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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007



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Role of sex steroid receptors in pathobiology of hepatocellular carcinoma

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Abstract

The striking gender disparity observed in the incidence of hepatocellular carcinoma (HCC) suggests an important role of sex hormones in HCC pathogenesis. Though the studies began as early as in 1980s, the precise role of sex hormones and the significance of their receptors in HCC still remain poorly understood and perhaps contribute to current controversies about the potential use of hormonal therapy in HCC. A comprehensive review of the existing literature revealed several shortcomings associated with the studies on estrogen receptor (ER) and androgen receptor (AR) in normal liver and HCC. These shortcomings include the use of less sensitive receptor ligand binding assays and immunohistochemistry studies for ER α alone until 1996 when ER β isoform was identified. The animal models of HCC utilized for studies were primarily based on chemical-induced hepatocarcinogenesis with less similarity to virus-induced HCC pathogenesis. However, recent *in vitro* studies in hepatoma cells provide newer insights for hormonal regulation of key cellular processes including interaction of ER and AR with viral proteins. In light of the above facts, there is an urgent need for a detailed investigation of sex hormones and their receptors in normal liver and HCC. In this review, we systematically present the information currently available on androgens, estrogens and their receptors in normal liver and HCC obtained from *in vitro*, *in vivo* experimental models and clinical studies. This information will direct future basic and clinical research to bridge the gap in knowledge to explore the therapeutic potential of hormonal therapy in HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies with limited treatment options. The major risk factors for HCC are chronic liver diseases with cirrhosis that include hepatitis B, hepatitis C, alcoholic liver disease and non-alcoholic steatohepatitis. Epidemiological reports indicate that regardless of etiologies, the incidence of HCC is higher in males than in females with the male: female ratio usually averaging between 2:1 and 4:1^[1]. This male predominance is further supported by the clinical observations that chronic liver disease progresses more rapidly to cirrhosis in males than females and therefore cirrhosis that leads to HCC development is largely considered to be the disease of men and postmenopausal women^[2]. In view of this remarkable gender disparity, various *in vitro* as well as *in vivo* studies have been initiated from time to time to explore the importance of sex hormones in HCC. However, the precise role of male and female sex hormones and their receptors in HCC remains still poorly understood. Androgens have been suggested to induce and promote HCC^[3] and altered androgen metabolism has been reported to be associated with HCC^[4]. In contrast, the role of estrogen in HCC has been controversial with evidence suggesting both carcinogenic and protective effects in the liver^[3,5-9]. Very limited information is currently available regarding the mechanism of estrogen and androgen action in normal liver as well as in HCC.

It is well known that estrogen and androgen mediate their biological functions by binding with a high affinity to specific receptors, the estrogen receptor (ER) and the androgen receptor (AR). Both ER and AR belong to the family of nuclear receptors that act as transcription factors and regulate the expression of several genes. Our present day knowledge of structure and function of these receptors is primarily attributed to the extensive research on ER and AR in cancer of reproductive organs. However, recent advances in molecular research reveal that sex hormones do play a significant role in normal physiology of various organs other than the organs of the reproductive system. Both androgens and estrogens regulate transcriptional activation of various molecules involved in key cellular processes such as generation of immune responses, cell proliferation and apoptosis through functional receptors localized in various sub-cellular organelles^[10-12].

The expression and functional status of AR and ER appear to play a significant role in the carcinogenesis of all hormone sensitive organs. However, liver has remained a less studied organ in the context of sex hormones and their receptors. Differential expression of wild type and variant forms of ER and AR has been reported in normal liver and HCC, indicating a strong link between sex hormones and pathogenesis of HCC^[13-19]. Recent *in vitro* studies also provide further evidence in support of AR and ER involvement in various cellular events as well as interaction with viral proteins in hepatitis B virus (HBV) and hepatitis C virus (HCV)-induced HCC^[20-25]. This review is focused on the compilation of the information so far available on the significance of AR and ERs in HCC and brings forth wide gaps in the existing knowledge to the notice of scientific world for future research.

SEX HORMONE RECEPTORS IN NORMAL HUMAN LIVER AND HCC

Estrogen receptor

The role of estrogen in modulating morphological and physiological features of liver became evident in early 1970s when a possible correlation between occurrence of hepatic neoplasms and use of oral contraceptives was suggested^[26,27]. In the reproductive system, estrogen is known to act by binding to specific cytoplasmic and nuclear receptors. Hence, search began to identify such a receptor in the liver. In 1978, Duffy and Duffy first reported the presence of ER in normal human liver^[28]. Subsequently, the presence of ERs in human HCC was demonstrated by Molteni *et al*^[29] followed by Friedman *et al*^[30] and Iqbal *et al*^[31]. Since then, a number of studies have been reported addressing the expression of ERs in normal as well as neoplastic liver tissues. The early studies used indirect methods of receptor detection based on ligand binding assays. Table 1 gives the details of these studies^[13,15,17,30-37]. These assays were quantitative and measured the amount of receptor in the samples as well as its affinity for the ligand in terms of dissociation

constants. The percentage positivity for ER expression varied significantly among different studies. These variations may be attributed to the differences in sample size, methodologies, ethnicity of the population studied, stage of the disease and underlying etiologies. Earlier studies by Friedman *et al*^[30] and Iqbal *et al*^[31] showed that ER content is similar in HCC and normal liver. In contrast, later studies consistently showed that the expression of ER is decreased in HCC tissue specimens as compared to normal liver tissue specimens or the non-tumor part of the liver^[17,32,38]. However, Eagon *et al*^[33] documented elevated levels of cytosolic ER in 3 of the 9 tumors as compared to non-cancerous tissues. Nuclear ER expression was found to be suppressed in all HCC samples as compared to normal samples^[33]. The major drawback of these studies was the use of binding assays for detection that do not provide any information on the subtype of ER, i.e. ER α and ER β as known today. It is important to study the relative expression of both isoforms of ER since ER α and ER β are known to have overlapping but quite distinct functions. There are few reports on direct detection of ER using specific antibodies. Table 1 gives the details of these studies^[32,39-47]. However, all these studies have employed either immunohistochemistry (IHC) or enzyme-immuno assays (EIA) using antibodies specific for only ER α isoform. The ER β isoform was identified later in 1996^[48] and information on the expression of ER β protein in HCC is lacking though few studies at mRNA levels have been documented.

Since the molecular characterization and cloning of ER α in the mid 1980s^[49,50], attempts have been made to determine the expression of ER in liver tumors at mRNA level. Table 2 gives the details of these studies^[18,19,40,45,51,52]. *In situ* hybridization using ER specific oligonucleotide sequence probe revealed that 11 out of 15 HCC tissue samples expressed ER mRNA^[40]. Interestingly, the same samples were found to be negative for ER protein by IHC, suggesting the use of more sensitive methods and more specific antibodies for detecting ER at protein level. Subsequently, the mRNA expression of ER in HCC tissues was studied in different populations by a highly sensitive method of reverse transcriptase-polymerase chain reaction (RT-PCR). Villa *et al*^[51] were the first to demonstrate the presence of wild type ER α in peritumoral and tumoral tissue of HCC patients using this technique. The use of RT-PCR further enabled the authors to detect a splice variant of ER α lacking exon 5 in the hormone binding domain^[18,51]. A similar splice variant has been described in breast cancer tissues to be associated with tumor pathogenesis^[53]. The significance of the variant ER α (vER α) in pathology, prognosis and treatment of HCC has also been studied. The presence of vER receptor is able to influence the natural history of patients with HCC by regulating tumor growth as well as patient survival. The presence of the liver vER α transcript in the tumor has been described to be the strongest negative predictor of survival in operable HCC patients^[54,55]. Furthermore, the presence of vER α was found to correlate with a higher

Table 1 Estrogen receptor expression in liver tissue samples from HCC patients

Subcellular localization	Method	ER subtype/Antibody source	n	Subjects	Positive cases	Country and area	Yr	Reference
Cytosolic	BA	NA	5	5 M	5 M	United States	1982	[30]
Cytosolic and nuclear	BA	NA	5	3 M, 2 F	3 M, 2 F	United Kingdom	1983	[31]
Cytosolic	IHC	NA, Anti ER Ab Hypolabs, Switzerland	10	NA	1	Singapore	1984	[39]
Cytosolic	BA	NA	30	29 M, 1 F	12	Japan	1986	[13]
Cytosolic and nuclear	BA	NA	8	6 M, 2 F	1 M	Japan	1986	[17]
Cytosolic	BA	NA	13	9 M, 4 F	1 F	Japan	1987	[32]
Cytosolic	EIA	Abbot ER-EIA monoclonal kit	13	9 M, 4 F	3 M, 2 F	Japan	1987	[32]
Cytosolic	BA	NA	19	19 F	7 F	Japan	1989	[15]
Cytosolic	BA	NA	66	52 M, 14 F	23 M, 3 F	Japan	1990	[34]
Cytosolic	BA	NA	6	4 M, 2 F	1 F	Japan	1990	[35]
Cytosolic	BA	NA	21	18 M, 3 F	9 M, 1 F	Japan	1991	[36]
Cytosolic and nuclear	BA	NA	9	6 M, 3 F	6 M, 2 F	Italy, United States	1991	[37]
Cytosolic and nuclear	BA	NA	9	6 M, 3 F	6 M, 2 F	Italy, United States	1991	[33]
NA	IHC	NA, Abott, ER-ICA	15	12 M, 3 F	0	Italy	1993	[40]
Cytosolic	EIA	NA, Abbot anti ER	26	18 M, 8 F	4	Spain	1993	[41]
Cytosolic	EIA	NA, Abbot anti ER	33	20 M, 13 F	8 M, 5 F	Germany	1997	[42]
Cytosolic	IHC	NA, ER monoclonal Ab, Dako	71	59 M, 12 F	15 M, 2 F	Hong Kong	1997	[43]
Cytosolic and nuclear	IHC	ER α , Santacruz	45	37 M, 8 F	21 (cytosolic) 11 (nuclear)	United States, Korea ¹	2004	[44]
NA	IHC	NA	28	NA	11	China	2004	[45]
NA	IHC	NA	66		3	Mexico	2007	[46]
Nuclear	IHC	ER α , Dako (ID5)	31	26 M, 5 F	12 M, 4 F	Spain	2007	[47]

ER: Estrogen receptor; BA: Binding assay; EIA: Enzyme immunoassay; IHC: Immunohistochemistry; n: Sample size; M: Male; F: Female; NA: Information not available; Ab: Antibody. ¹Study conducted in USA on patient population from Korea.

Table 2 Messenger RNA (mRNA) expression of estrogen receptor in liver tissue samples from HCC patients

Method	ER subtype	n	Subjects	Positive cases	Country	Yr	Reference
ISH	ER α Wt	15	12 M, 3 F	11	Italy	1993	[40]
RT-PCR	ER α Wt	14	7 M, 7 F	1 M, 7 F	Italy	1995	[51]
RT-PCR	ER α delta5 variant	14	7 M, 7 F	7 M, 3 F	Italy	1995	[51]
RT-PCR	ER α Wt	40	25 M, 15 F	16 M, 12 F	Italy	1998	[18]
RT-PCR	ER α delta5 variant	40	25 M, 15 F	20 M, 10 F	Italy	1998	[18]
RT-PCR	ER α Wt	42	35 M, 7 F	20 M, 5 F	Italy	2003	[19]
RT-PCR	ER α delta5 variant	42	35 M, 7 F	37	Italy	2003	[19]
RT-PCR	ER β Wt	42	35 M, 7 F	12 M, 4 F	Italy	2003	[19]
RT-PCR	ER α Wt	28	NA	25	China	2004	[45]
RT-PCR	ER α delta5 variant	28	NA	27	China	2004	[45]
RT-PCR	ER α Wt	32	23 M, 9 F	23 M, 9 F	Korea	2006	[52]
RT-PCR	ER α delta5 variant	32	23 M, 9 F	21 M, 9 F	Korea	2006	[52]
RT-PCR	ER β Wt	32	23 M, 9 F	26	Korea	2006	[52]

ER: Estrogen receptor; ISH: *In situ* hybridization; RT-PCR: Reverse transcriptase-polymerase chain reaction; Wt: Wild type; n: Sample size; M: Male; F: Female; NA: Information not available.

clinical aggressiveness of the tumor in comparison with the tumors characterized by wild-type ER α transcript. These tumors were responsive to megestrol and unresponsive to anti estrogen tamoxifen. High rates of vER α expression have been shown to be present in men at high risk of HCC development^[56,57]. In patients with chronic hepatitis and cirrhosis, the expression of vER α has been associated with higher oxidative stress-induced DNA damage and c-myc mRNA expression, a factor indicating increased genomic instability, augmented cytoproliferation and carcinogenesis^[5].

Using RT-PCR, in addition to ER α , the expression of the lately identified isoform of ER, i.e. ER β , has also been studied in HCC patients. Iavarone *et al*^[19] report that both ER β and ER α wild type receptors either alone or together with vER are co-expressed more frequently

in patients with chronic liver disease than in those with HCC. However, both ERs are similarly expressed in tumoral and extratumoral tissues of HCC patients^[19]. In this study, HBV-related tumors either expressed wild type ER α and ER β or expressed variant ER and ER delta 5 more often than HCV-related tumor, and HBV-related tumors showed a tendency towards loss of ER β expression as the disease progressed from chronic inflammatory liver disease to HCC^[19].

Breast cancer studies suggest that ER α :ER β expression ratio changes during carcinogenesis and is believed to play a role in tumor development^[58]. Recently, Wang *et al*^[52] studied the expression of ER α and ER β in HCC tissues of Korean population using RT-PCR, and assessed 32 tumoral and peritumoral tissues from HCC patients with underlying chronic HBV or HCV

Table 3 Estrogen receptor and androgen receptor expression in normal and non-cancerous liver tissue samples

Receptor protein/mRNA	Type of liver tissue	Subjects	Positive cases	Method	Country	Yr	Reference
Estrogen Receptor (ER)							
ER protein (cytosolic)	Normal liver tissue	4 F	4 F	BA	United Kingdom	1978	[28]
ER protein	Normal	3 F	3 F	BA	Germany	1978	[59]
ER protein	Normal	2 M	2 M	BA	United States	1982	[30]
ER protein	Normal	1 M, 5 F	1 M, 5 F	BA	Germany	1982	[60]
ER protein	Normal	3 M, 3 F	3 M, 3 F	BA	United States	1983	[61]
ER protein (cytosolic & nuclear)	Normal	2 M, 2 F	2 M, 2 F	BA	United Kingdom	1983	[31]
ER protein (cytosolic)	Surrounding liver tissue	30	13	BA	Japan	1986	[13]
ER protein (cytosolic & nuclear)	Non-cancerous tissue	7	3	BA	Japan	1986	[17]
ER protein (cytosolic & nuclear)	Normal	NA	NA	BA	United States	1987	[62]
ER protein (cytosolic)	Non-cirrhotic liver	5 M, 7 F	5 M, 7 F	BA, EIA	Japan	1987	[32]
ER protein	Normal	2	2		Japan	1988	[63]
ER protein (cytosolic)	Surrounding liver tissue	17	11	BA	Japan	1989	[15]
ER protein	Surrounding non-cancerous tissue	22	14	NA	Japan	1989	[64]
ER protein (cytosolic)	Surrounding normal liver	4 M, 1 F	4 M, 1 F	BA	Japan	1990	[35]
ER protein	Adjacent normal tissue	6 M, 3 F	6 M, 3 F	BA	Italy, United States	1991	[33]
ER protein (cytosolic)	Non-tumoral liver	18 M, 8 F	9 M, 2 F	BA	Spain	1993	[41]
ER mRNA	Non-tumorous liver tissue	13	7	ISH	Italy	1993	[40]
ER mRNA	Peri-tumor tissue	32	28	RT-PCR	Korea	2006	[52]
Androgen Receptor (AR)							
AR protein (cytosolic & nuclear)	Normal	2 M, 2 F	0	BA	United Kingdom	1983	[31]
AR protein (cytosolic & nuclear)	Non-cancerous tissue	6	1	BA	Japan	1986	[17]
AR protein (cytosolic)	Non-neoplastic liver tissues	17	11	BA	Japan	1989	[15]
AR protein	Surrounding non-cancerous tissues	21	7	NA	Japan	1989	[64]
AR protein (cytosolic)	Surrounding liver	9 M, 1 F	7 M, 1 F	BA	Japan	1990	[35]
AR protein	Adjacent normal tissue	6 M, 3 F	6 M, 3 F	NA	Italy, United States	1991	[33]
AR mRNA	Peri-tumor tissue	23 M, 9 F	23 M, 9 F	RT-PCR	Korea	2006	[52]

BA: Binding assay; EIA: Enzyme immunoassay; IHC: Immunohistochemistry; M: Male; F: Female; NA: Information not available.

infection and observed that wild type and variant ER α are expressed in all the samples. However, the expression of vER α is stronger in tumor than in peritumor tissues. Interestingly, ER β was found to be significantly over-expressed in HCV-infected HCC tissues as compared to HBV-infected HCC tissues. The differences in ER expression in HCV-infected HCC tissues compared to HBV-infected HCC tissues suggest different pathogenetic mechanisms. Overall from these studies there appears to be a change in co-expression pattern of ER α and ER β from cirrhosis to HCC development in both HBV- and HCV- related tumors. Thus, these studies provide further evidence in support of importance of wild type and variant ERs in HCC, suggesting detailed investigations in this area.

Androgen receptor

Like estrogens, androgens have also been reported to play an important role in liver carcinogenesis. Iqbal *et al*^[31] showed the presence of androgen receptors (AR) in HCC in 1983. However, it was not until 1985 that normal human liver was believed to express AR. In 1985, Nagasue and colleagues^[14] demonstrated the presence of AR in normal human liver as well as in tumor and non-tumor parts of HCC tissues. Since then, several reports showing protein and mRNA expression of AR in liver have been published. Table 3^[13,15,17,28,30-33,35,40,41,52,59-64] and Table 4^[14-17,31,33-36,39,41,47,52,65-71] give the details of these studies. In general, AR is found to be over expressed in liver tumor compared to the adjacent normal tissue^[15,17,33,36,38]. However, like ER expression studies,

majority of the early studies employed indirect binding assays to detect AR in the liver tissues. More precise quantitative methods of direct detection of AR protein are needed. Reports using antibody-based detection of AR in liver tissues are very sparse.

The mRNA levels of AR have been assessed in the non-cancerous and HCC tissues primarily by RT-PCR (Tables 3 and 4). In 1994, Negro and colleagues^[16] developed a non-radioisotopic *in situ* hybridization assay specific for human AR mRNA and found that 73% of HCC tissues could express variable amount of AR mRNA. However, normal hepatocytes were stained weakly in 42% of the non-neoplastic tissues. Though initial binding studies demonstrated higher AR levels in tumor tissues than in respective peri-tumoral part^[15,17,33,36,38], more recent observations based on mRNA expression do not reveal any significant differences in tumor and peri-tumor tissues. Taviani *et al*^[71] found higher AR mRNA levels in tumor than in the corresponding peri-tumoral tissue in a relatively small percentage of HCC samples, suggesting that AR mRNA levels are associated with the histological tumor differentiation showing a lower AR expression in poorly-differentiated HCC than in well-differentiated tumors. In contrast, AR levels in Korean population with HCC do not show differences between tumor and peri-tumor tissues using RT-PCR^[52]. Due to these conflicting reports on AR expression in HCC, there is a need for detailed investigation of AR mRNA as well as protein levels using more sensitive and accurate detecting quantitative methods.

Table 4 Androgen receptor protein and mRNA expression in liver tissue samples from HCC patients

	Subcellular Organelle	Method	n	Subjects	Positive	Country	Yr	Reference
AR protein	Cytosolic and nuclear	BA	5	3 M, 2 F	3 M, 2 F	United Kingdom	1983	[31]
	Cytosolic	IHC	10	NA	5	Singapore	1984	[39]
	Cytosolic	BA	19	19 M	14 M	Japan	1985	[14]
	Cytosolic and nuclear	BA	5	3 M, 2 F	3 M, 2 F	United Kingdom	1985	[65]
	Cytosolic and nuclear	BA	8	6 M, 2 F	2 M, 2 F	Japan	1986	[17]
	Cytosolic	BA	13	8 M, 5 F	8 M, 5 F	United Kingdom	1988	[66]
	Cytosolic	BA	19	19 F	7 F	Japan	1989	[15]
	Cytosolic	BA	45	31 M, 14 F	25 M, 6 F	Japan	1989	[67]
	Cytosolic	BA	11	9 M, 2 F	6 M, 1 F	Japan	1990	[35]
	Cytosolic	BA	21	18 M, 3 F	18	Japan	1991	[36]
	Cytosolic and nuclear	BA	9	6 M, 3 F	6 M, 3 F	Italy, United States	1991	[33]
	Cytosolic	BA	5	3 M, 2 F	3 M, 2 F	Japan	1992	[68]
	Cytosolic	BA	26	18 M, 8 F	14	Spain	1993	[41]
	Cytosolic	BA	43	30 M, 13 F	28	Spain	1995	[69]
	NA	BA	32			China	1998	[70]
AR mRNA	Nuclear	IHC	31	26 M, 5 F	18 M, 3 F	Spain	2007	[47]
		ISH	22	16 M, 6 F	13 M, 3 F	Italy	1994	[16]
		RT-PCR	38	24 M, 14 F	21 M, 13 F	Italy	2002	[71]
		RT-PCR	32	23 M, 9 F	23 M, 9 F	Korea	2006	[52]

AR: Androgen receptor; BA: Binding assay; IHC: Immunohistochemistry; ISH: *In situ* hybridization; RT-PCR: Reverse transcriptase-polymerase chain reaction; n: Sample size; M: Male; F: Female; NA: Information not available.

CLINICAL AND PATHOLOGICAL SIGNIFICANCE OF ESTROGEN AND ANDROGEN RECEPTORS IN HCC

Despite a wide variability observed in studies of ER and AR expression in HCC, attempts have been made to determine the significance of these receptors by correlating their levels with clinical and pathological parameters. Table 5^[13,15,34,42,55,69,70,72] and Table 6^[30,54,73-77] present the salient findings of such clinical studies.

In few earlier studies using binding assays, no correlation was found between ER protein expression and sex, age, alcohol abuse, serum alpha-feto protein, carcinoembryonic antigen, HBV markers or tumor histology^[15,34]. However, in subsequent reports, ER mRNA levels were shown to be associated with sex and viral etiology^[19,51,52]. Increased vER α expression has been demonstrated more often in males than in females with HCC in Italian population, suggesting a strong link of ER with a higher incidence of HCC in males^[19,51]. On the other hand, in Korean subjects no correlation has been found between the expression of vER α and HCC prevalence in males^[52]. Interestingly, a distinct difference in ER expression pattern was observed in HBV- and HCV-infected HCC patients. Delta 5 deletion variants of ER α (vER α) and ER β were found to be more often expressed in HBV-related tumors than in HCV-related tumors (67% *vs* 15%, $P < 0.0007$)^[19]. In contrast, Wang *et al*^[52] showed no remarkable difference in vER α levels in HCV- and HBV-infected HCC tissues (91.3% *vs* 100%). Nevertheless, a predominant expression of ER β has been reported in HCV-infected than in HBV-infected patients with HCC (95.7% *vs* 44.4%, $P < 0.05$), suggesting that ER β may play an important role in HCV-induced liver disease^[52].

In addition to gender and etiological factors,

tumor size, histopathology, operative mortality, tumor recurrence and survival after curative resection have also been studied in relation to ER expression in HCC^[34,42,55]. Nagasue *et al*^[34] showed that the large tumors are more commonly found in ER negative HCC patients and therefore the incidence of major hepatic resection is significantly higher in this group than in ER positive HCC patients. However, they did not report significant differences in histopathology of ER positive and ER negative tumors. Rates of mortality, tumor recurrence and long-term survival were also found to be similar in the two groups. In contrast to these observations, Jonas *et al*^[42] showed that in patients undergoing curative resection, the 1- and 2-year survival rates in ER positive group are substantially lower than in ER negative group^[42], suggesting that ER positive status has a negative effect on patient survival after curative resection of advanced HCC. Further, significantly longer survival rates have been reported in HCC patients with wild type ERs than in those expressing variant ERs^[55].

In contrast with ER expression, AR levels are strongly associated with intra hepatic recurrence of tumors. The 5-year survival of recurrence free HCC patients was shown to be 55% for AR negative, 24% for ER negative, 10% for ER positive and 0% for AR positive tumors^[67]. Similar findings have been reported by other researchers, suggesting a negative impact of AR positivity on tumor recurrence^[69]. It was reported that AR negative patients show significantly better survival than AR positive patients^[70]. Considering the tumor size, variable results have been documented in relation with AR expression. Boix *et al*^[69] showed that AR expression is significantly related to smaller tumor size while Zhang *et al*^[70] found that AR levels are positively correlated to tumor size.

Table 5 Studies on correlation of estrogen receptor and androgen receptor expression with clinical and pathological parameters in HCC patients

Receptor protein/ mRNA expression	Clinical parameter	n	Country	Salient findings	Yr	Reference
ER protein	Serum alpha-fetoprotein, carcinoembryonic antigen, HBV profile, tumor histology	30	Japan	No correlation with any parameter	1986	[13]
ER and AR protein	Serum alpha fetoprotein, HBV markers, histopathology	19	Japan	No correlation	1989	[15]
ER protein	Sex, age, alcohol abuse, underlying liver disease, hepatic functions	66	Japan	No correlation	1990	[34]
	Tumor size, hepatic resection			Large tumor size and higher rate of resection in ER-		
	Histopathology			No differences in ER+ and ER-		
	Operative mortality, tumor recurrence, long-term survival rate			Similar in ER+ and ER-		
ER and AR protein	Intrahepatic recurrence	78	Japan	AR expression strongly associated with intrahepatic recurrence. Weak association with ER expression	1995	[72]
ER protein	Survival after curative resection	28	Germany	Negative effect of an ER+ tumor on patient survival after curative resection	1997	[42]
Wild type and variant ER mRNA	Survival	96	Italy	Significantly long survival in patients with wild type ERs than variant ERs	2000	[55]
AR protein	Recurrence rate	45	Japan	Significantly higher recurrence rates in AR+ group than AR-	1989	[15]
	Survival rate			Significantly better survival rates in AR- patients than in AR+		
AR protein	Tumor size	43	Spain	AR expression was significantly related to smaller tumor size	1995	[69]
	Tumor recurrence			Higher tumor recurrence rates in surrounding tissues of AR+ than AR-		
AR protein	Tumor size and survival time	32	China	Survival rate correlated inversely with the levels of AR expression	1998	[70]
				AR levels had positive correlation with the tumor size		

CURRENT STATUS OF HORMONAL TREATMENT IN HCC

The association of estrogens and androgens in HCC observed in basic and clinical studies has led to initiation of various clinical trials on hormonal treatment of HCC. Differential clinical outcome was reported in these trials that have resulted in continued debate about the use of hormonal therapy in HCC. Table 6 presents a list of few clinical studies that utilized hormonal therapy in HCC patients. A systematic review of these clinical trials on therapeutic evaluation of anti-estrogen and anti-androgen agents in liver cancer has been recently compiled by Di Maio *et al*^[78]. The authors conclude that hormonal treatment should not be a part of the current management of HCC patients^[78,79]. However, in most of these clinical studies, various inherent factors may have contributed to the observed inconclusive results. Few of these may include faulty patient subset selection criteria, no monitoring of tumor ER and AR expression at the time of recruitment and also during treatment of these patients and lastly the type of hormonal treatment given to the patient. Therefore, the debatable potential of hormone therapy in HCC may finally be attributed to the lack of complete understanding of ER and AR expression and hormonal responsiveness in the liver and their involvement in development of HCC.

Currently, limited information is available on the functional significance of ER and AR in HCC. In the following sections, we review the *in vivo* animal studies

on liver carcinogenesis and *in vitro* studies on cell lines that have been conducted to understand the role of ER and AR in the liver.

ESTROGEN RECEPTOR AND ANDROGEN RECEPTOR STUDIES IN ANIMAL MODELS OF EXPERIMENTAL LIVER CARCINOGENESIS

Estrogen receptor studies

Several attempts have been made to establish the role of estrogen and its receptors in hepatocarcinogenesis using animal models. Rat is the most extensively used model to study liver carcinogenesis. Rat hepatocytes are known to express ERs. ER α is the predominant isoform expressed in rat hepatocytes while cholangiocytes express both ER α and ER β ^[80]. However, Inoue *et al*^[81] showed that the levels of ER β are higher than those of ER in cultured rat hepatocytes^[81]. Hepatic stellate cells from rats appear to contain mainly ER β ^[82]. Due to the lack of information about the existence of various ER isoforms, in most of the earlier studies, hepatic stellate cells did not differentiate ER into ER α and ER β . In addition, majority of the *in vivo* studies have been conducted in animal models of chemical carcinogenesis. Diethylnitrosamine (DEN) is the most commonly used carcinogen in rat and mouse models of HCC. The pathogenesis of HCC in DEN-induced carcinogenesis in

Table 6 Clinical outcome of hormonal therapeutic trials in HCC patients

Receptor protein/ mRNA expression	Clinical parameter	Treatment	n	Country	Salient Findings	Yr	Reference
ER protein	Tumor growth	Progestin	5	United States	Tumor regression in 2	1982	[30]
NA	Anti-tumor response	Tamoxifen 20 mg twice daily	33	United States	No complete or partial antitumor response	1990	[73]
	Survival time				Long term survival (18+ to 39+ mo) in 4 patients		
NA	Anti-tumoral effect	Tamoxifen 20 mg daily	120 (placebo = 62)	Spain	No-antitumor effect	1995	[74]
	Survival time				No significant differences in survival rate of placebo and treated groups		
Wild type and variant ER mRNA	Tumor size and growth rate	Tamoxifen 80 mg daily or Megestrol 160 mg daily	8	Italy	Growth rate 4 times higher in tumors expression variant ER than wild type ERs. Tumor regression to half size in patients with wild type ER following tamoxifen treatment. Megestrol slowed down tumor growth in tumors with variant ERs	1996	[54]
ER protein	Mortality rates	Tamoxifen	119 (placebo = 58)	China	No difference in 1 mo mortality rates and median survival in treated and control groups	2000	[75]
	Survival				No effect of ER expression on survival		
Variant ER mRNA	Tumor growth, survival	Megestrol 160 mg daily	24 placebo, 21 treated	Italy	Significantly slowed down tumor growth and improved survival in treated patients than placebo group	2001	[76]
NA	Survival rates	Tamoxifen 120 mg daily or 60 mg daily	329	Singapore	No positive effect on survival and increasingly negative impact with increasing doses	2005	[77]

NA: Information not available.

animal models differs from that in humans and therefore may not be directly comparable to human HCC^[83]. Nevertheless, the histology and genetic signatures are similar to human HCC and a striking gender disparity with male predominance is also observed in these animal models as seen in humans^[84]. In addition to DEN, acetylaminofluorene (AAF), di(2-ethylhexyl) phthalate (DEHP), peroxisome proliferator, arsenic and carbon tetrachloride have been used to induce HCC in various animal models^[85-90].

