post-neoadjuvant resected tissues by immunohistochemistry, except 9 cases in whom the residual tumor cells were few or even did not exist at all in post-neoadjuvant resected tissues. ER and PR levels were regarded as positive if there was at least 1% positive tumor nuclei staining. Additionally, the Allred score, which is the sum of the intensity score (0-3) and the proportion score (0-5), was calculated for the ER and PR. HR+ tumors were defined as those determined to be ER-positive and/or PR-positive. HER2-overexpressing tumors were defined as those with scores of 3+ according to the immunohistochemistry or gene amplification by silver *in situ* hybridization. Ki-67 labeling index in the tumor cells was measured by eyeball estimation and dichotomized into < 20% and ≥ 20%.

Among the 126 patients, 56 were available for formalin-fixed, paraffin-embedded tissue blocks of both biopsies and resected specimens at the time of this study. Each resected tissue sample was arrayed in three 1-mm diameter cores to minimize tissue loss and overcome tumor heterogeneity. Full sections of biopsy tissues and tissue microarray sections of

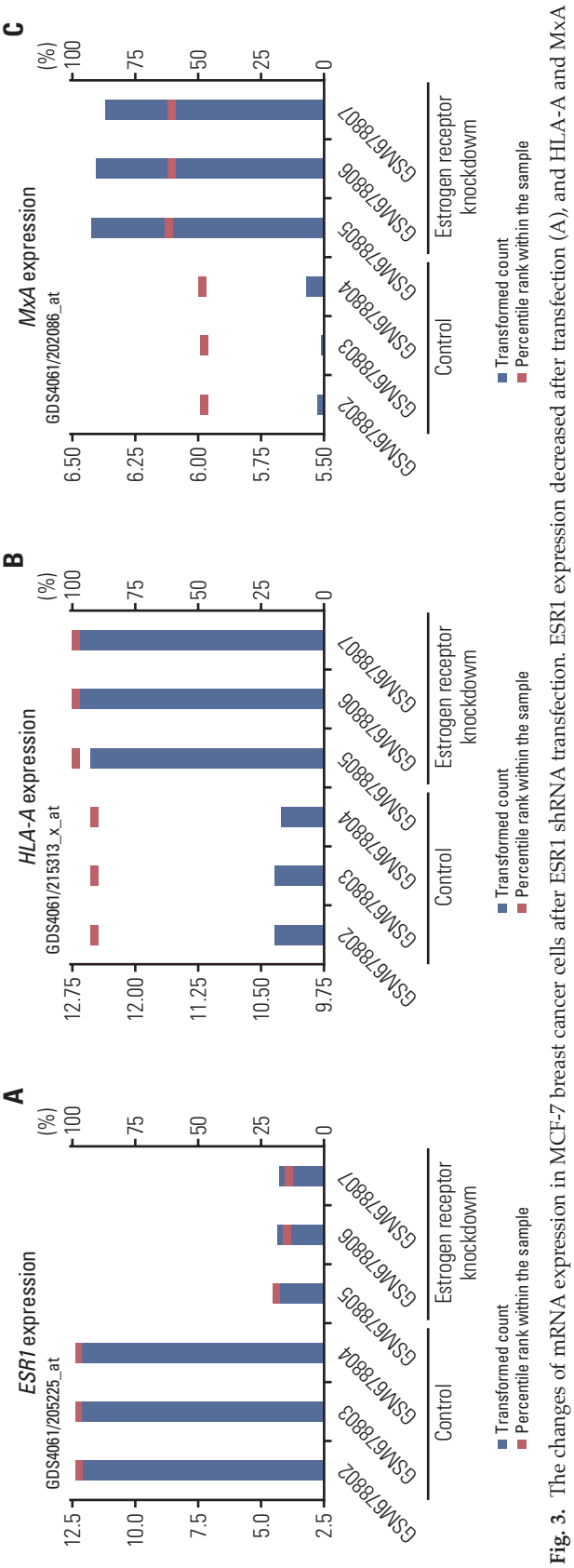

Fig. 3. The changes of mRNA expression in MCF-7 breast cancer cells after ESR1 shRNA transfection. ESR1 expression decreased after transfection (A), and HLA-A and MxA expressions increased after transfection (B, C). HLA, human leukocyte antigen.

the resected tissues were stained with an automatic immunohistochemical staining device (Benchmark XT, Ventana Medical Systems, Tucson, AZ). The HLA-ABC staining was semi-quantitatively evaluated as the H-score, which is the product of the actual percentage of positive-stained cells and the intensity score (0-3); the H-score can range from 0-300. We then categorized HLA-ABC expression in tumor cells as one of two levels (negative, H-score < 50; positive, H-score ≥ 50).

S2 Table summarizes the antibodies used for the immunohistochemical staining.

7. Statistical analysis

All statistical analyses were performed using R ver. 3.2.3 [19]. The Wilcoxon rank-sum test, chi-square test, Fisher exact test, and log-rank test were used as appropriate. All tests were two-sided, and statistical significance was set at 5%.

Results

1. The expression of HLA-ABC in breast cancer cell lines treated with ICI or IFN-γ

We evaluated the expression of the HLA-ABC protein in the ER-positive cell lines (MCF-7 and T47D) and in an ER-negative cell line (MDA-MB-231) by Western blot analysis. The baseline HLA-ABC protein expression was higher in the MDA-MB-231 cell line than in the MCF-7 and T47D cell lines (Fig. 1A). Next, we treated the MCF-7 cells with 1 to 10 μM ICI, which is an ER downregulator, for 24 or 48 hours. The ER protein expression decreased and HLA-ABC increased with ICI treatment under all conditions (Fig. 1B). When the MCF-7 cells were treated with 100 units/mL of IFN-γ with or without 1 nM of estradiol for 48 hours, the HLA-ABC protein expression was markedly increased (Fig. 1C).

2. The expression of HLA-ABC in the ER-negative breast cancer cell line transfected with hESR-GFP

We also observed changes in the HLA-ABC protein expression in the ER-negative MDA-MB-231 breast cell line. Twenty-four or 48 hours after transfection with the hESR-GFP plasmid, the MDA-MB-231 cells showed ER expression and decreased HLA-ABC protein levels (Fig. 2A). When the MDA-MB-231 cells were treated with 100 units/mL of IFN-γ with or without 1 nM of estradiol for 48 hours, HLA-ABC protein expression was markedly increased (Fig. 2B).

3. The change of *HLA-A* and *MxA* mRNA expressions in breast cancer cell lines in the GEO data

We also analyzed the *HLA-A* and *MxA* mRNA expressions

Table 1. Comparison of clinicopathologic variables according to neoadjuvant systemic therapy in breast cancer patients

Variable	Neoadjuvant systemic therapy		p-value[a)]
	Chemotherapy (n=65)	Estrogen modulator (n=61)	
Pre-neoadjuvant			
Age at diagnosis (yr)	43 (38-46)	42 (38-46)	0.784
cT			
1	12 (18.6)	7 (11.5)	0.522
2	42 (64.6)	42 (68.9)	
3	10 (15.4)	12 (19.7)	
4	1 (1.5)	0	
cN			
0	1 (1.5)	0	> 0.99
1	54 (83.1)	52 (85.2)	
2	4 (6.2)	4 (6.6)	
3	6 (9.2)	5 (8.2)	
ER Allred score in biopsy	8 (8-8)	8 (8-8)	0.467
Ki-67 labeling index in biopsy			
< 20%	24 (36.9)	18 (29.5)	0.488
≥ 20%	41 (63.1)	43 (70.5)	
HLA expression in biopsy			
Negative	17 (65.4)	24 (80.0)	0.243
Positive	9 (34.6)	6 (20.0)	
TIL level in biopsy	10 (0-12.5)	10 (0-20)	0.514
Post-neoadjuvant			
Histologic grade			
1	4 (6.9)	3 (5.0)	0.358
2	46 (79.3)	53 (88.3)	
3	8 (13.8)	4 (6.7)	
ypT			
0	7 (10.8)	1 (1.6)	< 0.001
1	32 (49.2)	12 (19.7)	
2	20 (30.8)	37 (60.7)	
3	6 (9.2)	9 (14.8)	
4	0	2 (3.3)	
ypN			
0	8 (12.3)	2 (3.3)	0.016
1	39 (60.0)	27 (44.3)	
2	15 (23.1)	23 (37.7)	
3	3 (4.6)	9 (14.8)	
Miller-Payne grade			
1	2 (3.1)	20 (32.8)	< 0.001
2	10 (15.4)	24 (39.3)	
3	36 (55.4)	14 (23.0)	
4	10 (15.4)	2 (3.3)	
5	7 (10.8)	1 (1.6)	
Pathologic complete response			
No	60 (92.3)	61 (100)	0.058
Yes	5 (7.7)	0	
LVI			
Absent	36 (55.4)	22 (36.1)	0.046
Present	29 (44.6)	39 (63.9)	

(Continued to the next page)

Table 1. Continued

Variable	Neoadjuvant systemic therapy		p-value[a)]
	Chemotherapy (n=65)	Estrogen modulator (n=61)	
ER Allred score in resected tissue	8 (7.25-8)	8 (7-8)	0.027
Ki-67 labeling index in resected tissue			
< 20%	50 (86.2)	36 (61.0)	0.004
≥ 20%	8 (13.8)	23 (39.0)	
HLA expression in resected tissue			
Negative	17 (68.0)	10 (35.7)	0.038
Positive	8 (32.0)	18 (64.3)	
TIL in resected tissue	0 (0-10)	10 (10-20)	< 0.001

Values are presented as median (IQR) or number (%). ER, estrogen receptor; HLA, human leukocyte antigen; IQR, interquartile range; LVI, lymphovascular invasion; TIL, tumor-infiltrating lymphocyte. [a)]p-values, calculated by Kruskal-Wallis test, chi-square test, or Fisher exact test.

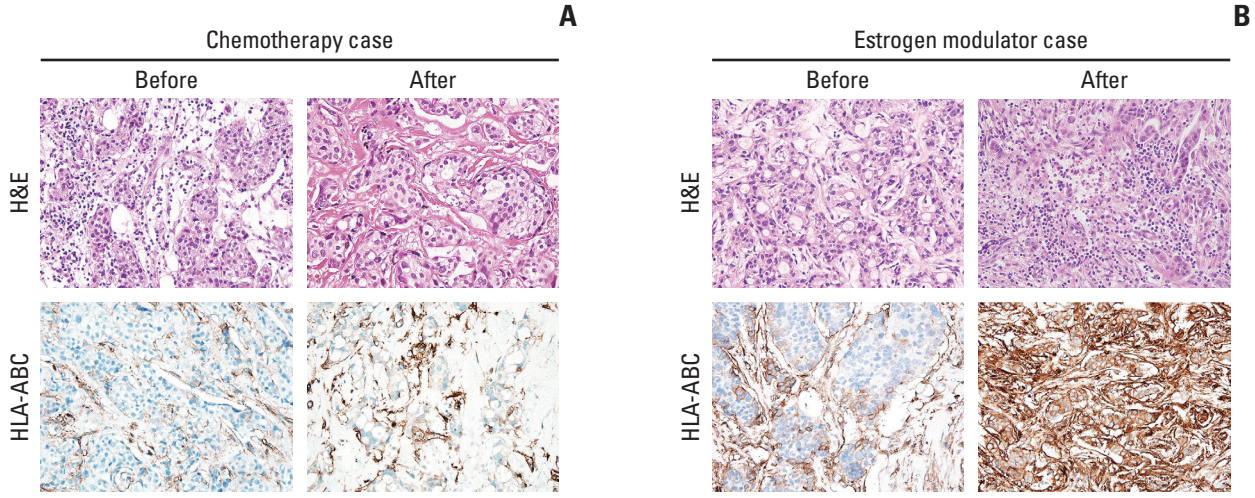

Fig. 4. The microscopic images of breast tissue before and after chemotherapy or estrogen modulator treatment. In the patient who received neoadjuvant chemotherapy (A), the quantity of TILs decreased and HLA-ABC expression was similar in the resection specimen compared with the pre-neoadjuvant biopsy specimens. By contrast, the TIL levels and HLA-ABC expression increased after estrogen modulator treatment in the patient who received estrogen modulator treatment (Nolvadex and Zoladex) (B) (A and B, ×400). HLA, human leukocyte antigen; TIL, tumor-infiltrating lymphocyte.

in breast cancer cells from the GEO database. When the MCF-7 cells were transfected with *ESR1* shRNA, the *ESR1* mRNA expression decreased, and the *HLA-A* and *MxA* mRNA expressions increased (Fig. 3). The fold changes were 0.360, 1.225, and 1.156 for *ESR1*, *HLA-A*, and *MxA*, respectively.

4. The changes of ER and HLA-ABC expressions, Ki-67 labeling index and TIL levels after neoadjuvant chemotherapy or estrogen modulators in breast cancer patients

The clinicopathologic characteristics of the 126 patients who randomly received chemotherapy (adriamycin and cyclophosphamide) or estrogen modulator treatment (tamoxifen and goserelin) as their neoadjuvant systemic therapy and following surgical resection were analyzed (Table 1). Before the neoadjuvant therapy, there were no differences between the two groups in patient age, cT and cN categories, ER Allred score, Ki-67 labeling index, and TIL levels in biopsy specimens. After neoadjuvant therapy, no significant difference was observed in the pCR ratio (p=0.058). However, the chemotherapy group showed significantly lower ypT and ypN categories (p < 0.001 and p=0.016, respectively) and a more reduced tumor burden according to the Miller-Payne grade (p < 0.001). The estrogen modulator group showed more frequent lymphovascular invasion (p=0.046), a

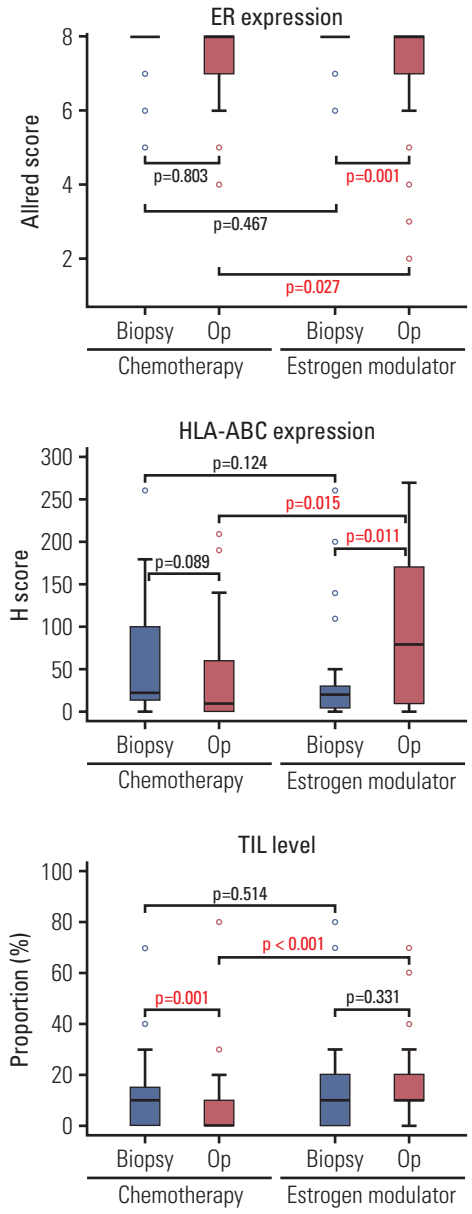

Fig. 5. The changes of ER and HLA-ABC expression and TIL levels. The TIL levels significantly decreased after chemotherapy, while the expression of ER decreased after estrogen modulator treatment. Compared with the chemotherapy group, HLA-ABC expression increased significantly after estrogen modulator treatment (Op). ER, estrogen receptor; HLA, human leukocyte antigen; Op, operation; TIL, tumor-infiltrating lymphocyte.

lower ER Allred score (p=0.027), higher Ki-67 labeling index (p=0.004) and higher TIL levels (p < 0.001) in resected specimens than the neoadjuvant chemotherapy group (Figs. 4 and 5).

Next, we evaluated HLA-ABC expression in both the biopsy and resection tissues of 56 patients by immunohistochemistry (Table 1). Before neoadjuvant therapy, the HLA-ABC positivity in tumor cells did not differ significantly between the two groups (p=0.243). After neoadjuvant systemic therapy, however, the estrogen modulator group showed higher HLA-ABC positivity than the chemotherapy group (p=0.038).

The overall survival and recurrence-free survival between the two groups did not differ significantly (p=0.396 and p=0.758, respectively) (S3 Fig.).

Discussion

Recently, the significance of TILs, particularly that of CD8+ cytotoxic T cells, in breast cancer has been revealed. Higher TIL level is known to be associated with longer patient survival and better response to chemotherapy [4,11,20]. Higher TIL level also correlates with programmed death-ligand 1 (PD-L1) expression [21], which may predict response to immune checkpoint inhibitor treatment [22]. Immune checkpoint inhibitors pembrolizumab and atezolizumab recently have been approved in TNBC with PD-L1 expression by FDA. However, HR+ breast cancers are still considered to be immunologically cold, and it is not yet hopeful whether immunotherapy can be effective in patients with those cancers [23].

The expression of MHC I proteins on the tumor cell surface is essential for CD8+ T cells to act, and several reports have documented the positive relationship between TIL levels and MHC I expression. We previously reported that TILs were more abundant in tumors with a stronger expression of HLA-ABC. We also reported that the HLA-ABC expression of the tumor cells was positively correlated with TIL levels in consecutive series of primary breast cancers and TNBC cohorts [10]. Although some may think that high HLA-ABC expression is due to a high mutation rate and more immunogenic mutations, we previously revealed that the total number of mutations was not associated with *HLA-A* expression in the tumor, and Spranger et al. [24] also reported that the density of nonsynonymous somatic mutations is not significantly associated with T cell related gene expression.

Instead, some evidence has indicated that MHC I expression is related to ER expression and IFN signaling. In our previous study, HLA-ABC expression was negatively associated with ER expression in a consecutive breast cancer cohort and normal breast tissue, and *HLA* mRNA expression was positively correlated with IFN-associated gene expression in a TCGA and Cancer Cell Line Encyclopedia data analysis [10]. We also reported that the high expression of IFN-mediator MxA in the tumor cells was positively associated with TIL levels, CD8+ cell number, and stronger HLA-ABC

expression and was an independent prognostic factor for better disease-free survival in breast cancer [12]. Although one article has reported that ERα signaling modulates IFN-γ inducible MHC II expression through class II transactivator in breast cancer cells [25], the mechanism of the relationship between HLA-ABC expression, ER activity, and IFN signaling in breast cancers has never been reported.

In the current study, we revealed that the expressions of HLA-ABC protein and *HLA-A* mRNA in breast cancer cells are negatively affected by ER signaling *in vitro*. We insist that this is a novel and important finding in addition to previously known tumor-intrinsic oncogenic pathways that have been suggested to be associated with the reduction of immune reactions, such as the WNT/β-catenin pathway, the mitogen-activated protein kinase pathway, the phosphoinositide 3-kinase/AKT pathway, MYC upregulation, and CDK4/6 activation [26]. The baseline HLA-ABC protein expression was higher in ER-negative cells than in ER-positive cells, and HLA-ABC protein expression was increased after ICI treatment and decreased after ER overexpression. *HLA-A* mRNA expression was also increased after *ESR1* shRNA transfection. We also revealed a positive association between HLA-ABC and IFN signaling and a negative association between ER and IFN signaling. Breast cancer cells showed increased HLA-ABC protein expression after IFN treatment and increased *MxA* mRNA expression after *ESR1* shRNA transfection. This is the first study that analyzed the relationship between HLA-ABC expression, ER activity, and IFN signaling in breast cancer *in vitro*.

We also analyzed the breast cancer tissues of patients who received either neoadjuvant chemotherapy or estrogen modulator therapy. We revealed that the HLA-ABC expression in breast cancer cells increased and ER expression decreased after estrogen modulator treatment compared with chemotherapy. Estrogen modulator treatment negatively regulate ER signaling in breast cancer. By combining the results of the *in vitro* experiments, our data suggested that ER signaling was downregulated by estrogen modulator treatment, and this caused the increase of HLA-ABC expression in tumor cells. We also analyzed the TIL levels in biopsies and resection tissues from the two groups. TIL levels were significantly decreased in resected tissue after neoadjuvant chemotherapy compared with biopsy tissue, which is concordant with a previous publication [27]. Although the HLA-ABC expression in tumor cells was increased in the estrogen modulator treatment group, we did not identify a significant change in TIL levels in this group. This is possibly due to the insufficient duration of neoadjuvant estrogen modulator therapy. Further studies with a longer duration of neoadjuvant treatment are necessary. However, estrogen modulator therapy group still showed significantly higher TIL level in resected tissue than chemotherapy group. Park et al. [28] reported that immune-stimulation after neoadjuvant chemotherapy is associated with pCR in breast cancer. Therefore, we suggest that higher HLA-ABC expression and TIL level in estrogen modulator therapy group may have a positive effect on prognosis of patients.

We found that Ki-67 labeling index was decreased both after neoadjuvant chemotherapy and after neoadjuvant estrogen modulator treatment, but chemotherapy group showed significantly lower Ki-67 labeling index than estrogen modular treatment group in resected tumor tissue. This might be explained by the fact that conventional chemotherapeutic agents are generally toxic to proliferative cells. Considering that chemotherapy group showed remarkable decrease of Ki-67 labeling index but did not show significant change of HLA-ABC expression, we suggest that increase of HLA-ABC expression in estrogen modulator group is rather associated with downregulation of ER signaling than decreased proliferation activity.

This study has several limitations. First, our data do not include *in vitro* results with estrogen treatment, the key molecule in ER signaling, or with tamoxifen treatment, an important selective ER modulator drug. Second, molecular mechanisms and mediators on how HLA-ABC expression and IFN signaling are regulated by ER signaling must be identified. Therefore, further experiments are necessary to more precisely define these molecular mechanisms. We suggest that more significant results might be obtained by future studies with more patient samples and a longer follow-up duration.

In conclusion, this study demonstrated that MHC I expression and TIL levels were affected by ER pathway modulation and IFN treatment. Therefore, we suggest that downregulation of ER signaling might induce immune reaction in HR+ breast cancer. Further studies elucidating the mechanism of MHC I regulation could suggest a way to boost TIL influx in cancer and increase the efficacy of immunotherapy in treatment of HR+ breast cancer patients.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Author Contributions

Conceived and designed the analysis: Kim HJ, Ahn SH, Lee HJ, Gong G.

Collected the data: Song IH, Kim YA, Heo SH, Bang WS, Park HS, Choi YH, Lee H, Seo JH, Cho Y, Jung SW.

Contributed data or analysis tools: Kim YA, Heo SH, Bang WS, Park HS, Lee HJ, Gong G.

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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

Acknowledgments
This work was supported by the Asan Institute for Life Sciences, Asan Medical Center, Seoul, Korea (2019IP0732).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1083
Cancer Res Treat. 2022;54(4):1121-1129

Original Article

The Pattern of Care for Brain Metastasis from Breast Cancer over the Past 10 Years in Korea: A Multicenter Retrospective Study (KROG 16-12)

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Purpose We aimed to investigate manifestations and patterns of care for patients with brain metastasis (BM) from breast cancer (BC) and compared their overall survival (OS) from 2005 through 2014 in Korea.

Materials and Methods We retrospectively reviewed 600 BC patients with BM diagnosed between 2005 and 2014. The median follow-up duration was 12.5 months. We categorized the patients into three groups according to the year when BM was initially diagnosed (group I [2005-2008], 98 patients; group II [2009-2011], 200 patients; and group III [2012-2014], 302 patients).

Results Over time, the median age at BM diagnosis increased by 2.2 years (group I, 49.0 years; group II, 48.3 years; and group III, 51.2 years; p=0.008). The percentage of patients with extracranial metastasis was 73.5%, 83.5%, and 86.4% for group I, II, and III, respectively (p=0.011). The time interval between BC and BM was prolonged in patients with stage III primary BC (median, 2.4 to 3 years; p=0.029). As an initial brain-directed treatment, whole-brain radiotherapy alone decreased from 80.0% in 2005 to 41.1% in 2014. Meanwhile, stereotactic radiosurgery or fractionated stereotactic radiotherapy alone increased from 13.3% to 34.7% during the same period (p=0.005). The median OS for group I, II, and III was 15.6, 17.9, and 15.0 months, respectively, with no statistical significance.

Conclusion The manifestations of BM from BC and the pattern of care have changed from 2005 to 2014 in Korea. However, the OS has remained relatively unchanged over the 10 years.

Key words Brain metastasis, Breast neoplasms, Overall survival, Pattern of care study

Introduction

Up to 40% of cancer patients with systemic disease experience brain metastasis (BM) [1]. The incidence of BM has been steadily increasing [2]. Some tumors have a high propensity to BM and the reported incidence are as follows: melanoma, 28.2%; lung, 26.8%; renal, 10.8%; and breast, 7.6% [3]. Given the worldwide high incidence of breast cancer [4], BM management of breast cancer (BC) patients is a crucial issue.

The treatment options for BM from BC consist of surgical resection and brain-directed radiotherapy (RT) [5]. Historically, whole-brain radiotherapy (WBRT) has been the first choice of treatment if BM is unresectable [5]. Concerning WBRT-induced neurocognitive toxicity, WBRT with memantine or hippocampal-sparing WBRT has been introduced [6,7]. And finally, as the results of several randomized trials comparing stereotactic radiosurgery (SRS) with or without WBRT, there has been a paradigm shift from WBRT to SRS, especially in limited BM [8-11].

Accompanying changes in RT, there has also been a breakthrough in systemic treatment. Several innovative cancer treatments such as molecular targeted therapy and immunotherapy have shown satisfactory results in metastatic BC patients [11-14]. With a higher control rate of extracranial disease, more patients are now presented with BM. Due to the low penetration efficacy of drugs into the blood-brain barrier [15], however, it is still an unmet clinical need to find effective systemic drugs for BM management. Although currently, there are a few systemic treatment options for BM [16], substantial progress would be expected and the treatment patterns for BM could be changed accordingly.

This is the first pattern-of-care study of patients with BM from BC in Korea past decade. In this study, we tried to find changes in the manifestations of BM, the evolution of treatment modalities, and improvement of overall survival (OS) during the decade of the study period.

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Received September 29, 2021 **Accepted** December 30, 2021 **Published Online** December 31, 2021
*Jae Sik Kim and Kyubo Kim contributed equally to this work.

Materials and Methods

1. Patients

Among a total of 730 patients with BM from BC who were enrolled in the Korean Radiation Oncology Group (KROG) 16-12 study from 17 high-volume institutions in Korea, 600 patients were identified with available initial BC stage from 2005 to 2014. The inclusion and exclusion criteria of the KROG 16-12 study were previously described [17].

The median follow-up duration was 12.5 months (interquartile range [IQR], 5.1-23.3). Based on the year of initial BM diagnosis, patients were classified arbitrarily into three groups: group I, from 2005 to 2008, n=98; group II, from 2009 to 2011, n=200; and group III, from 2012 to 2014, n=302, respectively.

We categorized tumor subtypes into three by the results

Table 1. Baseline characteristics according to the year of brain metastasis diagnosis

Characteristic	2005-2008 (group I)	2009-2011 (group II)	2012-2014 (group III)	p-value
No. of patients	98	200	302	
Age at primary BC (yr)	45.1 (39.2-51.9)	45.5 (37.8-52.8)	48.1 (41.8-53.7)	0.019
Age at BM (yr)	49.0 (41.4-56.1)	48.3 (39.8-56.2)	51.2 (45.6-57.4)	0.008
Interval of primary BC and BM (mo)	30.6 (14.4-48.9)	29.4 (16.5-56.4)	34.2 (19.2-54.0)	0.482
Tumor subtype				
HR+/HER2–	17 (17.3)	54 (27.0)	96 (31.8)	0.082
HER2+	46 (46.9)	87 (43.5)	126 (41.7)	
Triple-negative	35 (35.7)	59 (29.5)	80 (26.5)	
Initial BC stage				
Stage I	11 (11.2)	17 (8.5)	28 (9.3)	0.092
Stage II	39 (39.8)	56 (28.0)	99 (32.8)	
Stage III	35 (35.7)	70 (35.0)	94 (31.1)	
Stage IV	13 (13.3)	57 (28.5)	81 (26.8)	
ECOG				
0-1	69 (70.4)	130 (65.0)	197 (65.2)	0.601
2-3	29 (29.6)	70 (35.0)	105 (34.8)	
Primary tumor[a]				
Uncontrolled	23 (23.5)	39 (19.6)	82 (27.7)	0.092
Controlled	75 (76.5)	160 (80.4)	214 (72.3)	
Extracranial metastasis				
Absent	26 (26.5)	33 (16.5)	41 (13.6)	0.011
Present	72 (73.5)	167 (83.5)	261 (86.4)	
Symptoms				
No	14 (14.3)	24 (12.0)	34 (11.3)	0.725
Yes	84 (85.7)	176 (88.0)	268 (88.7)	
No. of BMs				
≤ 4	51 (52.0)	116 (58.0)	163 (54.0)	0.548
> 4	47 (48.0)	84 (42.0)	139 (46.0)	
Location of BM				
Supra- or infra-tentorial	51 (52.0)	109 (54.5)	134 (44.4)	0.068
Both	47 (48.0)	91 (45.5)	168 (55.6)	
Breast-GPA				
0-1.0	13 (13.3)	20 (10.0)	34 (11.3)	0.903
1.5-2.0	27 (27.6)	63 (31.5)	84 (27.8)	
2.5-3.0	47 (48.0)	89 (44.5)	139 (46.0)	
3.5-4.0	11 (11.2)	28 (14.0)	45 (14.9)	

Values are presented median (IQR) or number (%). BC, breast cancer; BM, brain metastasis; Breast-GPA, breast cancer-specific graded prognostic assessment; ECOG, Eastern Cooperative Oncology Group; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; IQR, interquartile range. [a]Available data only.

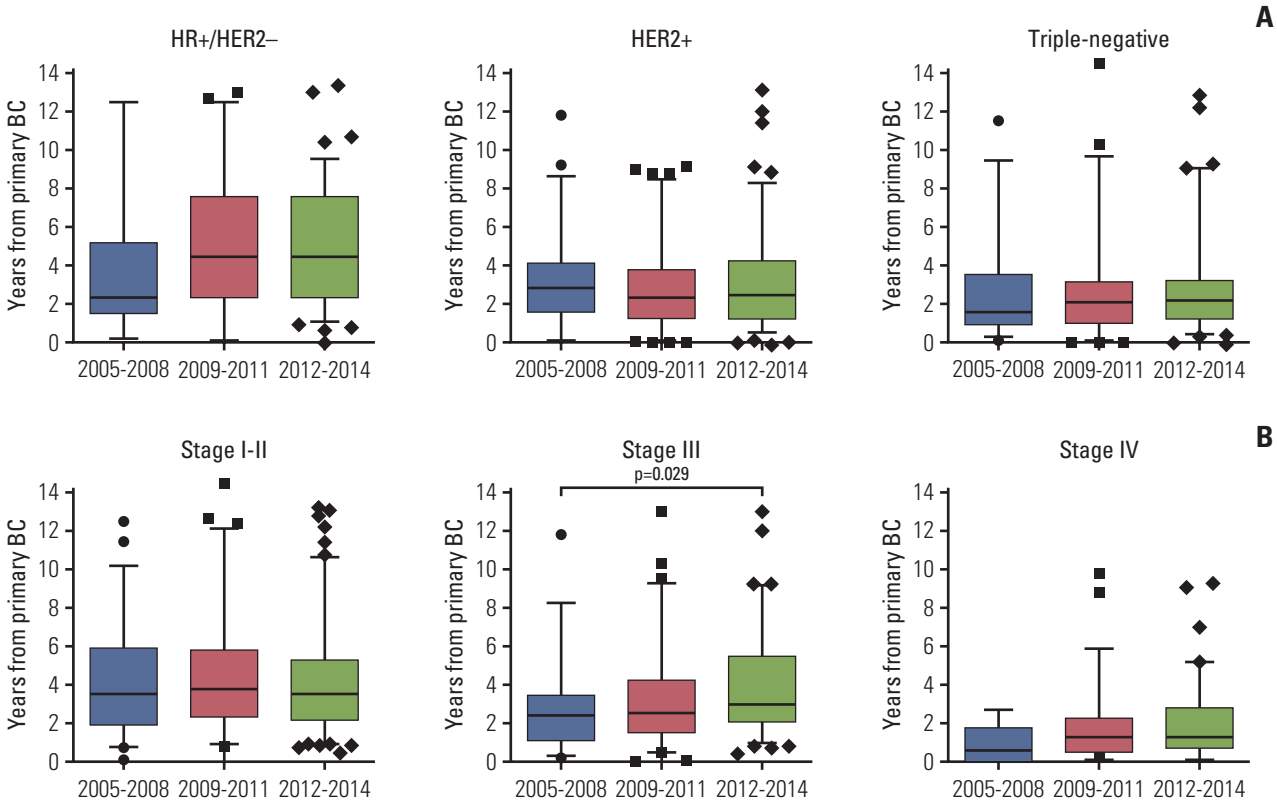

Fig. 1. Time interval between primary breast cancer and brain metastasis according to tumor subtypes (A) and initial stage of primary breast cancer (B). Box plots present median value with 5-95 percentile. Adjusted p-values were calculated using Tukey's multiple comparisons. BC, breast cancer; HER2, human epidermal growth factor receptor 2; HR, hormone receptor.

Table 2. Treatment distribution according to the year of brain metastasis

Characteristic	2005-2008 (group I)	2009-2011 (group II)	2012-2014 (group III)	p-value[a]
WBRT alone	64 (65.3)	114 (57.0)	160 (53.0)	0.036
SRS or FSRT alone	18 (18.4)	41 (20.5)	81 (26.8)	0.045
Op alone	3 (3.1)	7 (3.5)	12 (4.0)	0.657
Op or SRS or FSRT → WBRT	9 (9.2)	27 (13.5)	39 (12.9)	0.454
WBRT → SRS	1 (1.0)	2 (1.0)	1 (0.3)	0.358
Other brain-directed treatment	3 (3.1)	9 (4.5)	9 (3.0)	0.733
Subsequent systemic therapy	77 (78.6)	158 (79.0)	235 (77.8)	0.810
Anti-HER2 therapy[b]	23 (50.0)	54 (62.1)	66 (52.4)	0.846

Values are presented as number (%). FSRT, fractionated stereotactic radiotherapy; HER2, human epidermal growth factor receptor 2; Op, operation; SRS, stereotactic radiosurgery; WBRT, whole-brain radiotherapy. [a]p-value for trend, [b]In HER2+ patients.

of immunohistochemical staining of primary BC: hormone receptor (estrogen receptor and/or progesterone receptor)–positive/human epidermal growth factor receptor 2–negative (HR+/HER2–), HER2+, and triple-negative BC (TNBC). The initial stage of BC was described according to the seventh edition of the American Joint Committee on Cancer staging criteria. The BC-specific graded prognostic assessment (breast-GPA) score was calculated using three factors, Karnofsky performance status, tumor subtype, and age [18]. According to the breast-GPA score, we divided patients into four groups: GPA 0-1.0, 1.5-2.0, 2.5-3.0, and 3.5-4.0, respectively.

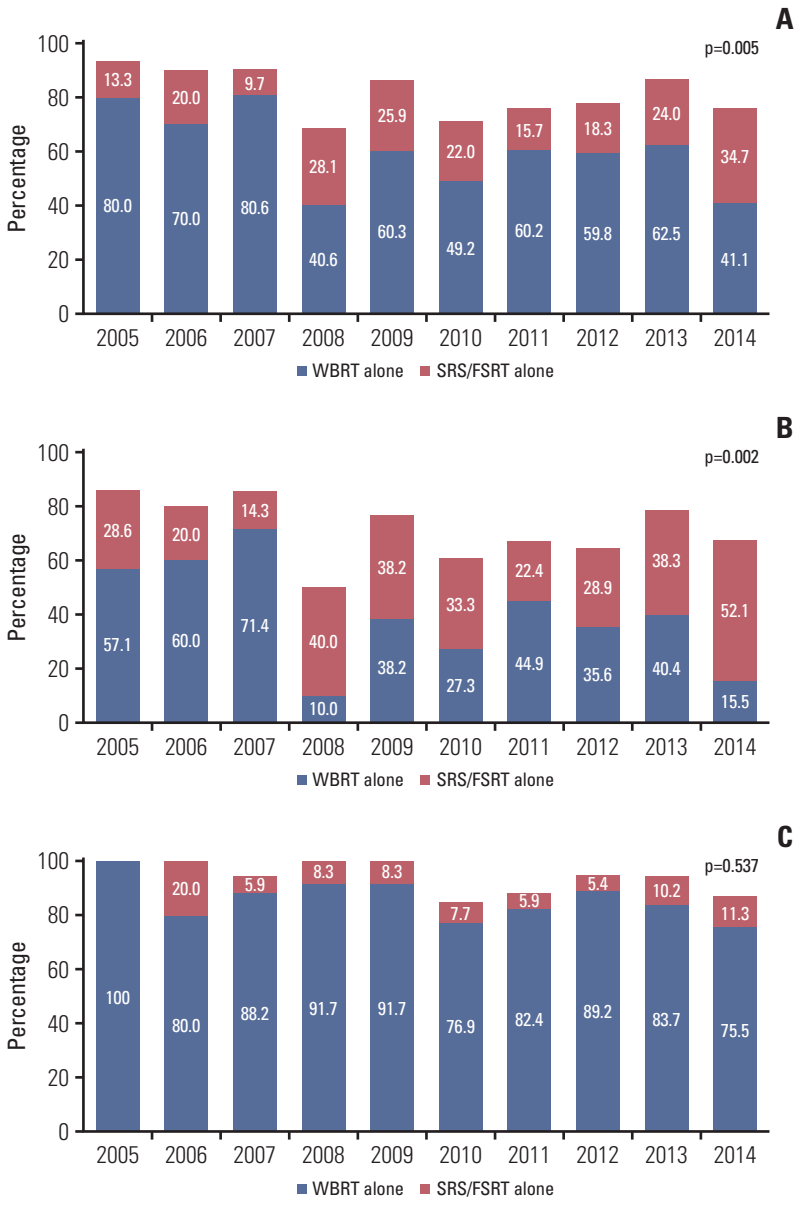

Fig. 2. Trend of radiotherapy for brain metastasis (BM) from breast cancer: all patients (A), patients with 1-4 BM (B), patients with more than 4 BM (C), according to tumor subtype (D), and according to breast cancer-specific graded prognostic assessment (E). FSRT, fractionated stereotactic radiotherapy; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; SRS, stereotactic radiosurgery; WBRT, whole-brain radiotherapy. *(Continued to the next page)*

2. Statistical analysis

Comparisons of continuous variables were done using a one-way anlaysis of variance or the Kruskal-Wallis test. Tukey's multiple comparison test was used for *post-hoc* analysis. For categorical data, chi-square or Fisher exact test was used. The Cochran-Armitage trend test was performed to calculate p-values for trend. OS was calculated from the date of BM diagnosis to that of any death, with the Kaplan-Meier method. And its difference between groups was compared using the log-rank test. A two-sided p-value less than 0.05 was considered statistically significant. Figures without a p-value mean no statistical significance. All analyses were carried out using the R statistical software ver. 4.1.0 (https://www.r-project.org/). Graphics except for the Kaplan-Meier curve were made by GraphPad-Prism Analysis software ver. 8.3.0 (San Diego, CA) or Microsoft Excel 2019 (Redmond, WA).

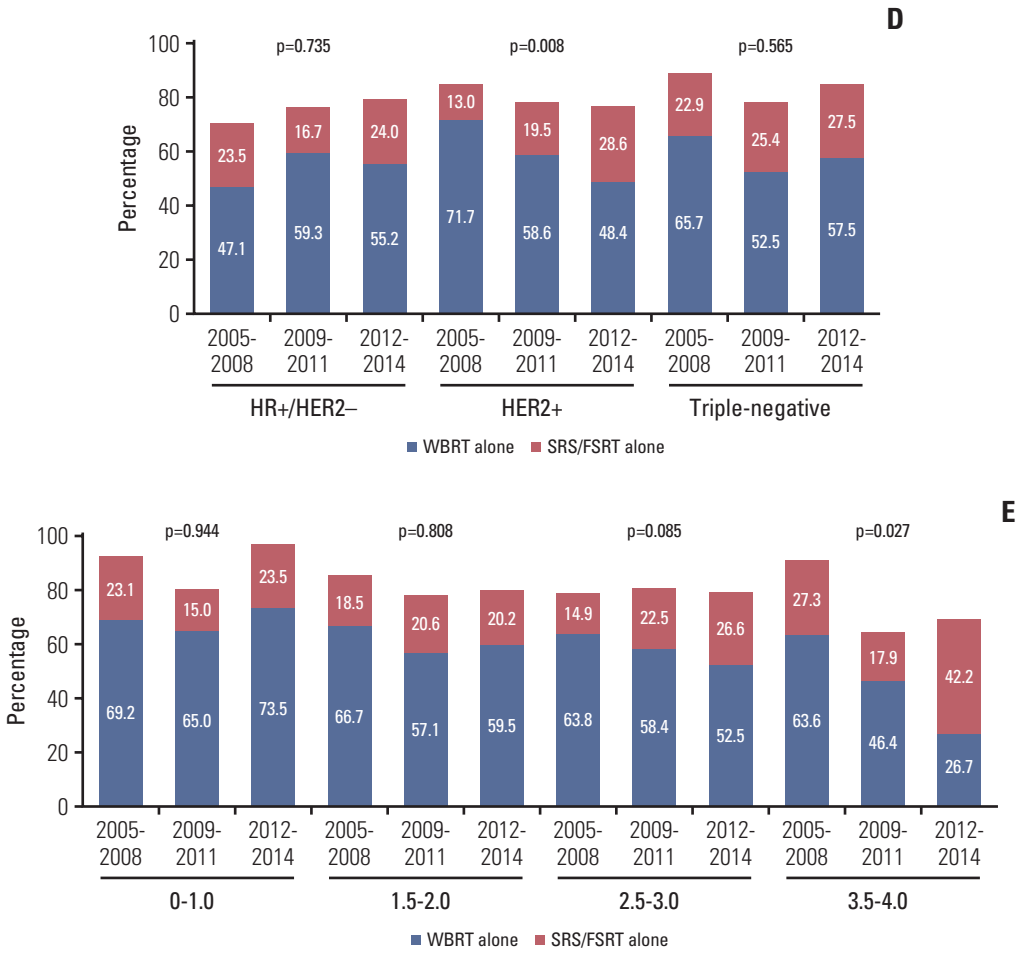

Fig. 2. *(Continued from the previous page)*

Results

Baseline characteristics among the three groups are compared in Table 1. During the study period, the median age at diagnosis of primary BC and BM has been increased from 45.1 to 48.1 years (p=0.019) and from 49.0 to 51.2 years (p=0.008), respectively. Without statistical significance, BM tended to develop latest in the group III. Regarding tumor subtypes, tumors with HR+/HER2– marginally increased their portion, meanwhile, those of other subtypes decreased (p=0.082). However, HER2+ occupied the largest portion during the study period. Patients with extracranial metastasis in group I, II, and III accounted for 73.5%, 83.5%, and 86.4%, respectively (p=0.011). Over 80% of each group had neurologic symptoms at BM diagnosis. There were no significant differences in intracranial tumor burden but, the largest number of patients in group III had BM in both tentorial regions, compared to that of group I and II (p=0.068). Overall, the distribution of breast-GPA showed no difference.

In terms of the change of the number of BM according to tumor subtypes, patients with HER2+ BM of 4 or less showed a tendency to increase more recent, nevertheless with no statistical significance (group I, n=21, 45.7%; group II, n=45, 51.7%; and group III, n=73, 57.9%, p for trend=0.134). In HR+/HER2–, the opposite trend was observed (group I, n=11, 64.7%; group II, n=31, 57.4%; and group III, n=50, 52.1%, p for trend=0.296), and no specific trend in TNBC.

Fig. 1 shows the interval between BC and BM based on tumor subtypes or the initial stage of BC. The changes of this interval according to the times were not found except for that of stage III patients. In these patients, the median time interval has steadily protracted from 2.4 to 3 years (p=0.029).

Brain-directed local treatment was immediately administered approximately 5 days after the initial diagnosis of BM (S1 Fig.). The largest portion of the initial brain-directed treatment was WBRT alone, followed by SRS or fractionated stereotactic radiotherapy (FSRT) alone (Table 2). However, WBRT decreased from 80.0% to 41.1% and SRS/FSRT alone

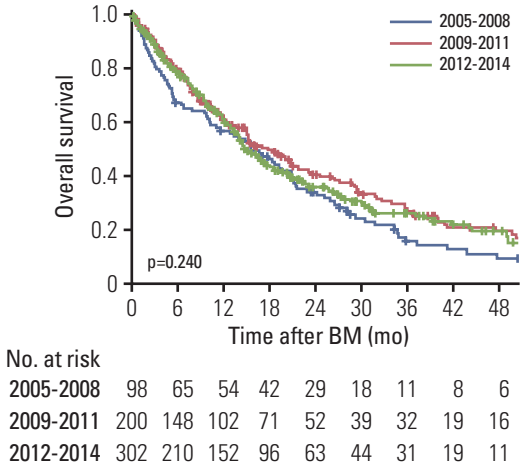

Fig. 3. Kaplan-Meier curve of overall survival according to the year of brain metastasis (BM).

increased from 13.3% to 34.7% over the past 10 years (p for trend=0.005) (Fig. 2A). These changes were prominent in patients with limited BM (1-4 BM, p for trend=0.002) (Fig. 2B), but not in those with BM > 4 (p for trend=0.537) (Fig. 2C). This paradigm shift in treatment strategy into SRS/FSRT alone was observed for patients with HER2+ BM (p for trend=0.008, Fig. 2D) and with breast-GPA scores of 3.5-4.0 (p for trend=0.027, Fig. 2E). About 80% of patients were treated with systemic treatment following brain-directed treatment. Especially, for patients with TNBC, the use of systemic therapy after initial brain-directed treatment did not increase (77.1% in group I, 78.0% in group II, and 68.8% in group III, respectively; p for trend=0.255). With respect to HER2+ patients, more than half of patients received anti-HER2 therapy. Among 417 patients with WBRT, only 21 patients received WBRT using 3-dimensional conformal RT or intensity-modulated RT (one patient was treated with hippocampal avoidance WBRT). Additional boost ranging from 3 to 25 Gy was administered after WBRT in 25 patients (19 patients after conventional WBRT) and a simultaneous-integrated boost was done in another patient.

For the entire cohort, OS did not change significantly from 2005 to 2014 (median, 15.6 months in group I, 17.9 in group II, and 15.0 in group III, respectively; p=0.240) (Fig. 3, S2 Table). The 1-year OS rate of group I, II, and III was 57.0%, 61.0%, and 61.0%, respectively. In subgroup analysis, shown in S2 Table, only patients with the highest breast-GPA scores improved their median OS by a factor of two from 15.5 to 30.0 months (p=0.03). According to tumor subtype, the initial stage of primary BC, number of BM, brain-directed treatment for initial BM as well as other breast-GPA groups, we did not find any improvement in OS.

Discussion

In our study, the proportion of older BM patients with extracranial metastasis significantly increased over the past 10 years. Regardless of tumor subtypes, the time of BM diagnosis has been prolonged after primary BC with stage III disease. The first choice of brain-directed treatment for BM was primarily WBRT alone, but the use of SRS/FSRT alone has been increased during the period, especially in limited BM, which had 1-4 BM. Also, subsequent systemic treatment was frequently given and emphasized that multidisciplinary approaches based on the individualized situation were important in these patients. Unfortunately, there has been no such dramatic improvement in OS over 10 years.

Median age at initial BM diagnosis increased by 2.2 years in the current study. However, due to the increased median age at primary BC, there was no statistically significant increment in the time interval from primary BC to BM. This result was contrary to the report by Nieder et al. [19] which found the significantly lengthened time to development of BM. They explained this result by the increased use of systemic treatment. While the study by Nieder et al. [19] had a time interval of more than 25 years, this conflicting result might also be related to the fact that our study described a change over a short period of 10 years.

However, in terms of the initial stage of BC, patients with stage III, high-risk localized disease, showed a longer period until the brain failure was experienced. This interval seemed to be increasing recently in stage IV patients as well. It has been known that the stage of BC, as well as subtypes, is a prognostic factor for the time from BC to BM [20]. Concerning that advanced stage is associated with an earlier BM development [20], it is important to note that the time to BM was prolonged in stage III-IV patients in this study. In addition, a greater portion of extracranial metastasis could reflect the effectiveness of systemic treatment.

We would readily expect early identification of asymptomatic and tiny BM in recent years on account of the progress of brain imaging modalities. However, over 80% of the included patients had neurologic symptoms in the present study. Furthermore, no changes in the number of BM at diagnosis and slightly more patients with BM in both supra- and infra-tentorial regions were found. This might result from the timing of brain imaging after patients have symptoms. Currently, controversies exist on the role of brain magnetic resonance imaging (MRI) as a screening tool for BM [21]. However, recent studies emphasize and favor the use of MRI because early detection of BM could be managed by SRS with less invasiveness and toxicities [21,22]. In view of cost-effectiveness, it is necessary to select the optimal candidates for BM screening.

Our significant observation was that WBRT accounted for the largest proportion among the brain-directed local therapies. However, its use was decreasing while the use of SRS increased. This was observed especially in patients with 1-4 BM and over half of these patients in 2014 were treated with SRS/FSRT alone. These findings coincide with previous reports [19,23,24]. Several factors have affected this paradigm shift in initial approaches for BM. Increased awareness of late toxicities after WBRT, including neurocognitive dysfunction, has made physicians avoid choosing WBRT in selected patients [10,25]. Recently amended guidelines recommending SRS for patients with limited BM have changed the choice of RT as well [11]. Besides, as the distinctive situation in Korea, the relaxed reimbursement guidelines for SRS of National Health Insurance Service might play a part since April 2007.

We performed analyses on the relation of the shift to SRS not only with the number of BM but also with tumor subtypes and breast-GPA. Among three subtypes, the first course of RT for HER2+ BM solely has preferred SRS/FSRT alone over WBRT. Although we could not determine the obvious reasons for this alteration, it might be affected by the synergism of the increasing number of patients with limited BM and SRS utilization in these patients. The reasons for the low number of BM at initial, especially in HER2+ patients, were not clear. Other possible causes of this propensity beyond our data should also be perceived. Although SRS/FSRT is being widely used in HER2+ BM, the risk of distant intracranial failure should be considered. The risk of new BM without initial WBRT was higher in HR+/HER2- followed by HER2+ subtypes according to our previous report for the new BM development after the initial brain-directed local treatment according to the tumor subtypes [26]. Contemporary patients with breast-GPA scores of 3.5-4.0 were largely treated with SRS/FSRT alone. In the next high breast-GPA group, WBRT was still mainly used, but the use of SRS/FSRT alone increased marginally. These indicated that SRS/FSRT was favored in patients with a better prognosis.

Overall, survival has not altered during the study period. This was disappointing but, from another point of view, might be an encouraging result. As Nieder et al. [19] described, recently treated patients had more extracranial metastasis and few options of systemic treatments since several systemic agents were heavily administered to these patients before BM diagnosis. Even though in this situation, 77.8% of patients in group III received systemic treatment after brain-directed treatment, and there was no decrease in OS rate at 1 year.

The current study has clear limitations; the retrospective design had inherent flaws such as selection bias and the cohort was relatively small compared to population-based studies. We did not look at the socioeconomic status of enrolled patients, which could influence decisions making of the treatment modality. A lack of detailed information on systemic treatment, especially chemotherapeutic agents or novel molecular targeted therapy, limited the interpretation of our analysis. In spite of these shortcomings, this study was currently the best way possible to show the evolving strategies of BM treatment in BC patients over the past 10 years in Korea, as it analyzed much more detailed data not covered in large-scale population-based studies.

In conclusion, presentations of BM from BC have profoundly changed from 2005 to 2014 in Korea. In accordance with these changes, management for BM has also been evolved. Still, WBRT had a large portion of the brain-directed treatment however, it has been reserved for salvage option after initial use of SRS/FSRT. Although patients with unfavorable features have been increasing, there has been no significant change in OS over the past decade. Patients with good prognostic factors showed an improvement in OS.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was approved by the institutional review board of each institution. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Because of the retrospective design of the analysis, requirement for obtaining informed consent of participants included in the study was exempted.

Author Contributions

Conceived and designed the analysis: Kim K, Kim IA.
Collected the data: Jung W, Shin KH, Im SA, Kim HJ, Kim YB, Chang JS, Kim JH (Jee Hyun Kim), Choi DH, Park YH, Kim DY, Kim TH, Choi BO, Lee SW, Kim S, Kwon J, Kang KM, Chung WK, Kim KS, Nam JH, Yoon WS, Kim JH (Jin Hee Kim), Cha J, Oh YK.
Contributed data or analysis tools: Kim JS, Kim K, Kim IA.
Performed the analysis: Kim JS, Kim K.
Wrote the paper: Kim JS, Kim K.
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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

Acknowledgments
This work was supported by grants from the Ministry of Science and Information & Communication Technology (NRF#2020R1A2-C2005141) to In Ah Kim.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1103
Cancer Res Treat. 2022;54(4):1130-1137

Original Article

Real-World Evidence of Trastuzumab, Pertuzumab, and Docetaxel Combination as a First-Line Treatment for Korean Patients with HER2-Positive Metastatic Breast Cancer

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Purpose Trastuzumab has markedly improved the survival outcomes of patients with human epidermal growth factor receptor 2 (HER2)–positive breast cancer, and dual blockade of HER2 using trastuzumab and pertuzumab in combination with taxanes (THP) has become a standard of care for HER2-positive metastatic breast cancer (MBC) worldwide since the CLEOPATRA trial. We assessed the outcomes of THP as a first-line treatment for Korean HER2-positive MBC patients in the real-world setting.

Materials and Methods Between August 2008 and October 2020, we identified 228 HER2-positive MBC patients who received THP as a first-line palliative chemotherapy. We analyzed survival outcomes, efficacy, and adverse events of THP retrospectively.

Results After a median follow-up duration of 28.7 months, median overall survival and progression-free survival were 58.3 months (95% confidence interval [CI], 36.6 to 80.0) and 19.1 months (95% CI, 16.2 to 21.9), respectively. Better survival outcomes were observed in patient who received docetaxel for more than six cycles. Patients exposed to anti-HER2 directed therapies in a perioperative setting had poor survival outcomes. The overall response rate was 86.8% with a complete response (CR) rate of 17.7%. Among responders, 16.7% of patients sustained THP over 35 months and showed better survivals and higher CR rates. Adverse events were comparable to those reported in previous studies.

Conclusion In a real-world context, clinical outcomes of Korean HER2-positive MBC patients treated with THP were similar to those of patients in the CLEOPATRA trial. Much longer follow-up results would be warranted.

Key words Metastatic breast cancer, HER2 positive, Trastuzumab, Pertuzumab, Docetaxel

Introduction

Breast cancer (BC) is the most common cancer in women and the leading cause of cancer death worldwide [1]. Among all BC patients, approximately 15%-20% present with overexpression of human epidermal growth factor receptor 2 (HER2), which is characterized by a progressive nature and a poor clinical outcome [2,3]. Advances in HER2-targeted treatment strategies such as trastuzumab, a humanized monoclonal antibody that targets the extracellular domain of HER2 and inhibits proliferation [4], have improved the survival outcomes of patients with HER2-positive metastatic breast cancer (MBC) [5]. However, despite the use of trastuzumab, more effective treatment options and strategies are required to address disease progression. Pertuzumab, one of the new HER2 targeting agents, inhibits HER2 by a different mechanism than trastuzumab [6], and provides better antitumor activity than trastuzamab alone due to blockade of HER2 signaling when co-administered with trastuzumab [7]. The CLEOPATRA trial investigated the use of pertuzumab, trastuzumab and docetaxel (THP) as a first-line treatment for HER2-positive MBC patients and reported significantly prolonged survival outcomes with manageable toxicities [8-12]. Due to the findings of this pivotal trial, dual HER2 antibody therapy plus taxane has become the first-line standard of care for treating HER2-positive MBC patients, showing median overall survival (OS) close to 5 years.

Although clinical trials are the gold standard for demonstrating the efficacy of treatment, the outcomes of well-designed clinical trials might not reflect the real-world situation due to the careful selection of patients. Thus, analysis of real-world data is required to produce long-term efficacy data of treatments to compensate the weaknesses of clinical trials. In this retrospective study, we evaluated the efficacy and safety of THP treatment as a first-line palliative chemotherapy for Korean patients with HER2-positive MBC based on the single institution experience in the real-world context.

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Received October 9, 2021 **Accepted** January 13, 2022 **Published Online** January 17, 2022

Materials and Methods

1. Patients and data collection

This is a retrospective study of HER2-positive MBC patients with treatment-naïve for their metastatic disease. We identified patients who received THP as a first-line palliative chemotherapy and collected data retrospectively from medical records and laboratory results in the BC registry of single institution in Korea, Samsung Medical Center from August 2008 through October 2020. Demographic information and clinical characteristics were abstracted including age, date of diagnosis, confirmed pathology, initial cancer stage, hormone receptor status, type of perioperative treatment, and type of surgery. Patients received 6 mg/kg of trastuzumab (after an initial 8 mg/kg loading dose), 420 mg pertuzumab (after an initial 840 mg loading dose), plus 75 mg/m^2 of docetaxel every 3 weeks. In order to alleviate hypersensitivity and adverse events caused by docetaxel, each patient receiving docetaxel took 8 mg of dexamethasone 6 times over 3 days from the night before THP treatment to the next day. The treatment was continued until disease progression or occurrence of unacceptable toxicities. For patients who developed toxic effects that contraindicated docetaxel administration during THP treatment, we omitted docetaxel and maintained dual anti-HER2 directed therapy. HER2 overexpression was defined as either three-positive or two-positive on immunohistochemistry (IHC) test. For a two-positive IHC test result, HER2 status was confirmed through additional tests such as fluorescent *in situ* hybridization or silver *in situ* hybridization. In the *in situ* hybridization test, a positive *HER2* gene amplification was defined as a *HER2*/centromere enumerator probe 17 ratio greater than 2.0.

2. Statistical analysis

OS was defined as the time from the initiation of THP treatment to the date of death from any cause and was censored at the date of last available follow-up. Progression-free survival (PFS) was measured from the initiation of THP treatment to progression or death from any cause, and was censored at the date of last available follow-up. The primary objective of this study was to evaluate survival outcomes, including median OS and PFS. Secondary objectives were to assess treatment efficacy by objective response rate (ORR), safety profiles of THP, and clinical outcomes of subsequent treatment after progression. ORR was defined as the proportion of patients who achieved a complete response (CR) or partial response as their best responses obtained during THP treatment. Response evaluation to treatment was assessed in patients with measurable lesions according to the Response Evaluation Criteria in Solid Tumors [13] using computed tomography and magnetic resonance imaging.

Table 1. Baseline demographics and disease characteristics at diagnosis

Characteristic	No. (%)
No. of patients	228
Age (yr)	
Median (range)	60 (26-78)
≤ 40	14 (6.1)
> 40 and ≤ 50	64 (28.0)
> 50 and ≤ 60	82 (35.9)
> 60	68 (29.8)
Menopausal status	
Pre-menopause	113 (49.5)
Post-menopause	107 (46.9)
Unknown	8 (3.5)
Hormone receptor status	
ER positive and/or PR positive	124 (54.3)
ER negative and PR negative	94 (41.2)
Unknown	10 (4.3)
***De novo* metastatic breast cancer**	123 (53.9)
Relapsed metastatic breast cancer	105 (46.0)
Curative surgery	96/105 (91.4)
Progression during neoadjuvant treatment	6/105 (5.7)
Unknown for surgery	3/105 (2.8)
Perioperative chemotherapy	83 (86.4)
Neoadjuvant chemotherapy	29
TCHP → Surgery → Herceptin	2
AC followed by TH → Surgery → Herceptin	25
AC followed by T → Surgery	2
Adjuvant treatment	54
AC followed by TH	32
TCH	5
AC followed by T	7
FAC	5
HTx. only	5
Exposure to HER-2 targeted therapy prior to THP treatment	
Yes	67 (29.3)
No	161 (70.6)
No. of docetaxel administration	
Median (range)	9 (1-28)
< 6	20 (8.7)
≥ 6	208 (91.2)
6-9	139 (66.8)
≥ 10	69 (33.1)

(Continued to the next page)

Treatment-related adverse events were assessed by review of medical records and evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse

Table 1. Continued

Characteristic	No. (%)
Disease-free interval (mo)	96
Median (range)	38.5 (6.5-1,387.5)
> 6 and ≤ 12	5/96 (5.2)
> 12 and ≤ 24	23/96 (23.9)
> 24	64/96 (66.6)
Non-available	4/96 (4.1)
Site of metastasis at the time of THP treatment	
Visceral metastasis	78 (34.2)
Bone metastasis	51 (22.3)
Brain metastasis	6 (2.6)

AC, adriamycin, cyclophosphamide; ER, estrogen receptor; FAC, fluorouracil, doxorubicin, cyclophosphamide; HTx., hormone therapy; PR, progesterone receptor; TCH, docetaxel, carboplatin, trastuzumab; TCHP, docetaxel, carboplatin, trastuzumab, pertuzumab; TH, docetaxel, trastuzumab; THP, docetaxel, trastuzumab, pertuzumab.

Events, ver. 5.0 [14]. For statistical analyses, demographics and patient characteristics were summarized by descriptive statistics, and the chi-square test was used for comparison of characteristics. The Kaplan-Meier method was used for univariate analysis of survival outcomes, and the log-rank test was used for comparisons. All data were analyzed using the Statistical Package for Social Sciences software ver. 24.0 (IBM Corp., Armonk, NY).

Results

1. Patient characteristics

We analyzed a total of 228 patients with MBC who received THP as a first-line palliative chemotherapy. Baseline characteristics of the patients are shown in Table 1. Median age at the time of THP treatment was 60 years (range, 26 to 78 years). Among 228 patients, 123 patients (53.9%) had *de novo* stage IV disease and 105 patients (46.0%) had relapsed MBC. Of the patients with recurrent disease, 96 patients (91.4%) underwent curative surgery, and six of nine patients

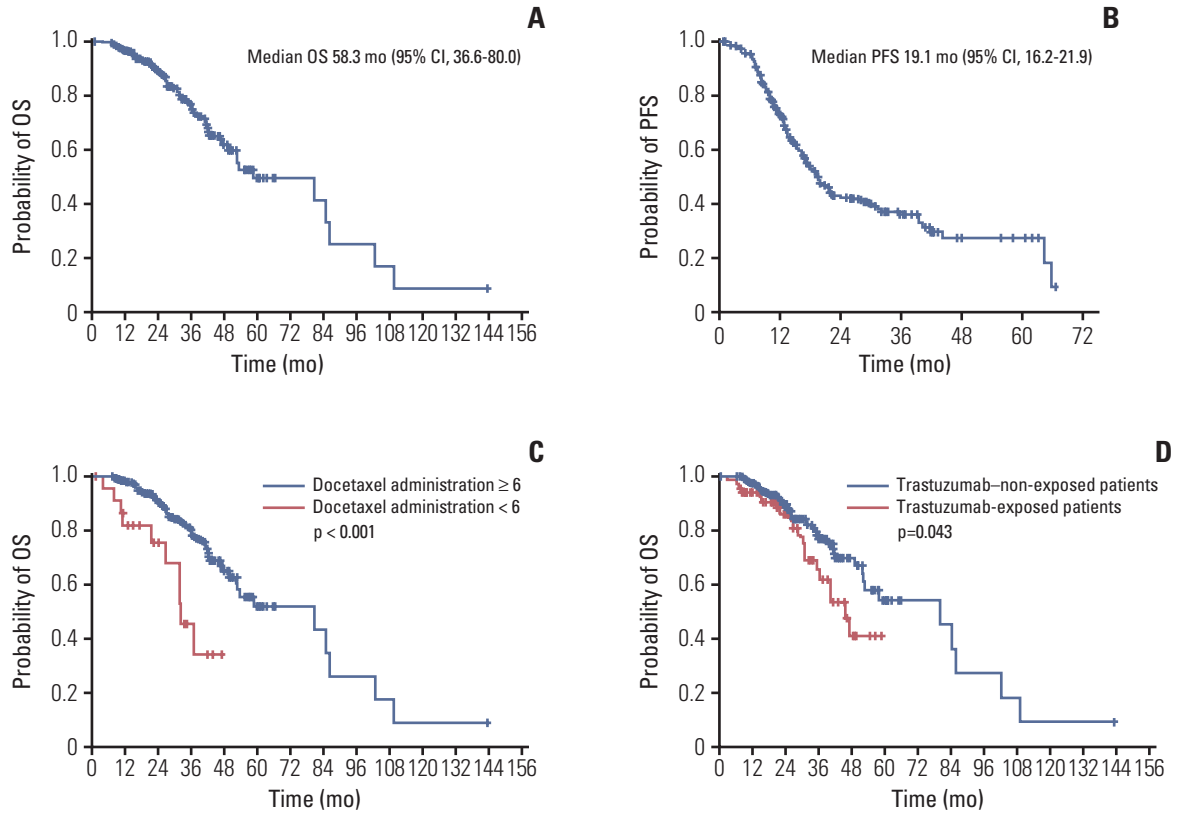

Fig. 1. OS (A) and PFS (B) after THP treatment. OS according to the number of docetaxel administrations (C) and exposure to trastuzumab prior to THP treatment (D). CI, confidence interval; OS, overall survival; PFS, progression-free survival; THP, docetaxel, trastuzumab, pertuzumab.

Table 2. Response rate of patients of first-line THP treatment with measurable lesions

Best response	No. (%) (n=220)
Complete response	39 (17.7)
Partial response	152 (69.0)
Overall response	191 (86.8)
Stable disease	25 (11.3)
Progressive disease	4 (1.8)

THP, docetaxel, trastuzumab, pertuzumab.

did not receive curative surgical treatment due to progressive disease during neoadjuvant chemotherapy. The treatment history of the remaining three patients was confirmed before THP treatment, but it was uncertain whether they were treated surgically. Of the 96 patients who underwent curative surgery, 83 (86.4%, 83/96) patients received perioperative treatment, including chemotherapy, radiotherapy, or hormonal therapy. While receiving perioperative treatment, a total of 67 patients, including three who did not undergo surgery due to progressive disease during neoadjuvant treatment, were exposed to anti-HER2 directed therapies (two patients received trastuzumab plus pertuzumab, 62 patients received trastuzumab alone). Most patients (n=208, 91.2%) received more than six cycles of docetaxel. At the time of diagnosis of MBC, visceral metastasis was presented in one-third of patients (78/228), and bone metastasis was presented in approximately 20% of patients (51/228). In contrast, only six patients (2.6%) had brain metastasis.

2. Survival outcomes of THP as a first-line treatment

For a median follow-up duration of 28.7 months (range, 0.7 to 143.5 months), median OS and PFS in our study were 58.3 months (95% confidence interval [CI], 36.6 to 80.0) and 19.1 months (95% CI, 16.2 to 21.9), respectively (Fig. 1A and B). In subgroup analysis, there was no difference in survival outcomes according to age, menstrual condition, status of hormonal receptor expression, or metastatic site. Patients who received docetaxel for more than six cycles along with anti-HER2 directed therapies had significantly improved survivals than patients who received less than six cycles of docetaxel ($p < 0.001$) (Fig. 1C). In our study, patients unexposed to anti-HER2 directed therapies prior to THP treatment (trastuzumab–non-exposed patients) had better survival outcomes than those patients already exposed (trastuzumab-exposed patients) (p=0.043) (Fig. 1D). Survival analysis did not reveal any significant difference between *de novo* MBC patients and relapsed MBC patients (S1A Fig.).

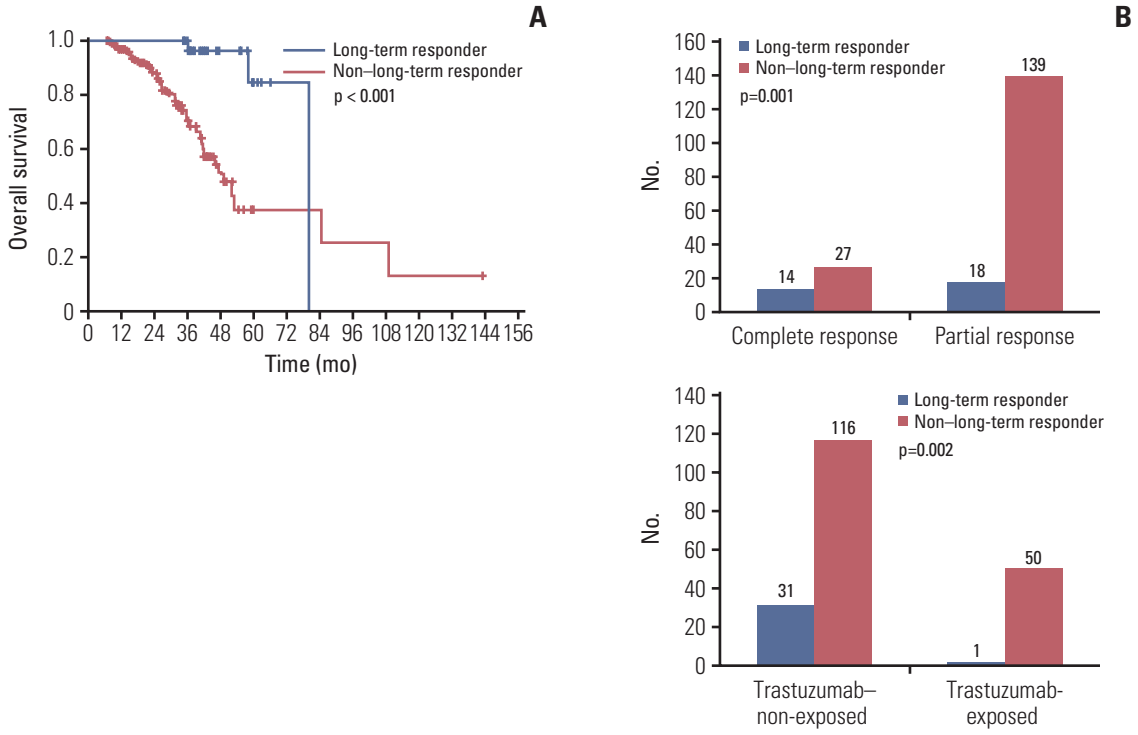

Fig. 2. Overall survival according to long-term responses (A). Comparison of response rates (B) and trastuzumab exposure (C) between long-term responders and non–long-term responders.

Table 3. Adverse events of THP treatment

	Grade 1-2	Grade 3	Grade 4
Hematopoietic adverse events			
Neutropenia	55 (24.1)	28 (12.2)	35 (15.3)
Febrile neutropenia	-	21 (9.2)	-
Non-hematopoietic adverse events	Grade 1-2	Grade 3-4	
Diarrhea	37 (16.2)	8 (3.5)	
Nausea	126 (55.2)	45 (19.7)	
Vomiting	105 (46.0)	31 (13.5)	
Mucositis	118 (51.7)	35 (15.3)	
Peripheral neuropathy	62 (27.1)	20 (8.7)	
Any kind of bacteremia	-	11 (4.8)	-

THP, docetaxel, trastuzumab, pertuzumab.

3. Response to THP treatment and safety outcomes

In 220 patients with measurable lesions, ORR was 86.8% (191/220) with a 17.7% CR rate (39/220) (Table 2). Median number of THP cycles was 19 (range, 2 to 88). After co-administration of docetaxel for a median of nine cycles (range, 1 to 28), we continued to use trastuzumab and pertuzumab as maintenance therapy with omission of docetaxel. The median duration of response who achieved objective response was 21.3 months (95% CI, 15.1 to 27.5) and 93.1% of responders (178/191) received docetaxel for more than six cycles. Among responders, 32 (16.7%) were long-term responders, defined as patients who sustained THP over 35 months. In our study, long-term responders had better survival outcomes and higher CR rates than non–long-term responders, and more long-term responders were observed in trastuzumab–non-exposed patients than other patient groups (Fig. 2, S2 Table). During THP treatment, 118 patients (51.7%) experienced any kind of neutropenia, and 63 patients (27.6%) experienced grade 3 or 4 neutropenia. In the neutropenic period, about 10% of patients had febrile events (21/228), and of patients who had febrile neutropenia, 11 patients had actual bacteremia. In addition to hematopoietic adverse events, patients who underwent THP therapy suffered from non-hematopoietic adverse events, including diarrhea, nausea, vomiting, and mucositis (Table 3). Among patients who suffered from any grade of peripheral neuropathy (n=62, 27.1%), 8.7% of patients had high-grade of neuropathy and required interventions such as medications, dose reduction (n=20), or cessation of docetaxel (n=12). Sixty deaths were reported among all enrolled patients; however, there was no death events related to THP treatment.

4. Efficacy of subsequent treatment after THP treatment

In our study, 131 patients (57.4%) who received first-line THP had progressive disease and excluding five patients who died or refused further treatment, 126 patients underwent subsequent treatment. Most patients received trastuzumab emtansine (T-DM1) (72.2%, 91/126) while other patients received capecitabine plus lapatinib (17.4%, 22/126) or conventional chemotherapy with anthracycline plus cyclophosphamide (6.3%, 8/126) as second-line treatments (S3 Table). Median OS and PFS of T-DM1 were 30.3 months (95% CI, 25.2 to 35.3) and 9.9 months (95% CI, 7.0 to 12.8), respectively (S4A and S4B Fig.). Among patients who received second-line T-DM1 therapy, 57.4% showed disease progression and underwent several salvage-line chemotherapies. In our study, 15 (11.9%, 15/126) patients who had progressive disease after THP treatment participated in clinical trials. Although there was no significant difference in survivals between the clinical trial group and the conventional chemotherapy group, the survival curve for the group of patients enrolled in clinical trials plateaued over time (S1D Fig.).

Discussion

We analyzed the real-world, single-center data from patients who underwent combination treatment with trastuzumab, pertuzumab, and docetaxel as a first-line chemotherapy for HER2-positive MBC. Survival outcomes in this study were comparable to those of previous ones, including several studies using real-world data [12,15-17]. In addition, in terms of ORR, our results were similar or better than those reported previously along with a higher CR rate of 17.9% [8,15]. Although safety outcomes in our study were consistent with those of previous studies, it should be considered that evaluation of toxicities was quite limited by the retrospective nature of this study. Although the proportion of long-term responders was smaller than that of the CLEOPATRA study

(14.0% vs. 29.6%, respectively) [12], long-term responders in our study showed higher CR rates than non–long-term responders (43.8% vs. 16.3%) (Fig. 2B), which was associated with better survival outcomes (median OS, 80.5 months vs. 49.5 months) (Fig. 2A), consistent with the findings of Wong et al. [18]. Furthermore, approximately 70% of patients who experienced progressive disease after first-line THP received T-DM1 as a second-line treatment. In this patient population, we performed survival analysis as a secondary objective. Survival outcomes including OS and PFS were similar to those of the EMILIA trial, which demonstrated the efficacy of T-DM1 as a secondary treatment (S4A and S4B Fig.) [19,20]. Analysis of adverse events of T-DM1 was beyond the scope of this study. Thus, we demonstrated the efficacy of T-DM1 as a subsequent treatment after THP in a real-world context.

In our study, trastuzumab-exposed patients had poorer survival outcomes with significantly fewer long-term responders than trastuzumab–non-exposed patients (Fig. 2C). In a past study, Uncu et al. [21] demonstrated clinical benefits through continuous HER2 blocking treatment with trastuzumab in patients receiving several anti-HER2 treatments. In addition, in patients exposed to trastuzumab as (neo-)adjuvant treatment, the efficacy of re-treatment with trastuzumab in relapse had been proved [22,23]. In contrast, a study conducted by Rier et al. [24] suggested that palliative anti-HER2 treatment in a group of patients treated with HER2 blockade therapy before and after surgery had reduced efficacy. Although few studies have directly compared the efficacy of first exposure versus re-challenge for trastuzumab, several studies had shown the efficacy of continued blockade of the HER2 pathway through other mechanisms in patients with recurrent or progressive disease treated with trastuzumab as an adjuvant or a palliative treatment [20,25]. Given that the use of anti-HER2 directed therapies for HER2-positive MBC patients is inevitable in subsequent treatment, it is important to distinguish between patients who will benefit from continued use of trastuzumab and those who require different HER2 blockade strategies, and it is important to determine the proper duration of maintenance anti-HER2 directed therapy in future studies.

According to recent studies, the proportion of *de novo* stage IV BC has increased to the extent that it accounts for more than 50% of MBC [18,26]. Although *de novo* stage IV BC accounted for more than 50% of BCs in this study, there were no survival benefits of *de novo* BC compared to recurrent BC in contrast to the previous study [26]. Furthermore, when we performed survival analysis of three patient groups: a *de novo* stage IV group, a trastuzumab–non-exposed rela-psed group, and a trastuzumab-exposed relapsed group, there was no significant difference in OS between the *de novo* stage IV group and the trastuzumab–non-exposed group (80.5 months [95% CI, 40.7 to 12.2] vs. 86.2 months [95% CI, not available (NA) to NA], p=0.360). However, the trastuzumab-exposed group had an OS of 46.6 months (95% CI, 36.8 to 56.0), which was lower than that of the other groups. There were more patients with no evidence of disease or long-term responders in the trastuzumab–non-exposed relapsed patient group than in the trastuzumab-exposed relapsed patient group. Other than that, there were no significant differences in overall response or duration of response to THP or subsequent treatment after THP. Considering the significant difference in OS upon trastuzumab exposure in patients with recurrent MBC (S1E Fig.), anti-HER2 agent use may have decreased efficacy following reuse. Therefore, when considering continuing anti-HER2 directed therapy after disease progression, it is necessary to consider the possibility of decreased efficacy in patients pre-exposed to anti-HER2 directed agents.

Taxanes, including paclitaxel and docetaxel, are commonly used standard therapeutic options for (neo-)adjuvant and palliative treatment of MBC [27,28]. Despite the benefits, limited doses of docetaxel are often given because of dose-dependent persistent peripheral neuropathy [29]. In this study, patients were divided into three groups according to the number of docetaxel doses: less than six, six to nine and more than nine. Most patients (91.2%) received six or more cycles of docetaxel based on the initial CLEOPATRA trial design (median cycles of docetaxel in the THP group and control group were eight in the CLEOPATRA trial) [11]. The leading cause of fewer than six cycles of docetaxel administration was unmanageable adverse events including anaphylaxis or infusion-related syndrome. Administration of docetaxel for more than six cycles had survival benefits in our study, but more than nine cycles of docetaxel treatment was not associated with better survivals (S1B and S1C Fig.), rather concerning severe toxicities including neutropenia ($p < 0.001$) and high-grade peripheral neuropathy (p=0.004). Although the optimal doses and cycles of docetaxel have not been established yet, at least six cycles of docetaxel administration are necessary to improve long-term survival outcomes.

Innovative HER2-targeted therapeutics such as trastuzumab deruxtecan, tucatinib, and margetuximab have recently been demonstrated to be effective against advanced HER2-positive BC [30-32], therefore better clinical outcomes are expected for HER2-positive MBC in the near future. Our single center-based retrospective study demonstrated that THP combination treatment of Korean patients with HER2-positive MBC is an effective first-line palliative chemotherapy with a good safety profile in the real-world context, consistent with the findings of the CLEOPATRA trial.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was approved by the institutional review board of Samsung Medical Center (IRB No. 2021-07-192) and was conducted in accordance with the Declaration of Helsinki. The requirement for informed consent was waived due to the retrospective nature of the study. We used only anonymized information from patients' medical charts. All research was carried out in accordance with relevant guidelines and regulations.

Author Contributions

Conceived and designed the analysis: Lee YP, Park YH.
Collected the data: Lee YP, Lee MS, Kim H, Kim JY, Ahn JS, Im YH, Park YH.
Contributed data or analysis tools: Lee YP, Lee MS, Kim H, Kim JY, Ahn JS, Im YH, Park YH.
Performed the analysis: Lee YP, Lee MS, Kim H, Kim JY, Ahn JS, Im YH, Park YH.
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Conflicts of Interest

Park YH reports grants from AstraZeneca, Pfizer, Eisai, Roche, Daiichi-Sankyo, Eli Lilly, Novartis, Hanmi, Merck and Alteogen. All other authors declare no competing interests.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.842
Cancer Res Treat. 2022;54(4):1138-1147

Original Article

Prevalence of Psychological Symptoms in Patients Undergoing Pancreatoduodenectomy and Results of a Distress Management System: A Clinic-Based Study

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Purpose Patients undergoing pancreatoduodenectomy are a high-risk group that requires psychosocial support. This study retrospectively reviewed the prevalence of psychological symptoms in patients undergoing pancreatoduodenectomy for periampullary neoplasm and the psychosocial referral rate after implementing full screening and triage algorithm for administering a distress management protocol based on the integrated supportive care system established in 2010.

Materials and Methods From September 2010 to December 2018, insomnia, anxiety, and depression were screened on the first day of admission (T1) and on the 10th postoperative day (T2). Patients with clinical levels of distress were referred to a mental health clinic for appropriate aftercare.

Results The adherence rate to routine screening was 82.7% (364/440). Among the 364 patients, the prevalence of insomnia, anxiety, and depression increased from 22.0% (T1) to 32.6% (T2, p=0.001), 29.1% to 33.6% (p=0.256), and 18.4% to 27.6% (p=0.001), respectively. Less than 45% of those with psychological symptoms expressed their needs for psychological supportive care. Among those with psychological symptoms at T2, clinical insomnia, anxiety, and depression were detected via in-depth evaluations among 77.2%, 38.1%, and 82.5% of patients, respectively. Patients who had two or more symptoms at T2 had a longer postoperative hospital stay, as compared to those with one or no symptoms (a median of 20.5 days vs. 18.0 days, p=0.006). Psychiatric consultation rate was 72.8% among patients with clinical psychological symptoms, and 74% of the consulted patients completed psychiatric intervention before discharge.

Conclusion Over one-third of the patients had psychological symptoms before and after pancreatoduodenectomy. Implementing a routine psychological symptoms screening with a systematic psychiatric referral protocol enhanced surgeons' responsiveness to patients' psychological symptoms.

Key words Neoplasms, Oncology, Periampullary cancer, Pancreatoduodenectomy, Psychological symptoms

Introduction

Patients with newly diagnosed/recurred cancer or those receiving active cancer treatment have several physical/psychological symptoms [1-3]. Given the impact of poorly managed distress on quality of life, targeted supportive care should be provided to patients with a higher risk of symptom burden, especially during the first few months of a diagnosis when the odds of moderate-to-severe psychological symptoms are high [4]. However, despite its importance for distress screening and management, patients' distress continues to be under-recognized. Distress screening is more effective when it is linked with mandatory intervention or referral [5].

Periampullary neoplasm is characterized by its grave prognosis and the need for a pancreatoduodenectomy, which has a high morbidity rate; both these aspects result in higher psychological stress among patients compared to other cancers. The prevalence of anxiety or depression is higher for those with pancreatic cancer than other cancer sites [1]; after six months of undergoing a pancreatectomy, 17%-33% and 7%-21% of disease-free survivors had anxiety or depression [6,7]. Additionally, 52% of the patients with periampullary cancer reported that they had an unmet need: psychologi-

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Received July 25, 2021 **Accepted** January 3, 2022
Published Online January 4, 2022

cal supportive care [8]. However, despite the high interest in pancreatoduodenectomy (due to its potential impact on the quality of life), few studies have focused on psychological distress that might occur after the operation [9]. While a recent study from the Ontario Cancer Registry reveals that 51.4% and 39.5% of patients report moderate-to-severe anxiety or depression during the first 12 months after pancreatoduodenectomy [10], the prevalence of psychological distress and need for supportive care that patients with periampullary neoplasm experience immediately after a pancreatoduodenectomy have not yet been sufficiently studied.

In South Korea, only 1.3% of patients had psychiatric consultations in 2006, reflecting the under-recognized burden of psychological distress that cancer patients experience [11]. Thus, distress management guidelines for Korean cancer patients were developed in 2009 [11]. Accordingly, an integrated supportive care system was established in 2010 at the Center for Liver and Pancreatobiliary Cancer, National Cancer Center, Korea, to provide multidisciplinary management for patients undergoing a pancreatoduodenectomy experience. A distress management protocol comprising a routine screening and referral system for psychological distress (e.g., insomnia, anxiety, and depression) was included in the integrated supportive care system. This study retrospectively investigates patients with periampullary neoplasm undergoing pancreatoduodenectomy, including the prevalence of psychological symptoms, related factors, and the responsiveness of surgeons to clinically significant symptoms after implementing the distress management protocol.

Materials and Methods

1. Integrated supportive care for patients undergoing pancreatectomy

As part of our institute's "critical pathways", we organized a multidisciplinary team of experts, including surgeons, dietitians, endocrinologists, anesthesiologists, physiatrists, psychiatrists, and psychologists, all having substantial experience in supportive care of cancer patients. Each specialization provided personalized care e.g., nutritional support, diabetes control, pain management, preoperative and postoperative rehabilitation, and psychiatric intervention to patients before and after pancreatectomy according to its own management protocols (S1 Fig.). Surgeons were responsible for the integrated management of the patients and made referrals to relevant specialists upon problem detection.

For psychological distress management, we developed a distress management protocol for patients undergoing pancreatoduodenectomy based on the Korean distress management guidelines [11]. This protocol included a routine screening and systematic psychiatric referral system for psychological symptoms based on the two-tiered and triage model. The patients' psychological symptoms were categorized into three domains (insomnia, anxiety, and depression), based on commonly experienced psychiatric symptoms in cancer patients [12]. Patients were routinely screened for symptoms at the time of admission (T1) and on the tenth postoperative day (T2). For those who had psychological symptoms at T2, an in-depth evaluation of their symptoms was performed on the same day. The screening and in-depth questionnaire were administered by a registered nurse, who assisted the patients in reading, understanding, and completing the questionnaire. Then, a clinical psychologist interpreted the test score, decided the pathway for care according to the algorithm, and recommended aftercare to the surgeon. The clinical psychologist recommended emotional support and the provision of educational medical information by the primary medical staff to patients with normal to mild distress levels. Referral for psychiatric intervention was provided to patients with mild-to-severe levels of distress. For patients who did not complete the in-depth evaluation, whether to refer to mental health services was decided based on the results of the T2 screening. Surgeons referred those with clinically significant symptoms to psychiatric consultation for receiving relevant management. The psychiatrists and/or psychologists visited the patients for examination and consultation. Patients who were referred to the mental health clinic received pharmacological and/or non-pharmacological intervention, such as psychotherapy and psychoeducation. The median hospital stay of the study population was 25 days (range, 12 to 123 days); hence, all the eligible patients were in admission when T2 screening was performed and the in-depth questionnaire was administered. The surgeons were responsible for supervising the registered nurse and making referrals to the psychiatrists and psychologists. A screening and consultation request system was implemented on the electronic medical records (EMR) system to enable communication among the experts. Screening results at T1 mainly served as the baseline, but occasionally, the patient was referred to a psychiatrist when it was determined that psychiatric treatment was required.

2. Study design

In this retrospective study, de-identified archival data was analyzed, while the serial data of patients who underwent a pancreatoduodenectomy for a periampullary neoplasm (from September 2010 to December 2018) were retrospectively reviewed. Patients whose operations were canceled, or who did not otherwise undergo planned pancreatoduodenectomy, were excluded from analysis. Among the 440 patients admitted for the procedure during the study peri-

od, 364 patients who completed screening of psychological symptoms at T1 and underwent pancreatoduodenectomy were included in the analysis.

3. Measures

Screening was performed at T1 and T2 by measuring psychological symptoms with the Korean version of the self-reported National Cancer Center Psychological Symptom Inventory (NCC-PSI) [12]. The NCC-PSI consists of six items on an eleven-point visual analogue scale that measures the severity and impact of insomnia, anxiety, or depression over the past week. One item enquires about the "need for help from mental health experts" for each symptom [12]. The cut-off score in the NCC-PSI for clinically significant insomnia, anxiety, and depression was five, four, and four points, respectively [12].

In-depth questionnaires were administered to patients who had NCC-PSI scores higher than the cutoff score at T2 to triage patients who needed priority psychiatric intervention. The severity of insomnia was measured using the Korean version of the Insomnia Severity Index (ISI) [13,14]. Each of the seven items was rated on a five-point Likert scale (0, not at all; 4, very severe), and the range of total scores for insomnia was interpreted as not being clinically significant (0-7 points), mild (8-14 points), moderate (15-21 points), or severe (22-28 points) [14]. Meanwhile, the severity of state anxiety was measured using the Korean version of the self-reported State-Trait Anxiety Inventory (STAI-X) [15,16]. Each of the 20 items was rated on a four-point Likert scale (1, almost never; 4, almost always), and the results for the patient's anxious state were classified as insignificant (20-51 points), mild (52-56 points), moderate (57-61 points), or severe (62-80 points) [16]. Finally, the level of depression over the past week was measured using the Korean version of the self-reported Beck Depression Inventory (BDI) [17,18]. Each of the 21 items was rated via four possible responses (0-3), and the severity of depression was categorized as insignificant (0-9 points), mild (10-15 points), moderate (16-23 points), or severe (24-63 points) [18]. By utilizing the cutoff score suggested by the scale, questionnaire results rated as mild-to-severe were considered clinically significant.

4. Demographic and clinical variables

The demographic and clinical data from the EMR were retrospectively collected, including the following: age; sex; the status of marriage, employment, and education; smoking and alcohol intake; past psychiatric history; medication; family history of cancer; insight into one's disease; cancer type; and the European Cooperative Oncology Group (ECOG) performance scale at the time of hospitalization. In addition, the following data were collected: patients' nutritional status (via a patient-generated subjective global assessment [PG-SGA]) at T1, any complications that developed within ten days after the operation, and the highest pain score on

Table 1. Patients' characteristics

Variable	Value (n=364)
Age (yr)	67 (59-72)
Male sex	237 (65.1)
Spouse: yes, currently	285 (81.0)
Employed: yes, currently	168 (46.2)
Education	
Primary or less	106 (29.1)
Secondary	188 (51.6)
University or higher	65 (17.9)
Missing	5 (1.4)
Smoking	
Yes, any point of time	189 (51.9)
Amount (pack-year)	31.0 (20.8-48.0)
Alcohol	
Yes, any point of time	188 (51.6)
Amount (g)	12.3 (4.1-40.3)
Past psychiatric history	
Insomnia	13 (3.6)
Depression	8 (2.2)
Malignancy: yes	342 (94.0)
Primary diseases	
Pancreatic head cancer	151 (41.5)
Distal common bile duct cancer	81 (22.3)
Ampulla of Vater cancer	83 (22.8)
Others	49 (13.5)
Insight of disease	342 (94.0)[a]
European cooperative oncology group performance scale	
0	237 (65.1)
1	101 (27.7)
2, 3, 4	26 (7.1)
Postoperative complication	137/261 (52.5)
Clavien-Dindo classification, grade ≥ III	53/261 (20.3)
Pancreatic fistula, grade ≥ B	32/261 (12.3)
Delayed gastric emptying, grade ≥ B	23/261 (8.8)
Hemorrhage requiring transfusion	7/261 (2.7)
Postoperative in-hospital mortality	1 (0.3)
Postoperative hospital stay (days)	19 (15-27)
Serum hemoglobin at T2 (g/dL)	10.1 (9.1-11.1)
Serum C-reactive protein at T2	6.2 (1.6-12.4)
Numeric rating scale of pain at T2	3 (1-5)

Values are presented as median (interquartile range) or number (%). [a]The other 22 patients had intraductal papillary mucinous neoplasms (n=14), adenomas (n=4), and other benign neoplasms (n=4).

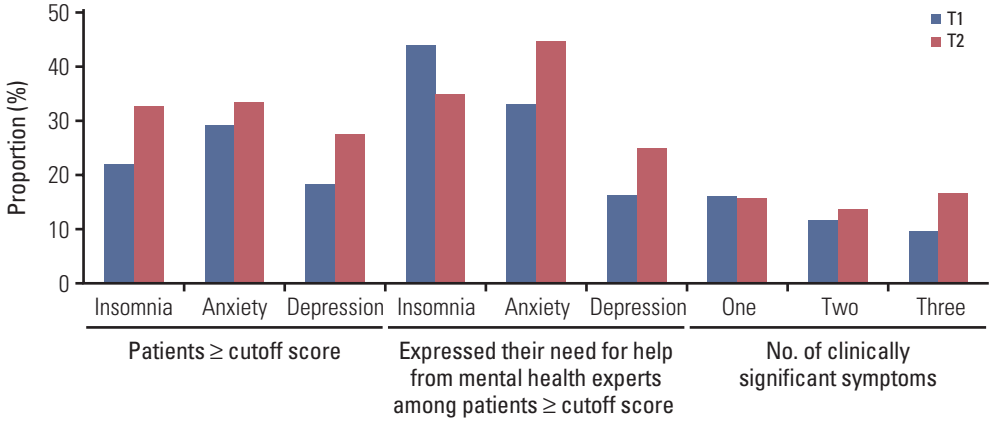

Fig. 1. Screening results on the first day of admission (T1) and the tenth postoperative day (T2) using National Cancer Center Psychological Symptom Inventory.

the 10th postoperative day (T2) as well as the postoperative hospital stay.

5. Statistical analyses

Continuous variables were expressed as median and interquartile range (IQR) and compared using Mann-Whitney U tests. The serial NCC-PSI scores at T1 and T2 were compared using Wilcoxon signed-rank tests. The categorical variables were compared using the chi-square or Fisher exact test, and the correlation was tested via Spearman's correlation test. All the statistical analyses, which were performed using STATA ver. 16.1 (StataCorp LLC, College Station, TX), were two-sided, and the statistical significance was defined as $p < 0.05$.

Results

1. Adherence to the protocol

The adherence rate for routine screening psychological symptoms at the time of admission was 82.7% (364/440) for all the patients who underwent a pancreatoduodenectomy during the study period. Of the 364 patients, 91.8% (n=334) completed their screening at T2. Response rates for the in-depth questionnaires were 80.7% (88/109, ISI), 75.0% (84/112, STAI-X), and 68.5% (63/92, BDI), respectively. Although all the questionnaires were administered one-on-one, patients tended to refuse to answer it due to their poor general condition or to drop out when the questionnaire was lengthy (e.g., STAI-X or BDI). In addition, psychiatric referrals were occasionally omitted because the referral process was not electronically automated and had to be handled by the surgeons (S2 Fig.).

2. Patients' characteristics

The median annual number of patients was 45 (IQR, 37 to 48), and their demographics are presented in Table 1. The patients with distal common bile duct cancer included a higher proportion of men (80.2% [n=65] vs. 63.6% [n=96, pancreatic head], 55.4% [n=46, ampulla of Vater], 61.2% [n=30, other types]; p=0.007), and the proportion of patients with an ECOG score that was higher than or equal to two was higher for those with pancreatic head cancer (11.3% [n=17] vs. 2.5% [n=2, distal common bile duct], 7.2% [n=6, ampulla of Vater], 2.0% [n=1, other types]; p=0.039). Other demographic factors were comparable across the primary sites of disease.

3. Screening results of psychological symptoms preoperation and postoperation

The prevalence of insomnia, anxiety, or depression at T1 was 22.0% (n=80), 29.1% (n=106), and 18.4% (n=67), respectively, and after the operation, these percentages increased by 10.6%, 4.5%, and 9.2% (Fig. 1). At T1 and T2, 37.9% (138/364 [T1]) and 46.4% (155/334 [T2]) of patients had psychological symptoms in at least one of the three domains. However, less than 15% and 20% of all patients at T1 and T2, respectively, expressed their "need for help from mental health experts" for their psychological symptoms through the items on the screening questionnaire (NCC-PSI). Among those with scores higher than the cutoff, less than 45% expressed the need for psychological assistance, and this frequency was even lower in patients with depression, compared to those with insomnia or anxiety. The presence of psychological symptoms at T1 and T2 showed significant correlation (r_s=0.173, p=0.002 [insomnia]; r_s=0.352, $p < 0.001$ [anxiety]; r_s=0.353, $p < 0.001$ [depression]). Each domain illustrated significant cross-correlation (insomnia and anxiety, $p < 0.001$; insomnia and depression, $p < 0.001$; anxi-

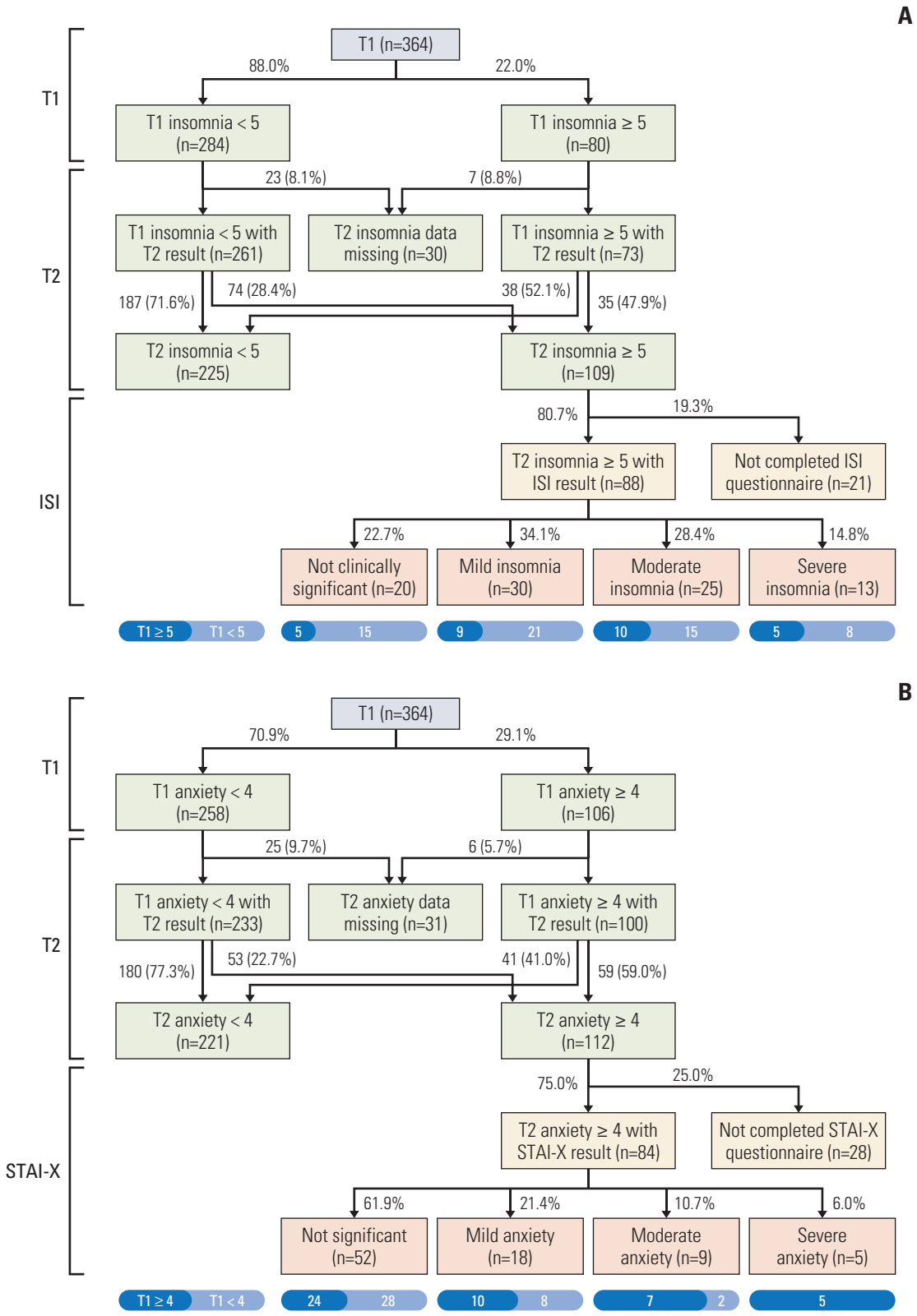

Fig. 2. Serial prevalence and severity of psychological symptoms at T1 and T2, and with in-depth questionnaires for symptomatic patients at T2: insomnia (A), anxiety (B), and depression (C). ISI, Insomnia Severity Index; STAI-X, Korean version of the self-reported State-Trait Anxiety Inventory. *(Continued to the next page)*

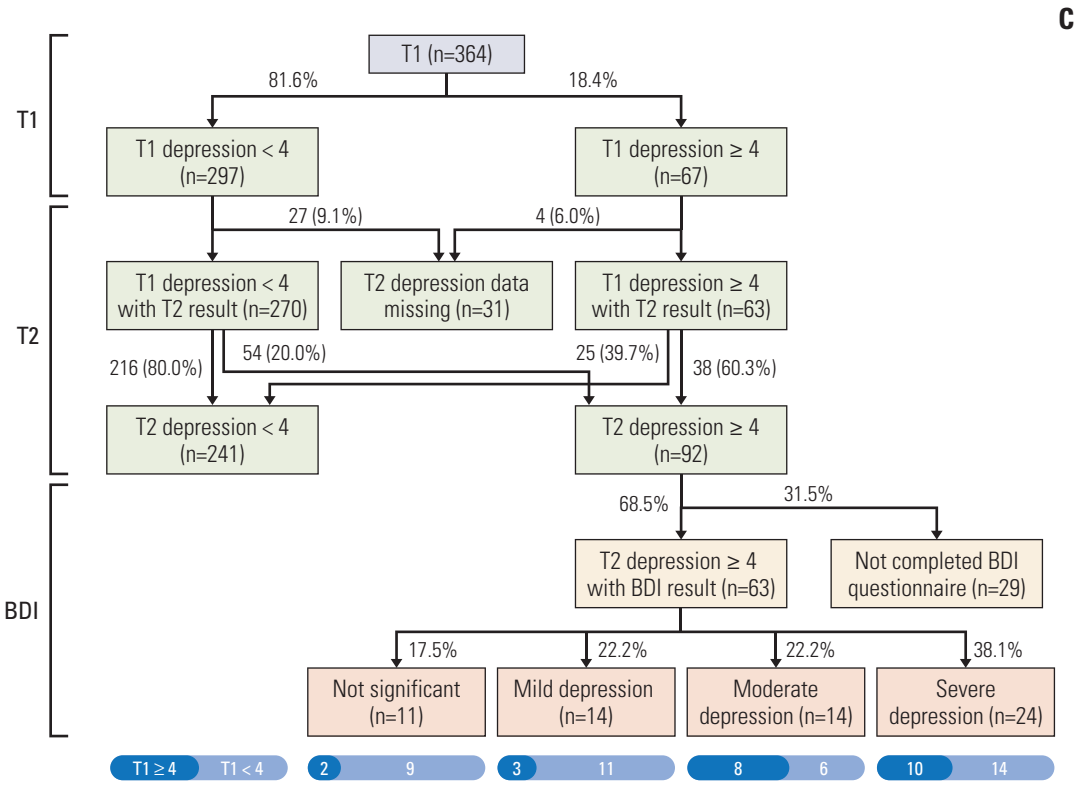

Fig. 2. *(Continued from the previous page)*

ety and depression, $p < 0.001$), especially the patients with depression who had concurrent anxiety, 92.5% (n=62) at T1 and 84.8% (n=78) at T2.

4. Results from in-depth evaluation on T2

For the screening test at T2, the patients who exceeded the cutoff score across the three domains were evaluated in-depth for the relevant domain. The ISI was completed for 80.7% (88/109) of eligible patients, and of these, 77.2% (n=68) had a mild-to-severe level of insomnia. As shown in Fig. 2A, 28.4% of patients who did not have clinically significant insomnia at T1 developed symptoms after the operation. Meanwhile, the STAI-X was completed for 75.0% (84/112) of eligible patients, and of these, 38.1% (n=32) had either a mild-to-severe level of state anxiety. Among the patients with clinically significant anxiety at T1, 59.0% (n=59) remained symptomatic after the operation (Fig. 2B). The proportion of patients who had anxiety at T1 was higher than those who did not (85.7% [n=12] vs. 14.3% [n=2], p=0.011) among 14 patients with moderate-to-severe anxiety. Finally, BDI was completed for 68.5% (63/92) of eligible patients, of whom 82.5% (n=52) had either a mild or higher level of depression. Sixty percent of the patients who experienced clinically significant depressive symptoms at T1 remained symptomatic after the operation (Fig. 2C).

5. Factors related to psychological symptoms

Related to the clinical level of psychological symptoms at T1 and T2, we analyzed pre- and postoperative factors listed in the "Demographic and clinical variables" section. At T1, insomnia was related to having a poor nutritional status, no previous history of alcohol intake, and a past history of depression/insomnia. Additionally, a history of depression was related to having anxiety/depression at T1 (Table 2).

The postoperative hospital stay was significantly longer for patients who had clinically relevant anxiety (median, 21 days [IQR 16-29] vs. 18 days [IQR 14-23]; p=0.001) or depression (median, 21 days [IQR 16-29] vs. 18 days [IQR 15-24]; p=0.007) at T2. Except for elevated serum C-reactive protein level in patients with clinically relevant anxiety (median, 8.5 [IQR 2.9, 13.4] vs. 4.7 [IQR 1.3, 10.6]; p=0.028), postoperative complications [any complications, Clavien-Dindo classification grade ≥ III, postoperative pancreatic fistula grade ≥ B, delayed gastric emptying grade ≥ B, postoperative hemorrhage requiring transfusion, superficial surgical site infection only], serum hemoglobin, serum C-reactive protein, and numeric rating scale of pain did not have statistical significance in relation to clinically relevant insomnia, anxiety or

Table 2. Factors associated with NCC-PSI scores over the cutoff values on baseline assessment (T1)

	No. (n=364)	Insomnia ≥ 5			Anxiety ≥ 4			Depression ≥ 4		
		Yes (n=80)	No (n=284)	p-value	Yes (n=106)	No (n=258)	p-value	Yes (n=67)	No (n=297)	p-value
Nutritional status (PG-SGA)										
Grade A	156	25 (16.0)	131 (84.0)	0.006	46 (29.5)	110 (70.5)	0.979	26 (16.7)	130 (83.3)	0.700
Grade B, C	136	40 (29.4)	96 (70.6)		40 (29.6)	95 (70.4)		25 (18.4)	111 (81.6)	
History of alcohol										
Yes	188	33 (17.6)	155 (82.4)	0.035	52 (27.8)	135 (72.2)	0.547	32 (17.0)	156 (83.0)	0.481
No	176	47 (26.7)	129 (73.3)		54 (30.7)	122 (69.3)		35 (19.9)	141 (80.1)	
History of depression										
Yes	8	5 (62.5)	3 (37.5)	0.015	5 (71.4)	2 (28.6)	0.024	5 (62.5)	3 (37.5)	0.007
No	356	75 (21.1)	281 (78.9)		101 (28.4)	255 (71.6)		62 (17.4)	294 (82.6)	
History of insomnia										
Yes	13	10 (76.9)	3 (23.1)	< 0.001	8 (61.5)	5 (38.5)	0.024	5 (38.5)	8 (61.5)	0.070
No	354	70 (19.9)	281 (80.1)		98 (28.0)	252 (72.0)		62 (17.7)	289 (82.3)	

Values are presented as number (%). Variables with insignificant statistical results were not listed in the table. NCC-PSI, National Cancer Center Psychological Symptom Inventory; PG-SGA, patient-generated subjective global assessment.

depression at T2.

Meanwhile, patients who had two or more symptoms at T2 had a longer postoperative hospital stay, as compared to those with one or no symptoms (a median of 20.5 [IQR, 16.0 to 29.0] vs. 18.0 [IQR, 14.3 to 24.0], p=0.006). Although, according to the number of symptoms, notably, the following factors were not significantly different: age (a median of 67.0 [IQR, 58.0 to 72.0] vs. 67.0 [IQR, 59.0 to 72.0], p=0.911) and complication grade (Clavien-Dindo classification ≥ grade 3, 45.1% [n=23] vs. 54.9% [n=28], p=0.344).

A multivariate analysis of the effect of psychological symptoms and postoperative complications on postoperative hospital stay revealed that clinically relevant anxiety at T2 (odds ratio, 4.930; 95% confidence interval [CI], 1.300 to 8.559; p=0.008), postoperative pancreatic fistula grade ≥ B (odds ratio, 13.115; 95% CI, 9.443 to 16.796; p < 0.001), and delayed gastric emptying grade ≥ B (odds ratio, 12.314; 95% CI, 8.036 to 16.591; p < 0.001) were significant factors related with postoperative hospital stay.

6. Psychological care after psychiatric referral

After the psychological symptoms screening at T2, 31.1% (n=104) of all patients had a psychiatric consultation, while 52.9% (n=82) who had psychological symptoms at T2 were provided this service as well. Overall, Among the 92 patients who had a mild-to-severe level of in-depth questionnaire scores, 72.8% (n=67) underwent psychiatric consultation.

Among the 104 patients who had in-patient referrals, 82.7% (n=86) and 57.7% (n=60) were treated with psychotherapy or medication, respectively. The median number of psychotherapy sessions was one (IQR, 1 to 2), while medication was prescribed for a median of 13 days (IQR, 6 to 29). The top three frequent in-hospital medications prescribed were zolpidem (28 patients; IQR, 3 to 10 days), quetiapine (19 patients; IQR, 12 to 32 days), and lorazepam (18 patients; IQR, 7 to 20 days). Twenty-seven patients required follow-ups at the out-patient clinic due to persistent symptoms; 100% (n=27) and 59.3% (n=16) received psychotherapy and medication, respectively. After discharge, the median number of psychotherapy sessions that were performed at the out-patient clinic was one (IQR, 1 to 3), while medication was prescribed for a median of 28 days (IQR, 22 to 43).

Discussion

The study results showed that about one-third of the respondents experienced insomnia, anxiety, or depression after pancreatectomy, which was comparable to the out-patient clinical data that was collected one month after the pancreatoduodenectomy [10]. However, the prevalence was higher than the prevalence experienced by patients 6 months after the operation [6], which is concordant with the decreasing level of psychological symptoms after surgery [10]. The prevalence of psychological symptoms observed in this study increased from 4.5% to 10.6% across the three symptoms after the pancreatoduodenectomy, which is unlike breast cancer patients, whose anxiety significantly decreased after their operation [19]. About 45% of the patients had psychological symptom comorbidly at T2 and those who had two

or more symptoms at T2 had a longer postoperative hospital stay, as compared to those with one or no symptoms. These findings imply that psychological supportive care should be provided at the onset of surgical treatment when the burden of psychological symptoms is the highest.

The risk factors of being younger or female has been reported as being associated with cancer patients' symptom burden [4], but it was not found to be significant in this study. Although psychological symptoms before surgery did not reveal direct correlations with postoperative outcomes, a considerable proportion of patients who had anxiety (59.0%) or depression (60.3%) at T1 remained symptomatic until T2, especially those with anxiety at T1, as they were more likely to develop moderate-to-severe anxiety by T2. Meanwhile, 82.5% of patients with depression at T2 had a mild-to-severe level of symptoms. Moreover, patients with anxiety or depression at T2 experienced a longer postoperative hospital stay. Therefore, patients screened as having either anxiety or depression perioperatively should be the main target of receiving improved psychological supportive care. Psychological symptom screening before surgery would help identify those who are at higher risk of developing symptoms after surgery.

This study revealed that only 44.6% and 25.0% of the patients who experienced clinical levels of anxiety or depression after operation expressed their need for help from mental health experts. This could be because of the patient's lack of awareness of their own symptoms or misunderstanding or prejudices about mental health services. Therefore, it is important for the clinician to recognize the patient's need for help, but above all, it is important to objectively evaluate psychological distress. The patient's needs can also be considered along with the scale scores for determining psychiatric referrals. Given the absence of routine screening in the majority of surgical wards, it is important to improve surgeons' understanding about the prevalence of patients' psychological symptoms before and after a pancreatoduodenectomy to address the latter's psychological needs. This is also supported by other research; in Australia, for example, only 15% of patients with periampullary cancer voluntarily sought psychological supportive care [8]. Similarly, in Korea, psychological distress, or psychiatric disorders, in cancer patients remain an under-recognized phenomenon [20]. Moreover, psychiatric referrals are still the only way for hospitalized patients to receive psychological interventions as part of their cancer treatment [11].

Importantly, the rate of psychiatric consultations was less than 2% in the authors' institution before the introduction of a routine screening for psychological symptoms with a systematic psychiatric consultation protocol [11]. After the protocol was implemented, the adherence rate for initial psychological symptom screening was 82.7%, and 52.9% of patients with psychological symptoms and 72.8% of patients with a mild-to-severe level psychological symptoms, specifically, were referred to psychiatrists for consultations. This is as high as in the United States, where since 2015, screening for psychosocial distress and relevant referrals have been made mandatory for cancer center accreditation by the American College of Surgeons Commission on Cancer [21].

Meanwhile, patient-reported outcomes (PROs) are one of the important outcome measures in terms of health care's quality, efficiency, and safety [22]. Recently suggested sets of PROs [23] or value-based, patient-centered outcomes [24] for pancreatic cancer have included a list of psychological/emotional symptoms, which should be validated and incorporated in future outcome-based research. Although it is difficult to prove a direct correlation between PROs and quantitative treatment responses, adopting PROs in clinical research may foster the provision of patient-centered health care by considering the patients' perspectives in both clinical decision-making as well as health policy formulation.

This study has several limitations. First, the study was a retrospective analysis. Therefore, reasons for not adhering to the newly implemented in-patient protocol including psychological screening and psychiatric consultation were not identified from both the patients' and surgeons' perspective. A possible underestimation of the rate of psychological symptoms may have occurred, as there were cases of patients not responding at all or refusing to participate in in-depth evaluation due to their poor condition. Psychiatric consultation rates might have been influenced by the surgeons' preferences given the long timeframe included in analysis. Additionally, since we carried out the research at a single site, its results are limited in generalizability. Although this study offers limited evidence to support the feasibility of a multicenter study, the observed adherence rate to the newly implemented in-patient protocol was 82.7%. This rate might serve as a baseline when planning future, larger scale studies. Third, having no control group, it was difficult to evaluate whether implementing a routine psychological screening using a systematic psychiatric consultation protocol improved the treatment outcomes for patients. Although more patients received psychiatric consultations and treatments than was the case in the past, it is difficult to conclude that these interventions enhanced early recovery postoperation. Fourth, the psychological symptoms were only evaluated for in-patients; therefore, long-term psychological outcomes could not be identified. Moreover, the improvement of psychological symptoms after psychological intervention could not be assessed quantitatively because of minimized out-patient visits due to the social taboo of visiting psychiatric clinics and human resource

limitations in administering questionnaires at surgical out-patient clinics. However, 74.0% of the patients who had an in-hospital referral did not require out-patient clinical follow-up after being discharged, suggesting that their symptoms had been relieved before discharge. Finally, this study did not evaluate the protocol's cost-effectiveness. Therefore, there is limited evidence to suggest that the protocol implemented in this study should be adopted in other hospitals or institutions.

In summary, more or less than 40% of the patients who had periampullary neoplasm experienced psychological symptoms before and after their pancreatoduodenectomy, respectively. After implementing a routine screening of psychological symptoms with a systematic psychiatric consultation protocol, surgeons' responsiveness to patients' psychological symptoms increased compared with the rate gleaned from available historical data. Seventy-three percent of the patients who experienced a mild-to-severe level of psychological symptoms received psychiatric consultations after the routine screening of psychological symptoms as part of the integrated supportive care protocol, while psychological management was completed during the admission of 74% of the patients. Future studies should evaluate both short-term and long-term trends associated with the epidemiology of psychological symptoms after pancreatoduodenectomy. Additionally, the feasibility and cost-effectiveness of routine psychological supportive care must be assessed on a larger scale using a control group to direct the future reform of daily surgical practice.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the human research committee of National Cancer Center, with exemption of written informed consent because of the retrospective study design (IRB No. NCC2019-0223).

Author Contributions

Conceived and designed the analysis: Kang MJ, Yu ES, Han SS, Kim JH.
Collected the data: Kang YH.
Performed the analysis: Kang MJ, Yu ES.
Drafting the article, critical revision, and final approval: Kang MJ, Yu ES, Kang YH, Park HM, Park SJ, Kim SW, Kim JH, Han SS.

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

We would like to thank Editage (www.editage.co.kr) for English language editing.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.885
Cancer Res Treat. 2022;54(4):1148-1156

Original Article

Role of Esophagectomy after Chemoradiation Therapy in Patients with Locally Advanced Squamous Cell Carcinoma: A Comparative Analysis Stratified by Clinical Response to Chemoradiation Therapy

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Purpose This study aimed to evaluate the long-term effect of esophagectomy in patients with esophageal squamous cell carcinoma (ESCC) by comparing the chemoradiotherapy (CRT)-only group and the trimodality treatment (TMT) group who received concurrent CRT followed by surgery.

Materials and Methods We included 412 operable ESCC patients treated with TMT or CRT between January 2005 and December 2015. The oncological outcomes of the two groups were compared using a weighted Cox proportional-hazards model with inverse probability of treatment weighting (IPTW).

Results The median survival time was 64 and 32 months in the TMT (n=270) and CRT (n=142) groups, respectively ($p < 0.001$). After IPTW, the median overall survival (OS) remained significantly higher in the TMT group than in the CRT group (61 months vs. 32 months, p=0.016). Moreover, the TMT group showed a better local recurrence–free rate (LRFR; $p < 0.001$) and distant metastasis–free rate (p=0.007). In the subgroup of patients with clinical complete response (cCR), the OS was not significantly different between the two groups, both before and after IPTW adjustment (p=0.35 and p=0.93). However, among non-cCR patients, the OS was significantly higher in the TMT group (64% vs. 45%, $p < 0.001$).

Conclusion In patients with locally advanced ESCC, TMT was superior to CRT in terms of OS and LRFR. Such difference was more prominent in the non-cCR subgroup. In patients who achieved cCR, esophagectomy was effective in improving LRFR but not OS, suggesting that esophagectomy may be omitted in complete responders.

Key words Esophageal neoplasms, Squamous cell carcinoma, Chemoradiotherapy, Trimodality treatment, Clinical complete response

Introduction

Esophageal cancer is the seventh most common malignancy and the sixth most common cause of cancer-related mortality globally [1]. Trimodality treatment (TMT) of chemoradiotherapy (CRT) followed by surgery is currently accepted as the standard treatment in locally advanced esophageal cancer [2-4]; however, some studies reported that surgical resection may be omitted without serious impact on survival in cases showing response to CRT [5,6]. This may be considered a reasonable approach considering the high rates of mortality and morbidity associated with surgical resection [2,7,8]. In addition, patients are often reluctant to undergo surgery after the completion of neoadjuvant CRT, especially when they show good clinical response to CRT, experience the disappearance of the main symptoms, or show incomplete recovery of performance after chemoradiation.

However, the term "response" includes a wide range of disease status in the real-world setting, from partial responses of a slight decrease in the disease status to complete disappearance of the tumor. Moreover, it may be difficult to evaluate the clinical response due to treatment-induced edema and esophagitis, which do not subside at the time of post-treatment reevaluation. Therefore, it is necessary to accurately define the criteria for "response", which is complicated due to the individual variability among clinicians in interpreting treatment responses. To minimize these variations and possible confusion, the use of the criteria of "complete response" may be more reasonable and practical despite the controversy on the evaluation methods.

We assume that surgical resection may be omitted only in responders to CRT, because patients may miss the optimal

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Received August 5, 2021 **Accepted** December 16, 2021 **Published Online** December 20, 2021

timing for surgical resection and cure. However, there is a limited amount of available data on the survival benefit of esophagectomy in patients who show a clinically good response after CRT for locally advanced esophageal squamous cell carcinoma (ESCC), and the available results are not consistent [9-12]. Therefore, in this study, we evaluated the long-term effect of esophagectomy in patients with ESCC who received concurrent CRT followed by surgery (TMT) in terms of their clinical response to CRT.

Materials and Methods

1. Study population

We identified 730 patients who were treated with TMT or CRT at our center between January 2005 and December 2015 for locally advanced esophageal cancer with squamous cell carcinoma. The inclusion criteria were as follows: (1) histologically confirmed resectable but advanced ESCC (cT2-4/anyN/M0 or anyT/N+/M0 stage), (2) medically operable status, (3) Eastern Cooperative Oncology Group (ECOG) performance status of ≤ 2, and (4) no history of thoracic surgery. The exclusion criteria were as follows: (1) double primary cancer, (2) suboptimal radiotherapy (RT) dose (< 38 Gy), and (3) insufficient follow-up duration (< 3 months) without oncologic event. Accordingly, 412 patients were included in this analysis (Fig. 1).

2. Evaluation

The initial diagnostic evaluation included detailed medical history, physical examination, laboratory blood analysis, esophagogastroduodenoscopy (EGD), endoscopic ultrasound, computed tomography (CT) scans of the chest and abdomen, esophagography, and positron emission tomography–CT (PET-CT) scan. Four weeks after the completion of CRT, treatment response was evaluated with EGD and biopsy, chest CT scan, and PET-CT.

Clinical complete response (cCR) after CRT was defined as the absence of residual tumor on endoscopy with biopsy and metabolic complete remission (CR) on PET scan. Metabolic CR was defined as the complete resolution of fluorodeoxyglucose uptake in the primary tumor and metastatic lymph nodes or indistinguishable initial tumor site from the surrounding tissue in cases of diffuse esophagitis with increased uptake within a radiation field.

After treatment, regular follow-up examinations were performed every 3 months during the first 2 years, and every 6 months thereafter until 5 years. Toxicities during and after treatment were evaluated using the Common Terminology Criteria for Adverse Events (ver. 4.03). Surgical complications were assessed by the Clavien-Dindo classification.

3. Treatment

Treatment strategies for locally advanced esophageal cancer were primarily determined by a multidisciplinary team. Several patients who could not be assessed by the multidisciplinary team were assessed by individual members of the

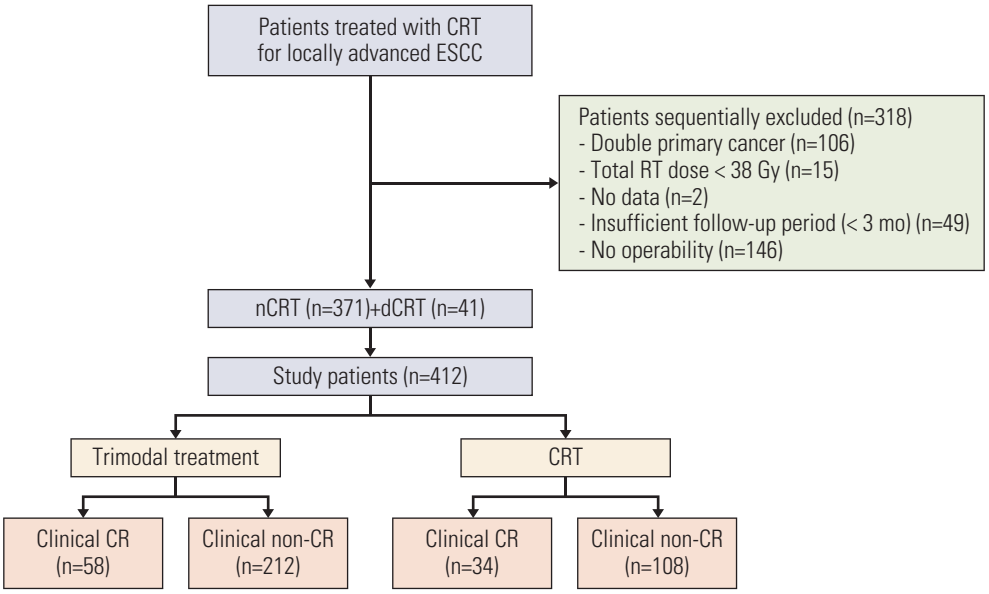

Fig. 1. Flow diagram of patient selection. CR, complete response; CRT, chemoradiotherapy; dCRT, definitive chemoradiotherapy; ESCC, esophageal squamous cell carcinoma; nCRT, neoadjuvant chemoradiotherapy; RT, radiotherapy.

Table 1. Patient characteristics

	Trimodality (n=270)	CRT (n=142)	p-value
Sex			
Male	256 (94.8)	135 (95.1)	0.91
Female	14 (5.2)	7 (4.9)	
Age (yr)			
≤ 60	127 (47.0)	39 (27.5)	< 0.001
> 60	143 (53.0)	103 (72.5)	
Mean±SD	61.36±7.09	66.14±8.8	< 0.001
ECOG score			
0-1	267 (98.9)	140 (98.6)	> 0.99
2	3 (1.1)	2 (1.4)	
Charlson-Deyo score			
0	199 (73.7)	88 (62.0)	0.029
1	56 (20.8)	42 (29.6)	
2	12 (4.4)	6 (4.2)	
3	3 (1.1)	6 (4.2)	
Alcohol			
No	33 (12.2)	18 (12.7)	0.89
Yes	237 (87.8)	124 (87.3)	
Smoking			
No	53 (19.6)	32 (22.5)	0.49
Yes	217 (80.4)	110 (77.5)	
T category			
≤ 2	112 (41.5)	63 (44.7)	0.53
> 2	158 (58.5)	78 (55.3)	
N category			
Negative	74 (27.4)	33 (23.2)	0.36
Positive	196 (72.6)	109 (76.8)	
Stage			
≤ II	100 (37.0)	56 (39.7)	0.60
> II	170 (63.0)	85 (60.3)	
Differentiation			
Well	33 (12.2)	15 (10.6)	0.021
Moderate	205 (75.9)	94 (66.2)	
Poor	24 (8.9)	22 (15.5)	
Tumor location			
Upper	35 (13.0)	25 (17.6)	0.21
Mid	127 (47.0)	55 (38.7)	
Lower	108 (40.0)	62 (43.7)	
Clinical response			
CR	58 (21.5)	34 (23.9)	0.57
Non-CR	212 (78.5)	108 (76.1)	

Continuous variables were compared using the t test, and categorical variables were compared using the Fisher's exact test or chi-squared test. CR, complete response; CRT, chemoradiotherapy; ECOG, Eastern Cooperative Oncology Group; SD, standard deviation.

multidisciplinary team, including thoracic surgeons, medical oncologists, gastroenterologists, and radiation oncologists.

The median prescribed radiation dose was 46 Gy for neo-adjuvant treatment and 54 Gy for definitive CRT. LightSpeed RT (GE Medical Systems, Palo Alto, CA) was used for CT simulation with intravenous contrast enhancement. The gross tumor volume (GTV) was delineated on each slice of the acquired CT images and was assisted by the information from PET-CT, chest CT, and EGD. During the period of 3D treatment, primary tumor and mediastinal lymph node were

treated with margins of 5 cm in the cranio-caudal direction and 2 cm in the lateral direction. The supraclavicular lymph nodes were included when the GTV existed in the upper thoracic esophagus, and the celiac trunk was included when the GTV was in the mid or distal thoracic esophagus. After the introduction of intensity-modulated radiotherapy (IMRT), the clinical target volume (CTV) margin was reduced to 3 cm in the S-I direction and 1 cm in the radial direction. The planning target volume margin was 7 mm in the radial direction and 10 mm in the cranio-caudal expansion of the CTV.

All patients were treated with capecitabine-cisplatin (XP) or 5-fluorouracil–cisplatin (FP) chemotherapy for concurrent CRT. For XP chemotherapy, patients received capecitabine 1,600 mg/m^2/day for 5 days plus cisplatin 30 mg/m^2/day on the first day, weekly. For FP chemotherapy, patients received cisplatin 60 mg/m^2/day on the first day plus 5-fluorouracil 1,000 mg/m^2/day on the second day for 4 days, every 3 weeks.

All patients were routinely evaluated for operability by an experienced thoracic surgeon. Surgery was performed 6 to 8 weeks after the completion of CRT using either the Ivor-Lewis or McKeown approach.

4. Statistical analysis

Continuous variables were compared using the t test, and categorical variables were compared using the Fisher exact test or chi-squared test. The rates of overall survival (OS), local recurrence–free rate (LRFR), and distant metastasis–free rate (DMFR) were estimated using the Kaplan-Meier method and compared using the log-rank test. Inverse probability treatment weighting (IPTW) analysis based on the propensity score was used to reduce the impact of selection bias and potential confounding factors. Propensity scores were calculated using a logistic regression model using the following variables: sex, age, Charlson-Deyo score, alcohol, smoking, ECOG performance status, tumor location, and tumor stage. The absolute standardized differences (STDs) were used to check the balance after IPTW, and weighted Cox regression models with robust standard errors were used for the comparison of survival after IPTW adjustment. Also, tests for interaction were performed to assess the heterogeneity of treatment effect among the cCR subgroups. p-values less than 0.05 were considered statistically significant. All statistical analyses were performed with SAS ver. 9.4 (SAS Institute Inc., Cary, NC) and R ver. 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

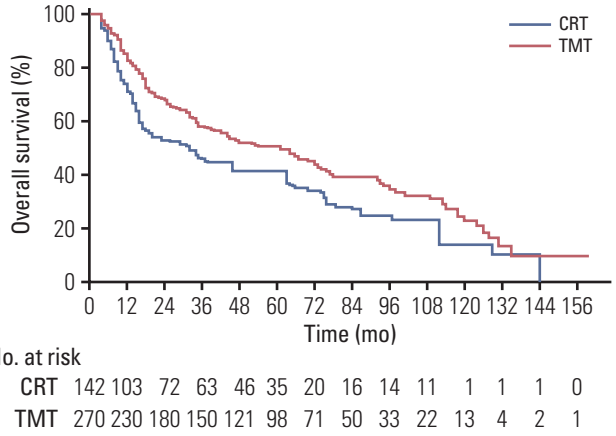

Fig. 2. Kaplan-Meier survival analysis for overall survival (OS) after inverse probability of treatment weighting adjustment. The OS of the trimodality treatment (TMT) group was significantly better than that of the chemoradiotherapy (CRT) group (p=0.016).

Table 2. Patterns of failure

	TMT	CRT	Total
cCR	58	34	92
Local recurrence	2 (3.4)	10 (29.4)	12 (13.0)
Distant metastasis	5 (8.6)	3 (8.8)	8 (8.7)
Both	4 (6.9)	5 (14.7)	9 (9.8)
Non-cCR	212	108	320
Local recurrence	24 (11.3)	30 (27.8)	54 (16.9)
Distant metastasis	18 (8.5)	14 (13.0)	32 (10.0)
Both	26 (12.3)	18 (16.7)	44 (13.8)

Values are presented as number (%). cCR, clinical complete response; CRT, chemoradiotherapy; TMT, trimodality treatment.

Results

1. Patient characteristics

Among the 412 study patients, 270 patients (65.5%) received TMT and 142 (34.5%) received CRT. The baseline characteristics of the patients are summarized in Table 1. The CRT group were older, had a significantly higher Charlson-Deyo score, and had a higher proportion of patients with poor differentiation. The balance of the variables was markedly improved after IPTW adjustment (S1 Table), with all absolute STDs after weighting being less than 0.1 except for the Charlson-Deyo score (STD=0.106).

2. Post-CRT response

After the completion of CRT, all patients were evaluated by EGD with/without biopsy, CT scan, and PET-CT. One

Table 3. Hazard ratios for oncological outcomes in the entire cohort

Oncologic outcomes	Method	HR[a]	95% CI	p-value
Overall survival	Univariate	0.627	0.488-0.805	< 0.001
	Multivariable-adjusted[b]	0.651	0.487-0.870	0.004
	IPTW-adjusted	0.693	0.514-0.933	0.016
Local recurrence–free rate	Univariate	0.362	0.252-0.519	< 0.001
	Multivariable-adjusted[b]	0.310	0.209-0.460	< 0.001
	IPTW-adjusted	0.352	0.235-0.528	< 0.001
Distant metastasis–free rate	Univariate	0.585	0.388-0.883	0.011
	Multivariable-adjusted[b]	0.474	0.303-0.740	0.001
	IPTW-adjusted	0.529	0.332-0.843	0.007

CI, confidence interval; HR, hazard ratio; IPTW, inverse probability of treatment weighting. [a]Chemoradiotherapy compared with trimodality treatment, [b]A multivariate analysis was performed using the variables used to calculate the propensity score.

Table 4. IPTW-adjusted hazard ratios for oncological outcomes in the subgroups stratified by clinical complete response

Clinical response	Oncologic outcomes	HR[a]	95% CI	p-value	p-value for interaction
cCR	Overall survival	1.027	0.561-1.880	0.93	0.13
	Local recurrence–free rate	0.247	0.097-0.624	0.003	0.44
	Distant metastasis–free rate	0.905	0.324-2.524	0.85	0.24
Non-cCR	Overall survival	0.610	0.463-0.805	< 0.001	
	Local recurrence–free rate	0.367	0.250-0.539	< 0.001	
	Distant metastasis–free rate	0.465	0.299-0.722	0.001	

cCR, clinical complete response; CI, confidence interval; HR, hazard ratio; IPTW, inverse probability of treatment weighting. [a]Adjusted hazard ratio, chemoradiotherapy compared with trimodality treatment.

patient did not undergo a PET-CT scan but was confirmed with a residual tumor on EGD. The median time from the last day of CRT to PET-CT was 0.9 months (range, 0.3 to 2.8). Metabolic CR was achieved in 241 patients (58.6%). Of the 412 patients, 119 (28.8%) showed a complete response on EGD. Among the 353 patients (85.7%) who underwent endoscopic biopsy, 324 (91.8%) had negative biopsy results.

A total of 92 (22.3%) patients showed cCR. In the TMT group, 119 (44.1%) patients had a pathologic complete remission (pCR), which was more common in the cCR group than in the non-cCR group (36/58 [62.1%] vs. 83/212 [39.2%], p=0.003).

3. Oncologic outcome

At the time of analysis (May 2020), 150 patients were alive (TMT, 109 [40.3%]; CRT, 41 [28.9%]). The median follow-up duration was 39.2 months (range, 5.3 to 164.0) in the entire cohort and 67.5 months (range, 13.6 to 164.0) in the living patients. The median OS duration was 64 months (95% confidence interval [CI], 44 to 74) and 32 months (95% CI, 18 to 38) for the TMT group and the CRT group, respectively (p < 0.001) (S2 Fig.). After IPTW adjustment, the TMT group still had a superior OS compared with the CRT group (p=0.016). The 2- and 5-year OS rates were 67% and 50% in the TMT group, and 52% and 41% in the CRT group, respectively (Fig. 2).

The patterns of failure are summarized in Table 2. Treatment failures were observed in 29 of the 92 cCR patients (31.5%) at the time of analysis. The sites of the first failure in the TMT group were distant metastasis in five (8.6%), local recurrence in two (3.4%), and both distant metastasis and local recurrence in four patients (6.9%).

In the CRT group, the major patterns of failure were local recurrence in 10 (29.4%), distant metastasis in three (8.8%), and both local recurrence and distant metastasis in five patients (14.7%). The TMT group had a significantly better LRFR and DMFR than did the CRT group (hazard ratio [HR], 0.362; 95% CI, 0.252 to 0.519; p < 0.001 and HR, 0.585; 95% CI, 0.388 to 0.883; p=0.011, respectively). These results were also observed in multivariate and IPTW-adjusted analyses (Table 3). The cumulative incidences of local recurrence and distant metastasis are shown in S3 Fig. The 2- and 5-year progression-free survival rates were 67% and 49% in the TMT group and 52% and 34% in the CRT group, respectively (p=0.003)

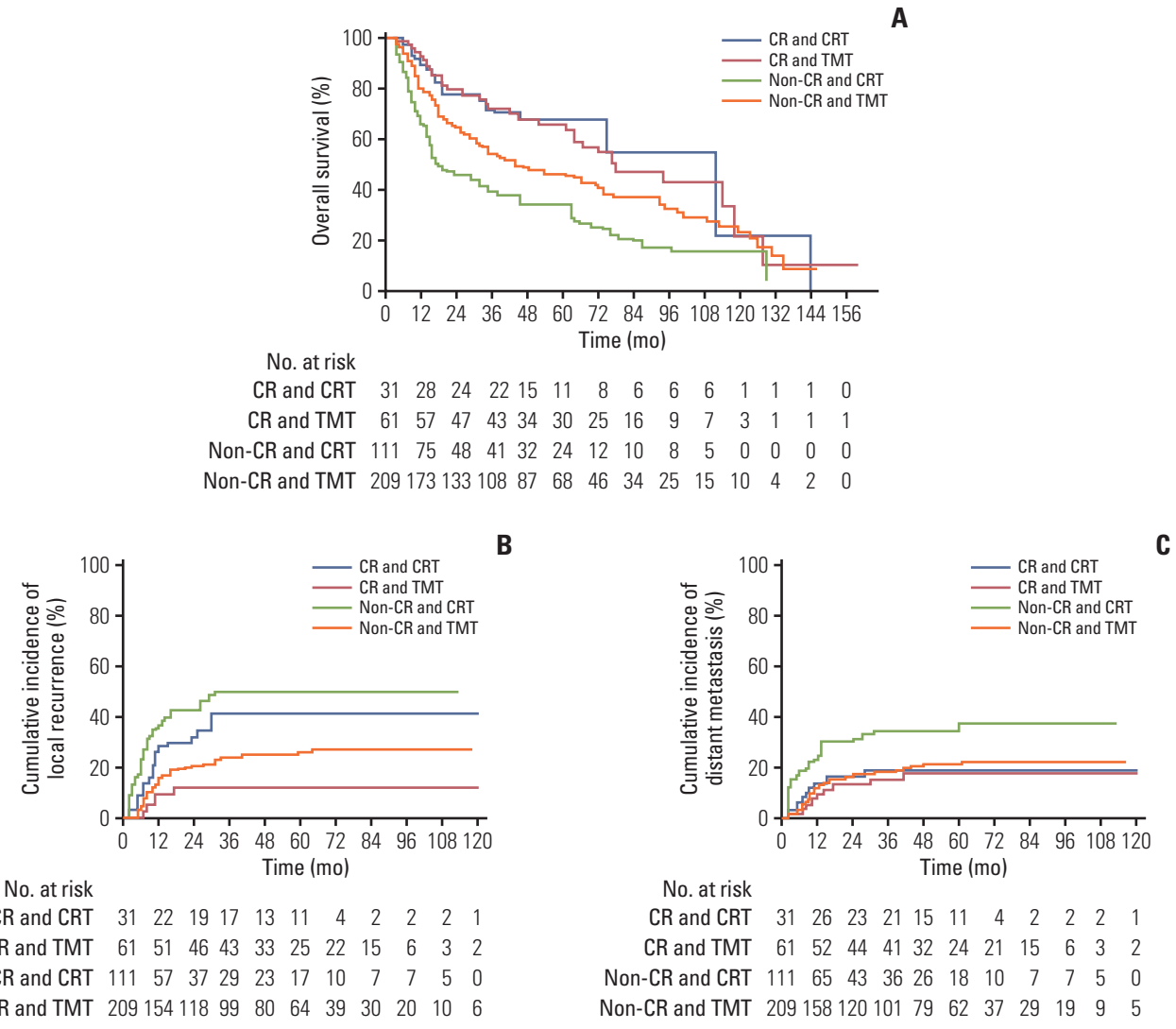

Fig. 3. Kaplan-Meier survival analysis for overall survival (A), local recurrence (B), and distant metastasis (C) after inverse probability of treatment weighting adjustment stratified by clinical complete response (cCR) and treatment. (A) In the cCR group, the overall survival of the trimodality treatment (TMT) group was comparable to that of the chemoradiotherapy (CRT) group (p=0.93); in the non-cCR group, the overall survival of the TMT group was significantly better than that of the CRT group (p < 0.001). (B) Local recurrence–free rate of the TMT group was significantly higher than that of the CRT group in both the cCR group (p=0.003) and the non-cCR group (p < 0.001).

(S4 Fig.). Of the 63 patients in the CRT group who developed local recurrence, 10 (15.9%) had salvage esophagectomy and the median time to salvage surgery was 6.7 months (range, 3.4 to 3.1 months). Of the 10 patients with salvage esophagectomy, eight (80%) achieved R0 resection and the median survival time after salvage surgery was 9.4 months (range, 3.7 to 31.4 months). Further details of salvage treatment are presented in S5 Table.

4. Subgroup analysis after IPTW

In the cCR group, OS and DMFR were not significantly different between the TMT group and the CRT group (HR, 1.027; 95% CI, 0.561 to 1.880; p=0.93 and HR, 0.905; 95% CI, 0.324 to 2.524; p=0.85, respectively) (Table 4). The 2- and 5-year OS rates were 79% and 65% in the TMT group and 77% and 67% in the CRT group, respectively (Fig. 3A). The 2- and 5-year distant metastasis rates were 13% and 18% for the TMT group and 16% and 19% for the CRT group, respectively. However, the LRFR was higher in the TMT group compared with the CRT group (p=0.003). The 1-, 3-, and 5-year local recurrence rates were 9%, 12%, and 12% in the TMT group and 28%, 41%, and 41% in the CRT group, respectively. There

Table 5. Treatment-related acute toxicity (grade ≥ 3)

	Grade	TMT group (n=270)	No.
From CRT	3	Dysphagia	2
		Odynophagia	2
		Hemorrhage	1
From surgery	3	Vocal cord palsy	19
		Pneumonia	9
		Infection	8
		Anastomosis site leakage	7
		Chylothorax	2
		Fistula	1
		Diaphragmatic hernia	1
		Cardiac toxicity	1
	4	Pneumonia	9
		Chylothorax	3
		Anastomosis site leakage	3
		Cardiac toxicity	2
		Infection	1

One case of grade 3 dysphagia was detected in the CRT group.
CRT, chemoradiotherapy; TMT, trimodality treatment.

was no significant interaction effect between TMT and CRT in terms of OS, LRFR, and DMFR (Table 4).

In the non-cCR subgroup, the TMT group showed significantly superior rates of OS, DMFR, and LRFR compared with the CRT group ($p < 0.001$, $p=0.001$, and $p < 0.001$, respectively) (Table 4). The 2- and 5-year OS rates were 64% and 46% in the TMT group and 45% and 33% in the CRT group, respectively (Fig. 3A). The 1-, 3-, and 5-year local recurrence rates according to the Kaplan-Meier curve were 15%, 23%, and 26% for the TMT group and 37%, 50%, and 50% for the CRT group, respectively (Fig. 3B). The 2- and 5-year distant metastasis rates were 17% and 21% for the TMT group and 32% and 36% for the CRT group, respectively (Fig. 3C).

5. Toxicity

Acute complications after TMT and CRT are summarized in Table 5. CRT did not result in any grade 4 toxicities; in contrast, surgery resulted in more severe complications including 18 cases of grade 4 toxicities. The 90-day postoperative mortality rate was 4.4% (12/270).

Discussion

Currently, TMT is regarded as the standard treatment for locally advanced esophageal cancer, with its role having been established by the results of randomized controlled trials (RCTs) on adenocarcinoma [2,4] and squamous cell carcinoma (SCC) [13]. However, considering the high rate of pathologic CR in patients with SCC (~50%), it may be possible to omit esophagectomy in good responders if they could be identified prior to surgical resection. In the real-world setting, the role of esophagectomy in good responders after CRT is yet to be firmly established. Many RCTs that compared between esophagectomy and no surgery reported that esophagectomy did enhance the survival rate in no responders but not in good responders [5,6]. Yet, those RCTs included partial responders and viable tumor cells might have remained in the esophagus and regional lymph nodes.

In order to focus on testing the possibility of safely omitting esophagectomy, remnant tumors in the mediastinum should be considered for performing a successful study. Accordingly, the study by Piessen et al. [9] was meaningful as it only enrolled cCR cases and reported the benefit of esophagectomy in terms of survival (58.9% vs. 33.4%, $p=0.001$); however, their study had limited generalizability because it excluded patients with short follow-up durations of 3 years or less, and included both adenocarcinoma and squamous cell carcinoma, which have different chemoradiosensitivity [14].

Other investigators also tried to identify the benefit of esophagectomy in cCR patients with SCC, but none has demonstrated significant advantages in terms of survival. Castoro et al. [10] investigated 77 SCC patients who achieved cCR after neoadjuvant CRT, and did not observe a significant difference in 5-year survival (50% vs. 57%, $p=0.99$). Chao et al. [11] also analyzed 150 SCC patients who underwent endoscopic CR after CRT and reported no significant survival benefit with esophagectomy. It should be noted that PET scan was not fully available during the study periods of the studies by Castoro et al. [10] and Chao et al. [11], and that the response evaluation was primarily dependent on endoscopy findings. Therefore, it is possible that their study patients might not have achieved cCR according to current standards that include metabolic response on PET scan, and might have had residual tumors in the extraluminal area such as the regional lymph nodes.

We have published a similar study with more advanced evaluation methods such as PET scan, and reported that while esophagectomy resulted in significantly improved disease-free survival, it did not result in a significant improvement in OS [12]. We reasoned that such statistically null findings in OS likely stemmed from the small number of patients, and therefore expected to find a positive result in this updated study with a larger cohort size. As a result, we found that esophagectomy was associated with a significantly better OS as well as LRFR and DMFR in the entire cohort. However, we could not find a significant beneficial effect of surgical resection in the subgroup analysis on patients with cCR, which

indicates that the improvement in LRFR was not directly translated to an improvement in OS. We could only reaffirm that esophagectomy was beneficial for patients without cCR.

Although the exact reason why we could not observe a significant difference in OS in the cCR patients is unclear, the following factors may have been involved. First, surgical mortality might have been involved in the nullification of the survival benefits of esophagectomy. For example, robot-assisted surgery was introduced to our center for esophageal cancer in 2010, and there may have had been a period of higher rates of complication and mortalities during the learning curves of the surgeons. Yet, the transient increase in mortality was not enough to offset the difference in LRFR. Second, salvage esophagectomy might reduce the mortality from local recurrence in CRT patients. As salvage esophagectomy was performed in 15.8% of cases with locally recurrent tumors and was not enough to explain the negative result, it is worth noting that 80% of those cases had R0 resection without any in-hospital mortality or death within 90 days after salvage surgery. Markar et al. [15] conducted a multicenter study that demonstrated the role of salvage esophagectomy, in which salvage esophagectomy after definitive CRT did not result in significant differences with TMT in terms of in-hospital mortality, 3-year overall survival, and disease-free survival in 848 patients [15]. Third, the characteristics of the CRT group (e.g., higher age, poor differentiation, and higher Charlson-Deyo score) may have predisposed the patients to poor prognosis despite the correction effort. Fourth, the effect of distant metastasis on survival in the cCR cases might have been strong, and surgery as a local treatment could be limited in significantly affecting the overall survival. However, as the rate of distant metastasis was around 9% in both groups (Table 2), the effect of distant metastasis on survival does not seem to be large enough to offset the increase in LRFR. Collectively, we assume that these four possible explanations are likely to have led to the null findings in survival in this study.

In terms of radiation dose, our patients received a neoadjuvant dose of around 45 Gy and some patients received 50-54 Gy for definitive aim. Some investigators insisted on the use of 60 Gy or higher for definitive CRT considering that local recurrence occurs in about half of patients as in our current study. Yet, there is not enough empirical evidence to change the current standard dose, which was established based on RCTs; in the near future, however, it may be feasible to administer higher doses through more advanced techniques such as IMRT and Proton. In order to determine the role of esophagectomy after CRT, we chose a rather narrow range of conventional radiation doses because we sought to perform response evaluation at one month after treatment. In order to use a higher radiation dose, a treatment break of 1 month should be considered prior to the additional dose, whose effect is not established at present.

The present study has several limitations. First, it is a retrospective analysis and the result might have been influenced by potential selection bias. Although IPTW-adjusted analysis was performed to adjust for the differences in patient characteristics, unobserved confounding factors may have still been present. Second, the rate of pCR was higher than that of cCR, which suggests that our criteria for cCR may have been overly strict and not representative of the real-world setting. Third, we focused on high-grade treatment-related toxicities and may have underestimated lower grade toxicities and their possible effects. Forth, histological confirmation of recurrent lesions could not be performed in every patient.

Despite these limitations, our study provides clinically meaningful results because the treatments were performed according to a prospectively established study protocol by an experienced multidisciplinary esophageal cancer team. Moreover, we applied the IPTW adjustment to perform a reliable analysis. Although some of our results did not support our initial hypothesis, this study showed that esophagectomy after CRT was associated with improved survival compared with CRT and we hope that our study may be used as a reliable reference for future studies.

Esophagectomy after CRT was associated with significantly better survival results and lower rates of local recurrence and distant metastasis rates compared with CRT. As such effects were more prominent in patients who did not achieve cCR, esophagectomy may be considered in such patients. In complete responders, however, the treatment decision should be made by considering the pros and cons of esophagectomy, which was effective for improving LRFR but not OS.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was approved by the institutional review board of Asan Medical Center (Seoul, Korea; IRB number: 2020-1562) and written informed consent was waived because of the retrospective study.

Author Contributions

Conceived and designed the analysis: Yu J, Kim JH.
Collected the data: Yu J, Song KJ, Jang JY, Jo YY, Yoo YJ.
Contributed data or analysis tools: Kim JH, Kim SB, Park SR, Kim YH, Kim HR, Lee HJ, Song HJ.
Performed the analysis: Yu J.
Wrote the paper: Yu J, Kim JH.
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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

Acknowledgments
We thank Dr. Joon Seo Lim from the Scientific Publications Team at Asan Medical Center for his editorial assistance in preparing this manuscript.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.997
Cancer Res Treat. 2022;54(4):1157-1166

Original Article

Aberrant DNA Methylation Maker for Predicting Metachronous Recurrence After Endoscopic Resection of Gastric Neoplasms

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Purpose This study aimed to investigate whether *MOS* methylation can be useful for the prediction of metachronous recurrence after endoscopic resection of gastric neoplasms.

Materials and Methods From 2012 to 2017, 294 patients were prospectively enrolled after endoscopic resection of gastric dysplasia (n=171) or early gastric cancer (n=123). When *Helicobacter pylori* was positive, eradication therapy was performed. Among them, 124 patients completed the study protocol (follow-up duration > 3 years or development of metachronous recurrence during the follow-up). Methylation levels of *MOS* were measured at baseline using quantitative MethyLight assay from the antrum.