Use of oral contraceptives and synthetic estrogens in women is reported to be a major risk factor for the development of hepatocellular adenoma, a benign liver tumor with malignant potential^[26,27]. Shimomura *et al*^[91] studied the role of ethinyl estradiol (EE) in inducing HCC in female rats, following EE treatment for 12 mo, 8% of rats developed HCC, revealing that EE causes mutations in hepatocytes leading to DNA adduct formation and induces HCC development in affected cells. The initial events in HCC, i.e. DNA adduct formation by EE, appear to be carried out in an ER independent manner since tamoxifen, a known selective estrogen receptor modulator (SERM), inhibited ER expression and suppressed HCC, but did not affect DNA adduct formation. Exogenous estrogens have also been shown to promote hepatocarcinogenesis induced by other agents^[92,93]. Campen *et al*^[92] documented that administration of 17- α ethinylestradiol in ovariectomized rats promotes DEN-induced carcinogenesis in a dose dependent manner. Further,

it was reported that synthetic female hormones act synergistically with ethanol to increase HCC incidence^[93]. Alcohol could affect HCC development due to EE by promoting changes in ER kinetics and expression as well as in DNA adduct formation.

Liver is the major site of estrogen metabolism^[94]. Alterations in sex hormone metabolism are also considered a critical factor determining the significance of sex hormones in the process of liver carcinogenesis. Eagon *et al*^[86,87] reported that the activity of male estrogen-metabolizing enzyme, estrogen 2-hydroxylase and male specific estrogen sequestering protein is reduced in liver, which explains the raised serum estradiol levels but the decreased hepatic activity of cytosolic and nuclear ER observed in DEHP-induced HCC in male Fischer 344 rats. The expression of cytochrome P450 enzymes that play an important role in estrogen metabolism have also been shown to be affected during hepatocarcinogenesis. Waalkes and colleagues^[88] have described the feminized pattern of P450 genes in male mice with HCC induced by exposure to arsenic in utero. The expression of female dominant CYP2A4 and CYP2B9 is increased whereas levels of male dominant CYP7B1 gene gets reduced in arsenic treated animals. Recent findings suggest that cytochrome P450 (CYP) is regulated by estrogen itself through the involvement of estrogen receptors^[94]. Nonetheless, elucidation of the exact mechanism of regulation of CYP isoforms by estrogen in liver needs further investigation.

In contrast with earlier animal studies that support

estrogens in promoting and inducing carcinogenesis, recent studies highlighted the protective role of estrogens in HCC development. Shimizu *et al*^[8] showed that estrogen can suppress chemical hepatocarcinogenesis induced by dimethylnitrosamine (DEN)-2-acetylaminofluorene (AAF) in partial hepatectomy (PH) model of hepatocarcinogenesis. In addition, estrogen has been shown to prevent the progression of liver disease to HCC. Estradiol treatment could reduce hepatic steatosis and restore the impairment in mitochondrial and peroxisomal fatty acid β -oxidation in aromatase-deficient mice which lack intrinsic ability to produce estrogen^[95]. Furthermore, estradiol treatment was also shown to result in a dose dependent suppression of hepatic fibrosis in hepatic fibrosis models of male rats^[96,97]. The mechanism of protective action of estrogens against progression of chronic liver disease has been recently reviewed by Shimizu and Ito^[7].

Recently, using a mouse model of DEN-induced hepatic carcinogenesis, Naugler *et al*^[83] described a molecular mechanism explaining the lower HCC susceptibility in females and the anti-inflammatory role of estrogen in preventing HCC development. The authors investigated the relationship between HCC development and gender dependent expression of interleukin-6 (IL-6). IL-6 is a proinflammatory cytokine that plays an important role in chronic hepatitis, the prerequisite for progression to cirrhosis and HCC. The serum IL-6 levels were higher in male mice than in female mice after administration of DEN, leading to a higher rate of liver cell proliferation in male mice. This effect can be further mediated by ER α , suggesting that ER β plays a little role in modulating the expression of IL-6.

Androgen receptor studies

The role of AR has also been studied in animal models of chemical induced carcinogenesis^[87,98-100]. In DEN treated Wistar rats, a 20-fold increase in hepatic AR concentration was reported in females, suggesting that increased hepatic AR concentration is correlated with accelerated tumor development in these animals, in which male rats showed a slower tumor development with no change in AR concentrations^[98]. Subsequent studies in the same model revealed that removal of ovary increases AR levels in the liver of female rats but testosterone treatment does not further enhance AR levels^[99]. On the other hand, normal adult males with intact testis or testosterone treatment maintain high levels of AR but in castrated rats estrogen treatment reduces AR expression^[99]. Animal studies demonstrated that the expression of both AR and ER increases during preneoplastic stages and that progression towards cancer development can suppress ER and maintain AR expression levels^[94,100].

Interestingly, anti-androgen treatment has been shown to reduce AR levels in liver as well as the size and number of tumors in male Spargue Dawley rat model of hepatocarcinogenesis^[101]. It has been shown that inhibition of AR positive HCC with anti-androgen

cypertone acetate in male mice involves cell cycle arrest and to some extent induction of apoptosis due to increased synthesis of transforming growth factor- β 1 (TGF- β 1)^[102]. In another model of chemical-induced liver carcinogenesis, inhibition of androgens using 5-alpha reductase inhibitors significantly suppressed HCC development in rats^[103]. Recent studies in a xenograft model of hepatocarcinogenesis in nude mice suggested that AR expression remains elevated until development of tumor and starts declining as the size of tumor increases^[104]. It is therefore proposed that androgen therapy may be ineffective after establishment of the tumor. Nevertheless, for better understanding and rationale design of hormone-based therapies, it is mandatory to study the role of ERs and AR in animal models mimicking the natural course of disease progression to HCC development as in humans. Currently available HBV and HCV transgenic mice depicting features close to human HCC pathogenesis, appear to be promising models for future *in vivo* studies.

SEX HORMONE RECEPTORS: GENOMIC AND NON-GENOMIC ACTIONS IN LIVER CELLS MEDIATING HEPATOCARCINOGENESIS

Estrogen receptor

Estrogen action and the role of ERs in carcinogenesis have been well documented in mammary carcinoma and the studies have revealed the involvement of estrogens in key cellular processes such as apoptosis, cell cycle, proliferation, oxidative stress and inflammation. The progress in understanding the role of estrogen in regulating various cellular events in liver carcinogenesis has been rather slow. However, the research conducted over recent years provides key insights in this direction.

The classical mode of estrogen action is the genomic mechanism in which ERs function as ligand-activated transcription factors^[105]. Activated ERs translocate to the nuclei and regulate the expression of specific target genes. These transcriptional regulations are achieved through interaction with estrogen responsive element (ERE) sequences located in the promoter region of the target gene^[106]. However, one third of the genes regulated by ERs in humans do not contain ERE-like sequences^[107]. ERs can also regulate the transcription of such genes without binding to DNA through protein-protein interactions with other transcription factors, such as AP-1 and Sp-1 in the nuclei^[108]. In addition, this transcriptional control at alternate response elements is also facilitated by non-genomic actions of estrogen. The non-genomic functions of estrogen are initiated by membrane-localized ERs and are associated with activation of various signaling pathways especially protein kinases^[109]. The functions of many transcription factors are regulated through protein kinase-mediated phosphorylation including CREB, NF- κ B and AP-1 and these transcription factors may thus be targets

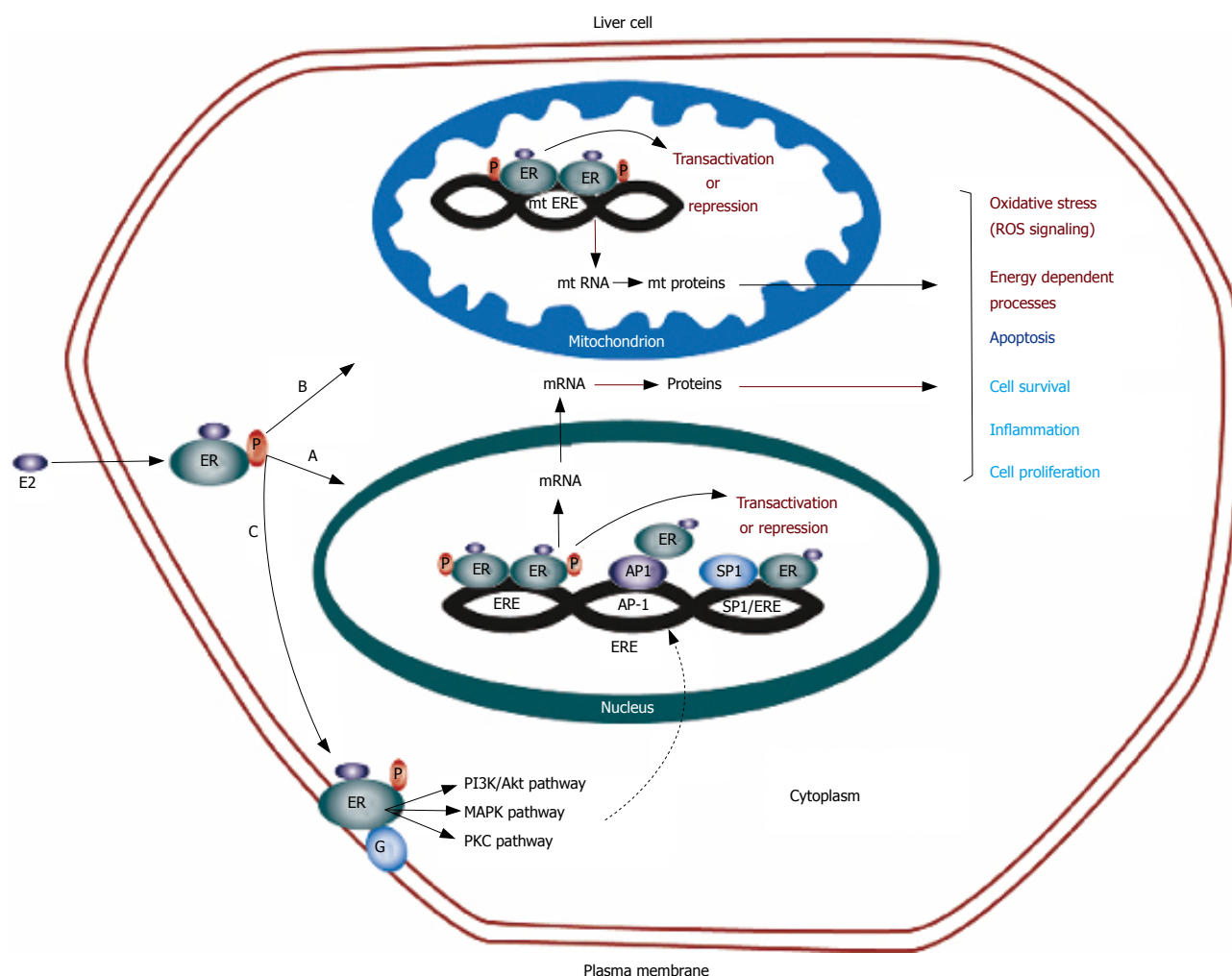


Figure 1 Genomic and non-genomic functions of estrogen mediated by estrogen receptors (ERs) localized in different sub-cellular organelles of a liver cell. A: Genomic actions include translocation of ligand-activated ERs to the nuclei for regulating gene transcription. This transcriptional control is carried out by binding to DNA at a sequence containing either full estrogen response element (ERE) site or an ERE half site adjacent to the binding site for another transcription factor like Sp1. An additional mechanism involves gene regulation at alternate response element through protein-protein interactions with other transcription factors (AP-1, CREB, NF- κ B); B: Activated ERs control mitochondrial gene transcription by binding to ERE like sequences (mt ERE) leading to modulation of mitochondrial functions including metabolism, oxidative stress and apoptosis; C: Membrane localized ERs are G-protein coupled receptors that activate various protein kinase pathways. These signal transduction cascades in turn regulate functions of many transcription factors resulting in modulation of expression of a number of genes involved in cell proliferation, survival, apoptosis and inflammation. Genomic and non-genomic actions of estrogen converge to exhibit a fine degree of control for the regulation of transcription by ERs in a liver cell. Modified from: Chen *et al*^[110] 2005.

for non-genomic actions of estrogens. This possible convergence of genomic and non-genomic actions at multiple response elements provides an extremely fine degree of control for the regulation of transcription for ERs (Figure 1)^[105,110]. In the following section, we discuss the findings of recent *in vitro* studies highlighting the significance of ER in mediating genomic and non-genomic actions of estrogen in liver cells to modulate the expression of a number of genes involved in cellular processes central to carcinogenesis.

Telomerase activation has been implicated in hepatocarcinogenesis and the expression of human telomerase reverse transcriptase (hTERT) that encodes for the catalytic subunit of the multicomponent enzyme telomerase hTERT is the prerequisite for telomerase activation^[111,112]. Several studies indicated that estrogen regulates transactivation of the hTERT gene by direct interaction of activated ER with an imperfect

ERE sequence in the hTERT promoter^[113]. Estrogen treatment has been shown to up-regulate the expression of hTERT mRNA and protein in three normal human hepatic cell lines (hc-cells, hNheps and WRL-68) expressing ER α to varying degrees^[90]. Furthermore, estrogen exposure prevents shortening of telomeres and decreases the number of cells undergoing senescence, indicating that estradiol acts as a positive modulator of the hTERT gene in the liver^[90]. However, the mechanism of ER-mediated transactivation of hTERT in the liver is not well understood. In contrast, in HepG2 cells, estrogen modulation of telomerase activity has been found to be regulated post-transcriptionally *via* the IP3/PKC pathway^[114,115]. IP3 production has been shown to be up-regulated by estrogens in HepG2 cells^[114]. Furthermore, estradiol-induced IP3/PKC- α production is dependent on either ER α or ER β expression in both HepG2 and Hela cells^[114]. It is

hypothesized that membrane ER-mediated IP3/PKC- α pathway represents an alternative signaling pathway utilized by cells when low ER levels are unable to activate classic ER-mediated genomic mechanisms as in HepG2 cells^[114].

A similar regulatory mechanism has been observed in case of estrogen modulation of expression of cyclin D1 gene in hepatoma cells. Cyclin D1, important for progression of cells through G1 phase of cell cycle, is a well defined target for estrogen action in mammary carcinoma^[116,117], although no detectable estrogen responsive element like sequence in the cyclin D1 gene promoter has been reported in these cells^[118]. The cyclin D1 mechanism identified in mammary carcinoma cells involves direct interaction of ER α and Sp1 or ER α and Ap-1^[119]. Interestingly, Marino *et al*^[23] demonstrated that in HepG2 cells, estrogen-induced activation of cyclin D1 transcription can occur independently of the transcriptional activity of ER. They further showed that the effect of 17-beta estradiol on HepG2 cells is mediated by activation of the MAPK/ERK pathway by membrane-localized ER that increases the expression of cyclin D1 gene through activation of AP-1 transcription factor^[23], suggesting that non-genomic signaling pathways play an the pivotal role in estrogen-mediated regulation of gene expression at multiple response elements.

Besides, modulating the molecules involved in cell cycle control and cell proliferation, estrogen has also been shown to regulate the expression of genes crucial for apoptosis of hepatocytes and dysregulation of apoptosis in hepatic cells is reported to be a significant factor in accelerating hepatocarcinogenesis or tumor progression in HCC^[120]. The Bcl-2 family of proteins regulates one of the key steps in the conserved apoptotic pathway. Among the members of this family, Bcl-2 and Bcl-xL act as inhibitors of apoptosis where as Bax and Bak promote apoptosis^[121,122]. Ethinyl estradiol is known to increase the levels of Bcl-2 protein in cultured female rat hepatocytes^[123]. Estradiol and idoxifene, two selective estrogen receptor modulators, are known to induce the expression of Bcl-2 protein in male rat liver tissues^[124]. Omoya *et al*^[9] and Inoue *et al*^[81] also demonstrated that estradiol is able to stimulate the expression of Bcl-2 and Bcl-xL and to suppress Bad expression in oxidative stress-induced early apoptotic rat hepatocytes. Similar findings have been recently documented in response to estradiol treatment of Huh-7 human hepatoma cells describing a dose dependent increase in expression of Bcl-2 and Bcl-xL and a reduction in Bad levels^[22]. No change was observed in expression of pro-apoptotic protein Bax. The regulation of Bcl-2 gene expression by estrogen in mammary carcinoma cells has been shown to be mediated indirectly through activation of Sp-1 transcription factor^[125]. However, the precise mechanism of Bcl-2 transactivation in hepatocytes has not been clearly understood.

One of the most interesting mechanisms of transcriptional regulation at alternate response elements by estrogen is through inhibition of transcription factor

NF- κ B. Studies demonstrating a mutually antagonistic cross-talk between these families of transcription factors have been recently reviewed^[126]. The ER has been shown to mediate opposition of NF- κ B functions at various levels by inhibiting the activation of signaling pathways, preventing nuclear translocation, blocking DNA binding or inhibiting recruitment of co-activators for transcription^[126]. Estrogen has been shown to bring about its anti-inflammatory and anti-oxidant effects on liver cells by suppressing the NF- κ B activity as evident from the following studies. It was reported that 17 beta-estradiol-bound ER α interferes with cytokine-induced activation of a NF- κ B reporter in HepG2 cells, suggesting that estrogen exerts its anti-inflammatory and protective effects on human liver cells^[127]. Moreover, in an *in vivo* model, estrogen treatment has been shown to block the induction of hepatic expression of inflammatory vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor- α (TNF- α), and regulate normal T-cell expression and secretion upon activation^[128]. In a mouse model of DEN-induced HCC, ER α was suggested to suppress IL-6 production, a pro-inflammatory molecule, through the involvement of the NF- κ B pathway^[83]. Estrogen has also been reported to suppress oxidative stress-induced reactive oxygen species (ROS) generation, lipid peroxidation, activation of AP-1 and NF- κ B as well as loss of Cu-Zn SOD activity in cultured rat hepatocytes^[29].

In addition to genomic and non-genomic actions of estrogen mediated by nuclear and membrane ER, mitochondria have also recently been identified as important targets of estrogen and ERs^[110]. Early binding studies on sub-cellular fractions indicated that ER is present in rat liver mitochondria^[129]. Both ER α and ER β have been reported to be present in the mitochondria of human HepG2 cells^[130-132]. The mitochondrial genome has been shown to contain sequences that have partial homology to the estrogen responsive elements^[132-134]. Both ER α and ER β bind to mitochondrial DNA and the binding can be increased by estradiol using mobility shift assays and surface plasmon resonance^[135]. These results suggest that estradiol is directly involved in the regulation of mitochondrial DNA transcription (Figure 1). Regulation of apoptosis and oxidative metabolism by estrogens in mitochondria may be important in the normal liver and in the development of HCC.

Ethinyl estradiol treatment has been shown to elevate the expression levels of mitochondrial DNA-encoded cytochrome C oxidase subunit III (CO III) and ATP synthase 6 *in vivo* as well as in HepG2 cells^[136]. This increased expression of mitochondrial transcripts is accompanied by increased mitochondrial superoxide production and respiratory chain activity that require cytochrome P450-mediated biotransformation of ethinyl estradiol and 17-beta estradiol to catechol metabolites^[136,137]. In addition to CO III, the levels of CO I and CO II encoded by mitochondrial DNA have also been found to be elevated in ethinyl estradiol treated female rat hepatocytes. This effect is accompanied

by increased mitochondrial superoxide production, high ATP levels and increased Bcl2 production, and is suggested to play a role in ethinyl estradiol-mediated inhibition of apoptosis^[123]. In contrast, 17-beta estradiol and 17-beta estradiol like compounds, diethylstilbestrol (DES), tamoxifen and genistein, have been found to induce apoptotic effects in human hepatoma Hep3B cell line^[138]. These compounds cause the leaking of cytochrome C from mitochondria and activation of caspase-3 in an ER dependent manner. In another study, the two isoforms, ER α and ER β , showed their opposing actions on apoptosis in a poorly differentiated HCC cell line HA22T^[139]. Over-expressed ER β but not ER α induces the expression of caspase-8 and TNF- α in HA22T cells in response to estradiol treatment, indicating that the death receptor-mediated apoptotic pathway is activated^[139].

Differential roles of ER α and ER β have also been observed in non-genomic actions of estrogen in the liver^[21,23]. There is indirect evidence that membrane ER may exist in human liver as the binding of gold tagged estrogen-BSA conjugate on the surface of clathrin-coated pits in HepG2 cells has been demonstrated by electron microscopic visualization^[140]. The non-genomic mechanism of action of sex steroids on the plasma membrane involves the activation of protein kinase cascades (Figure 1). Two major cascades, protein kinase C, and mitogen-activated protein (MAP) kinase are active and important in carcinogenic liver cells. Protein kinase C cascade and its second messenger IP3 are important in cell proliferation and have been discussed in this review in context of transcriptional regulation of hTERT expression by estrogen. The mitogen-activated protein (MAP) kinase cascade is another pathway that is regulated by the action of sex steroids at the plasma membrane. This complex signaling cascade involves three major pathways: ERK, p38, and JNK^[141]. In HepG2 cells, estradiol has been found to rapidly increase the phosphorylation of ERK^[21,23]. Naringenin, an anti-estrogenic flavonone, induces the activation of p38 in ER α containing HepG2 cells or in ER β containing human colon adenocarcinoma DLD-1 cells^[142], suggesting that naringenin has an antiestrogenic effect only on the ER α expressing cells, whereas it mimicks the estradiol effects on ER β expressing cells. The role of ER α and ER β in the regulation of MAP kinase cascade has been further studied in cell lines expressing either ER α or ER β ^[21]. It was found that estrogen-bound ER α can rapidly activate the ERK and AKT signal transduction pathways leading to cell cycle progression and inhibition of apoptosis, whereas estrogen-complexed ER β can induce rapid phosphorylation of p38 leading the cells to the apoptotic cycle and cell death. These studies further support the functional antagonism between ER α and ER β with respect to estrogen-induced cell proliferation and emphasize the need to study the independent and interactive role of both isoforms in hepatocarcinogenesis.

Androgen receptor

In comparison with ER, there is limited information

about genomic and non-genomic functions of AR in the liver. Like ER, AR has also been shown to regulate gene transcription by binding to androgen responsive sequences (ARE)^[143,144]. Yoon *et al*^[145] demonstrated that androgen can directly regulate the expression of transformation growth factor-beta 1 (TGF- β 1) through binding of AR to ARE in TGF- β 1 promoter, suggesting that such activation might regulate the progression of HCC in both human and animal models^[145]. Furthermore, AR has been shown to interact with a newly identified transcription factor, paternally expressed gene 10 (PEG 10) in hepatoma cell line^[146]. PEG 10 has growth promoting properties and is implicated in hepatocarcinogenesis^[147,148]. Dihydrotestosterone (DHT) promotes hepatoma formation in nude mice through PEG 10 activation. In addition, DHT treatment is shown to up-regulate hTERT expression in hepatoma cell lines in a PEG-10 dependent manner^[146]. These studies indicate that PEG-10-mediated transactivation of target genes by AR has an essential role in hepatocarcinogenesis.

To the best of our knowledge, AR has not been detected in the liver mitochondria. The information about the membrane localization of AR in human liver cells is also lacking. However, AR has been reported to occur in the plasma membranes of male rat liver^[149]. Androgens are also involved in the regulation of the MAP kinase signaling pathways as orchietomy of H-ras 12V transgenic mice decreases phospho-MEK and phospho-ERK in liver tissues. In addition, orchietomy reduces hepatotumorigenesis in male mice while ovariectomy increases phospho-MEK and phospho-ERK in liver tissue from female mice, but ovariectomy does not affect the incidence of tumorigenesis^[150]. Detailed investigations are urgently needed to confirm the existence of non-genomic signaling actions of androgens in liver and the role of AR in mediating these functions.

INTERACTION OF ER AND AR WITH VIRAL PROTEINS IN HBV AND HCV PATHOGENESIS

Chronic infection with HBV and HCV is the major cause of increasing incidence of HCC worldwide. Several reports support the role of HBV and HCV proteins in disturbing cellular homeostasis and causing malignant transformation of hepatocytes^[151,152]. Recent studies in hepatoma cell lines suggest the interactive role of ERs and AR with HBV and HCV proteins in viral pathogenesis.

Estrogen receptor and viral proteins

Han *et al*^[24] recently reported that HBV protein (HBx) interacts with ER α . HBx is a multifunctional protein involved in neoplastic transformation in cultured cells and can induce HCC in transgenic mice. HBx associates with both ER α and delta 5 deletion variant of ER α (vER α) and inhibits ER α transcriptional activity by recruiting histone deacetylase enzyme, HDAC-1^[24].

HDAC-1 belongs to the family of enzymes involved in deacetylation of hyperacetylated histone tails, leading to compaction of chromatin and transcriptional repression^[153]. Both HBx and ν ER α have additive effects on suppression of ER α transactivation^[24].

ER α has also been shown to interact with nonstructural (NS) 5B protein of HCV^[25]. NS5B is a RNA-dependent RNA polymerase, which plays a central role in viral genome replication^[154]. HCV replication takes place in a replication complex consisting of viral RNA and non structural proteins including NS5B^[155]. The replication complex forms on the surface of intracellular membranes including endoplasmic reticulum membrane and is associated with lipid rafts rich in caveolin 2 (CAV 2) on these membranes^[156-158]. Using chemical biology approach, Watashi *et al*^[25] demonstrated that ER α facilitates the interaction of NS5B with CAV 2 in lipid rafts and hence promotes the participation of NS5B in HCV replication complex. However, they did not find that ER β affects HCV replication in the same study^[25]. An important observation of the study is that tamoxifen inhibits ER α actions and suppresses HCV genome replication, further supporting the potential for anti-ER drugs in developing new anti-HCV strategies.

Androgen receptor and viral proteins

Like ER, HBx protein has also been shown to interact with AR. HBx functions as a positive transcriptional co-regulator to increase AR-mediated transcriptional activity. This transcription enhancement is increased in the presence of androgen in a concentration-responsive manner. However, HBx does not physically associate with ligand-bound AR in the nuclei, suggesting that HBx augments AR activity by increasing the phosphorylation of AR through HBx-mediated activation of the c-Src kinase signaling pathway^[159]. In contrast, Zheng *et al*^[160] demonstrated that HBx can physically bind to AR in the liver and alter the subcellular localization of AR both in the presence and absence of dihydrotestosterone (DHT). Further studies indicated that HBx can enhance the gene transactivation activity of AR by enhancing its DNA binding activity in a DHT-dependent manner.

CONCLUSION

Taken together, studies on hepatoma cell lines, HCC tissues and animal models of hepatocarcinogenesis, highlight the importance of sex hormones and their receptors in HCC pathogenesis. Further investigations are urgently needed to elucidate the precise mechanism of action of estrogens, androgens and their receptors in regulating normal liver physiology and pathophysiology of chronic liver diseases resulting in HCC.

A thorough re-examination of studies conducted so far to detect the expression of ER and AR in liver tissues is needed using newer specific, sensitive and quantitative methods. With the emerging significance of ER β and the availability of isoform specific antibodies, the relative levels of both ER α and ER β can be determined. The studies on mRNA expression in liver tissues have

demonstrated the presence of deletion mutants (variant forms) that need to be further validated at protein levels for establishing their significance in diagnosis and prognosis of HCC. Considering the male predominance of HCC and the wide gap in the information available on AR in liver, detailed mechanistic studies need to be conducted to reveal the mechanism of androgen function in normal liver and HCC. In addition, evaluation of ER and AR status at premalignant stages of chronic liver disease due to different etiological factors is required for critical understanding of their role in HCC pathogenesis.

Recent *in vitro* studies focusing on molecular interaction of hormonal receptors with viral proteins need to be further confirmed in *in vivo* animal models. Currently available HBV and HCV transgenic mouse models as well as human hepatocyte xenograft models can serve as a valuable preclinical tools to validate the importance of sex hormone receptors in chronic liver disease development and progression to HCC. Thus, with the availability of state of the art technologies, the time is ripe to embark on to move this important field forward. Well designed, systematic studies employing adequate tools to study ERs and ARs in chronic liver disease and HCC may contribute to the development of novel therapeutics or prognostic markers. These studies may also be further helpful in resolving controversies about the use of hormonal therapy for HCC.

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REVIEW

MYC and gastric adenocarcinoma carcinogenesis

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INTRODUCTION

A temporal decline in gastric cancer (GC) incidence has been seen in several countries, including Brazil^[1,2]. However, this cancer causes nearly one million deaths a year worldwide and is still a serious public health cancer^[3], especially in the Pará State, Northern Brazil, where mortality rates are higher than the national average rate^[2]. GC is usually diagnosed at advanced stages and the single curative therapy available requires surgical resection^[4].

Over 95% of gastric malignancies are adenocarcinomas^[5]. They are subdivided into two main histological types: well-differentiated or intestinal-type, and undifferentiated or diffuse-type^[6]. Intestinal-type gastric tumors predominate in high-risk geographic areas whereas diffuse-type tumors are more common in low-risk areas^[7].

The identification of peculiar genetic characteristics of gastric tumors may help predict prognosis of GC patients and allow more accurate therapeutic approaches. Genetic analyses of GC suggest that there occur structural and functional alterations of several oncogenes and tumor suppressor genes, as well as genetic instability^[8]. Additionally, GC has been an interesting carcinogenesis model. Evidence suggests that intestinal- and diffuse-type gastric carcinomas develop through distinct genetic pathways due to different genetic alterations identified in these histological types^[9,10].

MYC (C-MYC) oncogene has been described as a key element of several carcinogenesis processes in humans^[11]. In the present review, we focus on the deregulation of the MYC oncogene in gastric carcinogenesis.

Abstract

MYC is an oncogene involved in cell cycle regulation, cell growth arrest, cell adhesion, metabolism, ribosome biogenesis, protein synthesis, and mitochondrial function. It has been described as a key element of several carcinogenesis processes in humans. Many studies have shown an association between MYC deregulation and gastric cancer. MYC deregulation is also seen in gastric preneoplastic lesions and thus it may have a role in early gastric carcinogenesis. Several studies have suggested that amplification is the main mechanism of MYC deregulation in gastric cancer. In the present review, we focus on the deregulation of the MYC oncogene in gastric adenocarcinoma carcinogenesis, including its association with *Helicobacter pylori* (*H. pylori*) and clinical applications.

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Key words: MYC; Gastric adenocarcinoma; Gastric

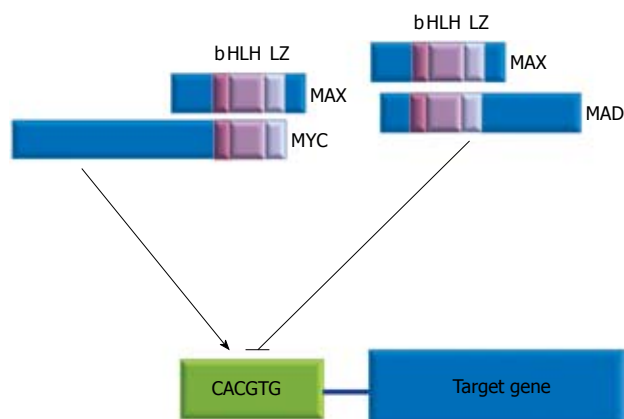


Figure 1 Activation of MYC target genes by the interaction between MYC:MAX or their repression by MAX:MAD. Domains that are common to each protein and are involved in heterodimerization are shown; b: Basic region; HLH: Helix-loop-helix; LZ: Leucine zipper. E-box sequence is shown in green.

MYC AND CANCER

MYC gene was found to be the cellular homolog of retroviral v-myc oncogene about 30 years ago^[12-14]. It is located on chromosomal region 8q24.1, has 3 exons^[15,16] and encodes a nuclear phosphoprotein^[17].

MYC has to heteromerize with MAX, a protein expressed constitutively, to acquire DNA-binding activity. MYC/MAX dimers are made viable by a basic region helix-loop-helix leucine-zipper motif (bHLH-Zip), conserved sequences in the carboxyl terminus of both proteins. MYC/MAX dimers bind to E-box sequence CACGTG in the promoters of specific target genes and stimulate their transcription^[18].

MYC has an effect on up to about 15% of genes in genomes of many organisms, from flies to humans^[19]. Groups of genes involved in cell cycle regulation, metabolism, ribosome biogenesis, protein synthesis, and mitochondrial function are over-represented in the Myc target gene network.

MYC also consistently represses genes involved in cell growth arrest and cell adhesion^[20]. Dominguez-Sola *et al.*^[21] recently showed that Myc interacts with the pre-replicative complex and localizes to early sites of DNA synthesis. Thus, it also has a direct role in the control of DNA replication.

MYC regulates transcription from its targets through several mechanisms, including recruitment of histone acetylases, chromatin modulating proteins, basal transcription factors and DNA methyltransferase^[22-26].

Protein products of *MYC* target genes go on to mediate the downstream effects of *MYC* on cell biology. MYC is then rapidly degraded, and the pathway switches to a transcriptionally repressive state when MAX dimerizes with a group of related bHLH-Zip proteins, the MAD family, that act as MYC antagonists^[27] (Figure 1).

MYC expression might be regulated transcriptionally (initiation and elongation), post-transcriptionally (mRNA stability and translation) or post-translationally (protein stability)^[28].

MYC is generally recognized as an important regulator of proliferation, growth, differentiation and apoptosis^[29,30]. Therefore, it is also accepted that the deregulation of *MYC* expression is a major event in cancer pathogenesis or progression. Deregulated expression of a wild-type MYC protein is sufficient to lead to cellular transformation *in vitro* and tumorigenesis *in vivo*^[31].

Recent studies have also found that MYC oncoprotein, in addition to its directly transforming role, can mediate genomic instability *via* the induction of reactive oxygen species and by promoting whole chromosome instability leading to tetraploidy and aneuploidy. MYC's ability to promote chromosomal instability is closely linked to its function as a transcriptional regulator^[32]. Our research group reported higher frequency of tetraploid clones in GC cell line^[33] and aneuploid cells in primary gastric tumor^[34,35].

Oncogenic alterations of *MYC* are commonly induced by events such as point mutations, gene amplification, chromosomal translocation, viral insertion at the *MYC* locus, and resistance of MYC protein to ubiquitin-mediated proteolysis and enhanced transcription or translation by other oncogenic signaling pathways^[30].

MYC AND GASTRIC CARCINOGENESIS

MYC overexpression has been described in over 40% of GC^[36]. We found that MYC protein was expressed in all cases of both intestinal- and diffuse-type gastric adenocarcinoma samples of individuals from Northern Brazil^[37]. Table 1^[38-59] shows the proportion of cases with MYC aberration in several GC studies.

Several studies have shown the association between MYC expression and histopathologic characteristics. Xu *et al.*^[51] and Yang *et al.*^[54] described a significantly higher expression of MYC in intestinal-type than in diffuse-type GC.

Kozma *et al.*^[50] and Yang *et al.*^[54] reported that higher MYC expression was associated with the presence of metastasis. Onoda *et al.*^[41] also found MYC mRNA levels were higher in metastatic than in primary lesions. Han *et al.*^[45] described that patients with high levels of MYC expression had poor disease-free survival. Therefore, MYC expression may represent an aggressive phenotype of GC.

MYC overexpression has also been seen in early GC when tumor invasion is confined to the mucosa or submucosa regardless of the presence of lymph node metastasis^[40,41,43,46,52,54,59]. Yang *et al.*^[54] found a significantly higher expression of MYC in advanced GC than in early stage GC. However, Onoda *et al.*^[41] reported that MYC expression was found to be more frequent and stronger in early than in advanced lesions. Other studies have not found this same difference.

Several studies demonstrated an increased MYC expression in pre-cancerous gastric lesions and its increased expression also has been associated with *Helicobacter pylori* (*H. pylori*) infection. *H. pylori* is defined as a carcinogen factor to gastric carcinoma infection by the International Agency for Research on Cancer (IARC)^[60].

Table 1 Several MYC studies in gastric cancer

Reference	Cause of MYC deregulation	Increased MYC	Number of cases	Rate (%) of cases with MYC deregulation
[38]	Overexpression	Protein	88	55
[39]	Overexpression	Protein	213	23.5
[40]	Amplification/Overexpression	DNA/Protein	31/51	12.9/41.2
[41]	Overexpression	RNA	51	68.6
[42]	Amplification	DNA	23	26
[43]	Amplification	DNA	21	48
[44]	Amplification	Protein	154	15.5
[45]	Overexpression	Protein	48 advanced/28 early	50/42
[46]	Overexpression	Protein	98 advanced/45 early	28/34
[47]	Amplification	DNA	51	24
[48]	Amplification	DNA	10	30
[49]	Overexpression	Protein	42 advanced/77 early	40.5/15.6
[50]	Amplification/Overexpression	DNA/Protein	23	26
[51]	Overexpression	Protein	30 advanced/ 6 early	63.3/50
[52]	Overexpression	Protein	35 advanced/74 early	34/16
[53]	Overexpression	Protein	84	88.1
[54]	Overexpression	Protein	63	52.4
[55]	Overexpression	Protein	65	61.5
[56]	Amplification	DNA/Protein	11	100
[37]	Amplification/Overexpression	DNA/Protein	7	100
[57]	Overexpression	Protein	204	43
[58]	Overexpression	Protein	71	42.3
[59]	Amplification/Overexpression	DNA/Protein	5 early	100

Chronic gastritis caused by *H pylori* infection may progress to intestinal metaplasia and even to GC^[61,62].

Tatsuta *et al*^[63] evaluated MYC mRNA expression by in situ hybridization in 31 elevated gastric lesions. Patients who had borderline lesions with and without MYC overexpression were followed up with repeated endoscopic examinations and gastric biopsies. The authors reported that well-differentiated elevated-type adenocarcinomas were detected in 46% of patients with elevated lesions that presented MYC overexpression during a follow-up period of about 15 mo (range, 2-32 mo) and that no cancers were found in patients with elevated lesions without MYC overexpression. These sample groups were significantly different. Therefore, MYC overexpression may provide a valuable tool for distinguishing between adenomas and well-differentiated elevated-type adenocarcinomas.

Xu *et al*^[51] noticed that MYC protein expression increased progressively as follows: chronic active gastritis, gastric ulcer, mild nonclassic proliferation, severe non-classic proliferation, early GC, and progressive GC.

Lan *et al*^[53] found that MYC expression was higher in GC than in chronic gastritis, intestinal metaplasia and dysplasia. MYC expression was higher in type III intestinal metaplasia with *H pylori* compared to the same metaplasia without infection and the positive rate in dysplasia with *H pylori* was higher than that without infection. Zhang *et al*^[55] also reported that MYC expression was higher in chronic atrophic gastritis with severe intestinal metaplasia than that with mild intestinal metaplasia. In chronic atrophic gastritis with severe intestinal metaplasia, MYC expression was higher in cases with *H pylori* infection than in those without infection. Higher MYC expression was also found in GC with *H pylori* infection than in that without infection.

Thus, MYC expression was coordinately up-regulated in *H pylori* infected GC and chronic atrophic gastritis with severe intestinal metaplasia. Authors have suggested that *H pylori* infection may affect MYC expression in gastric diseases, especially in chronic atrophic gastritis.

Several studies have shown that patients with preneoplastic and neoplastic gastric epithelial lesions are more likely to be infected by cagA positive strains. *H pylori* cagA is one of the most virulent strains of *H pylori*. Increased cancer risk is described in individuals infected by cagA-positive *H pylori* strains compared with those infected by cagA-negative *H pylori* strains and, in general, in those living in areas with a high rate of cagA-positive *H pylori* strains^[64]. Yang *et al*^[54] compared MYC expression in gastric tissues (intestinal metaplasia, dysplasia and GC) with and without *H pylori* cagA. These authors found that MYC expression was significantly higher in those lesions of type III intestinal metaplasia and dysplasia II-III with cagA than in those without cagA. Nardone *et al*^[64] also suggested that the increased prevalence of MYC expression was in agreement with the high prevalence of cagA positivity seen in the population studied.

Kim *et al*^[65] investigated the expression of MYC protein and mRNA in 22 patients with chronic gastritis who had been successfully treated for *H pylori*. Two endoscopic antral biopsies were taken before and 2 mo after *H pylori* eradication. The proportion of gastric antral epithelial cells expressing MYC protein was significantly lower after *H pylori* eradication. MYC mRNA expression was not changed by *H pylori* eradication. *H pylori* may affect cell cycle progression and carcinogenesis through post-translational effects on specific gene expression. Nardone *et al*^[64] also found that MYC expression disappeared after *H pylori* eradication.

In vitro studies have also confirmed that *H pylori* can

affect *MYC* expression. Yang *et al*^[66] described that *H pylori* induces apoptosis in human gastric adenocarcinoma cells mediated by an increased expression of *MYC* mRNA.

Epstein-Barr virus (EBV) is another infectious agent thought to contribute to cancerous transformation of human host cells. EBV infection is seen in about 10% of gastric adenocarcinoma cases^[49,58,67]. Ishii *et al*^[49] found *MYC* expression in early stages of EBV-positive GC was higher than that of EBV-negative GC, while *MYC* expression in advanced stages of EBV-positive GC was lower than that of EBV-negative tumors. It was inferred that EBV might cause the host cell to induce *MYC* expression in early cancer development, but then negatively affect *MYC* expression in advanced stages of cancers, making them less likely to have a natural regression *via* apoptosis. Lima *et al*^[58] also reported *MYC* low expression in EBV-positive GC samples. However, Luo *et al*^[67] have not found any correlation between EBV and *MYC* expression in GC, suggesting that EBV does not inhibit *MYC* expression in advanced stages of EBV-positive gastric cancer.

MECHANISMS OF MYC DEREGULATION IN GASTRIC CANCER

Copy number gains are frequently detected along chromosome 8 in gastric tumors^[43,48,56,68-73]. Suzuki *et al*^[43] described that chromosome 8 copy number was significantly higher in differentiated than undifferentiated types of GC. Our research group found 8q24.1 gain, where *MYC* is located, exclusively in intestinal subtype with metastasis by comparative genome hybridization (CGH)^[72]. However, Koo *et al*^[48] reported that amplifications in 8q region were more common in diffuse-type cancer.

Some studies have showed an association between *MYC* amplification and GC^[42-44,48]. We have also previously seen *MYC* amplification in intestinal adenocarcinoma by dual-color fluorescence in situ hybridization (FISH), such as homogeneously staining chromosomal regions and double minutes, supporting our CGH results^[56]. Our findings support that these two histological GC types follow different genetic pathways.

Our research group also found that all five early GC cases with *MYC* overexpression also had three signal to *MYC* gene by FISH assay, varying between 13% and 26% of cells/case^[59]. Suzuki *et al*^[43] found *MYC* amplification in all 6 early GC cases studied, varying between 19% and 89% of cells/case, and this rate was not significantly difference from that found in advanced GC samples. These findings suggest that *MYC* amplification can be a critical event to gastric carcinogenesis.

MYC translocation is frequently described in Burkitt's lymphoma. Few studies have also found translocation of the *MYC* locus associated with gastric carcinogenesis. Yamashita *et al*^[74] identified chromosomal translocations involved in 8q24 breakpoint by spectral karyotyping (SKY) analysis of established GC cell lines and cancerous ascitic fluids. In a previous study, our findings

suggested that translocations can be related to diffuse-type GC using FISH assay^[37,56].

Epigenetic events play a significant role in cancer development and progression. DNA methylation is the most studied epigenetic alteration. Some studies also have demonstrated that *MYC* hypomethylation, which leads to its activation, is significantly more common in GC samples than non-cancerous tissues^[75,76]. Fang *et al*^[77] and Weng *et al*^[78] suggest that folate level reduction is associated with upregulation of *MYC* expression and its promoter hypomethylation in GC.

FUTURE PERSPECTIVES

Proto-oncogenes have a major role not only in cancer development, but also in cancer therapies^[79]. *MYC* alteration is seen in the early gastric carcinogenesis progress. The detection of *MYC* locus amplification may be used as an auxiliary tool to GC diagnosis and as a predictor of GC aggressiveness.

MYC also could be used as a therapeutical target. Several experimental studies showed that *MYC* inactivation suppresses tumors in animal models, suggesting *MYC* as a molecular target in cancer treatment^[80-83].

Chen *et al*^[84] evaluated the effect of *MYC* expression inhibition by recombinant antisense *MYC* adenovirus (Ad-ASc-myc) infected SGC7901 human gastric carcinoma cells, which have *MYC* gene amplification, in the proliferation, apoptosis and growth processes of human gastric tumors in nude mice. It was found that *MYC* expression inhibition may strongly inhibit cell growth and induce apoptosis in SGC7901 cells. Proliferation of Ad-ASc-myc-infected SGC7901 cells was reduced by 44.1%. Studies involving tumorigenicity in nude mice and experimental therapy in nude mice model using Ad-ASc-myc also support these findings. These studies also suggest that Ad-ASc-myc overexpression may result in the elimination of tumor cells *via* apoptosis and proliferation inhibition, and therefore reduce tumor burden.

Inhibiting *MYC* expression can be a potential tool for GC treatment in tumors with *MYC* overexpression. *MYC*'s therapy target may help identifying more specific and less toxic therapeutic agents^[30].

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Insight into congenital absence of the portal vein: Is it rare?

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Abstract

Congenital absence of portal vein (CAPV) was a rare event in the past. However, the number of detected CAPV cases has increased in recent years because of advances in imaging techniques. Patients with CAPV present with portal hypertension (PH) or porto-systemic encephalopathy (PSE), but these conditions rarely occur until the patients grow up or become old. The patients usually visit doctors for the complications of venous shunts, hepatic or cardiac abnormalities detected by ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI). The etiology of this disease is not clear, but most investigators consider that it is associated with abnormal embryologic development of the portal vein. Usually, surgical intervention can relieve the symptoms and prevent occurrence of complications in CAPV patients. Moreover, its management should be stressed on a case-by-case basis, depending on the type or anatomy of the disease, as well as the symptoms and clinical conditions of the patient.

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Key words: Congenital absence of portal vein; Abernethy malformation; Focal nodular hyperplasia

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INTRODUCTION

The first account of congenital absence of the portal vein (CAPV) was given by John Abernethy in 1793^[1], based on a postmortem examination of a 10-mo-old female, which revealed the termination of portal vein in the inferior vena cava at the insertion level of the renal veins and multiple congenital abnormalities other than CAPV. It was reported that complete portosystemic shunts not perfusing the liver *via* portal vein are defined as type I, whereas partial shunts with a remaining degree of portal perfusion to the liver are defined as type II^[2]. Furthermore, type I is sub-classified into types Ia and Ib depending on the anatomy of portal vein. In type Ia, the splenic vein (SV) and superior mesenteric vein (SMV) drain separately in type Ia, while both drain together in type Ib after uniting to form a common trunk^[3]. Howard and Davenport^[4] suggested that the congenital diversion of portal blood away from the liver, by either an end-to-side or a side-to-side shunt, is known as the Abernethy malformation. Thus, CAPV associated with extra hepatic portocaval shunts can be referred to as an Abernethy type I malformation (Figure 1).

ANATOMY AND EMBRYOLOGY

The portal vein returns blood from the intraperitoneal section of the gastrointestinal tract and the spleen, pancreas, and biliary apparatus, while SV and SMV return blood from the portal vein. At the porta hepatis, the portal vein is subdivided into right and left branches (besides providing the quadrate lobe with an additional branch). These branches ramify to form small vessels that drain into the sinusoids^[5]. CAPV with an extrahepatic porto-caval shunt means that the mesenteric vasculature where splanchnic blood bypasses the liver through a congenital shunt vessel, completely drains into the systemic circulations, such as inferior vena cava, left renal vein, right atrium, iliac vein, left hepatic vein (HV), and azygos vein^[6-8]. Strictly speaking, CAPV is also characterized by complete absence of venules within the portal areas, which has been confirmed by liver biopsy^[9,10]. The case seems to be a complete portal vein agenesis^[10]. Similarly, complete absence of the portal

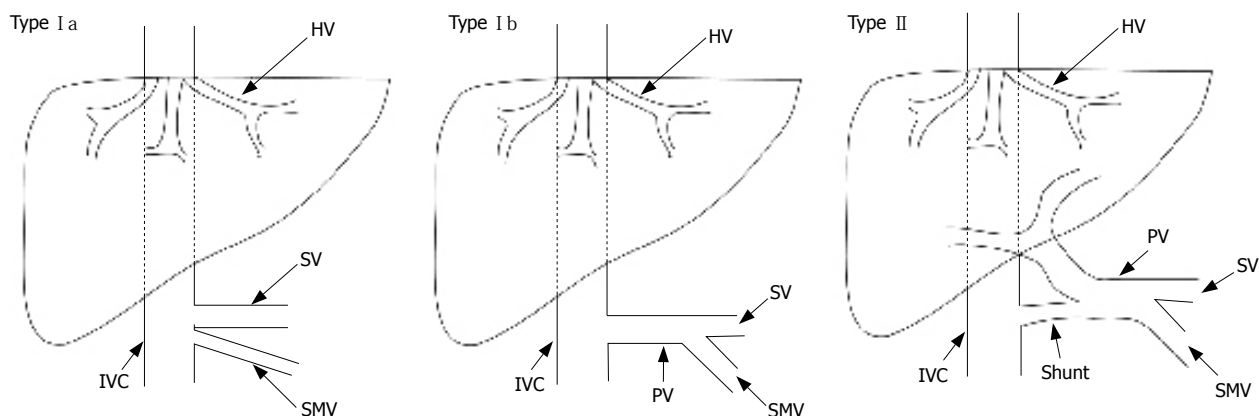


Figure 1 Schematic demonstration of different types of Abernethy abnormality^[2]. SMV: Superior mesenteric vein; HV: Hepatic vein; SV: Splenic vein; PV: Portal vein; IVC: Inferior vena cava.

vein with intrahepatic portal venules can be named portal vein atresia.

The portal vein develops embryologically between the 4th and 10th weeks^[10,11]. In the 4th week embryo, three paired venous systems are present: umbilical veins of chorionic origin, vitelline veins from the yolk sac and cardinal veins from the body of the embryo. Towards the end of the 4th week, three cross links are formed between the right and left vitelline veins^[12]. The intrahepatic portal veins develop from the superior link, while the extrahepatic portal vein forms during the selective involution of the caudal part of the right and left vitelline veins. Selective involution of these communications generates the fully developed portal vein^[11]. Primary failure to form this critical anastomosis will lead to complete or partial absence of the portal system. As a result, mesenteric and splenic venous flow cannot but drain into renal veins, HVs, or directly into the inferior vena cava (IVC)^[6-8,13], with relatively poor perfusion of the liver^[14].

CLINICAL MANIFESTATIONS

CAPV can cause a broad spectrum of clinical manifestations, which can be divided into two groups: concomitant congenital abnormalities and sequent syndromes. The former can be referred to as a congenital hepatopathy and a congenital cardiopathy, which can be explained by its close relation to the development of cardiovascular system.

Systemic shunting of the visceral venous return can lead to abnormal hepatic development, function and regeneration secondary to the absence of portal hepatotrophic factor, resulting in the development of focal nodular hyperplasia (FNH) and hepatic tumors^[15-17]. Other than CAPV, many cases are presented with liver abnormalities, including FNH^[7,15,18-30] or nodular regenerative hyperplasia (NRH)^[8,31-35]. Several patients have been found to have combined hepatocellular adenoma^[31,36,37]. These are generally considered benign parenchymatous lesions, and also have the potential to deteriorate into hepatoblastoma^[26,28] and hepatocellular carcinoma (HCC)^[12,20,38,39]. CAPV, though rare, could

be found along with chronic hepatitis^[20] and liver cirrhosis^[17]. Gocmen *et al*^[40] reported a 7-year-old boy with congenital hepatic fibrosis (CHF). Intrahepatic calcification was detected during prenatal diagnosis in a fetus. The liver volume is often larger than normal because of the regenerative or hyperplasia nodular change and sometime hepatomegaly might be observed^[15]. Moreover, the mass can cause a series of mechanical pressure symptoms, such as mild intermittent jaundice and pruritus^[35]. Nevertheless, the liver size may be small due to insufficient portal vein supply or lobular hypoplasia^[41,42]. Abnormal hepatic development, with aberrant lobation, absence of ligamentum teres and falciform ligament, has been noted in Hellweg's patients^[43]. Liver dysfunction (LD), frequently found in liver tumors, also occurs in patients without liver mass.

CAPV may result from an embryologic insult which causes defect of the cardiovascular system and complicated cardiogenesis could be affected by the insult or the systemic diversion of portal venous flow^[11]. Congenital cardiac diseases including atrial septal defect (ASD)^[2, 21,26,28,37,44,45] or patent foramen ovale^[43], ventricular septal defect (VSD)^[16,17,21,26,31,40], and patent ductus arteriosus (PDA)^[12,23,43,44] are frequently observed along with CAPV. Bellah *et al*^[45] have also detected a fatal congenital hypertrophic cardiomyopathy with ultrasound in an infant. Dextrocardia^[11] and Mesocardia^[44] that reported congenital cardiac abnormalities are rarely found in CAPV cases. Congenital stenosis of aortic valve^[11] and pulmonary artery valve^[45], found in a few CAPV cases, can cause tricuspid regurgitation^[44]. Even stenosis^[11] or coarctation^[12] has been observed in aorta. Most frequently encountered Cardiovascular lesions, most frequently encountered in CAPV patients with Goldenhar syndrome, are tetralogy of Fallot and VSD^[46]. Cardiomegaly has been noted in CAPV patients^[41,47], which may result from congenital insult or increased blood flow into the heart with a certain degree of congestive heart failure. A large number of non-heart abnormalities are found in all the cases reviewed^[3,9,14,27,34-36,39,48,49].

Besides CAPV, visceral or cutaneous vascular

malformations have been reported, such as double SV^[1], double inferior vena cava^[40], left sided IVC^[2,17,26], hepatic artery originating from superior mesenteric artery^[8,50], intrapulmonary shunting^[7], azygos and hemiazygos continuation^[1,17,43,48], and skin hemangioma^[14,43]. Obvious compensatory changes may be found in blood vessels, especially in veins returning visceral blood or ramus anastomoticus, such as the SV^[51], inferior mesenteric vein^[33,52], left renal vein^[44,53], azygos system^[30,51], IVC^[14,44,53], and right atrium^[14]. Hepatic artery is enlarged and hypertrophied^[1,19] due to the same reason.

Polysplenia^[1,2,4,17,26,54], megalosplenia^[14,24,51] and hypersplenism^[51] are the frequently encountered splanchnic abnormalities other than hepatic and cardiac abnormalities, which may be due to the embryonic impairment or portal hypertension (PH). The latter is a fault because CAPV accompanying PH has merely been observed in several cases^[9,16,55], which is inconsistent with our general deduction. One reasonable explanation is out of the congenital adaptation.

It was reported that CAPV patients could also have congenital biliary atresia (CBA)^[2,4,17,21], congenital choledochal cyst^[29], and intrahepatic gallbladder^[43]. The urinogenital system including cystic dysplasia of the kidneys^[22,24], bilateral ureteropelvic obstruction of the kidneys^[45], vesicoureteral reflux^[33], crossed fuses renal ectopia^[33,40], and hypospadias^[56], is also involved. Nonfunctioning pancreatic tumor^[25], ulcerative colitis (UC)^[33], juvenile polyposis^[21], inguinal hernia^[23,37], and even situs inversus viscerum^[2,4], are sporadically observed in CAPV patients.

The skeletal systematic abnormalities, such as radial hypoplasia^[33] and congenital absence of the first metacarpophalangeal complex of the right hand^[33], are noted in Grazioli's patients. Vertebral abnormalities or hemivertebra scoliosis^[15,40,57], and oculoauriculovertebral dysplasia or Goldenhar's syndrome^[16,26,28,58] exhibit thoracic hemivertebrae, right maxillary hypoplasia, mild micrognathia, and short fifth fingers, are not uncommon in CAPV patients.

Sequent syndromes, which can result in poor prognosis^[27], are mainly related to LD or liver abnormalities besides CAPV malformation. Although most cases do not possess any liver abnormalities^[1,3,9,27,43-45,47-49,54,57], CAPV patients suffer from different levels of LD^[2-4,9,15,16,20,23,24,26-29,34,37,38,44,47,55,56] possibly due to the lack of portal flow. Hepatic encephalopathy, hepatopulmonary syndrome (HPS), and hepatorenal syndrome are closely related to metabolic disorder because of liver lesions, including hyperammonemia^[3,27,41,59] and galactosemia^[20,27]. Toxic compounds produced in the digestion process only bypass the liver into the systemic circulation in CAPV patients, and are prone to cause hepatic encephalopathy^[23,26,27,34,40,49,56]. Mild CAPV patients present with cognitive retardation^[33] or mental retardation^[27], and their symptoms are merely drowsiness or delirium^[56]. Wakamoto *et al*^[23] reported the first case of sub-clinical porto-systemic encephalopathy (PSE) with CAPV. When it becomes worse, tremor

or orthostatic disturbance^[56], abscess in the brain^[60], even epilepsy^[38] and cerebellar meningioma^[55] may occur. If the porto-systemic shunt ratio is high enough, HPS^[27,30,60-63] may occur, including cyanosis^[30] of the hands, feet, and lips, digital clubbing and pectus excavatum^[60], bronchial asthma^[8,23] or hypoxemia-induced bronchial stenosis^[56], pulmonary hypertension^[56,64]. When the shunt ratio is over 90%, CAPV patients would have hematuria and proteinuria, namely hydropigenous nephritis^[65]. CAPV patients also could have chronic renal failure^[33,34]. Gonadal hormonal disorder^[33,47,66] can result in hypergalactocemia^[66-68], primary amenorrhoea and signs of virilization^[33]. Satoh's patients present with hyperandrogenism, insulin resistant hyperinsulinaemia, and hyperglycaemia^[47]. A few cases of PH^[9,16,55], rectal bleeding^[9,21,33,42], anaemia^[31,33] and peripheral edema^[21] have also been reported. All the metabolic disorders described above can lead to growth retardation^[21,40,56], small head or microcephaly^[14,38].