Results Median follow-up duration was 49.9 months. *MOS* methylation levels at baseline were not different by age, sex, and current *H. pylori* infection, but they showed a weak correlation with operative link on gastritis assessment (OLGA) or operative link on gastric intestinal metaplasia assessment (OLGIM) stages (Spearman's ρ=0.240 and 0.174, respectively; $p < 0.05$). During the follow-up, a total of 20 metachronous gastric neoplasms (13 adenomas and 7 adenocarcinomas) were developed. Either OLGA or OLGIM stage was not useful in predicting the risk for metachronous recurrence. In contrast, *MOS* methylation high group (≥ 34.82%) had a significantly increased risk for metachronous recurrence compared to *MOS* methylation low group (adjusted hazard ratio, 4.76; 95% confidence interval, 1.54 to 14.79; p=0.007).

Conclusion *MOS* methylation can be a promising marker for predicting metachronous recurrence after endoscopic resection of gastric neoplasms. To confirm the usefulness of *MOS* methylation, validation studies are warranted in the future (ClinicalTrials No. NCT04830618).

Key words Methylation, Stomach neoplasms, Second primary neoplasms, Recurrence

Introduction

Lung cancer is one of the most prevalent malignant neopl-Gastric cancer (GC) is the sixth most diagnosed cancer and the third leading cause of cancer mortality with 1,090,103 incident cases, and more than 768,793 deaths in 2020 [1]. *Helicobacter pylori* infection is associated with peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and GC.

H. pylori infection induces chronic inflammation, increased secretion of inflammatory cytokines, and aberrant DNA methylation including promoter CpG island hypermethylation and global DNA hypomethylation [2,3]. In result, prolonged *H. pylori* infection results in epigenetic field defect [4,5], suggesting that methylation could be a surrogate marker for GC [6,7]. Previously, we performed a genome-wide DNA methylation chip study in *H. pylori*–induced gastric carcinogenesis and identified several methylation markers [8]. Then we validated these methylation markers in a case-control study, and among the candidate genes, methylation of *MOS*, a, proto-oncogene, was associated with the duration of *H. pylori* exposure and the risk of GC [9]. Interestingly, *MOS* methylation decreased after *H. pylori* eradication in controls, but it remained significantly increased in patients with gastric dysplasia or GC even after *H. pylori* eradication [10].

In Korea, biannual upper gastrointestinal endoscopy is covered by national insurance for adults over 40 years of age to detect the early gastric cancer (EGC) before progression to advanced GC. This has led to an increase both in diagnosis and endoscopic resection (ER) of EGC [11]. *H. pylori* eradication after ER of EGC reduced the risk for metachro-

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Received September 6, 2021 **Accepted** January 17, 2022
Published Online January 18, 2022

nous recurrence [12]. However, many patients still develop metachronous gastric cancers or gastric dysplasia even after *H. pylori* eradication treatment [13,14]. Thus, there is a need for a surrogate marker that can predict the risk of GC after *H. pylori* eradication [15].

From this background, we performed a prospective cohort study to investigate whether *MOS* methylation can be useful for the prediction of metachronous recurrence after ER of gastric neoplasms.

Materials and Methods

1. Study subjects

The study was designed as a prospective cohort study. From 2012 to 2017, 294 patients were prospectively enrolled after ER of gastric dysplasia (n=171) or EGC (n=123). All lesions were assessed by endoscopy with biopsy before ER. Endoscopic mucosal resection or endoscopic submucosal dissection (ESD) was performed for gastric dysplasia and early gastric cancers which met the absolute indication (differentiated adenocarcinoma, intramucosal cancer, lesions < 20 mm, and no endoscopic evidence of ulceration). All lesions were curatively resected; if non-curatively resected, then the patients were not enrolled in the study. All subjects, who provided informed consent at the time of initial endoscopic treatment, were asked to complete a questionnaire under the supervision of a well-trained interviewer. The questionnaire included questions regarding demographic data (age, sex), socioeconomic data (smoking, alcohol, and education), their family history of GC in first-degree relatives, and history of *H. pylori* eradication therapy.

Among the 294 subjects, *MOS* methylation level at baseline could be determined in 261 patients from noncancerous gastric mucosae at antrum. When *H. pylori* was positive by CLOtest or histology at baseline or during the follow-up, eradication therapy was done. To evaluate whether *H. pylori* was eradicated, ^{13}C-urea breath testing was performed at least 4 weeks after completion of eradication therapy. The definition of the completion of the study protocol was (1) endoscopic and/or radiologic follow-up for more than 3 years, or (2) development of metachronous gastric neoplasm (gastric dysplasia or cancer) during the follow-up. Metachronous recurrence was defined as secondary dysplasia or cancers detected > 1 year after initial diagnosis. Finally, 124 of 261 subjects completed the study protocol and were included for the survival analysis.

2. Follow-up after endoscopic resection

All study subjects were closely followed up since recurrent tumors at previous ER sites can be easily detected on endoscopy with biopsy and treated during follow-up. Patients with local recurrence underwent further treatments, including repeated ESD, argon plasma coagulation, and gastrectomy based on pathology, and patients who refused treatment received supportive care.

All patients underwent endoscopy with biopsy within 6 months, then at 12 months after ESD to check for metachronous lesions or local recurrences. After 12 months, endoscopy with biopsy was performed annually. In case of EGCs, abdominal computed tomography scan was performed in the first year and biennially thereafter to detect lymph node or distant metastases.

3. *H. pylori* testing and histologic assessment

At each endoscopy, 12 biopsy specimens were obtained for histological analysis, *Campylobacter*-like organism test, to determine the presence of a current *H. pylori* infection. This methodology has been presented previously [10,16]. In brief, two biopsy specimens from the antrum and two from the corpus (1 from the lesser curvature, 1 from the greater curvature) were fixed in formalin to assess the presence of *H. pylori* by modified Giemsa staining and the degree of inflammatory cell infiltration, atrophy and intestinal metaplasia (all by hematoxylin and eosin staining). These histologic features of the gastric mucosa were recorded using the updated Sydney scoring system (0, none; 1, mild; 2, moderate; and 3, marked) [17]. One specimen from each of the lesser curvature of the antrum and the body was used for rapid urease testing (CLOtest, Delta West, Bentley, Australia). The remaining six noncancerous mucosal biopsy specimens (3 antrum and 3 body each) were immediately frozen at –70°C until DNA extraction.

4. Operative link on gastritis assessment and operative link on gastric intestinal metaplasia assessment staging

Operative link on gastritis assessment (OLGA) or operative link on gastric intestinal metaplasia assessment (OLGIM) stages were made by histological examination of gastric biopsy samples (antrum and corpus) following the updated Sydney System [18]. Two independent gastrointestinal pathologists, who were blinded to clinical information, assessed the biopsies. if there was a disagreement, the biopsies were assessed by a third pathologist again.

5. DNA extraction, bisulfite modification, and MethyLight assay

Genomic DNA was extracted directly from noncancerous antral biopsy specimens using sodium bisulfite. The methodology was reported previously [19]. Briefly, specimens were homogenized in proteinase K solution (20 mmol/L Tris–HCl [pH 8.0], 10 mmol/L ethylenediaminetetraacetic acid, 0.5%

sodium dodecyl sulfate, and 10 mg/mL proteinase K) using a sterile micropestle, followed by incubation for 3 hours at 52°C. DNA was isolated from homogenates using phenol/chloroform extraction and ethanol precipitation. Genomic DNA (1 μg) was bisulfite modified using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) by following the manufacturer's instructions. The methylation status of *MOS* from bisulfite-modified DNA samples was quantified using real-time polymerase chain reaction–based MethyLight technology. MethyLight, as a sensitive, high-throughput methylation assay, allows the highly specific detection of methylation using probes that cover methylation sites, as well as methylation-specific primers [20]. The primer and probe sequences used in the reaction are as follows: forward primer sequence, TTCACTCCAACGACCCTAATATCC; backward primer sequence, GGGAAAATTCGTTTCGGAGGTAG; probe oligo sequence, 6FAM-AATACGATACCCTCGCCCCTA-ACCCTACG-BHQ-1 [19]. The quantified level of *MOS* was reported as a percentage of methylated reference, which is the relative methylation ratio of the target gene to the ALU gene of a sample, divided by the ratio of the target gene to the *Alu* gene of sodium bisulfite and CpG methyltransferase (M-SssI)–treated sperm DNA, multiplied by 100.

6. Statistical analysis

For sample size calculation, the expected incidence of metachronous recurrence in low-risk group (low methylation group) is presumed to be 0.01 per year, and that in high-risk group increases by 4-fold. Assuming that the ratio of the number of the low-risk and high-risk individuals is 1:1, the number of patients in each group was calculated as 131 at a statistical power of 0.80 with a two-sided significance level of 0.05. Considering a dropout rate of ~10%, the sample size was determined as 290 (145 in each group).

Continuous variables were presented as mean±standard deviation. Categorical variables were presented as numbers with proportions. To compare continuous variables, Student t test was used. For categorical variables, chi-square test was used for analysis. For determining the optimal diagnostic cutoff value on predicting metachronous recurrence, receiver operating characteristic curve was used. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. For survival analysis, Kaplan-Meier curves for cumulative incidences were used with log-rank test. Cox proportional hazard model was adopted under adjustment with clinically important variables. All the statistical analyses were performed using R ver. 3.2.3 (The R Foundation for Statistical Computing, Vienna, Austria; http://www.r-project.org). All tests were two-sided and $p < 0.05$ were considered statistically significant.

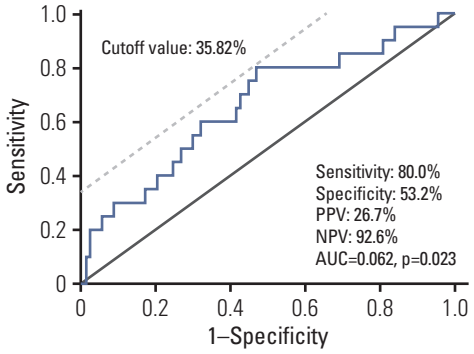

Fig. 1. Receiver operating characteristic curve analysis to determine a cutoff value of *MOS* methylation level to predict the risk for metachronous recurrence (n=124). Optimal cutoff value was 35.82% and sensitivity, specificity, PPV and NPV were 80.0%, 53.2%, 26.7%, and 92.6%, respectively. AUC, area under curve; NPV, negative predictive value; PPV, positive predictive value.

Results

1. Optimal cutoff value of *MOS* methylation level to predict metachronous recurrence

Among the study subjects who completed the study protocol (n=124), 20 metachronous gastric lesions (13 adenomas and 7 adenocarcinomas) were developed during the follow-up (median of the follow-up duration: 49.9 months [range, 13.1 to 96.2 months], median follow-up visits: 4.9 times). To determine the optimal cutoff value of *MOS* methylation level to predict metachronous recurrence, receiver operating characteristics curve analysis was performed (Fig. 1), and the optimal cutoff value was 35.82% (sensitivity, specificity, PPV, and NPV: 80.0%, 53.2%, 26.7%, and 92.6%, respectively.). In *MOS* methylation low group (n=74), eight metachronous recurrences (4 adenomas and 4 adenocarcinomas) were developed; in *MOS* methylation high group (n=50), 12 metachronous lesions (9 adenomas and 3 adenocarcinomas) were developed during the follow-up.

2. Characteristics of the study subjects at baseline

The clinical and pathological characteristics of the study subjects at baseline were summarized in Table 1. There was no significant difference between the methylation high group (*MOS* methylation level ≥ 35.82%) and the methylation low group (methylation level < 35.82%) except for follow-up duration and follow-up visits ($p < 0.001$), which was attributed to a higher metachronous recurrence in the methylation high group.

Also, the clinicopathological characteristics of the 124 patients completed the study protocol according to metachronous recurrence were presented in S1 Table. In patients

Table 1. Characteristics of the study subjects at baseline

Variable	Total (n=294)	*MOS* methylation level (n=261)		p-value
		Low (n=99)	High (n=162)	
Age (yr)	63.2±8.7	62.4±9.1	64.1±8.6	0.132
Male sex	200 (68.0)	68 (68.7)	108 (66.7)	0.735
Follow-up duration (day)	998.1±670.1	1,250.5±712.4	837.4±589.8	< 0.001
No. of endoscopic follow-up	3.6±2.4	4.4±2.7	3.1±2.1	< 0.001
***Helicobacter pylori* positive**	110 (37.4)	33 (33.3)	63 (38.9)	0.366
Current or ex-smoker	127 (43.2)	57 (47.9)	62 (52.1)	0.572
Current or ex-drinker	158 (53.7)	70 (48.3)	75 (51.7)	0.787
Family history of GC in 1° relatives	49 (17.4)	20 (20.6)	25 (16.2)	0.378
Body mass index (kg/m²)	24.4±3.2	24.3±3.5	24.4±3.0	0.678
Education				
Elementary-Middle-High	149 (65.6)	62 (72.1)	79 (64.2)	0.232
University	78 (34.4)	24 (27.9)	55 (35.8)	
Pathology				
Low-grade dysplasia	147 (50.0)	44(44.9)	77 (48.7)	0.662
High-grade dysplasia	24 (8.2)	7 (7.1)	14 (8.9)	
Adenocarcinoma	123 (41.8)	47 (48.0)	67 (42.4)	
OLGA stage				
Stage 0	26 (22.4)	11 (28.2)	13 (18.3)	0.760
Stage 1	28 (24.1)	8 (20.5)	18 (25.4)	
Stage 2	28 (24.1)	8 (20.5)	19 (26.8)	
Stage 3	22 (19.0)	8 (20.5)	13 (18.3)	
Stage 4	12 (10.3)	4 (10.3)	8 (11.8)	
OLGIM stage				
Stage 0	51 (17.3)	21 (21.6)	25 (16.0)	0.430
Stage 1	59 (20.1)	16 (16.5)	35 (22.4)	
Stage 2	87 (29.6)	34 (35.1)	44 (28.2)	
Stage 3	52 (17.7)	15 (15.5)	30 (19.2)	
Stage 4	35 (11.9)	11 (11.3)	22 (14.1)	
Synchronous EGCs/dysplasia[a)]	25 (10.2)	8 (8.7)	16 (10.9)	

Values are presented as mean±SD or number (%). p-values were calculated using chi-square test or Student's t test. The cutoff value (35.82%) of high or low *MOS* methylation levels was determined by receiver operating curve analysis. Statistically significant at $p < 0.001$. EGC, early gastric cancer; GC, gastric cancer; OLGA, operative link on gastritis assessment; OLGIM, operative link on gastric intestinal metaplasia assessment; SD, standard deviation. [a)]Synchronous lesions were defined as secondary dysplasia or cancers detected within 1 year after initial diagnosis.

with metachronous recurrence, initial pathology was low- or high-grade dysplasia rather than adenocarcinoma ($p < 0.001$), and synchronous lesions (dysplasia or EGCs) were more prevalent (p=0.053). OLGA and OLGIM stages were not different between the two groups ($p > 0.05$), but *MOS* methylation level was higher in patients with metachronous recurrence (p=0.009).

3. Association between *MOS* methylation level and clinical and histologic variables.

Next, we evaluated whether *MOS* methylation levels were different by age, family history of GC, synchronous gastric lesions, current *H. pylori* infection, and OLGA and OLGIM stages. There was no correlation between age and *MOS* methylation level (Pearson's correlation coefficient=0.063, p=0.312) (Fig. 2A). Family history of GC in 1° relatives, synchronous gastric neoplasms, current *H. pylori* infection did not affect *MOS* methylation levels ($p > 0.05$) (Fig. 2B-D). In contrast, *MOS* methylation levels correlated with OLGA or OLGIM stages (Spearman's ρ=0.240 and 0.174, respectively, both $p < 0.05$) (Fig. 2E and F).

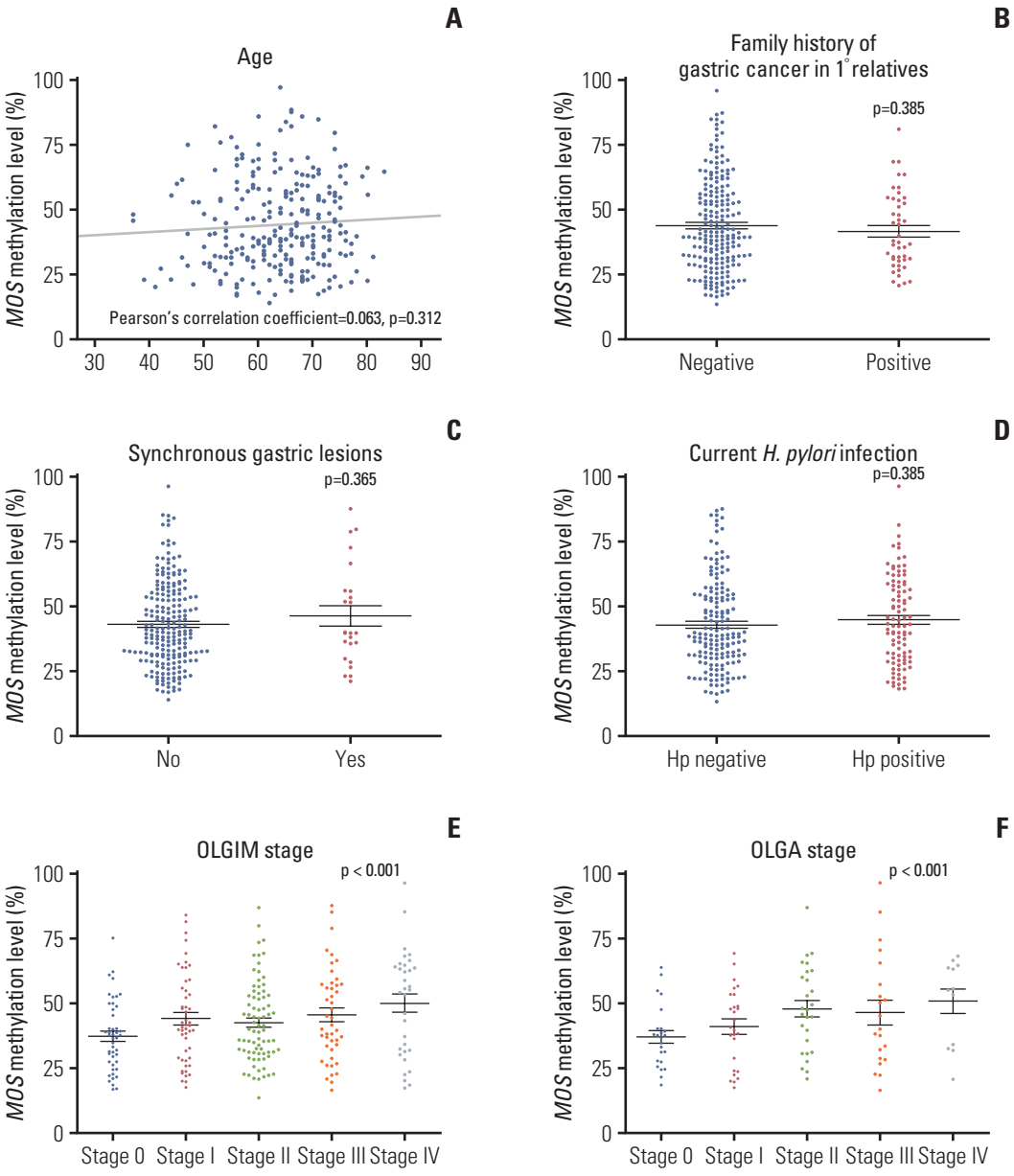

Fig. 2. *MOS* methylation levels according to age (A), family history of gastric cancer (B), synchronous gastric lesions (C), current *Helicobacter* pylori infection (D), and OLGA (E) and OLGIM (F) stages (n=261). OLGA, operative link on gastritis assessment; OLGIM, operative link on gastric intestinal metaplasia assessment.

4. Clinical implication of mucosal atrophy, intestinal metaplasia, and *MOS* methylation in the prediction of metachronous gastric recurrence after endoscopic resection

Then, we evaluated whether atrophic gastritis, intestinal metaplasia, or *MOS* methylation level could predict the metachronous recurrence after ER of gastric neoplasms (Table 2, Fig. 3). Kaplan-Meier curves for cumulative incidences of metachronous recurrence showed that presence or absence of atrophic gastritis and intestinal metaplasia did not predict the risk for metachronous recurrence in this high-risk population (Fig. 3A and C). Also, OLGA and OLGIM stages were not useful in predicting the risk (Fig. 3B and D); if the analysis was performed comparing low-risk (grade 0 to 2) and high-risk (grade 3 and 4) groups, it was not statistically significant (S2 Fig.).

In contrast, *MOS* methylation could be useful to determine the high-risk group in metachronous recurrence. That is, *MOS* methylation high group (≥ 34.82%) had a significantly

Table 2. Univariate and multivariate Cox proportional regression analyses of the metachronous recurrence (n=124)

	Crude HR (95% CI)	p-value	p for trend	Adjusted HR[a] (95% CI)	p-value[a]	p for trend
***MOS* high[b]**	4.73 (1.56-14.40)	0.006[c]		4.76 (1.54-14.79)	0.007[c]	
Atrophic gastritis	1.40 (0.18-10.88)	0.746		1.31 (0.16-10.75)	0.802	
Intestinal metaplasia	2.62 (0.35-19.74)	0.349		2.32 (0.30-18.29)	0.423	
***MOS* quartile**						
Q1	1 (reference)			1 (reference)		
Q2	1.64 (0.41-6.61)	0.485		1.42 (0.35-5.87)	0.624	
Q3	3.36 (0.87-13.03)	0.080		3.11 (0.78-12.46)	0.109	
Q4	3.53 (1.02-12.22)	0.047	0.027[c]	3.29 (0.94-11.53)	0.062	0.034[c]
OLGA stage						
0	1 (reference)			1 (reference)		
1	1.79 (0.18-17.56)	0.615		1.65 (0.13-20.16)	0.697	
2	0.61 (0.04-9.90)	0.728		0.57 (0.03-10.28)	0.705	
3	0.60 (0.04-27.31)	0.719		0.51 (0.02-10.59)	0.665	
4	1.56 (0.09-27.31)	0.761	0.689	1.63 (0.09-30.55)	0.745	0.677
OLGIM stage						
0	1 (reference)			1 (reference)		
1	3.26 (0.38-27.93)	0.282		2.95 (0.33-26.56)	0.334	
2	2.22 (0.27-18.54)	0.461		2.10 (0.24-18.20)	0.503	
3	2.21 (0.24-20.15)	0.481		1.75 (0.18-16.83)	0.627	
4	3.78 (0.42-34.44)	0.238	0.452	3.38 (0.34-33.17)	0.297	0.617

CI, confidence interval; HR, hazard ratio; OLGA, operative link on gastritis assessment; OLGIM, operative link on gastric intestinal metaplasia assessment. [a]Adjusted for age, sex, *Helicobacter pylori* infection status, and smoking, [b]The cutoff value (35.82%) of high or low *MOS* methylation levels was determined by receiver operating curve analysis. Atrophic gastritis and intestinal metaplasia were defined as the presence of histologic atrophy (score 1-3) and intestinal metaplasia (score 1-3), respectively, at either antrum or corpus by the updated Sydney scoring system, [c]Statistically significant.

increased risk for metachronous recurrence compared to *MOS* methylation low group (adjusted hazard ratio [HR], 4.76; 95% confidence interval [CI], 1.54 to 14.79; p=0.007) (Table 2). In adjusted Cox proportional regression model, the risk of metachronous recurrence significantly increased in the highest quartile level (Q4) compared with the lowest quartile level (Q1) (HR, 3.53; 95% CI, 1.02 to 12.22; p=0.047). However, this was not statistically significant after adjusting for age, sex, *H. pylori* infection, and smoking (p=0.062) (Table 2). Nevertheless, a significant increasing linear trend was observed between *MOS* methylation and the risk of meta-chronous recurrence (adjusted p for trend=0.034).

When the same analyses were performed in the entire cohort (n=261), the results were not different (S3 Table, S4 Fig.).

Discussion

This study showed that *MOS* methylation could be useful in predicting metachronous recurrence after *H. pylori* eradication in the high-risk patients who had undergone ER of gastric neoplasm. The patients who underwent ER of EGC or gastric dysplasia are regarded as a high-risk population of metachronous gastric neoplasms [15]. In the previous studies, the incidence of metachronous GC was reported to be 1.9%-25.3% when observed up to 4-7 years [21], and *H. pylori* eradication reduced the incidence of metachronous GC by ~50% [12]. However, metachronous recurrence still develops even after *H. pylori* eradication; thus, we need a surrogate marker for the risk of metachronous GC after *H. pylori* eradication [15].

Differentiated GCs are frequently found after *H. pylori* eradication, showing characteristic endoscopic features such as reddish depression; benign reddish depression is difficult to be distinguished from GC because of the histological alterations in the surface structures (non-neoplastic epithelium or epithelium with low-grade atypia) as well as multiple appearances of benign reddish depression [22]. Furthermore, submucosal invasive cancers were not infrequently found after *H. pylori* eradication despite of the annual endoscopic surveillance [22]. In this study, all cases of metachronous recurrence (n=20) were either gastric dysplasia or EGC; six of seven metachronous gastric cancers (85.7%) were differentiated gastric cancers, but three cases (42.9%) invaded submucosa.

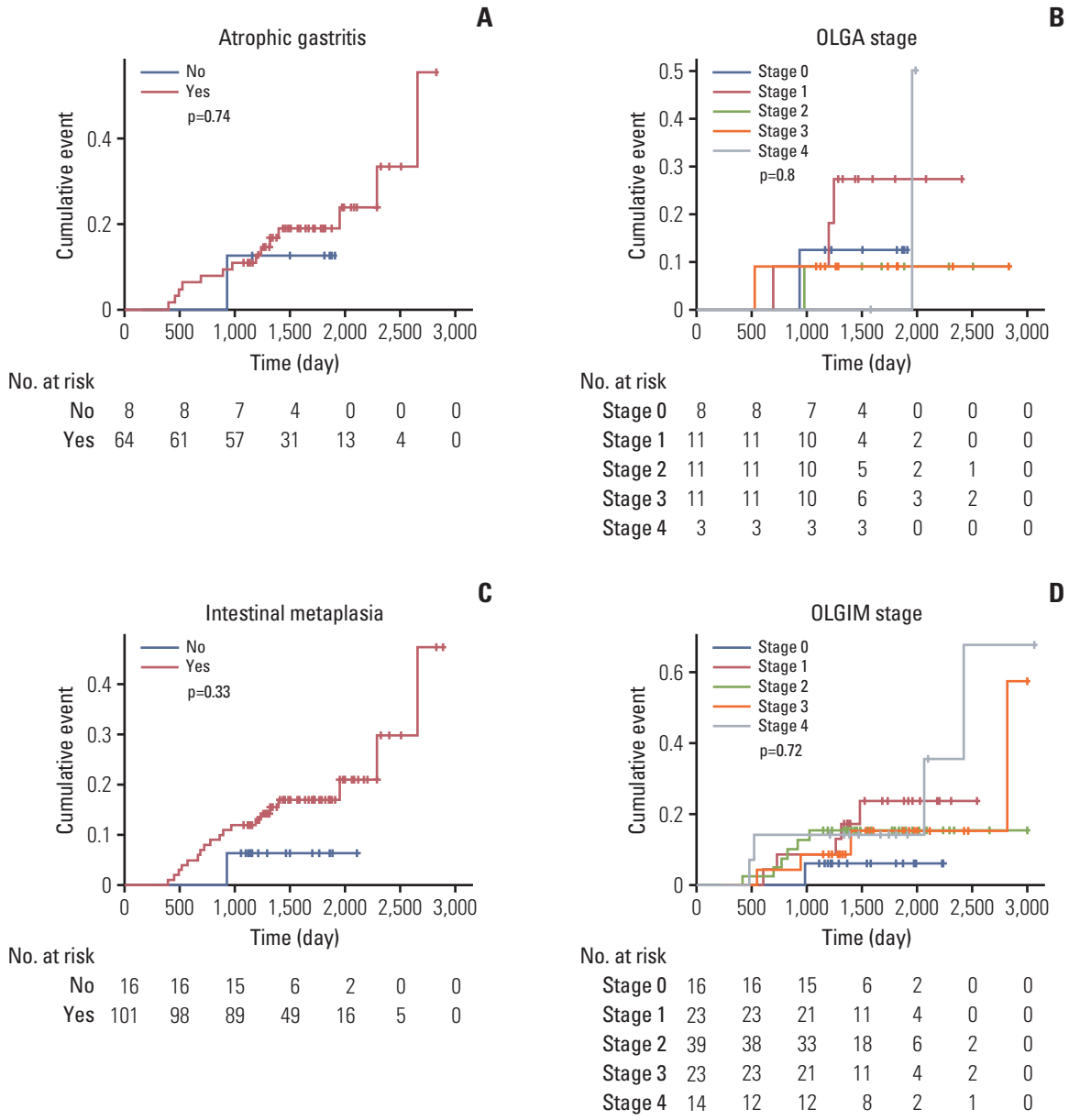

Fig. 3. Kaplan-Meier curves for cumulative incidences of metachronous recurrence according to atrophic gastritis (A), OLGA stage (B), intestinal metaplasia (C), OLGIM stage (D), *MOS* methylation status (E, F, n=124). Atrophic gastritis and intestinal metaplasia were defined as the presence of histologic atrophy (score 1-3) and intestinal metaplasia (score 1-3), respectively, at either antrum or corpus by the updated Sydney scoring system. The cutoff value (35.82%) of high or low level of *MOS* methylation was determined by receiver operating characteristic curve analysis. OLGA, operative link on gastritis assessment; OLGIM, operative link on gastric intestinal metaplasia assessment. *(Continued to the next page)*

There have been several studies that aberrant DNA methylation could be a surrogate marker for the risk of metachronous GC [6,23]. Previously, a Japanese group published the impact of aberrant DNA methylation accumulation on metachronous GC in a 5-year follow-up of a multicenter prospective cohort study [24,25]. They showed that the higher quartiles of methylation levels in *miR-124a-3*, *EMX1*, and *MKX6-1* showed an increased risk for metachronous GCs. Another study has shown that aberrant methylation of microRNA-34b/c is a predictive marker of metachronous GC risk [23].

In the present study, the rationale for choosing *MOS* meth-

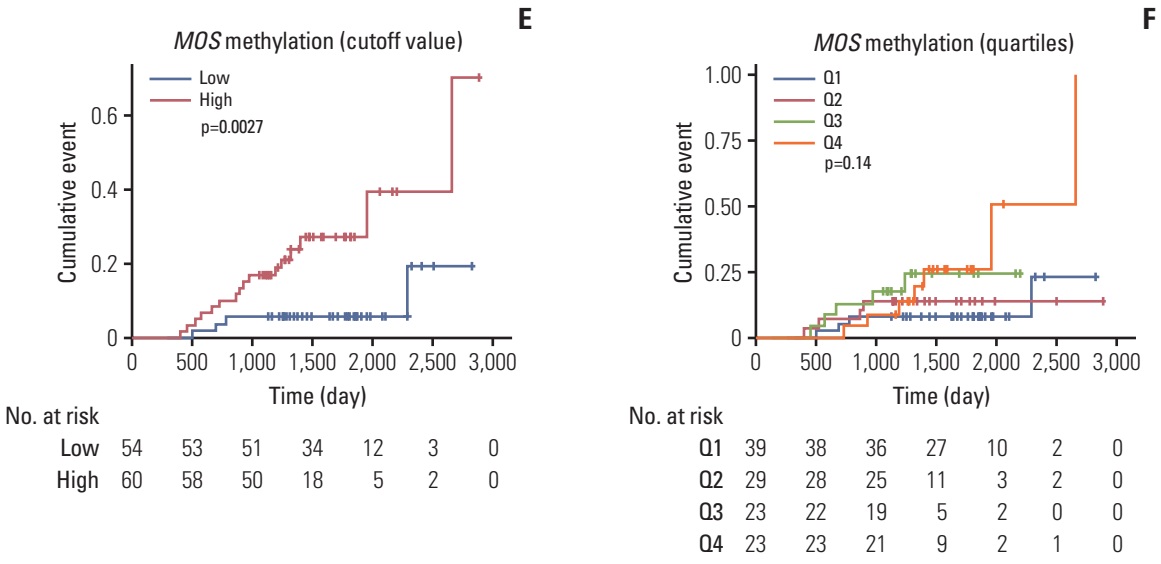

Fig. 3. *(Continued from the previous page)*

ylation as a marker is based on the results of previous studies. Previously, we evaluated the usefulness of several candidate methylation markers to define a high-risk group for GC [8]. Among them, methylation of *MOS* was associated with the duration of *H. pylori* exposure. *MOS* methylation was also increased in remote past infection in which *H. pylori* disappeared in gastric mucosa, and it was significantly increased in patients with GC regardless of *H. pylori* infection [9]. Interestingly, *MOS* methylation decreased after *H. pylori* eradication in controls, but it remained significantly increased in patients with gastric dysplasia or GC even after *H. pylori* eradication [10]. In a retrospective study, we have shown that *MOS* methylation levels at baseline were significantly higher among patients with metachronous gastric neoplasms [26].

We paid attention to the results of previous studies in that there are two types of methylation occurring in the gastric mucosa. One is temporary components of methylation (induced in progenitor or differentiated cells) and the other is permanent components (induced in stem cells) [2,4]. During active *H. pylori* infection, both temporary and permanent components of methylation increase as the duration of infection increases. When *H. pylori* infection discontinues, the temporary component will disappear, leaving only the permanent component. The remaining permanent components correlate with the risk of developing gastric cancers.

From this point of view, *MOS* methylation could be an ideal marker for predicting the risk of GC. The *MOS* methylation we analyzed in this study does not originate from the promoter region (promoter CpG island), but the exon region [8]. Although methylation of some marker genes is not directly involved in carcinogenesis, their methylation levels correlate with those of tumor-suppressor genes and thus GC risks. Methylation of a marker gene is not requisite for gastric carcinogenesis [4]. Methylation levels of *MOS* in GC tissues did not correlate with those in their background gastric mucosa. Rather, we found that hypomethylation of *MOS* in GC tissues was associated with tumor invasion, nodal metastasis, and undifferentiated histology, suggesting that *MOS* methylation occurs in a complex manner depending on the stages of gastric carcinogenesis [9].

In the present study, *MOS* methylation was not affected by age (Table 1, Fig. 2). Therefore, *MOS* methylation might not be an aging process. There was no significant difference in *MOS* methylation level between *H. pylori*–positive and –negative patients. This is because most of the subjects were high-risk patients in this study. Even if some of them had no evidence of active *H. pylori* infection at present, most of them might be in remote past *H. pylori* infection [27]. Likely, *MOS* methylation levels did not differ according to the presence or absence of synchronous gastric neoplasm.

In contrast, *MOS* methylation level positively correlated with OLGA and OLGIM staging (Fig. 2). Atrophic gastritis and intestinal metaplasia are not only important precancerous lesions of GC but have been reported to be significantly associated with the occurrence of metachronous GC [13,28]. In this study, however, OLGA and OLGIM stages failed to show the relations to metachronous recurrence. This might be attributed to the fact that the frequencies of patients with high OLGA and OLGIM stages (stage 3-4) at baseline were much lower than those reported in GC patients (Table 1). In contrast, we found that *MOS* methylation may predict the

risk of metachronous gastric neoplasms better than atrophy or metaplasia (Table 2, Fig. 3). Unlikely with the previous studies, the reason of insignificant results in atrophic gastritis and intestinal metaplasia might be attributed to the relatively small sample size; if the sample size is sufficiently large, significant results could be shown for atrophic gastritis and metaplasia as well. However, the fact that *MOS* methylation was found to be significantly related to the risk for metachronous recurrence despite the relatively small sample size in this study indicates that *MOS* methylation can be a more powerful marker to predict the recurrence of metachronous gastric neoplasms after endoscopic resection. Recently, we found that metachronous GC occurred in the 35 patients among 3,044 patients (1.1%) in the remaining stomach after curative gastric partial resection with GC [29]. In this population, the metachronous GC was only related to older age and surgical methods used. Thus, it might be valuable to perform further study whether the *MOS* methylation can be beneficial in predicting the metachronous recurrence after gastrectomy.

Our study has several limitations as the following. First, the sample size was relatively small. In addition, the dropout rate (follow-up loss within 3 years after initial endoscopic treatment) was much higher than expected (137/261, 52.5%). In South Korea, it is recommended that the patients be returned to the local clinic for screening endoscopy if there are no problems after endoscopic treatment. As a result, many subjects were dropped out, and only 124 subjects were followed up for more than 3 years. Thus, this study might be underpowered. Nevertheless, *MOS* methylation showed statistically significant results. In addition, the results were not different when the survival analyses were performed in the entire cohort (n=261) (S3 Table, S4 Fig.). However, the results of our study should be verified through a large prospective study. Second, serum gastrin-17, anti–*H. pylori* IgG antibody, and pepsinogen I/II levels were not measured in this study. They have been shown to be a surrogate marker of metachronous recurrence after ER of EGC [30,31]. Third, *H. pylori*–positive rate was relatively low (~37%) for the study population, which was EGC or dysplasia patients. It might be because most of the patients who were *H. pylori*–negative in this study were patients with a remote past infection. However, since OLGA and OLGIM stages were not high at baseline, there is a possibility that *H. pylori* infection rate was actually low. Fourth, the interpretation of OLGA and OLGIM staging should be cautious because gastric mucosae were not obtained at gastric angle. Furthermore, OLGA staging was possible in 110 of 261 (42.1%) patients only, because in many cases either antrum or corpus biopsy specimen was inappropriate to assess the degree of atrophy. Despite these limitations, the results of this study show the possibility of *MOS* methylation as a surrogate marker for metachronous gastric neoplasms, and also prove the importance of aberrant DNA methylation in gastric carcinogenesis.

In conclusion, *MOS* methylation can be a promising marker for predicting metachronous gastric neoplasms after ER of gastric neoplasms. To confirm the usefulness of *MOS* methylation, large prospective studies (validation studies) are warranted in the future.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study protocol was approved by the Ethical Committee at Seoul National University Bundang Hospital (IRB No. B1204/152-005). All study participants signed a consent form before enrolling in the study.

Author Contributions

Conceived and designed the analysis: Shin CM, Kim N, Lee DH.
Collected the data: Shin CM, Yoon H, Choi YJ, Park YS.
Contributed data or analysis tools: Kim N, Park JH, Lee DH.
Performed the analysis: Shin CM, Park JH.
Wrote the paper: Shin CM.
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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant for the Global Core Research Center (GCRC) funded by the Korea government (MSIP) (No. 2011-0030001).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1040
Cancer Res Treat. 2022;54(4):1167-1174

Original Article

Adjuvant Imatinib Treatment for 5 Years versus 3 Years in Patients with Ruptured Localized Gastrointestinal Stromal Tumor: A Retrospective Analysis

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Purpose Three years of adjuvant imatinib is the standard treatment for resected gastrointestinal stromal tumors (GISTs) with rupture, but the recurrence rate is prominently high. We aimed to investigate the efficacy and safety of 5-year adjuvant imatinib compared with 3-year treatment in patients with a ruptured GIST following surgical resection.

Materials and Methods A total of 51 patients were included in the analysis. The assessment of GIST rupture was based on Nishida's classification. Twenty patients who were diagnosed before November 2013 were treated with 5 years of imatinib, and 31 patients who were diagnosed after November 2013 were treated with 3 years of imatinib. We retrospectively compared the clinical outcomes of the two groups.

Results Baseline characteristics and the incidence of the adverse events were generally comparable between the two groups. During a median follow-up duration of 43.8 months and 104.2 months in the 3- and 5-year group, 8 and 9 patients had a disease recurrence, respectively. The 5-year group showed better recurrence-free survival (RFS) than the 3-year group. In multivariate analysis, low mitotic index was a significant independent favorable prognostic factor for RFS, while 5-year imatinib treatment was marginally associated with a favorable RFS.

Conclusion Five years of adjuvant imatinib treatment in patients with ruptured GIST was associated with favorable survival outcomes with manageable toxicity profiles. Our findings warrant validation and confirmation in future studies.

Key words Gastrointestinal stromal tumors, Rupture, Adjuvant chemotherapy, Imatinib

Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract [1]. Most GIST harbors an activating oncogene mutation in *KIT* (60%-70%) or platelet-derived growth factor receptor α (*PDGFRA*) (10%-15%) [2-4]. In the past two decades, the introduction of tyrosine kinase inhibitors (TKIs) has markedly improved the outcome of GIST. Imatinib mesylate, one of the selective TKIs that targets KIT and PDGRFA currently plays a crucial role in the management of GIST, both in the metastatic and adjuvant setting [5].

Because GIST is a soft, highly vascularized, and fragile tumor, it may rupture spontaneously or during surgical manipulation [6]. GIST rupture may cause spillage and dissemination of tumor cells into the intra-abdominal cavity, making it a significant adverse risk factor for tumor recurrence [5,7]. Currently, patients with GIST rupture are classified as a high-risk group for recurrence after resection according to the modified National Institutes of Health (NIH) Consensus Criteria [8].

Presently, 3-year adjuvant imatinib therapy is standard-of-care after resection for high-risk GIST patients including GIST rupture [5,9,10] based on the results of the Scandinavian Sarcoma Group (SSG) XVIII/Arbeitsgemeinschaft Internistische Onkologie (AIO) study [11]. However, the optimal duration of adjuvant therapy remains controversial [12]. Recently, the PERSIST trial, which evaluated the efficacy and tolerability of 5-year adjuvant imatinib in patients with resected GIST, reported the estimated 5-year recurrence-free survival (RFS) was 90% and suggested the role of long-term adjuvant therapy in preventing recurrence in GIST patients after resection [13]. Considering that the recurrence rate of patients with GIST rupture has been reported to be substantially higher than that of non-rupture patients after completion of 3 years of adjuvant imatinib therapy (55% vs. 14%) [14], an extended duration of adjuvant imatinib beyond 3 years could be considered to reduce the recurrence rate in patients with GIST rupture, but there has been a lack of evidence supporting this strategy.

At our institution, before the SSG XVIII/AIO study's results were reported, patients with GIST rupture were con-

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Received September 16, 2021 **Accepted** December 3, 2021 **Published Online** December 6, 2021
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sidered as metastatic and treated with imatinib with palliative intent for at least 5 years. When there was no evidence of gross lesions after 5 years of imatinib treatment, the patients discontinued imatinib treatment and went into active surveillance. Since the approval and reimbursement of 3-year adjuvant imatinib based on the results of the SSG XVIII/AIO study in Korea in November 2013, 3-year adjuvant imatinib has been applied thereafter to patients with GIST rupture following surgical resection. Therefore, we had an opportunity to assess the clinical outcomes according to different durations of imatinib treatment in patients with GIST rupture.

Materials and Methods

1. Study patients

Between 2006 and 2018, a total of 1,409 patients who underwent macroscopically complete resection for localized non-metastatic GIST were identified from the GIST registry of Asan Medical Center, Seoul, Korea. We defined tumor rupture or perforation according to the Nishida classification through a comprehensive review of the preoperative radiologic findings of computed tomography (CT) and operative reports [15]. The Nishida classification defines GIST rupture as one of the following features: tumor spillage or fracture, blood-stained ascites, gastrointestinal perforation, microscopic infiltration into an adjacent structure, piecemeal resection, or intralesional dissection and incisional biopsy. Mucosal defect, intraluminal tumor perforation or gastrointestinal bleeding, microscopic peritoneal penetration of tumor cells or iatrogenic peritoneal damage, or R1 resection were not regarded as tumor rupture. According to the definition, a total of 53 patients who were documented as rupture or perforation were identified. After excluding two patients (one patient participated in a clinical trial that investigated adjuvant imatinib for 2 years (NCT00278876), and the other patient had concurrent metastatic gastric cancer), 51 patients were included as the study population.

2. Adjuvant imatinib treatment

To assess the efficacy and safety according to the different durations of adjuvant therapy, we divided the patients into two groups according to the treatment duration: the 3-year group (n=31) and the 5-year group (n=20) (Fig. 1). In principle, patients were started with 400 mg of imatinib once daily, and the dose was modified based on the grade of toxicity. Patients were followed-up at 4 weeks after initiating imatinib treatment to evaluate the tolerability of the treatment. Thereafter, regular physical examination and laboratory assessments, CT scans of the abdomen and pelvis and chest radiographs were conducted every 3 months during the treatment duration and the first 2 years after stopping treatment, and every 6 months for the next 3 years if disease recurrence was not documented. Adverse events were identified by retrospective medical records review and evaluated according to the Common Terminology Criteria for Adverse Events, ver.

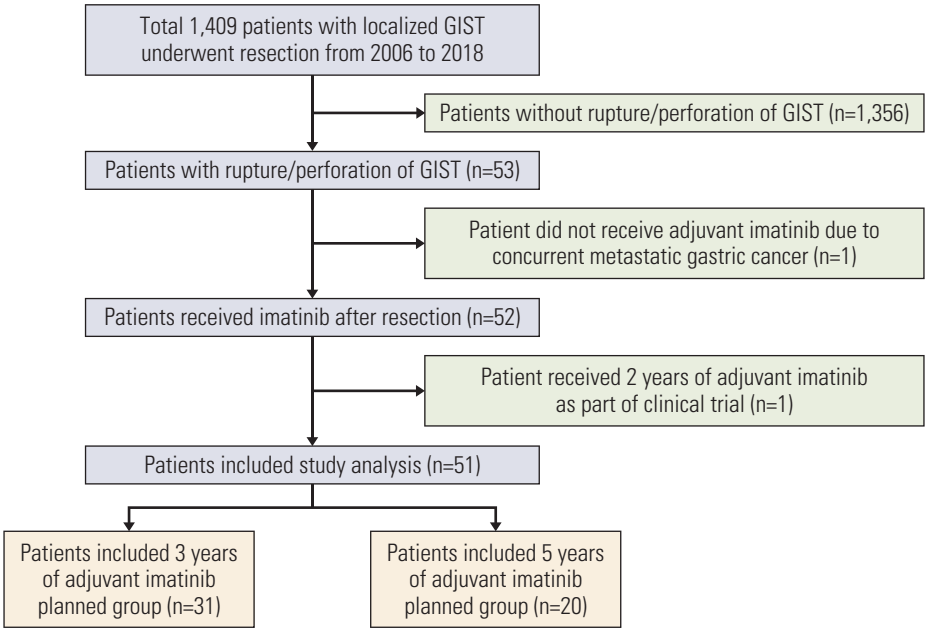

Fig. 1. Study flow diagram of patients who were included in the analysis. GIST, gastrointestinal stromal tumor.

Table 1. Baseline characteristics of the study patients

	3 Years of imatinib (n=31)	5 Years of imatinib (n=20)	p-value
Age (yr)			
≤ 60	22 (71.0)	12 (60.0)	0.612
> 60	9 (29.0)	8 (40.0)	
Age (yr)	58 (24-78)	57 (26-79)	0.992
Sex			
Male	19 (61.3)	15 (75.0)	0.478
Female	12 (38.7)	5 (25.0)	
Location of tumor			
Stomach	14 (45.2)	4 (20.0)	0.125
Non-stomach	17 (54.8)	16 (80.0)	
Small intestine	16	15	
Large intestine	0	1	
Peritoneum	1	0	
Largest diameter of tumor (cm)			
≤ 5	0	2 (10.0)	0.275
5.1-10	16 (51.6)	9 (45.0)	
> 10.0	15 (48.4)	9 (45.0)	
Largest diameter of tumor (cm)	10.0 (5.2-27.0)	10.0 (2.5-20.0)	0.877
Tumor mitotic count (/50 HPFs)			
≤ 5	14 (45.2)	8 (40.0)	0.859
6-10	4 (12.9)	4 (20.0)	
> 10	13 (41.9)	8 (40.0)	
Tumor mitotic count (/50 HPFs)	7 (1-125)	7 (0-50)	0.772
Tumor mutation type			
KIT exon 11 mutation	21 (72.4)	19 (95.0)	0.092
KIT exon 9 mutation	2 (6.9)	1 (5.0)	
Wild type for *KIT* and *PDGFRA*	4 (10.8)	0	
Others	4[a] (10.8)	0	

Values are presented as number (%) or median (range). HPF, high-power field. [a]*KIT* exon 17 mutation (n=1), not available (n=2), and undetermined (when the *KIT* mutation analysis revealed no mutation and *PDGFRA* mutation analysis was not conducted, n=1).

5.0. For the non-hematologic toxicities, only grade 3 or higher adverse events were analyzed since there was a limitation in the complete analysis of toxicities of a lesser degree in this clinical practice setting.

3. Statistical analysis

The chi-squared test or Fisher's exact test was used to analyze the categorical variables. RFS was defined as the time from the date of the operation to the date of the first radiologically documented disease recurrence or death from any cause, whichever occurred first. Overall survival (OS) was defined as the time from the date of the operation to the date of death from any cause or the last follow-up. The Kaplan-Meier method was used to estimate the OS and RFS, and a two-sided log-rank test was used to compare the treatment groups. Multivariate analysis using Cox's proportional hazards model was performed to evaluate the prognostic value

Table 2. Comparison of the treatment profiles

Treatment profile	3 Years of imatinib (n=31)	5 Years of imatinib (n=20)
Completion	17 (54.8)	12 (60.0)
Ongoing	8 (25.8)	0
Early discontinuation	6 (19.3)	8 (40.0)
Disease progression	2 (6.5)	0
Patient's choice	3 (9.6)	1 (5.0)
Adverse events	0	4 (20)
Lost to follow-up	1 (5.4)	1 (5.0)
Other medical conditions	0	2 (10.0)[a]

Values are presented as number (%). [a]Includes sepsis related to cellulitis (n=1), postoperation complication (enterocutaneous fistula, n=1).

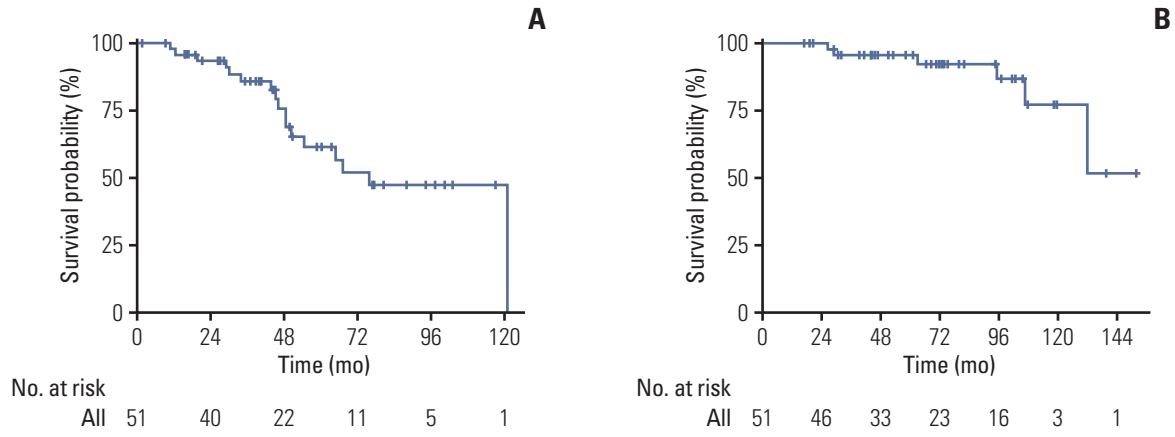

Fig. 2. Recurrence-free survival (A) and overall survival (B) of all included patients.

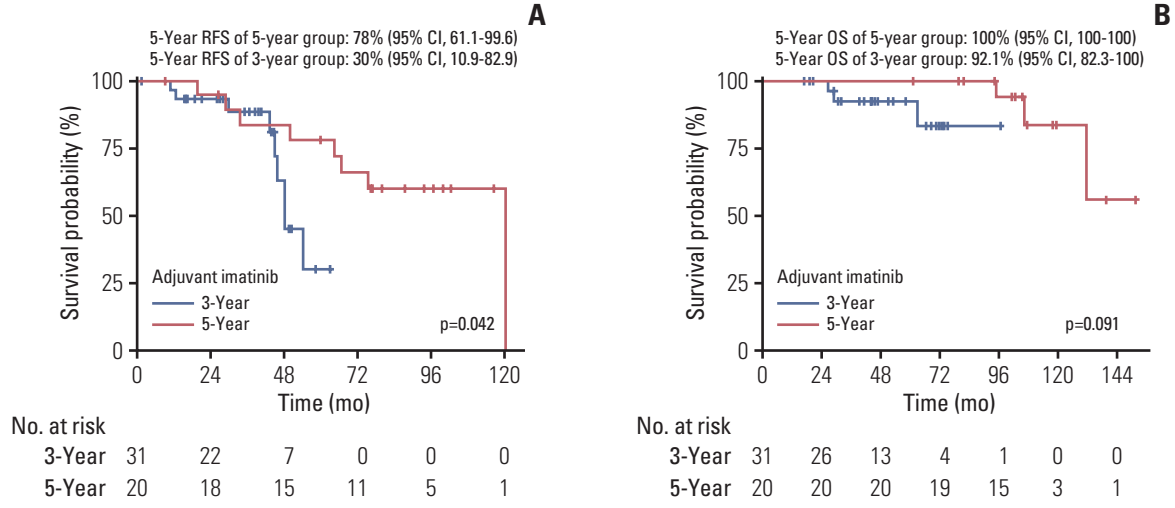

Fig. 3. Comparison of RFS (A) and OS (B) according to adjuvant imatinib treatment duration. CI, confidence interval; OS, overall survival; RFS, recurrence-free survival.

of the risk factors, including age, location of the tumor, largest tumor diameter, mitotic count, gene mutation type, and duration of treatment. A p-value < 0.05 was considered statistically significant. All data analysis was performed using R statistical software ver. 4.0.1 (R Core Development Team, Vienna, Austria).

Results

1. Characteristics of the study patients and tumor rupture

Table 1 shows the baseline characteristics of the study patients. All patients were Korean. Thirty-four patients (66%) were male, and the median age was 58 years (range, 24 to 79 years). There were no significant differences in the baseline characteristics between the 3-year group and the 5-year group, although the proportion of patients who had a non-stomach primary tumor tended to be higher in the 5-year group (80%) than the 3-year group (54.8%) (p=0.125). All patients were included in the high-risk group, according to the modified NIH criteria due to tumor rupture. According to the Armed Forces Institute of Pathology risk criteria, most of the patients were classified into the 6a group (S1 Table) [16]. The most common genotype was a *KIT* exon 11 mutation: 72% in the 3-year group and 95% in the 5-year group. There was no patient with the *PDGFRA* D842V mutation.

The features related to GIST rupture according to Nishida's classification are summarized in S2 Table. The most common type was tumor fracture and/or tumor spillage (n=13, 41%) and gastrointestinal perforation through the tumor (n=9,

Table 3. Univariate and multivariate analysis of recurrence-free survival

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (yr)				
≤ 60	Reference			
> 60	0.97 (0.34-2.81)	0.960	-	-
Location of tumor				
Stomach	Reference			
Non-stomach	0.61 (0.21-1.74)	0.353	-	-
Largest tumor diameter (cm)				
> 10 (median)	Reference			
≤ 10	0.87 (0.32-2.33)	0.783	-	-
Mitotic count (/50 HPFs)				
> 7 (median)	Reference		Reference	
≤ 7	0.31 (0.11-0.89)	0.030	0.20 (0.06-0.67)	0.009
Gene mutation				
Others	Reference		Reference	
KIT exon 11	0.34 (0.1-1.12)	0.076	0.38 (0.09-1.52)	0.170
Treatment duration (yr)				
3	Reference		Reference	
5	0.29 (0.09-1.01)	0.052	0.25 (0.06-1.05)	0.058

CI, confidence interval; HPF, high-power field; HR, hazard ratio.

45%) in the 3- and 5-year groups, respectively.

2. Treatment profiles

The median follow-up duration of the survivors was 43.8 months (range, 16.0 to 95.7 months) and 104.2 months (range, 60.0 to 149.3 months) in the 3- and 5-year group, respectively. Overall, 17 (54%) and 12 (60%) patients completed the scheduled treatment, and six (19.3%) and eight (40%) patients discontinued treatment early in the 3- and 5-year group, respectively (Table 2). The median adjuvant duration of the 5-year group was 60.0 months (range, 2.3 to 60.0 months), and the median adjuvant duration of the 3-year group except those with ongoing adjuvant treatment was 36.0 months (range, 0.78 to 36.0 months).

In the overall study population, disease recurrence was documented in 17 patients (9 in the 3-year group; 8 in the 5-year group) including two patients whose disease recurred while receiving adjuvant imatinib. Among them, three patients showed a locoregional recurrence (2 in the 3-year group and 1 in the 5-year group), and 14 patients showed distant recurrence (7 patients in each group). The most common distant recurrence site was the peritoneum (6 in the 3-year group and 4 in the 5-year group) followed by the liver (1 in the 3-year group and 3 in the 5-year group).

Most of the patients were treated with 400 mg imatinib once daily for first-line treatment after recurrence, except two patients whose disease recurred while they were receiving adjuvant imatinib. Of these two patients, one had a *KIT* exon 17 mutation and was treated with sunitinib, and the other had a *KIT* exon 11 mutation and was treated with 600 mg of imatinib.

3. Survival outcomes

The 5-year RFS and OS of all patients were 61.4% (95% confidence interval [CI], 46.4 to 81.2) and 95.7% (95% CI, 89.0 to 100), respectively (Fig. 2). In the survival analysis according to treatment duration, the 5-year group showed better 5-year RFS than the 3-year group (78% vs. 30%, p=0.042) (Fig. 3A), while OS was comparable between the two groups (Fig. 3B). Among patients who had a *KIT* exon 11 mutation (n=40), the 5-year group still showed a trend toward a better RFS (p=0.089; 4-year RFS, 83.3% vs. 61.5%, respectively) (S3 Fig.).

In the multivariate analysis, low mitotic count (≤ median) was independently associated with favorable RFS (hazard ratio [HR], 0.20; 95% CI, 0.06 to 0.67; p=0.009), while the 5-year imatinib treatment was marginally associated with a favorable RFS (HR, 0.25; 95% CI, 0.06 to 1.05; p=0.058) (Table 3).

4. Toxicity profiles and dose modification

Detailed toxicity profiles are described in S4 Table. There were no statistically significant differences in the frequency of toxicity between the 3-year and 5-year groups. Permanent dose modification (300 mg/day or 200 mg/day) was

required in four patients (12%) in the 3-year group, and three patients (15%) in the 5-year group. None of the patients in the 3-year group discontinued treatment due to toxicity; however, four patients in the 5-year group eventually discontinued treatment prematurely due to intolerable toxicity despite dose modification (n=3) or steroid therapy (n=1) (Table 2). All of the toxicities leading to treatment discontinuation in the 5-year group occurred within the first year of imatinib initiation; grade 3 skin toxicity (at 6 months), grade 3 nausea and vomiting (at 2 months), grade 3 neuropathy (at 9 months), and grade 3 fatigue (at 12 months).

Discussion

In this registry-based retrospective study, we analyzed the safety and efficacy profiles of 5 years of imatinib compared to the standard 3 years of imatinib for patients with GIST rupture following surgical resection. The 5-year imatinib treatment exhibited favorable RFS (78% vs. 30% at 5 years, p=0.042) with an association with a reduced risk of recurrence in multivariate analysis (HR, 0.25; p=0.058). Furthermore, the frequency of adverse events in the 5-year group was not significantly different from that of the 3-year group. To our knowledge, this is the first study to highlight the feasibility of extended imatinib therapy based on the systematic definition of GIST rupture proposed by Nishida et al. [15], which may provide evidence and support for conducting future prospective studies.

Joensuu et al. [17] showed that even among patients with the same high risk according to the modified NIH risk classification, the 10-year recurrence rate varied widely from 30% to 100%; the authors also showed that the recurrence rate of patients with rupture was higher than that of patients without rupture, regardless of tumor size, location, and mitosis count. Accordingly, a recent report on the real-world clinical outcomes of high-risk patients treated with adjuvant 3-year adjuvant imatinib showed that patients with rupture had a higher recurrence rate than did those without rupture even after adjuvant treatment [14]. These findings suggest that the prognosis of patients with GIST rupture differs from that of high-risk GIST patients without rupture, and that patients with GIST rupture may need more intensive or longer treatment to reduce the risk of recurrence.

While our study suggested the potential benefit of prolonging the duration of adjuvant imatinib from 3 to 5 years, the follow-up analysis of the SSG XVIII/AIO trial showed that 3-year imatinib treatment was not significantly associated with a reduced risk over 1-year adjuvant imatinib treatment in patients with GIST rupture [18]. Therefore, we assume that while 3-year administration of imatinib may not be sufficient to reduce the recurrence risk relative to 1-year treatment, prolonged adjuvant imatinib over 3 years appears to lead to a reduced risk of recurrence. Currently, two randomized clinical studies are ongoing to evaluate the clinical utility of prolonged adjuvant imatinib (NCT02413736, NCT02260505); however, these studies did not focus specifically on patients with GIST rupture. Given the low incidence of GIST tumor rupture and the associated difficulty of conducting a prospective study focusing on GIST tumor rupture patients [19,20], our results provide valuable insights into the optimal duration of adjuvant imatinib for patients with GIST rupture.

Generally, it is well known that the benefits of adjuvant imatinib are greater in patients with a *KIT* exon 11 mutation than in patients with a *KIT* exon 9 mutation [21], and there is no established strategy of adjuvant imatinib treatment for those with non-exon 11 mutations or wild-type GIST [1]. In this study, to avoid the potential bias caused by different genotypes, additional subgroup analysis was conducted only on patients who had *KIT* exon 11 mutation, and it revealed the same trend of RFS benefit from 5-year imatinib.

There are several concerns with prolonged imatinib treatment. First, long-term exposure to imatinib would have the potential to increase the frequency of adverse events. In our study, all toxicities leading to imatinib discontinuation occurred in the 5-year group. However, since all of the patients discontinued treatment due to toxicity within a year from the initiation of imatinib administration, the enrichment of patients who discontinued imatinib due to adverse events in the 5-year group does not appear to be attributable to prolonged imatinib administration. Besides, in our institution, there have been recent advances in the management of imatinib toxicities and improvement of treatment outcomes associated with them [22,23]. Considering that patients in the 5-year imatinib group were treated about 5 years earlier than those in the 3-year imatinib group, the 3-year group might have received better management for imatinib toxicities, and it could be another reason for differences in the proportion of early discontinuation between the two groups.

Patient medication adherence is an essential issue because it is highly associated with a successful treatment outcome [24]. In the PERSIST-5 trial, nearly 50% of patients discontinued imatinib earlier than the scheduled 5-year period; 20% of them discontinued treatment due to the patient's choice [13]. Therefore, to maintain medication adherence and obtain optimal treatment effects of adjuvant therapy, attending physicians should focus on educating patients about their disease status, the benefits of adjuvant therapy, and how to manage the side effects of imatinib.

One of the important aspects of our study is that we comprehensively applied the systematic definition of ruptured GIST by Nishida et al. [15]. Most of the previous studies of

GIST rupture have not clearly described how they defined GIST rupture, which may have contributed to the varying incidence of GIST rupture [6,15,17,25,26]. Future studies should be performed based on a systematic definition of GIST rupture such as Nishida's classification to accurately define the patient subgroup with rupture and avoid misclassifications.

Some limitations of this analysis should also be considered. First, because our study was retrospective and based on a single-center experience, our results may be subject to selection bias. In particular, the patient classification of our study was not prospectively determined, which may limit the interpretation and generalization of our findings. Another limitation is the small sample size. Indeed, the small number of study patients appears to be the main reason for the difference in RFS not reaching statistical significance in multivariate analysis. Nevertheless, considering the rarity of ruptured GIST, the number of patients included in our study was relatively larger than that of the previous studies of ruptured GIST [15,20,27,28]. Finally, we were not able to show an OS difference according to the treatment duration. Whether the RFS benefit of 5-year imatinib treatment could be translated into an OS benefit should be further confirmed with a longer follow-up.

In conclusion, 5 years of imatinib treatment following surgical resection of ruptured GIST may be feasible and associated with favorable survival outcomes with manageable toxicity profiles. Our findings warrant validation and confirmation in future studies.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was approved by the Institutional Review Board (IRB) of Asan Medical Center, Korea (approval number: 2021-1489), and the study was conducted according to the principles of the Helsinki declaration. Due to the nature of this retrospective study, the requirement of obtaining informed consent was waived by the IRB.

Author Contributions

Conceived and designed the analysis: Kang YK.
Collected the data: Ryu MH, Bang YH, Kim HD, Lee HE, Kang YK.
Contributed data or analysis tools: Kang S, Ryu MH, Bang YH, Kim HD, Kang YK.
Performed the analysis: Kang S, Ryu MH, Kim HD, Lee HE, Kang YK.
Wrote the paper: Kang S, Ryu MH, Kim HD, Kang YK.

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Conflicts of Interest

Nothing directly related to this work. Outside of this work, YKK has served as a consultant for ALX Oncology, Zymeworks, Amgen, Novartis, Macrogenics, Daehwa, Blueprint, Surface Oncology, BMS, and Merck (MSD).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1133
Cancer Res Treat. 2022;54(4):1175-1190

Original Article

Histopathologic and Molecular Biomarkers of PD-1/PD-L1 Inhibitor Treatment Response among Patients with Microsatellite Instability–High Colon Cancer

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Purpose Recent clinical trials have reported response rates < 50% among patients treated with programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) inhibitors for microsatellite instability–high (MSI-H) colorectal cancer (CRC), and factors predicting treatment response have not been fully identified. This study aimed to identify potential biomarkers of PD-1/PD-L1 inhibitor treatment response among patients with MSI-H CRC.

Materials and Methods MSI-H CRC patients enrolled in three clinical trials of PD-1/PD-L1 blockade at Asan Medical Center (Seoul, Republic of Korea) were screened and classified into two groups according to treatment response. Their histopathologic features and expression of 730 immune-related genes from the NanoString platform were evaluated, and a machine learning–based classification model was built to predict treatment response among MSI-H CRCs patients.

Results A total of 27 patients (15 responders, 12 non-responders) were included. A high degree of lymphocytic/neutrophilic infiltration and an expansile tumor border were associated with treatment response and prolonged progression-free survival (PFS), while mucinous/signet-ring cell carcinoma was associated with a lack of treatment response and short PFS. Gene expression profiles revealed that the interferon-γ response pathway was enriched in the responder group. Of the top eight differentially expressed immune-related genes, *PRAME* had the highest fold change in the responder group. Higher expression of *PRAME* was independently associated with better PFS along with histologic subtypes in the multivariate analysis. The classification model using these genes showed good performance for predicting treatment response.

Conclusion We identified histologic and immune-related gene expression characteristics associated with treatment response in MSI-H CRC, which may contribute to optimal patient stratification.

Key words Microsatellite instability, Colonic neoplasms, Immune checkpoint inhibitors, Biomarker, Transcriptome profiles, Histology, Machine learning

Introduction

Colorectal cancer (CRC) is a common neoplasm that accounts for approximately 10% of malignancies diagnosed worldwide, and about 20% of CRC patients are found to have stage IV disease at the time of the initial diagnosis [1,2]. The clinical outcomes of metastatic CRC have improved in the last few decades with a tailored approach of systemic treatment combining targeted agents with cytotoxic chemotherapy based on molecular biomarkers. With these advancements, the median overall survival of patients with stage IV CRC has been extended to 30 months [2].

Deficient mismatch repair (dMMR)/microsatellite instability–high (MSI-H) tumors account for about 5% of patients with stage IV CRC [3,4]. Microsatellite instability (MSI) is a genetic mutational signature of simple and short repeats of DNA sequences caused by the failure of cellular mismatch repair (MMR) systems, which is referred to as the dMMR status. While the majority of sporadic CRC cases with dMMR/MSI-H features arise from somatic epigenomic alteration, such as *MLH1* gene promoter methylation in the CpG island hypermethylator phenotype leading to silencing of *MLH1* expression, some cases arise in Lynch syndrome patients harboring germline mutations of MMR genes with additional hits leading to biallelic loss of MMR genes [5]. As the dMMR/MSI-H status leads to the accumulation of frameshift

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Received October 21, 2021 **Accepted** January 11, 2022
Published Online January 12, 2022

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mutations generating increased tumor neoantigen burden, the dMMR/MSI-H status was thought to be a predictive biomarker of response to immunotherapy. Accordingly, several phase 2 and phase 3 clinical trials have established dMMR/MSI-H as a predictive biomarker of response to immune checkpoint inhibitors (ICIs) for the treatment of metastatic CRC [6-8].

Although ICI treatment has been associated with encouraging results against dMMR/MSI-H metastatic CRC, a considerable portion of patients still do not respond to the treatment, and variable clinical outcomes have been reported from different clinical trials, with response rates to ICI in phase 2 clinical trials for pre-treated solid tumors ranging from 30% to 40% [6,7]. Although pembrolizumab as a front-line treatment was associated with a high response rate of 43.8% in a phase 3 trial comparing it with conventional chemotherapy, survival analysis revealed that about 40% of patients progressed within the first 4 months. This implies the presence of a subpopulation among dMMR/MSI-H CRC patients who show primary resistance to immunotherapy [8].

Accordingly, new biomarkers for predicting response or primary resistance to ICIs among dMMR/MSI-H CRC patients are needed to make better clinical decisions and better understand the mechanism of action or resistance to immunotherapy. In the study described and discussed herein, we comprehensively evaluated the characteristics of dMMR/MSI-H metastatic CRC according to the response to programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) blockade, including histopathologic features and immune-related gene expression characteristics.

Materials and Methods

1. Patients and study group

Patients diagnosed with dMMR/MSI-H colorectal adenocarcinoma and treated with ICIs at Asan Medical Center (Seoul, Republic of Korea) between October 2015 and February 2020 were screened for analysis. The patients were either treated with pembrolizumab or were enrolled in one of the phase 2 investigator-sponsored clinical trials of other ICIs, including NCT03150706 (avelumab for previously treated dMMR/MSI-H or *POLE*-mutant colorectal cancer) and NCT-03435107 (durvalumab for previously treated dMMR/MSI-H or *POLE*-mutant colorectal cancer) [9,10]. Patients were treated with the conventional ICI doses: intravenous pembrolizumab 200 mg every 3 weeks, avelumab 10 mg/kg intravenously every 2 weeks, and durvalumab 1,500 mg (20 mg/kg for patients with body weight ≤ 30 kg) intravenously every 4 weeks as described in each of the study protocols.

Among the screened patients, those who had finished the study treatment or had undergone disease evaluation once or more at the time of data collection (March 2020) were included in the study. The patients were then divided into two groups according to ICI treatment response (responder group vs. non-responder group). The responders were patients who received ICI treatment for > 4 months without progression after two consecutive disease evaluations within 8 to 9 weeks. The criteria were based on the results from a previous trial of pembrolizumab for pre-treated metastatic CRC patients. From the KEYNOTE-164 study, the median progression-free survival (PFS) was 4.1 months for dMMR/MSI-H metastatic CRC patients who received pembrolizumab after one or more prior treatments [11]. Patients who withdrew consent due to symptomatic deterioration before disease evaluation were included in the non-responder group.

2. Ascertainment of MSI status

Each patient was required to undergo a test for dMMR or MSI-H, or a next-generation sequencing (NGS) test for *POLE* mutation, which was also one of the eligibility criteria of the two trials. The tests for dMMR and MSI-H included MMR protein immunohistochemistry (IHC), polymerase chain reaction (PCR) fragment assay, and targeted NGS in which the MSI-H status was determined by a tumor mutational burden (TMB) ≥ 40 and an I-index (insertion/deletion mutation to whole mutation percentage) ≥ 9%, as previously described [12].

In current practice, the test methods for MMR and MSI-H are not standardized across patients, and previous studies have reported a considerable degree of discrepancy between MMR and MSI-H test results [9,13]. Therefore, to assure the MSI status of patients before biomarker analysis, we reviewed the results of IHC, PCR fragment assay, and targeted NGS. In cases of inconsistency among the results of IHC, PCR, and NGS, the MSI status was determined according to the results of a thorough review. NGS results were prioritized for determining the MSI status because our NGS testing based on TMB performs well even at low tumor cellularity (10% or more) compared with PCR testing, which requires a tumor cellularity > 20% to yield reliable results. IHC results are often affected by tissue quality, and misinterpretations of IHC results are known to be the most common cause of discrepancies between IHC and molecular testing [14]. Therefore, we prioritized NGS results over PCR and IHC results. For those without NGS results, a pathologist (J.K.) determined MSI status by reviewing the IHC and/or PCR results. Analyses of histopathologic characteristics and immune gene expression profiles were performed for each patient with a verified MSI-H status.

3. Clinical and histopathologic variables

Baseline clinical characteristics, including initial stage, previous treatments, mutational status of *KRAS*, *NRAS*, and *BRAF*, follow-up duration, and survival status, were obtained from the clinical trial database. Histopathologic features were evaluated, including histologic cancer subtypes, neutrophil infiltration grades, lymphocyte infiltration grades, tumor borders, Crohn-like lymphoid aggregate status, and lymphovascular invasion. Additional PD-L1 22C3 IHC (DAKO/Agilent, Santa Clara, CA) analyses were performed for cases with sufficient archival tissue for staining. PD-L1 results were interpreted by a pathologist (J.K.) by combined proportion score (CPS), defined as the ratio of all PD-L1–positive cells to viable tumor cells [15]. PD-L1 results were considered as positive if the CPS was ≥ 1.

$$CPS = \frac{No.\ of\ PD\text{-}L1\ positive\ cells}{No.\ of\ all\ viable\ tumor\ cells} \times 100$$

4. IHC for PRAME

Differential expression of *PRAME* according to treatment response was examined using tumor tissues obtained during surgery for routine diagnostic pathologic examinations were analyzed with IHC for PRAME using anti-PRAME antibody (1:1,000, rabbit monoclonal, clone EPR20330, catalog No. ab219650, Abcam, Cambridge, UK). Briefly, 4-μm-thick sections of formalin-fixed, paraffin-embedded (FFPE) tissues were obtained with a microtome, transferred onto silanized charged slides, dried for 10 minutes at room temperature, and incubated at 65°C for 20 minutes. The tissue sections were processed by heat-induced epitope retrieval method using Cell Conditioning 1 buffer for 64 minutes and incubated for 32 minutes with the anti-PRAME antibody in a BenchMark XT automatic immunostaining device (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions. Antigen-antibody reactions were visualized using the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems). Counterstaining was performed using Ventana Hematoxylin II for 12 minutes and Ventana Bluing Reagent for 4 minutes. Finally, all slides were removed from the stainer, dehydrated, and coverslipped for microscopic examination. Slides in which > 1% of cancer cells were immunostained for PRAME were considered as positive for PRAME.

5. Differential gene expression and pathway analyses

Total RNA was extracted from the FFPE tissues of each patient. Quality control (QC) of each sample was performed using a Denovix DS 11 AATI Fragment Analyzer (Wilmington, DE) to evaluate the quantity and condition of the isolated RNA before analysis. Total RNA of approximately 100 μg was used for gene expression analysis, and the input amount of total RNA was increased for samples with excess RNA strand fragmentation. Immune-related gene expression profiling was performed using the NanoString nCounter platform (NanoString Technologies, Seattle, WA) with a PanCancer Immune Profiling Panel composed of 730 immune-related genes and 40 internal reference genes. The prepared RNA was thawed just before analysis and mixed with the reporter code set and probe set in a hybridization buffer. The hybridization process was performed at 65°C for 16 to 24 hours and then moved to a NanoString nCounter preparation station for cleansing of inadequately hybridized probes, and the properly hybridized transcript-probe complexes were immobilized on the cartridge. Finally, the fixed samples on the cartridge were scanned and read by the NanoString nCounter Digital Analyzer (NCT-DIGT-120) and recorded as reporter code count files, which were analyzed in nSolver software (NanoString Technologies) for the QC process, including image QC, binding density QC, as well as positive and negative control QC. The expression levels of each gene in the samples with adequate QC data were normalized in the nSolver software using a positive control and housekeeping genes. The immune cell type was annotated based on the annotation file provided by NanoString Technologies for the nCounter PanCancer Immune Profiling Panel.