Routine clinical examination can find the above or other diseases in CAPV patients, suggesting that there are a large number of asymptomatic CAPV patients.

ETIOLOGY

Cardiovascular system

Embryologically, paired vitelline veins enter the embryo with yolk stalk, anastomose with each other around the developing duodenum forming a loop, and pass through the septum transversum to the sinus venosus. Portal venous system development occurs depending on selective apoptosis of the bilateral vitelline veins and their median links before entering the septum transversum^[33]. The whole process is complex and coinciding, any insult may affect the development resulting in a preduodenal portal vein, CAPV and duplications, as well as communications between the portal and pulmonary veins^[10,12,16,28]. The pathogenesis of CAPV may be attributed to excessive involution of the peri-intestinal vitelline venous loop^[10,13,22,69], or to total failure of the vitelline veins to establish the critical anastomosis with hepatic sinusoids^[4]. Behind the abnormalities, the initiative event may be referred to genetic mutation or chromosome variation as CAPV has been sometimes reported in conjunction with chromosomal disorders^[70], such as translocation (2,10)^[21] and turner syndrome (45, XO)^[6,71]. The associated extrahepatic portosystemic shunts may occur due to the persistent subcardinohepatic anastomosis with the vitelline veins. The subcardinohepatic anastomosis connects the vitelline vein that develops into the portal system and the right subcardinal vein that develops into the renal segment of the IVC, as well as forms the hepatic segment of the IVC, thus accounting for the high incidence of draining points at the suprarenal IVC^[3].

Cardiac malformations are frequently observed in patients with CAPV^[11,72], the close relationship between the development of vitelline veins and the heart in embryonic life may be responsible for the association

between cardiovascular malformations and CAPV^[12,40,45]. The cardiac abnormalities may result from a some embryogenic insults and compensate for the congestive effect of portal vein absence and shunting^[37], indicating that prenatal insult occurs during the concurrent development of the heart and gastrointestinal tract. However, it was also supposed that systemic shunt of the portal venous flow could adversely affect the hepatic and cardiac development and function^[11]. It was reported that concomitant atrial and ventricular septal defects related to CAPV may be attributed to a congenital adaptive change occurring during the development from the embryonic stage, which tends to compensate for the congestive effects of portal venous aplasia^[10]. CAPV can result in cardiomegaly or even congestive heart failure due to the shunts of blood flow^[10].

Liver

The importance of intact portal vein flow following liver resection or transplantation has been recognized both in experiments and in clinical practice^[46,73]. The lack of portal flow can affect the development, function and regenerative pability of the liver. The importance of certain substances, such as insulin and glucagon, is underscored because of CAPV^[73], and these substances are no longer supplied to the liver through the mesenteric blood flow and lead to hepatic hypoplasia because they help maintain the hepatic structure and function^[74-77]. The hepatic volume is extremely small compared with the standard one as detected by computed tomography (CT) volumetry^[56]. However, as far as the regenerative ability of the liver is concerned, such an assumption is questionable. In fact, in cases of CAPV, the regenerative ability appears to be normal after liver lobectomy and trisegmentectomy^[16,37].

CAPV is frequently observed with hepatic tumors and tumor-like conditions, such as FNH and NRH^[40], suggesting that intrahepatic changes due to hemodynamic imbalance participate in the development of liver tumors^[8]. In CAPV patients, liver is supplied only by the hepatic artery in the absence of the portal vein^[18]. Many CAPV patients have an enlarged and hypertrophied hepatic artery with a high flow^[1,19,50,54] as well as the absence of portal vein, or the presence of hypervascular liver tumor^[28]. Such conditions affect the development, function and regenerative capacity of liver, thus predisposing to the development of nodular dysplasia, hepatocarcinoma, or other benign and malignant hepatic tumors^[38]. It was reported that abnormal hepatic circulation is one of the etiological factors for hepatocellular hyperplastic nodular lesions^[78-80]. Both CAPV and other situations, such as Budd-Chiari syndrome, cause abnormal hepatic flow or peripheral portal venous thrombosis^[81,82]. HCC can occur in patients with chronic Budd-Chiari syndrome^[82]. Cells in the hyperplastic nodules contain fat deposits^[8,83,84], which can be differentiated from other masses.

Marois *et al*^[16] demonstrated that abnormal thin-walled vessels filled in a retrograde fashion from hepatic arteries, may be too weak to burden the changed flow.

FNH is an uncommon benign tumor-like lesion of well-circumscribed hyperplastic liver parenchyma, often with central stellate scars. These lesions are hypervascular and can be supplied exclusively with arterial blood^[79,85]. FNH, derived from acquired thrombosis, has been reported as well^[85]. NRH is due to obstruction or narrowing of portal branches caused by thrombosis or atrophy of areas with severely impaired blood flow, and hypertrophy of areas with a relatively mild impairment of blood flow, leading to nodular formation^[80].

Yoshidome *et al*^[86] observed morphological alterations in the liver parenchyma of patients with congenital portocaval shunts, and proposed that morphological changes in the liver of patients with cirrhosis and acquired portocaval shunts as well as HCC may be explained by a common mechanism, namely reduced portal flow. However, circulatory disturbance alone cannot explain the pathogenesis and the underlying unknown mechanism^[25]. Simple occlusion of the portal vein and a compensatory increase in arterial blood flow have been proved insufficient for nodule formation^[82]. Although rare, fibrosis may develop due to hemodynamic imbalance^[20], because flow disturbance only affects hyperplastic hepatic cells but not mesenchymal cells. CAPV occasionally involves HCC, and 40% of HCC patients have no cirrhosis or chronic liver disease^[87,88], suggesting that HCC is related to genetic alterations^[38]. Indeed, early genetic alterations or the common genetic pathways of hepatic tumors and CAPV would allow accurate comprehension of the commensalisms.

Intrahepatic bile ductules develop from the primitive ductal plate. It was reported that the portal vein plays a crucial role in the formation and remodelling of the ductal plate^[89]. Lack of remodeling of the ductal plates results in persistence of an excess of embryonic bile duct structures. This is why biliary atresia and choledochal cysts concur in CAPV patients.

PSE

When the portal vein is absent, toxic metabolites such as ammonia and bile acids collected from the gastrointestinal tract have to bypass the liver directly drainage into the systemic circulation, thus may initiate hepatic encephalopathy. Interestingly, PSE is rarely observed in CAPV patients with mild hyperammonemia and CAPV patients show no clinical manifestations of hepatic encephalopathy until they become obvious^[41]. Only a small number of CAPV cases present with subclinical PSE^[23,90-92]. Although PSE is not usually observed in CAPV patients, the serum ammonia level in such patients is not always highly elevated. In fact, a significantly low blood ammonia level in SMV is discovered in patients with CAPV^[36,37], suggesting that this low level might indicate the presence of a homeostatic control mechanism^[37]. The presence of compensatory alterations in intestinal bacterial flora has been suggested as an explanation^[9,15,16]. Kamiya^[36] analyzed intestinal flora in faeces of CAPV patients and healthy persons before operation, and did not isolate any microorganisms with a strong urease activity. However,

intestinal microorganisms isolated from the faeces of patients after operation produced as much urease *in vitro* as from healthy volunteers^[36], indicating that some inhibitory factors for urease-positive microorganisms may exist in the intestinal tract. It was reported that *lactulose* is effective on hyperammonemia of hepatic encephalopathy by inducing a remarkable growth of *Lactobacillus* to produce lactic acids which interfere with urease-producing microorganisms^[93,94]. It is also possible that proteolysis might be inhibited in the intestine of patients, causing decreased production of ammonia^[36]. Kavukcu *et al.*^[95] hold the opposite opinion as among the varieties of bacteria displaying urease activity, only three species have been detected: *Klebsiella pneumonia*, *Enterococcus avium*, and *Peptostreptococcus productus*. Moreover, the number of these bacteria is extremely small with no significant differences observed in the flora in fecal specimens obtained before, during, and after surgery^[95]. Another likely explanation is that PSE might be due to the increased sensitivity of an aging brain to ammonia and other toxic materials^[23] or that homeostatic control may gradually become disordered with increasing age^[52]. The brain sensitivity to ammonia or other toxic metabolites may increase with aging^[96]. Healthy brain may tolerate to high ammonia levels, while aging brain may not cope with high ammonia and other metabolites and develop symptoms^[96-98]. Such mechanisms may contribute to the delayed presentation with hyperammonemia-related encephalopathy^[99]. Moreover, another plausible one is that the thin anastomoses at birth slowly become large as the patient ages. The shunt ratio may play a certain role in the occurrence of symptoms^[100]. Certain special unknown mechanisms underlying CAPV lead to the delayed PSE.

Others

Bile acids are synthesized in the liver, secreted into bile ducts, and expelled into the intestinal lumen where they are reabsorbed into the systemic circulation *via* the lymphatic system followed by hepatic uptake from the portal vein, and then metabolized in the liver. However, when the portal vein is absent, these fatty acids, after absorption by the intestinal tract, assume the form of chylomicrons that are transported into the vena cava. Before arriving at the liver, blood from the vena cava reaches the capillaries of peripheral tissues, including adipose and muscular tissue where fat is accumulated, and this presumably constitutes the cause of obesity in such cases^[37]. Gitzelmann *et al.*^[67] detected patients with hypergalactosaemia along with congenital portosystemic shunts^[66-68], and proposed that high blood galactose found in newborns is useful for detecting this abnormality. Hypergalactosaemia might also result from insulin resistance (IR) which is correlated to LD^[47]. Imbalance between vasodilator and vasoconstrictor substances has been reported in CAPV patients with HPS^[27,30,60-63], and decrease in metabolism or synthesis of these substances in the liver is responsible for the imbalance^[60]. Mehrotra *et al.*^[101] observed extrahepatic portal vein obstruction in children with high serum

levels of growth hormone and somatostatin (IGF- I), and showed that there is some resistance to the action of growth hormones^[101]. Chronic anemia (secondary to loss of blood caused by bleeding and/or hypersplenism), and intestinal venous congestion with secondary malabsorption may interfere with the growth rate^[31]. Liver dysfunction causing hepatotrophic hormone deprivation also results in growth retardation^[40,59,102]. Takeichi^[34] reported a CAPV case of patient with chronic renal failure in a vicious circle of toxic materials, leading to severe encephalopathy with coma, waiting for liver transplantation (LT).

However, since CAPV cases have individual series of presentations, more or less, subtle or obvious, the pathology of these manifestations seems to be complicated and covered. Further study is needed to confirm its etiology by analyzing more entities.

IMAGEOLOGY

Imaging abnormality is often coincidentally discovered in children with portal vein disorder. Routine liver examination can reveal the extraordinary mass in majority of these children, triggering further imaging exploration. Portosystemic shunt diagnosis is usually based on clinicopathologic and portographic findings^[103]. Portography, ultrasonography, scintigraphy, CT and magnetic imaging are used in diagnosis of this portal vein disorder.

Doppler ultrasonography has been extensively used in evaluating vessels of the abdomen. It is most useful for determining flow direction and pattern besides liver tumor. However, the most important advantage of this technique is its noninvasiveness and no requirement for anesthesia. It yields much useful information about the detailed vascular anatomy as well as hemodynamics^[104]. Prenatal screening and intraoperative ultrasonography is especially superior to other techniques^[104]. Although color Doppler sonography first depicted the image of the absent portal vein in most cases, ultrasonography (US) may fail to accurately detect the associated extrahepatic shunts because of its subtle US features^[31]. Experience and good ultrasound system, knowledge of the examination protocol, and familiarity about the ultrasound anatomy of abdominal vessels and portal vein abnormalities, contribute to the accurate diagnosis of CAPV.

Accurate depiction of intra and extrahepatic vascular anatomy will undoubtedly guide management decisions and surgical or angiographic approaches^[21]. Cross-sectional imaging (CT and MR) is very helpful in depicting the course of portosystemic shunt and in identifying absent vessels and type of malformations^[42,50]. Currently, 3D-computed tomography angiography (3D-CTA) and magnetic resonance angiography (MRA) can confirm CAPV and visualize the portosystemic shunt^[50]. It was reported that multi-slice CTA displays even small vascular branches and has superior spatial resolution to MRA^[105]. The posterior or short gastric veins cannot be visualized

in patients with portal vein disorders by conventional angiographic portography, but can be clearly revealed by 3D-CT portography^[106]. However, a breath-holding technique has been recommended to prevent motion artifacts during scanning by Tsuji *et al*^[8] who assumed that if a patient is able to hold the breath, 3D-CTA can easily capture the entire abnormal vasculature during one breath holding. Unfortunately, CAPV predominately occurs in children difficult to hold breath that urges us to search the substitute. MRA is also a reliable and noninvasive diagnostic modality for the portal venous system^[49]. MR imaging can be used both in diagnosis of CAPV and in evaluation of focal hepatic lesions^[41]. In addition, previous reports indicate that the presence of a portosystemic shunt may cause lesions of middle cerebellar peduncles, which are responsible for cerebellar symptoms^[91]. MR imaging can be used to find cerebral lesions. It was recently reported that conventional high-resolution MR angiography seems unnecessary^[41], as the spatial resolution time of MR angiography is almost equivalent. Since 3D-MRA obtained from quality multiplanar reformatted images and volume-rendered images has the advantage of high temporal resolution, contamination from overlapping vascular structures can be avoided, flow dynamics can be assessed, and quality feature is obtainable even in young children with free breaths because the technique is relatively insensitive to motion artefacts^[41]. Perhaps, it will be widespread in the diagnosis of CAPV several years later.

Although cross-sectional imaging in most cases can approximately suggest the diagnosis of portal vein disorder, the definitive diagnosis can be made only with catheter angiography^[15] and by additional histological analysis of the hepatic parenchyma that demonstrates the absence of hepatic portal venules within the portal triad^[35]. Mesenteric portovenography, a usually indirect technique depicting the portal system anatomy, can clarify CAPV abnormality and extrahepatic shunts. In recent years, CO₂-wedged venography is considered a good and safe technique for demonstrating the portal circulation^[107]. It has such advantages over indirect portography obtained during visceral arteriography^[108] as only a venous puncture is required, free and wedged pressure measurements can be obtained with no iodinated contrast medium injected, and transvenous liver biopsy can be made at the same time. However, opacification of portal vein branches could not be obtained^[35].

Portovenography can facilitate measurement of the pressure gradient indicating vein blood flow and selected embolism shunts as a therapy. In dogs, transvenous retrograde portography^[109] is less invasive than operative mesenteric portography and allows measurement of portal pressures before and after temporary shunt ligation. It also helps differentiate rich-vessel tumor and confirm parenchymal magnetic resonance imaging (MRI) findings. Conventional angiography is not good for children, although it is fairly safe^[49,110].

Rectal portal scintigraphy plays an important part in suspected abnormalities of portal circulation and is

precise to quantitate portosystemic shunts and valuable for clinical diagnosis^[111]. During performing this kind of examination, shunt indexes (SI) are calculated, relative portal hemodynamics can be observed noninvasively, and portal collateral circulation can be detected as well^[112]. This technique is hopeful to be extensively applied in detecting CAPV if not expensive.

DIFFERENTIAL DIAGNOSIS

Definitive diagnosis of CAPV should exclude many seemingly resemble cases. For example, histologically confirmed absence of portal vein in the liver is mandatory in the diagnosis. Abernethy type II was previously misclassified as CAPV^[21]. Kerlan *et al*^[96] reported a case similar to CAPV, but surgery for closing the fistula between portal vein and inferior cava, revealed an intrahepatic portal vein. Absence of stigmata in patients with portal venous hypertension is an important clue to the final diagnosis^[40]. Radiologically, absence of the portal vein must be distinguished from portal vein thrombosis^[15,28], based on the absence of venous collaterals or other secondary signs of PH, such as splenomegaly or ascites. Compensatory hypertrophy of the hepatic artery may be present^[19]. Appel *et al*^[51] assumed that it is a secondary phenomenon, most properly due to thrombotic occlusion of the extrahepatic portal vein. However, gradual thrombosis of the portal vein stem may allow the development of collaterals without acute dramatic episodes, similar to CAPV. The term of portal vein "aplasia" or "agenesis" in such cases is inadequate since intrahepatic bile ducts are normal^[51,113]. Extrinsic compression of tumors, such as HCC and extrahepatic malignant tumor, especially pancreatic adenocarcinoma^[5], is another reason. If the portal flow is ceased, initial thrombus arises asymptotically, the only sign may be the formation of new vessels, which on Doppler US is known as "portal cavernoma" or "cavernomatous transformation" due to the blood volume at the site^[102]. It is not easy to differentiate this condition from Abernethy. It was reported that hepatic nodules with rich artery blood flow may prevent influx of portal blood resulting in increased sinusoidal pressure, the portal vein will not be visualized at portography^[82]. Owing to the progressive growth of tumor in the omentum and mesentery, increased portal flow would produce extrahepatic portosystemic venous shunts^[114].

During fetal life, ductus venosus is the continuation of umbilical vein, which directly inflows into the inferior vena cava, allowing blood returning through the umbilical vein to bypass the portal venous system^[115,116]. Failure to close patent ductus venosus within 2 wk after birth would lead to portosystemic encephalopathy or malformations, including congenital heart disease and minor abnormalities^[117]. Hepatic nodular lesions, such as FNH, and PSE, have been reported in patients with patent ductus venosus^[117,118]. Although the clinical manifestations of patent ductus venosus and CAPV are similar, the mechanism is different. The treatment of

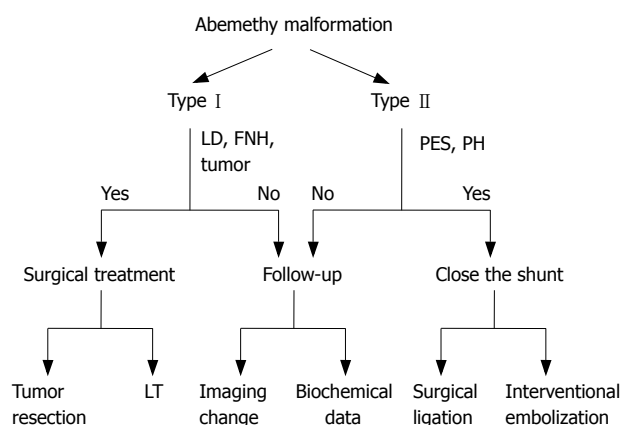


Figure 2 Schematic demonstration of decision-making advices for treatment of Abernethy malformation. LD: Liver dysfunction; FNH: Focal nodular hyperplasia; PSE: Porto-systemic encephalopathy; PH: Portal hypertension; LT: Liver transplantation.

CAPV needs liver transplantation and surgical ligation of the patent ductus venosus^[18].

It is difficult to differentiate hepatic benign lesions (FNH, FNH-like lesions, and NRH) from malignant lesions (HCC or hepatoblastoma) by imaging except in cases of such abnormal hepatic circulation while transvenous liver biopsy is a valuable alternative diagnostic tool^[35,119]. Intrahepatic portal vasculature abnormalities should be differentiated from congenital diseases, such as idiopathic non-cirrhotic PH, portal venous hypoplasia, or hepatic microvascular dysplasia. Histologic findings of these liver diseases are similar to those seen in congenital liver diseases. Thus, it is not possible to differentiate these diseases from congenital shunts^[120]. CAPV with PSE should be differentiated from mental disorder involving hyperammonemia or galactosemia due to metabolic deficiencies^[121].

Potential etiologies of extrahepatic shunts include shunt formation in association with PH and mesenteric adhesion due to prior abdominal surgery, abdominal trauma, and congenital shunts^[97,98,122]. As a result, congenital extrahepatic portosystemic shunts in CAPV patients must be confirmed by excluding the existence of the three liver diseases.

TREATMENT

Treatment options for extrahepatic portosystemic venous shunts strongly depend on the type of Abernethy abnormalities (Figure 2). Balloon-occluded retrograde transvenous obliteration (BRTO), embolization with metallic coils and surgical correction of the shunts are available^[97]. In type II patients previously diagnosed with CAPV, occlusion of the shunt is indicated in case of serious symptoms such as hepatic encephalopathy^[4] or lateral bleeding. The occlusion techniques include surgical ligation^[56,96,123] and interventional embolization^[124-126]. Otake *et al*^[99] used coils as embolic materials, because they can progressively occlude the shunt, avoiding acute overload of the portal venous system, and confirmed that there is no evidence

that the shunt vessels were recanalized after a two-year follow-up period. The treatment for venous shunts in type I patients without severe symptoms other than liver tumors and LDs is inactive, indicating that close clinical, biochemical, and imaging follow-up should be performed.

The treatment for such patients depends on the conditions of liver neoplasm, such as size and histology^[20,50]. It involves LT for hepatoblastoma^[26] and chemotherapy for hepatoblastoma after resection of the right hepatic lobe^[16]. The choice of treatment for liver tumor in CAPV patients is radical resection of the tumor^[39], although mostly it is benign, because the mass becomes larger and then progresses to malignancy^[18,26]. Morse *et al*^[28] reported a patient with CAPV who finally underwent LT for hepatoblastoma initially diagnosed as FNH 2 years ago^[26,28], suggesting that long-term follow-up and monitoring for malignancy are mandatory, even for benign tumors.

After tumor resection, liver regeneration is said to be dependent on hepatotropic factors in the portal venous blood^[37,73]. However, in the patients resected for liver tumor reported^[12,15,16,24,37], the resection was uncomplicated and the postoperative course uneventful, despite the absence of gut-derived hepatotropic factors to stimulate liver regeneration. Stimuli other than those transported with the portal blood stream must thus be sufficient to ensure an adequate postoperative liver regeneration in these patients^[39].

Some authors hold that increased blood flow comes mainly from the SV in patients with PH and hypersplenism or megalosplenism, indicating that partial splenic embolization can decrease the blood flow and pressure of the main portal vein, similar to the conjoint effects of splenectomy and devascularization^[127]. Surgical decompression is also recommended for selected children in order to promote their growth^[128].

CAPV has been thought to be asymptomatic and has no indication for LT, and only a few cases having been reported^[2,17,27,56,129]. More and more surgeons hold that LT is necessary when medical therapy cannot relieve CAPV-associated abnormalities, such as CAPV-associated cirrhosis caused by biliary atresia^[4,17], diffuse hepatoblastoma involving both lobes of the liver^[26], and severe portosystemic encephalopathy^[56]. No surgical method is available for reconstructing the portal structures of the native liver^[56,59]. Shinkai *et al*^[56] reported that LT is an effective surgical treatment for symptomatic CAPV patients when the disease is unresponsive to medical treatment, and believe that prophylactic LT is justified for patients with CAPV before the development of fatal pulmonary complications, such as pulmonary hypertension or HPS, which might complicate or preclude LT^[56]. Woodle *et al*^[17] successfully transplanted liver for a biliary atresia patient, and assumed that CAPV is not a contraindication for LT. Taoube *et al*^[130] performed the first paediatric liver transplant for a patient with portal venous agenesis, using the de piggy-back technique.

Recently, auxiliary partial orthotopic liver

transplantation (APOLT) was developed in order to reverse fulminant hepatic failure (FHF), which is advantageous over the orthotopic liver transplantation (OLT) and avoids eliminating regeneration of the native liver and a life-long immune suppression^[131]. It also has been utilized as an aid in small-for-size grafts to larger recipients during living donor liver transplantation (LDLT)^[131]. Soejima *et al*^[27] performed LT for a male patient using a left lateral segment graft from his mother to preserve his native right lobe. Configuration of the donor PV, hepatic artery, HV and bile duct was normal. The results indicate that APOLT is an ideal procedure for patients with CAPV^[27]. However, APOLT has certain drawbacks, such as portal steal phenomenon and potential risk of developing tumors in the remnant native liver.

Both the anatomy of portal vein and the function of liver can be restored, and other liver dysfunctions-associated complications may be relieved after LT, such as disappearance of high-intensity lesions in the brain^[59].

In conclusion, the prognosis of CAPV patients depends on congenital heart disease, liver disease, and the site of portosystemic shunts. The outcome of CAPV patients with no other abnormalities is different. A long-term follow-up including laboratory tests and image screening is recommended for CAPV patients^[20,35,50,126].

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LIVER CANCER

Fibrinogen-like protein 2/fibroleukin prothrombinase contributes to tumor hypercoagulability *via* IL-2 and IFN- γ

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protein expression in both THP-1 and HUVEC cell lines. One-stage clotting assays demonstrated that THP-1 and HUVEC cells expressing hfgl2 had increased procoagulant activity following cytokines stimulation.

CONCLUSION: The hfgl2 contributes to the hypercoagulability in cancer and may induce tumor angiogenesis and metastasis *via* cytokine induction.

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Key words: Fibrinogen-like protein 2/fibroleukin; Thrombin; Tumor; Coagulation; Cytokine

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Abstract

AIM: To examine the role of Fibrinogen-like protein 2 (fgl2)/fibroleukin in tumor development. Fgl2 has been reported to play a vital role in the pathogenesis in MHV-3 (mouse hepatitis virus) induced fulminant and severe hepatitis, spontaneous abortion, allo- and xenograft rejection by mediating "immune coagulation".

METHODS: Tumor tissues from 133 patients with six types of distinct cancers and the animal tumor tissues from human hepatocellular carcinoma (HCC) model on nude mice (established from high metastasis HCC cell line MHCC97LM6) were obtained.

RESULTS: Hfgl2 was detected in tumor tissues from 127 out of 133 patients as well as tumor tissues collected from human HCC nude mice. Hfgl2 was highly expressed both in cancer cells and interstitial inflammatory cells including macrophages, NK cells, and CD8⁺ T lymphocytes and vascular endothelial cells. Hfgl2 mRNA was localized in cells that expressed hfgl2 protein. Fibrin (nogen) co-localization with hfgl2 expression was determined by dual immunohistochemical staining. *In vitro*, IL-2 and IFN- γ increased hfgl2 mRNA by 10-100 folds and

INTRODUCTION

Fibrinogen-like protein 2 (fgl2)/fibroleukin, also called fgl2 prothrombinase, has recently been identified as a new member of fibrinogen-related protein superfamily, with the serine protease activity. Mouse fgl2 (mfgl2) and human fgl2 (hfgl2) are localized in chromosomes 5 and 7, respectively. The biological activity of fgl2 prothrombinase, similar to coagulating factor Xa, can directly catalyze prothrombinase into activated thrombinase, thereby, initiating a cascade coagulating reaction^[1]. Several studies indicate that fgl2 is involved in MHV-3 induced fulminant hepatitis and severe or fulminant viral hepatitis in human, spontaneous abortion and xenograft rejection by mediating pathological changes such as immune coagulation, fibrin deposition, and micro-thrombus^[2-5]. In addition to its primary role in homeostasis and blood coagulation, thrombin is a potent mitogen that dramatically increases the growth and metastasis potential of tumor cells. Both tissue factor (TF) and thrombin exert their influence on tumor angiogenesis and metastasis through clotting-dependent

and clotting-independent pathways^[6,7]. Fgl2 functions as a novel immune coagulant with the ability to generate thrombin directly. Therefore, we propose that fgl2 may contribute to tumor angiogenesis and metastasis through a clotting-dependent pathway.

In the present study, the authors investigated the expression and histological localization of hfgl2, co-localization of fgl2 with fibrin in cancer and the gene regulation of fgl2 upon cytokine induction, in the hope of providing a new point of view on the characteristic hypercoagulability of cancer and a novel anticoagulant target, the fgl2 gene.

MATERIALS AND METHODS

Patients' tumor tissues

Informed consent was obtained from all the participants, and the research protocol was reviewed and approved by the Institutional Review Board of Tongji Hospital, Wuhan, China. Patients were recruited at Tongji Hospital, and 133 tumor samples and their paired adjacent normal tissues were collected. The patients' characteristics are shown in Table 1. The specimens for RNA extraction were frozen in liquid nitrogen until studied. Specimens for immunohistochemical and *in situ* hybridization were fixed in 4% paraform.

Mice

Male BALB/c-nu/nu mice (Shanghai Silaike Animal Seed Center), 4–6 wk of age, with a body weight of 15.0–18.7 g, were kept in micro-isolated cages housed in Tongji Hospital and fed a standard lab chow diet and water *ad libitum*. Animals were divided into two groups: tumor-bearing mice (experimental group) and tumor-free mice (control group).

Cell and culture conditions

THP1 and HUVEC cell lines were purchased from Biology Treasure Center of Wuhan University. Human hepatocellular carcinoma (HCC) cell line MHCC97LM6 with high tendency of metastasis were purchased from Liver Cancer Institute, Fudan University, Shanghai. The HUVEC and MHCC97LM6 cell lines were cultured in Dulbecco modified Eagle medium (DMEM), and THP-1 cell lines were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco Life Technologies), 100 U/mL penicillin, and 100 mg/mL streptomycin and cultured at 37°C, 50 mL/L CO₂, and 95% humidity.