From the normalized gene expression levels from the NanoString nCounter assay using the PanCancer Immune Profiling Panel, differential gene expression analyses were performed in responders and non-responders by comparing the normalized expression levels of each gene by Wilcoxon rank-sum test. The nominal p-values were initially adjusted according to the false discovery rate (FDR); however, all genes had an FDR of > 0.05 due to the small sample size. Therefore, we considered using fold change (FC) and the genes with nominal p-values < 0.05 and $\log_2$-transformed fold change ($\log_2$FC) > 0.5 or < −0.5 were considered as candidate genes. To identify the functional ontology of the candidate genes, we performed unsupervised hierarchical clustering and gene set enrichment analysis.

6. Predictive modeling using machine learning and internal validation

Random forest (RF), a machine learning classification modeling approach, was utilized using the Python package sklearn v0.24.1 to generate a predictive model for classifying patients into PD-1/PD-L1 blockade response groups based on the genes with significant differential expression. Validation of the predictive model was performed according to the following steps: Step 1: For the i^{th} sample (i=1, …, n), divide the i^{th} sample from whole data as the training set and the remaining (n-1) patients as the validation set; Step

2: Apply classification models to the training set to fit a prediction model; Step 3: Apply the fitted prediction model to the validation set and calculate the predicted probabilities; Step 4: Repeat steps 1-3 for all n samples; Step 5: After completing the cross-validation, combine the predicted probability values of all samples calculated using the leave-one-out cross-validation (LOOCV) method. The overall accuracy was evaluated, and a single receiver operating characteristic curve was drawn, and the area under the curve (AUC) value was calculated.

7. Statistical analysis

For descriptive analysis of categorical variables, the chi-squared test or Fisher exact test was performed in R ver. 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria), as appropriate. The Wilcoxon rank-sum test was used to evaluate the significane of differences in continuous variables between groups. Survival was estimated with the Kaplan-Meier method and was compared by log-rank tests using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) or R ver. 4.0.3. PFS was defined from the initiation of the study treatment until objective disease progression according to Response Evaluation Criteria in Solid Tumor v1.1 or death due to any cause, whichever came first. Multivariate logistic regression analysis was performed in the response group using the logistf v1.24 package in R. Multivariate Cox regression analysis was performed for PFS using the survival v.3.2-7 package in R. Two-sided p-values < 0.05 were considered statistically significant.

Results

1. Patient screening and study design

A total of 50 patients who were enrolled and treated with PD-1/PD-L1 inhibitors between October 20, 2015, and February 27, 2020, at Asan Medical Center were screened. The median age was 59 years (range, 21 to 85 years), 40 patients (80%) were male, and all patients had Eastern Cooperative Oncology Group performance status of 0 or 1. Twenty-seven patients (54%) had initially metastatic disease at enrollment, while 23 patients (46%) had recurrence after surgical resection and adjuvant chemotherapy as needed. Nine patients (18%) received ICIs as the first-line regimen for palliative treatment, 18 patients (36%) as second-line therapy, and 23 patients (46%) as at least the third line. Among 33 patients with available tumor burden data, 17 (51.5%) had liver or lung metastases, and 26 patients (78.8%) had distant metastases elsewhere. Twenty-patients (40%) received pembrolizumab, while 13 (26%) and 17 patients (34%) received avelumab and durvalumab, respectively. The proportions of responders according to ICI were similar, although durvalumab was associated with the highest proportion of responders (9 patients, 52.9%) compared with pembrolizumab (8 patients, 40%) and avelumab (5 patients, 38.5%). At a median follow-up duration of 22.4 months, the median PFS was 3.7 months; 44% of patients (n=22) were categorized as responders (i.e., treated with ICIs for > 4 months without progression). The clinical characteristics of the 50 patients according to treatment response are summarized in S1 Table.

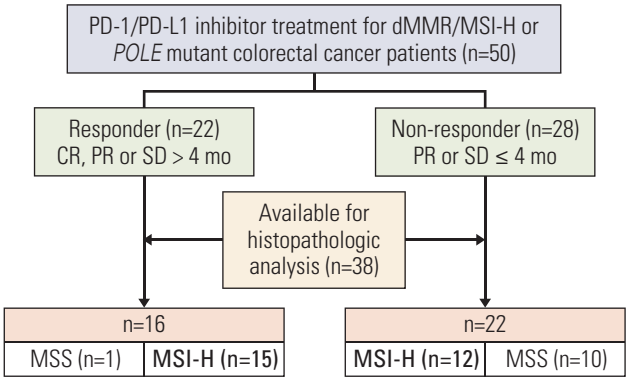

Fig. 1. Case selection and study design according to the response to immune checkpoint inhibitor treatment among patients with deficient mismatch repair (dMMR)/microsatellite instability–high (MSI-H) metastatic colorectal cancers. CR, complete response; MSS, microsatellite stable; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; PR, partial response; SD, stable disease.

Among the 50 patients, 27 patients (15 responders vs. 12 non-responders), who were verified as having a dMMR/MSI-H tumor and adequate archival tissue, were included in this study (Fig. 1). At a median follow-up duration of 32.4 months, the median PFS of the 27 patients was 32.8 months, and the objective response rate was 44.4% (95% confidence interval [CI], 27.6 to 62.7). Immune-related gene expression analysis using NanoString was performed for 19 patients with dMMR/MSI-H (11 responders vs. 8 non-responders) after quality assurance of the tissue RNA.

2. Histopathologic determinants of PD-1/PD-L1 blockade response in dMMR/MSI-H CRCs

Histopathologic tumor features were compared among 27 patients with confirmed dMMR/MSI-H status according to treatment response (15 responders vs. 12 non-responders) (Table 1). The histologic CRC subtype distribution was significantly different between the two groups (p=0.003, Fisher exact test), with most of the patients with mucinous adenocarcinoma or signet-ring cell carcinoma (Fig. 2A) in the non-responder group. Compared with the non-responder group,

Table 1. Comparison of histopathologic characteristics among patients with dMMR/MSI-H according to treatment response

Histopathologic characteristic	Responder (n=15)	Non-responder (n=12)	p-value
Histologic subtype			
Well-differentiated or moderately-differentiated	10 (66.7)	4 (33.3)	0.003
Poorly-differentiated	4 (26.7)	0	
Mucinous or signet-ring cell carcinoma	1 (6.6)	8 (66.7)	
Neutrophil infiltration grade			
0 or 1	8 (53.3)	11 (91.7)	0.043
2 or 3	7 (46.7)	1 (8.3)	
Lymphocyte infiltration grade			
0 or 1	6 (40.0)	12 (100)	0.001
2 or 3	9 (60.0)	0	
Crohn-like lymphoid aggregate			
Absent	6 (40.0)	9 (75.0)	0.120
Present	9 (60.0)	3 (25.0)	
Tumor border			
Expansile	8 (53.3)	0	0.003
Infiltrative	7 (46.7)	12 (100)	
Lymphovascular invasion			
Absent	8 (53.3)	7 (58.3)	> 0.99
Present	7 (46.7)	5 (41.7)	
PD-L1 status	12	7	
PD-L1 immunohistochemistry			
Negative	2 (16.7)	3 (42.9)	0.305
Positive	10 (83.3)	4 (57.1)	
Combined proportion score	5 (0-30)	5 (0-15)	0.290
***RAS* and *RAF* mutation**	13	11	
KRAS	7 (53.9)	5 (45.5)	0.827
NRAS	0	1 (9.0)	
BRAF V600E	1 (7.6)	0	
None	5 (38.5)	5 (45.5)	
Tumor mutational burden	7	6	
TMB (mutations/Mb)	110.9 (57.8-176.6)	101.6 (50.0-135.9)	0.656

Values are presented as number (%) or median (range). dMMR, deficient DNA mismatch repair; MSI-H, microsatellite instability–high; PD-L1, programmed death-ligand 1; TMB, tumor mutational burden.

patients in the responder group had abundant infiltration of immune cells, such as lymphocytes (p=0.001, Fisher exact test) and neutrophils (p=0.043, Fisher exact test) (Fig. 2A). We also found that the tumor border status was associated with treatment response, as an expansile tumor border (Fig. 2A) was significantly associated with treatment response (p=0.003, Fisher exact test); notably, none of the patients in the non-responder group had expansile borders. Interestingly, PD-L1 positivity and TMB were not significantly associated with the response to PD-1/PD-L1 blockade (Table 1). Also, there was no significant difference in the proportion of patients harboring *RAS* or *RAF* mutations between the two response groups (p=0.827, Fisher exact test). There were no significant differences in histopathologic features between dMMR/MSI-H tumors and microsatellite stable (MSS) tumors. TMB was significantly higher in dMMR/MSI-H tumors compared with MSS tumors, with median TMBs 104.7/Mb (range, 50.0/Mb to 176.0/Mb) and 12.5/Mb (range, 4.7/Mb to 17.2/Mb), respectively ($p < 0.001$, Wilcoxon rank-sum test) (S2 Table).

In accordance with the initial responsiveness to immunotherapy, PFS was significantly associated with specific histopathologic variables (Fig. 2C, S3 Fig.), as mucinous adenocarcinoma and signet-ring cell carcinoma were associated with a significantly shorter PFS compared with conventional adenocarcinoma (p=0.004, log-rank test). Higher neutrophil

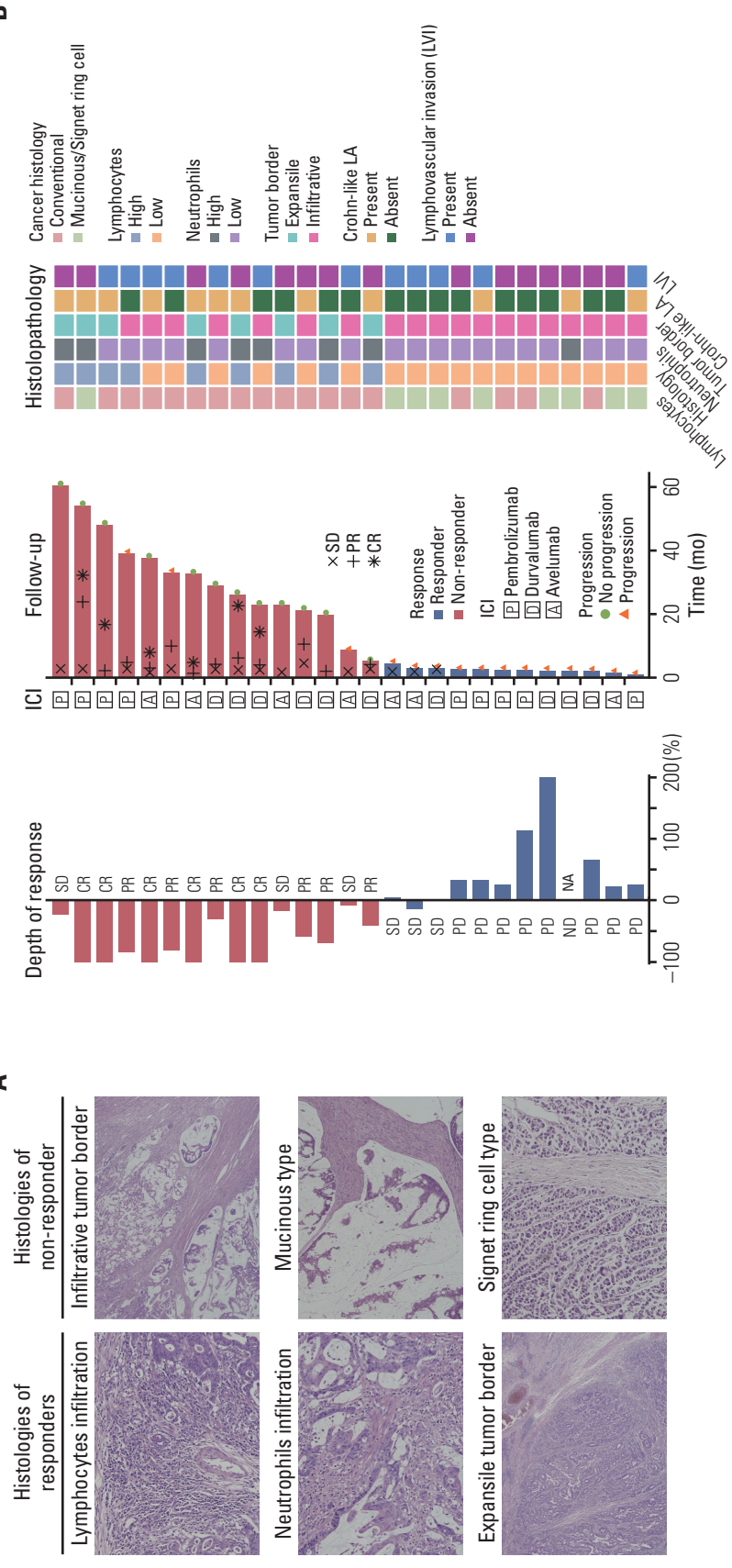

Fig. 2. Histopathologic features associated with ICI response among patients with dMMR/MSI-H CRC. (A) Representative histologic features of MSI-H CRCs among responders and non-responders. High degree of lymphocytic infiltration along the tumor border in the responder group (upper left, ×200). High degree of neutrophil infiltration grade along the tumor-stroma interface in the responder group (middle left, ×200). Expansile tumor border and surrounding of tumor cells by inflammatory cell infiltrates in the responder group (lower left, ×40). Infiltrative tumor border in the non-responder group (upper right, ×40). Mucinous adenocarcinoma with abundant extracellular mucin separating tumor cells from adjacent stroma in the non-responder group (middle right, ×100). Signet-ring cell carcinoma without signs of inflammatory cell infiltration along the tumor-stroma interface in the non-responder group (lower right, ×200). (B) Response status after ICI during follow-up and histopathologic features in patients with MSI-H CRCs. *(Continued to the next page)*

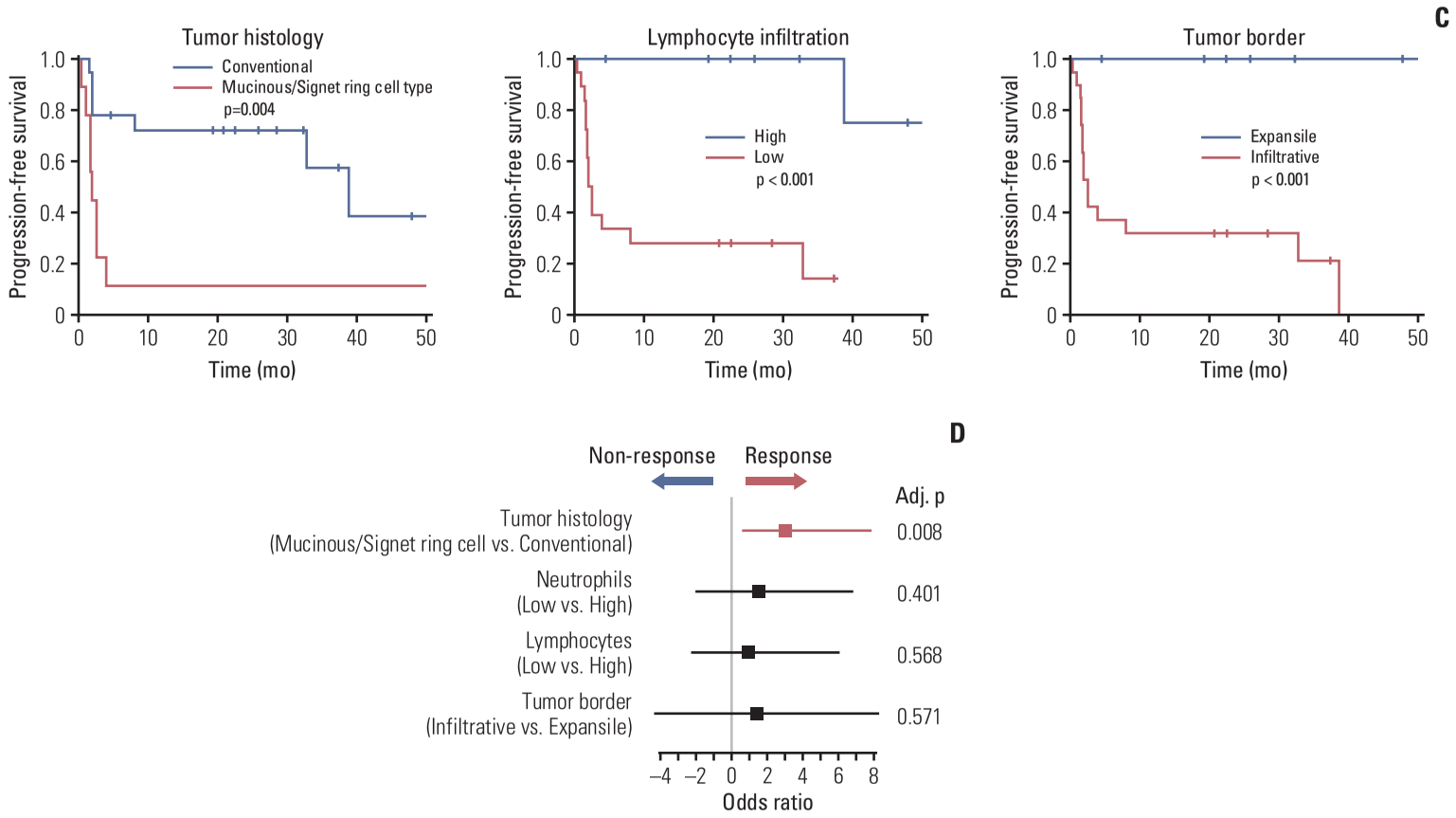

Fig. 2. *(Continued from the previous page)* (C) Progression-free survival (log-rank test) of patients with MSI-H CRCs after ICI according to tumor histology, lymphocyte infiltration, and tumor border. (D) Multivariable logistic regression analysis of treatment response with histopathologic variables. CR, complete response; CRC, colorectal cancer; dMMR, deficient mismatch repair; ICI, immune checkpoint inhibitor; LA, lymphoid aggregate; MSI-H, microsatellite instability–high; ND, not determined; PD, progressive disease; PR, partial response; SD, stable disease.

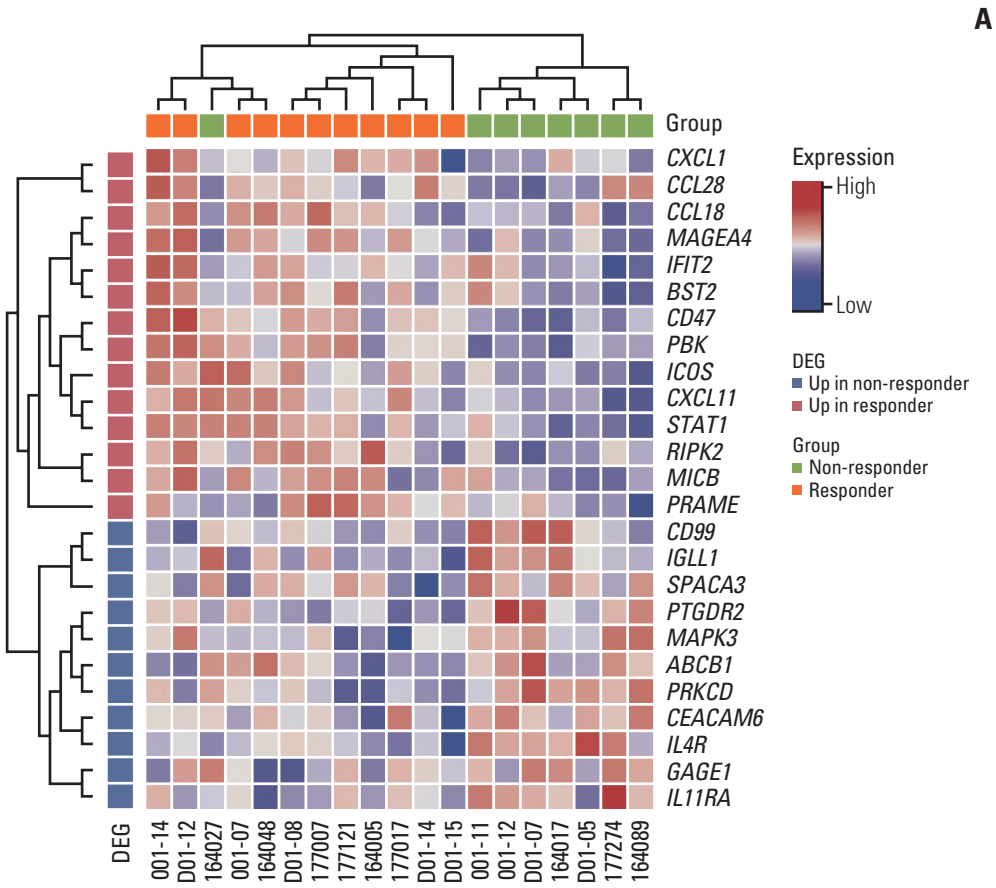

Fig. 3. Differential expression analysis of immune genes between the two groups (11 responders vs. 8 non-responders). (A) Heatmap of differentially expressed immune genes (absolute $\log_2$FC > 0.5 and p < 0.05 by Wilcoxon rank-sum test) between the two groups. *(Continued to the next page)*

infiltration grade (grade 2 to 3 vs. grade 0 to 1, p=0.016, log-rank test) and lymphocyte infiltration grade (grade 2 to 3 vs. grade 0 to 1, p < 0.001, log-rank test), presence of Crohn-like lymphoid aggregates (p=0.013, log-rank test), and expansile tumor border (p < 0.001, log-rank test) were associated with longer PFS. The presence of lymphovascular invasion was not associated with significant differences in PFS. In the multivariate logistic regression analysis, cancer histologic subtype (p=0.008) was independently associated with treatment response (Fig. 2D).

3. Differential expression of immune genes according to blockade responsiveness

Expression levels of the 730 immune genes in the PanCancer Immune Profiling Panel (NanoString Technologies) were compared according to treatment response. Immune cell type profiles were not significantly different between the two groups (S4 Fig.). At the individual gene level, 25 differentially expressed immune genes (p < 0.05, Wilcoxon rank-sum test and absolute $\log_2$FC > 0.5) were identified (Fig. 3A, S5 Table). Using these genes, we performed pathway enrichment analyses to compare the activation of immune-related molecular pathways between the two groups. Responders had elevated activity in pathways, such as those yielding interferon-γ (IFN-γ) and regulation of immune effector process, whereas non-responders had elevated activity in pathways associated with phagocytosis, positive regulation of macrophage activation, and immunoglobulin/B-cell–mediated immune responses (Fig. 3B). When more stringent criteria were applied (p < 0.05, Wilcoxon rank-sum test and absolute $\log_2$FC > 1.0), eight genes remained as differentially expressed (Fig. 3C), among which six genes (*PRAME*, *CCL18*, *CXCL1*, *BST2*, *CXCL11*, and *CCL28*) were specifically expressed in the responder group, and two genes (*CD99* and *ABCB1*) were specifically expressed in the non-responder group (Fig. 3D).

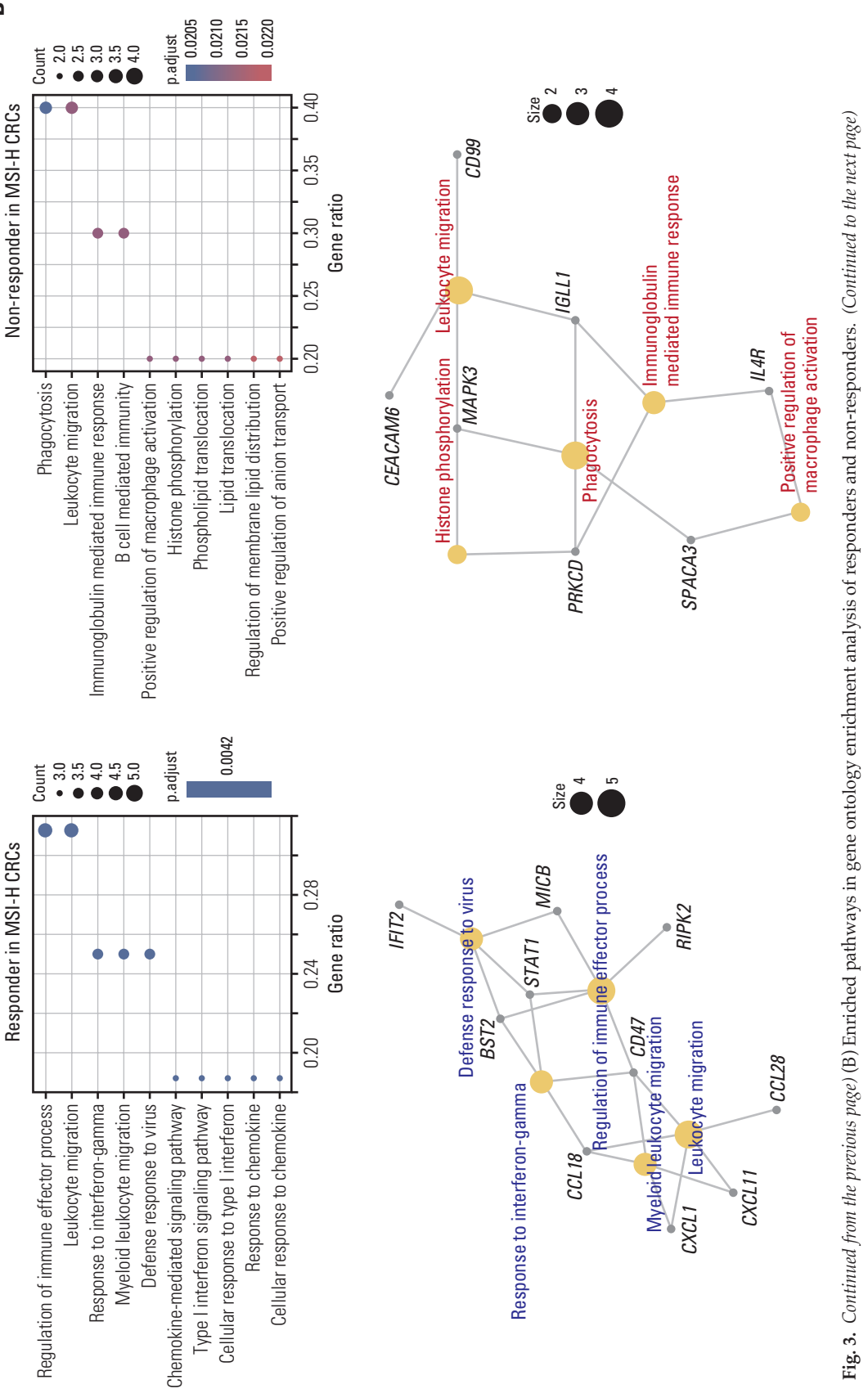

Fig. 3. *Continued from the previous page)* (B) Enriched pathways in gene ontology enrichment analysis of responders and non-responders. *(Continued to the next page)*

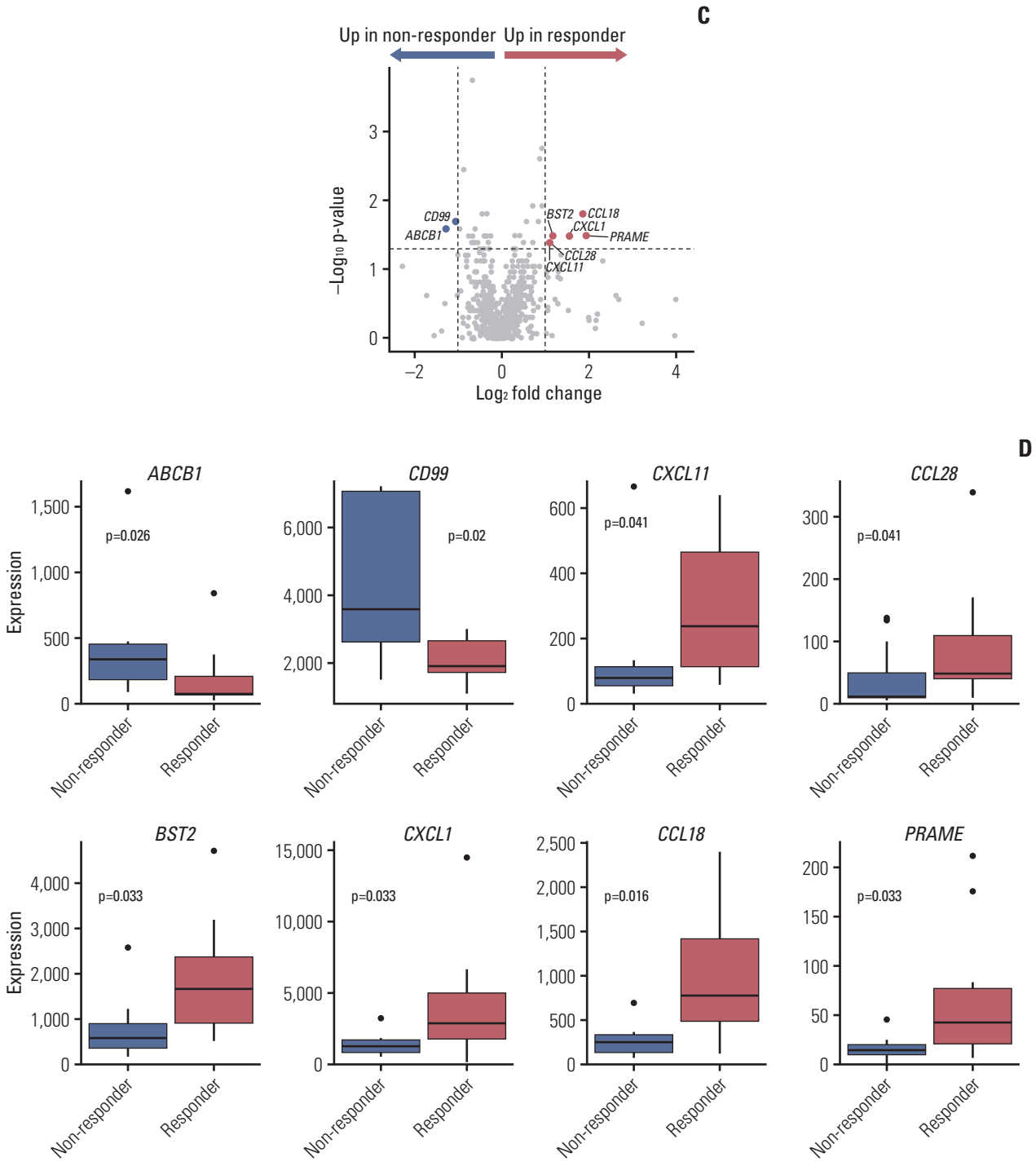

Fig. 3. *(Continued from the previous page)* (C) Volcano plots highlighting genes with significantly higher expression in the responder group and non-responder group (absolute $\log_2$FC > 0.5 and p < 0.05 by Wilcoxon rank-sum test). (D) The comparative expression levels of genes with significantly higher expression in the responder group (*BST2*, *CCL18*, *CCL28*, *CXCL1*, *CXCL11*, and *PRAME*) and the non-responder group (*ABCB1* and *CD99*). CRC, colorectal cancer; DEG, differentially expressed genes; MSI-H, microsatellite instability–high.

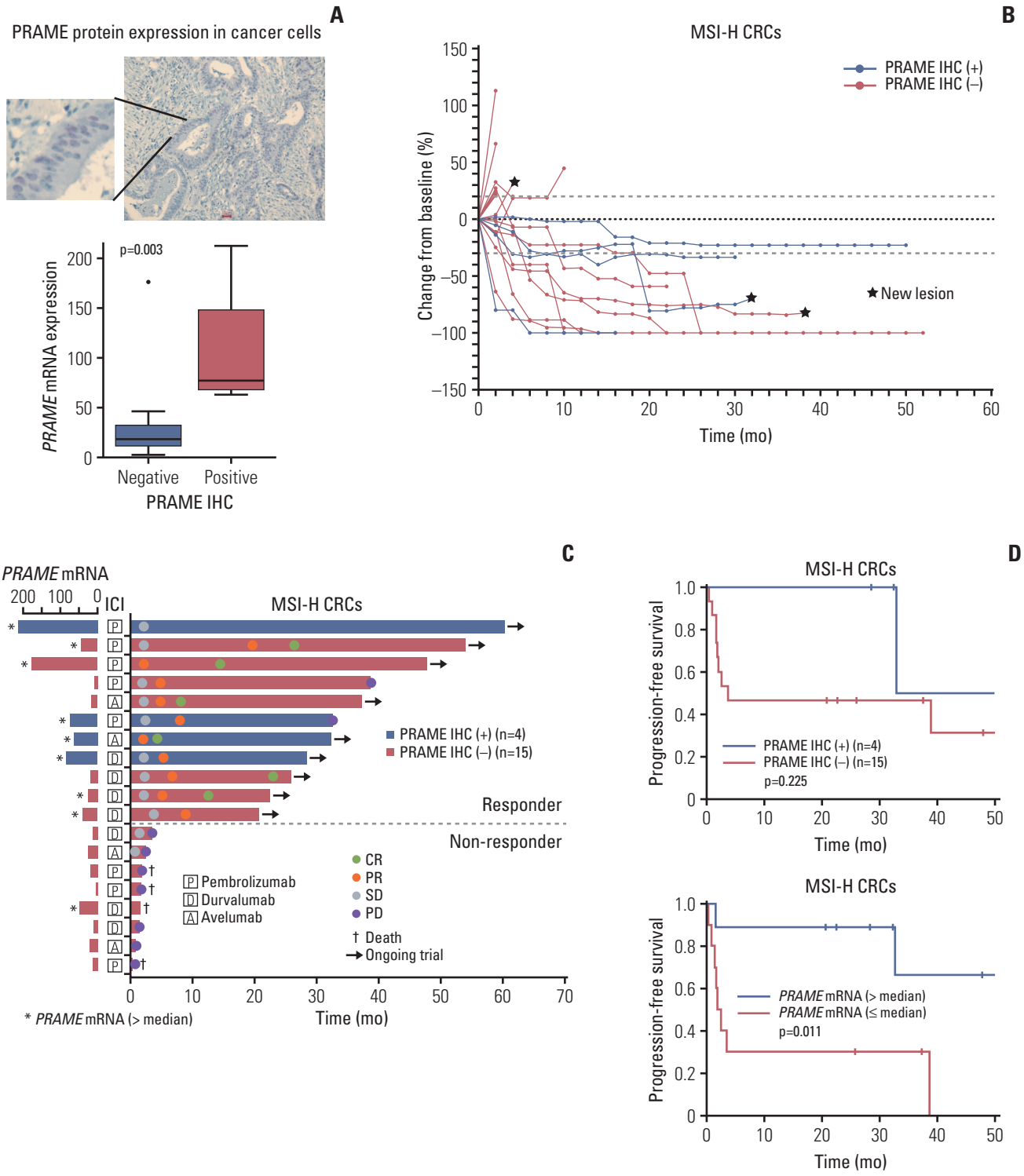

Fig. 4. Association between *PRAME* expression and response to ICIs. (A) Representative IHC results of PRAME with nuclear expression in CRC cells (×100) and significant correlation between protein expression and mRNA expression (p=0.0036, Wilcoxon rank-sum test). (B) Spider plot of the changes in the sum of target lesions from the baseline along ICI treatment with annotation of the PRAME IHC results. (C) Swimmer plot showing the clinical response and duration of ICI treatment with PRAME IHC results and *PRAME* mRNA expression levels. (D) Progression-free survival outcomes according to PRAME protein expression and *PRAME* mRNA expression among patients with MSI-H CRCs after ICI treatment (log-rank test). *(Continued to the next page)*

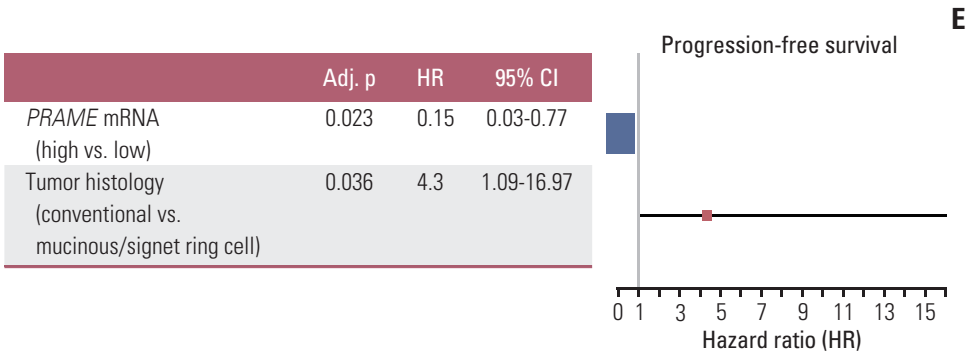

Fig. 4. *(Continued from the previous page)* (E) Multivariable Cox regression analysis for progression-free survival. CI, confidence interval; CR, complete response; CRC, colorectal cancer; ICI, immune checkpoint inhibitor; IHC, immunohistochemistry; MSI-H, microsatellite instability–high; PD, progressive disease; PR, partial response; SD, stable disease.

4. PRAME expression was associated with better response and prolonged survival

Of the six genes specifically expressed in the responder group, *PRAME* showed the highest FC (log_2FC=1.95). To examine the differential expression of *PRAME*, we performed IHC staining of PRAME (S6 Table). The IHC level of PRAME correlated well with the mRNA expression level from the NanoString panel, with a median normalized PRAME expression level of 76.6 (interquartile range, 66.8 to 146.4) among the PRAME-positive patients and 17.8 (interquartile range, 10.6 to 31.7) among the PRAME-negative patients (p=0.003, Wilcoxon rank-sum test) (Fig. 4A). All four PRAME-positive patients were in the responder group, and none were in the non-responder group (Fig. 4B and C). Moreover, except for one patient, all patients with a high mRNA expression of *PRAME* (i.e., higher than the median value) were in the responder group (Fig. 4C). High *PRAME* expression was associated with prolonged PFS compared with low *PRAME* expression (p=0.011, log-rank test) (Fig. 4D). Additionally, the prognostic significance of *PRAME* mRNA expression was independent of cancer histology (p=0.023, multivariate Cox regression) (Fig. 4E), thus showing that cancer histology was independently associated with ICI treatment response.

5. Predictive modeling of PD-1/PD-L1 blockade response based on immune-related gene expression

The PD-1/PD-L1 inhibitor response prediction model using an RF algorithm was built based on the top eight immune-related genes with differential expression, and the prediction model was validated using the LOOCV method (Fig. 5A). In accordance with the aforementioned finding of the association between *PRAME* expression and treatment response, *PRAME* had a high rank in terms of feature importance among the eight genes (Fig. 5B). The accuracy of the response prediction model was 0.84 (95% CI, 0.60 to 0.96), with a cross-validated AUC of 0.93, sensitivity of 0.91, specificity of 0.75, a positive predictive value of 0.833, and a negative predictive value of 0.857 (Fig. 5C).

Discussion

We investigated the unique histologic and gene expression features associated with treatment response to ICIs among patients with dMMR/MSI-H metastatic CRC. Although a large proportion of patients with dMMR/MSI-H metastatic CRC exhibit durable clinical benefits from ICI treatment, the degree of benefit likely varies by differences in tumor characteristics.

Comparison of the histopathologic features of MSI-H CRC according to ICI response revealed several notable responder characteristics. Lymphocytes and neutrophil infiltration of the tumor stroma, Crohn-like lymphoid aggregates, and expansile tumor borders were associated with good ICI responses, while infiltrative tumor borders with scanty immune cell infiltration and mucinous or signet-ring cell carcinoma subtypes were predominant features of non-responders. Of note, pure mucinous adenocarcinoma was associated with separation of tumor cells from peritumoral immune cells, and signet-ring cell carcinoma was associated with poor immune cell infiltration, which may underlie the poor response to ICI therapy. Some of the features are also known as traits of the MSI-H tumor itself, as rich infiltration of T lymphocytes, Crohn-like lymphoid aggregates, expansile tumor borders have been more frequently observed in MSI-H CRC than in MSS CRC [16,17]. Mucinous and signet-ring cell histology, which were independently associated with poor survival outcomes in the multivariate analysis, were also more common in association with MSI-H CRC (33.3%) than with MSS or MSI-L tumors (18.2%) (S2 Table);

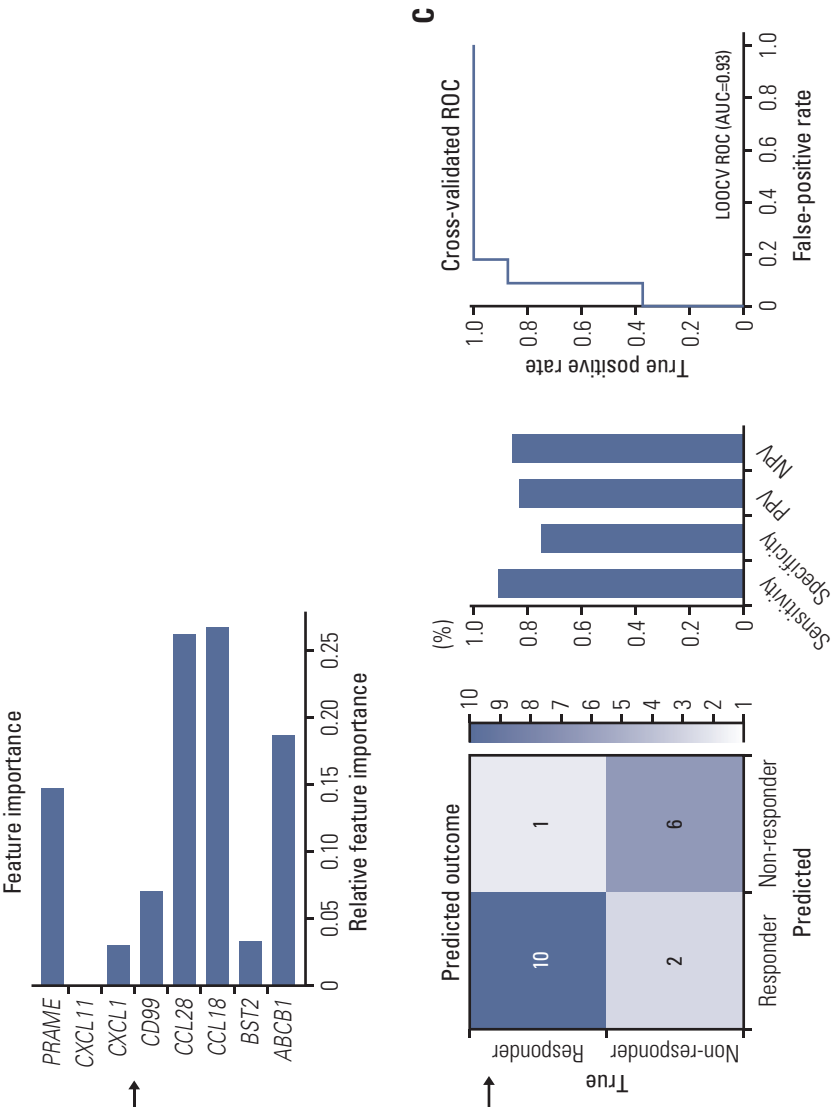

Fig. 5. Prediction modeling for treatment response to ICIs among patients with MSI-H CRCs. (A) Overview of the processes of prediction model building using RF based on immune-related gene expression and internal validation of the model using the LOOCV method. (B) Feature importance of input genes in modeling by RF. (C) Performance of the prediction model. AUC, area under the curve; CRC, colorectal cancer; DEG, differentially expressed genes; ICI, immune checkpoint inhibitor; LOOCV, leave-one-out cross-validation; MSI-H, microsatellite instability–high; NPV, negative predictive value; PPV, positive predictive value; RF, random forest; ROC, receiver operating characteristic.

these findings aligned with the proportions reported in previous studies (23.9% to 36%) [18,19]. Our study showed that careful histologic analyses focusing on the tumor-immune cell interaction could reveal useful histologic predictors of responsiveness to ICI therapy in MSI-H CRC.

A high density of tumor-infiltrating lymphocytes (TILs) is widely accepted as a prognostic factor in CRC and is also a strong predictor of immunotherapy response in many different types of cancers [20,21]. Generally, MSI-H tumors have higher TIL densities than MSS tumors; however, only 33% (9/27) of our study patients had grade 2 or 3 lymphocyte infiltration, none of whom were among the non-responders. In terms of Crohn-like lymphoid aggregates, a previous study showed that the formation of ectopic lymphoid tissues was correlated with better CRC survival outcomes, although none of the patients had received treatment with ICIs [22]. Although the association between neutrophil infiltration and response to immunotherapy or survival outcome is controversial, we found that a higher abundance of neutrophils in the tumor microenvironment was associated with the response to PD-1/PD-L1 blockade, which may imply that neutrophils can themselves be the target of PD-1/PD-L1 blockade, or they may play a role as anti-tumor inflammatory cells in CRC [23]. PD-L1 expression was not significantly associated with survival outcomes or response to PD-1/PD-L1 inhibitors, which is in line with the results from the CheckMate-142 study [7].

Although histopathologic analysis identified some distinct features of immunotherapy responders among MSI-H CRC patients, histopathologic features are difficult to standardize and quantify to apply as clinical biomarkers; rather, differentially expressed genes between responders and non-responders could be more useful as reliable biomarkers. We found that *PRAME* and several chemokine genes (*CXCL11*, *CCL18*, *CXCL1*, and *CCL28*) had significantly higher expression levels in the responder group. The accuracy of the response prediction model, which mainly consisted of *PRAME* and chemokine genes, was 0.842 (95% CI, 0.60 to 0.96), which is a favorable result that warrants further validation with a larger study sample. Several chemokines were also known to be upregulated in MSI-H tumors compared with MSS tumors. In a previous analysis using RNA sequencing data from the Total Cancer Genome Atlas data, *CXCL11* and *CCL18* showed significantly higher expression in MSI-H CRC compared with MSS CRC, and *CXCL1* was also highly expressed in a study using a multiplex cytokine assay [24,25]. Nonetheless, the association between chemokine gene expression and response to ICI therapy in MSI-H CRC had not been well established.

In a previous analysis of gene expression signatures for predicting the response of head and neck squamous cell carcinomas to ICI therapy, *CXCL11* was included in the signature as a predictor of response to ICIs [26]. *CXCL11* was also correlated with tumor-infiltrating T-cells and natural killer cells in a meta-analysis of gene expression studies that included 5953 solid tumor specimens [27]. From gene ontology enrichment analysis, one of the enriched pathways in the responder group was the IFN-γ response pathway. IFN-γ is thought to be related to inflammatory gene signatures and could be one of the biomarkers for immunotherapy response [20]. IFN-γ, with several chemokine gene expression signatures, has been shown to be predictive of the response to immunotherapy in patients with head and neck squamous cell carcinoma [26].

In this study, the *PRAME* gene yielded the largest $\log_2$FC between responders and non-responders and was thus subjected to validation at the protein level. *PRAME* was one of the first cancer/testis antigens identified in a melanoma cell line and is known to be expressed in many different solid tumors, including CRC and leukemia, with minimal expression in normal organs except the testes and endometrium [28]. In our study, IHC analysis identified PRAME expression in only 21% (4/19) of MSI-H patients—who were all responders—and correlated well with the level of *PRAME* gene expression.

Currently, there are only a few biomarkers associated with ICI response in dMMR/MSI-H CRC. In an analysis of 22 patients with metastatic dMMR/MSI-H CRC treated at five centers, high TMB was strongly associated with response to immunotherapy and better survival outcomes [29]. However, the predictive value of TMB for immunotherapy response should be further investigated and correlated with other molecular and immunologic aspects. In a study on the correlation of gene expression profiles and TMB with response to pembrolizumab in solid tumors, both high TMB and T-cell inflamed gene expression profiles were independently correlated with better pembrolizumab treatment outcomes (objective response rates of TMB^{high}/GEP^{high} vs. TMB^{high}/GEP^{low}, 37% to 57% vs. 11% to 42%) [30]; in that study, most of the MSI-H CRC patients had high TMBs, while only about a half of the patients had T-cell inflamed gene expression profiles [30].

There were several limitations to this study. This study was conducted at a single center with a relatively small number of patients who received different types of PD-1/PD-L1 inhibitors. One of the major limitations was that none of the differentially expressed genes identified in this study were significant when multiple testing correction using FDR was performed, although we considered FC as well as nominal p-values. Therefore, we acknowledge that there is a risk of false positivity in terms of the association between treatment response and the expression of any single gene. However, we

were able to show that the combinatory gene set, including *PRAME*, could predict the response to immunotherapy; we also observed high accuracy in the internal validation. However, the number of genes included in the differential expression analysis was also small, and none of the differentially expressed genes had a robust significance in terms of FDR. External validation of the differentially expressed genes and an RF classifier predictive model are needed, along with preclinical studies, to elucidate the molecular mechanisms underlying the results of the differential expression analysis. Moreover, there should be more efforts to utilize immunotherapy for patients with MSS CRC, who constitute the majority of the patients with metastatic CRC.

In conclusion, our study revealed the histopathologic characteristics and immunologic gene expression profiles associated with the response to PD-1/PD-L1 blockade among patients with dMMR/MSI-H metastatic CRC. We identified eight immune-related genes that could predict the response to PD-1/PD-L1 blockade, among which *PRAME* was differentially expressed in the responder group, with the highest absolute $\log_2$FC, and showed a good correlation with the IHC results. These results suggest the potential role of *PRAME* as a predictive biomarker of ICI response as well as histologic characteristics. Our study results may contribute to a better selection of candidates for immunotherapy and provide promising directions for further investigation on PD-1/PD-L1 blockade for MSI-H CRC.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

All procedures performed in studies involving human participants were performed in accordance with the ethical standards of the institutional review board of Asan Medical Center (#2019-0065) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The participants provided informed consents for the use of their clinical information and the analysis of their tumor samples through our translational study program (#2011-0511).

Author Contributions

Conceived and designed the analysis: Hyung J, Cho EJ, Sung CO, Kim SY.
Collected the data: Kim J, Sung CO, Kim SY.
Contributed data or analysis tools: Cho EJ, Kim JH, Sung CO, Kim SY.
Performed the analysis: Hyung J, Cho EJ, Kim J, Sung CO, Kim SY.
Wrote the paper: Hyung J, Kim J, Kim JH, Kim JE, Hong YS, Kim TW, Sung CO, Kim SY.

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Conflicts of Interest

This research was supported by HK inno.N Corp.

Acknowledgments

This research was also supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI18C2383).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.985
Cancer Res Treat. 2022;54(4):1191-1199

Original Article

Optimal Definition of Biochemical Recurrence in Patients Who Receive Salvage Radiotherapy Following Radical Prostatectomy for Prostate Cancer

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Purpose This study proposed the optimal definition of biochemical recurrence (BCR) after salvage radiotherapy (SRT) following radical prostatectomy for prostate cancer.

Materials and Methods Among 1,117 patients who had received SRT, data from 205 hormone-naïve patients who experienced post-SRT prostate-specific antigen (PSA) elevation were included in a multi-institutional database. The primary endpoint was to determine the PSA parameters predictive of distant metastasis (DM). Absolute serum PSA levels and the prostate-specific antigen doubling time (PSA-DT) were adopted as PSA parameters.

Results When BCR was defined based on serum PSA levels ranging from 0.4 ng/mL to nadir+2.0 ng/mL, the 5-year probability of DM was 27.6%-33.7%. The difference in the 5-year probability of DM became significant when BCR was defined as a serum PSA level of 0.8 ng/ml or higher (1.0-2.0 ng/mL). Application of a serum PSA level of ≥ 0.8 ng/mL yielded a c-index value of 0.589. When BCR was defined based on the PSA-DT, the 5-year probability was 22.7%-39.4%. The difference was significant when BCR was defined as a PSA-DT ≤ 3 months and ≤ 6 months. Application of a PSA-DT ≤ 6 months yielded the highest c-index (0.660). These two parameters complemented each other; for patients meeting both PSA parameters, the probability of DM was 39.5%-44.5%; for those not meeting either parameter, the probability was 0.0%-3.1%.

Conclusion A serum PSA level > 0.8 ng/mL was a reasonable threshold for the definition of BCR after SRT. In addition, a PSA-DT ≤ 6 months was significantly predictive of subsequent DM, and combined application of both parameters enhanced predictability.

Key words Prostatic neoplasms, Prostatectomy, Radiotherapy, Prostate-specific antigen

Introduction

Regular monitoring of serum prostate-specific antigen (PSA) is important during follow-up after curative treatment for prostate cancer, because an increased serum PSA level is usually the first sign of disease recurrence, preceding distant metastasis (DM) and prostate cancer-specific death by 7 and 15 years, respectively [1]. Biochemical recurrence (BCR) is defined as an increase in the serum PSA level above a particular value after curative treatment, depending on the type of treatment. Generally, when prostate cancer patients undergo radical prostatectomy (RP), a serum PSA level > 0.2 ng/mL is considered BCR, while in definitive radiotherapy, a serum PSA level > nadir+2.0 ng/mL is the widely adopted definition (the Phoenix definition).

Salvage radiotherapy (SRT) is the treatment of choice for patients who develop BCR during follow-up after RP. The percentage of prostate cancer patients who undergo SRT is relatively high, because > 30% of those who undergo RP eventually experience disease recurrence [2]. PSA monitoring remains an important method of evaluation during follow-up after SRT, and retrospective series have shown

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Received September 2, 2021 **Accepted** December 6, 2021
Published Online December 7, 2021

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that approximately 60%-75% of patients present with a biochemical response after SRT [3,4]. Although early detection of treatment failure after SRT is marked by elevated PSA levels, no widely accepted consensus has been reached on the optimal definition of BCR in SRT patients. Despite the need, few studies have sought the optimal definition of BCR for prediction of clinical outcomes after SRT.

The Korean Radiation Oncology Group (KROG) 18-01 protocol was designed to evaluate the efficacy of SRT after RP in patients with localized prostate cancer, based on data from more than 1,000 patients included in a multi-institutional database. Using this study population, we determined the optimal PSA levels and kinetics for prediction of the probability of DM, a critical event that contributes to cancer-specific mortality. Hence, this study was performed to propose the optimal definition of BCR after SRT.

Materials and Methods

Data from 1,117 consecutive patients with prostate cancer who received postoperative radiotherapy after RP between 2001 and 2012 at 19 institutions participating in the KROG 18-01 protocol were collected. The inclusion and exclusion criteria and evaluation methods for the KROG 18-01 protocol have been described previously [5]. Of the subjects, 579 patients were excluded because they received androgen deprivation therapy (ADT) perioperatively or concurrently with or after SRT. Among the 538 (48.1%) remaining hormone (ADT)-naïve patients, 205 experienced post-SRT PSA elevation and 333 did not. These 205 patients included those with histories of re-salvage treatment after SRT (n=177), or persistent PSA elevation after SRT (n=6), or re-elevation after reaching the post-SRT nadir (n=22). The flow of subjects through the study is summarized in Fig. 1.

The details of treatment of the patients included in the KROG 18-01 protocol have been described previously [5]. Briefly, all patients received SRT following RP, and a median RT dose of 66.7 Gy (interquartile range [IQR], 64.6 to 70.0) was delivered to the treatment target encompassing the prostate and seminal vesicle bed. After completion of SRT, patients' serum PSA levels were measured at regular follow-up evaluations every 3 months for 1 year, every 6 months for the next 4 years, and every 12 months thereafter. When PSA elevation was detected after 1 year, the evaluation interval reverted to 3 months. The median interval between SRT and post-SRT PSA elevation was 37.8 months (IQR, 17.5 to 66.0). For assessment of PSA kinetics, the PSA doubling time (PSA-DT) was calculated using at least three PSA measurements obtained at a 3-month interval before re-salvage treatment after SRT. The PSA-DT is the number of months required for the PSA level to double and may be associated with prostate cancer cell proliferation [6].

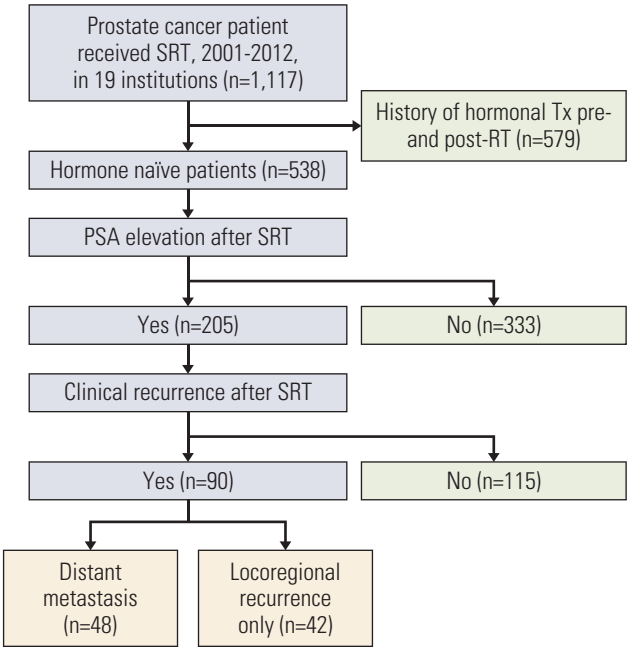

Fig. 1. The flow chart of the study subjects. PSA, prostate-specific antigen; SRT, salvage radiotherapy; Tx, therarpy.

The primary endpoint of this study was to determine the PSA parameters predictive of DM following SRT. Patients lost to follow-up were censored at the last known date on which they were alive. The ability of various definitions of BCR to predict DM was tested using the absolute serum PSA level and the PSA-DT before re-salvage treatment. The PSA-DT was not calculated for 18 patients (8.7%) due to a lack of adequate serial PSA measurements.

The Mann-Whitney U, chi-square, and Fisher exact tests were used to analyze the clinicopathological variables, as appropriate. Survival curves were plotted using the Kaplan-Meier estimator, and the log-rank test was used to compare survival curves between groups. The probability of DM according to the BCR definition was assessed with Harrell's c-index (also known as the concordance index), which is commonly used to evaluate risk models in a survival analysis in which data may be censored. p-values < 0.05 were considered significant, and all reported p-values are two-sided. IBM SPSS software ver. 27 (IBM Corp., Armonk, NY) was used to perform the statistical analyses.

Table 1. Patient characteristics (n=205)

Characteristic	Value
Age (yr)	65 (60-69)
Gleason score sum	
6	6 (2.9)
7	120 (58.0)
8	33 (15.9)
9	48 (23.2)
Pathologic staging	
pT2	57 (27.5)
pT3	140 (67.6)
pT4	10 (4.8)
RM status	
Negative	86 (41.5)
Positive	121 (58.5)
PSA information	
Initial PSA (ng/mL)	11.7 (7.0-19.2)
Pre-SRT PSA (ng/mL)	0.5 (0.3-0.9)
Post-SRT PSA nadir	
< 0.2 ng/mL	138 (67.3)
≥ 0.2 ng/mL	67 (32.7)
Absolute PSA reduction after SRT	
Increased	33 (16.1)
< 0.5 ng/mL decrease	104 (50.7)
≥ 0.5 ng/mL decrease	68 (33.2)
Interval between SRT to post-RT nadir[a] (mo)	5.49 (3.03-10.58)

Values are presented as median (IQR) or number (%). IQR, interquartile range; PSA, prostate-specific antigen; RM, resection margin; RT, radiotherapy; SRT, salvage radiotherapy. [a]Twelve pts the date of nadir was same as the date of post-SRT PSA elevation.

Results

1. Patient and tumor characteristics

The characteristics of the 205 patients with post-SRT PSA elevation are summarized in Table 1. The most common Gleason's score for the pathologic specimens was seven (58.0%), followed by nine (23.2%) and eight (15.9%). More than 70% of patients were diagnosed with pT3 or more advanced disease. More than half of the patients had positive resection margins. For these patients, the median serum PSA values at the time of initial diagnosis and SRT were 11.7 ng/mL (IQR, 7.0 to 19.2) and 0.5 ng/mL (IQR, 0.3 to 0.9), respectively. The median follow-up times from the days of RP and SRT were 120.3 months (IQR, 92.9 to 143.6) and 99.2 months (IQR, 75.9 to 121.8), respectively. Of the 205 patients with post-SRT PSA elevation, 90 (43.9%) developed clinical recurrence; of these, 48 (23.4%) developed DM and 42 (20.5%) had only locoregional recurrences. Among those who developed DM, the median lag time between the time of post-SRT PSA elevation and DM was 17.2 months (IQR, 2.2 to 43.8). The PSA-DT was assessable for 187 patients (91.2%).

2. BCR definition using serum PSA values

Nine definitions of BCR based on serum PSA levels ranging from 0.4 ng/mL to the nadir+2.0 ng/mL, were evaluated. Depending on the definition used (Table 2), the number of diagnoses of BCR after SRT ranged from 79 (PSA level > nadir+2.0 ng/mL) to 172 (PSA level > 0.4 ng/mL). The 5-year probability of DM ranged from 27.6% (PSA level > 0.4 ng/mL) to 33.7% (serum PSA level > 2.0 ng/mL). Among the nine definitions, the probability of DM was significantly higher based on the following five definitions compared to the counterparts: serum PSA level > 0.8 ng/mL, a PSA level > 1.2 ng/mL, a PSA level > 2.0 ng/mL, a PSA level > nadir +0.5 ng/mL, and a PSA level > nadir+2.0 ng/mL. The difference in the 5-year probability of DM became significant when BCR was defined as a serum PSA level of 0.8 ng/ml or higher (1.0, 1.2, and 2.0 ng/mL). Survival curves for the probabilities of DM based on two representative definitions (PSA level > 0.8 ng/mL and > 2.0 ng/mL) are depicted in Fig. 2. Harrell's c-index values for DM prediction using these definitions of BCR ranged from 0.526 to 0.589 (Table 2). To define BCR based on serum PSA levels, a level > 0.8 ng/mL was a useful threshold for prediction of DM, with a c-index value of 0.589 (95% confidence interval [CI], 0.525 to 0.654).

3. Definition of BCR using PSA-DT

Five definitions of BCR based on PSA-DTs of 3-24 months were evaluated. Depending on the definition used (Table 3), the number of diagnoses of BCR after SRT ranged from 40 (PSA-DT ≤ 3 months) to 165 (PSA-DT ≤ 24 months); the 5-year probability of DM ranged from 22.7% (PSA-DT ≤ 24 months) to 39.4% (PSA-DT ≤ 3 months). Among the five definitions, the probability of DM was significantly higher for a PSA-DT ≤ 3 months and a PSA-DT ≤ 6 months. Survival curves for the probability of DM based on the two representative definitions based on PSA-DT (≤ 3 months and ≤ 6 months) are depicted in Fig. 3. Harrell's c-index values for prediction of the probability of DM using the PSA-DT ranged from 0.510 to 0.660 (Table 3); a PSA-DT ≤ 6 months had the highest c-index value (0.660 [95% CI, 0.582 to 0.738]).

4. Combined use of the serum PSA level and the PSA-DT to predict DM

Two different DM probability patterns, illustrated in Figs. 2 and 3, were observed. Definition of BCR using serum PSA levels resulted in a relatively linear pattern of increased DM over time (Fig. 2), whereas definition of BCR using the PSA-

Table 2. Various BCR definitions using serum PSA value and its predictability of subsequent DM

BCR definitions by PSA values	No. of patients (%)	5-Year probability of DM (%)	p-value[a]	Harrell's c-index (95% CI)
> 0.4 ng/mL				
Yes	172 (83.9)	27.6	0.118	0.541 (0.489-0.593)
No	33 (16.1)	9.6		
> 0.6 ng/mL				
Yes	149 (72.7)	29.9	0.052	0.565 (0.502-0.629)
No	56 (27.3)	9.4		
> 0.8 ng/mL				
Yes	139 (67.8)	31.7	0.011	0.589 (0.525-0.654)
No	66 (32.2)	8.1		
> 1.0 ng/mL				
Yes	125 (61.0)	30.8	0.056	0.562 (0.487-0.636)
No	80 (39.0)	13.5		
> 1.2 ng/mL				
Yes	116 (56.6)	33.4	0.006	0.588 (0.513-0.663)
No	89 (43.4)	11.9		
> 2.0 ng/mL				
Yes	101 (49.3)	33.7	0.011	0.572 (0.493-0.651)
No	104 (50.7)	14.8		
> Post-SRT nadir+0.5 ng/mL				
Yes	145 (70.7)	30.1	0.022	0.583 (0.520-0.645)
No	60 (29.3)	12.5		
> Post-SRT nadir+1.0 ng/mL				
Yes	110 (53.7)	29.7	0.159	0.526 (0.446-0.605)
No	95 (46.3)	18.5		
> Post-SRT nadir+2.0 ng/mL				
Yes	79 (38.5)	33.4	0.027	0.550 (0.472-0.629)
No	126 (61.5)	19.0		

BCR, biochemical recurrence; CI, confidence interval; DM, distant metastasis; PSA, prostate-specific antigen; SRT, salvage radiotherapy.
[a]Log-rank test.

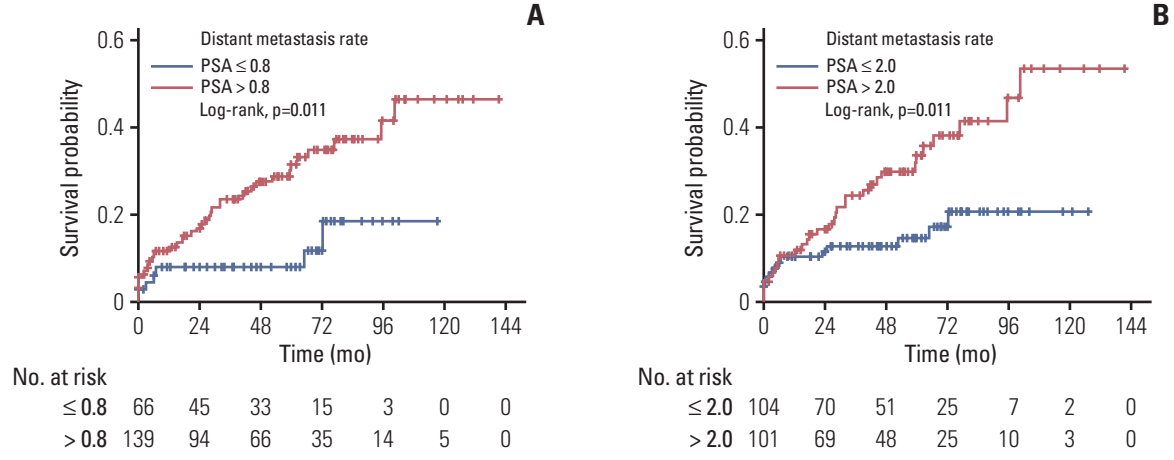

Fig. 2. The survival curves of probability of distant metastasis by biochemical recurrence definitions using serum prostate-specific antigen (PSA) value: (A) serum PSA > 0.8 ng/mL, (B) serum PSA > 2.0 ng/mL.

Table 3. Various BCR definitions using PSA doubling time and its predictability of subsequent DM

BCR definitions by PSA-DT	No. of patients (%)	5-Year probability of DM (%)	p-value[a)]	Harrell's c-index (95% CI)
≤ 3 months				
Yes	40 (21.4)	39.4	0.020	0.607 (0.524-0.689)
No	147 (78.6)	16.7		
≤ 6 months				
Yes	76 (40.6)	33.7	0.001	0.660 (0.582-0.738)
No	111 (59.4)	13.7		
≤ 12 months				
Yes	127 (67.9)	25.3	0.060	0.585 (0.526-0.644)
No	60 (32.1)	16.7		
≤ 18 months				
Yes	155 (82.9)	23.3	0.456	0.523 (0.472-0.574)
No	32 (17.1)	17.4		
≤ 24 months				
Yes	165 (88.2)	22.7	0.692	0.510 (0.467-0.553)
No	22 (11.8)	20.5		

BCR, biochemical recurrence; CI, confidence interval; DM, distant metastasis; PSA, prostate-specific antigen; PSA-DT, PSA doubling time.
[a)]Log-rank test.

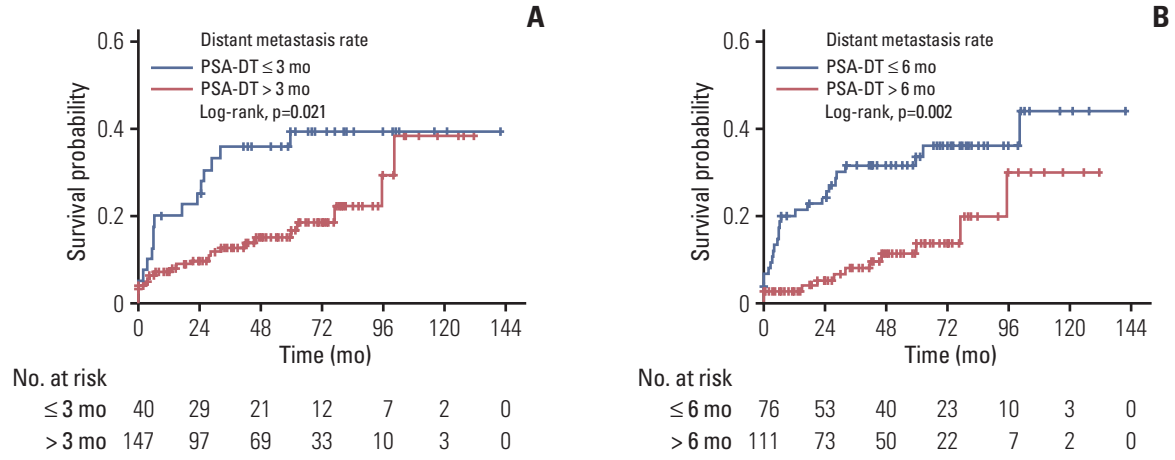

Fig. 3. The survival curves of probability of distant metastasis by biochemical recurrence definitions using prostate-specific antigen doubling time (PSA-DT): (A) PSA-DT ≤ 3 months, (B) PSA-DT ≤ 6 months.

DT yielded a pattern in which DM occurred in a relatively large percentage of patients during the early period (within 1 year after BCR diagnosis) and then plateaued after about 4 years. Definition of BCR using the PSA-DT and PSA levels tended to yield good short-term and long-term prediction of DM, respectively. Thus, these two parameters complemented each other. The 5-year probability of DM was determined based on combination of the absolute PSA level and PSA-DT (Table 4). Four absolute PSA levels (0.8, 1.2, 2.0, and the nadir 0.5 ng/mL) were combined with a PSA-DT ≤ 6 months. For patients meeting both PSA parameters (higher absolute PSA level and shorter PSA-DT), the probability of DM was 39.5%-44.5%. For those meeting one of the two PSA parameters, the probability was 14.3%-22.6%. For patients who did not meet either PSA parameter, the probability was 0.0%-3.1%. Survival curves of the probability of DM obtained with the combination of the two representative definitions, such as PSA > 0.8 ng/mL with a PSA-DT ≤ 6 months and PSA > 2.0 ng/mL with a PSA-DT ≤ 6 months, are presented in Fig. 4. Patients with PSA-DTs ≤ 6 months had a notably greater probability of DM within 1-2 years in both subgroups defined according to PSA values (> 0.8 and ≤ 0.8 ng/mL) (Fig. 4A). In contrast,

Table 4. Probability of DM according to combination of PSA values and PSA-DT

Combination		No. of patients (%)	5-Year probability of DM (%)	p-value[a]
Absolute PSA	PSA-DT (mo)			
> 0.8 ng/mL	≤ 6	54 (28.9)	39.6	0.001
	> 6	74 (39.6)	18.8	
≤ 0.8 ng/mL	≤ 6	22 (11.8)	18.2	
	> 6	37 (19.8)	0.0	
> 1.2 ng/mL	≤ 6	47 (25.2)	43.1	0.001
	> 6	62 (33.3)	21.2	
≤ 1.2 ng/mL	≤ 6	29 (15.5)	17.6	
	> 6	48 (25.8)	2.1	
> 2.0 ng/mL	≤ 6	39 (20.9)	44.5	< 0.001
	> 6	56 (29.9)	22.6	
≤ 2.0 ng/mL	≤ 6	37 (19.8)	22.5	
	> 6	55 (29.4)	1.9	
> Nadir+0.5 ng/mL	≤ 6	61 (32.7)	39.5	0.004
	> 6	79 (42.4)	18.6	
≤ Nadir+0.5 ng/mL	≤ 6	15 (8.0)	14.3	
	> 6	31 (16.6)	3.1	

DM, distant metastasis; PSA, prostate-specific antigen; PSA-DT, PSA doubling time. [a]Log-rank test.

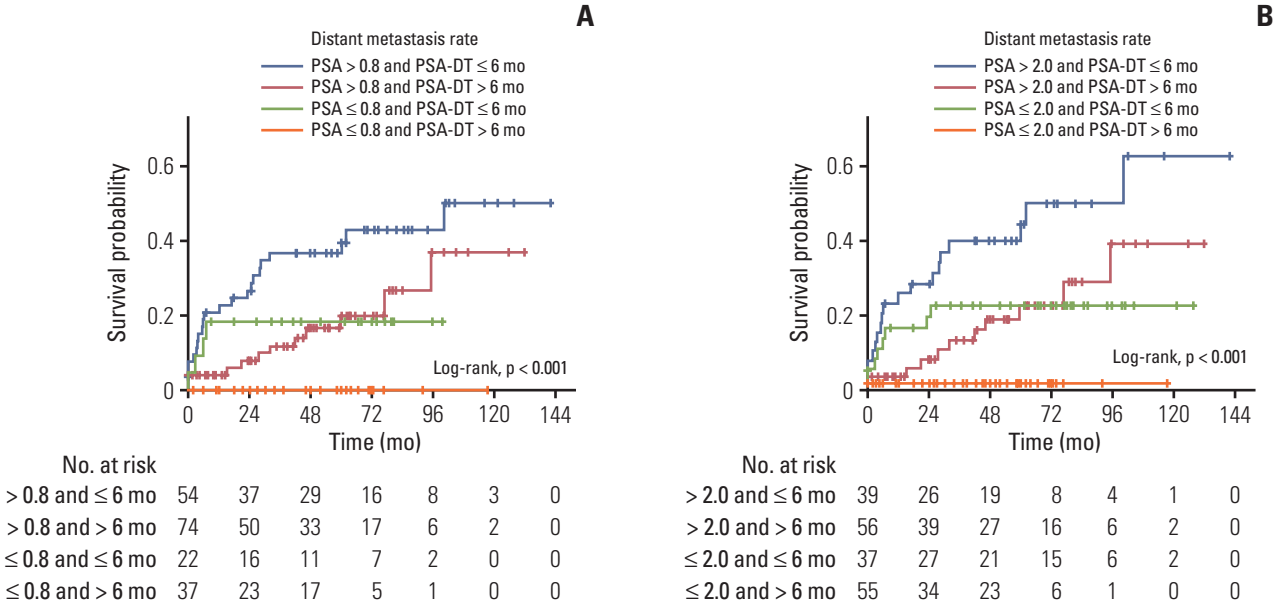

Fig. 4. The survival curves of probability of distant metastasis by biochemical recurrence definitions using combination of prostate-specific antigen (PSA) 0.8 ng/mL and prostate-specific antigen doubling time (PSA-DT) 6 months (A) and PSA 2.0 ng/mL and PSA-DT 6 months (B).

for patients with PSA levels > 0.8 ng/mL, the difference in the probability of DM within 2 years was minimal compared to those with PSA levels ≤ 0.8 ng/mL, but increased gradually after 2 years in both the PSA-DT ≤ 6 months and > 6 months subgroups. As a result, patients who met two PSA parameters had a ≥ 40% 5-year probability of DM according to the combinations assessed. Similar patterns were observed in patients with PSA levels > 2.0 and < 2.0 ng/mL (Fig. 4B).