Tumor cell inoculation and quantification of pulmonary metastatic foci

MHCC97LM6 cell lines were cultured *in vitro* by sub-confluent passage in DMEM. Sub-confluent tumor cells were washed with phosphate-buffered saline (PBS), detached by a brief exposure to a 0.125% trypsin and 0.02% EDTA solution, washed in serum-containing media, and then resuspended in cold serum-free medium to get the single cell suspension. The 95% viability of

Table 1 General data and pathologic diagnosis of hfgl2 positive samples

Diagnosis	Case	Gender		Age (yr)	Subtype	Metastasis
		F	M			
Colon carcinoma	21	12	9	58.27 ± 10.27	AC 21	17
Breast cancer	20	0	20	49.70 ± 11.06	IDC 20	16
Lung cancer	20	17	3	55.17 ± 12.53	SCC 5	19
					SqC 5	
					AC 7	
					AdCa 3	
Gastric cancer	26	13	13	55.8 ± 15.88	AC 26	20
Esophageal carcinoma	18	15	3	56.44 ± 8.63	SqC 15	13
					AC 3	
Cervix cancer	22	0	22	39.14 ± 6.48	SqC 16	22
					AC 6	

AC: Adenocarcinoma; IDC: Infiltrating ductal carcinoma; SCC: Small cell carcinoma; SqC: Squamous carcinoma; AdCa: Adenosquamous carcinoma.

the tumor cells was determined by trypan blue exclusion. The cells were kept in an ice bath until transplanted into mice. A single cell suspension of 9×10^6 cells in 100 μ L serum-free media was injected subcutaneously into the dorsal scapular skin of nude mice using a 27-gauge needle. Injection with the same volume of serum-free media served as the negative control. Once a tumor was clearly visible, it was measured daily and the volume estimated by the formula $V = ab^2/2$, where a = longest diameter, b = shortest diameter. After 36 d, the nude mice were sacrificed and the tumors and other organs including brain, heart, lung, liver, kidney, spleen, and small intestine were removed and rinsed in PBS. Aliquot of the tissue specimens were frozen in liquid nitrogen for RNA extraction. Other aliquots were fixed in 4% paraform and prepared for immunohistochemical studies. The lungs were separated into individual lobes and the number of metastatic foci was counted under a microscope with HE stain.

Immunohistochemical staining of fgl2 prothrombinase

Immunohistochemical staining was used to assess fgl2 expression in tumor tissue and HUVEC and THP-1 cell lines. Tissues were fixed with 4% paraform, processed into paraffin, and sectioned. Then they were rehydrated with 0.1 mol/L PBS (pH 7.4) and endogenous peroxidase. Nonspecific binding was blocked by sequential incubation of the sections in 10% hydrogen peroxidase solution for 10 min followed by 10% normal goat serum in PBS at room temperature for 30 min. Thereafter tissue or cultured cell slices were incubated with a polyclonal antibody against fgl2 at a dilution of 1/300 in PBS at 4°C for 16 h. Subsequently, sections were incubated with immunoperoxidase-conjugated goat IgG fraction to rabbit IgG Fc (Zhongshan Company) at room temperature for 15 min, followed by three washes in PBS. The secondary antibody, an anti-rabbit IgG linked to peroxidase, was incubated with 3,3'-diaminobenzidine chromagen and counterstained with hematoxylin.

Fibrin in human malignant tumor tissues

Fibrin was detected with the use of a rabbit-anti-fibrinogen antibody (Dako Cytomation). This reagent is known to react with fibrinogen and fibrin in mouse and human tissues. The technique used for detection of fibrin was the standard avidin-biotin complex (ABC) method. The biotinylated secondary antibody was an anti-rabbit IgG linked to peroxidase incubated with 3,3'-diaminobenzidine chromagen, followed by counterstaining with hematoxylin.

Dual immunohistochemical staining of hfgl2 and fibrin

Dual staining for hfgl2 and fibrin on the same tissue was performed using a Vectastain ABC kit (Vector Laboratories), with second Abs labeled with AP or HRP, respectively.

Immunohistochemical staining of macrophages, T lymphocytes, NK cells, and vascular endothelial cells

Antibodies against CD68, CD57, CD4, CD8 and a monoclonal antibody against von Wille brand factor antigen (NeoMarkers) were individually used at a dilution of 1:50-1:100 in PBS to detect macrophages (Kupffer cells), NK cells, T lymphocytes, and vascular endothelial cells using immunoperoxidase staining *via* similar methodology described above.

Western blot analysis

Cells were solubilized in lysis buffer containing 10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% TritonX-100, at 4°C for 30 min. The cell lysates were subjected to centrifugation at $10000 \times g$ at 4°C for 1 min. The supernatants were saved and their protein contents were measured. Thirty mg lysate protein was loaded onto 12% SDS-polyacrylamide gels. After the proteins were separated, they were transferred to a NC membrane. The membrane was blocked and probed with a polyclonal antibody against fgl2 at a dilution of 1:200 in 5% milk in TBS. After washing with TBS and 0.5% Tween-20, the blot was incubated with secondary antibodies conjugated to horseradish-peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence (ECL) reagent (Pierce).

In situ hybridization

A digoxigenin-11-UTP (Dig-UTP) (Roche)-labeled cDNA probe was cut by EcoRI following subcloning of a 169-bp fragment of mfgl2 cDNA, representing nt 756 (ACTGTGACA ...) to 924 (... GAGTAAGGA), into pCR2.1 vector (Invitrogen Life Technologies). The Dig-UTP-labeled probe concentration was determined by immunoenzymatic reaction with chemiluminescent detection, and the probes were stored at -80°C. Tissue sections were deparaffinized in 100% xylene and 100% alcohol, followed by prehybridization in 50% formamide and $2 \times$ SSC at room temperature for 1 h. The hybridization mixture consisted of 50% deionized formamide, 5% dextran sulfate, 250 µg salmon sperm DNA per milliliter, and 2 µg Dig-labeled cDNA probe

per milliliter in $2 \times$ SSC. The hybridization mixture with the probe was denatured by heating in an 85°C water bath for 5 min, chilled on ice for 1 min, and added to tissue sections for hybridization at 42°C overnight. Post-hybridization washing in a series of dilutions of SSC was followed by application of 3% blocking reagent at room temperature for 30 min. After a brief wash in Tris-HCl buffer (pH 7.5), sections were incubated with polyclonal anti-Dig Fab, conjugated to alkaline phosphatase (AP; Boehringer Mannheim), and diluted 1/500 in Tris-HCl buffer. Unbound antibody was removed by two 5-min washes with Tris-HCl buffer. A purple reaction product was developed using AP substrate, 5-bromo-4-chloro-3-indolyl-phosphate, and NBT to sections at room temperature for 120 min. Sections were counterstained with methylene green and mounted with Per mount for viewing.

RNA preparation and quantitative real-time PCR

Total RNA was isolated from tumor specimens and cell lines using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of RNA were determined by measuring the absorbance at 260 nm and 280 nm. Subsequently, the cDNAs were synthesized. The nucleotide sequences of the primers for PCR amplification of 169 bp fragment of fgl2 were the following: sense primer, 5'-ACTGTGACATGGAGACCATG-3', and antisense primer, 5'-TCCCTTACTCTTGGTCAGAAAG-3'. The amplified 571 bp fragment of GAPDH cDNA was used as an internal control to ensure equal loading and first strand synthesis with forward primer, 5'-ATCACCATCTTCCAGGAG-3' and reverse primer, 5'-TGCTTCCACCACCTTCTTG-3'. In the PCR reaction the DNA was amplified over 36 cycles, denatured at 94°C for 40 s, annealed at 60°C for 45 s, and extended at 72°C for 60 s. The real-time PCR reactions were performed using a SYBR green PCR kit (Biotium) in Roche Sequence Detection System. Specificity of the PCR reaction was verified by dissociation-curve analysis and agarose gel electrophoresis. Fgl2 mRNA relative quantification was assigned by reference to standard curve analysis.

Cytokine treatment

THP-1 and HUVEC cell lines were maintained in medium containing 10% FBS in six well plate for 72 h until they reached sub-confluence. Then they were incubated with IL-2 (100 U/mL) or IFN-γ (200 U/mL) in medium for 4 h, 8 h, 12 h and 24 h before they were collected for immunohistochemical staining and real-time PCR studies.

Prothrombinase assays (PCA)

Samples to be assayed for PCA were washed three times with unsupplemented RPMI 1640 and resuspended at a concentration of 10^6 /mL. The cells were then subjected to three cycles of freeze-thawing to obtain maximal total cellular procoagulant activity. Milliunits of PCA were determined from a standard curve generated by serial log dilutions of a standard rabbit brain thromboplastin

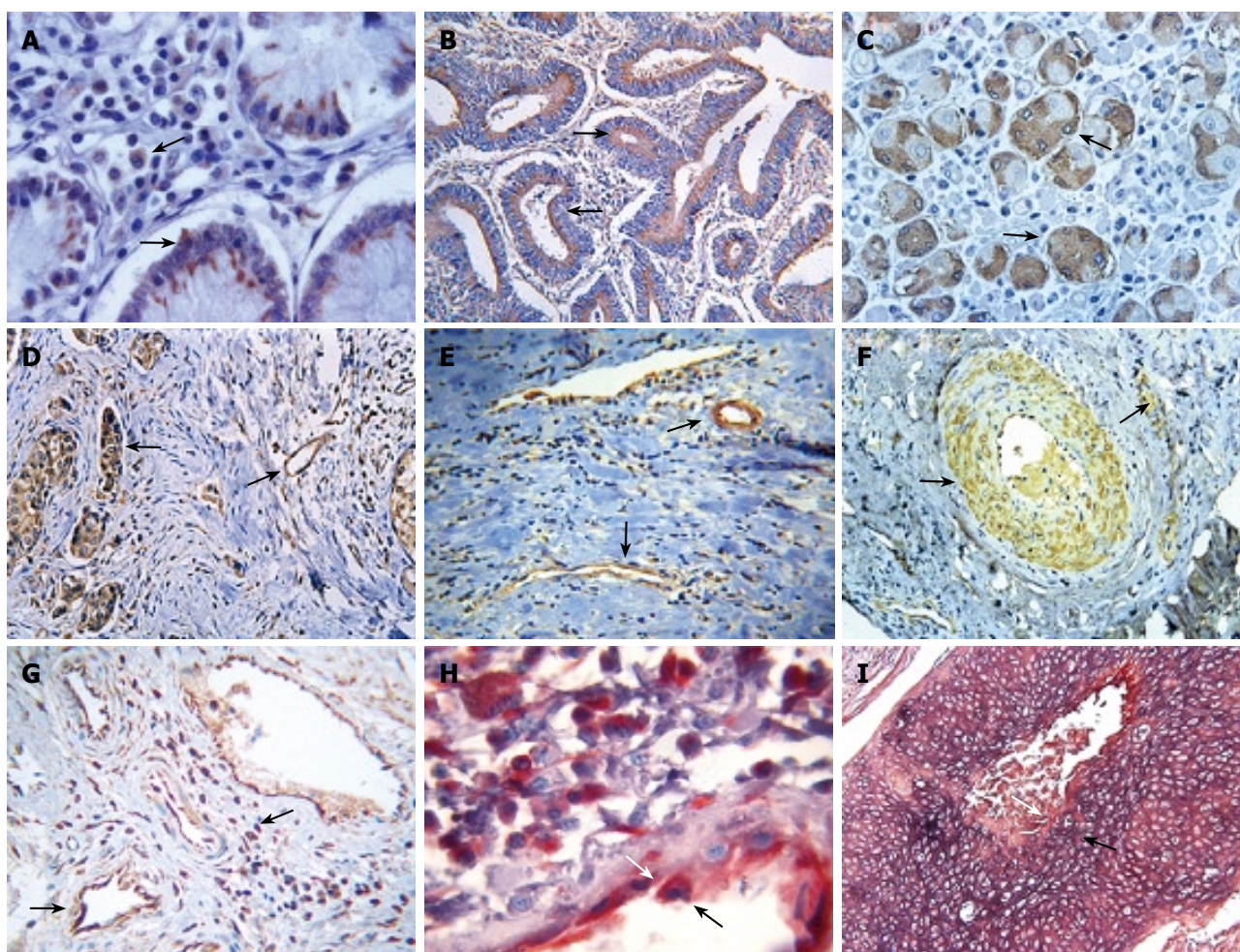


Figure 1 Immunohistochemical analysis of hfgl2 prothrombinase and fibrin in tumor tissues. Fgl2 was verified by immunohistochemistry in colon cancer (A, $\times 400$), esophageal cancer (B, $\times 200$), gastric cancer (C, $\times 400$), breast cancer (D, $\times 200$), lung cancer (E, $\times 200$) and cervix cancer (F, $\times 100$). Fibrin deposition was stained for colon cancer (G, $\times 200$). Dual staining of hfgl2 (indigo) and fibrin in colon cancer (H, $\times 1000$) and cervix cancer (I, $\times 400$) displayed the co-localization of hfgl2 (indigo) and fibrin (scarlet) expression. Cells expressed fgl2 protein and fibrin were detected with antibodies specific for fgl2 (black arrows) and fibrin (white arrow), respectively.

(Sigma) to determine functional shorting of the spontaneous clotting time of normal citrated human platelet-poor plasma. After addition of cellular sample, 0.1 mL of normal plasma and 0.1 mL of 25 mmol/L CaCl_2 were added and clotting time was visually determined by the appearance of white precipitate after incubation at 37°C . Human plasmas deficient in specific clotting factors such as factor II or factor X (ADI/DELLWIN) were also used as substrate in the clotting assay in place of normal human plasma.

Statistical analysis

Quantitative data were expressed as mean \pm SD. Statistical analysis was performed by one-way analysis of variance with $P < 0.05$ considered statistically significant.

RESULTS

Fgl2 expression in tumor tissues from patients

The study population was composed of 133 patients, of whom 107 patients were noted clinically to have metastasis (Table 1). Tumor tissues of the patients were examined for fgl2 expression at both the

mRNA and protein levels by *in situ* hybridization and immunohistochemical staining respectively. The normal tissue surrounding the tumor tissue was used as control. Fgl2 was present in cancer cells as well as interstitial infiltrated and vascular endothelium cells of the microvasculature (Figure 1A-F). There was significantly upregulated hfgl2 expression with cancers when compared with those in no magnificent tumor tissues which showed little or no fgl2 expression (data not shown). Dual staining of hfgl2 and fibrin displayed the co-localization of these two molecules, indicating the contribution of highly expressed hfgl2 protein to the hypercoagulability (Figure 1G-I). *In situ* hybridization showed a similar pattern of hfgl2 staining in tumor tissues of the patients (Figure 2).

Cellular source of fgl2 and fibrin deposition in tumor tissues

As shown with staining of serial tumor sections, the majority of CD68+, CD57+, CD8+, and vascular endothelial cells displayed increased expression of fgl2 protein in tumor tissues of the patients (Table 2 and Figure 3).

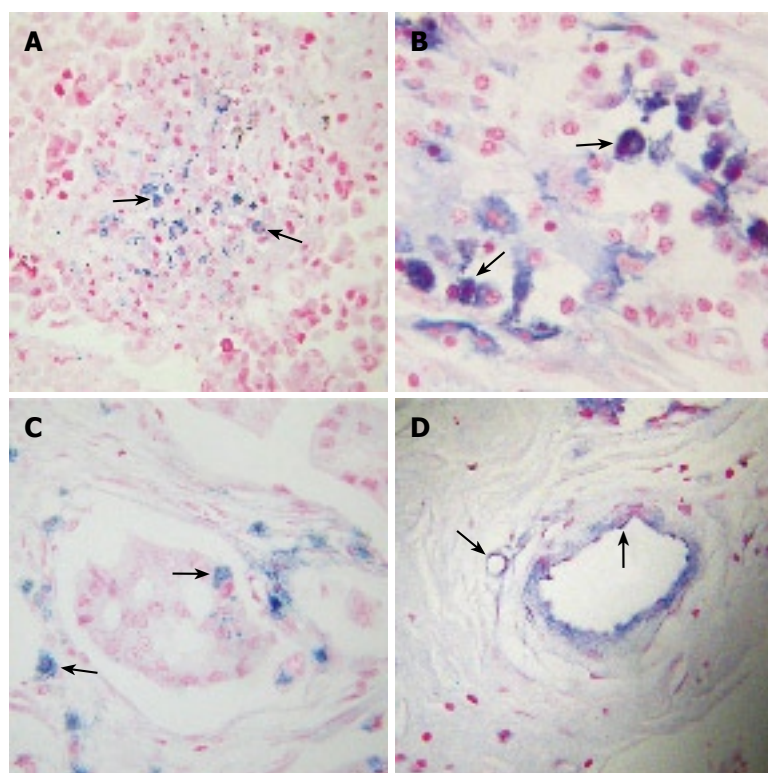


Figure 2 Hfgl2 mRNA detection in human tumor tissues by in situ hybridization. **A:** Hfgl2 mRNA was stained purple in lung small cell carcinoma, hfgl2 mRNA present in the nests of tumor cells, some tumor cells secreted melanin (x 400); **B:** Submucosa of colon descendens tubular adenocarcinoma, hfgl2 mRNA present in the infiltrated cells and single migrating tumor cells (x 400); **C:** Invasive ductal breast carcinoma, hfgl2 mRNA localized to nest tumor cells, exfoliated tumor cells and infiltrated cells around the duct (x 400); **D:** Submucosa of gastric adenocarcinoma, hfgl2 mRNA localized to tumor blood vessel endothelium (x 400). Arrows indicate hfgl2 mRNA locations.

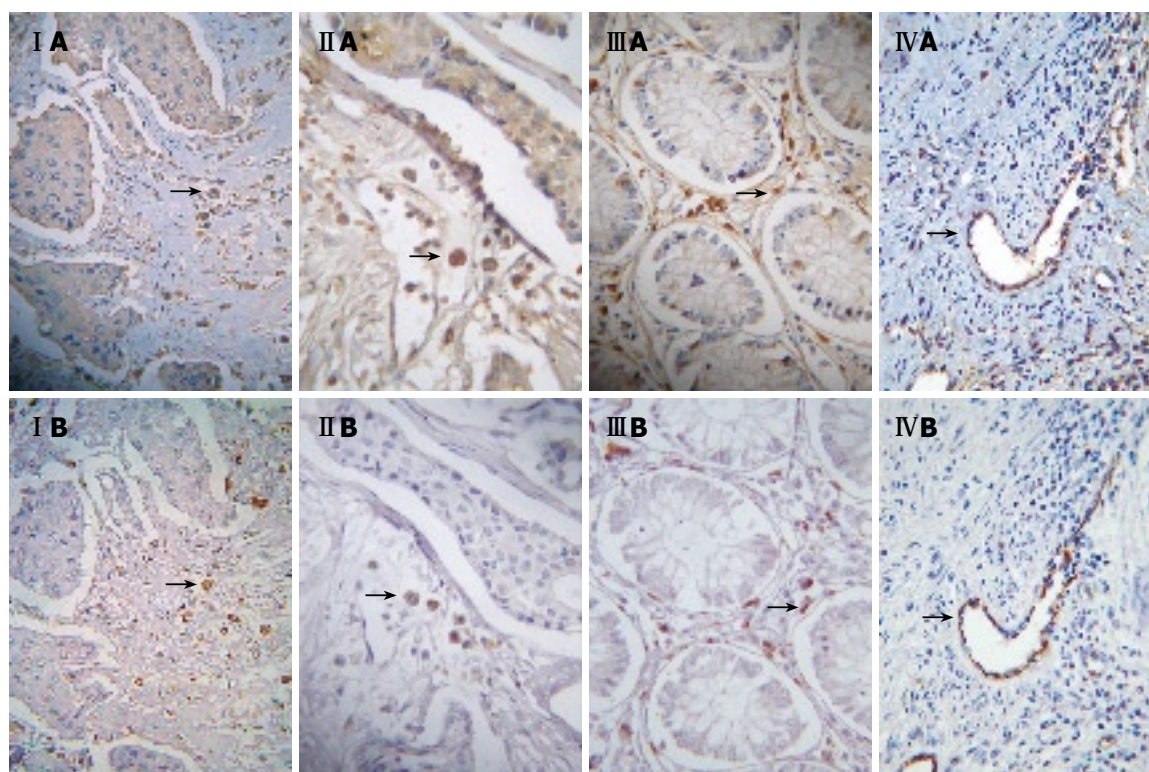


Figure 3 I A to IV A was stained with fgl2 antibody (black arrows) displaying the fgl2 protein in infiltrative cells. I B to IV B was a serial section of I A to IV A, arrows indicate the staining of CD68, CD57, CD8 and vWF respectively.

Hfgl2 expression in tumor tissue from HCC nude mouse model

In the HCC nude mouse model, no evidence of histoincompatibility or tumor rejection was observed based on the rapid development of visible tumors after injection (100% of mice within 3 d). The steady

growth of the tumors was found in MHCC97LM6 mice (Figure 4 I A and B) whereas tumors were not observed in the control group. Almost all MHCC97LM6 mice developed on site palpable tumors and metastatic foci in lung tissues within 7 d of injection (Figure 4 I C). Further studies showed that mfgl2 (mouse fgl2) expression

Table 2 Histological localization and involved cell type in hfgl2 positive samples

Tumor type	Case	Hfgl2 positive	Histological localization and cases				
			Tumor cell	CD57 ⁺	CD3 ⁺ , CD8 ⁺	CD68 ⁺	VWF
Colon carcinoma	21	21	19	20	18	21	17
Breast cancer	20	20	18	20	20	20	18
Lung cancer	22	20	18	20	19	20	18
Gastric cancer	26	26	25	26	13	26	25
Esophageal carcinoma	19	18	18	16	15	18	14
Cervix cancer	25	22	20	21	21	22	22

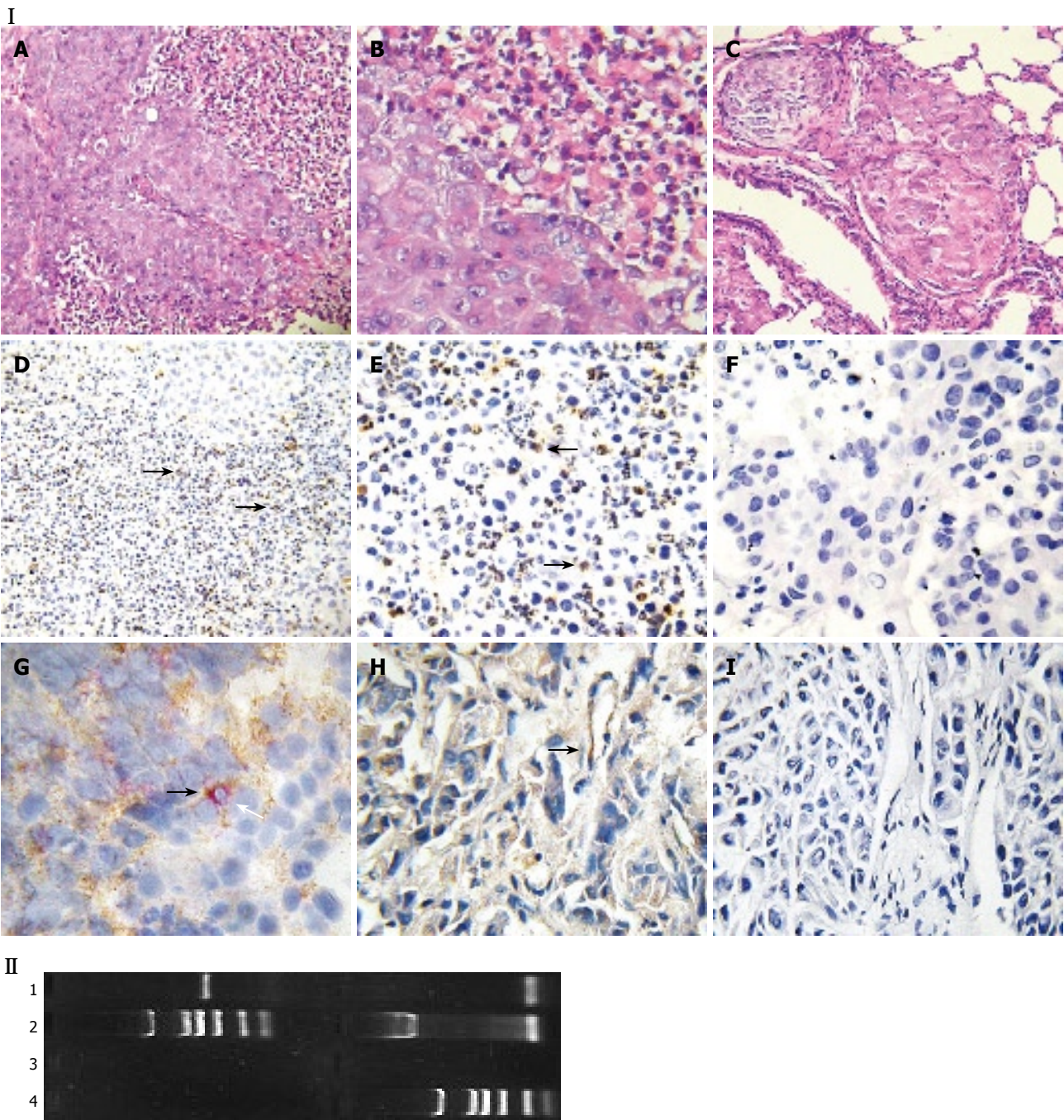


Figure 4 Fgl2 expression evidenced in mouse tumor tissue. Male BALB/c-nu/nu mice were subcutaneously injected MHCC97LM6 cell lines and tumor tissues were harvested 36 d later. Panel I : **A-C**, HE staining in tumor tissue at injection site (**A**, x 200; **B**, x 400) and lung metastatic tumor tissue (**C**, x 200); **D-I**: mfgl2 expression in tumor tissue of human hepatocellular carcinoma (HCC) nude mice model (**D**, SP x 200; **E**, SP x 400; **G**, dual staining of mfgl2 and marker of macrophages; **H**, dual staining of mfgl2 and marker of endothelial cells; **F** and **I**, negative controls). Arrows in **D** and **E** indicate fgl2 positive cells, arrows in **G** and **F** indicate the fgl2 positive macrophage and endothelial cells, respectively. Panel II : mfgl2 mRNA expression in tumor tissue (1), PCDNA3.1-fgl2 plasmid as positive control (2), PCDNA3.1 as negative control (3) and DL-2000 marker (4).

in interstitial inflammatory cells and vascular endothelial cells (Figure 4 I D-H). Furthermore the fgl2 was detected at mRNA level in the tumor tissue (Figure 4 II).

Increased hfgl2 expression and PCA in IL-2 or IFN- γ stimulated HUVEC and THP-1 cell lines

Endothelium original HUVEC and mononuclear original THP-1 cells were used to investigate the regulatory expression of fgl2 in response to various tumor cytokines involved in tumor development *in vitro*. RT-PCR analysis demonstrated minimal constitutive fgl2 mRNA levels in both cell lines, but increased in response to stimulation with IL-2 or IFN- γ (data not shown). This primary observation was further demonstrated by real-time PCR, which showed a 10-100 fold increase of fgl2 mRNA copies following stimulation of IFN- γ or IL-2 (Figure 5 III). Immunohistochemical staining and Western-blotting also detected upregulated hfgl2 protein expression upon stimulation of cytokines (Figure 5 I and II). The functional measurement of fgl2 protein was carried out by one-stage clotting assay expressed as PCA. Both HUVEC and THP-1 cells displayed basal levels of PCA with a significant increase following IL-2 or IFN- γ stimulation in parallel with fgl2 protein expression (Figure 6A). The induced PCA was independent of factor X, but closely associated with factor II, thus demonstrating the PCA was induced by increased expression of fgl2 protein (Figure 6B).

DISCUSSION

The association between thrombosis and cancer was observed by Professor Armand Trousseau in 1865, who noted that patients who present with idiopathic venous thromboembolism (VTE) frequently harbor an occult cancer. We now believe that there are two key mediators of this link: one being the thrombin^[6] whose broad substrate specificity supports a variety of cellular effects relevant to tumor growth and metastasis; and the other being the tissue factor (TF)^[7], the primary initiator of the coagulation cascade, whose rather ubiquitous presence as a transmembrane receptor on a variety of nucleated cells confers responsibility for the generation of cell-surface thrombin in many pathologic situations *via* both clotting-dependent and clotting-independent mechanisms. TF and thrombin are capable of inducing angiogenesis, the process of generating new blood vessels from preexisting vessels, which is essential for tumor growth and metastasis.

We and many others have described a new procoagulant other than tissue factor and thrombin: fgl2 prothrombinase, a member of the fibrinogen superfamily, which was primarily reported to be produced by activated macrophages, T cells, and endothelial cells. Mouse fgl2 (mfgl2) and human fgl2 (hfgl2), were localized in chromosomes 5 and 7, respectively^[1,3,8]. Fgl2 is a 64-70 kDa, type 2 transmembrane protein containing a C-terminal FRED (fibrinogen related extracellular domain). The

fgl2 amino acid sequence is 36% homologous to the β and γ fibrinogen chains^[9]. There is 78% homology between human and mouse fgl2 with 90% homology in their C-terminal domains containing FRED^[3]. Fgl2 functions as a strong prothrombinase which directly cleaves prothrombin to thrombin leading to fibrin deposition in the absence of factor VII or factor X^[10]. The direct prothrombinase activity of fgl2 is implicated in the pathogenesis of several inflammatory disorders including fulminant hepatitis and severe hepatitis, allo- and xeno-graft rejection^[4,11,12]. Furthermore, its role is also evidenced in murine and human cytokine induced fetal loss^[5,13-15] and neonatal death from contractile dysfunction and rhythm abnormalities during embryonic and postnatal development^[16]. The observations that neutralizing Abs to mfgl2 prevent both fibrin deposition and death from MHV-3 infection support its role as a coagulant^[17]. Recent studies have shown that inhibition of reticuloendothelial cell mfgl2 expression through the use of gene-targeted fgl2-deficient (fgl2-/-) mice or targeted fgl2 gene with antisense mfgl2 results in the prevention of MHV-3-induced fibrin deposition, liver injury, and death^[2,18].

Our study shows that fgl2 prothrombinase was expressed in malignant tumor tissues including colon, breast, lung, gastric, esophageal, and cervical tissues from patients and in HCC nude mouse models. Up-regulation of fgl2 gene expression is evident not only in cancer cells, but also in interstitial infiltrated cells including macrophages, NK cells, CD8⁺ T lymphocytes, and vascular endothelial cells. Dual staining shows that fibrin (nogen) uniformly co-localized with fgl2 protein. In breast cancer, fgl2 is present predominantly in the same cellular types in which TF was expressed^[19]. Other studies have further shown fibrin (nogen) co-localization with TF expression. Cross-linked fibrin (XLF) was found within the endothelium of angiogenic vessels of invasive breast cancer specimens, but not within the vessels of benign breast tumors in histological specimens from the patients^[20]. The similar expression patterns of TF and fgl2 have led us to hypothesize that both fgl2 and TF may be responsible for the coagulation cascade in cancer. Fgl2 and TF cleavage of prothrombin to thrombin results in fibrin deposition in the tumor microenvironment (TME). Thrombin-catalyzed, XLF formation is a characteristic histopathological finding in many human and experimental tumors^[21].