Discussion

After curative treatment for prostate cancer, proper definition of BCR is essential to enable earlier assessment of treatment failure and timely administration of salvage treatment. Two different definitions of BCR, based on the presence or absence of the prostate after definitive therapy, have been widely accepted. For example, BCR is defined as a postoperative increase in the PSA level ≥ 0.2 ng/mL after RP and as post-RT PSA elevation ≥ nadir+2 ng/mL. We assumed that the purpose of SRT was to sterilize microscopic cancer cells in the prostate bed after RP. Thus, we speculated that a particular PSA level between 0.2 ng/mL and the nadir+2.0 ng/mL would be a reasonable candidate for a definition of BCR after SRT. Various PSA levels, such as a single value of 0.4 ng/mL [7,8] and the nadir+0.3 ng/mL [9], have been used in previous studies of the efficacy of SRT and adopted as definitions of BCR in measurements of BCR-free survival.

For the first, we set the probability of DM as the primary endpoint to assess the predictive ability of various definitions of BCR, based on a report on the International Intermediate Clinical Endpoints in Cancer from the Prostate Working Group [10]. In that report, metastasis-free survival was a strong surrogate for overall survival in patients with localized prostate cancer [10]. Gharzai et al. [11] also demonstrated the surrogacy of metastasis-free survival as an intermediate clinical endpoint for prostate cancer, and reported that improvements in local failure rates alone are less likely to translate into improvements in overall survival, presumably because local recurrence can be indolent or curable by salvage therapy [10]. In previous studies conducted to establish a definition of BCR after RP, the probability of DM was adopted as the primary endpoint [12]. As described in the "Results", locoregional recurrence after SRT was also observed as many as subsequent DM. As seen in S1 Table, there was no significant difference between locoregional recurrence (–) and (+) cases in terms of serum PSA level and PSA-DT, especially showed relatively longer PSA-DT compared to DM. According to the previous study which tested the association between post-prostatectomy PSA-DT and type of recurrence [13], they also demonstrated short PSA-DT of DM and long PSA-DT of locoregional recurrence. Therefore, we'd like to suggest that the definitions of BCR in our study would not be optimal for the prediction of subsequent locoregional recurrence after SRT.

The ability of serum PSA levels ranging from 0.4 ng/mL to the nadir+2.0 ng/mL to predict DM was tested. Among them, a serum PSA level of 0.8 ng/mL was the most useful single PSA value for prediction of DM after SRT. Use of this threshold did not result in an overwhelmingly higher c-index value relative to the use of other values, such as 1.2 and 2.0 ng/mL. However, 0.8 ng/mL was the lowest PSA value that resulted in a significant difference in the probability of DM, and its c-index value was higher than those for 1.2 and 2.0 ng/mL. If a diagnosis of BCR could be made at a lower PSA level (0.8 ng/mL vs. 1.2 or 2.0 ng/mL), re-salvage treatment could be initiated earlier, before further progression. As described, c-index values of PSA 0.8 ng/mL and nadir+0.5 ng/mL were 0.589 (95% CI, 0.525 to 0.654) and 0.583 (95% CI, 0.520 to 0.645), respectively, and we believe that both values are valid to predict DM. However, the interval between SRT and BCR was shorter for the group with PSA > 0.8 ng/mL (mean±standard deviation, 43.89±34.10 months) than the group with nadir+0.5 ng/mL (47.40±35.89 months). In addition, the 5-year probability of DM was high (12.5%), even in patients with PSA levels ≤ nadir+0.5 ng/mL. Therefore, we decided to pick cutoff value of PSA > 0.8 ng/mL predicting DM for a subsequent early intervention in our study.

We also confirmed that the PSA-DT, particularly a PSA-DT ≤ 6 months, is an important measurement for a post-SRT definition of BCR, with the highest c-index value. In a previous study, the rate of PSA increase was notably greater in patients who subsequently developed DM. As suggested by Hanks et al. [14], the mathematical expression of the PSA-DT may be a useful indicator of recurrent prostate cancer tumor biology and the speed of PSA increase. No study has involved assessment of the PSA-DT in cases of post-SRT PSA elevation like ours, but several studies have been conducted to evaluate the prognostic value of the PSA-DT at the time of the first BCR after RP. According to Jackson et al. [15], a PSA-DT < 6 months before receipt of SRT for a postoperative BCR was a significant prognostic factor for metastasis and cancer-specific death. Nevertheless, definition of post-SRT BCR using the PSA-DT alone is limited because the PSA-DT sometimes cannot be calculated and is a weak predictor of long-term events (Fig. 3).

The ability to predict subsequent DM improved with the combined use of the serum PSA level and the PSA-DT. These parameters may complement each other, as the definitions of BCR based on them showed strength in long-term and short-term predictions of DM, respectively (Fig. 4). The 5-year probability of DM was approximately 40% or more for patients meeting two PSA parameters (PSA-DT ≤ 6 months and PSA level > 0.8 ng/mL), and extremely low for patients who did not meet either PSA parameter. Although our results should be interpreted with caution, we suggest that patients with PSA-DTs > 6 months should be observed closely until their PSA level reaches 2.0 ng/mL, at which point the 5-year probability of DM in this study was only 1.9%.

The significance of our study derives from the examination of a large population over a long follow-up period, with well-performed PSA monitoring coupled with clinical

examinations before and after SRT. The limitations of this study are due primarily to the multi-institutional, retrospective nature of the data. We selectively analyzed patients with prostate cancer who had received SRT and had post-SRT PSA elevation. This approach may have introduced unrecognized selection biases. The most challenging aspect of this study was that some patients with BCR are administered re-salvage hormonal therapy before they show further PSA elevation predictive of subsequent DM. For this reason, we assessed the PSA-DT in this retrospective analysis. Even when the last serum PSA level before re-salvage treatment is relatively low, the likelihood of subsequent metastatic progression can be assumed to be high when the PSA-DT is remarkably short. In addition, re-salvage hormone therapy may influence pattern or time sequence of subsequent DM developments. However, designing a prospective study to assess the optimal definition of BCR would be difficult because of the protracted time between BCR and detectable clinical recurrence, the need for a large population because of the relatively low clinical recurrence rate after curative treatment, and ethical issues with the delay of re-salvage treatment until macroscopic clinical recurrence.

In conclusion, various serum PSA levels and the PSA-DT were assessed to propose an optimal definition of BCR for prediction of subsequent DM. Based on our results, a PSA level > 0.8 ng/mL is a reasonable threshold for the definition of post-SRT BCR. In addition, a PSA-DT ≤ 6 months was significantly predictive of subsequent DM, and the combined use of the serum PSA value and the PSA-DT enhanced the predictive ability. To our knowledge, this report is the first to propose an optimal definition of BCR for patients who receive SRT following RP. A more universal definition of BCR is needed for these patients. We believe that use of this optimal definition of BCR can lead to the best management of prostate cancer and ultimately improve the clinical outcomes of these patients.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The Institutional Review Board of the National Cancer Center approved this protocol (NCC 2018-0116). The protocols of the other participating institutions were approved by their respective institutional review boards. The data were managed by the assignment of hospital-specific case numbers and anonymization. Data analysis was performed centrally at the National Cancer Center of Korea. The requirement for written informed consent was waived due to the retrospective nature of the study.

Author Contributions

Conceived and designed the analysis: Lee SU, Park W, Cho KH.
Collected the data: Lee SU, Kim JS, Kim YS, Cho J, Choi SH, Nam TK, Jeong SM, Kim Y, Choi Y, Park W, Cho KH.
Contributed data or analysis tools: Lee DE.
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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

The authors thank Dr. Jin Hee Kim at Keimyung University Dongsan Medical Center, Dr. Jun-Sang Kim at Chungnam National University College of Medicine, Dr. Ji Young Jang at Chosun University Hospital, Dr. Su Jung Shim at Eulji Hospital, Dr. Young Suk Kim at Jeju National University Medical Center, Dr. Dong Soo Lee at Uijeongbu St. Mary's Hospital, and Dr. Ah Ram Chang at Soonchunhyang University Hospital for patient accrual.

This study was supported by a National Cancer Center Grant (NCC 1910300-3) and the Korean Radiation Oncology Group. The funding source had no role in the study design, data curation, or analysis and interpretation of data.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.828
Cancer Res Treat. 2022;54(4):1200-1208

Original Article

Frequency of Mismatch Repair Deficiency/High Microsatellite Instability and Its Role as a Predictive Biomarker of Response to Immune Checkpoint Inhibitors in Gynecologic Cancers

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Purpose This study was to investigate the frequency of mismatch repair deficiency/high microsatellite instability (MMRd/MSI-H) in gynecologic malignancies and the efficacy of immune checkpoint inhibitors (ICIs) in patients with recurrent gynecologic cancers according to MMR/MSI status.

Materials and Methods We conducted a multi-center retrospective review on the patients who were diagnosed with gynecologic cancers between 2015 and 2020. Their clinicopathologic information, results of immunohistochemistry staining for MLH1/MSH2/MSH6/PMS2 and MSI analysis, tumor response to treatment with ICIs were investigated.

Results Among 1,093 patients included in the analysis, MMRd/MSI-H was most frequent in endometrial/uterine cancers (34.8%, 164/471), followed by ovarian, tubal, and peritoneal cancers (12.8%, 54/422) and cervical cancer (11.3%, 21/186). When assessed by histology without regard for cancer types, the frequency of MMRd/MSI-H was 11.0% (38/345) in high-grade serous adenocarcinoma, 38.6% (117/303) in endometrioid adenocarcinoma, and 30.2% (16/53) in carcinosarcoma. A total of 114 patients were treated with ICIs at least once. The objective response rate (ORR) was 21.6% (8/37) in cervical cancer, 4.7% (2/43) in ovarian cancer, and 25.8% (8/31) in endometrial/uterine cancers. Univariate regression analysis identified MMRd/MSI-H as the only significant factor associated with the ORR (28.9% [11/38] vs. 11.8% [9/76]; odds ratio, 3.033; 95% confidence interval, 1.129 to 8.144; p=0.028).

Conclusion The frequency of MMRd/MSI-H is moderate to high in gynecologic cancers in the Korean population. MMRd/MSI-H could be effective predictive biomarkers in gynecologic cancers of any type.

Key words Gynecologic neoplasms, Immune checkpoint inhibitors, Microsatellite instability, Mismatch repair, Recurrence

Introduction

The introduction of immune checkpoint inhibitors (ICIs) has led to a revolutionary change in the oncology field far beyond their remarkable clinical efficacy. In recent years, various ICIs have resulted in an improvement in the overall survival (OS) of patients with a broad range of advanced cancers [1,2]. However, for most types of cancer, only a minority of patients experience a durable response from such treatments while most patients do not benefit significantly. Therefore, attention has been paid to the identification and development of predictive biomarkers of response to ICIs, and more in-depth and comprehensive studies have been conducted in recent years [3,4]. Among the most widely investigated predictive biomarkers of response to ICIs, microsatellite instability (MSI) and defective mismatch repair (MMRd), universal screening tools for identifying Lynch syndrome [5], have been shown to be significant biomarkers for a favorable response to ICIs [6-8].

Mismatch repair deficient tumors have a unique genetic signature, harboring hundreds to thousands of somatic mutations that encode potential neoantigens. These susceptible mutations in repetitive DNA sequences, termed microsatellites, result in high levels of microsatellite instability (MSI-H) [9]. This signature results from primary bi-allelic defects in genes that govern DNA mismatch repair. These tumors arise in individuals with hereditary genetic syndromes, the so-called Lynch syndrome, or more often as sporadic diseas-

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Received July 18, 2021 **Accepted** December 10, 2021
Published Online December 13, 2021

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es. Tumors with MMRd represent approximately 4% of all diagnosed cancers [10,11]. These tumors vary in frequency across different cancer types. Also, in patients with gynecologic cancers, they occur at a rate of 17%-31% in endometrial cancer, 1%-3% in ovarian cancer, and 2%-4% in cervical cancer [10,11].

The phase II KEYNOTE-158 study of pembrolizumab, an anti–programmed death-1 (PD-1) monoclonal antibody and an ICI, in patients with previously treated, advanced non-colorectal MSI-H/MMRd cancers reported an objective response rate (ORR) of 34.3% (80/233) [7]. In a meta-analysis of 14 studies comprising 939 patients with pre-treated MSI-H tumor, ICIs showed high efficacy that was independent of the tumor type and specific ICI type used, showing a pooled ORR of 41.5% [12]. Pembrolizumab was approved by the United States Food and Drug Administration in May 2017 for the treatment of patients with any type of MSI-H/MMRd solid tumors that have progressed following prior treatment. This marked the first approval of a tumor-agnostic cancer therapy in which treatment is based on a common tumor biomarker rather than the anatomic site of origin. Therefore, it became clear that accurate identification of patients with MMRd/MSI-H tumors is essential for not only screening the genetic background of patients, but also making appropriate therapeutic decisions during disease recurrence.

The frequencies of MMRd/MSI-H in pan-cancer have been reported in several studies [10] and there have been some reports of their frequency in gynecologic cancer patients. However, real-world data comparing the ORR according to MMR/MSI status have not yet been reported in gynecologic cancers. In the present study, we retrospectively assessed the frequency of MMRd/MSI-H in Korean gynecologic cancer patients, and investigated the effect of ICI therapy in recurrent gynecologic cancer with MMRd/MSI-H.

Materials and Methods

1. Study design and patients

We conducted a multi-center, retrospective study at three tertiary academic medical institutions in South Korea. We reviewed the medical records of patients who were diagnosed with gynecologic cancers between January 2015 and December 2020. The collected data included the patient demographics and clinical data on pathologic results, including the results of immunohistochemistry (IHC) staining for MLH1/MSH2/MSH6/PMS2, and MSI analysis. A total of 1,093 patients were included in investigating the frequency of MMRd/MSI-H in gynecologic cancers. Among these patients, we further reviewed the clinicopathologic and radiologic records of those diagnosed with recurrent or persistent gynecologic cancer who underwent treatment with ICIs for at least one cycle. Patients who were treated with ICIs underwent intravenous administration of 200 mg of pembrolizumab every 3 weeks or 3 mg/kg of nivolumab every 2 weeks until disease progression, unacceptable toxicity, or patient withdrawal. The study protocol was approved by the institutional review board of each participating institution (CHA IRB 2020-12-034).

2. Tumor testing

The tumor MMR status was determined by examining the loss of protein expression via IHC staining of four MMR enzymes. Tumors with loss of MMR expression in at least one of those four markers were defined as MMRd. MSI status was determined by the polymerase chain reaction (PCR)–based MSI analysis of DNA from normal and tumor tissues. The analysis was performed using five mononucleotide loci (BAT25, BAT26, NR21, NR24, and Mono27) or five mixed mononucleotide and dinucleotide loci (BAT25, BAT26, D17S250, D2S123, and D5S346) according to the institution's established method. Specimens were classified as MSI-H if at least two allelic loci sizes shifted among the five microsatellite markers analyzed. Tumors were classified as MMRd/MSI-H if either MMRd and/or MSI-H were seen. Tumor programmed death-ligand 1 (PD-L1) expression was analyzed using the PD-L1 IHC 22C3 antibody (Agilent Technologies, Inc., Santa Clara, CA) to determine the tumor proportion score (TPS), defined as the percentage of viable tumor cells, or using the PD-L1 IHC 22C3 pharmDx assay (Agilent Technologies, Inc., Carpinteria, CA) to determine the combined positive score (CPS), defined as the ratio of PD-L1–positive cells (tumor cells, lymphocytes, and macrophages) to the total number of viable tumor cells multiplied by 100. PD-L1 positivity was defined as a TPS $\geq$ 1% or a CPS > 1.

3. Assessments of response and safety

Baseline tumor assessment was performed before the start of treatment, and response was evaluated via abdominopelvic and/or chest computed tomography scans performed at least every 3 months. Additional imaging studies were performed at the clinician's discretion if a patient's clinical symptoms deteriorated. Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) ver. 1.1 by a gynecologic oncologist at each institution. Safety was assessed by retrospectively reviewing charts of laboratory test results and physical examination to detect any possible adverse events (AEs), which were evaluated according to the Common Terminology Criteria for AEs, ver. 4.03.

Table 1. Frequency of MMRd and/or MSI-H in gynecologic cancers by origin

	No.	MMRd/MSI-H	Frequency (%)
Cervix/Vulvar/Vagina cancer	195	25	12.8
Cervix cancer	186	21	11.3
Vulvar cancer	6	2	33.3
Vagina cancer	3	2	66.7
Ovarian/Peritoneal/Tubal cancer	422	54	12.8
Ovarian	385	50	13.0
Epithelial ovarian cancer	377	50	13.3
Non-epithelial ovarian cancer	8	0	0.0
Peritoneal cancer	25	3	12.0
Fallopian tubal cancer	12	1	8.3
Endometrial/Uterine cancer	471	164	34.8
Endometrial cancer	373	139	37.3
Uterine sarcoma	98	25	25.5
Gestational trophoblastic neoplasia	5	2	40.0
Total	1,093	245	22.4

MMRd, mismatch repair deficiency; MSI-H, microsatellite high.

Table 2. Frequency of MMRd and/or MSI-H in gynecologic cancers by histology regardless of origin

	No.	MMRd/MSI-H	Frequency (%)
High-grade serous carcinoma	345	38	11.0
Endometrioid adenocarcinoma	303	117	38.6
Squamous cell carcinoma	74	7	9.5
Carcinosarcoma	53	16	30.2
Endocervical adenocarcinoma	52	10	19.2
Clear cell carcinoma	51	14	27.5
Mucinous carcinoma	36	8	22.2
Leiomyosarcoma	31	7	22.6
Neuroendocrine carcinoma	30	3	10.0
Mixed adenocarcinoma	20	5	25.0
Endometrial stromal sarcoma	14	2	14.3
Adenosquamous carcinoma	13	0	0.0
Mesonephric adenocarcinoma	12	7	58.3
Low grade serous carcinoma	10	1	10.0

MMRd, mismatch repair deficiency; MSI-H, microsatellite high.

4. Outcomes

The primary endpoints were the frequency of MMRd/MSI-H tumors in gynecologic cancers and the ORR, defined as the proportion of patients with complete response (CR) or partial response (PR), as assessed using RECIST ver. 1.1. The secondary endpoints included the duration of response, defined as the time from the response to tumor progression or death, whichever occurred first; progression-free survival (PFS), defined as the time from the start of treatment to tumor progression or death, whichever occurred first; and the OS, defined as the time from the start of treatment to death from any cause.

5. Statistical analysis

Efficacy and safety profile analyses included all patients who underwent at least one cycle of treatment. The data were summarized using descriptive statistics or contingency tables for demographic and baseline characteristics, response measurements, and safety. Patients without response data were considered to be non-responders. The duration of response, PFS, and OS were estimated using the Kaplan-Meier method. Univariate logistic regression analyses were performed to identify factors affecting the ORR. All statistical analyses were performed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL). Analysis items with p-values less than 0.05

were considered statistically significant.

Results

1. Frequency of MMRd/MSI-H

A total of 1,093 patients were included in the analysis. According to the origin of cancer, the frequencies of MMRd/MSI-H were 11.3% in cervical cancer (21/186), 12.8% in ovarian, tubal and peritoneal cancers (54/422), and 37.3% in endometrial cancer (139/373) (Table 1). When assessed by the types of histology regardless of the anatomical cancer origin, the frequency was the highest in mesonephric adenocarcinoma (58.3%, 7/12), 38.6% in endometrioid adenocarcinoma (117/303), 30.2% in carcinosarcoma (16/53), and 27.5% in clear cell carcinoma (14/51) (Table 2). The frequencies of MMRd/MSI-H were 22.1% (216/976) in tumors with non-sarcoma histology, 24.1% (27/112) in tumors with sarcoma histology, and 40% (2/5) in gestational trophoblastic neoplasia (GTN) (S1 Table).

2. Clinicopathologic characteristics

A total of 114 out of 1,093 patients were treated with ICIs for recurrence at least once. The clinicopathologic characteristics of these patients are listed in Table 3. The median age was 54 years (range, 21 to 86 years). Among them, 41.2% (47/114) had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤ 1, and 73.7% (84/114) had stage III or IV disease at the initial diagnosis. In total, eight tumor types were represented among the patients, most commonly ovarian, cervical, endometrial, and uterine corpus (mainly uterine sarcoma) cancers. PD-L1 expression was assessed in 93 patients (81.6%), 65 (69.9%) of whom were PD-L1 positive. Thirty-eight patients (33.3%) had MMRd/MSI-H tumors. The remaining 76 patients were identified as MMR proficient (MMRp)/microsatellite stable (MSS), but received ICI either because their tumor profiles showed PD-L1 positivity or their tumor histology types corresponded to those that have demonstrated response to ICI. The median sum of the target lesions size was 60 mm (range, 10 to 1,230 mm). The median number of lines of prior chemotherapy, including neoadjuvant chemotherapy, was two (range, 1 to 7). The specific agents of ICIs administered were pembrolizumab (88.6%, 101/114) and nivolumab (11.4%, 13/114). As of February 28, 2021, at the time of data cutoff, the median follow-up time was 4.9 months (range, 0.1 to 36.8 months). Eighty-five patients (74.6%) had discontinued ICIs, most commonly due to disease progression. The patients underwent a median of 4 cycles (range, 1 to 40 cycles) of chemotherapy with ICIs.

Table 3. Baseline clinico-pathologic characteristics of the patients treated with immune checkpoint inhibitors (n=114)

	No. (%)
Age, median (range, yr)	54 (21-86)
ECOG performance status	
0-1	47 (41.2)
2-4	67 (58.8)
FIGO stage at diagnosis	
I/II	22 (19.3)
III/IV	84 (73.7)
N/A	8 (7.0)
Origin of cancer	
Cervix	37 (32.5)
Vulvar	1 (0.9)
Ovary/Peritoneum/Fallopian tube	43 (37.7)
Endometrium	23 (20.2)
Uterine corpus	8 (7.0)
Gestational trophoblast	2 (1.8)
PD-L1 expression[a]	
≥ 1	65 (57.1)
< 1	28 (24.6)
N/A	21 (18.4)
MMRd and/or MSI-H	38 (33.3)
MMRp and/or MSS	76 (66.7)
Target lesion size, median (range, mm)[b]	60 (10-1,230)
No. of previous lines of chemotherapy	
1	29 (25.4)
2	39 (34.2)
3	18 (15.8)
4	15 (13.2)
≥ 5	13 (11.4)
Type of immune checkpoint inhibitors	
Pembrolizumab	101 (88.6)
Nivolumab	13 (11.4)

ECOG, Eastern Cooperative Oncology Group; FIGO, International Federation of Gynecology and Obstetrics; MMRd, mismatch repair deficiency; MMRp, mismatch repair proficiency; MSI-H, microsatellite high; MSS, microsatellite stable; N/A, non-available; PD-L1, programmed death-ligand 1. [a]Determined by either the tumor proportion score (TPS) or the combined positive score (CPS), [b]Sum of the diameters of the target lesions.

3. Antitumor activity

In the total population (n=114), five patients (4.4%) achieved CR and 15 (13.2%) achieved PR, resulting in an ORR of 17.5% (Table 4). Among the patients who achieved an objective response, the median time to response was 2.4 months (range, 0.8 to 17.3 months) and the median duration of response was not reached (range, 2.2 to 33.0 months). Among patients with MMRd/MSI-H tumors (n=38), the ORR was 28.9% (3 CRs and 8 PRs). Among patients with

Table 4. Tumor responses assessed by RECIST v.1.1 (n=114)

	Total population	MMRd/MSI-H group	MMRp/MSS group
Antitumor activity	114	38	76
Best overall response			
CR	5 (4.4)	3 (7.9)	2 (2.6)
PR	15 (13.2)	8 (21.1)	7 (9.2)
SD	23 (20.2)	4 (10.5)	19 (25.0)
PD	58 (50.9)	16 (42.1)	42 (55.3)
Not able to be assessed	13 (11.4)	7 (18.4)	6 (7.9)
Objective response rate	20 (17.5)	11 (28.9)	9 (11.8)
Disease control rate	43 (37.7)	15 (39.5)	28 (36.8)
Time to response (mo)			
Median (range)	2.4 (0.8-17.3)	3.7 (0.8-17.3)	1.9 (1.4-3.5)
Duration of response (mo)			
Median (range)	Not reached (2.2-33.0)	Not reached (2.3-33.0)	Not reached (2.2-32.0)

Values are presented as number (%) unless otherwise indicated. CR, complete response; MMRd, mismatch repair deficiency; MMRp, mismatch repair proficiency; MSI-H, microsatellite high; MSS, microsatellite stable; PD, progressive disease; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumor; SD, stable disease.

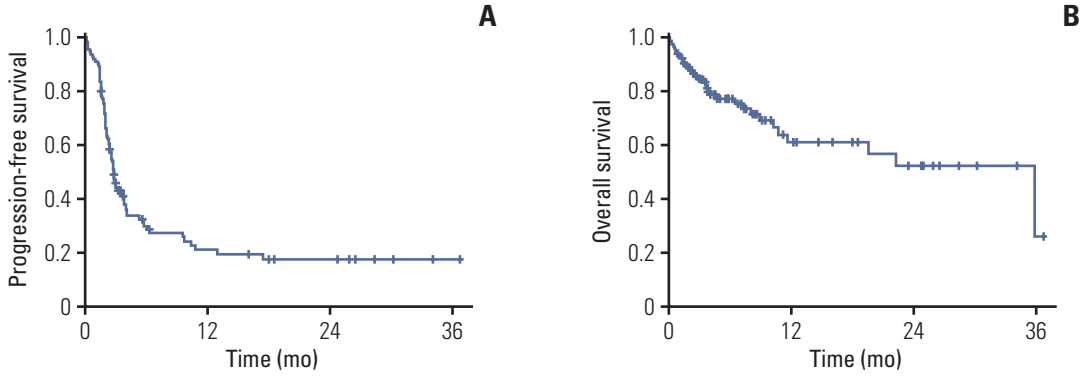

Fig. 1. Kaplan-Meier estimates of survival in the total study population (n=114): progression-free survival (A) and overall survival (B).

MMRp/MSS tumors (n=76), the ORR was 11.8% (2 CRs and 7 PRs) (Table 4).

The response to treatment with ICIs was assessed by anatomical cancer origins and the results are summarized in S2 Table. The ORR was 4.7% (2/43) in ovarian cancer, 21.6% (8/37) in cervical cancer, 26.1% (6/23) in endometrial cancer, 25.0% (2/8) in uterine corpus cancer, and 100.0% (2/2) in GTN. Among patients with MMRd/MSI-H tumors (n=38), the ORR was 33.3% for endometrial cancer (5/15), 33.3% (2/6) for uterine corpus cancer, 14.3% (1/7) for ovarian cancer, and 12.5% (1/8) for cervical cancer (S3 Table).

At the time of data cutoff, 86 (75.4%) patients in the total population had experienced disease progression or death. The median PFS was 2.8 months (95% confidence interval [CI], 2.4 to 3.2), and the estimated PFS rates at 6 and 12 months were 30.1% and 21.4%, respectively (Fig. 1A). Thirty-three patients (28.9%) in the total population had died. The median OS was 35.9 months (95% CI, 16.1 to 55.7) in the total population (Fig. 1B). The OS rates at 6 and 12 months were 77.1% and 61.1%, respectively.

4. Prognostic factors

We compared the ORR according to different clinical parameters, including age, tumor origin, the number of previous lines of chemotherapy, ECOG status, PD-L1 positivity, MMRd/MSI-H status, and tumor size (Table 5). MMRd/MSI-H status was the only significant factor found in the univariate regression analyses (odds ratio, 3.033; 95% CI, 1.129 to 8.144; p=0.028).

Table 5. Logistic regression analysis of predictive factors for the objective response rate

	Univariate analysis	
	OR (95% CI)	p-value
Age (yr)		
< 60	1	
≥ 60	0.711 (0.236-2.139)	0.544
Origin of tumor		
Cervix	1	
Ovary	0.274 (0.067-1.122)	0.072
Uterine	1.467 (0.490-4.392)	0.493
No. of prior lines of chemotherapy		
≤ 2	1	
> 2	0.759 (0.278-2.077)	0.592
ECOG performance status		
≤ 1	1	
> 1	0.554 (0.196-1.567)	0.266
MMRd/MSI-H		
MMRp and/or MSS	1	
MMRd and/or MSI-H	3.033 (1.129-8.144)	0.028
PD-L1 status		
< 1	1	
≥ 1	3.569 (0.754-16.899)	0.109
Tumor burden (cm)[a]		
< 2	1	
≥ 2 and < 5	1.739 (0.299-10.104)	0.538
≥ 5 and < 10	1.538 (0.266-8.890)	0.630
≥ 10	2.207 (0.417-11.669)	0.352

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; MMRd, mismatch repair deficiency; MMRp, mismatch repair proficiency; MSI-H, microsatellite high; MSS, microsatellite stable; OR, odds ratio; PD-L1, programmed death-ligand 1.
[a]Sum of the diameters of the target lesion.

5. Safety

Treatment-related AEs of any grade and treatment-related AEs of grade ≥ 3 were reported in 42.1% and 4.4% of patients, respectively (S4 Table). There were no treatment-related deaths. The most common AEs of any grade were hypothyroidism (10.5%), anemia (8.8%), fatigue (7.0%), and skin rash (3.5%). The AEs of grades 3/4 were hypothyroidism, anemia, renal insufficiency, colitis, and thrombocytopenia.

Discussion

In two previous studies that evaluated MSI with next-generation sequencing-based methods using data from the Cancer Genome Atlas [10], the frequency of MSI-H was reported to be 3.5%-3.8% for all carcinomas. The frequencies of MSI-H ranged from 28.3% (75/265) to 31.4% (170/542) in endometrial cancer, 1.4% (6/437) to 3.2% (14/436) in ovarian cancer, and 2.3% (7/305) to 2.6% (8/305) in cervical cancer. In the present study, the frequencies of MMRd/MSI-H were 37.3% (139/373) in endometrial cancer, 13.3% (50/377) in epithelial ovarian cancer, and 11.3% (21/186) in cervical cancer (Table 1). The frequency of MMRd and/or MSI-H in endometrial cancer was comparable to those reported in previous studies, and the frequency of ovarian and cervical cancers was higher than that previously reported. However, a previous study which used the classical PCR-based MSI method, which is the same method used in the present study, reported MSI-H rates of 10% for ovarian cancer [13] and 8% for cervical cancer [14]. Therefore, it is possible that these results could be influenced by the difference in MSI analysis methods and reporting methods. Overall, it is noteworthy that endometrial cancer has the highest MMRd/MSI-H frequency.

Mesonephric adenocarcinoma is a rare malignant tumor of the female genital tract, which originates from Wolffian duct remnants. It has been reported to carry a worse prognosis even in the early stages [15,16]. Although the MMRd/MSI-H frequency in mesonephric adenocarcinoma in the present study was 58.3% (7/12), previous studies reported that the frequency of MMRd or MSI-H in mesonephric adenocarcinoma was low [15,16]. This discordance might arise from the small number of cases and the absence of a central pathology review in the present study. Although data are lacking on the response rates of mesonephric adenocarcinoma to ICIs, treatment with ICIs in MMRd/MSI-H mesonephric adenocarcinoma can be considered.

Previous studies reported that the frequency of MMRd/MSI-H in uterine carcinosarcoma was as low as 3.5% (2/57) [11]. However, in the present study, the frequencies of MMRd/MSI-H were 30.2% (16/53) in carcinosarcoma and 22.6% (7/31) in leiomyosarcoma, which were relatively higher (Table 2). In the treatment of gynecologic sarcoma which usually carries a poor prognosis and has no effective therapeutic options at recurrence, it would be helpful to assess the MMRd/MSI-H status and consider treatment using ICIs. GTN comprises a unique group of diseases that arise from the malignant transformation of fetal trophoblasts, cells that originate from the placenta. Recent studies found strong expression of PD-L1 in GTN [17,18] and the frequency of MMRd/MSI-H was 40% (2/5) in the present study (Table 1). The therapeutic response to ICIs in treating chemo-resistant GTN was reported to be favorable [19,20], and there are two ongoing clinical trials on the treatment of chemo-resistant GTN with ICIs (NCT03135769 and NCT04303884).

The mismatch repair pathway plays a crucial role in repairing DNA replication errors. Deficiencies in MMR proteins

that cause MSI-H lead to the accumulation of mutations and the generation of neoantigens that might stimulate the antitumor immune response [7]. Tumors with MMRd could induce immune evasion by immune checkpoints, allowing them to escape from the tumor-specific T-cell response [21]. Therefore, using a monoclonal antibody to inhibit immune checkpoints might be an effective therapeutic approach to reversing immune suppression and re-activating the immune system in MMRd/MSI-H tumors regardless of cancer type.

In gynecological cancers regardless of MMRd/MSI-H status, the ORR of anti-PD-1 inhibitors was reported to be low (4%-23%). The respective rates were 4%-12% in cervical cancer [22], 8%-15% in ovarian cancer [23,24], and 13%-23% in endometrial cancer [25,26]. A meta-analysis of 14 studies comprising 939 patients with pre-treated MSI-H cancer reported that the pooled ORR of ICIs was 41.5% (95% CI, 34.9 to 48.4), the pooled median PFS was 4.3 months (95% CI, 3.0 to 6.8), and the pooled median OS was 24 months (95% CI, 20.1 to 28.5) [12]. Another previous study reported that the ORR was 34.3% with a median PFS of 4.1 months and a median OS of 23.5 months among 233 patients representing 27 MMRd/MSI-H tumor types [7]. In that study, the ORRs in endometrial cancer and ovarian cancer were 57.1% (28/49) and 33.3% (5/15), respectively.

In the present study, the ORR of the total population (n=114) was 17.5% and that of the MMRd/MSI-H group (n=38), representing five gynecologic cancer types, was 28.9% (Table 4). The ORRs were 33.3% (5/15) for endometrial cancer with MMRd/MSI-H and 14.3% (1/7) for ovarian cancer with MMRd/MSI-H (S3 Table). The median PFS of MMRd/MSI-H group (n=38) was 2.3 months (95% CI, 0.6 to 4.1), and the median OS was not reached (data not provided). Although it is difficult to directly compare the results of this study with those of other prospective studies, we observed a low overall ORR of ICIs for MMRd/MSI-H tumors. This difference could be influenced by the difference between prospective and retrospective study designs, and by the relatively high MMRd/MSI-H rates observed in the present study. Despite this difference, it was possible to confirm the statistical difference in ORR between MMRd/MSI-H and MMRp/MSS patients.

In the present study, we examined the effects of several factors on ORR: age, cancer type, number of prior lines of chemotherapy, ECOG status, MMRd/MSI-H status, PD-L1 positivity, and tumor size. MMRd/MSI-H was shown to be the only significant factor in the univariate analysis (odds ratio, 3.033; 95% CI, 1.129 to 8.144; p=0.028). Other factors showed no statistically significant associations (Table 5). PD-L1 protein expression on tumor or immune cells has also emerged as a potential predictive biomarker for sensitivity to ICIs [27]. In the present study, the association between PD-L1 expression and ORR could not be confirmed (Table 5). A high tumor mutational burden is another emerging agnostic biomarker with a wider range than MMRd/MSI-H in cancers of any type [28]. Further investigations on such potential biomarker and others are warranted to expand the understanding of profound immune response in malignant diseases.

According to a recent meta-analysis [29], AEs of any grade occurred in 65.8% of patients receiving an ICI, and 16.6% of patients experienced AEs of grade ≥ 3. In the present study, AEs of any grade occurred in 42.1% of patients, and 4.4% of patients experienced AEs of grade ≥ 3. The frequency of AEs in this study was relatively low, which is likely due to the limitations of a retrospective study conducted using chart reviews. Minor AEs might not have been recorded.

The limitations of this study mainly stem from its retrospective design. The lack of independent central pathologic review could also be a confounding factor. Accordingly, there may have been differences in the methods of MMRd/MSI testing and the interpretation of the results among the pathologists at each institution. The frequency of MMRd/MSI-H was higher than those reported in previous studies. The absence of a difference in disease control rate between the MMRd/MSI-H and MMRp/MSS groups (Table 4) might be due to the high MMRd/MSI-H frequency in this study. In addition, MMRd and MSI tests were not performed in all patients. Some patients underwent only one of the two tests. Therefore, it is difficult to conclude that the accurate MMRd/MSI-H frequency was reflected in the present study. Also, the response assessment could not be centralized by an independent central radiologic review. We did not assess the immune response based on the immune RECIST or immune-related RECIST. Although none of the 114 patients raised concerns regarding potential pseudoprogression or hyperprogression even when assessed by the RECIST, the implementation of immune-related response criteria might have portrayed different results. There may also have been differences in the interpretation of the results depending on the types of ICI (pembrolizumab or nivolumab) although both agents belong to the same category and act as anti-PD-1 antibodies. The potential discrepancy between the TPS and CPS to predict response to anti–PD-1/PD-L1 therapy is another limitation. Due to practical issues, the institutions in the present study have adopted different scoring methods. The relatively short follow-up period (median, 4.9 months) is another limitation of the study. Unlike prospective studies, in real-world practice, patients with poor general condition (ECOG PS ≥ 2) are treated with ICIs as the last attempt with short life expectancies. In the present study, more than half (58.8%, 67/114) of the patients had an ECOG PS ≥ 2 (Table 3). As non-responders with poor general condition mostly died

soon after treatment with ICIs, the study resulted in a short follow-up period.

Nevertheless, to the best of our knowledge, the present retrospective study of a relatively large, mainly Asian cohort, is the first to evaluate MMRd/MSI-H status as a predictive biomarker for ICIs in gynecologic cancers in a real-world setting. Compared to the known very low MMRd/MSI-H frequencies of ovarian and cervical cancer, in the present study, a relatively high frequency of > 10% was observed. This shows that treatment with ICIs is a potential therapeutic alternative in patients with gynecologic cancers with MMRd/MSI-H. Recently, the combination of ICI and multi-kinase inhibitors has received attention in the treatment of MSS/MMRp tumors, which have a much higher proportion compared to MMRd/MSI-H tumors. Combined therapy comprising pembrolizumab and lenvatinib (an oral multi-kinase inhibitor) for MSS/MMRp recurrent endometrial cancer has been found to yield favorable outcomes among 37.2% (35/94) of patients [30]. As such study, new combination therapeutic strategies are also being specified for MSS/MMRp tumors.

The present study has shown that the frequency of MMRd/MSI-H in gynecologic cancers is moderate to high in Korea. MMRd/MSI-H status was confirmed to be a predictive biomarker for ICI therapy in gynecologic cancers. Further studies are warranted to discover other predictive biomarkers for ICI therapy in gynecologic cancer.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was approved by the institutional review board (CHA IRB 2020-12-034) and adhered to the principles in the Declaration of Helsinki. A waiver to require informed consent was obtained.

Author Contributions

Conceived and designed the analysis: Choi MC, Lee JW, Lee C.
Collected the data: Noh JJ, Kim MK, Choi MC, Lee JW, Park H, Jung SG, Joo WD, Song SH, Lee C.
Contributed data or analysis tools: Noh JJ, Kim MK, Choi MC, Lee JW.
Performed the analysis: Choi MC, Lee JW, Park H, Jung SG, Joo WD.
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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.963
Cancer Res Treat. 2022;54(4):1209-1218

Original Article

Genomic Correlates of Unfavorable Outcome in Locally Advanced Cervical Cancer Treated with Neoadjuvant Chemoradiation

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Purpose Neoadjuvant therapy modality can increase the operability rate and mitigate pathological risks in locally advanced cervical cancer, but treatment response varies widely. It remains unclear whether genetic alterations correlate with the response to neoadjuvant therapy and disease-free survival (DFS) in locally advanced cervical cancer.

Materials and Methods A total of 62 locally advanced cervical cancer (stage IB-IIA) patients who received neoadjuvant chemoradiation plus radical hysterectomy were retrospectively analyzed. Patients' tumor biopsy samples were comprehensively profiled using targeted next generation sequencing. Pathologic response to neoadjuvant treatment and DFS were evaluated against the association with genomic traits.

Results Genetic alterations of *PIK3CA* were most frequent (37%), comparable to that of Caucasian populations from The Cancer Genome Atlas. The mutation frequency of genes including *TERT*, *POLD1*, *NOS2*, and *FGFR3* was significantly higher in Chinese patients whereas *RPTOR*, *EGFR*, and *TP53* were underrepresented in comparison to Caucasians. Germline mutations were identified in 21% (13/62) of the cohort and more than half (57%) had mutations in DNA damage repair genes, including *BRCA1/2*, *TP53* and *PALB2*. Importantly, high tumor mutation burden, *TP53* polymorphism (rs1042522), and *KEAP1* mutations were found to be associated with poor pathologic response to neoadjuvant chemoradiation treatment. *KEAP1* mutations, *PIK3CA-SOX2* co-amplification, *TERC* copy number gain, and *TYMS* polymorphism correlated with an increased risk of disease relapse.

Conclusion We report the genomic profile of locally advanced cervical cancer patients and the distinction between Asian and Caucasian cohorts. Our findings highlight genomic traits associated with unfavorable neoadjuvant chemoradiation response and a higher risk of early disease recurrence.

Key words Uterine cervical neoplasms, Neoadjuvant therapy, Pathologic response, Disease-free survival, DNA damage repair

Introduction

Cervical cancer is the fourth most common cancer diagnosed among females and every year leads to more than half-million new cases as well as over 300,000 deaths worldwide [1]. Despite recent advances in prevention, diagnosis and treatment, clinical outcome of cervical cancer patients remains poor in the developing countries [2]. While the incidence of cervical cancer in developed countries has more than halved over the past decades, a surge in cervical cancer incidence was recently reported in China [3]. On the macro level, insufficient pap smear screening and human papillomavirus (HPV) vaccination are the major culprits of this international disparity [4]. On the molecular level, there may also exist differences in the mutational landscape of cervical cancer between Chinese and the Western populations, which may reveal clues of carcinogenesis mechanism and susceptibility among different ethnic groups [5].

The primary treatment strategy for patients with early-stage cervical cancers, particularly stage IA-IB1, is radical hysterectomy with or without radiation or chemotherapy [6]. Multiple treatment regimens have been actively explored and proposed for high-risk early-stage (stage IB-IIA) cervical cancer patients [7,8]. Neoadjuvant brachytherapy and

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Received August 27, 2021 **Accepted** January 13, 2022
Published Online January 17, 2022

chemotherapy followed by radical surgery showed an efficacy non-inferior to standard chemoradiation treatment and a more favorable toxicity profile in stage IB2-IIA cervical cancer [7]. Despite a high three-year disease-free survival (DFS) rate of 90%, there was a portion of patients who failed to respond to the therapy. Identification of potential biomarkers predicting poor treatment response in these patients is much needed.

In this study, we compared the genetic landscape of cervical cancer between Chinese and the Western populations to understand the differences in potential tumorigenesis mechanisms and identified associations between specific genetic alterations and poor treatment response to neoadjuvant therapy.

Materials and Methods

1. Study design and patients

This was a single-institution retrospective study that enrolled a total of 62 patients who were diagnosed of cervical cancer from 2016 to 2019 and received treatment at Shandong Cancer Hospital, Jinan, Shandong, China. The study was approved by the Institutional Review Board/Ethics Committee of Shandong Cancer Hospital. All patients provided written informed consent prior to sample collection.

Patients were included for analysis according to the following criteria: (1) cervical cancer patients with histologically confirmed International Federation of Gynecology and Obstetrics (FIGO) stage IB1-IIA (FIGO 2009) [9]; (2) age ≥ 18 years old; (3) pathological subtypes were squamous cell carcinoma (SQCC), adenocarcinoma (ADC) or adenosquamous carcinoma (ASC), excluding special types of tumors, such as clear cells carcinoma; (4) Eastern Cooperative Oncology Group performance status score of 0-2. Patients voluntarily joined this study, signed informed consent and provided diagnosis and treatment data after cancer diagnosis before entering the group, good compliance, and cooperation with follow-up visits.

Patients were excluded for analysis when (1) potential radiation field overlap caused by previous radiotherapy; (2) patients could not undergo routine imaging examination; (3) any signs of severe or uncontrolled systemic diseases that the researchers believe may significantly affect the patient's risk/benefit balance, including hepatitis B, hepatitis C and human immunodeficiency virus.

2. Clinical data and samples

Patients' clinical data were carefully reviewed, including age, pathological grade, imaging examination (computed tomography, magnetic resonance imaging or positron emission tomography–computed tomography, etc.) with or without lymph node metastasis, tumor stage, immunohistochemical results, course of disease, location and size of lesions, performance status score, family history. Paraffin samples of tumors were biopsied before and after radiotherapy and chemotherapy for next generation sequencing and pathological response assessment, respectively. Ten millileters of venous blood was collected from each patient after chemoradiotherapy and was kept in the purple lid EDTA anticoagulant blood collection tube (BD, Franklin Lakes, NJ). The white blood cell or normal tissue adjacent to tumor was used as control of tumor samples.

3. Treatment

All patients received one cycle of chemotherapy (paclitaxel plus cisplatin) and brachytherapy ([500-700] cGy×[1-2] fraction) before the radical cervical cancer resection (extensive hysterectomy and pelvic lymph node dissection and salpingo-oophorectomy or abdominal para-aortic lymphadenectomy). The radical surgery was followed by adjuvant chemotherapy (three cycles), brachytherapy and irradiation (5,040 cGy/28 fraction). A detailed treatment regimen of each patient's neoadjuvant and adjuvant chemotherapy was provided in S1 Table. DFS was defined as the time from neoadjuvant chemoradiotherapy until the time of tumor relapse or the date of the last follow-up.

4. Pathological assessment

The tumor samples were taken and subject to hematoxylin and eosin (H&E) staining protocol after chemoradiotherapy to evaluate their pathologic response to treatment. H&E slides of sections of tumors after treatment were evaluated by pathologists blinded to the patient information. At least 1 section was taken every centimeter of tumor along its greatest diameter. About 5 to 30 slides were examined for each patient. The percentage of residual viable tumor was determined by dividing the estimated cross-sectional area of viable tumor foci by total cross-sectional areas evaluated on each slide [10,11]. An average (mean) value of the percent of residual viable tumor was determined for each patient. Histologic parameters analyzed include inflammation, necrosis, fibrosis, giant cell reaction, foamy macrophages, and cholesterol cleft granuloma.

5. DNA extraction and library preparation

Sample processing and genomic profiling were performed in a Clinical Laboratory Improvement Amendments (CLIA)- and the College of American Pathologists (CAP)-accredited laboratory (Nanjing Geneseeq Technology Inc., Nanjing, China) as previously described [12,13]. In brief, genomic DNA from tumor specimen and control samples were extrac-

ted and quantified by Qubit 3.0. Library preparations were performed with KAPA Hyper Prep Kit (KAPA Biosystems, Wilmington, MA). Target enrichment was performed using customized xGen lockdown probes (Integrated DNA Technologies, Coralville, IA) targeting 474 cancer- and radiotherapy response-relevant genes (Radio-tron gene panel, Nanjing Geneseeq Technology Inc.) (S2 Table). The hybridization capture reaction was performed with Dynabeads M-279 (Life Technologies, San Diego, CA) and xGen Lockdown hybridization and wash kit (Integrated DNA Technologies) according to manufacturer's protocols. Captured libraries were on-beads polymerase chain reaction (PCR) amplified with Illumina p5 and p7 primers in KAPA HiFi HotStart ReadyMix (KAPA Biosystems), followed by purification using Agencourt AMPure XP beads. Libraries were quantified by quantitative real-time PCR using KAPA Library Quantification kit (KAPA Biosystems). Library fragment size was determined by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

6. Targeted next generation sequencing and data processing

Sequencing was performed on the Illumina HiSeq4000 platform (Illumina, San Diego, CA) followed by data analysis as previously described [12,13]. In brief, sequencing data were analyzed by Trimmomatic [14] to remove low-quality (quality < 15) or N bases, and then mapped to the human reference genome hg19 using the Burrows-Wheeler Aligner (https://github.com/lh3/bwa/tree/master/bwakit). PCR duplicates were removed by Picard (available at: https://broadinstitute.github.io/picard/). The Genome Analysis Toolkit (GATK) (https://software.broadinstitute.org/gatk/) was used to perform local realignments around indels and base quality reassurance. Single nucleotide polymorphisms (SNPs) and indels were analyzed by VarScan2 [15] and HaplotypeCaller/UnifiedGenotyper in GATK, with the mutant allele frequency cutoff as 0.5% for tissue samples, 0.1% for cell-free DNA samples, and a minimum of three unique mutant reads. Common SNPs were excluded if they were present in > 1% population frequency in the 1000 Genomes Project or the Exome Aggregation Consortium (ExAC) 65000 exomes database. The resulting mutation list was further filtered by an in-house list of recurrent artifacts based on a normal pool of whole blood samples. Gene fusions were identified by FACTERA [16].

Tumor mutation burden (TMB) was calculated based on the number of non-silent somatic mutations per megabase coding region sequenced. Microsatellite (MS) status of tumor sample was determined on the overall stability of MS loci covered by the sequencing panel (Radiotron, Nanjing Geneseeq Technology Inc.) using a proprietary in-house developed microsatellite instability (MSI) analysis pipeline. Briefly, a total of 108 mononucleotide repeats were evaluated and a subset of 52 loci with a minimum of 15-bp repeats were eventually identified as the MSI determination sites in the targeted sequencing region, including those conventional MSI detection sites such as BAT-25, BAT-26, NR-21, NR-24, and MONO-27. A site is considered qualified for analysis only if > 100× coverage depth. A sample was reported as microsatellite instable ("MSI") if ≥ 40% of the qualified MS loci display instability, or as "MSS (microsatellite stable)" if < 40% of the qualified MS loci display instability, as previously described [17].

Table 1. Clinical characteristics of cervical cancer patients

	Chinese (n=62)	Caucasian[a] (n=82)
Age (yr)		
> 44	35 (56)	46 (56)
Median (range)	47 (26-66)	45 (20-80)
Clinical stage		
IB	34 (55)	72 (88)
IIA	28 (45)	10 (12)
Histological type		
Squamous cell carcinoma	51 (82)	61 (74)
Others	11 (18)	21 (26)
Residual viable tumor (%)		
0-10 (major response)	13 (21)	n/a
10-50 (partial response)	22 (35)	n/a
> 50	27 (44)	n/a

Values are presented as number (%) unless otherwise indicated. n/a, not applicable. [a]The Caucasian cohort data were derived from The Cancer Genome Atlas database.

7. Statistical analysis

Categorical variables were compared between mutation carriers and non-carriers using the Fisher exact test. The subgroup analysis of TMB was performed using Student's t test. The Kaplan-Meier method was used for DFS analysis, and statistical significance was assessed using the log-rank test. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using R ver. 3.4.4 (R Software, R Foundation for Statistical Computing, Vienna, Austria). Gene pathways were analyzed using ReactomePA R package [18].

Results

1. Patient overview

A total of 62 locally advanced cervical cancer patients (stage IB-IIA) who received neoadjuvant chemoradiation

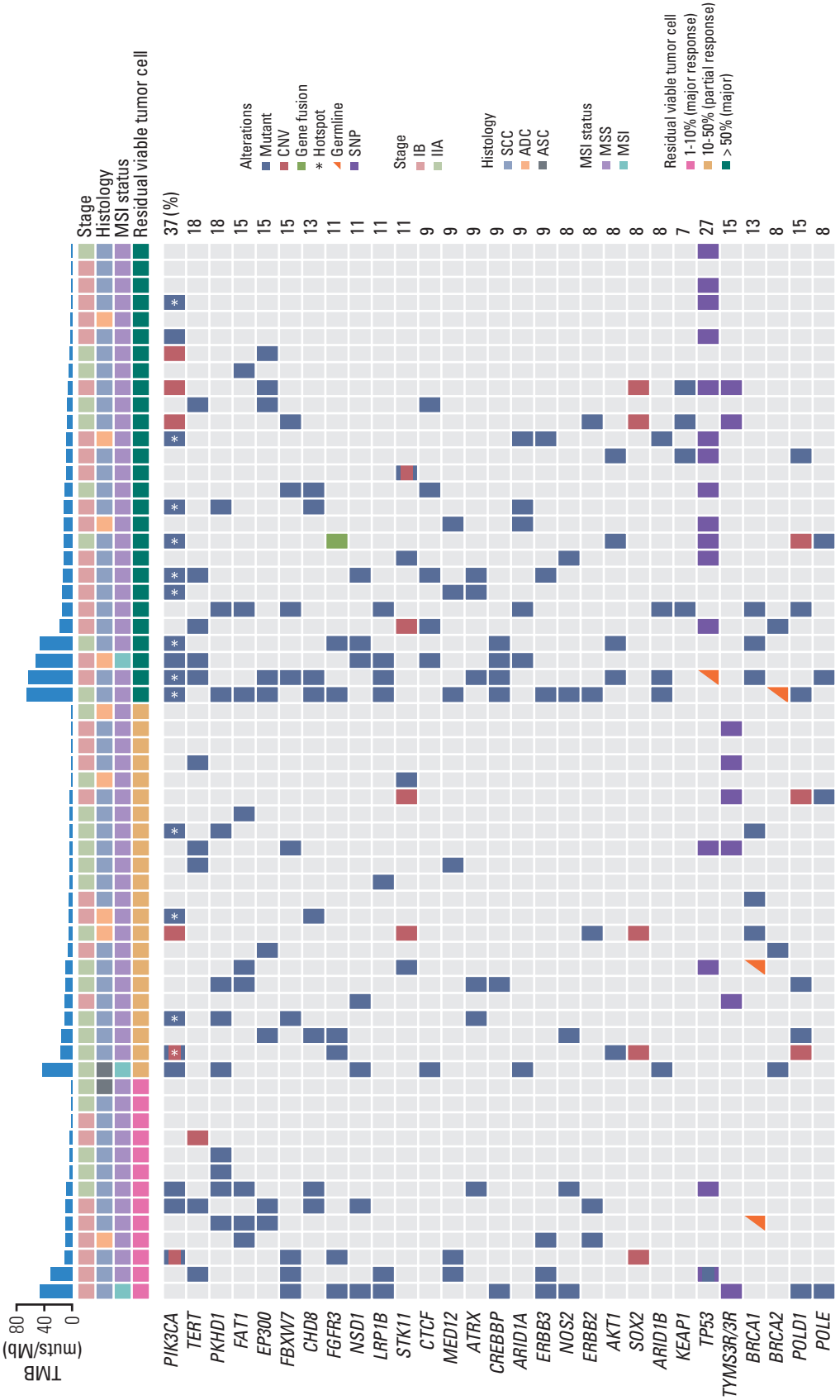

Fig. 1. Distribution of gene alterations correlated with pathologic response. Gene alterations and patient clinical characteristics were shown at the top and bottom, respectively. Patients were separated into three groups, of which H&E stains exhibited < 10%, 10%-50% and 50%-100% viable tumor cells. The *BRCA1/2*, *POLD1*, and *POLE* were genes related to targeted therapy or immunotherapy. *PIK3CA* hotspot mutations on E542, E545, and H1047 were marked by white asterisks. The 0%-10%, 10%-50%, and 50%-100% viable tumor cells represent major, partial and poor pathologic response, respectively. ADC, adenocarcinoma; ASC, adenosquamous carcinoma; CNV, copy number variation; MSI, microsatellite instability; MSS, microsatellite stability; SCC, squamous cell carcinoma; SNP, single nucleotide polymorphism; TMB, tumor mutation burden.

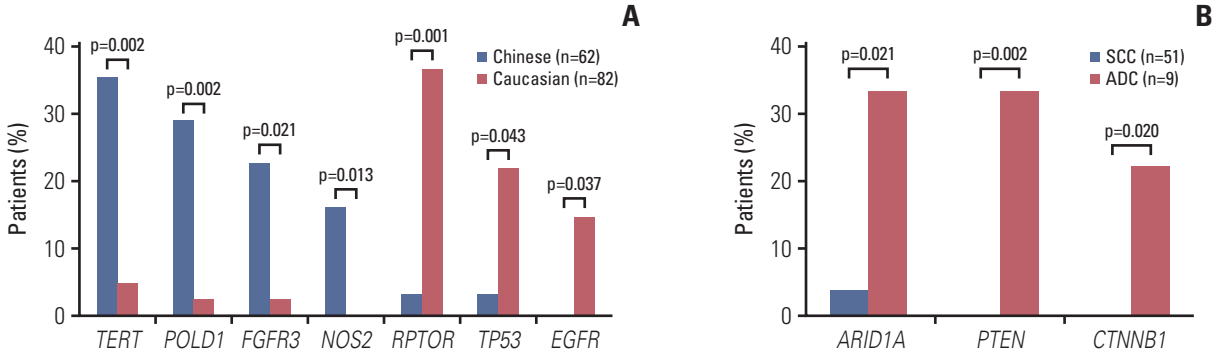

Fig. 2. Genetic alterations enriched in Chinese, Caucasian patients or cervical adenocarcinoma (ADC). (A) Gene alterations significantly enriched in Chinese (blue) and Caucasian (red) patients were shown on the left and right respectively. (B) Gene mutations associated with cervical adenocarcinoma were shown in red. SCC, squamous cell carcinoma.

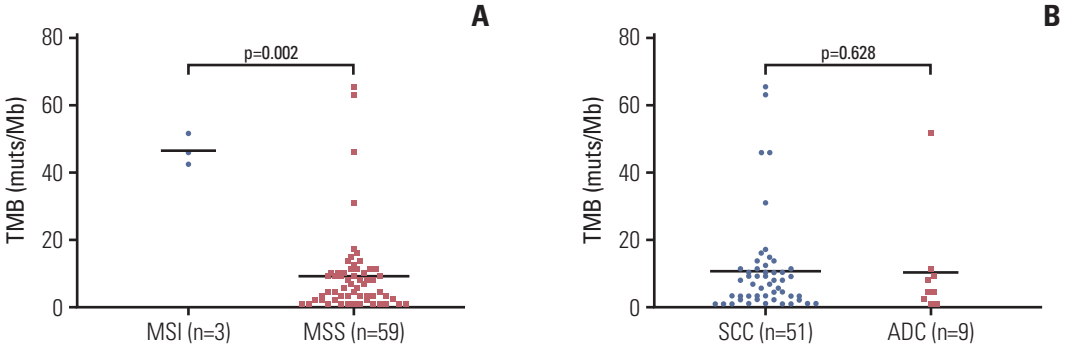

Fig. 3. Association of tumor mutation burden (TMB) with microsatellite instability in cervical cancer. Comparison of TMB levels in patients separated by microsatellite instability status (A) and histological types (B). ADC, adenocarcinoma; MSI, microsatellite instability; MSS, microsatellite stable; SCC, squamous cell carcinoma.

plus radical hysterectomy in Shandong Cancer Hospital from 2016 to 2019 were retrospectively reviewed in this study (Table 1). The median age of the cohort was 47 years (range, 26 to 66 years). Approximately 55% of patients were diagnosed of stage IB disease. SQCC accounted for ~82% of the cohort, with the remaining subjects being ADC (~15%) and ASC (~3%). Most patients (44/62, 71%) were classified as high-risk HPV types including 16, 18, 31, 33, 45, 52, and 58 [19], with the HPV type 16 being most common (30/62, 48%), while the remaining 18 patients remained unknown for the HPV type (S1 Table).

2. Genomic characteristics of locally advanced cervical cancer

We first characterized the mutational landscape of those 62 locally advanced cervical cancers through comprehensive genomic profiling by using targeted next generation sequencing (see "Materials and Methods"). The median depth of coverage was 974× (range, 322× to 2,159×), and the median coverage depth after removing PCR duplicates was 525× (range, 190× to 1,504×) (S3 Table). As shown in Fig. 1, *PIK3CA* represented the most frequently mutated gene of which mutations were detected in 37% of the cohort, comparable to what was reported in a Caucasian population of 82 cervical cancer patients from The Cancer Genome Atlas (TCGA) [20] (S4 Table), followed by *TERT* (18%) and *PKHD1* (18%). We acknowledge that there was a difference in the clinical stage between the TCGA dataset and the current cohort (Table 1), but no significant difference of mutation frequencies between IB and IIA patients was observed in either cohort. More than half of mutations identified in *PIK3CA* were hotspot mutations located in exons 9 and 20, including E542, E545, and H1047, which were involved in inhibitory interaction with regulatory subunit (E542 and E545) and membrane association (H1047) [21]. *PIK3CA* amplification was also detected in approximately 10% (6/62) of the cohort, and of note, three patients had multiple *PIK3CA* aberrations (Fig. 1). *TERT*, *POLD1*, *NOS2*, and *FGFR3* genes were fre-

Table 2. Germline mutant patient characteristics

Patient ID	Age (yr)	Stage	Histology	Gene	AA change	Variant type
CC_006	35	IIA	ADC	*MPL*	W398X	Nonsense variant
CC_007	44	IIA	SCC	*MMP1*	A330LfsX45	Frame shift variant
CC_015	41	IB	SCC	*PMS1*	K894RfsX17	Frame shift variant
CC_019	36	IB	SCC	*AXIN2*	R714W	Missense variant
CC_028	60	IB	SCC	*BRCA1*	c.4358-2A>G	Splice variant
CC_028	60	IB	SCC	*BRIP1*	S618*	Nonsense variant
CC_034	66	IB	SCC	*EPCAM*	L78R	Missense variant
CC_036	59	IIA	SCC	*PALB2*	S537L	Missense variant
CC_039	50	IB	SCC	*MUTYH*	Y453C	Missense variant
CC_039	50	IB	SCC	*TP53*	A86V	Missense variant
CC_056	43	IIA	SCC	*BRCA2*	S2414*	Nonsense variant
CC_072	38	IB	ADC	*FANCE*	S157Kfs*21	Frame shift variant
CC_079	41	IIA	SCC	*BRCA1*	S451Lfs*20	Frame shift variant
CC_081	44	IB	ADC	*MLH1*	S295G	Missense variant
CC_111	42	IB	SCC	*FANCM*	L923Cfs*3	Frame shift variant

ADC, adenocarcinoma; SCC, squamous cell carcinoma.

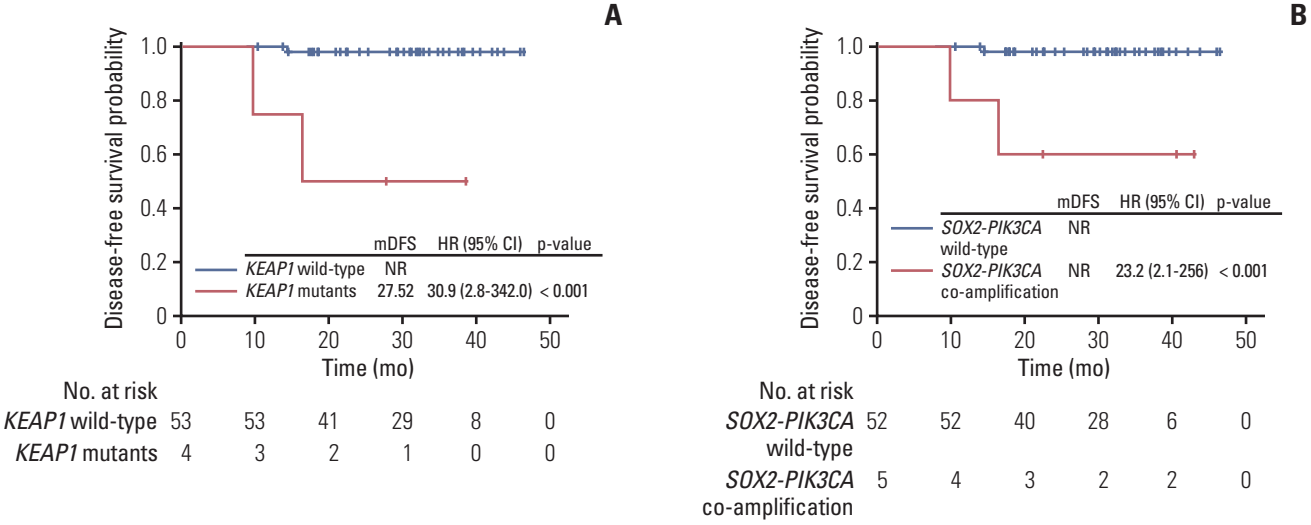

Fig. 4. Association of *KEAP1* mutation and *SOX2-PIK3CA* co-amplification with high cervical cancer recurrence risk. Poor disease-free survival was observed in patients harbouring *KEAP1* mutation (A) or *SOX2-PIK3CA* co-amplification (B). CI, confidence interval; HR, hazard ratio; mDFS, median disease-free survival; NR, not reported.

quently altered in Chinese cervical cancer, whereas *RPTOR*, *EGFR*, and *TP53* gene variants were significantly enriched in Caucasian cervical cancer (Fig. 2A). *ARID1A*, *PTEN*, and *CTNNB1* gene mutations were frequently observed in cervical ADC (Fig. 2B).

Furthermore, the TMB (median TMB: 46 muts/megabase [Mb]) of microsatellite unstable (MSI) cervical cancer patients (n=3) was significantly higher than MSS patients (median TMB: 9.2 muts/Mb) (Fig. 3A), although the MSI subgroup size was restricted. No significant differences of TMB were observed between histology subgroups as to SQCC or ADC (Fig. 3B).

In addition, germline mutations were detected in 21% (13/62) of the cohort (Table 2). The median age of the patients who carried germline mutations was 43 years, who were younger than those without germline mutations by an average of 6 years. Most of the patients (77%, 10/13) carried nonsynonymous mutations of genes including *TP53*, *BRCA2*, *BRIP1*, *BRCA1*, *FANCM*, *MUTYH*, *FANCE*, and *PALB2*, which play parts in DNA damage repair pathways (Table

2). Particularly, *BRCA2*, *BRIP1*, *BRCA1*, and *PALB2* that were involved in homology-directed repair process were detected in four patients (31%) (Table 2).

3. Genomic traits related to poor neoadjuvant chemoradiation response and higher disease relapse risk

The pathologic response to neoadjuvant chemoradiation was evaluated by quantifying the percent of residual viable tumor following the neoadjuvant chemoradiation (see "Materials and Methods"). Twenty-two patients (35%) showed partial pathologic response to neoadjuvant therapy (< 50% residual viable tumor), and thirteen patients (21%) demonstrated major pathologic response (< 10% viable tumor cells) [10,11], including two patients who showed complete pathologic response (Table 1, S1 Table). Univariate analysis showed that Kelch-like ECH-associated protein 1 (*KEAP1*) mutations (p=0.031), TMB-high (TMB-H) (p=0.011) and *TP53* polymorphism (rs1042522 P72R, p=0.007) were significantly associated with poor pathologic response to neoadjuvant chemoradiation in those patients (S5 Table). The *PIK3CA* mutations had a trend to associate with poor pathologic response (S5 Table). Multivariate analysis showed that *TP53* polymorphism was an independent factor that correlated with poor pathologic response (p=0.014) but not necessarily with poor DFS (hazard ratio [HR], 1.8; 95% confidence interval [CI], 0.2 to 20.8; p=0.616).

As of manuscript writing, the median follow-up time of the cohort was 31 months. Most of the patients (95%) remained relapse-free up till the data cutoff date. Five patients were lost to follow up after surgery. Firstly, major pathologic response (MPR) patients demonstrated better DFS than non-MPR patients although not significantly (S6 Fig.). Secondly, we were able to identify four genomic alterations that were significantly associated with poor DFS, including *KEAP1* mutations (n=4; HR, 30.9; 95% CI, 2.8 to 342; p < 0.001) (Fig. 4A), *SOX2*-*PIK3CA* co-amplification (n=5; HR, 23.2; 95% CI, 2.1 to 256; p < 0.001) (Fig. 4B), thymidylate synthase triple repeats (3R/3R) polymorphism (n=8; HR, 12.8; 95% CI, 1.2 to 142; p=0.007) (S7A Fig.), and *TERC* copy number gain (n=3; HR, 45.6; 95% CI, 4.1 to 507; p < 0.001) (S7B Fig.).

Discussion

In this study, we characterized genetic alteration of 62 cervical cancer cases in China and compared their molecular profile with that of Caucasian cervical cancer patients in TCGA database. *PIK3CA* was the most frequently mutated gene in cervical cancer regardless of racial groups, suggesting a universal dependence of cervical cancer on phosphoinositide 3-kinase (PI3K)/AKT signal pathway. Both datasets highlighted three mutation hotspots in *PIK3CA* gene, including E542, E545, and H1047 which accounted for half of mutation sites. Interestingly, several genes' mutation frequency differed significantly in Chinese and Caucasian cervical cancer. The three genes predominantly mutated in Caucasian population were *RPTOR*, *EGFR*, and *TP53*, all associated with PI3K/AKT pathway. The genes mainly mutated in Chinese patients were *TERT*, *POLD1*, *NOS2*, and *FGFR3*. *TERT* and *POLD1* were associated with telomere maintenance in cells. It has been reported that HPV type 16 E6 could activate *TERT* gene transcription [22], suggesting a close relationship between HPV infection and *TERT* expression. Gene amplification, rearrangement and protein expression of *TERT* were associated with poor clinical outcome in human cancers including thyroid cancer, glioma, and neuroblastoma [23]. Further clinical investigation is needed to evaluate the effect of *TERT* promoter mutation on survival of cervical cancer patients.

Our finding of the enrichment of germline mutations in DNA repair pathway agrees with prior evidence [24]. However, the mutation patterns of cervical cancer differed among studies, likely due to the difference in cohort size and racial/genetic background. In our study, over 60% of the germline mutations were truncation, frameshift, or splicing variants deleterious to protein function, suggesting tumor suppressor role of these genes and importance of inactivation of DNA repair pathway in tumorigenesis. Noteworthy, four patients (31%) carried mutations of genes involved in the homology-directed DNA repair process, yielding a homologous recombination deficiency phenotype, which strongly resembled the results showed in other gynecological cancers including breast, ovarian, and endometrial cancer [25]. So far, several poly(ADP-ribose) polymerase inhibitors (PARPi) have been approved by the US Food and Drug Administration in *BRCA1*/2-mutant ovarian and breast cancer. Given that, the clinical utility of PARPi in cervical cancer is worth exploration, either alone or in combination with chemotherapy or targeted therapy. The early onset of cancer was found in germline-mutant patients in this study, supporting the critical role of DNA repair gene mutations in carcinogenesis as previously described [26,27].

To date, two randomized phase III trials, NCT00193739 [28] and EORTC Protocol 55994 [29], were designed to compare the neoadjuvant chemotherapy followed by surgery with the standard regimen (concurrent chemoradiation) for FIGO IB-IIA cervical cancer patients, although the latter study has not yet reported its final results. According to both studies, neoadjuvant chemotherapy followed by surgery was not superior to the standard regimen in terms of 5-year DFS or overall survival (OS), while NCT00193739 showed that the neoadjuvant approach had a more favorable safety profile.

Nonetheless, the neoadjuvant chemotherapy followed by surgery for FIGO IB-IIA cervical cancer was permitted in National Comprehensive Cancer Network (NCCN) guidelines (ver. 1.2021). Furthermore, the neoadjuvant brachytherapy and chemotherapy to radical hysterectomy was included in the clinical practice guideline in China and has shown promising efficacy [7]. It reduced the size of stage IB2-IIA cervical cancer and enabled radical surgery, achieving an overall survival comparable to standard chemoradiation as well as a more favorable side-effect profile. However, there were still 10% of patients whose tumor progressed after the treatment which may partly be attributed to the poor response to neoadjuvant chemoradiotherapy. Taken together, in view of these limited datasets, further research is warranted to investigate the potential clinical benefit of neoadjuvant chemotherapy in early-stage cervical cancer.

Furthermore, the identification of biomarkers predicting response to neoadjuvant therapy as well as tumor recurrence would enable early detection of recurrence and maximize therapeutic window for patients. *KEAP1* mutations have been reported to occur commonly in diverse cancer types including lung cancer (both ADC and SQCC), colon ADC, and endometrial carcinoma [30]. Prior studies have shown that *KEAP1* mutations promote cell proliferation in tumors and may also give rise to resistance to chemotherapy [31,32], consistent with what we found in this study that *KEAP1* mutations were associated with poor pathologic response to neoadjuvant chemoradiation and an increased risk of early disease relapse in cervical cancer. Furthermore, our data showed that *SOX2* and *PIK3CA* were co-amplified in five patients (four SQCCs and one ADC). *SOX2* and *PIK3CA* are localized in proximity on the chromosome 3q26. This result is consistent with what was recently reported by Voutsadakis [33]. The amplification of 3q26 has also been reported in other cancer types, including head and neck [34], lung [35], and oropharyngeal squamous cell carcinomas [36], further corroborating our findings. Prior studies demonstrated that *PIK3CA* amplification was associated with shorter survival in lung [37], esophageal [38] and nasopharyngeal SQCC [39], and *SOX2* amplification was reported to be associated with clinical progression in squamous lung cancer [35]. Consistently, in this study, we report that in cervical cancer, particularly SQCC, *SOX2-PIK3CA* co-amplification was significantly associated with poor pathologic response to neoadjuvant chemoradiation and worse disease-free survival.

In addition, according to Li et al. [40], a genome-wide SNPs study of 596 patients with stage IA2-IIIB cervical cancer, four SNPs exhibited strong association with response to neoadjuvant chemotherapy in overall survival (OS) or DFS. In this study, we performed genomic profiling by using targeted next generation sequencing, and through univariate analysis, we found that *TP53* polymorphism (P72R, rs1042522), *KEAP1* mutations and TMB-H were associated with poor pathologic response to neoadjuvant therapy as measured by the proportion of residual viable tumor, and *TP53* remained significantly correlated by a multivariant correction. Though *TP53* rs1042522 and wild-type subgroups did not differ significantly in OS or DFS in either cohorts, our data suggest that the association between *TP53* polymorphism (P72R) and resistance of chemoradiotherapy also existed in cervical cancer in addition to what has previously been reported for head and neck cancer [41]. In addition, in June 2020, U.S. Food and Drug Administration expanded the approval of pembrolizumab (anti–programmed death-1) to include any cancer with TMB-H. Our previous work has shown that TMB-H (≥ 10 muts/Mb) was associated with favorable response to immune checkpoint blockade in lung cancer [42]. Thus, it seems rational that TMB-H cervical cancer patients can be considered for immunotherapy particularly in view of their poor pathologic response to chemoradiotherapy.