Fgl2 induces angiogenesis by generating thrombin. Thrombin dramatically increases the growth and metastatic potential of tumor cells *via* clotting dependent and independent mechanisms. The fibrin matrix that develops around tumors provides a provisional proangiogenic scaffold that supports vessel formation and stimulates endothelial cell proliferation and migration through clotting dependent mechanisms. Clotting independent mechanisms are thought to be mediated *via* proteolytic cleavage of the PARs and subsequent activation of G-protein-coupled signal transduction cascades, leading to the upregulation of many angiogenesis-related genes, including VEGF,

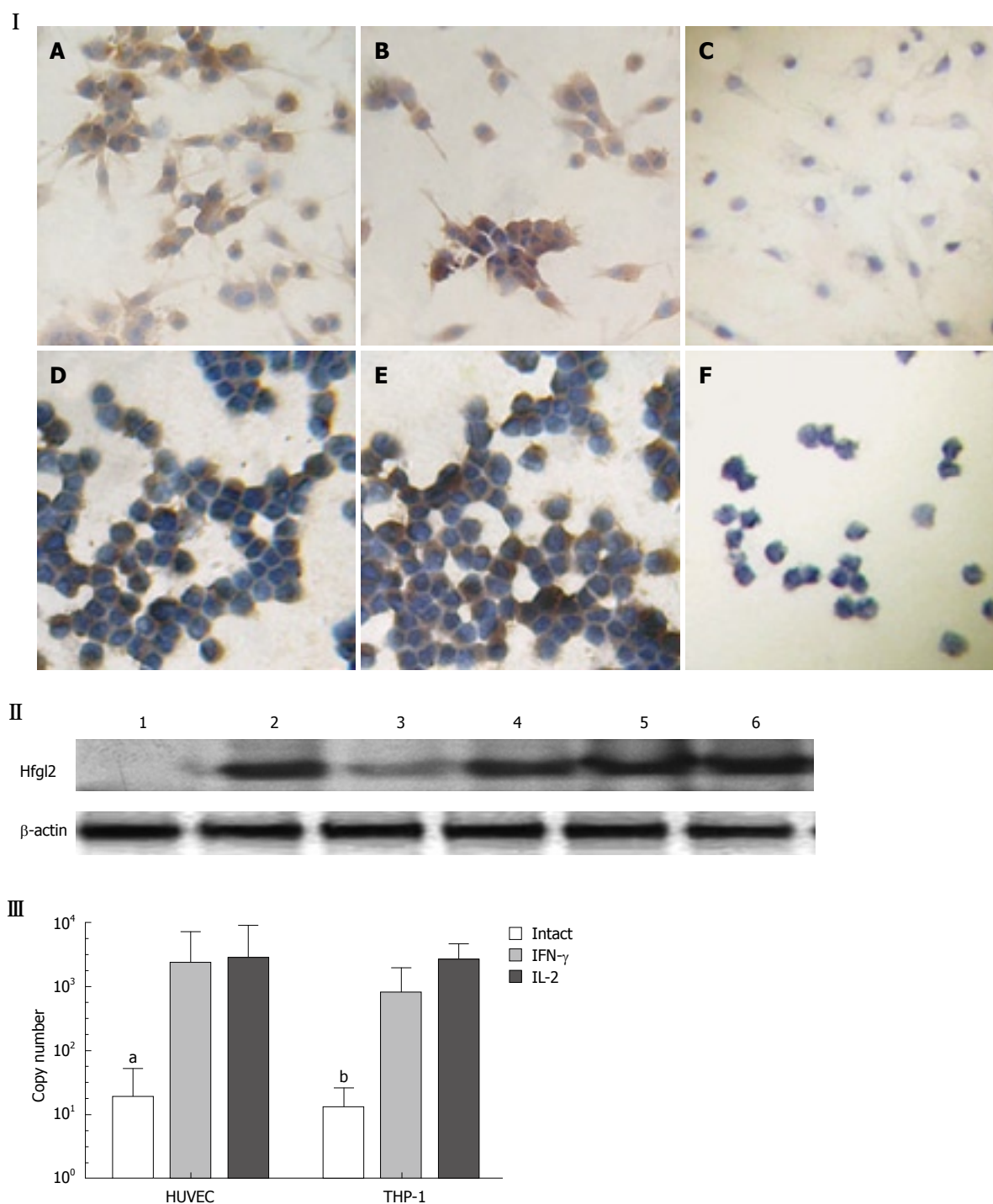


Figure 5 Increased expression of fgl2 after IFN- γ or IL-2 stimulation. **I**: HUVEC and THP-1 cells were treated with IFN- γ (200 U/mL) and IL-2 (100 U/mL) and stained with antibodies specific for hfgl2. **A**: IFN- γ (200 U/mL, 12 h) + HUVEC (SP, x 200); **B**: IL-2 (100 U/mL, 8 h) + HUVEC (SP, x 200); **C**: NS + HUVEC (SP, x 200); **D**: IFN- γ (200 U/mL, 12 h) + THP-1 (SP, x 200); **E**: IL-2 (100 U/mL, 8 h) + THP-1 (SP, x 200); **F**: NS + THP-1 (SP, x 200). **II**: Western blotting analysis of hfgl2 expression after IFN- γ (200 U/mL) and IL-2 (100 U/mL) stimulation for 12 h and 8 h on HUVEC and THP-1 cells. 1: Intact HUVEC; 2: IFN- γ + HUVEC; 3: Intact THP-1; 4: IFN- γ + THP-1; 5: IL-2 + HUVEC; 6: IL-2 + THP-1. **III**: Real-time PCR analysis of inducible hfgl2 mRNA after stimulation for 4 h. ^a P = 0.0329, vs HUVEC alone; ^b P = 0.0059, vs THP-1 alone.

VEGF receptors, TF, bFGF, and MMP-2^[22-24]. These genes can create a number of pleiotropic responses, such as change in endothelial cell shape, increased vascular permeability, increased endothelial cell proliferation, and increased proteolysis, all of which contribute to increased tumor angiogenesis.

The pathogenic role of fgl2 is not entirely understood as only one pathway of fgl2 activation has

been studied so far. In murine hepatitis viral infection, nucleocapsid protein induces transcription of fgl2 through the transcription factor hepatic nuclear factor 4 α and its cognate receptor^[25,26]. HBV X and core protein was shown to induce hfgl2 expression through a host factor c-Ets-2 and MAPK signal pathway^[27]. In transplantation, fgl2 transcription appears to be regulated by cytokines. Macrophage induction of fgl2 is

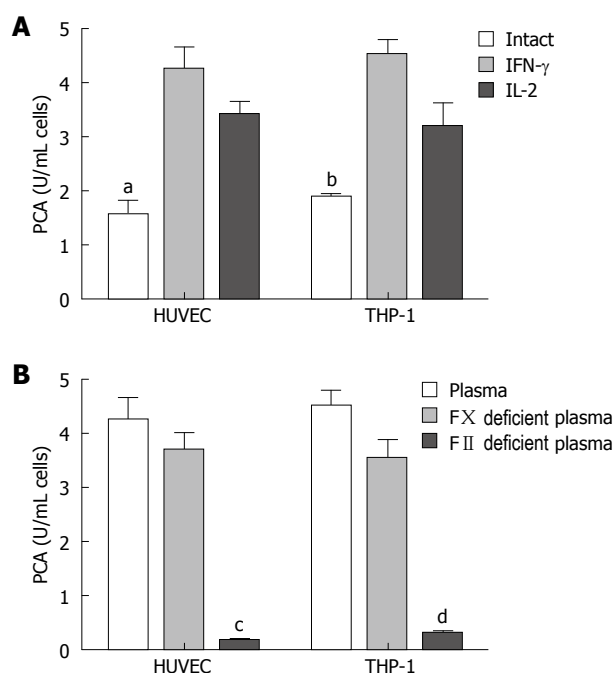


Figure 6 Increase of procoagulant activity (PCA) in HUVEC and THP-1 cells after IFN- γ and IL-2 stimulation dependent on FII but not FX. HUVEC and THP-1 cells received IFN- γ (200 U/mL) and IL-2 (100 U/mL) for 12 h and the PCA was assayed. IFN- γ and IL-2 increased PCA and this effect is preserved in FX poor plasma whereas is absent in FII poor plasma. ^a $P = 0.0026$, vs HUVEC alone; ^b $P = 0.0037$, vs THP-1 alone; ^c $P = 0.0002$, FII deficient plasma group vs normal plasma group or FX deficient plasma group; ^d $P = 0.0001$, FII deficient plasma group vs normal plasma group or FX deficient plasma group.

induced by IFN- γ , whereas preliminary data suggest that fgl2 transcription in endothelial cells occurs in response to TNF- α but not IFN- γ ^[28].

Our study has also shown that cultured HUVEC and THP-1 cells activated by IFN- γ or IL-2 demonstrated induction of hgl2 expression and enhanced activation of human prothrombin. The induced PCA activity was independent of factor X, but closely associated with factor II. These results suggest that macrophages are attracted to invading tumors and subsequently release cytokines that later induce fgl2 expression in cancer. Increased fgl2 expression may activate thrombin, to exert its effect on tumor angiogenesis and metastasis through clotting-dependent and independent mechanisms. Additional studies in molecular pathways for induction of fgl2 in cancer are presently underway in our laboratory.

The fgl2 protein described here is a membrane bound prothrombinase. The recent discovery of a secreted form of fgl2 (sfgl2) produced by T regulatory cells has potent immune modulatory effects on the adaptive immune system. Sfgl2 was reported to prevent maturation of dendritic cells (DC) by inhibiting NF- κ B nuclear translocation, expression of CD80 and MHCII, by inhibiting T cell proliferation in response to CD3/CD28, Concanavalin A, and allo-antigens. These observations have provided a potential explanation for many of the biological functions influenced by fgl2 protein^[29-31] in our laboratory. fgl2 was also found in the extracellular matrix in malignant tumor tissue samples.

This suggests the involvement of sfgl2 protein. Further studies are necessary to solve this conundrum.

In this study, we first reported the highly expressed fgl2 prothrombinase in a variety of tumor tissues both from patients and an animal model. Tumor related cytokines IFN- γ and IL-2 lead to the induction of hfgl2 expression and enhanced activation of human prothrombin. These observations suggest that fgl2 prothrombinase, in conjunction with thrombin and tissue factor, may contribute to tumor hypercoagulability and possibly to angiogenesis and metastasis. In turn, fgl2 may serve as a novel target for intervention of tumor development.

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COMMENTS

Background

Fibrinogen-like protein 2 (fgl2)/fibrinogen, also called fgl2 prothrombinase, has been found recently and belongs to fibrinogen-related protein superfamily. Fgl2 prothrombinase has serine protease activity. Human fgl2 gene is mapped at chromosome 7q11.23. Biological activity of the product of fgl2 prothrombinase, similar to coagulating factor Xa, can directly catalyze prothrombinase into activated thrombinase, initiating cascade coagulating reaction. Several studies abroad indicate that mouse fgl2 has been involved in MHV-3 induced fulminant hepatitis, spontaneous abortion and xenograft rejection by mediating "immune coagulation", fibrin deposition and microthrombus leading to the pathological changes.

Research frontiers

In addition to its primary role in hemostasis and blood coagulation, thrombin is a potent mitogen capable of inducing cellular functions. Thrombin can dramatically increase the growth and metastatic potential of tumor cells, thus it should be of great importance in the behavior of cancer. Both tissue factor (TF) and thrombin exert their influence on tumor angiogenesis and metastasis through clotting-dependent and clotting-independent mechanisms. Fgl2 functions as a novel immune coagulant with the ability to generate thrombin directly.

Innovations and breakthroughs

Fgl2 highly expressed in tumor cells and activated interstitial infiltrated cells, which may contribute to the characteristics of hypercoagulability and in turn induces tumor angiogenesis and metastasis.

Applications

In present study, the authors investigated hfgl2 expression and its histological localization in cancer, which will provide a new point of view on the characteristic hypercoagulability of cancer and efficacious anticoagulant therapy in cancer treatment.

Peer review

It has been proved that fgl2 functions as an immune coagulant with the ability to cleave prothrombin to thrombin directly and there are relationships between thrombosis and cancer. The aim of this study was to investigate the role of fgl2 in tumor development. They found that Hfgl2 was detected in tumor tissues from 127 out of 133 patients as well as tumor tissues collected from human HCC nude mice and IL-2 and IFN- γ could increase hfgl2 mRNA *in vitro*. It is an interesting subject and results were clearly described.

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CLINICAL RESEARCH

Rectal administration of d-alpha tocopherol for active ulcerative colitis: A preliminary report

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Author contributions: Mirbagheri SA developed the main hypothesis of the study, performed endoscopic studies and clinical examinations, supervised and edited the paper; Mirbagheri SA, Nezami BG, and Assa S designed the research; Nezami BG and Assa S followed the patients and recorded the data; Hajimahmoodi M determined the plasma level of α -tocopherol in recruited patients; Nezami BG and Assa S analyzed the data and wrote the paper.

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reported adverse events or was hospitalized due to worsened disease activity.

CONCLUSION: This preliminary report suggests that rectal d- α tocopherol may represent a novel therapy for mild and moderately active UC. The observed results might be due to the anti-inflammatory and anti-oxidative properties of vitamin E.

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Key words: Vitamin E; Ulcerative colitis; Inflammatory bowel disease; Enema; Activity index

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Mirbagheri SA, Nezami BG, Assa S, Hajimahmoodi M. Rectal administration of d-alpha tocopherol for active ulcerative colitis: A preliminary report. *World J Gastroenterol* 2008; 14(39): 5990-5995 Available from: URL: <http://www.wjgnet.com/1007-9327/14/5990.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.5990>

Abstract

AIM: To investigate the anti-oxidant and anti-neutrophil recruitment effects of rectal d-alpha (d- α) tocopherol administration on mild and moderately active ulcerative colitis (UC).

METHODS: Fifteen patients with mild and moderately active ulcerative colitis were enrolled in an open-label study of d- α tocopherol enema (8000 U/d) for 12 wk. All patients were receiving concomitant therapy with 5-aminosalicylic acid derivatives (5-ASA) and/or immunomodulator medications. Endoscopic evaluation was performed at baseline and after 4th and 12th weeks. Disease activity was measured with the Mayo disease activity index (DAI) and remission was defined as DAI of ≤ 2 with no blood in stool. Clinical response was defined as a DAI reduction of ≥ 2 .

RESULTS: At the end of 12th week, the average DAI score significantly decreased compared to the beginning of the study (2.3 ± 0.37 vs 8 ± 0.48 , $P < 0.0001$). One patient was withdrawn after 3 wk for being unavailable to follow-up. On the 4th week of therapy, 12 patients showed clinical response, 3 of whom (21.4%) achieving remission. After 12 wk, all 14 patients responded clinically to the therapy and remission was induced in 9 of them (64%). No patient

INTRODUCTION

Inflammatory bowel disease (IBD) comprises a group of chronic, lifelong, immuno-inflammatory disorders, characterized by flare-ups due to increased inflammatory activity of the intestinal mucosa, interspersed with asymptomatic periods of remission^[1]. Although the etiology of IBD remains unknown, it is believed that the generation of an exaggerated intestinal immune response to otherwise innocuous stimuli plays a key role in the pathophysiology of this intestinal disorder^[2]. IBD is mainly characterized by upregulation of synthesis and release of different proinflammatory mediators, including reactive oxygen and nitrogen metabolites, eicosanoids, platelet-activating factor and cytokines. All of these mediators actively contribute to the pathogenic cascade that initiates and perpetuates the inflammatory response of the gut. Thus, the treatment strategy for IBD focuses on eliminating these causal inflammatory triggers and mediators.

Unfortunately no disease-specific treatment for IBD has yet emerged, and the best strategy to effectively downregulate the exacerbated immune response is likely

to interfere with multiple stages of the inflammatory cascade^[3]. In fact, the drugs currently used for the management of human IBD, i.e. 5-aminosalicylic acid derivatives, immunosuppressives and systemic or local glucocorticoids, exert their beneficial effects through a combination of different mechanisms^[4]. On the other hand, even the most effective drugs used in inflammatory bowel disease are only successful in about two-thirds of patients^[5,6], while these drugs are not devoid of potentially serious side effects that limit their use in a further substantial proportion of patients^[7,8].

It is now well established that vitamin E is a major lipophilic antioxidant in cellular membranes with excellent antioxidant activities^[9,10] which protects membrane lipids from peroxidation^[11,12] by scavenging not only chain carrying peroxy radicals but also singlet oxygen and superoxide anion radicals^[13]. This is especially interesting in the case of ulcerative colitis (UC), considering the pivotal role of oxygen free radicals in the genesis of mucosal damage. Additionally, the production of reactive oxygen species increases by the colonic mucosa in patients with UC^[14-16]; and inhibition of lipid peroxidation or scavenging of oxygen free radicals produces valuable preventive and therapeutic strategies for IBD^[12].

Thus, given the recent evidence suggesting anti-inflammatory properties for vitamin E^[17,18], d- α (d- α) tocopherol, as the dominant vitamin E isomer in plasma with the highest biopotency, may be expected to reduce the development of tissue injury in IBD. In support of this hypothesis, we report the preliminary results of an ongoing open-label case series study on clinical and endoscopic changes of disease severity in patients with active UC who received daily rectal doses of d- α tocopherol for at least 12 wk.

MATERIALS AND METHODS

Inclusion and exclusion criteria

We recruited 15 volunteer UC patients (5 males, 10 females) between February 2006 and February 2008, seen in our university-based gastroenterology practice. The study protocol was submitted to the university ethics committee and written informed consent was obtained from all participating patients. All enrolled patients had active disease, limited to sigmoid at the beginning of the study. In order to minimize observation errors, all diagnoses were made on the basis of a combination of clinical, endoscopic and histological criteria by a single faculty member gastroenterologist. Only patients with mild and moderately active UC despite a minimum of 4 wk of therapy with at least 3 g/d of an oral 5-ASA compound were eligible for inclusion to whom we permitted concomitant therapy with azathioprine or 6-mercaptopurine as long as the patient had been receiving the medication for a minimum of 24 wk and was on a stable dose for a minimum of 12 wk before enrolment. No patient received concomitant therapy with corticosteroids or rectally administered therapies of any kind. Use of anti-diarrheal medications was not

permitted and no patient had taken additional non-steroidal anti-inflammatory drugs (NSAIDs) and/or antibiotics during the 3 mo preceding their enrolment.

Patients were excluded if they had evidence of infectious colitis, history of an active malignancy, previous surgical procedure except for cholecystectomy or appendicitis, and contraindication to flexible sigmoidoscopy or biopsy. Further exclusion criteria included present or recent pregnancy, and concomitant serious illness such as history of diabetes mellitus, hypertension, severe liver disease and cardiac failure.

Study design

Disease activity was assessed using Mayo Disease Activity Index (DAI)^[19]. This index is calculated by summing the scores of four factors, each of which are graded on a scale from 0 to 3. The four features of disease activity are stool frequency, bleeding, physician's assessment of disease activity, and mucosal appearance. The maximum potential score is 12 points, with mild and moderate disease activity defined as a score below 10. Only patients with mild or moderate active UC based on DAI score were selected.

A primary clinical and colonoscopic evaluation was performed in all cases. Patients were then assessed clinically every 2 wk, and the following endoscopic evaluations were performed after 4th and 12th weeks. Therefore, it was not possible to calculate a DAI score at each time point. However, we computed a modified DAI (mDAI) score that includes all components of the full DAI score except the endoscopic appearance of the bowel. The maximum potential score in the mDAI is 9.

Meticulous laboratory evaluations were conducted for all participants at the time of recruitment, including determination of the hematocrit value, white blood cell and platelet count, liver function tests, ERS, stool exam and culture, and serum level of vitamin E. Serum samples were stored at -80°C until analysis. Vitamin E was measured, after extraction with methanol, by HPLC, with UV detection at 294 nm. Methanol, deionized water and butanol (90:4:6) were used as mobile phase and the column was Eurospher 100 C₈ (4.6 mm \times 25 cm). Re-evaluation of these tests was done each month until completion of study^[20].

All patients were trained to rectally apply the sufficient amount of liquid vitamin E (0912, NOW Foods, IL, USA) equivalent to 8000 IU (15 mL) using specific instrument for enema (Enema irrigator disposable, Model: D-201, Taiwan Snatch Co., Taiwan; Figure 1) every night at home and were advised to lie for at least 15 min in left supine position after administration. During the first 12 wk all patients were given diary sheets containing multiple choice questions on stool characteristics including the number of defecation per day, stool consistency and type of blood excretion at each defecation episode. Patients were asked to complete each sheet on its day and bring them back at next visit (every 2 wk). After the first 12 wk we stopped obtaining diary sheets from patients and continued the follow-up by monthly visits only and endoscopic



Figure 1 Specific instrument for enema (Enema irrigator disposable). Fifteen mL of the liquid vitamin E was rectally administered in our patients using this disposable enema irrigator.

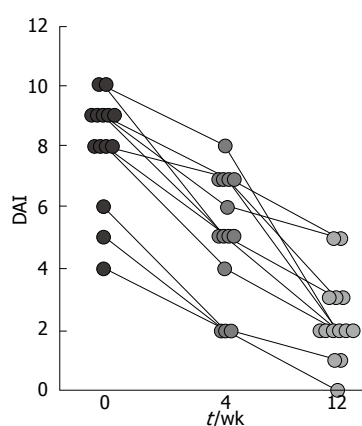


Figure 2 Individual changes in disease activity from week 0 to 12, assessed by the Mayo Disease Activity Index, in subjects who received d- α Tocopherol enema. One patient who was withdrawn early from the study is not included. There was a significant decrease in the mean DAI score for patients after 4th and 12th weeks of therapy ($P < 0.0001$).

studies every 3 mo. This second phase of study is still in progress.

Definition of outcomes

Patients with a final DAI score of ≤ 2 points were considered to have achieved remission. A clinical response was defined as a reduction in the DAI of ≥ 2 points. Clinical relapse was defined as the occurrence or worsening of symptoms, accompanied by an increase in the DAI score to 4, necessitating a change in therapy (addition of rectal therapies such corticosteroids or 5-ASA, surgery, *etc*). Refractory patients were those who had no significant improvement from their baseline, despite 12 wk of drug application.

Statistical analysis

Descriptive data are reported as percentages and medians and ranges. For statistical analysis, the 1-way analysis of variance (ANOVA) followed by Tukey post hoc was used. When appropriate, The Student *t* distribution was employed to compare 2 groups. Average DAI and mDAI scores at different time points were compared using the Mann-Whitney *U* test. All analyses used two sided tests of statistical significance with a significance level of 0.05.

RESULTS

Fifteen patients (5 males) were enrolled in the study

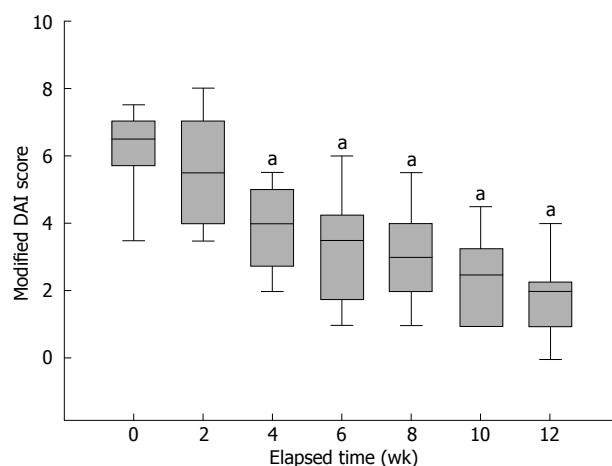


Figure 3 Change in the modified DAI. A modified DAI score, including all components of the complete DAI other than the endoscopic appearance is calculated at each observation point. By week 4 the mean modified DAI score significantly decreased compared to baseline (week 0) scores. $^aP < 0.0001$ vs baseline.

and one patient left the study due to permanent change of his city of residence. Therefore the analysis was performed on 14 patients. The age of patients was 33 ± 9.6 years (mean \pm SE) ranging from 21 to 55 years old, and the duration of UC was 4.7 ± 1.6 years. At enrolment, all patients had active disease with mild and moderate disease activity restricted to sigmoid (30 cm maximum involvement). All patients were receiving treatment at the time of entry into the study, consisting of sulphasalazine in 4 cases and mesalazine in the others. Two patients were also receiving azathioprine. No patient was on corticosteroids, antimicrobials or NSAIDs. At enrollment, the median Mayo DAI score was 8 (range, 4 to 10). Only 3 patients had mild disease activity, while the other 11 patients had moderate UC. On the 12th week, 9 patients had disease of mild severity and 5 had moderately active disease (Figure 2).

As summarized in Figure 2, clinical response was observed in 12 patients on the 4th week while clinical remission was induced only in 3 patients. After 12 wk, all patients responded clinically to therapy, with 9 of them going to clinical remission. In a secondary analysis, we examined the change in the DAI and mDAI scores over time (Figures 2 and 3). The mean DAI score before and on the 4th and 12th week of therapy were 8 ± 0.48 , 5.1 ± 0.54 and 2.3 ± 0.37 , respectively. Mann-Whitney *U* test revealed significant decrease in the mean DAI scores on 4th and 12th week of therapy comparing to the baseline ($P = 0.01$ and $P < 0.0001$, respectively) as shown in Figure 2. The average mDAI score started to decrease significantly on second week (Figure 3). As shown in Figure 4, mDAI score was significantly lower than at baseline for patients by week 4 and remained significantly lower for the remainder of the study ($P < 0.0001$ at weeks 4, 6, 8, 10 and 12, respectively). During the course of study there was no case of worsening disease activity or report of serious adverse event.

At the end of 12 wk, 12 patients elected to continue to receive d- α tocopherol, while 2 patients left the

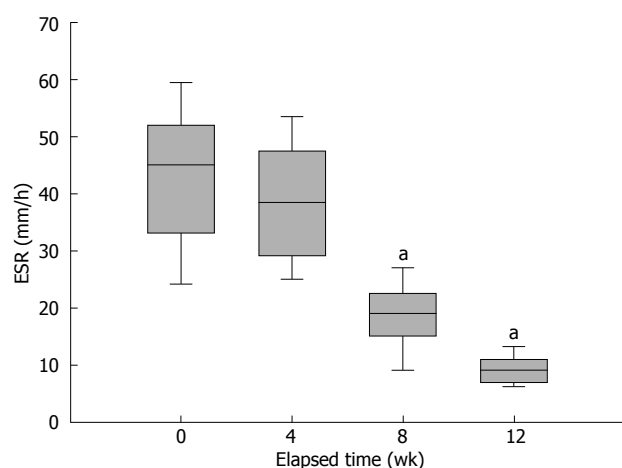


Figure 4 Individual changes in ESR from week 0 to 12, in our patients. There was a significant decrease in the mean ESR on the 8th and the 12th week of therapy compared to the beginning of the study. ^a $P < 0.0001$ vs baseline (0 wk).

survey due to personal reasons despite achieving a desirable response and satisfaction with therapy. All patients are assessed monthly for symptoms of disease activity and undergo endoscopic studies every 3 mo. The overall follow-up of patients in our study is approaching its mean of 8 mo by the time of composing this article. Interestingly, there have been no recurrences in patients who remained on therapy, while 2 flare-ups occurred in patients who left the study, after 4 wk and 7 wk.

As shown in Figure 4 the mean value of baseline ESR was 42.26 ± 11.7 while it was 9.33 mm/h on 12th week, showing a significant decrease ($P < 0.0001$). Eleven patients had moderate anemia at baseline (defined as haematocrit < 35 in men and < 40 in women) for which supplemental iron and folic acids were prescribed. At the end of the 12 wk, a low haematocrit was detected in only 3 patients, and it increased from 35.3 ± 6 to 43.9 ± 6 after 12 wk. The serum level of α -tocopherol did not change significantly after 12 wk compared to that of baseline (7.2 ± 4.2 and 6.7 ± 1.1 , respectively), and all patients had serum levels less than or within the normal range (5–12 $\mu\text{g/dL}$).

DISCUSSION

This case series provides evidence suggesting that local administration of d- α tocopherol might exert protective effects against UC in patients with mild and moderately active disease. In our study, rectal administration of d- α tocopherol significantly reduced the clinical disease activity indices and eliminated further need to corticosteroid therapy in otherwise non-responsive UC patients.

Recent studies demonstrated that plasma levels of α -tocopherol decrease after infection, trauma, burns, and inflammatory reactions, indicating that this antioxidant is exhausted during acute tissue injury^[21,22]. Similar concept exists for inflammatory bowel disease, indicating that anti-oxidants are intimately involved in the process of active IBD, for example ingested high doses of vitamin E as an anti-oxidant along with other anti-oxidants

reduce the extent of tissue damage in IBD, and tissue antioxidant levels are shown to be decreased in UC patients^[23–25]. Systemic α -tocopherol in conjunction with selenium is also shown to reduce the severity of IBD in chemically induced colitis in rats^[26]. Therefore, it is tempting to hypothesize that increasing the exposure of affected intestinal mucosa to this anti-oxidant might strengthen the defense capacity of the affected gut.