In conclusion, we report the comprehensive genomic profiles of locally advanced cervical cancer patients and the distinction between Asian and Caucasian populations. Our findings also highlight genomic traits associated with unfavorable neoadjuvant chemoradiation response and increased risk of early disease recurrence. This study has a few limitations. Firstly, a retrospective cohort study design was used, and the cohort size remained limited. Secondly, the study presented a relatively shorter follow-up period in comparison to previous studies. Thirdly, an external dataset with the clinical characteristic of the residual viable tumor would be ideal for the validation of response biomarkers. Future efforts should focus on validating these results in prospectively designed studies of larger patient sample size.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was approved by the Institutional Review Board/Ethics Committee of Shandong Cancer Hospital and Institute (Reference No. SDTHEC201901007). Written informed consent was obtained from each patient prior to sample collection.

Author Contributions

Conceived and designed the analysis: Wei Y, Yuan S.
Collected the data: Wei Y, Wei C, Chen L, Liu N.
Performed the analysis: Wei Y, Qu Q, Yin JC, Pang J, Fang Z, Wu X, Wang X, Mu D.
Wrote the paper: Wei Y, Qu Q, Yin JC, Pang J, Fang Z.
Study supervision: Shao Y, Yu J, Yuan S.

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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

Acknowledgments
We would like to thank the patients and family members who gave their consent on presenting the data in this study, as well as the investigators and research staff involved in this study.
This study was partially funded by Natural Science Foundation of China (NSFC81872475, NSFC81372413, NSFC81972683), National key Research and Development Program (2018YFC1313200), Shandong Natural Science Foundation Joint Fund (ZR2019LZL018), Shandong Key Research and Development Plan (2017CXGC1209 and 2017GSF18164) and the Outstanding Youth Natural Science Foundation of Shandong Province (JQ201423), Jinan Clinical Medicine Science and Technology Innovation Plan (201704095), National Key Research and Development Program of China (2016YFC0904700).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1010
Cancer Res Treat. 2022;54(4):1219-1229

Original Article

Identification of Patients with Recurrent Epithelial Ovarian Cancer Who Will Benefit from More Than Three Lines of Chemotherapy

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Purpose This study aimed to identify patients who would benefit from third and subsequent lines of chemotherapy in recurrent epithelial ovarian cancer (EOC).

Materials and Methods Recurrent EOC patients who received third, fourth, or fifth-line palliative chemotherapy were retrospectively analyzed. Patients' survival outcomes were assessed according to chemotherapy lines. Based on the best objective response, patients were divided into good-response (stable disease or better) and poor response (progressive disease or those who died before response assessment) groups. Survival outcomes were compared between the two groups, and factors associated with chemotherapy responses were investigated.

Results A total of 189 patients were evaluated. Ninety-four and 95 patients were identified as good and poor response group respectively, during the study period of 2008 to 2021. The poor response group showed significantly worse progression-free survival (median, 2.1 months vs. 9.7 months; $p < 0.001$) and overall survival (median, 5.0 months vs. 22.9 months; $p < 0.001$) compared with the good response group. In multivariate analysis adjusting for clinicopathologic factors, short treatment-free interval (TFI) (hazard ratio [HR], 5.557; 95% confidence interval [CI], 2.403 to 12.850), platinum-resistant EOC (HR, 2.367; 95% CI, 1.017 to 5.510), and non-serous/endometrioid histologic type (HR, 5.045; 95% CI, 1.152 to 22.088) were identified as independent risk factors for poor response. There was no difference in serious adverse events between good and poor response groups (p=0.167).

Conclusion Third and subsequent lines of chemotherapy could be carefully considered for palliative purposes in recurrent EOC patients with serous or endometrioid histology, initial platinum sensitivity, and long TFIs from the previous chemotherapy regimen.

Key words Epithelial ovarian carcinoma, Drug therapy, Recurrence, Survival, Treatment response, Prognosis

Introduction

Epithelial ovarian cancer (EOC) is a fatal gynecologic malignancy, and its incidence has steadily increased over the past decade [1,2]. It was estimated that 225,000 new cases of invasive cancer involving the ovary would be diagnosed worldwide in the year 2008; however, in 2020, an estimated 313,959 new cases occurred worldwide [2,3]. Most patients with EOC are diagnosed at advanced stage and experience disease recurrence despite extensive cytoreductive surgery and platinum-based chemotherapy. Approximately 70% to 80% of patients show initial response to platinum-based chemotherapy [4]. However, the median progression-free survival (PFS) in patients with advanced ovarian cancer is about 18 months, and recurrence occurs in more than 50% of patients within 2 years of completion of first-line therapy [5]. Due to the high recurrence rate, most patients are subject to repetitive treatment cycles and regimen changes [6].

Palliative chemotherapy is an important treatment option for patients with incurable advanced-stage cancer, and the rationale for treatment during disease progression has its pros and cons. The rationale for treatment would be to provide symptom palliation, maintain stable disease (SD), and for the opportunity to use newer agents with possibly fewer cumulative toxicities [7-9]. In fact, there has been an increase in the administration of palliative chemotherapy to patients with end-stage cancers due to the development of anticancer drugs that are highly effective and less toxic than conventional drugs [10]. In advanced non–small cell lung cancer (NSCLC), the development and availability of new chemotherapeutic agents has led to an increase in the number of lines of chemotherapy administered to patients, subsequently resulting in an increase in the length of time for patients to receive chemotherapy [11]. Similar treatment trends have

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Received September 9, 2021 **Accepted** November 15, 2021 **Published Online** November 17, 2021
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been shown in gynecologic cancer patients [8]. However, repetitive administration of palliative chemotherapy may be harmful to patients' quality of life depending on factors such as the timing of treatment, type of drugs administered, and toxicity [10]. Moreover, the delay in the referral to palliative care services can hinder adequate end-of-life treatment [12]. This leads to prolonged chemotherapy with lack of demonstrable benefits, low rates of hospice-care use, and increased interventions that result in emergency room (ER) visits, hospitalizations, or admissions to intensive care units (ICUs) at the end of life [10]. Therefore, timely discontinuation of chemotherapy and referral can lower healthcare costs and improve the quality of life of patients nearing the end of their lives [13].

However, it is difficult to estimate the survival duration of heavily treated recurrent ovarian cancer patients, and no indicator has been identified yet for the identification of those who may benefit from palliative chemotherapy [14]. Therefore, the objective of this study was to investigate the real-world survival outcomes in EOC patients who received third, fourth, and fifth-line chemotherapy due to disease progression. Furthermore, we aimed to identify patients who actually benefited from chemotherapy in regards to survival, so that we can select potential responders and preemptively avoid unnecessary chemotherapy administration and consider timely transfer to palliative and hospice care in poor responders at the end of life.

Materials and Methods

1. Study population

From the institution's ovarian cancer cohort database, we identified the following patients: (1) those with EOC who had received cytoreductive surgery and platinum-based chemotherapy for first-line chemotherapy; and (2) those who completed third, fourth, or fifth-line chemotherapy between June 2008 and March 2021. We excluded the patients who had insufficient clinicopathologic data or lost to follow-up. The *International Statistical Classification of Disease, 10th revision* (ICD-10) code of EOC is C56.

2. Data collection

By reviewing patients' medical records and pathologic reports, we collected clinicopathologic data such as age, serum cancer antigen 125 (CA-125) levels, International Federation of Gynecology and Obstetrics (FIGO) stage, histologic type and grade, extent of debulking surgery, and regimens and cycles of chemotherapy.

Patients were divided into the following three groups based on the latest line of chemotherapy that was administered during study period (to avoid duplicate patients): third-line, fourth-line, and fifth-line-chemotherapy groups. We compared the survival outcomes in these three groups. Further, depending on their treatment response, patients were defined as a good response group if their best overall response to treatment was complete response (CR), partial response (PR) or SD. Patients were categorized as a poor response group if the best overall response was progressive disease (PD) or if they expired before the treatment response assessment. Data on relevant factors related to survival outcomes and treatment-related adverse events were collected for these two groups of patients. The best overall response was defined as the best response recorded from the start of treatment until disease progression/recurrence. The final overall response was defined as the response recorded after the last dose of chemotherapy. Treatment-free interval (TFI) was defined as the time between the end of chemotherapy regimen and subsequent relapse. Chemotherapy regimens were decided by the gynecologic oncologists in consideration of the previous treatment regimens administered to the patients, the Korean National Health Insurance coverage, and adverse events that had previously occurred in patients.

3. Tumor assessment

PFS and overall survival (OS) were defined as the time to recurrence from the start of chemotherapy and the time from the start of chemotherapy until death, respectively. Disease control rate (DCR; percentage of patients who achieved CR, PR, or SD after receiving chemotherapy), and chemotherapy-induced toxicities were also evaluated. All patients underwent computed tomography (CT) scans every three cycles during chemotherapy. Tumor assessment was performed according to the Response Evaluation Criteria in Solid Tumors (ver. 1.1) using CT [15]. During the surveillance, CT scans were routinely performed every 3 to 4 months for the first 2 years, every 4 to 6 months for the next 2 years, and annually thereafter, or when symptoms or examination findings were suspicious for recurrence. Tumor markers were also used to evaluate treatment efficacy and response. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events (ver. 4.0) and classified as either hematologic or non-hematologic toxicity [16]. A serious adverse event (SAE) was defined as life-threatening event, persistent or significant disability, or hospitalization event during chemotherapy. The frequency of SAE was also documented.

4. Statistical analysis

Student's t, Mann-Whitney U, and Kruskal-Wallis tests were used for analyzing continuous variables, and dichotomous variables were compared with the chi-square and

Table 1. Clinicopathologic characteristics of study population according to treatment response of chemotherapy

Characteristic	Total (n=189)	Good response group (n=94)	Poor response group (n=95)	p-value
Age at initial diagnosis (yr)	54.0±10.8	53.8±9.9	53.3±11.6	0.760
Histologic type				
Serous	148 (78.3)	77 (81.9)	71 (74.7)	0.030
Endometrioid	15 (7.9)	10 (10.6)	5 (5.3)	
Clear cell	13 (6.9)	3 (3.2)	10 (10.5)	
Mucinous	7 (3.7)	1 (1.1)	6 (6.3)	
Mixed	3 (1.6)	2 (2.1)	1 (1.1)	
Undifferentiated	2 (1.1)	0	2 (2.1)	
Others	1 (0.5)	1 (1.1)	0	
Grade				
1	5 (2.6)	2 (2.1)	3 (3.2)	0.852
2	20 (10.6)	9 (9.6)	11 (11.6)	
3	156 (82.5)	80 (85.1)	76 (80.0)	
Undifferentiated	1 (0.5)	0	1 (1.1)	
Unknown	7 (3.7)	3 (3.2)	4 (4.2)	
FIGO stage				
I	8 (4.2)	2 (2.1)	6 (6.3)	0.261
II	7 (3.7)	5 (5.3)	2 (2.1)	
III	118 (62.4)	62 (66.0)	56 (58.9)	
IV	56 (29.6)	25 (26.6)	31 (32.6)	
Results of initial debulking surgery				
No residual tumor	92 (48.7)	50 (53.2)	42 (44.2)	0.255
Residual tumor < 1 cm	58 (30.7)	29 (30.9)	29 (30.5)	
Residual tumor ≥ 1 cm	39 (20.6)	15 (16.0)	24 (25.3)	
CA-125 at baseline (IU/mL)	809.0 (3-24,720)	846.0 (3-24,720)	795.0 (6-8,940)	0.437
Age at the latest recurrence (yr)	56.9±10.9	57.9±9.9	55.9±11.9	0.220
CA-125 at the latest recurrence (IU/mL)	164.5 (3-7,700)	128.0 (3-7,700)	202.5 (8-7,188)	0.010
TFI (mo)	1.38 (0.3-93.1)	3.58 (0.5-93.1)	1.05 (0.3-12.1)	< 0.001
Platinum sensitivity				
Sensitive	126 (66.7)	74 (78.7)	52 (54.7)	< 0.001
Resistant	63 (33.3)	20 (21.3)	43 (45.3)	
***BRCA* mutational status**				
BRCA1/2 mutation	19 (10.1)	14 (14.9)	5 (5.3)	0.182
BRCA1/2 wile-type	53 (28.0)	30 (31.9)	23 (24.2)	
Not tested	117 (61.9)	50 (53.2)	67 (70.5)	
Days from last chemotherapy to death	108 (6-1,738)	139.5 (31-1,738)	99 (6-1,181)	0.026

Values are presented as mean±SD, number (%), or median (range). CA-125, cancer antigen 125; FIGO, International Federation of Gynecology and Obstetrics; SD, standard deviation; TFI, treatment-free interval.

Fisher exact tests. Categorical data were presented as number and percentage, and numerical data were presented as median and range or mean and standard deviation. Survival analyses were performed using the Kaplan-Meier method, and results were compared using the log-rank test and Cox proportional hazards regression models and calculated the adjusted hazard ratios and 95% confidence intervals (CIs). Associations between categorical variables and treatment responses to third, fourth, and fifth-line chemotherapy were evaluated using binary logistic regression analysis. Statistical analyses were performed using SPSS ver. 25.0 (IBM Corp., Armonk, NY). p-values < 0.05 were considered statistically significant.

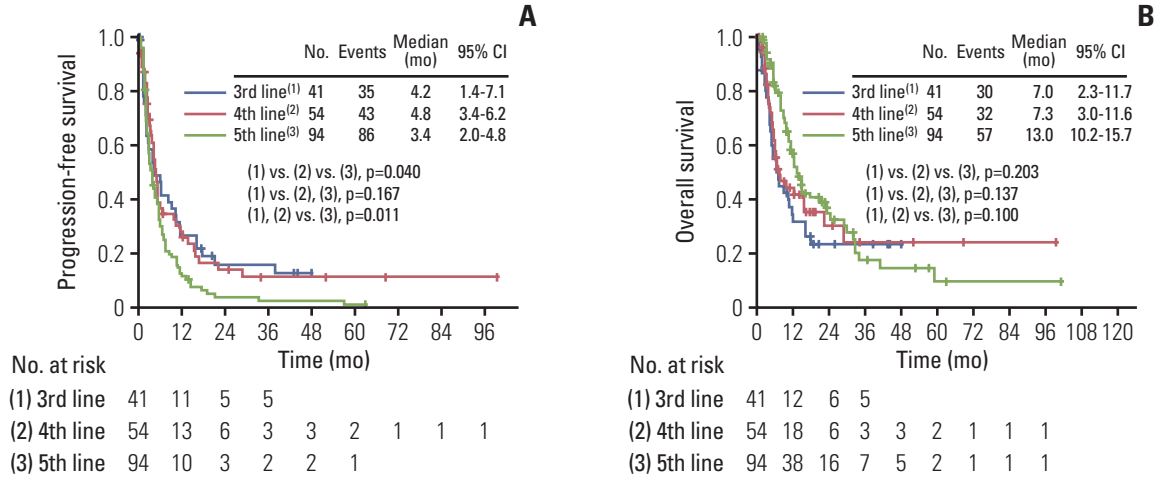

Fig. 1. Comparisons of survival outcomes in the 3rd line, 4th line, and 5th line chemotherapy groups. (A) Kaplan-Meier curves for progression-free survival in the 3rd line, 4th line, and 5th line chemotherapy groups; p-values of comparisons between two groups: (1) vs. (2), p=0.916; (1) vs. (3), p=0.040; (2) vs. (3), p=0.034; Number at risk is the number of patients who are at risk of recurrence after 3rd, 4th, or 5th line chemotherapy. (B) Kaplan-Meier curves for overall survival in the 3rd line, 4th line, and 5th line chemotherapy groups; p-values of comparisons between two groups: (1) vs. (2), p=0.483; (1) vs. (3), p=0.078; (2) vs. (3), p=0.282. Number at risk is the number of patients who are at risk of death after 3rd, 4th, or 5th line chemotherapy. CI, confidence interval.

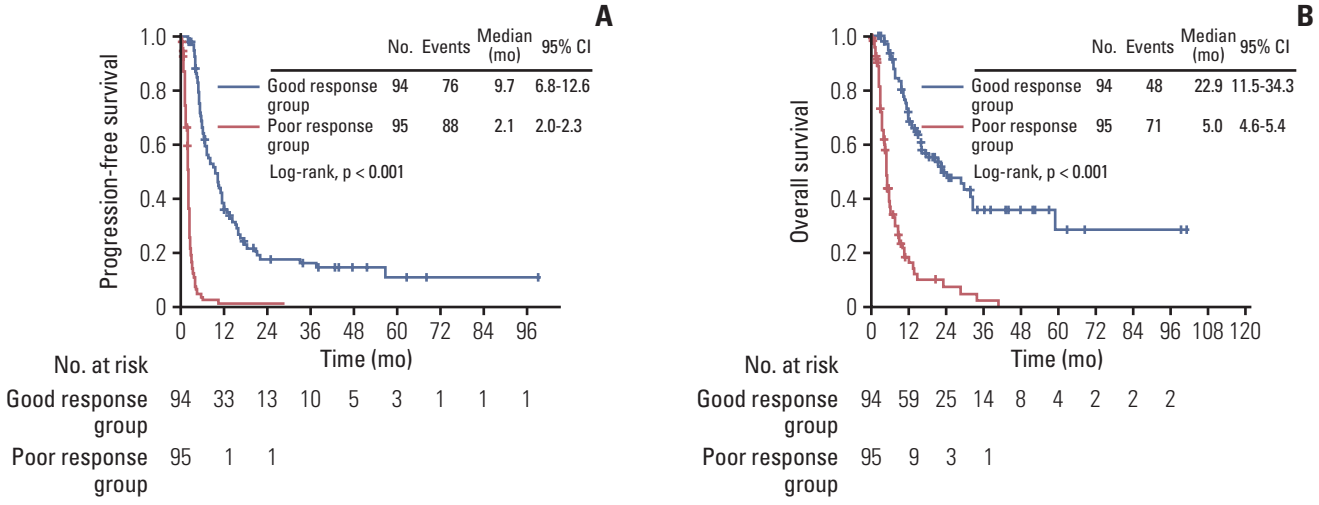

Fig. 2. Comparisons of survival outcomes in the good response group and poor response group. (A) Kaplan-Meier curves for progression-free survival in the good response group and poor response group. Number at risk is the number of patients in good and poor response group who are at risk of recurrence after 3rd, 4th, or 5th line chemotherapy. (B) Kaplan-Meier curves for overall survival in the good response group and poor response group. Number at risk is the number of patients in good and poor response group who are at risk of death after 3rd, 4th, or 5th line chemotherapy. CI, confidence interval.

Results

1. Patient characteristics and treatment response

A total of 189 consecutive patients who received third, fourth, or fifth-line chemotherapy during the study period were identified (S1 Fig.). Of them, 94 and 95 patients were categorized as good and poor response groups, respectively, based on their best overall response to treatment. There were no clinicopathologic differences between the two groups except for the histologic type, CA-125 level at the latest recurrence, TFI, and platinum sensitivity (Table 1). Median days from the last chemotherapy to death in the entire cohort was

Table 2. Univariate and multivariate analyses for progression-free and overall survival in study population

Characteristic	Progression-free survival						Overall survival					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	aHR	95% CI	p-value	HR	95% CI	p-value	aHR	95% CI	p-value
Age at recurrence (yr)												
≤ 56 vs. > 56	0.912	0.671-1.239	0.555	0.958	0.673-1.363	0.811	0.914	0.637-1.311	0.626	0.742	0.494-1.115	0.151
Histologic type												
Endometrioid	1 (reference)			1 (reference)			1 (reference)			1 (reference)		
Serous	1.845	0.967-3.521	0.063	1.259	0.603-2.632	0.540	1.901	0.879-4.110	0.103	1.354	0.516-3.551	0.538
Others	2.764	1.312-5.827	0.008	1.466	0.651-3.302	0.356	3.969	1.675-9.404	0.002	3.121	1.140-8.550	0.027
FIGO stage												
< IIIC vs. ≥ IIIC	1.390	0.971-1.989	0.072	1.286	0.775-2.136	0.331	1.434	0.933-2.205	0.100	0.996	0.540-1.839	0.990
Previous TFI (mo)												
≤ 3 vs. > 3	2.179	1.559-3.046	< 0.001	1.326	0.841-2.090	0.225	2.455	1.626-3.708	< 0.001	1.473	0.863-2.512	0.155
CA-125 at recurrence (IU/mL)												
≤ 500 vs. > 500	2.233	1.549-3.217	< 0.001	1.664	1.100-2.518	0.016	2.309	1.550-3.438	< 0.001	1.766	1.114-2.800	0.016
Residual tumor after debulking surgery												
No vs. Yes	0.823	0.604-1.121	0.216	0.739	0.504-1.084	0.122	1.459	1.009-2.109	0.044	1.073	0.691-1.668	0.753
Line of chemotherapy												
3rd line	1 (reference)			1 (reference)			1 (reference)			1 (reference)		
4th line	1.031	0.659-1.613	0.895	0.917	0.555-1.516	0.736	0.849	0.515-1.400	0.522	0.897	0.503-1.601	0.714
5th line	1.516	1.017-2.260	0.041	1.727	1.097-2.718	0.018	0.678	0.435-1.056	0.086	0.533	0.317-0.895	0.017
Malignant ascites												
No vs. Yes	1.172	0.731-1.877	0.510	0.766	0.435-1.349	0.356	1.271	0.725-2.229	0.402	1.112	0.532-2.321	0.778
Malignant pleural effusion												
No vs. Yes	0.764	0.495-1.181	0.226	1.026	0.636-1.655	0.916	1.555	0.948-2.549	0.080	1.075	0.636-1.816	0.788
Distant metastasis												
No vs. Yes	0.867	0.611-1.229	0.423	1.093	0.731-1.633	0.665	1.194	0.798-1.786	0.388	1.456	0.911-2.326	0.117
Platinum resistant												
Sensitive vs. Resistant	1.956	1.418-2.700	< 0.001	1.174	0.772-1.783	0.453	3.085	2.140-4.448	< 0.001	1.904	1.210-2.996	0.005
Response group												
Good response vs. Poor response	8.379	5.832-12.038	< 0.001	8.472	5.232-13.720	< 0.001	5.134	3.476-7.583	< 0.001	4.202	2.599-6.792	< 0.001

aHR, adjusted HR; CA-125, cancer antigen 125; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; TFI, treatment-free interval.

Table 3. Univariate and multivariate analysis of risk factors associated with poor response group

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-value	aHR (95% CI)	p-value
Line of chemotherapy				
3rd line	1 (reference)		1 (reference)	
4th line	1.158 (0.513-2.611)	0.724	2.088 (0.705-6.182)	0.184
5th line	1.261 (0.604-2.630)	0.537	2.041 (0.733-5.680)	0.172
Age at recurrence (yr)				
≤ 56 vs. > 56	0.900 (0.509-1.593)	0.717	1.099 (0.534-2.264)	0.797
CA-125 at recurrence (IU/mL)				
≤ 500 vs. > 500	2.592 (1.287-5.217)	0.008	1.820 (0.773-4.284)	0.170
Previous TFI (mo)				
> 3 vs. ≤ 3	5.376 (2.744-10.535)	< 0.001	5.557 (2.403-12.850)	< 0.001
Stage				
< IIIC vs. ≥ IIIC	1.612 (0.823-3.158)	0.164	1.244 (0.484-3.201)	0.650
Malignant ascites				
No vs. Yes	1.390 (0.574-3.368)	0.465	1.008 (0.302-3.361)	0.989
Malignant pleural effusion				
No vs. Yes	1.831 (0.791-4.239)	0.158	1.455 (0.467-4.532)	0.517
Histologic type				
Endometrioid	1 (reference)		1 (reference)	
Serous	1.844 (0.601-5.657)	0.285	1.865 (0.558-6.230)	0.311
Others	5.429 (1.366-21.570)	0.016	5.045 (1.152-22.088)	0.032
Distant metastasis				
No vs. Yes	1.226 (0.641-2.343)	0.538	1.054 (0.452-2.458)	0.902
Platinum resistant				
Sensitive vs. Resistant	3.060 (1.616-5.792)	0.001	2.367 (1.017-5.510)	0.046

aHR, adjusted HR; CA-125, cancer antigen 125; CI, confidence interval; HR, hazard ratio; TFI, treatment free interval.

108 days, with the median of 99 days (range, 6 to 1,181 days) in the poor response group. Baseline patient characteristics according to each line of chemotherapy and details on previous chemotherapy regimens are summarized in S2 Table and S3 Table. No significant differences were noted in clinical characteristics in patients who received third (n=41), fourth (n=54), or fifth-line (n=94) chemotherapy.

Chemotherapy responses after each line of chemotherapy are shown in S4 Table. Of the 189 patients, the treatment response of 167 patients was assessed since 22 patients expired before their treatment response could be assessed. The DCR among all patients was 49.7%; and there was no statistically significant difference among the third, fourth, and fifth-line chemotherapy groups (p=0.179). With respect to final overall response, the third-line chemotherapy group had the highest proportion of patients who achieved the objective response of CR, PR, or SD (31.7%) compared to later lines, with statistically significant differences among the three groups (p=0.031). The total number of patients who died before the completion of treatment was 27 (14.3%); without statistical difference among the three groups (p=0.070).

2. Comparison of survival outcomes

Median PFS in the third-line, fourth-line, and fifth-line chemotherapy groups was 4.2 months (range, 1.4 to 7.1 months), 4.8 months (range, 3.4 to 6.2 months), and 3.4 months (range, 2.0 to 4.8 months), respectively, with statistically significant differences among the three groups (p=0.040) (Fig. 1A). However, median OS in the third, fourth, and fifth-line chemotherapy groups was 7.0 months (range, 2.3 to 11.7 months), 7.3 months (range, 3.0 to 11.6 months), and 13.0 months (range, 10.2 to 15.7 months), respectively (Fig. 1B). There was no statistically significant difference among the three groups (p=0.203).

According to the treatment response, median PFS in the good response and poor response groups was 9.7 months (range, 6.8 to 12.6 months) and 2.1 months (range, 2.0 to 2.3 months), respectively (Fig. 2A), with significantly higher response in the good response group (p < 0.001). The OS in the good response group was also longer than the poor response group, with the median OS of 22.9 months (range, 11.5 to 34.3 months) and 5.0 months (range, 4.6 to 5.4 months), respectively (p < 0.001) (Fig. 2B).

Table 4. SAE between good response group and poor response group

Factor	Good response group (n=94)	Poor response group(n=95)	p-value
SAE	47 (50.0)	38 (40.0)	0.167
Neutropenia ≥ grade 3	36 (38.3)	15 (15.8)	< 0.001
Other hematologic AE ≥ grade 3	9 (9.6)	6 (6.3)	0.434
Non-hematologic SAE	14 (14.9)	26 (27.4)	0.049
Admission through ER	13 (13.8)	26 (27.4)	0.030
ICU admission	1 (1.1)	1 (1.1)	> 0.999
Malignant ileus	5 (5.3)	10 (10.5)	0.282
Septic shock	0	3 (3.2)	0.246

Values are presented as number (%). AE, adverse event; ER, emergency room; ICU, intensive care unit; SAE, serious adverse event.

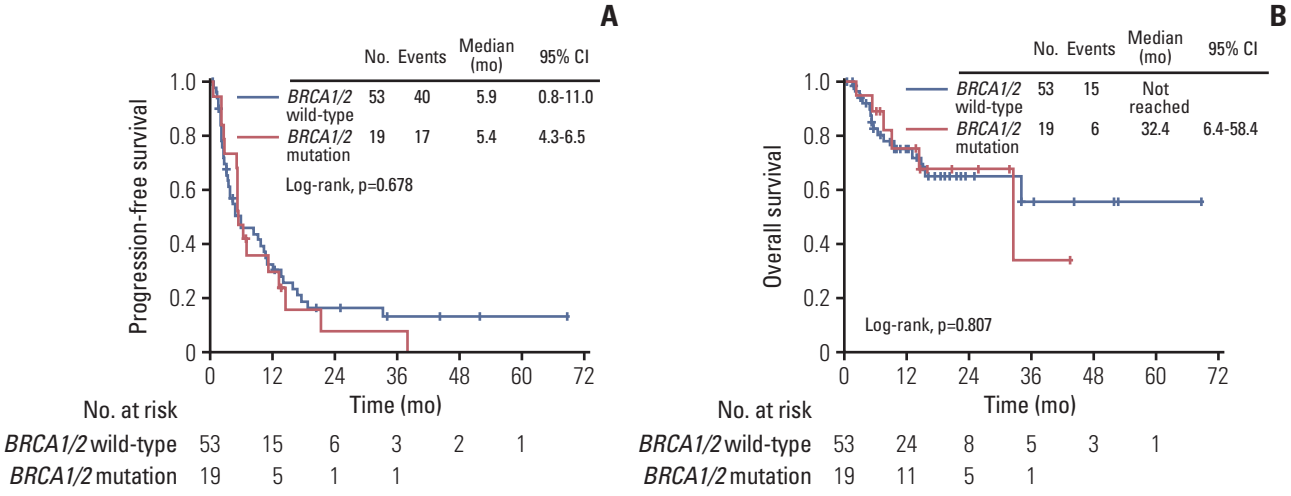

Fig. 3. Comparisons of survival outcomes in the *BRCA* wild-type group and *BRCA* mutation group. (A) Kaplan-Meier curves for progression-free survival in the *BRCA* wild group and *BRCA* mutation group. Number at risk is the number of patients in *BRCA* wild-type group and *BRCA* mutation group who are at risk of recurrence after 3rd, 4th, or 5th line chemotherapy. (B) Kaplan-Meier curves for overall survival *BRCA* wild group and *BRCA* mutation group. Number at risk is the number of patients in the *BRCA* wild-type group and *BRCA* mutation group who are at risk of death after 3rd, 4th, or 5th line chemotherapy. CI, confidence interval.

Multivariate analysis adjusted for age at recurrence, histologic type, FIGO stage, previous TFI, CA-125 at recurrence, residual tumor after debulking surgery, line of chemotherapy, malignant ascites, malignant pleural effusion, distant metastasis, and platinum sensitivity revealed that PFS in the good response group was significantly better than that in the poor response group (hazard ratio [HR], 8.472; 95% CI, 5.232 to 13.720) (Table 2). Similarly, OS in the good response group was significantly better than that in the poor response group (HR, 4.202; 95% CI, 2.599 to 6.792).

3. Predictive factors for poor responses

Univariate analyses revealed that poor responses occurred significantly more commonly among patients with higher CA-125 at recurrence (> 500 IU/mL, p=0.008), shorter TFIs (≤ 3 months, p < 0.001), platinum-resistant EOC (p=0.001), or non-serous/endometrioid (non-S/E) EOC (p=0.016). In multivariate analysis, we found that short TFIs, platinum-resistant EOC, and non-serous and non-endometrioid EOC were independent risk factors of poor response (Table 3).

4. Adverse events

With respect to the incidence of SAEs, there was no statistically significant difference between the good and poor response groups (p=0.167) (Table 4). Compared to the proportion of patients in the poor response group in whom neutropenic events of grade ≥3 occurred (15.8%), that in the good-response group was significantly higher (38.3%) (p < 0.001). The proportion of patients who were admitted through the ER due to treatment-related conditions was sig-

nificantly greater (27.4%) in the poor response group than the good response group (13.8%, p=0.030). A detailed comparison of the two groups with respect to SAEs is shown in Table 4.

5. Subgroup analysis

A total of 72 patients among all patients underwent somatic or germline *BRCA* test. There was no significant difference in PFS and OS according to the *BRCA* mutation. A detailed comparison of *BRCA* mutation group and *BRCA* wild-type group is shown in Fig. 3.

Discussion

In this study, the median PFS in the good and the poor response groups receiving three or more lines of chemotherapy for recurrent EOC was 9.7 and 2.1 months, respectively. Platinum sensitivity, longer TFI following the last chemotherapy regimen, and endometrioid/serous histology were revealed as independent factors for survival benefit after third and subsequent lines of chemotherapy.

The decision making on whether to continue with palliative chemotherapy in heavily treated ovarian cancer patients is difficult and requires careful consideration. In a study conducted in Italy, 66% of patients with NSCLC, breast, colorectal, and gastric cancer received chemotherapy during the last 3 months of their lives, and 33% of them received anticancer treatment during the last month of life [17]. In a recent study of gynecologic cancer patients who died between 2006 and 2010 after receiving palliative chemotherapy without hospice care, the mean frequency of palliative chemotherapy during the last 6 months of life was 3.84 times, which increased to 4.93 times between 2011 and 2015 [8]. The National Comprehensive Cancer Network guidelines recommend that patients should be referred to palliative care specialist and consider hospice care if there is evidence of worsening prognosis, including the decline in performance status to 3 or worse, or uncontrolled symptoms and distress despite anticancer therapy [18]. This is based on previous studies that showed the positive effect of early referrals (> 3 months before the occurrence of death) to palliative care services on fewer ER visits, decreased number of hospitalizations, and admissions to ICUs [19]. Nevertheless, the guidelines are not fully met in the real-world clinical practice due to the lack of data on survival outcomes of heavily treated patients and difficulty in predicting prognosis. In our study, the median time from the last chemotherapy to death was 108 days, with the shortest interval of 6 days, which suggests the need for continuous patient assessment for timely referral to end-of-life care.

Studies have been conducted to assess the survival benefit and response rates after multiple lines of chemotherapy in order to decide the timing of chemotherapy discontinuation and transition to hospice care [20]. In NSCLC patients on second-line chemotherapy, the survival improvement has been reported to be about 2 months [21]. In contrast, the response rate of third or fourth-line chemotherapy was only 0%-2% [22]. In this case, cytotoxic therapy would not be useful unless it is used for exceptionally emergent purposes. However, studies are lacking in regard to the specific criteria for palliative care referral in gynecologic oncologic patients or survival data of recurrent EOC after 2nd line therapy. In a retrospective study of platinum-resistant/refractory EOC patients, 60.2%, 27.0%, and 7.7% of patients were platinum-resistant after the first, second, and third-line chemotherapy, respectively [23]. The overall response rate was 30.6%, and the median progression-free interval (PFI) and OS was 16 and 48 weeks in patients with second-line chemotherapy after onset of platinum resistance [23]. In our study, the overall response rate was higher (49.7%) and the PFI was also longer with the median PFS of 4.2 months after third-line chemotherapy. Higher proportion of platinum-sensitive relapse patients (66.7%) included in our study may explain the difference in outcomes. Regarding treatment response, it is noteworthy that more than half of patients experienced PD in each chemotherapy lines, and less than 30% showed stable disease or higher. Expectedly, the rate of PD was the highest in the fifth-line chemotherapy group.

There are several prognostic tools to predict life expectancy in cancer patients. Among them, Palliative Prognostic Index is used as a useful prognosticator of life expectancy to distinguish patients who require palliative care referral [24]. The accuracy of prognostication can be further improved by the concurrent use of the Glasgow Prognostic Score and the Carlson Comorbidity Index [25]. These scales commonly use patients' performance status in addition to symptoms and/or serum markers. Although the patient's general performance status is crucial for successful maintenance of chemotherapy, recent evidence showed that chemotherapy use among chemotherapy-refractory metastatic cancer patients did not provide benefit to survival nor quality of life in the final week of life. Moreover, chemotherapy appeared to be most harmful to those patients with good performance status [26]. In this study, we aimed to distinguish good response group from the poor from a retrospective database, so that patients with poor response could preemptively avoid unnecessary exhaustive treatments. The 'good response' group was categorized according to the best objective response of SD or higher after the third and subsequent lines chemotherapy. These patients had serous and endometrioid histology, lower CA-125 level at recurrence, longer TFI, and initial plat-

inum sensitivity. This finding is consistent with other studies, one of them being a nomogram study to predict survival after recurrence in patients with recurrent ovarian cancer [27]. In their study, the time to recurrence showed strong significance in the nomogram for predicting survival (adequacy index=0.85). The median OS in patients with time to recurrence less than 6 months was 9.8 months and those longer than 36 months was 44.8 months (log-rank test $p < 0.001$). In terms of overall survival, residual disease, stage, histology, and age have been suggested as relevant factors [27]. In our cohort, residual disease, stage, and age were not significant factors for survival and this difference may lie in the pretreatment history, since the studied patients in our cohort were heavily treated patients of more than three lines of chemotherapy. The risk factors for poor response were short TFI less than 3 months, non-serous/endometrioid histology, and platinum resistance. Although platinum sensitivity is known as one of the most important prognostic factors in recurrent ovarian cancer, further research on individual biomarkers of platinum resistance is needed since majority of patients become platinum-resistant with subsequent relapses.

In recurrent ovarian cancer treatment, toxicity and quality of life should always be weighed together with the benefit from the cytotoxic therapy. Adverse events that lead to ER visits, hospital death, ICU admissions, and long hospital stays contribute to a poor quality of life near the end of life [28,29]. In our study, the rate of SAE was 50% in the good response group and 40 percent in the poor response group, without statistical significance. Non-hematologic SAE and ER visits and were significantly higher in the poor response group, although the proportion of patients with grade 3 or higher neutropenia was more frequently observed in the good response group. Despite being a frequent adverse effect of chemotherapy, there are some reports on the role of chemotherapy-induced neutropenia (CIN) as a favorable prognostic marker in different malignancies such as breast, gastric, non-small cell lung, and pancreatic cancer [30]. However, the timing of CIN onset and its effect in heavily treated patients may be different from the existing studies which mostly addressed early onset or CIN development during primary treatment. Nevertheless, active supportive management with granulocyte-stimulating factors in high-risk patients may be beneficial, especially in those with good treatment response.

The limitation of this study is its retrospective design; furthermore, only patients treated at a single institution were included in this study which results may not be suitable for generalization. Also, quality of life analysis was not performed, which is one of the markers to determine the continuation or cessation of treatment, especially for patients with PD. In addition, only 38 percent of patients in this cohort had been tested for *BRCA* mutation and therefore could not be adequately assessed whether *BRCA* contributes to treatment response in heavily treated patients. The role of genetic and molecular markers in regard to palliative care may be an important area of study in the coming years, especially in the recent era of immuno-oncology. The strength of this study is the comprehensive data on the chemotherapeutic agents used and toxicities including severe adverse events, which will provide insights to the outcomes of conventional cytotoxic treatment. Also, this is one of very few studies to report factors associated with good treatment responses in patients with EOC who received three or more line of chemotherapy. Therefore, we believe that the response rates shown at later lines of chemotherapy in this study may contribute as baseline information when counseling patients with palliative treatment options.

In conclusion, recurrent EOC patients with initial platinum sensitivity, longer treatment-free intervals, and endometrioid and serous histology are associated with good responses to third and subsequent lines of chemotherapy. Continuation of treatment beyond third line should be carefully considered in selected patients for palliative purposes, with timely discussions on goal-directed care.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This retrospective cohort study was conducted after the approval by the Institutional Review Board (IRB) of Seoul National University Hospital (SNUH) (No. 1811-159-989). As this study was a retrospective study, it was exempted from informed consent.

Author Contributions

Conceived and designed the analysis: Seol A, Yim GW, Lee M.
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Performed the analysis: Seol A, Yim GW.
Wrote the paper: Seol A, Yim GW, Lee M.
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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

Acknowledgments
This work was supported by grants from the National Research Foundation of Korea funded by the Ministry of Science and ICT (grant number: 2020R1G1A1005711) and the Cooperative Research Program of Basic Medical Science and Clinical Science of Seoul National University College of Medicine (grant number: 800-20210297).

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1011
Cancer Res Treat. 2022;54(4):1230-1239

Original Article

Outcome of Intensive Therapy for Children with Relapsed Acute Myeloid Leukemia: A Single Institution Korean Study

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Purpose Approximately 30%-40% of pediatric acute myeloid leukemia (AML) patients relapse. In this study, we analyzed the outcome and prognostic factors of relapsed AML patients who had previously received first-line therapy at our institution.

Materials and Methods The study group consisted of 50 patients who had been diagnosed with AML from April 2009 to December 2018, and then showed first relapse. Thirty-two of the patients (64%) had previously received allogeneic hematopoietic stem cell transplantation (HSCT) in first complete remission (CR).

Results Forty-five of the patients (90%) received intensive chemotherapy upon diagnosis of relapse, and 76% (34/45) of these patients achieved a second CR. Estimated 5-year overall survival for these 45 patients was 44.9%±7.6%. Time from diagnosis to relapse, extramedullary involvement (EMI) at diagnosis, core binding factor AML, and complex karyotype were significant prognostic factors; in multivariate study, both time from diagnosis to relapse and EMI at diagnosis proved significant. There was no difference in 5-year disease-free survival between patients previously treated with chemotherapy only and those who received HSCT in first CR (52.4%±14.9% vs. 52.6%±11.5%). Of the 19 patients who achieved second CR after previous allogeneic HSCT in first CR and subsequent relapse, 11 were treated with chemotherapy only, and seven survive disease-free.

Conclusion Intensive therapy allowed for long-term survival in 40%-50% of patients, and 50% of patients who achieved second CR, regardless of prior treatment modalities in first CR. Intensive treatment may allow for salvage of a significant portion of patients with relapsed pediatric AML.

Key words Acute myeloid leukemia, Children, Relapse, Hematopoietic stem cell transplantation, Extramedullary involvement

Introduction

Despite improvements in survival for pediatric acute myeloid leukemia (AML), relapse remains the most important cause of treatment failure [1]. The long-term overall survival (OS) rate for relapsed patients reported previously was less than 30% [2-4], whereas more recent studies have shown incremental improvement to 30%-40% [5-8]. In contrast to changes in outcome, the key prognostic factors for relapsed AML patients have remained consistent irrespective of study group and period. An aggregate of risk factors reported in these studies predicting better survival include a longer duration from diagnosis to relapse, favorable genetic features of the leukemic blast, prior omission of allogeneic hematopoietic stem cell transplantation (HSCT) in first complete remission (CR), and treatment with allogeneic HSCT in second CR [2-8]. In addition, one study found that the early response of relapsed AML to salvage therapy, as determined by the bone marrow (BM) blast percentage on day 28, was the most significant prognostic factor [9].

With this background, our main objective in this study was to determine outcome and important prognostic factors for relapsed pediatric AML patients diagnosed during a period of 10 years at our institution.

Materials and Methods

1. Patient group

The study received approval from our institutional review board. Patients diagnosed with AML at the Department of Pediatrics, The Catholic University of Korea from April 2009 to December 2018, who had received first-line therapy at our institution, and then subsequently relapsed were included. Primary refractory patients, and those diagnosed with a non-AML secondary malignancy after initial diagnosis and treatment for AML were excluded. The final study group consisted of 50 relapsed patients (Table 1). Seventeen of the patients had previously been reported as part of different studies [10,11].

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Received September 9, 2021 **Accepted** December 16, 2021 **Published Online** December 17, 2021

The median age at diagnosis was 10.6 years (range, 0.5 to 18.8 years). The most common genetic abnormality was *RUNX1-RUNX1T1* fusion, detected in 13 patients (26%). Seventeen patients (34%) had extramedullary involvement (EMI) at diagnosis, including leukemic involvement confirmed through cerebrospinal fluid study (n=3) and EMI diagnosed through imaging (n=14). Six of 13 patients with *RUNX1-RUNX1T1* fusion had EMI diagnosed through imaging, as well as two of three patients with –5/del(5q) abnormality. The remaining patients with imaging-based EMI had the following genetic abnormalities: complex karyotype (n=2), normal karyotype (n=2), *RBM15-MKL1* (n=1), and other non-complex (n=1).

2. First-line chemotherapy

The first-line chemotherapy regimens that the patients received were reported previously [11]. Patients diagnosed from 2008 to 2011 received Regimen 2008, while those diagnosed from 2012 onwards received AML 2012 chemotherapy as part of a multi-center clinical trial. Both treatment regimens classified patients into three risk groups (broadly low-, intermediate- and high-risk groups) on the basis of genetic abnormalities of the leukemic blast and response to initial chemotherapy. All patients in Regimen 2008 and intermediate-risk patients in AML 2012 underwent allogeneic HSCT in first CR if they had a human leukocyte antigen (HLA) matched donor. All high-risk patients in AML 2012 received allogeneic HSCT in first CR, regardless of presence of an HLA-matched donor. In the relapse study group, initial risk group classification was as follows: low eight (16%), intermediate 19 (38%), high 23 (46%). Thirty-two patients (64%) had received allogeneic HSCT in first CR.

Of the 14 core binding factor (CBF) AML patients, eight initially received chemotherapy only: for Regimen 2008, three low-risk patients who did not have an HLA-matched donor; for AML 2012, four intermediate-risk patients (*KIT* mutation (+) [n=3], delayed CR [n=1]) who did not have an HLA-matched donor, and one low-risk patient. Six patients received allogeneic HSCT in first CR: for Regimen 2008, two low-risk patients with an HLA-matched donor; for AML 2012, three intermediate-risk patients (*KIT* mutation (+)) with an HLA-matched donor, and one high-risk patient (concurrent FMS-like tyrosine kinase 3 [*FLT3*]–internal tandem duplication [ITD] mutation (+)).

3. Study objectives

Key objectives of the study were to initially determine the estimated probability of OS in the main study group of 50 patients. A second objective was to determine OS and risk factors for OS in the subgroup of patients who initially received intensive chemotherapy after diagnosis of relapse. We analyzed the influence of the following risk factors determined at initial diagnosis of AML in these patients on OS: patient sex, age at diagnosis, initial white blood cell (WBC) count, EMI at diagnosis, CBF AML, presence of *FLT3*-ITD, complex karyotype, chemotherapy regimen, achievement of first CR after 1 course of remission induction chemotherapy, allogeneic HSCT in first CR. We further analyzed the impact of the following variables on OS: period of relapse and time from diagnosis to relapse. We also attempted to determine whether the following relapse-specific variables affected OS: age and WBC count at relapse, as well as, CBF AML, *FLT3*-ITD mutation, complex karyotype at relapse. We also calculated the disease-free survival (DFS) for patients who achieved a second CR, and the OS and DFS of the two subgroups of patients who either relapsed after receiving chemotherapy only, or relapsed after treatment with allogeneic HSCT in first CR. Finally, we calculated the event-free survival (EFS) for patients who underwent post-relapse HSCT, and compared outcome according to type of conditioning regimen.

4. Statistical analysis

Comparison of key prognostic factors at relapse (WBC count at relapse, and genetic abnormalities at relapse [CBF AML, *FLT3*-ITD, complex karyotype status]) between those who received chemotherapy only in first CR, and those who received allogeneic HSCT in first CR was done with the Mann-Whitney and chi-square tests. OS was determined from the date of relapse to death or last follow-up, while DFS was determined from the date of second CR to subsequent relapse, death, or last follow-up. The EFS of the patients who received post-relapse HSCT was calculated from the time of HSCT to relapse, death, or last follow-up. OS, DFS, and EFS were calculated with the Kaplan-Meier method. Univariate and multivariate study of risk factors for OS were done with the log-rank test and Cox proportional hazard regression, respectively. Patient follow-up was done up till December 31, 2020. p-values < 0.05 were considered significant.

Results

1. Diagnosis of relapse

For the overall study group, the median time from diagnosis to relapse was 12.2 months (range, 2.8 to 62.2 months). Sites of relapse were as follows: BM 41, extramedullary (EM) 2, BM and EM combined 7. Although 34 of 48 evaluable patients (71%) showed cytogenetic changes of the leukemic blast from diagnosis to relapse, changes in the recurrent genetic abnormalities with prognostic relevance were only found in six patients: normal karyotype to *BCR-ABL1* (n=1),

Table 1. Patient characteristics

Characteristic	No. (%) (n=50)
Sex	
Male/Female	31 (62.0)/19 (38.0)
Age at diagnosis, median (range, yr)	10.6 (0.5-18.8)
Initial WBC count, median (range, ×10⁹/L)	17.10 (1.01-287.06)
EMI at diagnosis[a)]	
Yes/No	17 (34.0)/33 (66.0)
Genetic abnormalities[b)]	
RUNX1-RUNX1T1	13 (26.0)
FLT3-ITD	7 (14.0)
KMT2A rearrangement	3 (6.0)
–5, del(5q)	3 (6.0)
CBFB-MYH11	1 (2.0)
DEK-NUP214	1 (2.0)
FUS-ERG	1 (2.0)
RBM15-MKL1	1 (2.0)
NPM1	1 (2.0)
Biallelic *CEBPA*	1 (2.0)
Other complex karyotype[c)]	8 (16.0)
Normal	5 (10.0)
Others	5 (10.0)
Initial treatment regimen	
Regimen 2008/AML 2012	21 (42.0)/29 (58.0)
First CR after 1 course of remission induction	
Yes/No	40 (80.0)/10 (20.0)
Risk group	
Low/Intermediate/High	8 (16.0)/19 (38.0)/23 (46.0)
Allogeneic HSCT in first CR	
Yes/No	32 (64.0)/18 (36.0)
Time from diagnosis to relapse, median (range, mo)	12.2 (2.8-62.2)

CR, complete remission; EMI, extramedullary involvement; HSCT, hematopoietic stem cell transplantation; WBC, white blood cell. [a)]Leukemic blasts in initial cerebrospinal fluid study (n=3), or myeloid sarcoma-like extramedullary involvement detected by imaging (n=14), [b)]Classification based on dominant genetic abnormality for patients with cooperating mutations, [c)]Defined as three or more unrelated chromosomal abnormalities in the absence of 1 of the World Health Organization–designated recurrent genetic abnormalities.

FLT3-ITD (+) to *FLT3*-ITD (–) (n=1), complex karyotype to monosomy 7 (n=1), complex karyotype to non-complex karyotype (n=2), non-complex karyotype to complex karyotype (n=1). One patient with concurrent *RUNX1-RUNX1T1* fusion and *FLT3*-ITD mutation showed loss of *FLT3*-ITD mutation at relapse but retained the key *RUNX1-RUNX1T1* fusion.

Table 2. Chemotherapy regimens utilized for first reinduction chemotherapy

Chemotherapy regimen	No. (%) (n=45)
Fludarabine 30 mg/m²/day, days 1-5 Cytarabine 2-3 g/m²/day, days 1-5 G-CSF, days 0-4 ±Idarubicin 12 mg/m²/day, days 1-3 IT cytarabine	41 (91.1)
Cytarabine 2 g/m² twice daily, days 1-5 Etoposide 100 mg/m², days 1-5 IT cytarabine	1 (2.2)
Cytarabine 3 g/m² twice daily, days 1-2, 8-9 Asparaginase 6,000 units/m², days 3, 10	1 (2.2)
Cytarabine 3 g/m² twice daily, days 1-2	1 (2.2)
Cytarabine 1.5 g/m²/day, days 1-4[a)] Idarubicin 12 mg/m²/day, days 1-3 Sorafenib 200 mg/m² twice daily, days 1-7	1 (2.2)

G-CSF, granulocyte colony stimulating factor; IT, intrathecal. [a)]As detailed in Ravandi et al. [12].

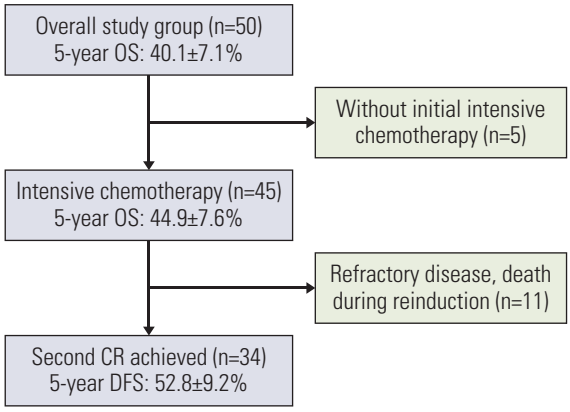

Fig. 1. Flow chart of relapsed acute myeloid leukemia study group. CR, complete remission; DFS, disease-free survival; OS, overall survival.

2. Treatment of relapse

Five patients did not begin treatment with intensive chemotherapy upon diagnosis of relapse: two patients who did not receive curative therapy and failed to achieve second CR, two patients who reached second CR after decrease and cessation of immunosuppression only, and one patient who received local radiotherapy only for treatment of isolated EM relapse.

Of the 45 patients who received intensive chemotherapy, 41 patients were treated with a combination of fludarabine, cytarabine, and granulocyte colony stimulating factor with or without idarubicin (FLAG±IDA) as the first reinduction

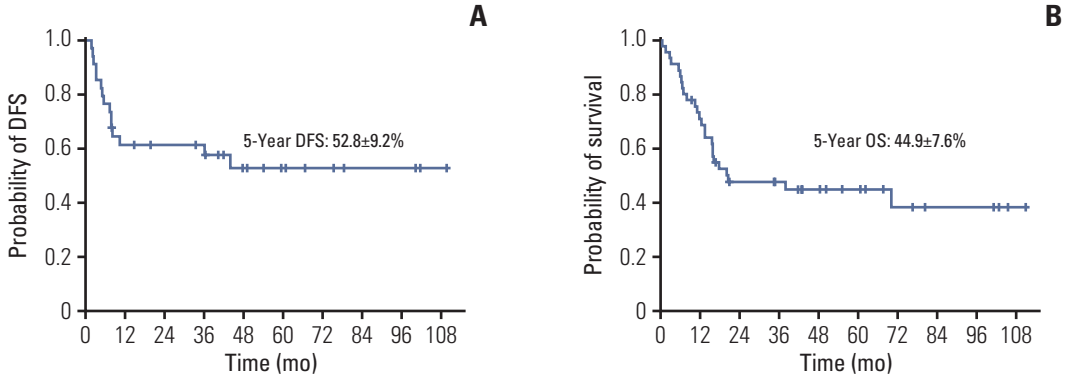

Fig. 2. (A) Estimated disease-free survival (DFS) for the 34 patients who received intensive chemotherapy and achieved second complete remission. (B) Estimated overall survival (OS) for the 45 patients who received intensive chemotherapy.

regimen (Table 2). FLAG±IDA resulted in second CR in 28 patients (68%), while none of the other four reinduction strategies resulted in second CR. The rate of second CR after the first course of chemotherapy was 62% (28/45), while the final, overall rate of second CR was 76% (34/45). Six patients achieved second CR after more than one salvage attempt, including four patients after the second reinduction, and one patient who showed CR of EM relapse after three courses of chemotherapy. One patient who failed to achieve second CR after two courses of reinduction chemotherapy went on to receive a second allogeneic HSCT without remission, and achieved second CR after transplant.

3. Outcome for overall study group and for those who received initial intensive chemotherapy

For the overall study group of 50 patients, the estimated probability of 5-year OS was 40.1%±7.1% (20/50) (Fig. 1). Among the subgroup of 45 patients who received initial intensive chemotherapy upon diagnosis of relapse, 11 patients died during reinduction chemotherapy or from refractory disease, and failed to achieve second CR. Among the 34 patients who achieved second CR, 13 patients experienced a second relapse, and two patients died from treatment-related causes in second CR, resulting in a 5-year DFS of 52.8%±9.2% (19/34) (Fig. 2A). The 5-year OS for the 45 patients treated with initial intensive chemotherapy was 44.9%±7.6% (20/45) (Fig. 2B), with a median duration of follow-up of 36.6 months (range, 6.0 to 111.2 months) for those who achieved a second CR.

Regarding risk factors for OS in these 45 patients determined at the time of diagnosis, time from diagnosis to relapse, EMI at diagnosis, presence of CBF AML and presence of complex karyotype proved significant (Table 3, Fig. 3A-D). When undertaking multivariate study with the two most significant variables, time from diagnosis to relapse and EMI at diagnosis, both factors proved significant (time from diagnosis to relapse: hazard ratio [HR], 2.66; 95% confidence interval [CI], 1.13 to 6.28; p=0.025; EMI at diagnosis: HR, 2.33; 95% CI, 1.02 to 5.31; p=0.044).

Regarding risk factors determined at the time of relapse, persistent CBF AML and *FLT3*-ITD mutation at relapse were significant factors influencing OS (S1 Table). In multivariate study, *FLT3*-ITD mutation at relapse was significant (HR, 3.35; 95% CI, 1.19 to 9.43; p=0.022).

4. Outcome according to treatment strategy in first CR

1) Initial chemotherapy only

Of the 45 patients who received intensive reinduction chemotherapy upon relapse, 18 had been previously treated with chemotherapy only without HSCT (Fig. 4). Fifteen of these 18 patients achieved second CR (83%), all of whom proceeded to allogeneic HSCT. For these 15 patients, the median number of post-relapse chemotherapy courses prior to HSCT was 2 (range, 2 to 3), and the median time from relapse to HSCT was 3.5 months (range, 2.4 to 4.6 months). Four patients relapsed post-HSCT and two patients died of treatment-related causes in CR, resulting in a 5-year DFS of 52.4%±14.9% (9/15). For the overall subgroup of 18 patients treated with chemotherapy only prior to relapse, the 5-year OS was 50.8%±12.9% (9/18).

2) Allogeneic HSCT in first CR

Twenty-seven of 45 patients who received intensive chemotherapy upon relapse had received allogeneic HSCT in first CR. The *FLT3*-ITD mutation was found at relapse solely in these patients who had received HSCT in first CR, while there was no difference in other key prognostic factors found at relapse when comparing patients who had received HSCT in first CR with those who had received chemotherapy only in first CR (S2 Table).

Table 3. Univariate study of risk factors for 5-year OS in patients initially treated with intensive chemotherapy upon relapse diagnosis

	Patients (deceased)	5-Year OS (±SE) (%)	p-value
Sex			
Male	26 (13)	53.3±9.9	0.363
Female	19 (12)	31.7±11.7	
Age at diagnosis (yr)[a)]			
< 11	23 (13)	46.8±10.6	0.596
≥ 11	22 (12)	42.6±11.1	
WBC at diagnosis (×10⁹/L)[a)]			
< 17	22 (11)	54.5±10.6	0.539
≥ 17	23 (14)	35.5±10.5	
EMI at diagnosis			
No	31 (14)	55.7±9.3	0.012
Yes	14 (11)	21.4±11.0	
CBF AML			
No	31 (21)	34.0±8.8	0.025
Yes	14 (4)	69.6±12.7	
***FLT3*-ITD**			
No	37 (19)	49.4±8.5	0.057
Yes	8 (6)	25.0±15.3	
Complex karyotype			
No	35 (16)	52.3±8.7	0.030
Yes	10 (9)	20.0±12.6	
Chemotherapy regimen			
Regimen 2008	17 (13)	29.4±11.1	0.051
AML 2012	28 (12)	54.3±9.9	
First CR after 1 course of remission induction			
No	9 (6)	33.3±15.7	0.670
Yes	36 (19)	47.9±8.6	
HSCT in first CR			
No	18 (9)	50.8±12.9	0.617
Yes	27 (16)	40.7±9.5	
Period of relapse			
2010-2014	23 (17)	30.4±9.6	0.072
2015-2020	22 (8)	62.2±10.6	
Time from diagnosis to relapse[a)]			
< 12 mo	22 (17)	27.3±9.5	0.008
≥ 12 mo	23 (8)	62.6±10.6	

CBF AML, core binding factor acute myeloid leukemia; CR, complete remission; EMI, extramedullary involvement; HSCT, hematopoietic stem cell transplantation; OS, overall survival; SE, standard error; WBC, white blood cell. [a)]Cutoff threshold based on median values.

Nineteen of 27 patients achieved second CR (70%), and there was no difference in 5-year DFS when comparing the HSCT in first CR and chemotherapy only subgroups (5-year DFS 52.6±11.5% vs. 52.4±14.9%, p=0.572). Four of these 19 patients proceeded to second allogeneic HSCT in second CR at a median time from relapse to HSCT of 4.2 months (range, 3.5 to 5.8); of these patients, one patient relapsed and the remaining three patients survive without event.

Of the remaining 15 patients, 14 were treated with chemotherapy only, either completing the planned treatment (n=11), or until early second relapse (n=3), while one patient received a second allogeneic HSCT without CR.

For the 11 patients who completed treatment with chemotherapy only, the median number of chemotherapy courses was 4 (range, 2 to 4). Seven survived without further event, and the genetic abnormalities of these patients at initial diagnosis were as follows: *RUNX1-RUNX1T1* (n=3), *FLT3*-ITD (n=2), and normal karyotype (n=2) (Table 4). For these seven patients, changes in key genetic abnormalities at relapse were observed in two patients (*FLT3*-ITD (+) with

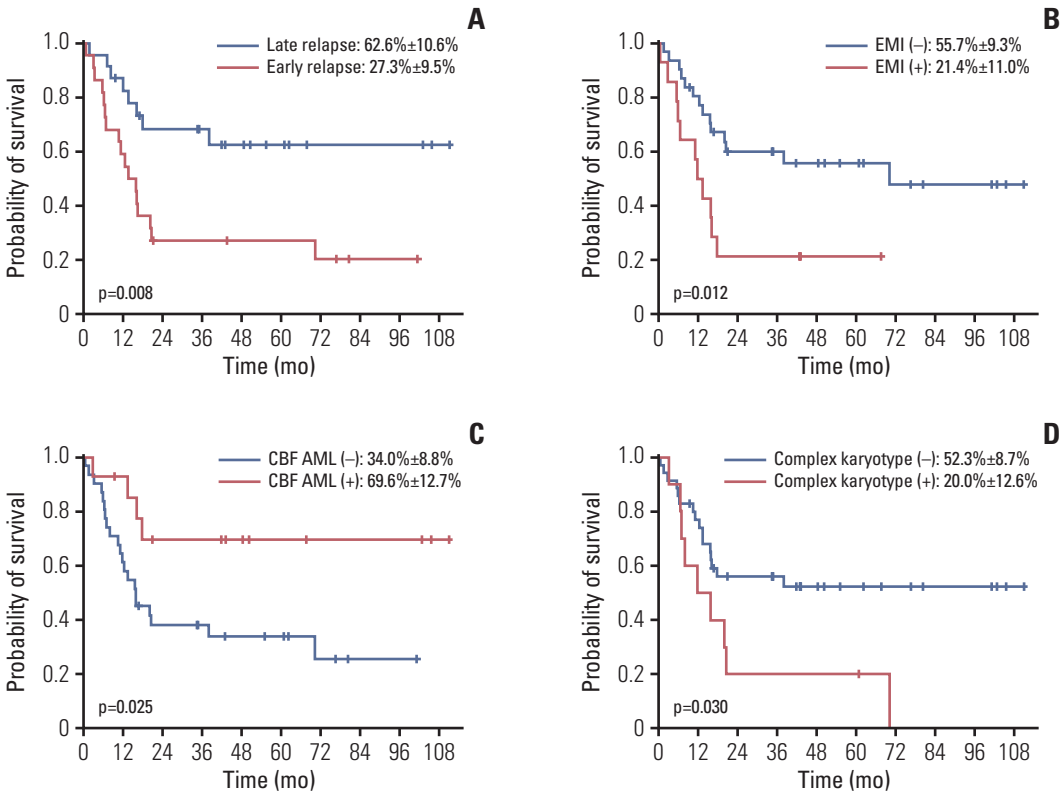

Fig. 3. Estimated overall survival for the 45 patients who received intensive chemotherapy according to time from diagnosis to relapse (< 12 months from diagnosis to relapse vs. ≥ 12 months from diagnosis to relapse) (A), extramedullary involvement (EMI) at diagnosis (B), presence of core binding factor (CBF) acute myeloid leukemia (AML) (C), and presence of complex karyotype (D).

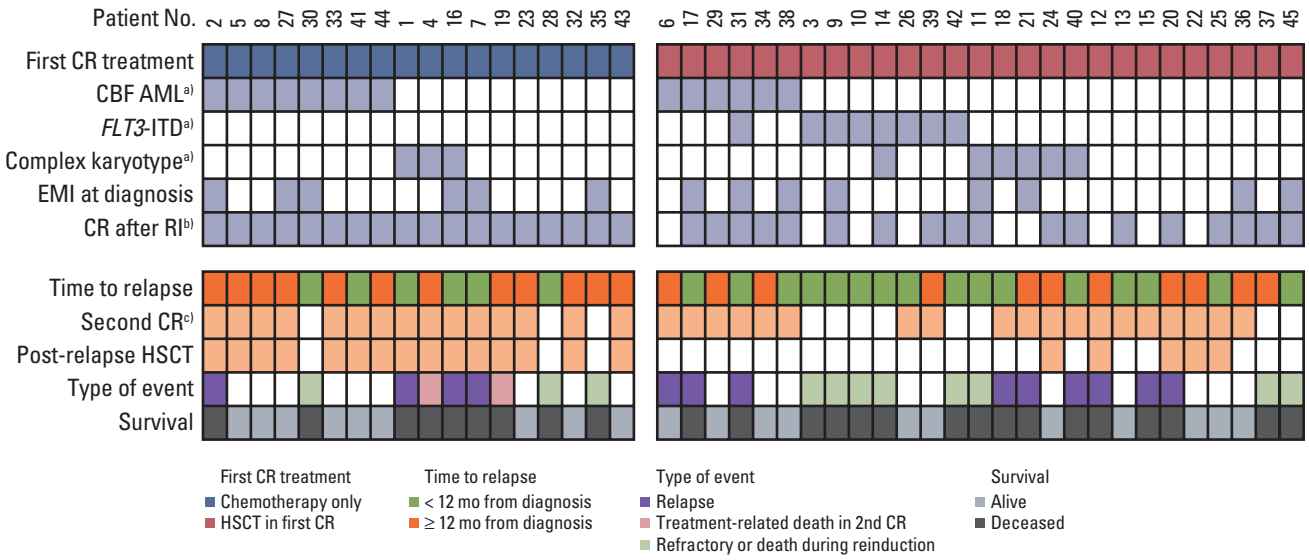

Fig. 4. Disease characteristics, treatment in first complete remission (CR), response to reinduction chemotherapy and outcome for the 45 patients who received intensive chemotherapy. CBF, core binding factor; RI, remission induction. [a]Genetic abnormalities at diagnosis of acute myeloid leukemia (AML), [b]Achieved complete remission after one course of chemotherapy after initial diagnosis, [c]Patient 20 did not achieve second CR after reinduction chemotherapy, and only achieved second CR after the second allogeneic hematopoietic stem cell transplantation (HSCT).

Table 4. Key characteristics of the patients who relapsed after allogeneic HSCT in first CR and survive disease-free after a chemotherapy only strategy

Patient No.	EMI at diagnosis	Time from diagnosis to relapse (mo)	Genetics at diagnosis	Genetics at relapse	GVHD after relapse	Treatment after relapse	Survival (mo)[a]
13	No	10.7	Normal karyotype	Normal karyotype	No	FLAG-Ida → FLAG (×2)	102
26	No	9.4	*FLT3*-ITD (+)	*FLT3*-ITD (–)	No	FLAG-Ida (×2) → FLAG (×2)	77
29	No	23.4	*RUNX1-RUNX1T1*	*RUNX1-RUNX1T1*	No	FLAG (×4)	49
34	No	23.1	*RUNX1-RUNX1T1*	*RUNX1-RUNX1T1*	No	FLAG (×4)	42
36	Yes	14.3	Normal karyotype	Non-complex karyotype	Yes	FLAG (×2)	43
38	Yes	8.6	*RUNX1-RUNX1T1*	*RUNX1-RUNX1T1*	No	FLAG-Ida → FLAG (×3)	43
39	No	15.5	*FLT3*-ITD (+)	*FLT3*-ITD (+)	Yes	FLAG (×4)	35

CR, complete remission; EMI, extramedullary involvement; FLAG±Ida, fludarabine, cytarabine, G-CSF±idarubicin; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation. [a]From relapse to last follow-up.

Table 5. Characteristics of post-relapse allogeneic HSCT

	No. (%) (n=20)
Disease status	
Second CR	19 (95.0)
Relapsed	1 (5.0)
Donor type	
MSD	2 (10.0)
MUD	3 (15.0)
HFD	15 (75.0)
Cell source	
BM	2 (10.0)
PBSC	18 (90.0)
Conditioning intensity	
Myeloablative	20 (100)
Conditioning type	
Bu-Flu-ATG[a]	7 (35.0)
TBI-Bu-Flu±(ATG or PTCy)[b]	13 (65.0)

ATG, anti-thymocyte globulin; BM, bone marrow; Bu, busufan; CR, complete remission; Flu, fludarabine; HFD, haploidentical family donor; HSCT, hematopoietic stem cell transplantation; MSD, matched sibling donor; MUD, matched unrelated donor; PBSC, peripheral blood stem cells; PTCy, post-transplantation cyclophosphamide; TBI, total body irradiation. [a]Busulfan 130 mg/m^2/day for 4 days, fludarabine 40 mg/m^2/day for 4 days, rabbit ATG 2.5 mg/kg/day for 3 days for unrelated donor HSCT, 2.5 mg/kg/day for 4 days for HFD HSCT, [b]Total body irradiation dose of 800 cGy over 2 days, busulfan 130 mg/m^2/day for 2 days, fludarabine 40 mg/m^2/day for 4 days, rabbit ATG (thymoglobuline) 1.25-2.5 mg/kg/day for 3 days for unrelated donor HSCT, 1.25 mg/kg/day for 4 days for HFD HSCT. For PTCy, 50 mg/kg/day for 2 days.

normal karyotype to *FLT3*-ITD (–) with non-complex karyotype [n=1] and normal karyotype to non-complex karyotype [n=1]), resulting in the following at relapse: *RUNX1-RUNX1T1* (n=3), non-complex karyotype (n=2), *FLT3*-ITD (+) (n=1), and normal karyotype (n=1). The one patient who showed persistent *FLT3*-ITD mutation at relapse survives disease-free after 4 cycles of FLAG chemotherapy without FLT3 inhibitor therapy.

For the overall subgroup of patients who relapsed post-allogeneic HSCT in first CR, the 5-year OS was 40.7%±9.5% (11/27), with no difference in OS when compared with patients treated with chemotherapy only prior to relapse (p=0.617).

5. Post-relapse allogeneic HSCT

Overall, 20 patients (40%) received allogeneic HSCT after relapse at a median of 3.7 months from relapse (range, 2.4 to 5.8 months), 15 as the first transplant, and five as the second transplant after receiving HSCT in first CR. A haploidentical

family donor (HFD) was utilized in 15 of 20 HSCTs (Table 5), with rabbit anti-thymocyte globulin (ATG, thymoglobuline, Sanofi, Paris, France)-based T cell depletion given in 13 patients, and post-transplantation cyclophosphamide in the remaining two patients. Since 2014, we have utilized a conditioning regimen of total body irradiation (TBI) 800 cGy, busulfan (Bu, 130 mg/m^2/day for 2 days) and fludarabine (Flu, 40 mg/m^2/day for 4 days) for relapsed AML patients, whereas the previous conditioning regimen consisted of Bu (130 mg/m^2/day for 4 days) and Flu (40 mg/m^2/day for 4 days). Patients who received a TBI-Bu-Flu regimen had better outcome than those who received the previous Bu-Flu regimen (5-year EFS, 72.5%±14.1% for TBI-Bu-Flu vs. 28.6%±17.1% for Bu-Flu; p=0.041), although the different doses of ATG administered for each conditioning regimen may also have influenced transplant outcome (Table 5). Evaluating a limited number of patients, those who received a matched sibling donor or matched unrelated donor HSCT had better 5-year EFS than those who received an HFD HSCT (80.0%±17.9% vs. 47.6%±14.3%, p=0.369).

Discussion

Of the 34 patients who achieved second CR, 28 patients reached CR after the first course of reinduction chemotherapy, while 32 patients overall achieved CR within two courses of reinduction chemotherapy. Hence, the vast majority of patients who achieved second CR did so within the initial attempts of salvage chemotherapy, as reported previously for relapsed or refractory AML [13].

Several reinduction regimens may be given for relapsed AML, with none being standard therapy. The majority of patients in our study received FLAG±IDA, the efficacy of which has been shown for relapsed patients [14]. This reinduction strategy resulted in second CR in 68% of patients treated with FLAG±IDA. Utilization of targeted or novel therapies such as gemtuzumab ozogamicin or venetoclax may further increase the rate of second CR [15,16].

In terms of outcome, the 5-year OS rates of 40.1%±7.1% and 44.9%±7.6% for the overall study group and for those who received reinduction chemotherapy, respectively were similar to those reported in more recent studies [5-7]. Time from diagnosis to relapse was the most significant prognostic factor for OS, reflecting consensus on the key role of this variable in post-relapse survival [2-7].

Of note, we found that patients with EMI at diagnosis had significantly worse outcome than those who lacked EMI. Studies on the role of EMI in pediatric AML outcome are conflicting. Recent studies based on a large number of patients showed that patients with EMI had a higher risk of induction death, and that EMI was not a prognostic factor for patients who undergo HSCT [17,18]. The incidence of EMI at diagnosis in our study group was 34%, higher than the 23% found in both recent studies [17,18]. Our strategy of active imaging-based surveillance for EMI at diagnosis as reported previously may have contributed to a greater incidence of EMI in our study group [11].

In terms of genetic abnormalities, we were able to confirm improved outcome in relapsed CBF AML patients, as shown in previous studies [5,6]. Furthermore, patients with a complex karyotype had lower survival compared with those lacking this genetic abnormality. Although past studies have shown that high-risk genetic features at diagnosis, such as the *FLT3*-ITD mutation, may result in poor outcome after relapse [6,19], none has focused on the prognostic role of complex karyotype in relapsed AML. The potential adverse effect of a complex karyotype on outcome of newly diagnosed pediatric AML lacks the consensus observed for other poor prognosis genetic abnormalities. However, a study of 454 pediatric AML patients showed that patients with a complex karyotype had significantly worse EFS than other patients [20]. Further study with a larger number of patients is necessary to confirm whether novel prognostic factors in the relapsed pediatric AML setting, such as EMI or a complex karyotype at diagnosis, define a high risk group of patients with inferior outcome after relapse. Regarding the influence of relapse-specific variables on patient outcome, persistent *FLT3*-ITD mutation at relapse predicted worse OS in multivariate study, consistent with the established poor prognosis of this genetic abnormality.

The data in this study derive from patients initially treated for AML using two consecutive, institutional protocols, Regimen 2008 and AML 2012 [11]. In both of these treatment regimens, many high-risk patients underwent allogeneic HSCT in first CR, while intermediate-risk patients also received allogeneic HSCT if they had HLA-matched donors. Hence, in our relapsed patient study group, the majority of patients (64%) had received allogeneic HSCT in first CR, in contrast to recent studies on relapsed, pediatric AML in which the proportion of patients who had received HSCT in first CR was a clear minority [5,7].

In the subgroup of patients who had been treated with chemotherapy only prior to relapse, all patients who achieved second CR proceeded to HSCT after a median of two chemotherapy courses. A previous study also showed that relapsed AML patients who received 2 cycles of pre-transplant chemotherapy had better OS than those who received 1 or 3 or more cycles of chemotherapy, possibly by lowering disease burden while minimizing treatment-related toxicity prior to transplant [21].

For patients who relapse after allogeneic transplant in first

CR, curative treatment incorporates a second HSCT. A recent study based on 333 AML children who relapsed after HSCT showed 4-year OS rates of 14% for the entire cohort and 31% for 122 children who received a second HSCT [22]. In our study group, the decision as to whether a patient would proceed to a second transplant was individualized, rather than based on a pre-planned strategy. Factors contributing to the decision were our attempts to minimize the number of patients who proceeded to second transplant during the treatment period of our study, due mostly to concern for late effects. We also aimed to forgo a second transplant for the good prognosis CBF AML patients who had received allogeneic HSCT in first CR.

As a result, in our subgroup of patients who relapsed after allogeneic HSCT in first CR, most of the patients who achieved a second CR completed a salvage strategy based on chemotherapy only (11 of 19 patients), with a median number of four chemotherapy courses, rather than receiving a second HSCT; of these 11 patients, seven survive disease-free. Some of the patients who were cured with a chemotherapy-only strategy had favorable genetic features, such as *RUNX1-RUNX1T1*. As these low risk patients would likely not have received allogeneic HSCT in first CR in other cooperative studies, whether this possibility of achieving disease-free status without a second HSCT is specific to our institutional context, rather than being broadly applicable requires further evaluation.

For all relapsed patients, allogeneic HSCT in second CR is a key component of curative therapy. Most of the 20 patients who proceeded to allogeneic HSCT after relapse received an HFD transplant. Also, patients who received a conditioning regimen consisting of TBI-Bu-Flu had better outcome than those who received Bu-Flu, although the differing doses of ATG given in each conditioning regimen confound the comparison. The feasibility of TBI-Bu-Flu in the HFD transplant setting was shown in adult AML patients [23]. Further studies are necessary to confirm the efficacy of this regimen in high-risk or relapsed pediatric AML patients.

Overall, there was no difference in either 5-year OS or DFS between the chemotherapy only and first CR HSCT subgroups who received intensive reinduction chemotherapy, in contrast to previous reports which showed that HSCT prior to relapse had a significant, negative effect on outcome [4,5]. In an earlier study, some of the patients who relapsed early after HSCT received supportive care only rather than intensive chemotherapy, contributing to the discrepancy in survival between post-HSCT and transplant-naïve relapsed patients [4]. Our study supports the role of intensive chemotherapy post-relapse in curing a significant proportion of patients, regardless of prior treatment methods. We also note that with regards to patients who relapsed after allogeneic HSCT, 5-year OS and DFS have improved since our previous report on a historical cohort (OS, 41% vs. 32%; DFS, 53% vs. 33%), with the caveat that some of the patients in this past cohort had received the first HSCT in second CR [10].

We emphasize the main limitations of our single institution study: that is, retrospective in nature, and based on a small number of patients. However, we confirmed the well-established prognostic role of duration from diagnosis to relapse in our relapsed AML study group. Further study is necessary to validate whether factors such as EMI or a complex karyotype at diagnosis can be added to the variables that may affect outcome post-relapse. Our results also indicate that for relapsed pediatric AML patients, intensive therapy may result in long-term survival in 40%-50% of patients, and in 50% of patients who achieve second CR, irrespective of prior treatment modalities in first remission.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study received approval from the ethical review board of Seoul Saint Mary's Hospital, The Catholic University of Korea (IRB No. KC20RISI0627). Requirement for patient consent was waived.

Author Contributions

Conceived and designed the analysis: Lee JW, Cho B.
Collected the data: Lee JW.
Performed the analysis: Lee JW, Yoo JW, Kim S, Jang PS, Chung NG, Cho B.
Wrote the paper: Lee JW, Yoo JW, Kim S, Jang PS, Chung NG, Cho B.

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

We express heartfelt gratitude to the nursing staff associated with the Division of Hematology/Oncology, Department of Pediatrics, The Catholic University of Korea, and, above all, to the patients and their families.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1194
Cancer Res Treat. 2022;54(4):1240-1255

Original Article

Whole-Genome and Transcriptome Sequencing Identified *NOTCH2* and *HES1* as Potential Markers of Response to Imatinib in Desmoid Tumor (Aggressive Fibromatosis): A Phase II Trial Study

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Purpose Desmoid tumor, also known as aggressive fibromatosis, is well-characterized by abnormal Wnt/β-catenin signaling. Various therapeutic options, including imatinib, are available to treat desmoid tumor. However, the molecular mechanism of why imatinib works remains unclear. Here, we describe potential roles of *NOTCH2* and *HES1* in clinical response to imatinib at genome and transcriptome levels.

Materials and Methods We identified somatic mutations in coding and noncoding regions via whole-genome sequencing. To validate the genetic interaction with expression level in desmoid-tumor condition, we utilized large-scale whole-genome sequencing and transcriptome datasets from the Pan-Cancer Analysis of Whole Genomes project. RNA-sequencing was performed using prospective and retrospective cohort samples to evaluate the expressional relevance with clinical response.

Results Among 20 patients, four (20%) had a partial response and 14 (66.7%) had stable disease, 11 of which continued for ≥ 1 year. With gene-wise functional analyses, we detected a significant correlation between recurrent *NOTCH2* noncoding mutations and clinical response to imatinib. Based on Pan-Cancer Analysis of Whole Genomes data analyses, *NOTCH2* mutations affect expression levels particularly in the presence of *CTNNB1* missense mutations. By analyzing RNA-sequencing with additional desmoid tumor samples, we found that *NOTCH2* expression was significantly correlated with *HES1* expression. Interestingly, *NOTCH2* had no statistical power to discriminate between responders and non-responders. Instead, *HES1* was differentially expressed with statistical significance between responders and non-responders.

Conclusion Imatinib was effective and well tolerated for advanced desmoid tumor treatment. Our results show that *HES1*, regulated by *NOTCH2*, as an indicator of sensitivity to imatinib, and an important therapeutic consideration for desmoid tumor.

Key words Fibromatosis aggressive, Imatinib mesylate, Clinical trial phase II, Computational biology, Whole-genome sequencing, Transcriptome

Introduction

A desmoid tumor (aggressive fibromatosis) is a fibroproliferative neoplasm arising from deep connective tissues. A stepwise approach including active surveillance is established as no metastatic potential and spontaneous tumor regression are observed [1]. Excision is the mainstay of treatment, but the postsurgical recurrence rate is high [2,3]. For unresectable or recurrent desmoid tumors, a variety of systemic therapeutic options are available, including tamoxifen, nonsteroidal anti-inflammatory drugs (NSAIDs), interferons, and chemotherapy [4-8]. Recently, sorafenib induced durable response and led to its approval for the disease treatment [9]. Imatinib is a new treatment option in unresectable, progressive, or recurrent desmoid tumors [10-12]. In addition to its promising efficacy (6%-15% response rate), the favorable tox-

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Received November 8, 2021 **Accepted** January 13, 2022
Published Online January 17, 2022

icity profiles support its long-term use for salvage treatment. In imatinib-responsive diseases, such as gastrointestinal stromal tumors or chronic myeloid leukemia, specific mutations or chromosomal translocations have been reported [13-15]. However, in desmoid tumors, no molecular abnormalities in imatinib-sensitive kinases have been observed [10,11]. Therefore, molecular mechanisms by which this rare tumor responds to imatinib are poorly understood.

In this study, we conducted a multicenter phase II trial to evaluate the efficacy of imatinib in patients with relapsing or progressive desmoid tumors (ClinicalTrials.gov, NCT02495519, registered July 13, 2015 retrospectively registered). To understand the molecular basis of the clinical responses to imatinib, we performed whole-genome sequencing to identify potential markers. Owing to limited insights gained from protein-coding mutations, we extended our analyses to noncoding regulatory regions. Our gene-wise recurrence model using 1,009 pan-cancer whole-genome data indicated that *NOTCH2* regulatory mutations are associated with the response of desmoid tumors to imatinib. We further evaluated the significance of *NOTCH2* in transcriptome analysis using RNA-sequencing data. We discovered that *HES1*, a well-known downstream target of Notch signaling pathway, is directly associated with imatinib sensitivity.

Materials and Methods

1. Patients and treatment

Patients with advanced desmoid tumors, defined as patients with radiographic progression after previous treatment, were eligible for prospective phase II study. Key inclusion criteria were as follows: age ≥ 10 years, Eastern Cooperative Oncology Group performance status of 0 to 2, and adequate hematologic and renal function. Patients were treated with 400 mg of imatinib mesylate (Glima, Boryung Pharmaceutical Co., Ltd., Seoul, Korea) daily until progression or unacceptable toxicity. Toxic effects were graded according to the National Cancer Institute—Common Toxicity Criteria v. 4.03. Disease was assessed every 8 weeks for the initial 32 weeks and then every 16 weeks according to RECIST (Response Evaluation Criteria in Solid Tumors) v1.1 [16]. Briefly, patients who experienced grade 3/4 toxicity or intolerable grade 2 toxicity stopped treatment and then restarted at a reduced dose (300 mg/day or 200 mg/day). Surgically resected formalin-fixed paraffin-embedded (FFPE) tissue samples obtained prior to radiotherapy or chemotherapy were subjected to transcriptome sequencing (Fig. 1). Of those, four cases were treated with imatinib and the remaining 20 were treatment naïve.

2. DNA extraction and quality assessment

Whole genome sequencing was performed using pretreatment tumor excision samples as well as matched blood samples. Briefly, 4-mm-thick sections with a tumor content of ≥ 80% were obtained, and ≥ 2 μg of DNA was extracted using the Maxwell 16 FFPE Plus LEV DNA Purification Kit (Promega, Madison, WI). For peripheral blood mononuclear cells (PBMCs), genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

3. DNA library construction and whole-genome sequencing

Library preparation was performed using the TruSeq Nano DNA Library Preparation Kit (Illumina, San Diego, CA) following the manufacturer's instructions. Illumina utilizes a unique "bridged" amplification reaction on the surface of the flow cell. A flow cell containing libraries was prepared using the cBot Fluidics Station and was then loaded into the HiSeq X-10 sequencer (Illumina) for automated cycles of extension and imaging. Sequencing-by-Synthesis cycles were repeated to achieve a paired-end read length of 2×150 bp.

4. RNA library construction and whole transcriptome sequencing

Total RNAs were extracted and purified from frozen tumor samples with ReliaPrep FFPE Total RNA Miniprep System (Promega) according to the manufacturer's procedures. Amount of RNA and its quality were checked on an Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA). For analysis of RNA-sequencing data, we prepared mRNA sequencing libraries as paired-end reads with a length of 100 bases using the SMARTer Stranded Total RNA-seq kit v2-Pico Input Mammalian according to the manufacturers' protocols. Briefly, mRNA molecules were purified and fragmented from 2 μg of total RNA. The libraries were sequenced as paired-end reads (2×150 bp) using the NovaSeq 6000 (Illumina).

5. Whole genome data processing

To process whole-genome sequencing data of desmoid tumors, we adopted the Genome Analysis Tool Kit (GATK) v3.7 best practice provided by the Broad Institute [17]. Briefly, we mapped qualified paired-end reads to the human reference genome (hg19) with Burrows-Wheeler Aligner 0.7.15 [18]. Subsequently, we filtered polymerase chain reaction duplicates using Picard tools 2.8.2 to remove potential bias that occurred during sequencing processes. Then, we performed recommended procedures, such as local realignment and base quality recalibration to extract analysis-ready reads.

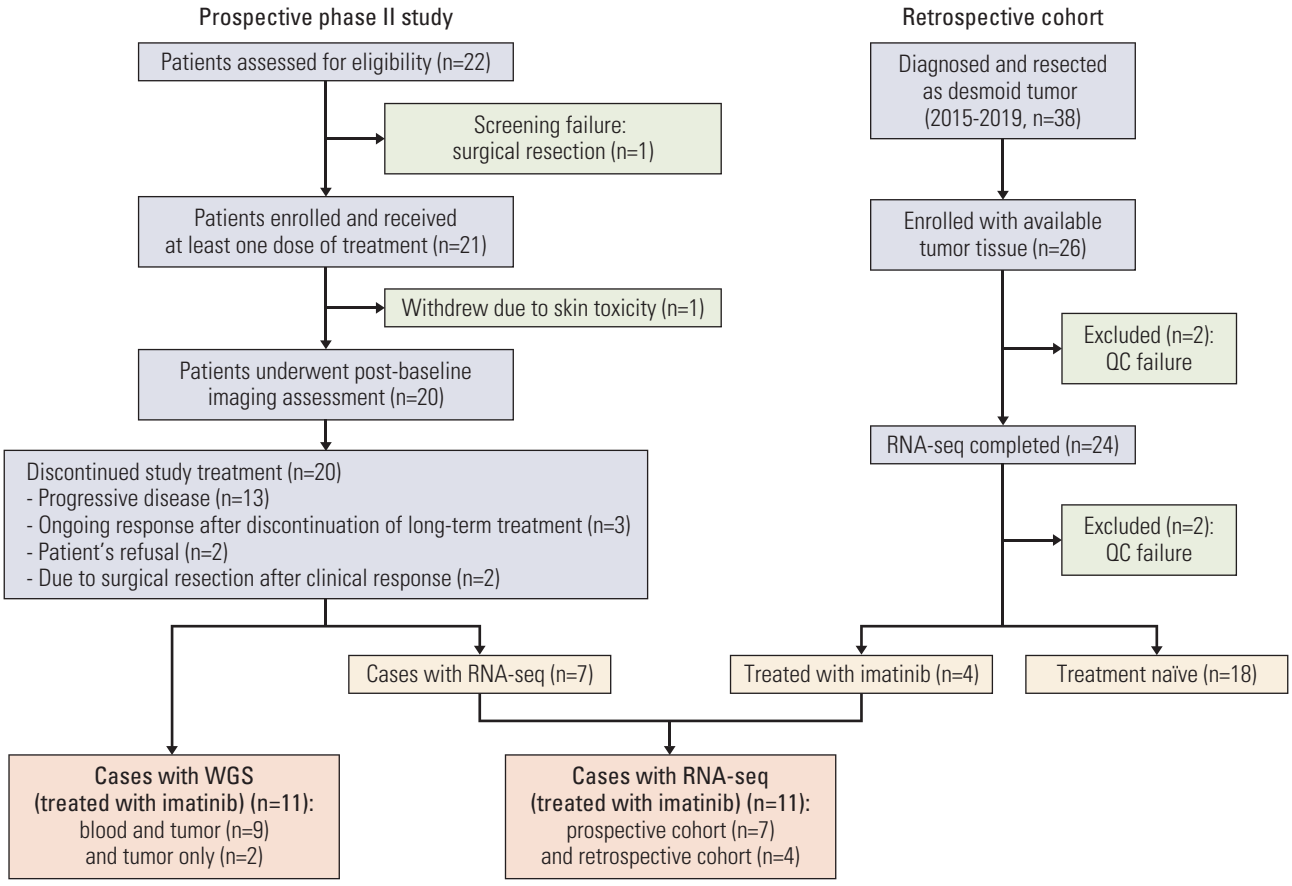

Fig. 1. Consort diagram of desmoid tumor patients included in this study. Prospective phase II study with treatment of imatinib included 20 patients after excluding two patients with clinical concerns. Tumor samples from 11 patients, which passed stringent quality check, were whole-genome-sequenced. RNA-sequencing (RNA-seq) was performed for 29 cases, including seven in phase II study and 22 in retrospective cohort, of which 11 cases were treated with imatinib. QC, quality control; WGS, whole-genome sequencing.

6. Somatic variant detection

MuTect2 [19] of the GATK pipeline with default parameters was used to identify somatic single nucleotide variants (SNVs) and small insertions/deletions (indels). The processed whole-genome-sequencing data for tumor and matched normal samples (PBMCs) were used in BAM format as inputs for Mutect2 [19]. Somatic variants were annotated using ANNOVAR [20]. Some candidate variants were manually inspected using Integrative Genomics Viewer [21]. Population-level allele frequencies of candidates were obtained using Genome Aggregation Database (gnomAD) [22]. For two samples with tumor data only, normal sample data for one of the nine other patients was used as matched normal control for variant calling. A sample that was sequenced in the same batch with a read depth of greater than 30 was used. The variants were further filtered using gnomAD to obtain putative somatic mutations in the tumor-only samples.

7. Scoring gene-wise recurrence of functional variants

Our previously developed gene-wise recurrence model was used [23]. Conventionally, mutations are considered recurrent if and only if they occur at the same genomic location across multiple samples. Mutations are considered oncogenic when their recurrence exceeds a certain threshold [24]. However, this definition of recurrence is inappropriate for analyses of noncoding regions owing to their vast size. Thus, we consider mutations recurrent if they occur in functional regions of the same gene, even if they are not recurrent in a site-specific manner. In particular, we focused on mutational events in cis-regulatory regions of a mammalian gene dispersed across a long range in the genome [25]. Genes were defined based on the GENCODE v.19 gene set mapped to GRCh37 [26].

To identify coding and noncoding mutations with significant functional consequences, deleterious effects of each SNV were predicted using two algorithms, Combined

Annotation-Dependent Depletion (CADD) [27] and Deleterious Annotation of genetic variants using Neural Networks (DANN) [28]. Both models were trained to distinguish benign variants from deleterious variants [27,28]. For multiple mutations in the same gene, the one with the highest score for deleteriousness was selected to represent the functional consequence.

8. Reference whole-genome and transcriptome datasets

To characterize the functional effects of *NOTCH2* noncoding mutations, a large-scale pan-cancer dataset consisting of somatic variants from whole-genome sequencing data and transcriptome data for tumor and matched normal samples were used. VCF files for somatic variant calling and gene expression matrices containing FPKM (fragments per kilobase of transcript per million mapped reads)-upper quantile values were obtained from the Pan-Cancer Analysis of Whole Genomes (PCAWG) Project [29].

9. RNA-sequencing data processing and quality control

We generated RNA-sequencing data of 31 desmoid tumor patients. We removed adapter sequences using Cutadpt [30], and aligned the trimmed reads using STAR [31] with hg19. Gene expression was quantified using RSEM [32]. Quality control check at pre-alignment step was conducted using FASTQC and at post-alignment step using RSeQC [33]. Quality control (QC) results were visualized with MultiQC [34]. At post-alignment step, we noticed two patients with potential problems in read distribution, and infer experiment criteria. Thus, we excluded those samples from future analysis.

10. Bioinformatics and statistical analyses

The chi-square test was used to assess correlations between marker status and clinical significance. All correlation analysis was conducted using spearman correlation. To assess statistical significance between responders and non-responders of imatinib, we calculated Mann-Whitney U test. All tests were two-sided and $p < 0.05$ is considered significant. Cleveland, scatter and box plots were generated by using ggplot2 R package and matplotlib python package.

To conduct enrichment analysis, we adopted two approaches. First of all, we identified genes that are significantly correlated with imatinib sensitivity and used those genes as input for EnrichR [35]. As an alternative step, we conducted Gene Set Enrichment Analysis (GSEA) between responders and non-responders using C2, C5, C6, and Hallmark MSigDB gene sets [36]. C5 Notch category was defined as Notch-related terms present in C5 category.

Progression-free survival was calculated from start date of imatinib to date of progression or death and progression-free rate at 16 weeks (PFR 16) was defined as proportions of patients without progression at 16 weeks, analyzed using the Kaplan-Meier method (SPSS ver. 18.0, SPSS Inc., Chicago, IL).

Results

1. Sample set, patient outcomes, and toxicity

Total of 21 patients was enrolled between April 2014 and October 2015. One patient withdrew, leaving 20 patients (Fig. 1, S1 Table). Three patients (7, 8, and 13) had a known diagnosis of familial adenomatous polyposis (FAP). Fifteen patients (75%) underwent one previous surgery. Most of the patients had been treated with non-surgical procedures, including radiotherapy (n=6, 30.0%) and/or chemotherapy (n=12, 60.0%).

Of the 20 evaluable patients, four (20%) had partial responses (PR) to treatment with durations of 6.7, 26.8 (Fig. 2A), 30.3, and 35.1 (Fig. 2B) months. One PR case had FAP. The PR duration was longer than 1 year for three patients. Fig. 2C provides a waterfall plot of the best response; 14 patients (66.7%) had stable disease (SD), and the clinical benefit rate was 90.0%. The median time to progression was 21.4 months (range, 2.8 to 40.7 months) and PFR 16 was 85% (Fig. 2D).

In terms of toxicity, 400 mg of imatinib was well tolerated with expected grade 3/4 toxicities: neutropenia (n=1), anorexia (n=1), vomiting (n=1), and fatigue (n=1) (S2 Table). Three patients had a one dose level reduction (300 mg/day), and one patient had a reduction of two levels (200 mg/day) owing to toxicity.

2. Results of whole-genome sequencing

After confirming adequate DNA quantities, whole genome sequencing was performed for 11 samples. Two samples (patients 11 and 13) without matched control data were excluded from the primary analysis and used for extensional validation only (Fig. 1). The average read depth was greater than 21.46 (range, 21.46 to 54.05) (S3 Table). By implementing the GATK pipeline from the Broad Institute, we identified 832-4,110 SNVs and indels per sample and used ANNOVAR for annotation (S4 Table).

Next, we examined mutational signatures from annotated variants to identify types of mutational processes [37]. Signature 1A was dominant, accounting for around 88% of signatures (S5 Table), indicating that desmoid tumor variants primarily arise due to errors in replicative polymerases in the DNA repair pathway [37]. The majority of mutations were in intronic regions. Mutations in coding regions were primarily missense and synonymous variants (S6 Fig.).

We also investigated alterations of cancer driver genes registered in the COSMIC database [38] and detected *CTNNB1*

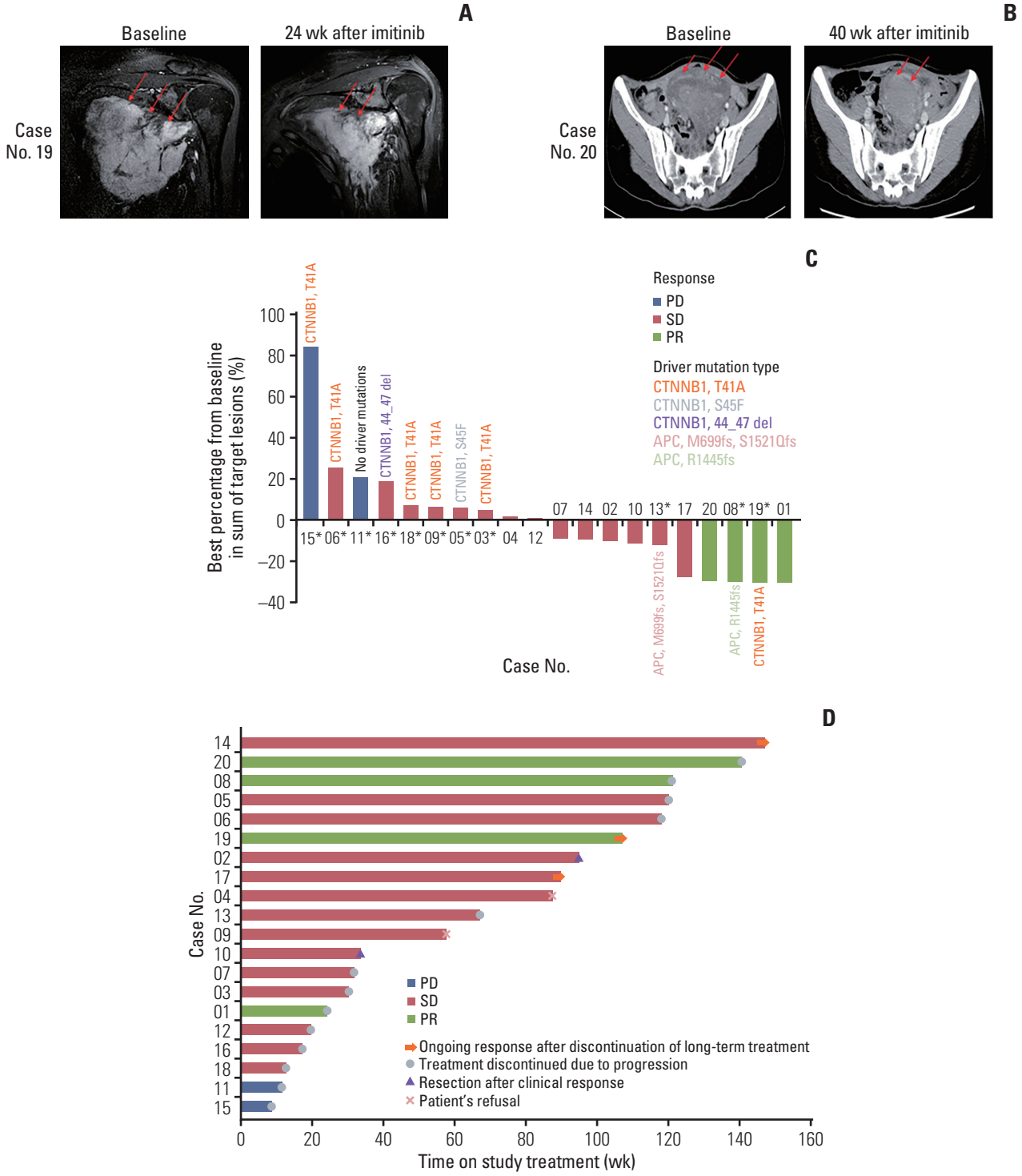

Fig. 2. Representative pre- and post-treatment imaging scans to show antitumor activity of imatinib in desmoid tumors. Soft tissue lesion in the shoulder of patient 19 (A) and pelvis of patient 20 (B) exhibited a significant size reduction after 24 and 40 weeks of imatinib treatment (red arrow). (C) Relative change in tumor volume of patients (n=20) over time. Asterisk indicates a sample with whole-genome sequencing data. The label at the end of the bar shows mutational information in known driver genes, *CTNNB1* and *APC*, for desmoid tumor. (D) Swimmer plot. Each lane represents a single patient's data. X-axis represents the duration of treatment for each patient. PD, progressive disease; PR, partial response; SD, stable disease.

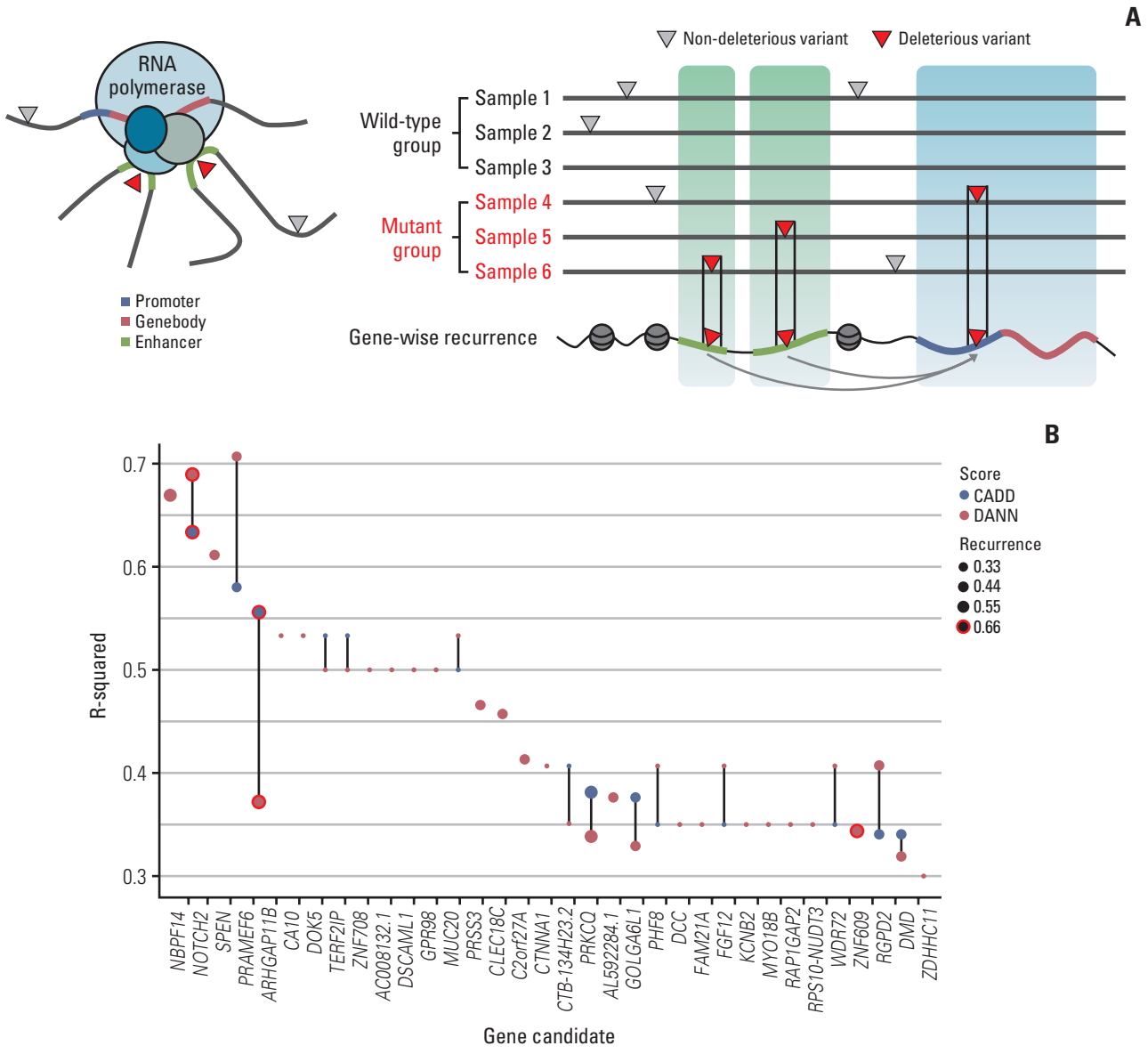

Fig. 3. Gene-wise recurrence of noncoding mutations. (A) Definition of functional noncoding mutations and schematic overview of the gene-wise recurrence model. Functional noncoding mutations in regulatory regions, such as enhancers, affect the expression level of the target gene. Mutations outside of functional regions were excluded from our analysis. Functional mutations were identified as recurrent if they occur in regulatory regions converging of the same gene via enhancer-promoter chromatin interactions across multiple patients. Deleteriousness of functional mutations in mutant groups was quantified using Combined Annotation-Dependent Depletion (CADD) and Deleterious Annotation of genetic variants using Neural Networks (DANN). (B) Cleveland plot shows correlation coefficients (R^2) for the relationship between tumor volume change and deleteriousness score, and recurrence for each gene in desmoid tumor patients (n=9). Genes are ordered by the magnitude of R-squared value. Only protein-coding genes with a recurrence value of 2 or greater are shown.
(Continued to the next page)

mutations in eight out of 11 samples, while two patients harbored adenomatous polyposis coli (*APC*) mutations. *CTNNB1* and *APC* mutation sites of patients are summarized in Fig. 2C. All mutations affected phosphorylation sites necessary for the proper degradation of β-catenin [39-41]. Remarkably, no other mutations in *COSMIC* cancer driver genes were detected, emphasizing the prominent role of the Wnt/β-catenin signaling pathway in desmoid tumor progression [8].

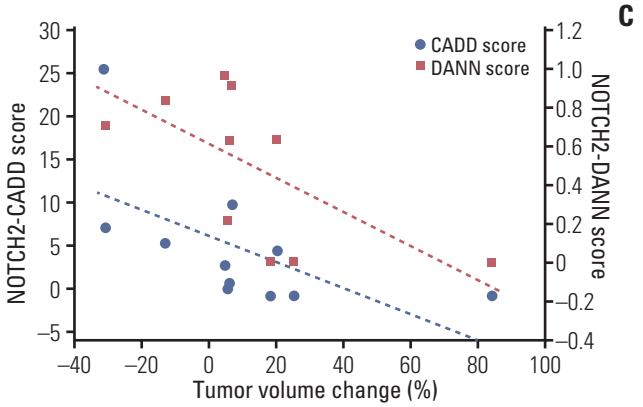

Fig. 3. *(Continued from the previous page)* (C) CADD scores for *NOTCH2* mutations according to tumor volume changes were plotted with the regression line shown in red (R=–0.716, p=0.013). DANN scores for *NOTCH2* mutations were also plotted with the regression line shown in blue (R=–0.831, p=0.006). Each point represents a patient's sample.

3. Gene-wise recurrence analysis of potential cancer-associated genes

To identify potential cancer-associated mutations in desmoid tumors, we employed gene-wise recurrence analysis of mutations in noncoding regions, according to previously developed method [23]. Briefly, the model assumes that mutations in multiple patients are recurrent if they affect the same gene (Fig. 3A). Recurrently affected genes were defined as those with mutations in at least two samples. This strategy allowed us to evaluate noncoding regions with potentially significant impacts on gene regulation with limited sample size. *NOTCH2*, *RGPD2*, and *ARHGAP11B* were identified as strong candidates (Fig. 3B).

4. Correlation between gene-wise scores and imatinib sensitivity

We examined the association between the change in tumor volume after imatinib treatment and the deleteriousness score of recurrently mutated genes. In case of *NOTCH2*, tumor volume changes were highly correlated with both CADD (R=–0.797, p=0.01) and DANN scores (R=–0.831, p=0.006) (Fig. 3B and C, S7 Table). This correlation was maintained even when two tumor-only samples (patient 11 and 13) were included (Fig. 3C). According to the annotated information of somatic variants in *NOTCH2*, three (patients 3, 5, and 8) and four (patients 9, 11, 13, and 18) variants were intergenic and intronic, respectively, while only one (patient 19) variant was nonsynonymous (S8 Table). Using gnomAD database, we found that the allele frequencies of the *NOTCH2* variants were extremely low (0%-1.13%), indicating that they were likely somatic. These results suggest that the regulation of *NOTCH2* at the gene level may contribute to the desmoid tumor response to imatinib.

5. Regulatory role of *NOTCH2* mutations

To evaluate the regulatory effects of *NOTCH2* mutations, we interrogated RNA sequencing and somatic mutation profiles from PCAWG datasets (n=1,009). For comparison across cancer types, we transformed the *NOTCH2* expression levels to Z-scores within each cancer type and identified samples with *NOTCH2* mutations. Considering the role of *CTNNB1* missense mutations in desmoid tumors [8], we further selected both-*NOTCH2*-*CTNNB1* mutants in the PCAWG data (Fig. 4A, S9 Table).

NOTCH2 expression levels were more highly correlated with CADD scores for both-*NOTCH2*-*CTNNB1* mutation group (R=0.607, p=0.013) than for *NOTCH2* mutation-only group (R=0.178, p=0.01) (Fig. 4B). The same trends were observed when DANN score was used for variant scoring (R=0.467) (Fig. 4C), although the correlation was only marginally significant (p=0.068). These results suggest that genetic interactions between *NOTCH2* noncoding mutations and *CTNNB1* missense mutations may influence the *NOTCH2* expression level.

6. Role of NOTCH family members and *HES1* in imatinib sensitivity

To validate significance of our finding, we analyzed RNA-sequencing data of 29 desmoid tumor patients (Fig. 1, S10 Table). We focused on Notch family members, including *NOTCH2*, and *HES1*, a marker of stemness [42] that has been implicated as a target of Notch signaling pathway [43] and marker of imatinib sensitivity [44]. We first investigated whether expression levels of Notch family members and *HES1* are correlated. We calculated spearman correlation between the genes, and discovered that *NOTCH2* expression was significantly correlated with *HES1* expression (p=0.0091568) while expressions of other Notch genes were not (Fig. 5A). This recapitulates the association between *HES1* and *NOTCH2* detected in whole-genome sequencing analysis. We also noticed that all Notch family members are significantly correlated with each other (S11 Fig.). This suggests that other Notch family members can potentially participate in regulation of *HES1* expression although *NOTCH2* is most directly associated with *HES1*.

Next, we examined whether expression of Notch family members or *HES1* is associated with imatinib response. We classified patients into responders (PR, n=5) or non-responders (SD/progressive disease, n=6) on the basis of clinical implementation, and compared expression values between two groups. Strikingly, we found that none of Notch family members had statistical power to distinguish two groups.

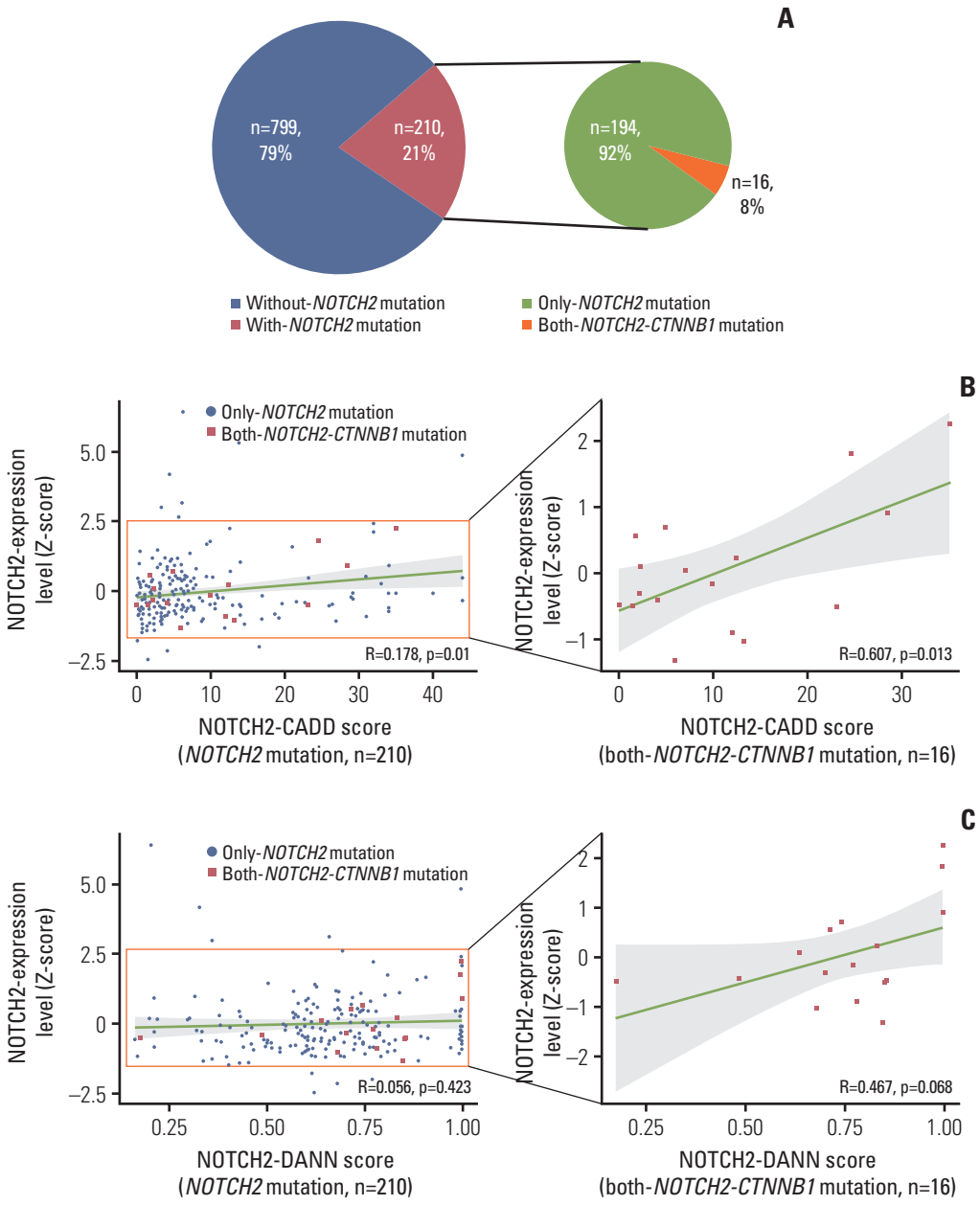

Fig. 4. Correlation between the deleteriousness of *NOTCH2* noncoding-mutations and expression level of *NOTCH2* in Pan-Cancer Analysis of Whole Genomes cohort. (A) Proportions of patients with *NOTCH2* mutations (n=210) and with both *NOTCH2* and *CTNNB1* missense mutations (n=16). (B) Correlation between Combined Annotation-Dependent Depletion (CADD) score and *NOTCH2* expression level in the group with *NOTCH2* mutations (left side) and in selected samples harboring both *NOTCH2* and *CTNNB1* missense mutations (right side). (C) The correlation analysis was repeated using Deleterious Annotation of genetic variants using Neural Networks (DANN) score in the group with *NOTCH2* mutations (left side) and in selected samples with both *NOTCH2* and *CTNNB1* missense mutations (right side).

Intriguingly, *HES1* was significant in discriminating patients' response (p=0.028) (Fig. 5B). This is in accordance with report that overexpression of *HES1* sensitizes cells to imatinib in chronic myeloid leukemia model [44]. Furthermore, we calculated spearman correlation between response rate and expression levels. Initially, we found no correlation between *HES1* or Notch family genes with tumor volume change, with marginal significance in *HES1* (p=0.070). However, after removing outlier (defined as patient with highest gene expression), we found that *HES1* was significantly correlated

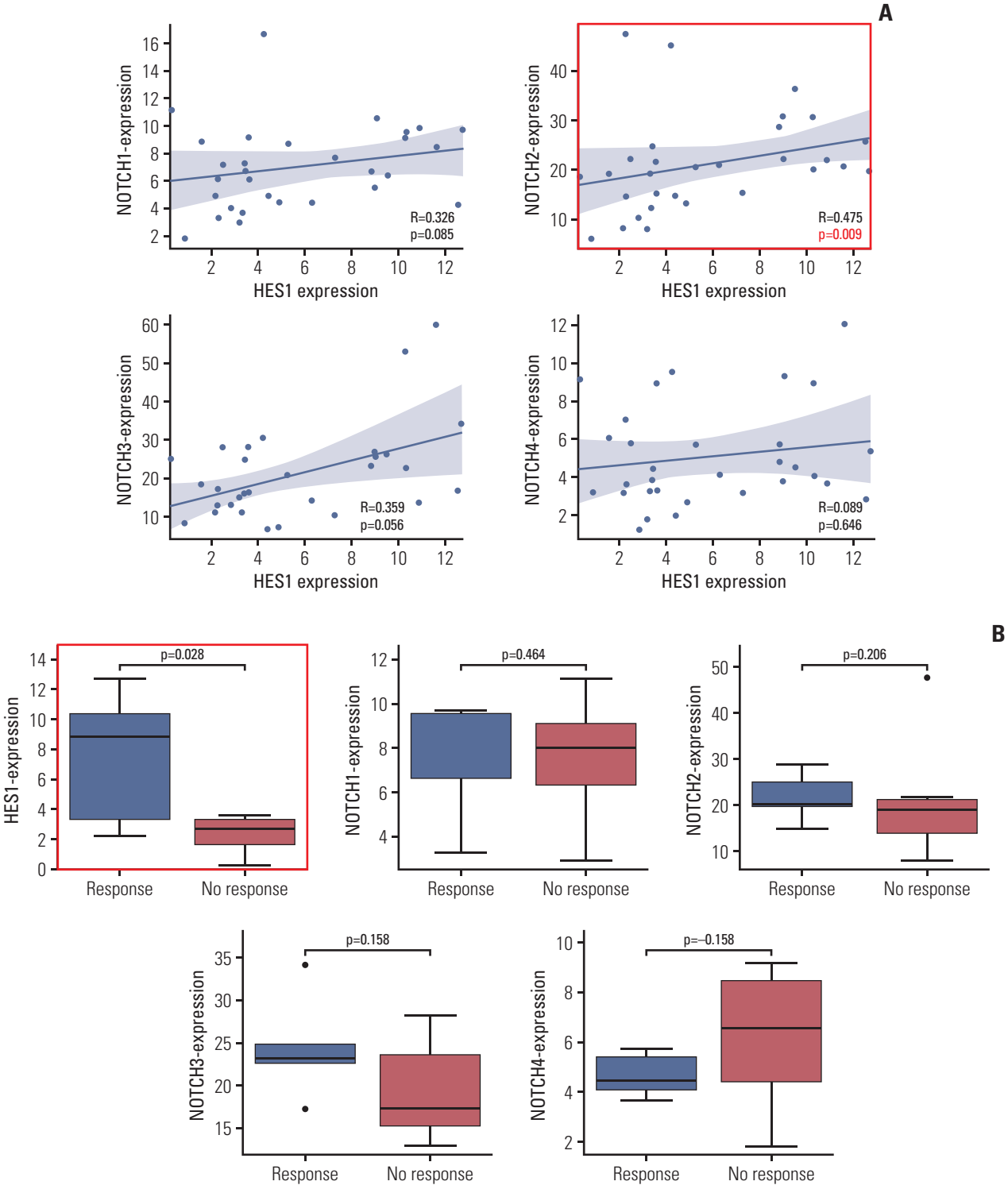

Fig. 5. Expressional association between Notch family and *HES1* genes, and its clinical significance in desmoid tumor samples (n=29). (A) Co-expression between HES1 and one of Notch family genes, including *NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4*, respectively. Correlation analysis was calculated using spearman and all expression levels are in transcripts per million values. (B) Expressional differences of *HES1*, *NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4*, between responders (n=5) and non-responders (n=6) after treatment of imatinib. Statistical significance was determined using Mann-Whitney U test. *(Continued to the next page)*

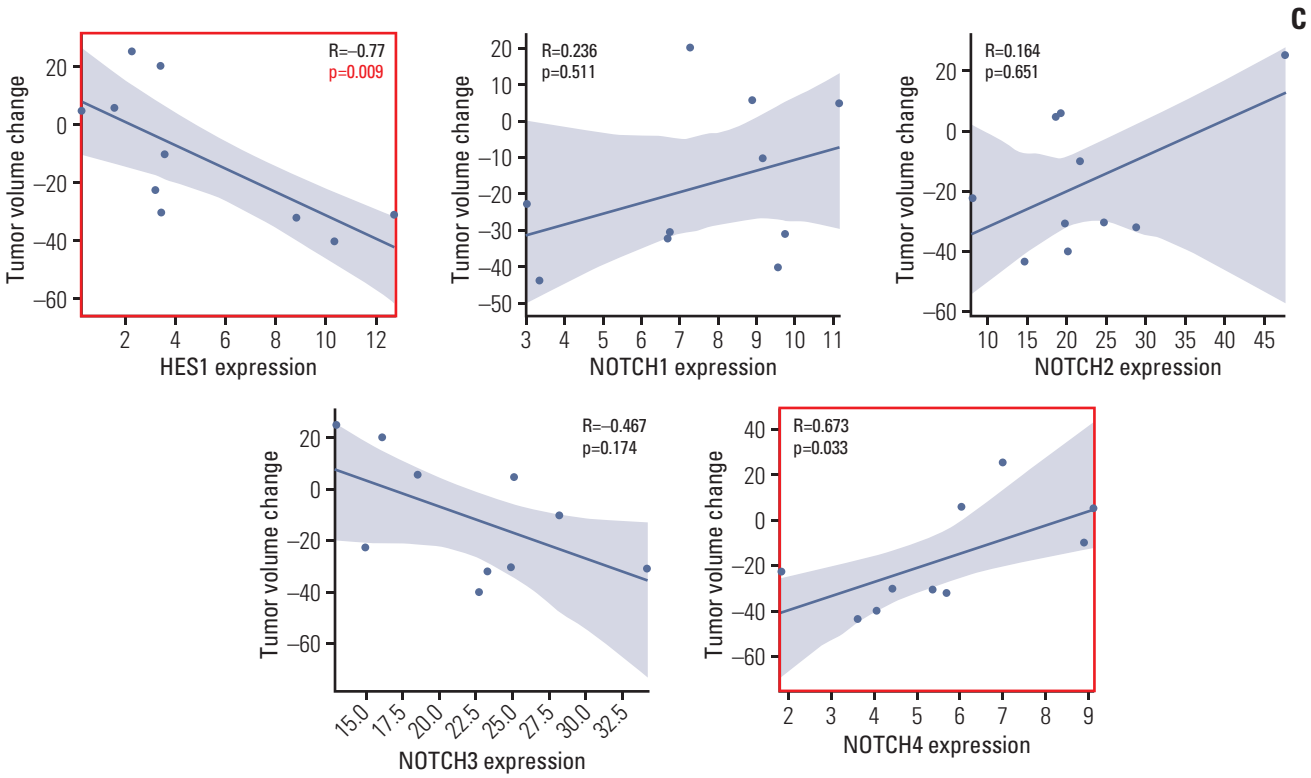

Fig. 5. *(Continued from the previous page)* (C) Correlation between tumor volume change, indicating the imatinib-response, and gene expression level: *HES1* and Notch family members. Plots depict correlation analysis after removing one outlier. All red square lines indicate statistical significance ($p < 0.05$).

with clinical response (p=0.009) (Fig. 5C). Still, none of Notch members, except *NOTCH4*, achieved statistical significance. Overall, *HES1* was the most significant marker of imatinib sensitivity.

7. Enrichment analysis of response-associated genes

To identify biological pathways that are associated with imatinib sensitivity, we first conducted GSEA between responders and non-responders (Fig. 6A). Differentially expressed genes were enriched in diverse biological pathways including muscle cell cellular homeostasis, negative regulation of myoblast differentiation, and skeletal tissue regeneration, angiogenesis and regulation of oxidative phosphorylation. We also performed enrichment analysis using genes that are significantly correlated with imatinib sensitivity (Fig. 6B). Similar terms, such as fibroblast growth factor binding, muscle cell migration, and oxidative phosphorylation, were enriched. Among these terms, we found that glucose metabolism and mitochondrial respiration have been closely linked to imatinib sensitivity [45] and are upregulated in naïve pluripotent stem cells [46] in previous researches. In addition, angiogenesis is a well-known feature of mesenchymal stem cells [47]. Collectively, we propose that these terms, such as oxidative phosphorylation and angiogenesis, and *HES1* all point to the significance of mesenchymal stem cell population that are prone to imatinib in desmoid tumor.

Discussion

Using whole-genome and transcriptome sequencing, we performed integrative molecular characterization of desmoid tumor in patients receiving imatinib treatment. Our analyses suggest *HES1* overexpression, potentially regulated by *NOTCH2*, can serve as a predictor of the clinical response to imatinib in desmoid tumor patients. To our knowledge, this is the first integrative study to characterize molecular determinants of the response to imatinib in desmoid tumor.

Our coding-region analyses recapitulated previous findings on the prevalence of *CTNNB1* and *APC* mutations [48]. Unfortunately, these mutations were not associated with clinical responses to imatinib. However, we discovered that mutations in noncoding regulatory regions of *NOTCH2* are positively correlated with the clinical response to imatinib.

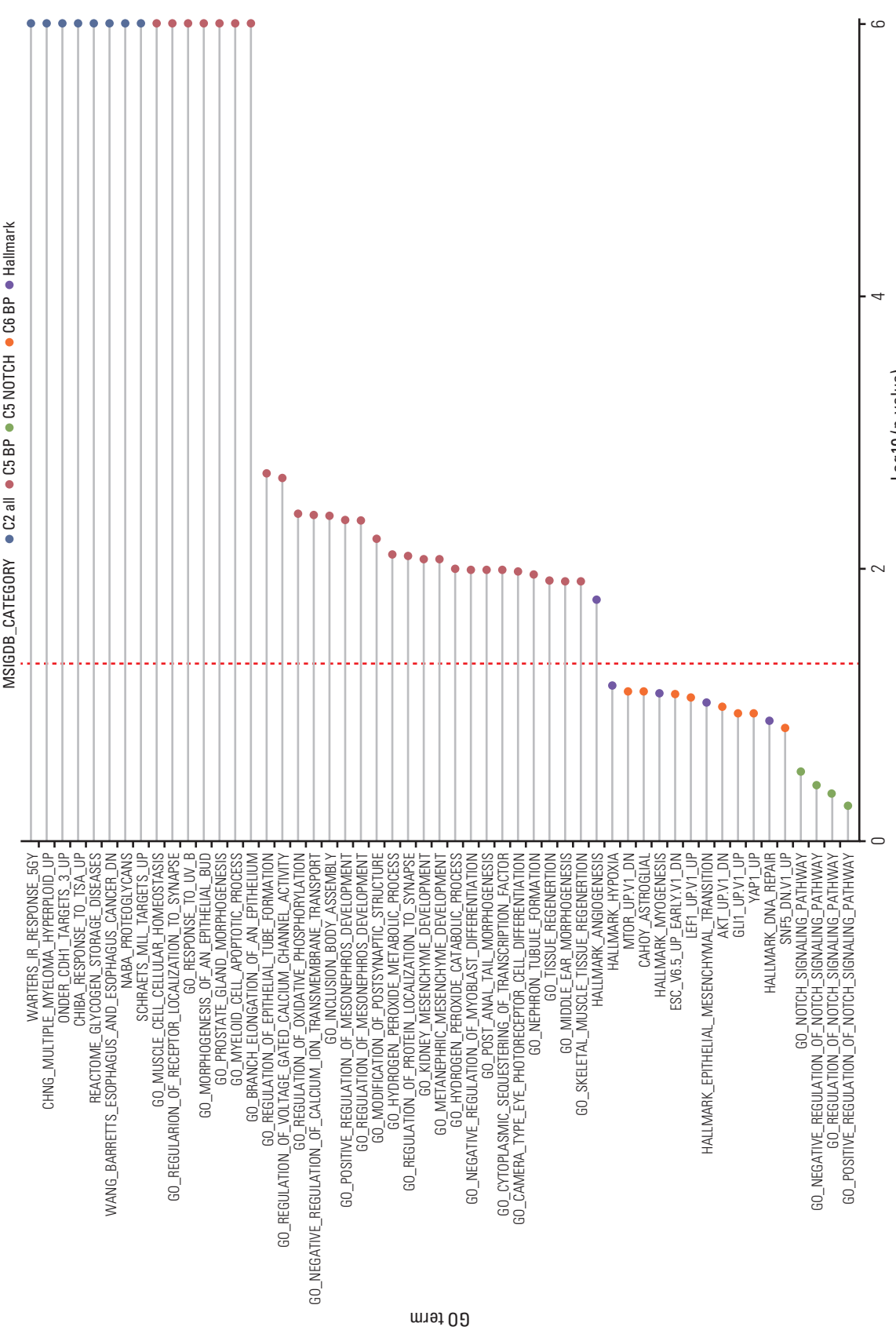

Fig. 6. (A) Gene Set Enrichment Analysis (GSEA) for differential genes with statistical importance ($p < 0.05$) between responders (n=5) and non-responders (n=6). Gene Ontology terms on y-axis indicate molecular signatures database (MSigDB), of which five gene sets are shown in legend: C2, as curated gene sets; C5 BP, as gene sets from biological process ontology; C5 NOTCH, as gene sets associated with Notch pathway; C6 BP, as oncogenic signatures of biological process; Hallmark, as gene sets to represent specific biological states or processes and show consistent expression. Most significant terms for each category are depicted. *(Continued to the next page)*

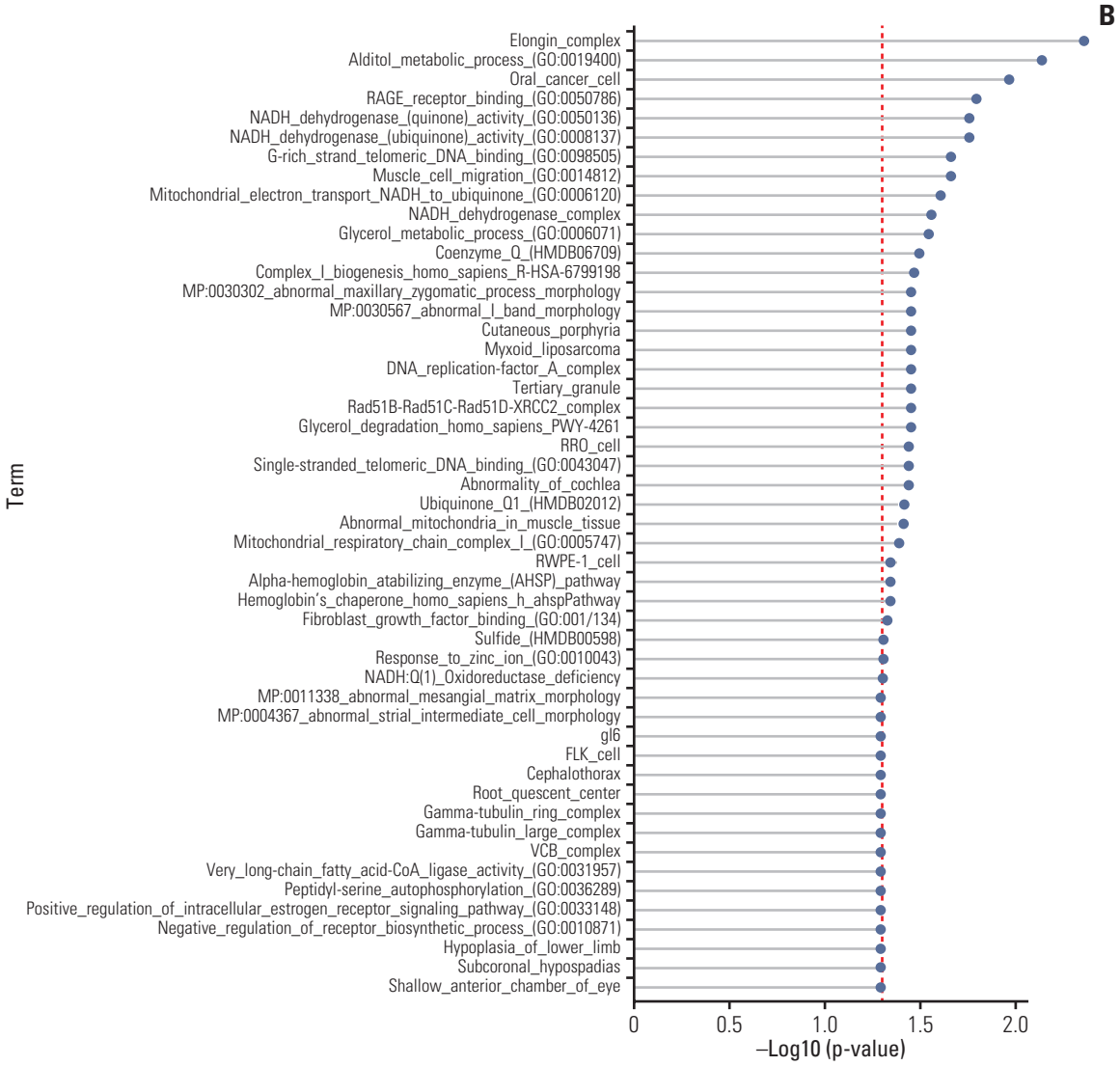

Fig. 6. *(Continued from the previous page)* (B) GSEA result by using EnrichR with genes that are significantly correlated with imatinib sensitivity. Red vertical lines indicate p-value of 0.05.

Moreover, our investigation of PCAWG samples revealed that noncoding mutations in *NOTCH2* regions increase expression. Importantly, *NOTCH2* expression was significantly correlated with *HES1*, and *HES1* was indicative of imatinib sensitivity in our desmoid cohort. We noticed that removal of one outlier restored statistical significance when assessing correlation between *HES1* and tumor volume change. Still, our correlation analysis suggests that *HES1* expression is the major determinant of imatinib sensitivity. Other minor factors of imatinib sensitivity need to be determined to fully elucidate mechanism of imatinib in desmoid tumor. Taken together, we suggest that *HES1* and *NOTCH2* overexpression is a predictor of the anti-cancer effects of imatinib on desmoid tumors.

Our study is not the very first attempt to investigate the significance of Notch signaling in desmoid tumor [43,49]. Based on multiple evidences, small molecule inhibitors, such as γ-secretase inhibitor, siRNA, and monoclonal antibody against Notch pathway were designed to treat desmoid tumor [50]. Studies have confirmed that activation of *NOTCH2* leads to overexpression of *HES1* and are accompanied by proliferation, immature morphology and aggressiveness in acute kidney injury model [51] and hepatocellular carcinoma model [52]. Of note, Notch signaling and *HES1* have been associated with response to imatinib in chronic myeloid leukemia cells [44,53], providing robust evidence for Notch-*HES1* axis in mechanism of imatinib in desmoid tumor. Surprisingly, however, no research reported its asso-

ciation with clinical response of imatinib in desmoid tumor until now.

The mechanism underlying the tumor response to imatinib is still not fully understood. In agreement with our findings, multiple lines of evidence support their significance in desmoid tumor. In a preclinical study, imatinib inhibits Notch signaling by increasing the proteosomal degradation of intracellular Notch. Furthermore, a primary effector of Notch signaling, *HES1*, decreased as the imatinib concentration increased [54]. In a neurodegenerative condition, Alzheimer disease, imatinib results in the dose-dependent inhibition of γ-secretase activity [55]. Similarly, NSAIDs, another standard treatment for desmoid tumors, alter γ-secretase activity [56]. Taken together, these findings support the predictive value of Notch and *HES1* as therapeutic strategy.

The Notch pathway exhibits crosstalk with the Wnt signaling cascade [57] and is involved in the regulation of tumor microenvironments and the maintenance of cancer stem cells [58-60]. With *NOTCH1* activation, desmoid tumors showed high expression levels of *NOTCH1* and its downstream transcription factor *HES1* [43], of which transcriptional activity is dependent on *NOTCH2* [61]. Thus, targeting γ-secretase to prevent Notch cleavage has been suggested as a novel therapeutic approach [62,63]. A phase II trial of the γ-secretase inhibitor PF-03084014 demonstrated a promising efficacy, with a response rate of 29% for patients with progressive desmoid tumors [63]. Furthermore, the efficacy of PF-03084014 is high in tumors with elevated expression of genes in the Notch and Wnt pathways [64].

We sought to explain the increased correlation between *NOTCH2* mutation scores and *NOTCH2* expression levels when considering the *CTNNB1* mutation status. A previous study has shown that the TCF4/β-catenin complex binds to the promoters of Notch signaling pathway genes, including *NOTCH2* [65]. In the absence of the TCF4/β-catenin complex, the transcription machinery cannot be assembled at regulatory regions and fails to induce *NOTCH2* expression, thereby preventing *NOTCH2* regulatory mutations from exerting effects. We speculate that the hyperactivity of the TCF4/β-catenin complex induced by *CTNNB1* msissense mutations leads to the constitutive activation of Notch signaling. This allows *NOTCH2* regulatory mutations to alter gene expression levels, leading to a high correlation between deleteriousness and transcript levels. Further investigations, including functional studies, are needed to validate the mode of action of imatinib.

As mentioned earlier, mutation status of driver genes failed to discriminate patients who will respond to imatinib. We overcame this hurdle with analysis of noncoding mutations, providing rationale to investigate Notch signaling and its downstream target *HES1* to interpret molecular mechanism of imatinib. Thus, significance of noncoding mutation confers huge advantage to whole-genome sequencing data over whole-exome sequencing data. Despite such significance, we acknowledge several limitations of our study. First of all, we are aware that limited sample size hinders more comprehensive study of desmoid tumor. For example, although we were initially unable to obtain statistical significance for *NOTCH2* in Fig. 5B (responder versus non-responder analysis), removal of outlier restored statistical significance (p-value from 0.2 to 0.07), proving that *NOTCH2* is a biologically meaningful biomarker. Rarity of desmoid tumor obscured the statistical power, and increased sample size will endow power to rescue unrecognized candidates. Also, we admit lack of functional study. Collectively, multiple evidences point out to Notch-*HES1* axis in various studies, its significance in response to imatinib, and role of *HES1* in stemness. Thus, future work should validate both clinical and biological significance of *HES1* in desmoid tumor to gain insight into this aggressive rare tumor.

In conclusion, using whole-genome sequencing with gene-wise recurrence model and transcriptome of desmoid tumor, we propose that overexpression of *NOTCH2* and *HES1* is the marker of sensitivity for the anti-cancer effects of imatinib on desmoid tumors. Our results suggest that *HES1* should especially be considered in clinical settings when using imatinib to treat this rare and challenging disease.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study was approved by the institutional review board (IRB No. 2013-1417-001). Retrospective cohort study of 24 additional patients with desmoid tumor was approved by the institutional review board (IRB No. 2020-2244-001). All patients provided written informed consent. This study was conducted in accordance of Declaration of Helsinki.

Author Contributions

Conceived and designed the analysis: Choi JK, Kim HS.
Collected the data: Lee YH, Lee J, Ahn JH, Kim SH (Se Hyun Kim), Kim SH (Seung Hyun Kim), Kim TI, Yun KH, Park YS, Kim JE, Lee KS.
Contributed data or analysis tools: Kwon J, Lee JH.
Performed the analysis: Kwon J, Lee JH.
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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.944
Cancer Res Treat. 2022;54(4):1256-1267

Original Article

Predictive Parameters of Febrile Neutropenia and Clinical Significance of G-CSF Receptor Signaling Pathway in the Development of Neutropenia during R-CHOP Chemotherapy with Prophylactic Pegfilgrastim in Patients with Diffuse Large B-Cell Lymphoma

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Purpose Pegfilgrastim is widely used to prevent chemotherapy-induced neutropenia (CIN) and febrile neutropenia (FN) in patients with diffuse large B-cell lymphoma (DLBCL). We investigated the predictive factors affecting CIN and FN incidence in patients with DLBCL receiving rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) chemotherapy with pegfilgrastim and conducted experiments to find reason for the occurrence of CIN even when pegfilgrastim was used.

Materials and Methods We reviewed the CIN and FN events of 200 patients with DLBCL. Based on these data, we investigate the association with predictive factor and the levels of granulocyte-colony stimulating factor (G-CSF) receptor signaling pathway markers (pSTAT3, pAKT, pERK1/2, pBAD, and CXCR4) in bone marrow (BM) samples isolated from patients with DLBCL.

Results FN was significantly associated with stage III/IV (hazard ratio [HR], 12.74) and low serum albumin levels (HR, 3.87). Additionally, patients with FN had lower progression-free survival (PFS; 2-year PFS, 51.1 % vs. 74.0%) and overall survival (OS; 2-year OS, 58.2% vs. 85.0%) compared to those without FN. The occurrence of CIN was associated with overexpression of G-CSF receptor signaling pathway markers, and expression levels of these markers were upregulated in BM cells co-cultured with DLBCL cells. The rate of neutrophil apoptosis was also higher in neutrophils co-cultured with DLBCL cells and was further promoted by treatment with doxorubicin.

Conclusion Our findings suggest that high DLBCL burden may alter the BM environment and G-CSF receptor signaling pathway, even in chemotherapy-naïve state, which may increase CIN frequency during R-CHOP chemotherapy.

Key words Neutropenia, G-CSF receptor, Chemotherapy, Pegfilgrastim, Diffuse large B-cell lymphoma

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common histological subtype of non-Hodgkin lymphoma (NHL) [1]. Although the treatment outcomes of DLBCL have drastically improved since the introduction of rituximab, an anti-CD20 monoclonal antibody, in the early 2000s [2,3], chemotherapy-induced neutropenia (CIN) and febrile neutropenia (FN) continue to be responsible for treatment-related deaths [4,5]. In addition, the success of curative treatment can be compromised by reduced or delayed chemotherapy after CIN [6,7].

To prevent these adverse events, current guidelines recommend the prophylactic use of recombinant human granulocyte-colony stimulating factor (G-CSF) [8,9], which reduces the incidence of CIN [10,11] and acts primarily by activating the G-CSF receptor. Although the specific mechanisms underlying G-CSF signaling remain unclear, some intracellular signaling pathways such as Janus kinase (JAK)/signal transducer and activator of transcription protein (STAT) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) have been shown to be involved in apoptosis and cell survival and differentiation [12,13].

Pegfilgrastim is a long-acting form of G-CSF that is synthesized by adding a polyethylene glycol molecule to G-CSF (filgrastim) to reduce the proteolytic degradation and renal

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Received August 23, 2021 **Accepted** December 30, 2021
Published Online December 31, 2021

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clearance of filgrastim, resulting in a more stable molecule with a reduced need for frequent dosing. Furthermore, the neutrophil-regulated clearance mechanism maintains a constant concentration of pegfilgrastim in the body until it has recovered from neutropenia [14].

Studies have shown that pegfilgrastim can reduce neutropenia-related complications, including CIN and FN, when used as a primary prophylactic after chemotherapy. Additionally, treatment once-per-cycle with pegfilgrastim was found to yield more favorable results than a daily regimen of filgrastim administration [15,16]. Consequently, pegfilgrastim has been used widely to prevent CIN in cancer patients; however, there is limited data regarding the efficacy of pegfilgrastim to prevent FN in cancer patients receiving chemotherapy, particularly patients with DLBCL receiving rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) immunochemotherapy.

A previous study found that 48% of the patients with DLBCL experienced grade 3/4 neutropenia and 16% developed neutropenic fever during R-CHOP chemotherapy with pegfilgrastim [17]. Therefore, we aimed to identify predictive factors affecting the incidence of CIN and FN during R-CHOP chemotherapy with pegfilgrastim in patients with DLBCL and to find out why prophylactic pegfilgrastim does not prevent CIN during chemotherapy. To achieve these goals, we investigated the molecular and cellular roles of G-CSF receptor signaling in CIN during chemotherapy with prophylactic pegfilgrastim treatment in patients with DLBCL.

Materials and Methods

1. Patients and samples

We reviewed the medical records of 200 patients with DLBCL who underwent R-CHOP chemotherapy followed by prophylactic pegfilgrastim treatment from three medical centers in South Korea. We collected comprehensive baseline patient characteristics, including disease- and host-related factors, as well as data about CIN and FN events after the first cycle of R-CHOP chemotherapy and treatment outcomes. Since prophylactic pegfilgrastim treatment has been covered by the National Health Insurance system in South Korea since 2015, all patients diagnosed with DLBCL between 2015 and 2018 were enrolled in the study. All enrolled patients received R-CHOP chemotherapy at an initial dose determined according to their characteristics and disease status, with pegfilgrastim administered at least 24 hours after the end of chemotherapy. Blood samples for laboratory testing were collected on day 1 and again 7±3 days after chemotherapy to determine the occurrence of neutropenia and the nadir value of absolute neutrophil count (ANC). Daily laboratory tests were performed for inpatients, and for outpatients, laboratory tests were performed at outpatient clinic visits scheduled 1 week after chemotherapy. CIN is generally

Table 1. Study patient demographics and clinical characteristics

Characteristic	No. of patients (%)
Age, median (range, yr)	62 (16-88)
Sex	
Male	118 (59.0)
Female	82 (41.0)
ECOG	
0-1	165 (82.5)
≥ 2	34 (17.0)
Unknown	1 (0.5)
IPI risk group	
Low (0-1)	68 (34.0)
Low-intermediate (2)	44 (22.0)
High-intermediate (3)	43 (21.5)
High (4-5)	44 (22.0)
Unknown	1 (0.5)
Ann Arbor stage	
I	23 (11.5)
II	55 (27.5)
III	24 (12.0)
IV	98 (49.0)
B symptoms	52 (26.0)
Bulky disease	26 (13.0)
Extranodal involvement	126 (63.0)
Single lesion	62 (49.2)
Lesion ≥ 2	64 (50.8)
LDH elevation	133 (66.5)
Hans criteria for DLBCL	
GCB	53 (26.5)
Non-GCB	127 (63.5)
Unknown	20 (10.0)
Double express	
None	7 (3.5)
Double	31 (15.5)
Unknown	162 (81.0)
Bone marrow involvement	34 (17.0)
Median cellularity (%)	40
Chromosome	
Normal	155 (77.5)
Abnormal	34 (17.0)
Unknown	11 (5.5)
Initial chemotherapy dose	
Standard	125 (62.5)
Reduced	75 (37.5)

DLBCL, diffuse large B-cell lymphoma; ECOG, Eastern Cooperative Oncology Group; GCB, germinal center B-cell; IPI, International Prognostic Index; LDH, lactate dehydrogenase.

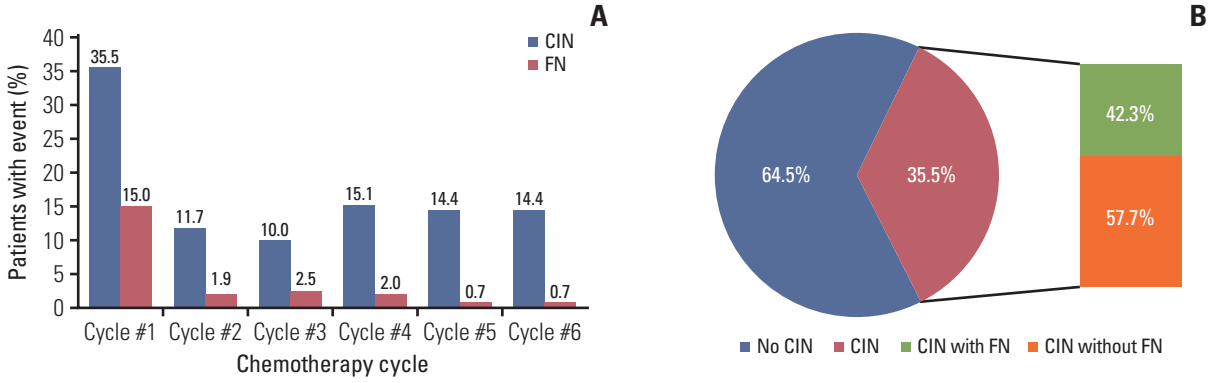

Fig. 1. CIN and FN events. (A) CIN and FN events on each R-CHOP chemotherapy cycle. (B) Analysis of CIN and FN events at first chemotherapy cycle of diffuse large B-cell lymphoma patients. CIN, chemotherapy-induced neutropenia; FN, febrile neutropenia; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone.

characterized as a decreased ANC < 2,000/μL in peripheral blood. In this study, grade 3/4 (ANC < 1,000/μL) neutropenia was defined as CIN. Fever was defined as a single oral temperature measurement of ≥ 38.3°C, or a temperature of ≥ 38°C sustained over a 1-hour period. FN was defined as the occurrence of fever in a state of neutropenia with an ANC of < 500/μL or < 1,000/μL that was expected to decrease to < 500/μL within 48 hours.

2. Cell culture and antibodies

Three different cell lines (human bone marrow [BM], murine BM, and OCI-Ly-1 [RRID: CVCL_1879]) were used in this study. Human BM samples from patients with DLBCL were aspirated prior to the first dose of R-CHOP chemotherapy, cryopreserved in the National Biobank of Korea, and analyzed after thawing according to the appropriate guidelines. Human bioresources deposited in the National Biobank of Korea must be accompanied by 'Consent to Research on Human Materials' or 'Consent to Donation on Human Materials' in accordance with 'Bioethics and Safety Act Enforcement Rules.'

Murine BM cells were collected from 7-week-old C57BL/6 mice and red blood cells were removed using ammonium chloride solution. BM cells were then resuspended in Dulbecco's modified Eagle medium (cat No. #LM001-05, Welgene, Gyeongsan, Korea), supplemented with 10% fetal bovine serum (FBS; cat No. #SH30084.03, HyClone Laboratories, Logan, UT) and incubated overnight at 37°C in 5% CO_2.

The OCI-Ly1 cell line was kindly provided by Dr. Ricardo Aguiar from the University of Texas Health Science Center at San Antonio and maintained in Roswell Park Memorial Institute-1640 medium (cat No. #30027.01, HyClone Laboratories) supplemented with 10% FBS at 37°C in 5% CO_2. The OCI-Ly1 cell line was authenticated using short tandem repeat profiling (Cosmogen Tech., Seoul, Korea) within the last 3 years and all experiments were performed using mycoplasma-free cells.

The following antibodies were used in this study: anti-phospho AKT (1:1,000 dilution, cat No. #9271, Cell Signaling Technology [CST], Danvers, MA), anti-phospho STAT3 (1:1,000, cat No. #9131S, CST), anti–phospho extracellular signal-regulated kinase (ERK) 1/2 (1:1,000, cat No. #9101, CST), anti–phospho BCL2-associated agonist of cell death (BAD; 1:1,000, cat No. #9295, CST), and anti–C-X-C chemokine receptor type 4 (CXCR4; 1:1,000, cat No. #ab1670, Abcam, Burlingame, CA).