Vitamin E, particularly α -tocopherol, exerts a number of non-anti-oxidant functions, some associated with inhibition of protein kinase C (PKC) such as inhibition of platelet aggregation, and others independent of PKC such as the expression of intercellular adhesion molecule-1 (ICAM-1, CD54), integrins and CD36^[27,28]. α -tocopherol also inhibits respiratory burst in human macrophages via a mechanism involving PKC inhibition, followed by attenuation of p47 (phox) phosphorylation and membrane translocation^[29].

Increased NF- κ B activity is found in inflamed intestinal mucosa; and factors implicated in IBD, such as TNF- α , LPS, and IL-1, are potent activators of NF- κ B^[30]. On the other hand, reactive oxygen species, the hypothetical mediators of IBD flare-ups, have been implicated in the stimulation of the signal transduction pathway involving NF- κ B. Many current therapies for IBD act at least in part through the inhibition of NF- κ B or through inhibition of signals that activate NF- κ B. Interestingly, α -tocopherol is proved to inhibit NF- κ B activation in rat Kupffer cells and a human monocytic cell line^[31,32].

One of the proinflammatory genes regulated by NF- κ B is ICAM-1 (cell surface glycoprotein, playing a critical role in mediating leukocyte-endothelial and a marker of active inflammation). Gulubova *et al*^[33] demonstrated a marked upregulation of endothelial E-selectin, ICAM-1, and VCAM-1 expression in the inflamed colonic mucosa and submucosa in active UC. Infiltration of neutrophils into colonic mucosa is a central event in the acute phase of UC for which cell adhesion molecules are necessary^[34]. Accordingly, by blocking the activity of NF- κ B, which leads to decreased production of proinflammatory cytokines, anti-inflammatory properties of α -tocopherol might be explained. Therefore, α -tocopherol in high local doses might also affect the binding affinity between the neutrophil and the endothelial cell by decreasing the expression of adhesion molecules on the endothelial cells.

Systemic markers of inflammation, such as ESR, CRP, platelet count and white blood cell count are commonly used in clinical practice, but correlation to ongoing intestinal inflammation is poor^[35]. Therefore, we monitored the consecutive changes in ESR (Figure 4) and complete blood count (CBC) of our patients. These results show a significant conversion of ESR and quantitative increase in haematocrit.

To the best of our knowledge, this is the first data on remission inducing properties of vitamin E. This study also confirms the feasibility and acceptability of rectal administration of vitamin E in patients with IBD. Except for one patient who left the study due to difficulty for

follow-up, all patients completed the three month course of study required for primary assessments. Furthermore, 12 patients are still taking medication and participate in the regular evaluations. By the time of composing this article, the average course of treatment is approaching 8 mo. Disease activity was rated as inactive (clinical remission) in 64%. Comparing these results with the beginning of study demonstrates a significant reduction in disease severity of UC patients ($P < 0.0001$) starting after the 4th week of therapy. As shown in Figure 3, symptoms started to improve after 3 wk. This result is comparable with conventional therapies for IBD such as mesalazine or sulfasalazine. Overall satisfaction of patients despite the uncomfortable method of nightly rectal administration of d- α tocopherol reflects that the desirable outcomes outweigh its difficulty in application.

Elevated and toxic levels of plasma vitamin E were a concern in our study, since there was no previous study measuring the systemic absorption of vitamin E when applied intrarectally. Thus, plasma levels of vitamin E were measured to eliminate concerns about vitamin E overdosage and results revealed that the mean vitamin E level was not significantly different from that of before the study. This shows that despite the high doses of rectal vitamin E administered every night, little is absorbed and no concern remains about the overdosage of vitamin E in patients.

In the setting of a case series study we only recruited UC patients with strictly defined criteria. Our hypothesis was the increased local effects of vitamin E in UC, in which the pathology is mainly restricted to mucosal layer. In addition to the substantial physical and financial burden of IBD on patients, it is often difficult to continue treatment due to decreased therapeutic effects or adverse reactions over time^[7,8]. In this regard, use of vitamin E may expand the choices available for treatment of IBD, simplifying prescription or therapeutic technique.

The nature of our study casts limitations to implicate definite clinical results from this report, since case series cannot measure and do not eliminate the placebo effect. However, the best expected placebo effect is reported to be 16%-52% in rectal therapies of IBD which is still far lower than our results^[6]. Best UC therapies only have 60% to 80% success rates, while we gained 100% clinical response with over 60% remission induction in our patients on the 12th week. This is especially important considering that these results are achieved without prescribing any synthetic agent, and we eliminated the need to corticosteroids in patients otherwise resistance to therapy.

Taken together, case series like this study are best used as a source of hypotheses for investigation by stronger study designs. Thus, future researchs should aim at testing the efficacy of natural vitamin E enema in a well controlled study to measure its exact effect on reducing risk of flare-ups with the minimum confounding factors. This evidence suggests that vitamin E reduced the development of colon inflammation. The observed effect seems to be due to antioxidative and

anti-inflammatory effects of vitamin E which is potentially taking effect by local administration. Based on our preliminary results vitamin E might show considerable promise as a new therapeutic modality for IBD.

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COMMENTS

Background

The exact etiology of inflammatory bowel disease (IBD) is not yet understood, however it is believed that the generation of an exaggerated intestinal immune response to otherwise innocuous stimuli plays a key role in the pathophysiology of this intestinal disorder. IBD is mainly characterized by upregulation of synthesis and release of different pro-inflammatory mediators all of which actively contribute to the pathogenic cascade that initiates and perpetuates the inflammatory response of the gut. Thus, the current treatment strategy for IBD focuses on eliminating these causal inflammatory triggers and mediators.

Research frontiers

Vitamin E is a major lipophilic antioxidant in cellular membranes with excellent antioxidant activities which protects membrane lipids from peroxidation by scavenging not only chain carrying peroxy radicals but also singlet oxygen and superoxide anion radicals. This is especially interesting in case of ulcerative colitis (UC), considering the pivotal role of oxygen free radicals in the genesis of mucosal damage. Given the recent evidence suggesting anti-inflammatory properties for Vitamin E, d-alpha (d- α) tocopherol, as the dominant vitamin E isomer in plasma with the highest biopotency, may be expected to reduce the development of tissue injury in UC.

Innovations and breakthroughs

This case series provides evidence for the first time that local administration of d- α tocopherol might exert protective effects against UC. The authors have carefully followed the serial alterations of patients' disease activity index, along with few other markers of disease severity and have shown that rectal administration of d- α tocopherol significantly reduces the clinical disease activity indices which eliminated further need to corticosteroid therapy in otherwise non-responsive UC patients with mild and moderately active disease. The observed effect seems to be due to antioxidant and anti-inflammatory effects of vitamin E which is potentially taking effect by local administration.

Applications

The results of this interesting study suggests that natural antioxidants like d- α tocopherol might show considerable promise as new therapeutic modalities for IBD, with apparently lower side effects and complications compared to the current therapies.

Peer review

This is the first study to address the immunoregulatory effects of d- α tocopherol in a dominant Th1 response disease. The outstanding results demonstrate that natural isomer of vitamin E, reduces the extent of macroscopic mucosal damage and clinical severity of related syndromes in UC patients, when applied intra-rectally for as short as 4 wk.

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BASIC RESEARCH

Dietary glycine blunts liver injury after bile duct ligation in rats

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significantly in the group fed glycine. Focal necrosis was observed 2 d after BDL. Glycine partially blocked the histopathological changes. Incubation of Kupffer cells with DCA led to increased intracellular calcium that could be blocked by incubation with glycine. However, systemic blockage of Kupffer cells with gadolinium chloride had no effects on transaminase release. Incubation of isolated hepatocytes with DCA led to a significant release of LDH after 4 h. This release was largely blocked when incubation with glycine was performed.

CONCLUSION: These data indicate that glycine significantly decreased liver injury, most likely by a direct effect on hepatocytes. Kupffer cells do not appear to play an important role in the pathological changes caused by cholestasis.

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Key words: Glycine; Bile duct ligation; Cholestasis; Kupffer cells; Serum alanine transaminase; Deoxycholic acid

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Abstract

AIM: To investigate the effects of (dietary) glycine against oxidant-induced injury caused by bile duct ligation (BDL).

METHODS: Either a diet containing 5% glycine or a standard diet was fed to male Sprague-Dawley (SD) rats. Three days later, BDL or sham-operation was performed. Rats were sacrificed 1 to 3 d after BDL. The influence of deoxycholic acid (DCA) in the presence or absence of glycine on liver cells was determined by measurement of calcium and chloride influx in cultivated Kupffer cells and lactate dehydrogenase (LDH) activity was determined in the supernatant of cultivated hepatocytes.

RESULTS: Serum alanine transaminase levels increased to about 600 U/L 1 d after BDL. However, enzyme release was blunted by about two third in rats receiving glycine. Release of the alkaline phosphatase and aspartate aminotransferase was also blocked

INTRODUCTION

Chronic cholestasis liver diseases lead to liver injury and ultimately progress to portal fibrosis, cirrhosis, and end-stage liver disease requiring liver transplantation. They include primary sclerosing cholangitis, primary biliary cirrhosis, extrahepatic biliary atresia, idiopathic adulthood ductopenia, idiopathic neonatal hepatitis, Byler's disease, and arteriohepatic dysplasia^[1-4]. Various drugs, total parenteral nutrition, sarcoidosis, chronic liver transplant rejection, and graft-versus-host disease may also cause chronic cholestasis^[5-7]. Currently the most promising therapy for chronic cholestatic liver diseases is ursodeoxycholic acid^[8], that may delay liver disease progression, but cannot prevent liver injury or fibrosis^[9].

The pathophysiology of cholestasis induced liver injury and fibrosis remains unclear. One possible mechanism is that hepatic accumulation of hydrophobic bile acids causes oxidative stress in the liver^[10]. Previous studies showed that hepatic mitochondria generate reactive oxygen species when isolated hepatocytes are exposed to hydrophobic bile acids^[10,11]. This mitochondrial free radical production may be an important mechanism of cholestatic liver injury. However, the major source of free radicals remains unclear. One possible cell type responsible for the generation of free radicals could be the Kupffer cells, the resident macrophages of the liver. They are involved in disease states, such as endotoxin shock^[12], alcoholic liver diseases^[13], and other toxicant-induced liver injury by releasing eicosanoids, inflammatory cytokines (IL-1, IL-6, TNF- α), and free radical species^[14].

Glycine, a simple nonessential amino acid, is a well-known inhibitory neurotransmitter in the central nervous system that acts *via* a glycine-gated chloride channel and has been shown to be protective against hypoxia, ischemia, and various cytotoxic substances^[15-17]. Furthermore, it was demonstrated that dietary glycine protected both, the lung and the liver against lethal doses of endotoxin in the rat^[18] and improved graft survival after liver transplantation^[19].

Based on pharmacological data^[15-17], a glycine-gated chloride channel was detected in Kupffer cells and other macrophages^[20] that influenced the activation process of these cells. Glycine binds to and opens a chloride channel at the cell membrane, causing cell hyperpolarization that subsequently blocks calcium influx^[20]. Thus it prevents the activation of intracellular signaling cascades.

Accordingly, we hypothesized in this study that dietary glycine has a protective effect in liver injury after bile duct ligation (BDL) by preventing activation of Kupffer cells.

MATERIALS AND METHODS

Animal husbandry and diet treatment

Adult male Sprague-Dawley (SD) rats (200-250 g) were housed four to a cage in a facility approved by the Association for the Accreditation and Assessment of Laboratory Animal Care International. Three days before surgery, rats were randomly assigned to two experimental groups and fed either a semisynthetic powdered diet (Teklad test diets, Madison, WI, USA) containing 5% glycine and 15% casein (glycine group) or 20% casein (control group). After surgery, each rat continued to receive its assigned diet throughout the entire experimental period. All animals received humane care in compliance with guidelines approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

BDL

Rats underwent BDL and transection or sham operation under ether anesthesia, as described elsewhere^[21]. Briefly,

the common bile duct was located through a midline abdominal incision, double ligated near the liver, and transected between ligatures. Control rats received sham operation underwent the same procedure except that the bile duct was only gently manipulated, but not ligated or sectioned. Some rats were given gadolinium chloride (GdCl₃; 20 mg/kg body weight iv 24 h before BDL) to selectively deplete Kupffer cells. Rats were sacrificed 1 to 3 d after BDL or sham operation for further investigations ($n = 5-6$ per group).

Clinical chemistry and histology

Blood samples were collected from the tail veins at times indicated. Serum alkaline phosphatase (ALP), alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), and bilirubin were measured using analytic kits from Sigma (St. Louis, MO, USA). On the day of death, each rat was anesthetized with pentobarbital sodium (75 mg/kg ip), the abdomen was opened, and the portal vein was cannulated with a 20-gauge cannula. The liver was rinsed using a syringe containing 10 mL physiological saline, followed by slow infusion of 5 mL 10% buffered formaldehyde (VWR International, West Chester, PA, USA). After 48 h in fixative, paraffin sections were prepared and stained with hematoxylin-eosin. Liver pathology was scored in a blinded manner based on a scoring system described by Nanji *et al*^[22] (inflammation and necrosis: 1 focus per low-power field: 1+; 2 or more foci: 2+).

Preparation and culture of Kupffer cells and hepatocytes

Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll (Sigma, Taufkirchen, Germany) as described elsewhere^[23] with slight modifications^[24]. Briefly, the liver was perfused through the portal vein with Ca²⁺- and Mg²⁺-free HBSS at 37°C for 10 min at a flow rate of 20 mL/min. Subsequently, perfusion was with HBSS containing 0.02% collagenase IV (Sigma) at 37°C for 10 min. After the liver was digested, it was excised and cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze and the filtrate was centrifuged two times at 70 g for 3 min at 4°C to remove parenchymal cells. The nonparenchymal cell fraction (mostly Kupffer cells) in the supernatant was washed with buffer and centrifuged at 650 g for 7 min at 4°C. Cell pellets were suspended in buffer and centrifuged on a density cushion of Percoll (25% and 50%) at 1800 g for 15 min at 4°C. The Kupffer cell fraction was collected, centrifuged at 650 g for 7 min and suspended again in buffer. Viability of cells was determined by Trypan blue exclusion. Purity (> 90%) of Kupffer cell cultures was evaluated by morphological observation and by phagocytic uptake of FITC-labeled 1 μ m latex-beads. Kupffer cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FCS and antibiotics/antimycotics (100 U/mL of penicillin G, 100 μ g/mL of streptomycin sulfate, and 0.25 μ g/mL amphotericin B; Sigma) at 37°C in a 10% CO₂-containing atmosphere. Nonadherent cells were removed after 30 min by replacing the culture medium.

The parenchymal cell fraction (mostly hepatocytes) was also isolated by Percoll (50%) centrifugation as described previously^[25] and cultured at 1×10^6 cells/well in RPMI 1640 medium (Sigma) containing 10% heat-inactivated fetal bovine serum and antibiotics as described above. Cells were cultured for 24 h before used for further experiments.

Measurement of intracellular Ca^{2+} concentration in Kupffer cells

Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured fluorometrically using the fluorescent calcium indicator dye fura-2. KC (1×10^6 cells/plate) were incubated in modified HBSS (mHBSS; in mmol/L): 110 NaCl, 5 KCl, 0.3 Na_2HPO_4 , 0.4 KH_2PO_4 , 5.6 glucose, 0.8 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 NaHCO_3 , 1.26 CaCl_2 , 15 HEPES, pH 7.4 containing 5 $\mu\text{mol/L}$ fura-2 AM (Molecular Probes, Eugene, OR, USA) at room temperature for 45 min. Coverslips plated with Kupffer cells were rinsed and placed in chambers with mHBSS at room temperature. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm and emission at 510 nm were monitored in individual Kupffer cells. A Nikon inverted fluorescent microscope interfaced with dual-wavelength fluorescent photometer (Intracellular Imaging, Cincinnati, OH, USA) was used to ratiometrically determine $[\text{Ca}^{2+}]_i$. Data were collected and analyzed using InCyt software (Intracellular Imaging).

Determination of lactate dehydrogenase (LDH) release in hepatocytes

Twenty-four hours after isolation, hepatocytes were stimulated with deoxycholic acid (DCA; 0.1 mmol/L; Sigma) or normal saline in the presence or absence of glycine (1 mmol/L; Sigma). After 4 h of culture, supernatant was collected and LDH assays were performed *via* standard enzymatic techniques as described elsewhere^[26].

Measurement of radiolabelled chloride influx by Kupffer cells

Assays for uptake of ^{36}Cl used an adaptation of a method described for neurons by Schwartz *et al*^[27] and modified by Morrow and Paul^[28]. Briefly, 2×10^6 Kupffer cells were plated on coverslips in 60 mm² culture dishes and incubated as described above. After 24 h, media was replaced with HEPES buffer (20 mmol/L HEPES, 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO_4 , and 2.5 mmol/L CaCl_2 , pH 7.4) and allowed to equilibrate for 10 min at room temperature. Coverslips were gently blotted dry and incubated in a petri dish with 2 mL of buffer containing 2 mCi/mL ^{36}Cl in the presence of glycine (1 mmol/L) and/or DCA (0.01 and 0.1 mmol/L) for 5 s. Chloride influx was linear between 2-10 s; thus, a 5 s incubation time was chosen for all experiments. Chloride influx was terminated by washing the coverslip with ice-cold buffer for 3 s followed by a second wash for 7 s^[28]. Coverslips were placed in scintillation vials, and protein was solubilized by adding

1.6 mL NaOH (0.2 mol/L) for 2 h. An aliquot (0.16 mL) was collected for determination of protein by the method of Lowry *et al*^[29]. Ecolume (10 mL) was added and radioactivity was determined by standard scintillation spectroscopy.

Statistical analysis

Data are presented as mean \pm SD. ANOVA and the Student-Newman-Keuls post hoc tests were used for the determination of statistical significance between treatment groups, and $P < 0.05$ was selected before the study as the level of significance.

Boxplots illustrate median values and interquartile distance. The error bars represent the 5th and 95th percentiles.

RESULTS

Effects of dietary glycine on serum enzymes and histology after BDL

In untreated rats fed a standard chow diet, serum alanine transaminase (ALT) levels average 40 U/L and were not significantly altered by sham operation (data not shown). One day after BDL, ALT increased to 541 U/L (Figure 1A), and remained elevated at day 2 and 3 after BDL with 599 U/L and 543 U/L, respectively. When rats were treated with dietary glycine, ALT levels increased to 248 U/L one day after BDL (Figure 1A). On day 2 and 3, ALT levels were also significantly decreased compared to bile duct-ligated rats fed a control diet (232 U/L on day 2 and 161 U/L on day 3). Serum aspartate aminotransferase (AST) and alkaline phosphatase (AP) levels, which were also measured at day 1, 2, and 3 after BDL, revealed similar results as ALT (Figure 1B and C).

Normal liver architecture was observed in rats on a control and a glycine diet after sham operation (Figure 2A). Two days after BDL focal necrosis and white blood cell infiltration were detected in livers of rats receiving a standard diet (Figure 2B). These pathological changes were partially blocked in rats receiving dietary glycine (Figure 2C).

Influence of DCA on calcium and chloride influx in cultivated Kupffer cells in the presence or absence of glycine

Intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in cultured Kupffer cells was determined fluorometrically with the calcium indicator fura-2 as described in MATERIALS AND METHODS. After the addition of 0.01 or 0.1 nmol/L DCA, $[\text{Ca}^{2+}]_i$ levels increased as expected over the investigated time period of 10 min (Figure 3). Glycine (1 mmol/L) added 3 min before DCA inhibited this increase/induction in $[\text{Ca}^{2+}]_i$. Glycine alone had no detectable effect on $[\text{Ca}^{2+}]_i$ (data not shown).

The glycine-gated chloride channel mediates the influx of chloride and hyperpolarizes the cells^[30] thereby preventing DCA-induced increases of $[\text{Ca}^{2+}]_i$ (Figure 3). Indeed, glycine (1 mmol/L) caused a significant, about 4-fold influx of radiolabeled chloride in the presence of DCA (0.01 or 0.1 nmol/L) (Figure 4).

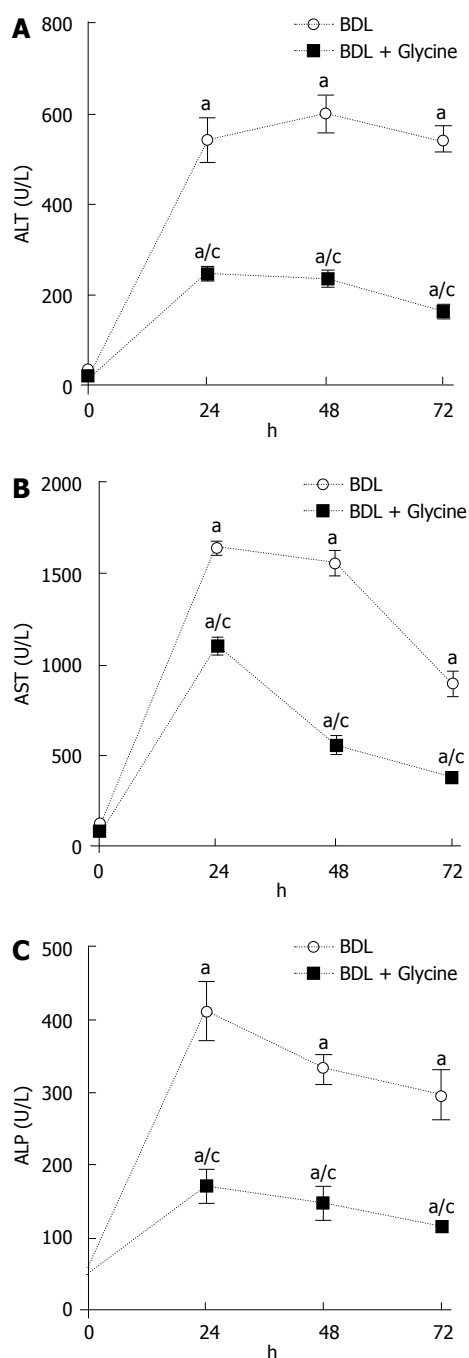


Figure 1 Dietary glycine blunts increased liver enzyme release after BDL. Blood was collected at the time points indicated. Serum alanine aminotransferase (A), aspartate aminotransferase (B), and alkaline phosphatase (C) activity was determined using commercial kits. Values are mean \pm SD ($n = 5-6$ in each group). ^a $P < 0.05$ vs sham operation; ^c $P < 0.05$ vs bile duct-ligated rats fed control diet.

This effect of glycine was significantly reduced by the classical glycine-gated chloride channel antagonist strychnine (data not shown).

Effects of Kupffer cell elimination on serum enzymes after BDL

To investigate whether Kupffer cells play an important role in cholestatic liver injury, rats were treated with GdCl_3 that selectively depletes Kupffer cells, before BDL. Suppression of Kupffer cells with GdCl_3 neither

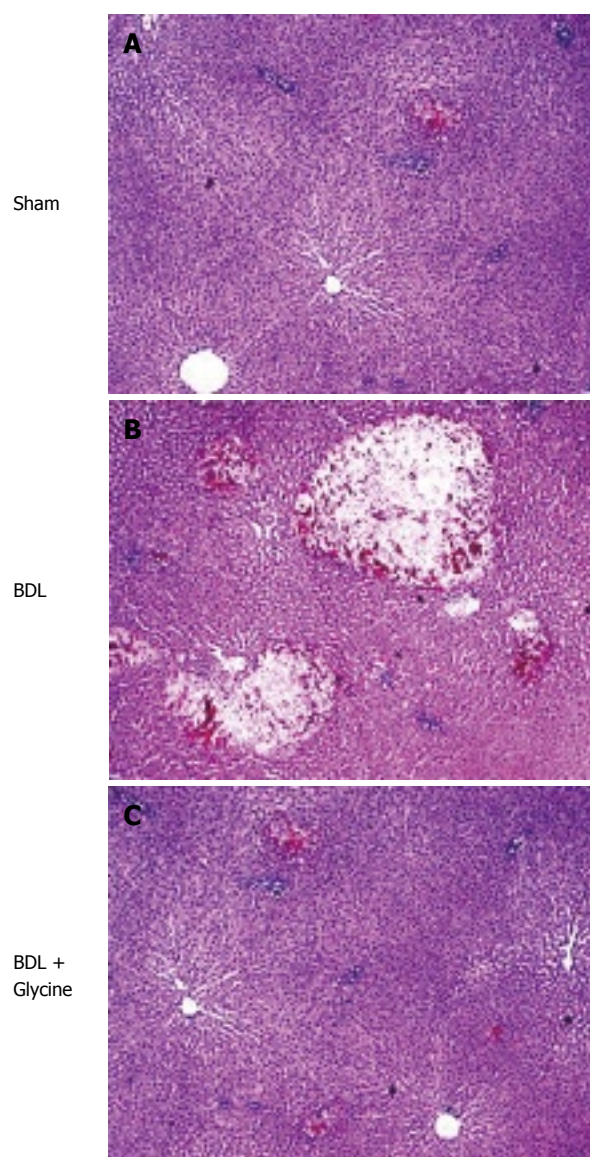


Figure 2 BDL induced focal necrosis that was blunted by dietary glycine. Shown are representative images ($\times 75$). A: Sham operation with control diet; B: BDL with control diet; C: BDL with dietary glycine.

blunted ALT release (Figure 5) nor attenuated focal necrosis after BDL (data not shown), confirming our previously published data^[31]. In detail, ALT levels increased up to $779 \text{ U/L} (\pm 53)$ 24 h after BDL. Pretreatment with GdCl_3 one day before BDL had no effect on this transaminase release after BDL ($870 \pm 78 \text{ U/L}$).

Influence of DCA on LDH release in isolated hepatocytes in the presence or absence of glycine

Incubation of isolated hepatocytes with DCA (0.1 mmol/L) led to a specific release of LDH (Figure 6) over the investigated time period (1 h incubation time: $101 \pm 8 \text{ U/L}$; 2 h incubation time: $112 \pm 7 \text{ U/L}$; 3 h incubation time: $119 \pm 8 \text{ U/L}$; 4 h incubation time: $149 \pm 18 \text{ U/L}$). This release was significantly blocked when glycine was simultaneously added (1 h incubation time: $42 \pm 7 \text{ U/L}$; 2 h incubation time: $77 \pm 7 \text{ U/L}$; 3 h incubation time: $92 \pm 8 \text{ U/L}$; 4 h incubation time:

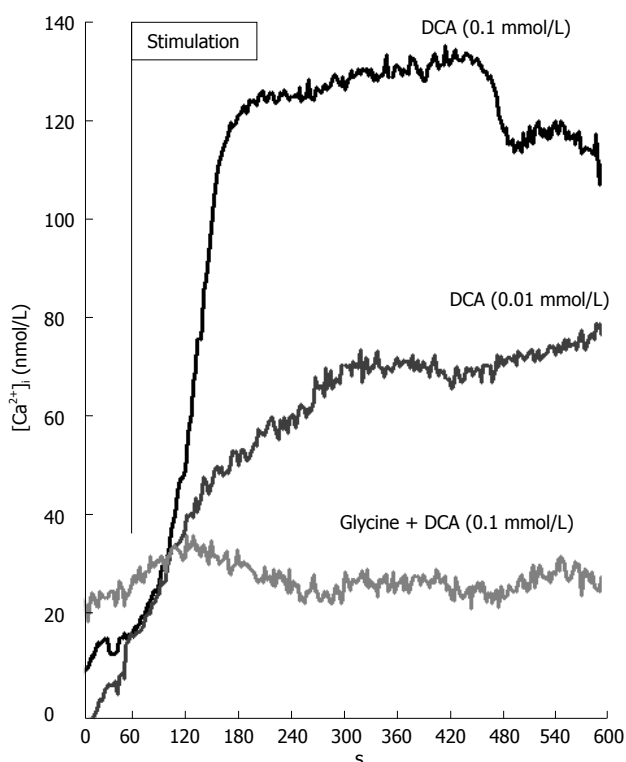


Figure 3 Glycine prevents DCA-induced rise of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in Kupffer cells. $[\text{Ca}^{2+}]_i$ was measured fluorometrically. Data are representative of 6 independent experiments.

79 ± 6 U/L). Incubation with glycine or saline alone had almost no effect on the release of LDH (Figure 6).

DISCUSSION

Chronic cholestatic liver diseases are one of the leading indications for liver transplantation in children and adults^[1,2]. Therefore, new strategies to reduce the pathological changes caused by (chronic) cholestasis are needed, because current therapies, such as ursodeoxycholic acid^[9], do not prevent liver injury. Oxidative stress and activation of Kupffer cells are probably involved in the pathogenesis of liver injury caused by cholestasis. Glycine has been shown to be an anti-inflammatory amino acid acting *via* inhibitory effects on several white blood cells, including Kupffer cells^[15-19]. Glycine activates a chloride channel, leading to cell hyperpolarization and a concomitant blocking of calcium influx *via* a voltage dependent calcium channel^[20]. Accordingly, we hypothesized that the activation of Kupffer cells and the associated free radical formation after BDL could be blocked by glycine, thus leading to a decreased liver injury.

Dietary glycine blunts liver injury due to BDL

In confirmation of previous work from our and several other laboratories^[21,31-35], BDL caused hepatic enzyme release (Figure 1) and focal cell necrosis (Figure 2), as expected. However, hepatic enzyme release (ALT, AST, AP) was significantly blunted and histopathological changes were partially blocked in the group receiving

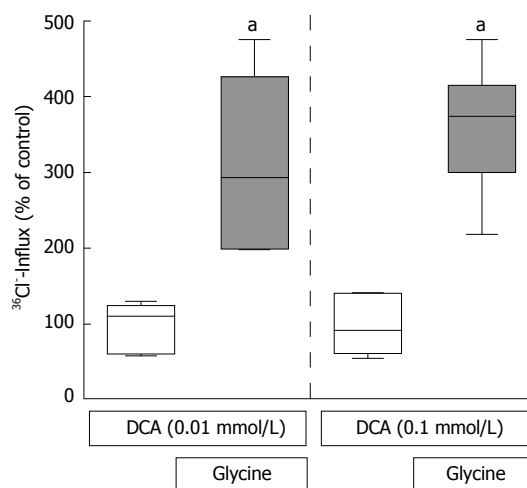


Figure 4 Glycine stimulates influx of radiolabeled chloride in Kupffer cells. Data are expressed as % of control. Values are mean \pm SD and are representative of at least 6 individual experiments. ^a $P < 0.05$ vs DCA (= control) group.