3**. Western blotting**

Protein levels were determined by western blotting. Briefly, cells were harvested, washed with 1× phosphate-buffered saline, and lysed in radioimmunoprecipitation assay buffer (Elpis Biotech, Daejeon, Korea) mixed with sodium vanadate (1 mM, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), β-glycerol phosphate (50 mM, Sigma-Aldrich, Merck KGaA), protease inhibitor (1×, G-Biosciences, St. Louis, MO), EDTA (5 mM, G-Biosciences), and β-mercaptoethanol (142 mM, Bioworld Technology, St. Louis Park, MN). Lysates were resolved on 10% or 15% polyacrylamide gels, transferred onto polyvinylidene difluoride membranes, and then blocked with 1% bovine serum albumin (MP Biomedicals, Irvine, CA) reconstituted in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature. The membranes were probed overnight with primary antibodies at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Signals were then visualized using enhanced chemiluminescent reagent (EzWestLumi plus, ATTO, Tokyo, Japan) and protein levels were analyzed using Lumino-

Table 2. Analysis of risk factors affecting chemotherapy-induced neutropenia and febrile neutropenia during the first chemotherapy cycle

Characteristic	Chemotherapy-induced neutropenia				Febrile neutropenia			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Age	0.99 (0.98-1.02)	0.594	-	-	0.99 (0.96-1.02)	0.446	-	-
Female sex	2.03 (1.13-3.66)	0.019	1.45 (0.72-2.94)	0.299	2.48 (1.12-5.49)	0.025	2.22 (0.86-5.71)	0.089
Ann Arbor stage ≥ 3	3.56 (1.83-6.94)	< 0.001	1.13 (0.39-3.22)	0.825	24.01 (3.2-180.32)	0.002	12.74 (1.61-100.74)	0.016
B symptoms	2.64 (1.38-5.28)	0.004	1.62 (0.76-3.46)	0.216	2.97 (1.27-6.96)	0.012	1.42 (0.52-3.93)	0.495
ECOG ≥ 2	2.75 (1.30-5.84)	0.008	0.82 (0.25-2.66)	0.738	5.39 (2.29-12.67)	< 0.001	1.30 (0.37-4.55)	0.682
IPI ≥ 3	2.93 (1.61-5.34)	< 0.001	1.93 (0.93-4.03)	0.078	6.73 (2.61-17.36)	< 0.001	0.96 (0.23-4.82)	0.955
Bulky mass	1.29 (0.53-3.13)	0.576	-	-	0.71 (0.20-2.52)	0.590	-	-
Extranodal involvement	2.26 (1.19-4.28)	0.012	0.72 (0.21-2.5)	0.603	6.46 (1.88-22.11)	0.003	2.09 (0.17-26.33)	0.569
BM involvement	3.75 (1.74-8.07)	0.001	3.16 (1.33-7.53)	0.009	5.43 (2.31-12.75)	< 0.001	2.55 (0.94-6.92)	0.065
Elevated LDH	1.68 (0.88-3.20)	0.117	-	-	5.26 (1.53-18.07)	0.008	3.06 (0.58-16.08)	0.186
B2MG	1.07 (0.94-1.21)	0.294	-	-	1.17 (1.01-1.35)	0.031	0.69 (0.19-2.48)	0.571
Albumin < 3.3	6.35 (2.34-15.29)	< 0.001	3.47 (1.27-9.48)	0.015	9.04 (3.71-22.06)	< 0.001	3.87 (1.40-10.72)	0.009
Double express	1.81 (0.30-10.8)	0.517	-	-	1.44 (0.15-14.32)	0.756	-	-
Ki-67	0.98 (0.97-1.00)	0.078	-	-	1.0 (0.98-1.02)	0.967	-	-
EBV	2.96 (0.79-11.09)	0.107	-	-	4.42 (1.12-17.38)	0.034	-	-
Non-GCB type	1.09 (0.56-2.12)	0.806	-	-	0.86 (0.26-2.05)	0.733	-	-
Chemotherapy dose	0.71 (0.39-1.30)	0.269	-	-	0.96 (0.43-2.14)	0.919	-	-

B2MG, beta-2 microglobulin; BM, bone marrow; CI, confidence interval; EBV, Epstein-Barr virus; ECOG, Eastern Cooperative Oncology Group; GCB, germinal center B-cell; HR, hazard ratio; IPI, International Prognostic Index; LDH, lactate dehydrogenase.

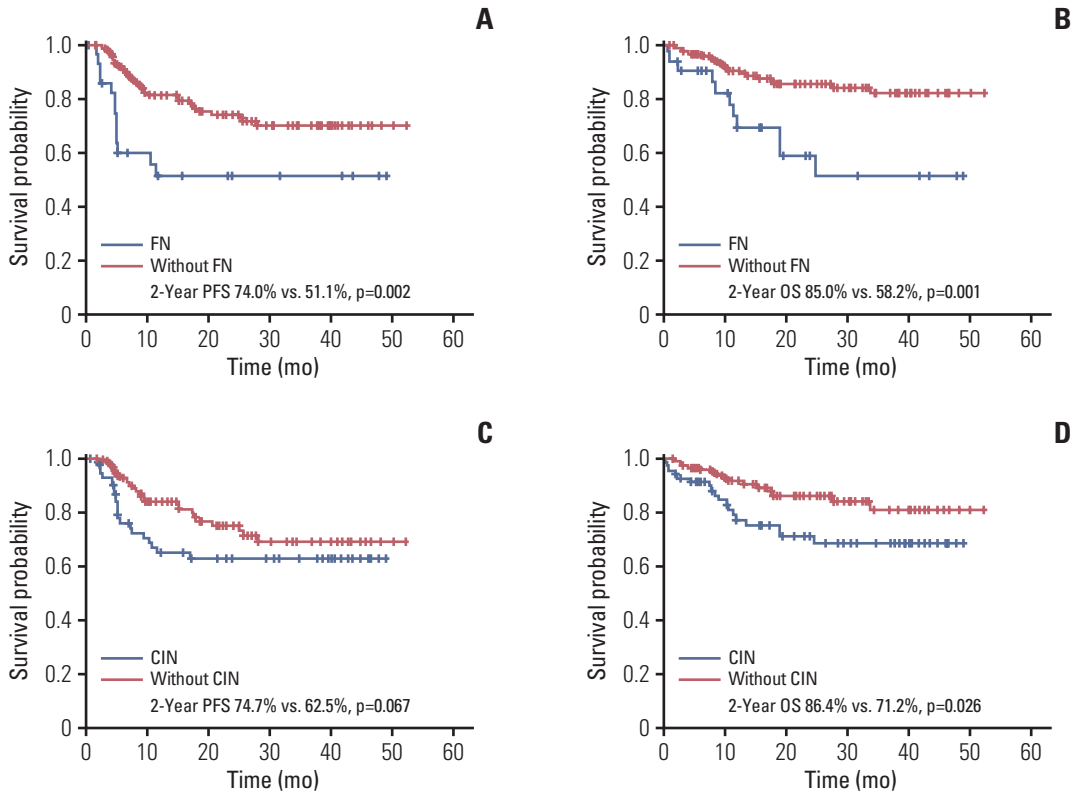

Fig. 2. Effect of CIN and FN on the progression-free survival (PFS, A and C) and overall survival (OS, B and D) Kaplan-Meier curves showing the PFS and OS of patients with newly diagnosed diffuse large B-cell lymphoma according to the FN events (A, B) and CIN (C, D) in the first cycle of chemotherapy. CIN, chemotherapy-induced neutropenia; FN, febrile neutropenia; OS, overall survival; PFS, progression-free survival.

Graph II image analysis software (WSE-6100, ATTO). Protein expression was quantified relative to β-actin using ImageJ v1.52a. All western blotting data were the result of at least three independent experiments.

4. Flow cytometry

The rate of apoptosis was investigated in murine BM cells co-cultured with OCI-Ly1 cells and murine BM cells alone with or without chemotherapy. To determine whether DLBCL cells affected neutrophil apoptosis during chemotherapy, 1×10^7 cells/mL murine BM cells were co-cultured with 5×10^6 OCI-Ly1 cells in 6-well Transwell plates and incubated at 37°C for 24 hours. The cells were then treated with 2.5 or 5.0 μM of doxorubicin and incubated for a further 24 hours under the same conditions. Next, BM cells were harvested and stained with fluorescein isothiocyanate-conjugated rat anti-CD11b (cat No. #561688, BD Biosciences, San Jose, CA), allophycocyanin-conjugated rat anti-mouse Ly-6G (cat No. #560599, BD Biosciences), and propidium iodide (cat No. #51-66211E, BD Biosciences) according to the manufacturer's instructions. Neutrophils were identified using key cell surface markers ($CD11b^+$ $Ly6G^+$) and apoptosis was determined by propidium iodide using flow cytometry (BD FACSCanto II software).

5. Statistical analysis

Patient characteristics were described using descriptive statistics. Categorical variables were compared using the chi-square test or Fisher exact test, whereas continuous variables were examined using a t test or u test depending on the data distribution. Stepwise conditional logistic regression analysis was used to control for the effects of confounding variables and identify independent risk factors for CIN and FN. Meaningful risk factors with a p-value of < 0.05 at the univariate level were included in the multivariate logistic model. Kaplan-Meier survival analysis was used to generate survival curves, which were compared using the log-rank test. All clinical data analyses were performed using SPSS ver. 22.0 (IBM Corp., Armonk, NY). All *in vivo* data were analyzed using one-way analysis of variance in Microsoft Excel (Microsoft, Redmond, WA) and Prism (GraphPad, San Diego, CA). p-values < 0.05 were considered significant.

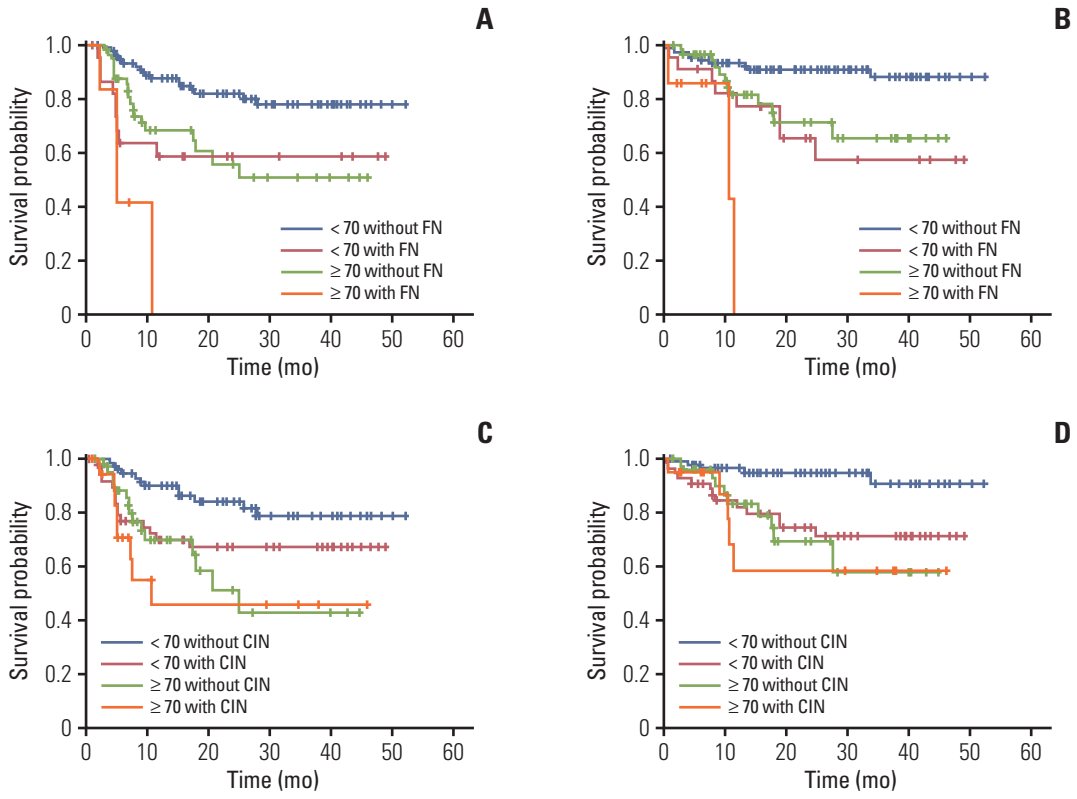

Fig. 3. Effect of CIN and FN on the progression-free survival (PFS, A and C) and overall survival (OS, B and D) according to patient's age. Kaplan-Meier curves showing the PFS and OS of patients with newly diagnosed diffuse large B-cell lymphoma according to the age and CIN (A, B) or FN events (C, D) in the first cycle of chemotherapy. CIN, chemotherapy-induced neutropenia; FN, febrile neutropenia; OS, overall survival; PFS, progression-free survival.

Results

1. Patient characteristics

The median age of the 200 DLBCL patients included in this study—at the time of diagnosis—was 62 years (range, 16 to 88 years) and 165 patients (82.5%) had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1. According to the International Prognostic Index (IPI), 87 (43.5%) patients were in high-intermediate or high-risk groups and approximately half of the patients (n=98, 49%) had Ann Arbor stage IV disease. Fifty-two patients (26.0%) experienced B symptoms and 126 (63.0%) manifested extranodal lesions. BM infiltration of lymphoma cells was confirmed in 34 patients (17.0%) and the median BM cellularity was 40%. Thirty-four patients (17.0%) presented abnormal chromosome test results. According to Han's criteria, 53 patients (26.5%) had germinal center B-cell-like (GCB) DLBCL and 127 (63.5%) had non-GCB DLBCL. Owing to old age or poor general condition, 75 patients (37.5%) received a reduced dose of chemotherapy (Table 1).

2. CIN and predictive factors

Among the 200 patients enrolled in this study, initial CIN and FN events were commonly observed during the earlier treatment cycles, particularly after the first chemotherapy cycle. Seventy-one patients (35.5%) experienced CIN during their first chemotherapy cycle, among which 30 (15.0%) experienced FN; however, the incidence of CIN and FN was lower during subsequent chemotherapy cycles (Fig. 1).

Univariate analysis revealed that female sex, stage III/IV, B symptoms, poor performance status, IPI, extranodal involvement, BM involvement, and low albumin levels correlated significantly with CIN. Moreover, multivariate analysis indicated that BM involvement (hazard ratio [HR], 3.16; 95% confidence interval [CI], 1.33 to 7.53; p=0.009) and low albumin levels (HR, 3.47; 95% CI, 1.27 to 9.48; p=0.015) was associated with CIN events.

Further univariate analysis indicated that female sex, stage III/IV, B symptoms, ECOG performance status, IPI, extranodal involvement, BM involvement, elevated lactate dehydrogenase, β2-microglobulin, and low serum albumin levels were strong predictive factors for FN, among which stage

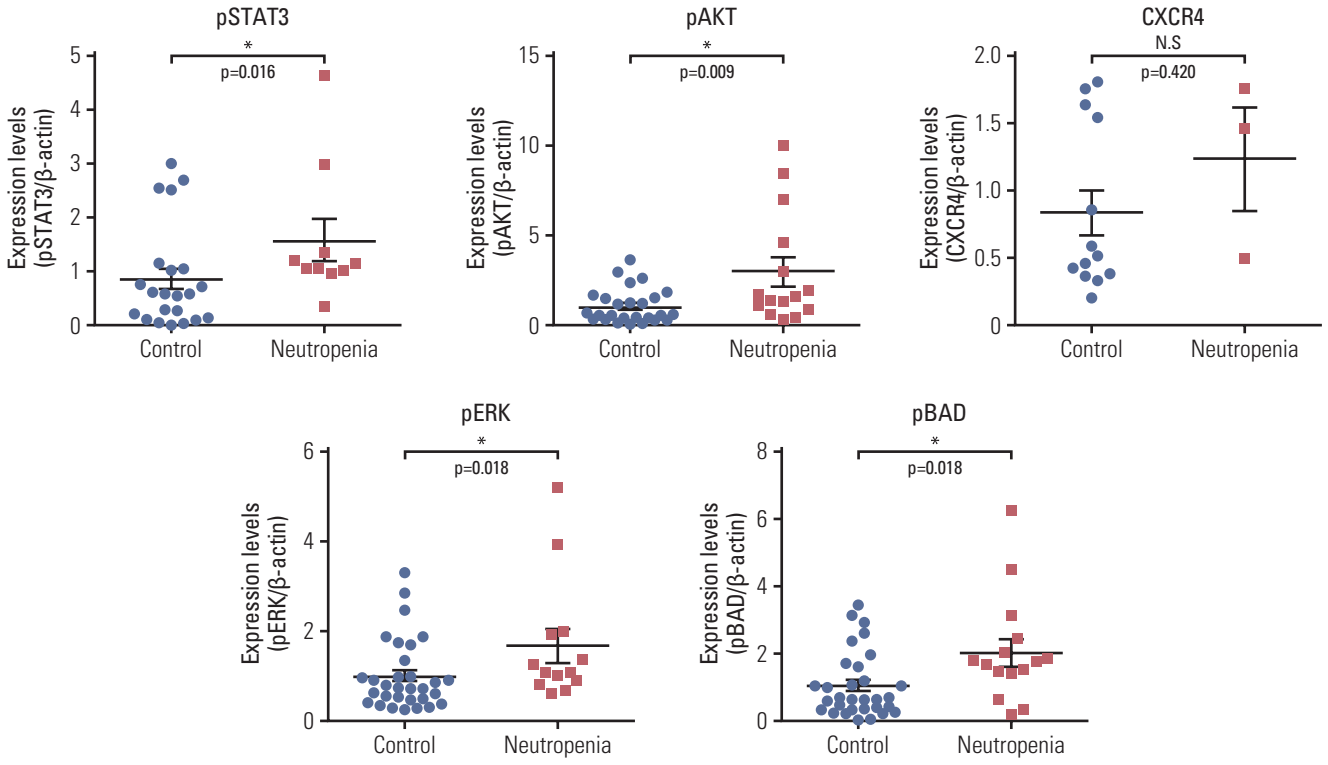

Fig. 4. Levels of phospho-STAT3, phospho-AKT, phospho-ERK, phospho-BAD, and CXCR4 in bone marrow samples from DLBCL patients with neutropenia were higher than in those without. Western blot analysis of phospho-STAT3, phospho-AKT, phospho-ERK1/2, phospho-BAD and CXCR4 levels in bone marrow samples of DLBCL patients with or without CIN were performed. Band densities of the western blots were quantified by Image software and densitometry results were shown as a dot graph. Each graph represents as mean±SD. AKT, protein kinase B; BAD, BCL2-associated agonist of cell death; CIN, chemotherapy-induced neutropenia; CXCR4, C-X-C chemokine receptor type 4; DLBCL, diffuse large B-cell lymphoma; ERK, extracellular signal-regulated kinase; SD, standard deviation; STAT, signal transducer and activator of transcription protein. *$p < 0.05$.

III/IV (HR, 12.74; 95% CI, 1.61 to 100.74; p=0.016) and low serum albumin levels (HR, 3.87; 95% CI, 1.40 to 10.72; p=0.009) were significantly associated with FN based on multivariate logistic regression (Table 2, S1 Table).

3. CIN, FN, and survival outcomes

Survival analysis revealed that patients with CIN had a lower progression-free survival (PFS; 2-year PFS, 62.5% vs. 74.7%; p=0.067) and overall survival (OS; 2-year OS, 71.2% vs. 86.4%; p=0.026) than those without CIN. Additionally, patients with FN also had a lower PFS (2-year PFS, 51.1% vs. 74.0%; p=0.002) and OS (2-year OS, 58.2% vs. 85.0%; p=0.001) than those without FN (Fig. 2). Analysis according to patient age showed that even if the patients' age was lower than 70 years, the PFS and OS of patients with FN were worse compared with those of patients without FN and similar to those aged 70 years or older without FN. Furthermore, all patients over 70 years of age with FN experienced relapse or died within 12 months of R-CHOP chemotherapy (Fig. 3).

4. DLBCL cells influence the BM environment in chemotherapy-naïve patients

We examined the protein levels of several markers related to proliferation (pSTAT3, pAKT, pERK1/2), apoptosis (pBAD), and neutrophil release (CXCR4) in chemotherapy-naïve BM cells from patients with DLBCL. Western blotting showed that the expression of pSTAT3, pAKT, pERK1/2, pBAD, and CXCR4 was increased in patients with CIN compared to that in patients without CIN (Fig. 4).

As high pSTAT3, pAKT, pERK1/2, pBAD, and CXCR4 expression in BM cells was found to be associated with the occurrence of CIN, we sought to confirm these observations *in vitro* by co-culturing OCI-Ly1 cells with C57BL/6 murine BM cells using a Transwell system. We found that the expression of pSTAT3, pAKT, and pBAD proteins was upregulated in BM cells co-cultured with OCI-Ly1 cells compared to that in cells cultured alone (Fig. 5). Together with our clinical data, these results indicate that DLBCL cells may influence the BM environment, i.e., neutrophil proliferation, apoptosis, and

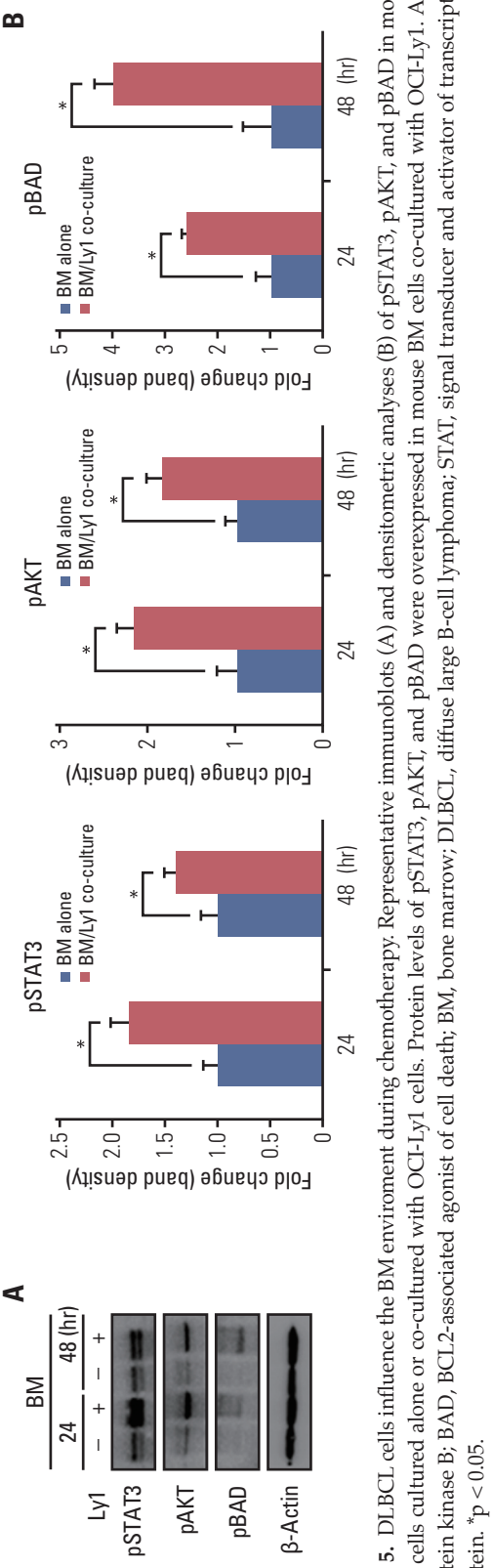

Fig. 5. DLBCL cells influence the BM enviroment during chemotherapy. Representative immunoblots (A) and densitometric analyses (B) of pSTAT3, pAKT, and pBAD in mouse BM cells cultured alone or co-cultured with OCI-Ly1 cells. Protein levels of pSTAT3, pAKT, and pBAD were overexpressed in mouse BM cells co-cultured with OCI-Ly1. AKT, protein kinase B; BAD, BCL2-associated agonist of cell death; BM, bone marrow; DLBCL, diffuse large B-cell lymphoma; STAT, signal transducer and activator of transcription protein. *$p < 0.05$.

differentiation even before chemotherapy and as a result, may affect the occurrence of CIN.

5. DLBCL cells promote neutrophil apoptosis

To investigate whether DLBCL cells affected neutrophil apoptosis with or without chemotherapy, we examined the rate of apoptosis in neutrophils co-cultured with OCI-Ly1 cells and/or treated with doxorubicin using fluorescence-activated cell sorting (FACS) analysis. We observed that the rate of apoptosis was higher in neutrophils co-cultured with OCI-Ly1 cells than that in neutrophils alone. The rate of apoptosis increased further after doxorubicin treatment (Fig. 6). Together, these results suggest that DLBCL cells regulate neutrophil apoptosis with or without chemotherapy, and this action can be further enhanced by chemotherapy.

Discussion

In this study, the analysis of clinical data was focused on the first cycle of chemotherapy and advanced stage DLBCL. We demonstrated that in most cases, CIN (41.8%) and FN (71.4%) developed during the first cycle of chemotherapy with prophylactic pegfilgrastim treatment, whereas the incidence of CIN (10%-15.1%) and FN (0.7%-2.5%) was much lower from the second cycle onwards (Fig. 1A). A Japanese study also reported similar results, wherein the incidence of FN was 9.1% (73.7% of the cases) in the first cycle [18]. Although prophylactic pegfilgrastim appeared to reduce the risk of FN during chemotherapy, more than one-third of patients with DLBCL continued to experience CIN and around 42.3% developed FN (Fig. 1B), thus increasing the frequency of treatment-related mortality. Therefore, it is important to verify risk factors for both CIN and FN, especially during the first treatment cycle.

The independent candidate risk factors associated with FN in this study included Ann Arbor stage III/IV and hypoalbuminemia; however, previous studies have reported age > 65 years, poor performance status, BM involvement, body mass index < 23 kg/m^2, renal and cardiovascular disease, relative dose intensity > 80%, and no G-CSF prophylaxis as risk factors for FN [4,19-22]. Our center recently treated elderly patients with DLBCL (> 65 years) with a reduced dose of R-CHOP; therefore, old age was not considered as a risk factor for FN in this study. Multiple studies have shown that advanced disease status is a significant predictor of FN in various cancers, including NHL and breast, ovarian, lung, colorectal, and prostate cancers [23,24]. Consistent with our findings, another Korean study also demonstrated that a high Ann Arbor stage was a risk factor for CIN and FN [25]. Therefore, we investigated why advanced DLBCL (e.g., stage

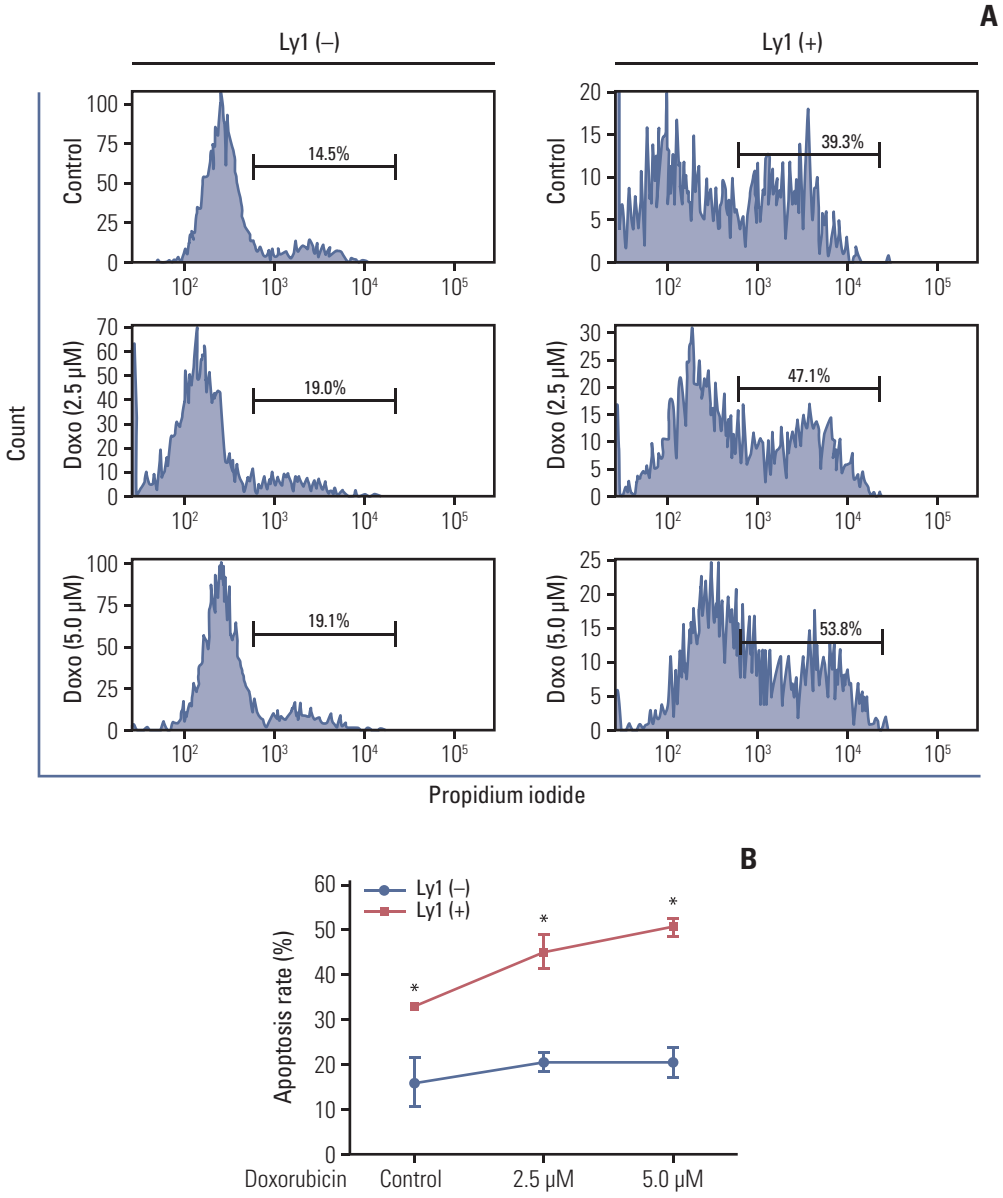

Fig. 6. DLBCL cells promote apoptosis of neutrophil during chemotherapy. Apoptotic rates of neutrophil were measured by propidium iodide followed by FACS analysis in bone marrow cells co-cultured with OCI-Ly1 for 24 hours and treated with doxorubicin (2.5 μM and 5.0 μM) for another 24 hours. (A) FACS data from a representative group is shown. (B) Quantitative data obtained from three independent FACS experiments in each group is plotted. DLBCL, diffuse large B-cell lymphoma; FACS, fluorescence-activated cell sorting. *$p < 0.05$.

III/IV or IPI ≥ 3) is a high-risk factor for CIN and FN, even with prophylactic pegfilgrastim use during R-CHOP chemotherapy.

Although G-CSF has no intrinsic tyrosine kinase activity, ligand binding induces a conformational change that leads to the activation of several downstream pathways, including JAK/STAT, PI3K/AKT, and mitogen-activated protein kinase (MAPK)/ERK [26-28]. Particularly, the activated G-CSF receptor mediates JAK phosphorylation, which then phosphorylates the tyrosine residues of the G-CSF receptor and STAT proteins, among which STAT3 plays a pro-differentiation role in myeloid lineage development. The growth-stimulatory effects of G-CSF–driven granulopoiesis depend on G-CSF/G-CSF receptor binding and the subsequent activation of STAT signaling pathways, including STAT3 activation. Indeed, in DLBCL, pSTAT3 expression is associated with an advanced stage as well as multiple extranodal sites of involvement [29], while high pAKT levels are also

associated with a more advanced stage, two or more sites of extranodal involvement, and a higher IPI risk score [30]. Our study confirmed these results by identifying that G-CSF receptor signaling was associated with a high incidence of CIN/FN, advanced stage, and extranodal involvement (S2 Table).

Consequently, we hypothesized that advanced DLBCL influences and pre-sensitizes G-CSF receptor intracellular signaling pathways, including JAK/STAT, PI3K/AKT, and MAPK/ERK by activating cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α, which reversibly inhibit the proliferation and differentiation of myeloid progenitor cells into neutrophils via pegfilgrastim. In this study, we determined the expression of cell proliferation and survival markers such as pSTAT3, pAKT, pERK, and pBAD in BM cells isolated from DLBCL patients before chemotherapy. Intriguingly, these markers were overexpressed in patients with CIN compared to those without CIN after chemotherapy, thus supporting our hypothesis.

We also investigated whether DLBCL cells directly affected the BM environment to activate the intracellular G-CSF receptor signaling pathway. Our *in vitro* data from OCI-Ly1 cells and murine BM cells showed that pSTAT3, pAKT, and pBAD were overexpressed in BM cells co-cultured with Ly1 cells compared to BM cells cultured alone. Therefore, DLBCL cells may directly affect the proliferation and survival of BM progenitor cells via G-CSF receptor downstream pathways.

Consequently, we examined whether apoptosis of BM progenitor cells from patients with advanced DLBCL was affected by lymphoma cells during chemotherapy. FACS analysis demonstrated that the rate of apoptosis was higher in the neutrophil population gated on $Ly6G^{+}CD11b^{+}$ in murine BM cells co-cultured with OCI-Ly1 compared to those cultured alone. Furthermore, the highest rate of neutrophil apoptosis was observed when BM cells co-cultured with OCI-Ly1 were treated with doxorubicin. These results suggest that DLBCL cells induce neutrophil apoptosis and thus may affect neutrophil survival before chemotherapy.

In conclusion, we identified that advanced stage DLBCL was associated with independent predictive factors for CIN and FN even with pegfilgrastim support. It may be possible that cytokines secreted from lymphoma cells affect the BM environment and G-CSF receptor signaling pathway, which finally results in CIN and FN, even with prophylactic use of pegfilgrastim. It can be concluded that more CIN and FN events occur in the first cycle of chemotherapy, when DLBCL imposes a huge burden.

Therefore, future studies should investigate the mechanisms underlying the interaction between DLBCL cells and BM environment in detail to prevent CIN in patients with DLBCL. Additionally, our findings suggest that pSTAT3, pAKT, pERK, and pBAD expression in BM cells isolated from patients with DLBCL before chemotherapy could serve as a valuable indicator for preventing neutropenia.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

This study was approved by the the Institutional Review Board of Pusan National University Hospital (1901-024-075). Since the data analyzed were obtained from medical records and did not include personal information, informed consent from patients was not required. All animal protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Pusan National University.

Author Contributions

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

This work was supported by a 2-Year Research Grant of Pusan National University. We are grateful to the doctors who collected and analyzed the clinical data.

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pISSN 1598-2998, eISSN 2005-9256
https://doi.org/10.4143/crt.2021.1168
Cancer Res Treat. 2022;54(4):1268-1277

Original Article

Pegfilgrastim Prophylaxis Is Effective in the Prevention of Febrile Neutropenia and Reduces Mortality in Patients Aged ≥ 75 Years with Diffuse Large B-Cell Lymphoma Treated with R-CHOP: A Prospective Cohort Study

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Purpose Febrile neutropenia (FN) can cause suboptimal treatment and treatment-related mortality (TRM) in diffuse large B-cell lymphoma (DLBCL) patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP).

Materials and Methods We conducted a prospective cohort study to evaluate the effectiveness of pegfilgrastim prophylaxis in DLBCL patients receiving R-CHOP, and we compared them with the PROCESS cohort (n=485).

Results Since January 2015, 986 patients with DLBCL were enrolled. Pegfilgrastim was administered at least once in 930 patients (94.3%), covering 90.3% of all cycles. FN developed in 137 patients (13.9%) in this cohort (23.7% in the PROCESS cohort, p < 0.001), and 4.2% of all cycles (10.2% in the PROCESS cohort, p < 0.001). Dose delay was less common (≥ 3 days: 18.1% vs. 23.7%, p=0.015; ≥ 5 days: 12.0% vs. 18.3%, p=0.023) in this cohort than in the PROCESS cohort. The incidence of TRM (3.2% vs. 5.6%, p=0.047) and infection-related death (1.8% vs. 4.5%, p=0.004) was lower in this cohort than in the PROCESS cohort. The 4-year overall survival (OS) and progression-free survival (PFS) rates of the two cohorts were not different (OS: 73.0% vs. 71.9%, p=0.545; PFS: 69.5% vs. 68.8%, p=0.616). However, in patients aged ≥ 75 years, the 4-year OS and PFS rates were higher in this cohort than in the PROCESS cohort (OS: 49.6% vs. 33.7%, p=0.032; PFS: 44.2% vs. 30.3% p=0.047).

Conclusion Pegfilgrastim prophylaxis is effective in the prevention of FN and infection-related death in DLBCL patients receiving R-CHOP, and it also improves OS in patients aged ≥ 75 years.

Key words Pegfilgrastim, Prophylaxis, Diffuse large B-cell lymphoma

Introduction

Rituximab, cyclophosphamide, doxorubicin, and prednisolone (R-CHOP) is the standard treatment for patients with diffuse large B-cell lymphoma (DLBCL) and cures 60%-70% of patients [1,2]. Since the advent of chemoimmunotherapy, there has been no major breakthrough in terms of the front-line treatment for DLBCL. Therefore, supportive measures to reduce preventable treatment-related mortality (TRM) are very important for optimal treatment outcomes. One of the most important side effects of R-CHOP is febrile neutropenia (FN) caused by bone marrow suppression. TRM as well as suboptimal treatment may occur because of treatment delay, unplanned dose reduction, and discontinuation of chemotherapy due to FN [3]. In addition, FN causes additional medical costs in terms of need for hospitalization and antibiotic therapy [4,5]. Therefore, proper prevention and treatment of FN are crucial for achieving the best treatment outcomes with R-CHOP.

The reported incidence of FN with R-CHOP therapy varies (18%-23.8%) in many studies [1,3,6,7]. It was higher in patients who had risk factors such as older age, poor performance status, advanced disease, comorbidities, low serum albumin level, low baseline blood cell counts, low body surface area/body mass index, and absence of granulocyte colony-stimulating factor (G-CSF) prophylaxis [8-11]. As per clinical guidelines, primary prophylaxis with G-CSF or pegfilgrastim is recommended for patients planning to undergo chemotherapy if the assessed risk of FN is higher than 20% [6,12]. However, in real-world practice, primary prophylaxis is underutilized notwithstanding clinical guidelines [13]. For primary prophylaxis, use of pegfilgrastim is desirable as it needs to be administered only once per cycle because of its neutrophil-mediated pharmacokinetics, whereas other

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Received October 30, 2021 **Accepted** December 29, 2021 **Published Online** December 30, 2021
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G-CSFs needs to be administered on a daily basis [14-16]. In addition, pegfilgrastim prophylaxis was associated with a reduced risk of neutropenia-related or all-cause hospitalization relative to filgrastim prophylaxis in a large-scale retrospective analysis [17]. Mean per-cycle neutropenia-related costs were also lower with pegfilgrastim than with filgrastim [16].

As pegfilgrastim prophylaxis became available for cancer patients in Korea in 2014, and the reported incidence of FN was higher than 20% among Korean DLBCL patients treated with R-CHOP [7], we prospectively collected data on primary prophylaxis with pegfilgrastim (GIRAFFE-B cohort) and compared them with the data obtained from a previous cohort enrolled in the same setting (PROCESS cohort) [18,19] to evaluate the benefits of pegfilgrastim prophylaxis in DLBCL patients treated with R-CHOP (ClinicalTrials.gov Identifier: NCT02474550).

Materials and Methods

1. Patients

The current cohort included patients with newly diagnosed DLBCL of any subtype according to the World Health Organization 2008 classification, who were planning to receive standard R-CHOP 21 as the primary treatment, planning to receive pegfilgrastim prophylaxis, aged 18 or older, and who had provided written informed consent. Patients with primary central nervous system (CNS) lymphoma or other concomitant malignancies that needed treatment or who had previous chemotherapy or radiotherapy were excluded. The study was conducted at 24 hospitals belonging to the Consortium for Improving Survival of Lymphoma (CISL) in Korea. The protocol was reviewed and approved by the institutional review board of each participating center, and all patients provided written informed consent before treatment initiation. The PROCESS cohort recruited patients from 27 hospitals belonging to the CISL between August 2010 and August 2012 to investigate CNS involvement in DLBCL. It also recruited newly diagnosed DLBCL patients with similar inclusion criteria as those used for the current cohort, except that it included patients aged ≥ 20 years and R-CHOP was administered without G-CSF prophylaxis [18].

2. Treatments

Patients were treated with the standard R-CHOP 21 regimen [1]. After enrollment, patients received up to 6-8 cycles of R-CHOP therapy. The number of cycles was reduced to 3-4 cycles in patients with stage I/II disease that was completely resected or who were about to undergo radiotherapy. CNS prophylaxis was performed at physician's discretion. Patients received pegfilgrastim 6 mg (Neulasta, Amgen Manufacturing Ltd., Juncos, Puerto Rico) injection subcutaneously at least 24 hours after the completion of chemotherapy. Dose modification of the standard R-CHOP therapy and deferral of treatment were performed according to the physician's decision. Concomitant use of prophylactic antibiotics was also allowed according to the institutional policies.

3. Study endpoints

The primary endpoint was the incidence of FN during and within 30 days of R-CHOP treatment.

FN was defined as a single oral temperature ≥ 38.3°C or ≥ 38.0°C for ≥ 1 hour with a neutrophil count of ≤ 0.5×10^9/L or a neutrophil count ≤ 1.0×10^9/L which was predicted to fall below 0.5×10^9/L. The secondary endpoints were as follows: (1) the delivery of planned treatments including dose delay of > 3 days and > 5 days, dose reduction of > 20%, relative dose intensity (RDI) of doxorubicin and cyclophosphamide, and average RDI (ARDI); (2) infection-related death and

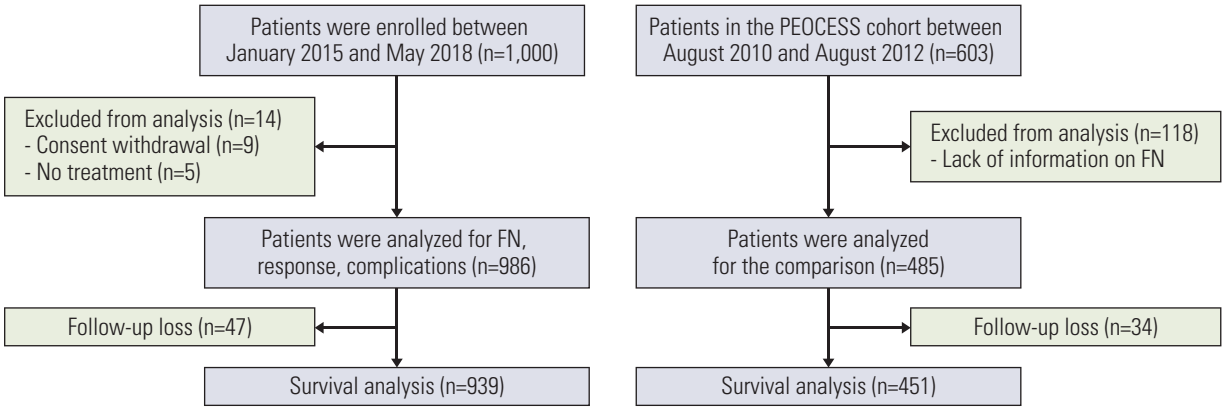

Fig. 1. Flow of the study.

TRM within 6 months from the start of treatment; and (3) response to R-CHOP treatment, overall survival (OS) and progression-free survival (PFS). RDI for each agent was defined as the proportion of the standard dose intensity delivered. ARDI was calculated by averaging the delivered RDIs of cyclophosphamide and doxorubicin. OS was calculated from the time of diagnosis to the date of the last follow-up or death from any cause. PFS was calculated from the date of diagnosis to the date of relapse or progression, the last follow-up, or death from any cause. Response to therapy was evaluated according to the Lugano response criteria for non-Hodgkin lymphoma (NHL) [20]. Follow-up data including survival and disease status were updated and centrally reviewed every 6 months.

4. Statistical analysis

Statistical analysis was performed using SPSS ver. 25.0 software program for Windows (IBM Corp., Armonk, NY). We used a Fisher's exact test to identify the associations between categorical variables. The time variable was estimated based on Kaplan-Meier curves and compared using a log-rank test. A Cox-regression hazard model was used for univariate and multivariate analyses. To define the risk factors for FN, a multivariate analysis was performed including variables with a $p < 0.1$ in a univariate analysis. A two-sided $p < 0.05$ was considered statistically significant. Propensity score matching (PSM) using parameters included in international prognostic index (IPI) and sex was performed to compare survival between the cohort of this study and the PROCESS cohort to minimize selection bias. In the present study, 1:1 nearest neighbor matching was performed using SPSS.

Results

1. Characteristics of patients

A total of 1,000 patients were enrolled in the GIRAFFE-B cohort from January 2015 to May 2018. Of these, nine patients withdrew consent, and five patients did not receive R-CHOP treatment. Thus, 986 patients who received at least one cycle of R-CHOP were included in the analysis. From the PROCESS cohort, 485 patients who had information available regarding the incidence of neutropenia and FN were included (Fig. 1). The overall characteristics of the analyzed patients did not deviate from those of all patients in the PROCESS cohort [18].

The characteristics of the patients in both cohorts are summarized in Table 1. The median age of the patients included in this study was 62 years (range, 19 to 86 years), which was 3 years higher than in the PROCESS cohort (range, 20 to 89 years). However, the proportion of patients aged ≥ 65 years and ≥ 75 years was not significantly different between the two cohorts.

Table 1. Characteristics of patients

Characteristic	This cohort (n=986)	PROCESS cohort (n=485)	p-value
Age (yr)			
Median (range)	62 (19-86)	59 (20-89)	
≥ 65	423 (42.9)	185 (38.1)	0.090
≥ 75	166 (16.8)	69 (14.2)	0.226
Sex			
Male	555 (56.3)	275 (56.7)	0.911
Female	431 (43.7)	210 (43.3)	
ECOG			
0, 1	901 (91.4)	429 (88.6)	0.108
≥ 2	85 (8.6)	55 (11.4)	
Stage			
1, 2	478 (48.5)	239 (49.3)	0.824
3, 4	508 (51.5)	246 (50.7)	
Extranodal sites			
0 or 1	620 (62.9)	314 (64.8)	0.488
≥ 2	366 (37.1)	171 (35.2)	
BM involvement			
No	877 (88.9)	429 (88.5)	0.792
Yes	109 (11.1)	56 (11.5)	
LDH elevation			
No	467 (47.4)	252 (52.0)	0.096
Yes	519 (52.6)	233 (48.0)	
IPI			
0 or 1	362 (36.7)	217 (44.7)	0.075
2	254 (25.8)	100 (20.6)	
3	203 (20.6)	91 (18.8)	
4 or 5	167 (16.9)	77 (15.9)	
Albumin (mg/dL)			
≥ 3.5	738 (74.8)	370 (76.3)	0.608
< 3.5	248 (25.2)	115 (23.7)	

Values are presented as number (%) unless otherwise indicated.
BM, bone marrow; ECOG, Eastern Cooperative Oncology Group; IPI, international prognostic index; LDH, lactate dehydrogenase.

2. FN and TRM with pegfilgrastim prophylaxis

Pegfilgrastim prophylaxis was administered at least once to 930 patients (94.3%) in this cohort. The overall prophylaxis rate was 90.3% (4,831 doses in 5,348 cycles). The overall incidence of FN was 4.2% (222 events in 5,348 cycles) in this cohort (Table 2). Throughout the treatment course, 137 patients (13.9%) experienced at least one episode of FN in this cohort, whereas 23.7% (115/485) of the patients experienced FN in the PROCESS cohort, with an overall incidence of 10.2% (264 events in 2,581 cycles). Forty-five patients (32.8%)

Table 2. Incidence of neutropenia, FN, and TRM according to pegfilgrastim prophylaxis

Characteristic	This cohort	PROCESS cohort	p-value
Neutropenia (grade 4)			
Patients	286 (29.0)	335 (69.1)	< 0.001
Cycles	626 (11.7)	193 (39.8)	< 0.001
FN			
Patients	137 (13.9)	115 (23.7)	< 0.001
Cycles	225 (4.2)	264 (10.2)	< 0.001
TRM	32 (3.2)	27 (5.6)	0.047
Infection-related	18 (1.8)	22 (4.5)	0.004
Patients age ≥ 75 yr	5 (3.0)	8 (11.6)	0.023
Non-infectious[a)]	14 (1.4)	5 (1.0)	0.535

Values are presented as number (%). FN, febrile neutropenia; TRM, treatment-related mortality within 6 months of the initiation of treatment. [a)]TRM of non-infectious causes includes intracranial hemorrhage, cardiac event, pneumothorax, acute exacerbation of chronic pulmonary obstructive disease, hepatic failure and bowel infarction.

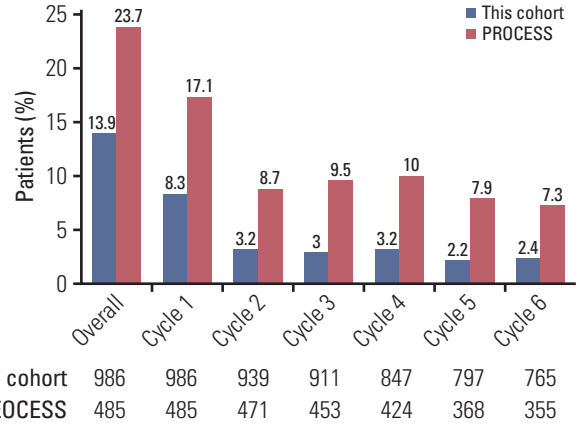

Fig. 2. Incidence of febrile neutropenia according to pegfilgrastim prophylaxis.

Table 3. Delivery of R-CHOP according to pegfilgrastim prophylaxis

Chemotherapy	This cohort	PROCESS cohort	p-value
Completion of 6 cycles	765 (77.6)	355 (73.2)	0.069
Dose delay			
> 3 days	179 (18.2)	115 (23.7)	0.015
> 5 days	119 (12.1)	80 (16.5)	0.023
Dose reduction > 20%			
Doxorubicin	192 (19.5)	75 (15.5)	0.062
Cyclophosphamide	131 (13.3)	73 (15.1)	0.378
ARDI ≥ 80%[a)]	722 (73.2)	344 (70.9)	0.353

Values are presented as number (%). ARDI, average relative dose intensity; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone. [a)]ARDI was calculated by averaging the delivered. Relative dose intensities of cyclophosphamide and doxorubicin.

experienced multiple episodes of FN in this cohort. In the PROCESS cohort, 49 patients (42.6%) had multiple episodes, which included up to six events. Grade 4 neutropenia was reported in 286 patients (28.8%) and occurred in about 11.7% of the cycles in this cohort, which was significantly lower than that in the PROCESS cohort (Table 2). FN developed at a median of 7 days after treatment (range, 5 to 30 days). The incidence of FN was highest in the first cycle (8.3%) and decreased in the subsequent cycles. The risk reduction rate of FN with pegfilgrastim prophylaxis was higher in subsequent cycles than in the first cycle (Fig. 2).

In this cohort, TRM was significantly lower than that in the PROCESS cohort (3.2% vs. 5.6%, p=0.047), which was mainly due to lower incidence of infection-related death (1.8% vs. 4.5%, p=0.004) (Table 2). In particular, eight patients (11.6%) died of infection among patients aged ≥ 75 years in the PROCESS cohort, but only five deaths (3%) were infection-related in this cohort (p=0.023). TRM of non-infectious causes was not different between the two groups (1.4% vs. 1.0%, p=0.535), which includes intracranial hemorrhage, cardiac event, pneumothorax, acute exacerbation of chronic obstructive pulmonary disease, hepatic failure and bowel infarction.

3. R-CHOP treatment with pegfilgrastim prophylaxis

Overall, 765 patients (77.6%) completed six cycles of R-CHOP in this study, which was not significantly different from that in the PROCESS cohort (73.2%, p=0.069) (Table 3). In terms of dose delay, 179 patients (18.1%) experienced dose delay of > 3 days, and 119 patients (12%) had a dose delay of > 5 days (Table 3). In the PROCESS cohort, more patients experienced treatment delay (> 3 days in 23.7% and > 5 days in 16.5%) than that in this cohort. As for dose reduction of > 20%, this cohort showed a slightly higher doxorubicin reduction tendency compared to the PROCESS cohort (19.5% vs. 15.5%, p=0.062). This is because 60 patients (6.1%) were treated with R-miniCHOP [21] and more patients initially started R-CHOP at a reduced dose of doxorubicin (18.9%) and cyclophosphamide (12.4%) in this cohort than did patients in the PROCESS cohort. However, in the subsequent cycles, only 0.6% and 0.9% of patients experienced a dose reduction of > 20% for doxorubicin and cyclophosphamide, respectively. In contrast, fewer patients started treatment with a reduced dose of doxorubicin and cyclophosphamide

Table 4. Univariate analysis of risk factors for FN according to pegfilgrastim prophylaxis

Characteristic	This cohort	p-value	PROCESS cohort	p-value
Age (yr)				
< 65	47 (8.3)	< 0.001	38 (12.7)	< 0.001
≥ 65	90 (21.3)		77 (41.6)	
Sex				
Male	66 (11.9)	0.041	59 (21.5)	0.197
Female	71 (16.5)		56 (26.7)	
BM involvement				
No	124 (13.8)	0.615	100 (23.3)	0.616
Yes	13 (11.9)		15 (26.8)	
Ann Arbor stage				
I-III	62 (11.4)	0.461	51 (20.1)	0.242
IV	75 (17.3)		64 (27.2)	
LDH elevation				
No	47 (10.1)	0.003	35 (18.6)	0.036
Yes	90 (17.2)		80 (26.9)	
ECOG PS				
0 or 1	110 (12.2)	< 0.001	90 (21.0)	< 0.001
≥ 2	27 (31.8)		25 (44.6)	
Extranodal sites				
0 or 1	82 (13.2)	0.447	74 (23.6)	0.914
≥ 2	55 (15.0)		41 (24.0)	
Albumin (mg/dL)				
< 3.5	64 (25.7)	< 0.001	40 (33.6)	0.004
≥ 3.5	73 (9.9)		75 (20.5)	
Baseline ANC (/μL)				
< 1,500	3 (12.5)	> 0.99	4 (28.5)	0.750
≥ 1,500	134 (13.9)		111 (23.6)	
Baseline Hb (mg/dL)				
< 12	58 (13.3)	0.644	55 (26.4)	0.236
≥ 12	79 (14.4)		60 (21.7)	

Values are presented as number (%). ANC, absolute neutrophil count; BM, bone marrow; ECOG PS, Eastern Cooperative Oncology Group performance status; FN, febrile neutropenia; Hb, hemoglobin; LDH, lactate dehydrogenase.

(6.8% and 6.2%, respectively) in the PROCESS cohort, but more patients experienced a reduction of doxorubicin and cyclophosphamide (8.7% and 8.9%, respectively) in the subsequent cycles. Accordingly, the proportion of patients with ARDI of ≥ 80% did not differ between the two cohorts (73.2% in this cohort vs. 70.9% in the PROCESS cohort, p=0.353) (Table 3).

4. Risk factors for FN in patients treated with or without pegfilgrastim prophylaxis

We analyzed the risk factors for FN according to the use of pegfilgrastim prophylaxis. In the PROCESS cohort, age ≥ 65 years, Eastern Cooperative Oncology Group (ECOG) performance status ≥ 2, lactate dehydrogenase (LDH) elevation, and albumin level < 3.5 mg/dL were significant risk factors for FN in the univariate analysis (p < 0.05) (Table 4). In the multivariate analysis, age (hazard ratio [HR], 4.308; 95% confidence interval [CI], 2.719 to 6.826) and performance status (HR, 2.500; 95% CI, 1.072 to 5.832) were significant (Table 5). In this cohort, age ≥ 65 years, female sex, ECOG performance status ≥ 2, LDH elevation, and albumin level < 3.5 mg/dL were significantly associated with FN in the univariate analysis. In the multivariate analysis, age ≥ 65 years (HR, 2.550; 95% CI, 2.719 to 6.826), female sex (HR, 1.505; 95% CI, 1.068 to 2.316), ECOG performance status ≥ 2 (HR, 2.376; 95% CI, 1.380 to 4.092), and albumin level < 3.5 mg/dL (HR, 2.987; 95% CI, 2.017 to 4.396) remained significant.

5. Treatment outcome and survival

The overall response rate with R-CHOP in this cohort was

Table 5. Multivariate analysis of risk factors for FN according to pegfilgrastim prophylaxis

Characteristic	This cohort			PROCESS cohort		
	HR	95% CI	p-value	HR	95% CI	p-value
Age ≥ 65 yr	2.550	1.757-3.903	< 0.001	4.308	2.719-6.826	< 0.001
Female	1.505	1.068-2.316	0.037	-	-	-
ECOG	2.376	1.380-4.092	0.002	2.500	1.072-5.832	0.034
LDH	1.408	0.938-2.115	0.099	1.289	0.768-2.162	0.336
Albumin < 3.5 mg/dL	2.978	2.017-4.396	< 0.001	1.808	0.943-3.485	0.074

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; FN, febrile neutropenia; HR, hazard ratio; LDH, lactate dehydrogenase.

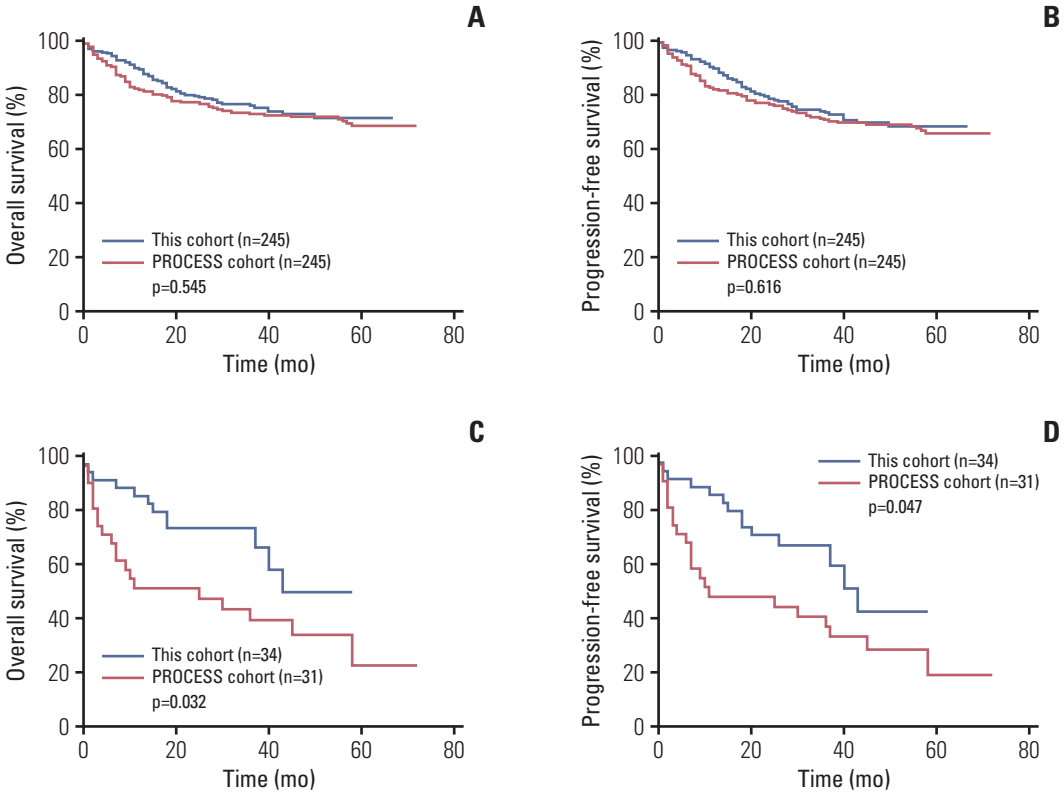

Fig. 3. Comparison of survival of the two cohorts after propensity score matching: overall survival (A), progression-free survival (B), overall survival of patients aged ≥ 75 years (C), and progression-free survival of patients aged ≥ 75 years (D).

90.9% (complete response, 80.6%; partial response, 10.3%), which was not different from that in the PROCESS cohort [18]. At the time of analysis, 707 patients (71.7%) who were being followed up were alive, with a median follow-up duration of 44 months. The four-year OS and PFS rates in this cohort were 73.9% and 69.4%, respectively. In the PROCESS cohort, 451 patients (93.0%) were available for full survival analysis, with median follow-up duration of 47 months for the survivors. We performed PSM to compare survival between the two cohorts. After PSM with the parameters of IPI and sex, the OS and PFS rated of 245 patients from each cohort were compared (S1 Table). The 4-year OS and PFS rates of the two cohorts after PSM were not significantly different (OS: 73.0% in this cohort vs. 71.9 in the PROCESS cohort, p=0.545; PFS: 69.5% in this cohort vs. 68.8% in the PROCESS cohort, p=0.616) (Fig. 3A and B). However, for patients aged ≥ 75 years, the 4-year OS and PFS rates of this cohort were higher than those of the PROCESS cohort (OS:

49.6% vs. 33.7%, p=0.032; PFS: 44.2% vs. 30.3% p=0.047) (Fig. 3C and D).

Discussion

The prophylactic effect of pegfilgrastim has not been fully evaluated throughout the cycles of standard R-CHOP in DLBCL patients as previous studies included heterogeneous disease groups (NHLs), diverse regimens (CHOP, R-CHOP, and others), use of pegfilgrastim only in the first cycle, or mixed use of G-CSF or pegfilgrastim [9,10,22-24]. In real-world practice, primary prophylaxis is not fully performed [13]. As pegfilgrastim prophylaxis has been available in Korea since 2014, we were able to compare the incidence of FN and treatment outcomes of DLBCL patients treated with R-CHOP between two cohorts collected during different time periods. Although this study was not a randomized trial, the two cohorts compared in this study were homogenous in terms of ethnicity, disease, treatment regimen, and other clinical characteristics. In addition, more than 90% of all cycles of R-CHOP were delivered with pegfilgrastim prophylaxis in this cohort, which was not given to the historical control cohort. Thus, we believe that the evidence provided by this study is valuable for evaluating the benefits of pegfilgrastim prophylaxis.

In this study, the proportion of patients who experienced FN throughout cycles of R-CHOP treatment was significantly lower than that in the PROCESS cohort (13.9% vs. 23.7%, $p < 0.001$) (Fig. 2). This was similar to the result of a previous study (16%) that retrospectively analyzed the incidence of FN with pegfilgrastim prophylaxis in aggressive NHLs [25]. As in previous studies, the incidence of FN was highest in the first cycle (8.3%) despite the use of pegfilgrastim prophylaxis [7,26]. Therefore, pegfilgrastim prophylaxis is essential for all patients in the first cycle of R-CHOP, and patients should be monitored around the 7th day of R-CHOP treatment when FN occurs most frequently. Although the overall incidence of FN significantly decreased in the subsequent cycles in both cohorts, continuous use of pegfilgrastim prophylaxis in this cohort reduced the incidence of FN by more than 50% compared to that in the PROCESS cohort. Subsequently, the incidence of TRM (3.2% vs. 5.6%, p=0.047) and infection-related deaths (1.8% vs. 4.5%, p=0.004) was significantly lower in this cohort than in the PROCESS cohort, which signifies a substantial benefit in the treatment of DLBCL patients.

In the risk factor analysis, age and performance status were consistently associated with the occurrence of FN in both cohorts, as in previous studies [8-10]. Even with pegfilgrastim prophylaxis, FN occurred in more than 20% of patients aged ≥ 65 years (21.3%), with poor performance (ECOG ≥ 2) (31.8%) and albumin level < 3.5 mg/dL (25.7%) in this cohort (Table 4). As pegfilgrastim prophylaxis is not sufficient for these patients, it is desirable to use antibiotic prophylaxis as well. In addition, those who experience FN should be considered at high risk for it in the subsequent cycles, because these patients tended to have multiple episodes in the subsequent cycles in both cohorts (32.8% in this cohort and 42.6% in the PROCESS cohort), even up to six events in six cycles.

In terms of treatment delivery, more patients received treatment on schedule in this cohort pegfilgrastim prophylaxis than in the PROCESS cohort (Table 3). However, as the median age of patients recruited in this cohort was 3 years higher than in the PROCESS cohort (62 years vs. 59 years), more patients started R-CHOP at a reduced dose in the former than in the latter. Therefore, the proportion of patients receiving ARDI of ≥ 80% did not differ between the two cohorts (Table 3). However, it is likely that ARDI could be increased by pegfilgrastim prophylaxis. This assumption is supported by a recent analysis from Japan, where pegfilgrastim prophylaxis improved dose delivery in elderly patients [24]. Given that reduced dose intensities have been associated with poor outcomes in most studies [27,28], maintaining dose intensity with pegfilgrastim prophylaxis may improve overall treatment outcomes.

As the population continues to age, increased number of elderly patients need to be treated for lymphoma [29]. Therefore, the treatment of elderly patients with DLBCL, for whom cytotoxic R-CHOP is still the standard therapy, is becoming very important. A recent meta-analysis showed that unlike in younger patients, dose intensity is not strongly correlated with better survival in elderly patients with DLBCL (≥ 80 years) due to higher TRM with higher dose intensity [27]. Therefore, finding a balance between treatment intensity and toxicity is the most important factor in treating elderly patients. From this point of view, prevention of FN is crucial. In this study, more elderly patients started treatment with a lower dose of chemotherapy than in the previous cohort since the introduction of R-miniCHOP in 2011 [21]. The incidence of TRM and infection-related death among patients aged ≥ 75 years in this cohort was significantly lower than that in the PROCESS cohort with the prophylactic effect of pegfilgratsim (Table 2), which improved the OS rate of patients age ≥ 75 years in this cohort compared to that in the PROCESS cohort (Fig. 3B).

Based on the findings of this study and previous studies, the benefits of pegfilgrastim primary prophylaxis can be summarized as follows: (1) it reduces the incidence of FN and need for hospitalization [17,23,25]; (2) it provides better dose delivery [24,25]; (3) it is convenient as only a single injection is required and is more cost-effective compared to secondary prophylaxis, daily use of G-CSF, or treatment

of FN [4,23,30]; and (4) it is associated with fewer infection-related deaths and better survival outcomes, especially in elderly DLBCL patients. Although OS benefit was not observed in the entire group, the current study supports more active use of pegfilgrastim prophylaxis in DLBCL patients treated with R-CHOP.

In conclusion, primary prophylaxis with pegfilgrastim significantly reduced the incidence of FN and infection-related death in DLBCL patients treated with R-CHOP, and it improved OS in patients aged ≥ 75 years.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The protocol was reviewed and approved by the institutional review board of each participating center, and all patients provided written informed consent before treatment initiation; Gachon University Gil Medical Center: GCIRB2015-74, Gyeongsang National University Hospital: GNUH 2015-02-012, Dongsan Medical Center: DSMC 2015-03-015, Korea University Anam Hospital: ED15024(AN15024-001), Kosin University Gospel Hospital: KUGH 2017-09-008, National Cancer Center: NCC 2015-0091, Dong-A University Medical Center: DMC 15-037, Pusan National University Hospital: H-1701-011-051, Samsung Medical Center: SMC 2014-07-181, Seoul National University Hospital: H-1504-119-667, Asan Medical Center: S2015-0302-0001, Soonchunhyang University Hospital: SCHUH 2015-05-007, Soonchunhyang University Bucheon Hospital: SCHBC 2015-07-008, Wonju Severance Christian Hospital: CR315002-002, Ajou University Hospital: AJIRB-MED-OBS-14-254, Yeungnam University Medical Center: YUMC 2015-03-016, Korea Cancer Center Hospital: K-1409-001-001, Ulsan University Hospital: UUH 2015-11-007, Inje University Sanggye Paik Hospital: SGPAIK 2015-06-002, Inje University Ilsan Paik Hospital: ISPAIK 2015-07-002, Inje University Busan Paik Hospital: BS 2015-0110, Jeonbuk National University Hospital: CUH 2015-02-034, Chonnam National University Hwasun Hospital: TMP-2015-023, Chung-Ang University Hospital: C2015048(1506), Chungnam National University Hospital: CNUH 2015-10-060, Chungbuk National University Hospital: CBNUH 2015-03-006, Hallym University Sacred Heart Hospital: HUH 2015-I043.

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Conflicts of Interest

This study was supported by the Kyowa Kirin Korea Co., Ltd.

Acknowledgments

This study was supported by the Consortium for Improving Survival of Lymphoma (CISL).

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We, the editors of Cancer Research Treatment (CRT), have strived toward the goal to make CRT a high quality journal. The CRT's impact factor of this year has risen to 5.036, which could not have been achieved without the peer reviewers' unselfish contribution of their valueless time and effort. Their thorough insights and constructive critiques have helped to maintain high standard of research articles published in CRT.

We owe a lot to the following peer reviewers during 2021 and 2022 and would acknowledge their enormous contribution with our most heartfelt appreciation.

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49th Annual Meeting of Korean Cancer Association & 9th International Cancer Conference

제49차 대한암학회 학술대회 및 제9차 국제암컨퍼런스

June 15(Thu) – 16(Fri), 2023

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대한암학회
Korean Cancer Association

Anyfusion® ACPi system

주사기 직진운동을 회전운동화한 실린더 카트리지 핵심원천 기술
실린더식 주사(항암)제 조제 시스템


PCA Balloon 조제


TPN 조제


파우더 조제

정확성

- 실린더식 의약품 주입 펌프와 주사기 직진운동을 회전운동화한 실린더 펌프용 카트리지 기술(국제특허)
- 음압 양압상쇄 약물 추출 도입용 Needle형 CSTD (국제특허)
- 사람이나 로봇이 주사기와 주사바늘을 Vial용기에 찌르고 빼는 기능을 기계화한 CAM(Closed system Assembly Machine)

편리성

- 전 과정 Full Closed System으로 독성이 강한 약물에 노출되는 의료인의 스트레스와 위험 해소
- 실린더식 의약품 주입 펌프와 실린더 펌프용 카트리지 기술(국제특허)로 간단한 버튼조작으로 조제가 가능
- Needle형 CSTD기술로 음압과 양압으로 인해 주사기와 Needle을 사용하여 반복적인 약물 추출과 도입을 하였던 의료인의 손과 팔의 피로도 획기적 개선
- 더 안전하고 효과적인 무균실에서 약물의 Admixing 환경 제공

경제성

- 음압 양압상쇄 약물 추출 도입용 Needle형 CSTD (국제특허)로 미세분말포집 전량추출로 환경오염과 폐기약물 최소화로 경제적 비용 해소
- Anyfusion ACPI Kit Set 보험 등재
- 기존 무균실과 BSC 시설 사용으로 새로운 공간 구축 비용 절약
- 사용자 친화적인 Customizing, 낮은 유지관리비, 신속한 A/S, 로봇대비 합리적인 가격

안전성

- 조제에서 주입까지 Full Closed System으로 약물의 오염예방 면역력이 약한 환자를 보호
- 독성이 강한 약물의 노출과 누출, 유증기 흡입으로부터 의료인 보호, 의료인의 주사침 자상등의 의료사고 차단

활용성

- 로봇으로 불가능한 TPN 조제, 5FU 조제
- PCA BALLOON 충전
- Powder 제재 조제 가능

디자인

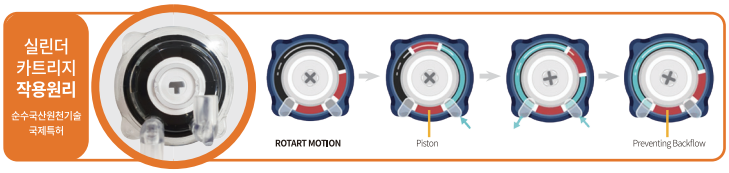
- 기존 BSC 시설 사용 가능
- 가로 x 세로 x 높이(20mm x 37.5mm x 55mm)
- BSC(biological safety cabinet) 시설 구축비 절약

ACPi Kit Set

Anti-Cancer drug Preparation and Infusion System

실린더 카트리지 주요 특장점

- 주사기의 직진운동을 회전운동화한 도넛형 실린더 구조 내 듀얼 피스톤 방식
- 두개의 피스톤을 교차 회전하여 약물을 흡입 및 배출하는 원리
- 정밀제어로 ±1%대 오차 정확성
- 신속조제, 넓은조제범위(95% 이상)
- 조제에서 주입까지 소모품 Anyfusion ACPi Kit Set이 Full closed system
- None BSC 가능 (검증필요)



ACPi Couple System

- ACPi V-100 두대를 이용하여 항암제와 수액을 3way를 통하여 동시에 조제 System
- 5FU 조제

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EGFR T790M 변이 NSCLC의 효과적인 표적 치료, 강력한 억제력, 양호한 내약성[1-3]

EGFR, Epidermal Growth Factor Receptor; **NSCLC**, Non-Small-Cell Lung Cancer

References 1. 렉라자® 제품설명서. 2021.01.18. **2.** Myung-Ju Ahn, et al. Lazertinib in patients with EGFR mutation-positive advanced non-small-cell lung cancer: results from the dose escalation and dose expansion parts of a first-in-human, open-label, multicentre, phase 1 - 2 study. Lancet Oncol. 2019 Dec;20(12):1681-1690. **3.** Myung-Ju Ahn, et al. Lazertinib in patients with EGFR mutation-positive advanced non-small-cell lung cancer: results from the dose escalation and dose expansion parts of a first-in-human, open-label, multicentre, phase 1 - 2 study. Lancet Oncol. 2019 Dec;20(12):1681-1690.(Supplementary appendix).

[효능·효과] 이전에 EGFR-TKI로 치료받은 적이 있는 EGFR T790M 변이 양성 국소 진행성 또는 전이성 비소세포폐암 환자의 치료.

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✓ 글로벌 임상 데이터[1, 2] 통해 유효성과 안전성 입증한
중증 호중구 감소증 치료제

✓ 다수의 ISO 인증을 받은 바이오 플랜트에서 생산한
우수한 품질의 바이오 의약품

Hanmi 한미약품

References

1. Schwartzberg LS, Bhat G, Peguero J, et al. Eflapegrastim, a Long-Acting Granulocyte-Colony Stimulating Factor for the Management of Chemotherapy-Induced Neutropenia:Results of a Phase III Trial. Oncologist. 2020 Aug;25(8):e1233-e1241. **2.** Cobb PW, Moon YW, Mezei K, et al. A comparison of eflapegrastim to pegfilgrastim in the management of chemotherapy-induced neutropenia in patients with early-stage breast cancer undergoing cytotoxic chemotherapy(RECOVER): A Phase 3 study. Cancer Med. 2020 Sep;9(17):6234-6243.

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무료 다운로드

광범위한 암 연구 애플리케이션에 활용 가능한 간소화된 포괄적인 워크플로우

암은 단순히 한 가지 요인으로 인해 발생한다고 할 수 없는 복잡한 질병입니다. 암 세포와 암 미세환경(microenvironment) 간의 동적인 상호작용은 유전체(genome), 후성유전체(epigenome), 전사체(transcriptome), 단백체(proteome) 등 모든 수준의 세포조절(cellular regulation)에 영향을 줍니다. 차세대 시퀀싱(next-generation sequencing, NGS)과 마이크로어레이(microarray)는 이러한 암의 다차원적 복잡성에 관한 포괄적인 정보를 제공하는 우수한 분석 도구로, 연구자들의 암에 대한 이해의 폭을 크게 넓혀주었습니다.
암 연구의 폭넓은 어플리케이션에 대해서, 그 이점이나 워크플로우 등을 정리한 가이드를 확인해 주세요.

목차

- NGS 기반 암 연구 워크플로우의 개요
- 모든 옴을 동시에 분석하는 단일세포 시퀀싱
- 유전체 분석 방법
- 후성유전체 분석 방법
- 전사체 분석 방법

아래의 QR코드를 스캔하셔서 다운로드 받으세요.