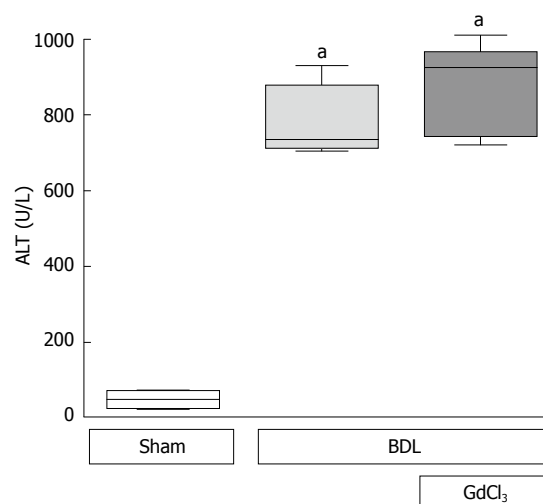


Figure 5 Elimination of Kupffer cells does not prevent elevated ALT release after BDL. Rats were given gadolinium chloride (GdCl_3 ; 20 mg/kg body weight) or saline 24 h before BDL or sham operation was performed. Values are mean \pm SD ($n = 5-6$ in each group). ^a $P < 0.05$ vs sham-operated group.

dietary glycine. Incubation of Kupffer cells with DCA led to increased intracellular calcium that was inhibited by incubation with glycine (Figure 3), most likely thru a glycine stimulated influx of chloride (Figure 4). However, systemic blockage of Kupffer cells with gadolinium chloride had no effect on transaminase release (Figure 5), indicating a minor, if any role of Kupffer cells in the pathophysiology of experimental cholestasis. Incubation of hepatocytes with DCA *in vitro* led to a significant release of LDH that was reduced by glycine (Figure 6).

How does dietary glycine decrease cholestasis-induced liver injury?

The protective effects of glycine are probably due to its direct effect on target cells or mediated by inhibition of inflammatory cell activation. Glycine appears to exert several protective effects, including anti-inflammatory, immunomodulatory and direct cytoprotective actions.

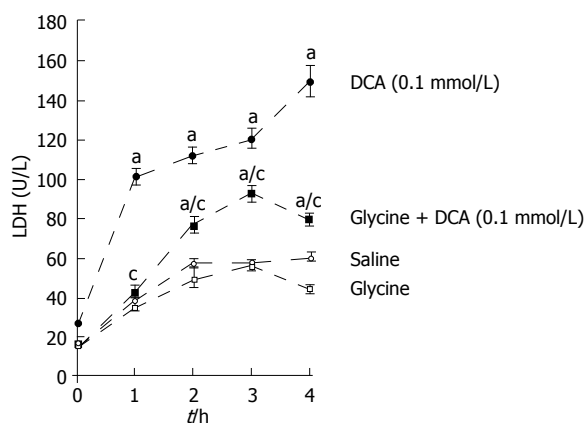


Figure 6 Glycine reduces DCA-induced release of LDH in isolated hepatocytes. Cells were isolated and stimulated with DCA (0.1 mmol/L) or saline in the presence or absence of glycine (1 mmol/L). Values are mean \pm SD ($n = 5-6$ in each group). ^a $P < 0.05$ vs saline or glycine group; ^c $P < 0.05$ vs DCA treated hepatocytes.

The underlying mechanisms are not completely understood. Glycine decreases oxidative stress^[15-19] by different and partly indirect mechanisms that prevent reactive oxygen species formation. Furthermore, glycine protects renal tubular cells, hepatocytes and endothelial cells against injury from hypoxia, ischemia-reperfusion and ATP depletion^[16,36-39]. Most studies show that glycine protects plasma membrane integrity, but does not restore ATP levels or affect intracellular pH^[16,38-40].

Activation of the glycine-gated chloride channel is another widely postulated mechanism for the effects of glycine. The glycine receptor exists in a wide variety of cells, beside its typical occurrence at the postsynaptic neuronal membranes of the spinal cord. Besides endothelial cells and renal proximal tubular cells^[17,41], cells involved in inflammatory and immune responses, such as macrophages, monocytes, neutrophils, and T lymphocytes, express a glycine receptor^[18,20,42-44]. Glycine acts thru its receptor on inflammatory cells, such as Kupffer cells, to suppress activation of transcription factors and the formation of free radicals and inflammatory cytokines. In the plasma membrane, glycine appears to activate a chloride channel^[20,30] that stabilizes or hyperpolarizes the plasma membrane potential (Figure 4). As a consequence, agonist-induced opening of L-type voltage dependent calcium channels and the resulting increases in intracellular calcium ions are suppressed (Figure 3), which may account for the immunomodulatory and anti-inflammatory effects of glycine. By preventing Kupffer cell activation, a decreased formation of inflammatory and fibrogenic mediators may be achieved. However, the role of Kupffer cells in fibrosis is controversial. Destruction of Kupffer cells attenuated liver fibrosis caused by carbon tetrachloride^[45]. By contrast, in a rat model of reversible biliary obstruction, inactivation of Kupffer cells impaired collagen metabolism and inhibited the resolution of fibrosis^[46]. Kupffer cells release many mediators, like TNF- α , TGF- β , human growth factor, PDGF, and reactive oxygen species^[47,48] that activate stellate cells

leading to fibrosis. TNF- α production and NF- κ B activation increase during cholestasis^[49,50]. Activation of NF- κ B, probably due to oxidative stress, could lead to expression of TNF- α . However, suppression of Kupffer cell function with GdCl₃, a treatment that blocks carbon tetrachloride-induced fibrosis, did not attenuate injury caused by cholestasis (Figure 5) confirming previous work from our laboratory^[51]. This finding indicates that Kupffer cells most likely do not play a prominent role in cholestasis-induced fibrosis *in vivo* and that glycine does not work exclusively by inhibiting Kupffer cell activation.

Recent work suggested that liver parenchymal cells at least contain a glycine dependent receptor. In isolated hepatocytes, glycine blocks the increase in intracellular calcium due to PGE2 and phenylephrine, an α 1-type adrenergic receptor agonist^[51]. Low-dose strychnine partially reverses the inhibition by glycine. When extracellular chloride is omitted, glycine is much less effective in preventing increases in intracellular calcium due to PGE2. These data suggested that hepatoprotection by glycine is, in part, due to its direct effect on hepatocytes *via* regulating of intracellular calcium^[51]. Consistent with these earlier findings, a direct effect of glycine on LDH release in isolated hepatocytes was observed in the present study after DCA challenge (Figure 6). Nevertheless, the effect of the conjugation of glycine and the used “secondary bile acid” DCA on the ability to lyse cells directly and solubilize cellular and membrane components should be also considered.

In conclusion, we demonstrated that hepatic injury, due to BDL, is significantly reduced by dietary glycine. Moreover, the data indicate that glycine decreases liver injury under the conditions of experimental cholestasis thru a direct effect on hepatocytes. Surprisingly, Kupffer cells do not appear to play a major role in the pathological changes caused by cholestasis.

COMMENTS

Background

Chronic cholestasis leads to liver injury and will ultimately progress to portal fibrosis, cirrhosis and end-stage liver disease requiring liver transplantation. Oxidative stress and activation of Kupffer cells are probably involved in liver injury caused by cholestasis. The nonessential amino acid glycine has been shown to be anti-inflammatory in several injury models, acting via inhibitory effects on several white blood cells, including Kupffer cells. Additionally, it activates a chloride channel, leading to cell hyperpolarization and a concomitant blocking of calcium influx into the cell via a voltage dependent calcium channel.

Research frontiers

Ursodeoxycholic acid is currently the most promising therapy for chronic cholestatic liver diseases; however, it cannot prevent fibrosis. How cholestasis induces liver injury and fibrosis remains unclear. One possible mechanism is that accumulation of hydrophobic bile acids causes oxidative stress in the liver, leading to tissue injury, fibrosis and finally liver cirrhosis. One possible cell type responsible for the generation of free radicals could be the Kupffer cells, the resident macrophages of the liver. It is known that destruction of Kupffer cells by gadolinium chloride or transduction of Kupffer cells by recombinant adenovirus can protect the liver against injury. However, the role of Kupffer cells in fibrosis is controversial. Destruction of Kupffer cells attenuated liver fibrosis caused by carbon tetrachloride. By contrast, in a rat model of reversible biliary obstruction, inactivation of Kupffer cells impaired collagen metabolism and inhibited the resolution of fibrosis.

Innovations and breakthroughs

Recent studies demonstrated that dietary glycine protected both the lung and

liver against lethal doses of endotoxin in the rat and improved graft survival after liver transplantation. Based on pharmacological data a glycine-gated chloride channel could be detected in Kupffer cells and other macrophages that influence the activation process of these cells by preventing the activation of intracellular signaling cascades.

Applications

The aim of this study was to investigate the effects of (dietary) glycine against oxidant-induced injury caused by bile duct ligation (BDL). The findings suggested that glycine significantly decreased liver injury, most likely by a direct effect on hepatocytes. Kupffer cells do not appear to play an important role in the pathological changes caused by cholestasis.

Terminology

Glycine, a simple nonessential amino acid, is a well-known inhibitory neurotransmitter in the central nervous system that acts via a glycine-gated chloride channel and has been shown to be protective against hypoxia, ischemia, and various cytotoxic substances. Kupffer cells, which are derived from monocyte/macrophage cell lineage, are the resident macrophages of the liver. Although they represent about 80% of the total fixed macrophage population, they are less than 5% of the total hepatic cell population. Kupffer cells play a critical role in the pathogenesis of several disease states, including endotoxin shock and alcoholic liver disease, because they release physiologically active substances such as eicosanoids, inflammatory cytokines, and many free radical species leading to localized tissue injury.

Peer review

This is an interesting study. It investigated the effects of (dietary) glycine against oxidant-induced injury caused by BDL.

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BASIC RESEARCH

Facilitating effects of berberine on rat pancreatic islets through modulating hepatic nuclear factor 4 alpha expression and glucokinase activity

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Abstract

AIM: To observe the effect of berberine on insulin secretion in rat pancreatic islets and to explore its possible molecular mechanism.

METHODS: Primary rat islets were isolated from male Sprague-Dawley rats by collagenase digestion and treated with different concentrations (1, 3, 10 and 30 $\mu\text{mol/L}$) of berberine or 1 $\mu\text{mol/L}$ Glibenclamide (GB) for 24 h. Glucose-stimulated insulin secretion (GSIS) assay was conducted and insulin was determined by radioimmunoassay. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cytotoxicity. The mRNA level of hepatic nuclear factor 4 alpha (*HNF4 α*) was determined by reverse transcription polymerase chain reaction (RT-PCR). Indirect immunofluorescence staining and Western blot analysis were employed to detect protein expression of *HNF4 α* in the islets. Glucokinase (GK) activity was measured by spectrophotometric method.

RESULTS: Berberine enhanced GSIS rather than basal insulin secretion dose-dependently in rat islets and showed no significant cytotoxicity on islet cells at the concentration of 10 $\mu\text{mol/L}$. Both mRNA and protein

expressions of *HNF4 α* were up-regulated by berberine in a dose-dependent manner, and GK activity was also increased accordingly. However, GB demonstrated no regulatory effects on *HNF4 α* expression or GK activity.

CONCLUSION: Berberine can enhance GSIS in rat islets, and probably exerts the insulinotropic effect *via* a pathway involving *HNF4 α* and GK, which is distinct from sulphonylureas (SUs).

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Key words: Berberine; Sulphonylureas; Hepatocyte nuclear factor 4 alpha; Glucokinase; Pancreatic islet

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Wang ZQ, Lu FE, Leng SH, Fang XS, Chen G, Wang ZS, Dong LP, Yan ZQ. Facilitating effects of berberine on rat pancreatic islets through modulating hepatic nuclear factor 4 alpha expression and glucokinase activity. *World J Gastroenterol* 2008; 14(39): 6004-6011 Available from: URL: <http://www.wjg-net.com/1007-9327/14/6004.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6004>

INTRODUCTION

Type 2 diabetes mellitus is a complex and heterogeneous disorder caused by the interaction of hereditary and environmental factors, pathophysiologically characterized by insulin resistance and functional defects in insulin release from pancreatic β cells.

Sulphonylureas (SUs) are the most commonly prescribed insulin secretagogues. These drugs act *via* augmentation of insulin secretion from pancreatic β cells. The SU receptor-1 on the ATP-sensitive potassium channels (K_{ATP} channels) is occupied by SU leading to closure of the potassium channels and subsequent opening of calcium channels, resulting in exocytosis of insulin granules^[1]. Yet, the maintenance of satisfactory long-term glycaemic control in patients undergoing SU therapy is usually restricted by increased risk of

hypoglycemia coupled with declined insulinotropic activity due to desensitized β cells to the agents^[2,3]. Therefore, new types of insulinotropic substances with an alternative action profile are in demand.

Berberine, the major active constituent of Chinese herb *Rhizoma Coptidis*, is being used to treat diabetes for decades, showing obvious therapeutic actions with few reported side effects. Previous studies have demonstrated that berberine modulates cholesterol through increasing low-density lipoprotein receptor mRNA stability^[4], reduces body adiposity and increases insulin sensitivity partly through activating AMP-activated protein kinase^[5], and improves glucose metabolism *via* induction of glycolysis^[6], implying a promising future for berberine in the therapy of diabetes. Moreover, a new research further revealed that berberine also possessed insulinotropic property in isolated pancreatic islets^[7]. However, the underlying mechanism is not fully understood.

Recently, the hepatocyte nuclear factors (HNFs) transcriptional regulatory networks were identified in pancreatic islet tissue, providing insight into the molecular basis of abnormal β cell function. It was deemed that three members of HNFs family, HNF4 α , HNF1 α and HNF6, were at the center of the connected network, operating cooperatively to regulate numerous developmental and metabolic functions in human pancreatic islets. It was also revealed that HNF4 α was bound to about 11% of the genes represented on the DNA microarray in pancreatic islets^[8,9]. The occupancy by HNF4 α of a substantial fraction of expressed genes suggests that HNF4 α is a widely acting transcription factor and crucial for development and proper secretory function of pancreatic β cells. Furthermore, Bartoov-Shifman *et al*^[10] found that HNF4 α could activate insulin gene directly, through a previously unrecognized cis element. Clinical researches have indicated that mutations in gene encoding HNF4 α result in maturity-onset diabetes of the young type 1, characterized by autosomal dominant inheritance, early onset and impairment of glucose-stimulated insulin secretion (GSIS)^[11]. And two independent laboratory studies demonstrated that β -cell-specific HNF4 α knock-out mice exhibited impaired GSIS and deficient intracellular calcium response to glucose or SU^[12,13].

Increasing evidences suggest an essential role for HNF4 α in the maintenance of proper secretory function of pancreatic β cell and glucose metabolism. In this study, we hypothesize that berberine facilitates insulin secretion through a pathway involving HNF4 α in pancreatic islets. We introduce Glibenclamide (GB) as a control to compare the action profile of berberine with SUs, and to explore the possible molecular mechanism.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 250-300 g [Grade SPF, Certificate No. SCXK (E2004-0007)] were purchased from the Experimental Animal Center, Tongji

Medical College, Huazhong University of Science and Technology. The rats were housed at 22°C, and 60%-70% relative humidity, with 12 h light/dark cycle. The rats were given free access to food and tap water. All rats received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Tongji Medical College, Huazhong University of Science and Technology.

Drug preparation

Berberine hydrochloride (Sigma, St Louis, MO, USA) and GB (Alexis CO, San Diego, CA, USA) were dissolved in dimethyl sulfoxide (DMSO, Amresco, TX, USA), with a final concentration of DMSO 0.01% (v/v) in the culture medium.

Islet isolation and culture conditions

Primary pancreatic islets were isolated as previously reported^[14,15]. In brief, rats were anesthetized with intramuscular pentobarbital injection, 8-10 mL ice-cold Hanks' balanced salt solution (HBSS) containing 0.75 mg/mL type V collagenase (Sigma, St Louis, MO, USA) was injected *via* pancreatic duct, extended pancreas was removed and digested in a 38°C water bath for 8-10 min. Then digestion was terminated by 30 mL ice-cold HBSS with 10% fetal bovine serum (Gibco, USA), and the suspension was filtered through a 600 μ m screen to discard the undigested tissue. After twice washes with HBSS, islets were purified by Ficoll-400 (Amersham Pharmacia Biotech, Uppsala, Sweden) discontinuous gradient centrifugation at 800 \times g for 20 min at 4°C, and hand-picked under dissecting microscope. About 250-400 islets were yielded from each pancreas. The purity of islets was evaluated by dithizone (DTZ, Sigma, St Louis, MO, USA) staining^[16], and the viability was assessed according to the acridine orange/propidium iodide (AO/PI, Sigma, St Louis, MO, USA) fluorescent staining method^[17]. Freshly isolated islets were first cultured overnight at 37°C in a 50 mL/L CO₂-950 mL/L air atmosphere in serum-free RPMI 1640 (Hyclone, Gaithersburg, MD, USA) containing 2% (w/v) bovine serum albumin fraction V (BSA, Amresco, TX, USA), 11.1 mmol/L glucose, 5 mmol/L glutamine, 1 mmol/L sodium pyruvate, 100 IU/mL penicillin, 100 μ g/mL streptomycin and 15 mmol/L HEPES. Then islets were cultured for 24 h in various experimental media containing 1, 3, 10 and 30 μ mol/L berberine or 1 μ mol/L GB. Normal control was also set by incubating islets with medium in the absence of berberine or GB.

GSIS assay

For evaluation of insulin secretion, islets were washed twice with Krebs-Ringers Bicarbonated HEPES [KRBH, containing 120 mmol/L NaCl, 4.8 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 25 mmol/L NaHCO₃, 10 mmol/L HEPES, 2.8 mmol/L glucose, 0.5% BSA (w/v), pH 7.4] at the end of the incubation. Batches of 10 size-matched islets (six replicas per condition) were transferred into

1.5 mL Eppendorf tubes, and pre-incubated for 30 min at 37°C in KRBH with 2.8 mmol/L glucose. Subsequently, islets were incubated in KRBH supplemented with either 2.8 mmol/L or 16.7 mmol/L glucose for 1 h at 37°C. Aliquots of supernatant were collected after gentle centrifugation and stored at -20°C for insulin determination by radioimmunoassay kit (Beijing Institute of Atomic Energy, China).

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay

Cytotoxicity of berberine and GB on islet cells was tested by a colorimetric assay that detected the conversion of MTT (Sigma, St Louis, MO, USA) into the formazan by the mitochondrial enzyme succinate dehydrogenase in viable cells^[18]. After *in vitro* treatment, islets were dissociated into single cells by incubation in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free KRBH containing 5 mmol/L EDTA and 0.25 mg/mL trypsin for 10 min at 37°C with gentle shaking, and then resuspended in RPMI 1640. Islet cells were cultured in a 96-well plate supplemented with 0.5 mg/mL MTT. After 4 h incubation, the insoluble formazan crystals within islet cells were extracted by DMSO, and absorbance was measured by ELX800 Universal Microplate Reader (BioTek Instruments Inc, USA) at wavelength of 630 nm.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Semi-quantitative RT-PCR was performed to determine the mRNA level of HNF4 α in the islets. Total RNA was extracted from about 300 islets by the Trizol Reagent Kit (Gibco, USA) according to the manufacturer's instructions. Two μg of RNA from each sample was then reverse-transcribed into first-strand cDNA in 25 μL solution using Oligo (dT) Primers and MMLV reverse transcriptase (Promega, Madison, WI, USA). PCR reaction was performed in a standard 25 μL reaction solution contained 3 μL cDNA, 0.5 μL each of sense and anti-sense primers. Sequence-specific primers for cDNA amplification were as follows: HNF4 α (product 464 bp, sense 5'-GCAGTGCCTGGTAGACAAAGATA-3'; anti-sense 5'-AGTGCCGAGGGACGATGTAG-3') and the housekeeping gene β -actin (product 213 bp, sense 5'-AGATCTGGCACCACCTTCTAC-3'; anti-sense 5'-TCAGGATCTTCATGAGGTAGTCT-3'). Reaction conditions for HNF4 α were as follows: predenaturing at 95°C for 5 min, denaturing at 95°C for 1 min, annealing at 60°C for 50 s, extending at 72°C for 1 min, 35 cycles, with final extending at 72°C for 10 min. Amplification of β -actin was performed by predenaturing at 94°C for 5 min, denaturing at 94°C for 1 min, annealing at 55°C for 50 s, extending at 72°C for 50 s, 30 cycles and final extending at 72°C for 10 min. PCR products were electrophoresed through 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. Band intensity was calculated densitometrically using the SensiAnsys software (Shanghai PeiQing Science & Technology, China).

Immunofluorescence

Indirect immunofluorescence assay was performed as previously described with a few modifications^[19,20]. In brief, islets were placed into 1.5 mL Eppendorf tubes, fixed in 4% paraformaldehyde/10 mmol/L PBS for 30 min, followed by a 3 h permeabilization with 0.3% Triton-X100/10 mmol/L PBS. Subsequently, islets were blocked with 5% fetal bovine serum/0.2% Triton-X100/10 mmol/L PBS overnight at 4°C and then equilibrated in antibody dilution buffer (1% BSA/0.2% Triton X-100/10 mmol/L PBS) twice for 20 min at room temperature. Primary antibodies used were as follows: goat anti-rat HNF4 α IgG (1:300, Santa Cruz, San Diego, CA, USA), Guinea pig anti-insulin antibody (1:300, Sigma, St Louis, MO, USA), and the incubation was carried out for 16 h at 4°C. The secondary antibodies were Cy3 conjugated rabbit anti-goat IgG (1:400, Sigma, St Louis, MO, USA) and FITC conjugated rabbit anti-guinea pig IgG (1:250, Sigma, St Louis, MO, USA), incubation was performed for 1 h at room temperature. Finally, islets were mounted with 50% glycerol/10 mmol/L PBS, smeared onto glass slides and subjected to Confocal Laser Scanning Microscope (Olympus FV500, Japan). The excitation wavelengths for Cy3 and FITC were 552 nm and 488 nm, and the emission wavelengths were 565 nm and 525 nm, respectively. To avoid variability in fluorescent intensity caused by depth-related changes and wide range of islet diameters, a single section image was taken at the depth 1/3 the distance between the upper islet surface and its maximum diameter. Ten areas from each cover slip were randomly selected and analyzed by the HMIAS-2000 Imaging System (Champion Medical Imaging Co., Wuhan, China).

Western blot analysis

Primary pancreatic islets treated with the various experimental conditions were lysated in buffer containing 25 mmol/L Tris, 1% SDS, 5% β -mercaptoethanol, 10 mmol/L EDTA, 20 mmol/L PMSF, 10 mg/L aprotinin, 10 mg/L leupeptin, 10 mg/L antipain, 8 mol/L urea. Homogenates were centrifuged at $12000 \times g$ for 15 min at 4°C, supernatants were harvested. Protein concentrations were measured by the method of Bradford^[21], with Coomassie brilliant blue staining kit (Jiancheng Biology Institute, Nanjing, China). And 50 μg total protein for each group was boiled for 5 min in sample buffer [50 mmol/L Tris·Cl (pH 6.8), 100 mmol/L DTT, 2% SDS, 0.1% bromochlorophenol blue, 10% glycerol] and separated by 12% SDS-PAGE. Thereafter, proteins were transferred electrophoretically onto a polyvinylidene fluoride membrane. Before immunostaining, the membranes were blocked with 5% non-fat milk in Tris-buffered saline and 0.1% Tween (TBST) overnight at 4°C, followed by incubation with appropriate dilutions of the primary specific antibody goat anti-rat HNF4 α IgG (1:500, Santa Cruz Biotechnology Inc, CA, USA) in 5%BSA/TBST at 4°C for 16 h. The secondary antibody was horseradish

peroxidase (HRP) conjugated rabbit anti-goat IgG (1:2000, Sigma, St Louis, MO, USA), incubation was carried out at room temperature for 2 h. GAPDH was probed with anti-GAPDH IgG as a loading control. Immunodetection was performed using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). Protein bands on films (Eastman Kodak, Rochester, NY, USA) were analyzed by densitometry (Bio-Rad, Hercules, USA) using "Quantity One" quantitation analysis software program.

Glucokinase (GK) activity assay

GK activity was measured by spectrophotometric assay as previously described^[22]. Briefly, islet were washed twice with PBS, approximately 150 islets from each group were homogenized (30 strokes) in 200 μ L lysis buffer containing 20 mmol/L K_2HPO_4 , 5 mmol/L dithiothreitol, 1 mmol/L EDTA, and 110 mmol/L KCl, followed by sonication (20 KHz, 60 W) for 3×10 s on ice. The homogenate was then centrifuged at $12000 \times g$ for 10 min, and the supernatant fraction was used for GK determination. Then 10 μ L of the supernatant was added to 100 μ L reaction buffer, containing 50 mmol/L HEPES/HCl (pH 7.6), 100 mmol/L KCl, 7.4 mmol/L $MgCl_2$, 15 mmol/L β -mercaptoethanol, 0.5 mmol/L NAD^+ , 0.05% BSA (w/v), 2 IU/mL glucose-6-phosphate dehydrogenase, and 5 mmol/L ATP. The assay was conducted for 1 h at 30°C, and reaction was stopped by adding 1 mL of 500 mmol/L $NaHCO_3$ buffer (pH 9.4). In each assay, blanks were obtained by incubating 0.5 or 100 mmol/L glucose in the absence of ATP. Absorbance was measured at 340 nm, correction for hexokinase activity was applied by subtracting the activity measured at 0.5 mmol/L glucose from the activity measured at 100 mmol/L glucose. Protein concentrations were determined by the Bradford assay.

Statistical analysis

All the data were expressed as mean \pm SD, and analyzed with SPSS 13.0 software by one-way analysis of variance (ANOVA) LSD-*t* and SNK-*q*. $P < 0.05$ was considered statistically significant.

RESULTS

Purity and viability of freshly isolated islets

The purity of the freshly isolated islets was estimated by the percentage of DTZ-positive islets (crimson red) in the preparation. According to the method, an approximate 95% purity was assessed. For viability evaluation, islets were exposed to AO/PI, and subjected to fluorescent microscopy (Nikon ECLIPSE TE2000-U, Japan) with viable cells stained green while nonviable cells bright red. The viability exceeded 90% as assessed by the method (Figure 1).

Effects of berberine and GB on insulin secretion

In the experiment, we employed static incubation assay

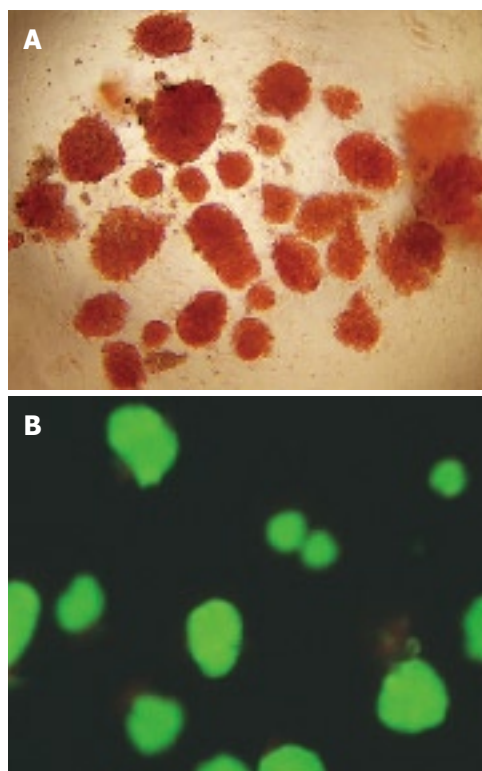


Figure 1 Quality assessment of freshly isolated islets. **A:** DTZ staining of islets under an inverted microscope (x 10); **B:** AO/PI double fluorescence staining of islets under a fluorescent microscopy (x 10).

to further examine the facilitative effect of berberine on islet insulin secretion. Islets were first incubated with 2.8 mmol/L glucose, and then challenged with 16.7 mmol/L glucose. All samples were determined by immunoradioassay. As Figure 2A depicted, islets in control group exhibited a normal response to glucose stimulation, with insulin secretion of 7.21 ± 1.43 vs 30.50 ± 5.17 (mIU/L per hour per 10 islets). Treatment of islets with 1 μ mol/L GB potently elevated basal insulin secretion ($P < 0.01$), while inhibited GSIS by about 3 folds relative to normal control ($P < 0.05$). In contrast, although none of the four berberine groups showed any promoting effects on basal insulin secretion, treatment with 1, 3, 10 μ mol/L berberine resulted in dose-dependently increased GSIS ($P < 0.05$ or $P < 0.01$), still no enhancement was observed in islets of 30 μ mol/L berberine group.

Cytotoxicity of berberine and GB on islet cells

We used MTT assay to analyze cytotoxicity caused by berberine and GB. Results were expressed as percentage of formazan absorbance relative to control value. It was observed that, 30 μ mol/L berberine inhibited formazan absorbance by about 2 folds compared to the control, indicating significant cytotoxicity on islet cells ($P < 0.01$). None of the other groups demonstrated significantly diminished absorbance (Figure 2B).

Effects of berberine and GB on HNF4 α gene expression

In RT-PCR experiment, it was observed that at concentrations lower than 10 μ mol/L, berberine

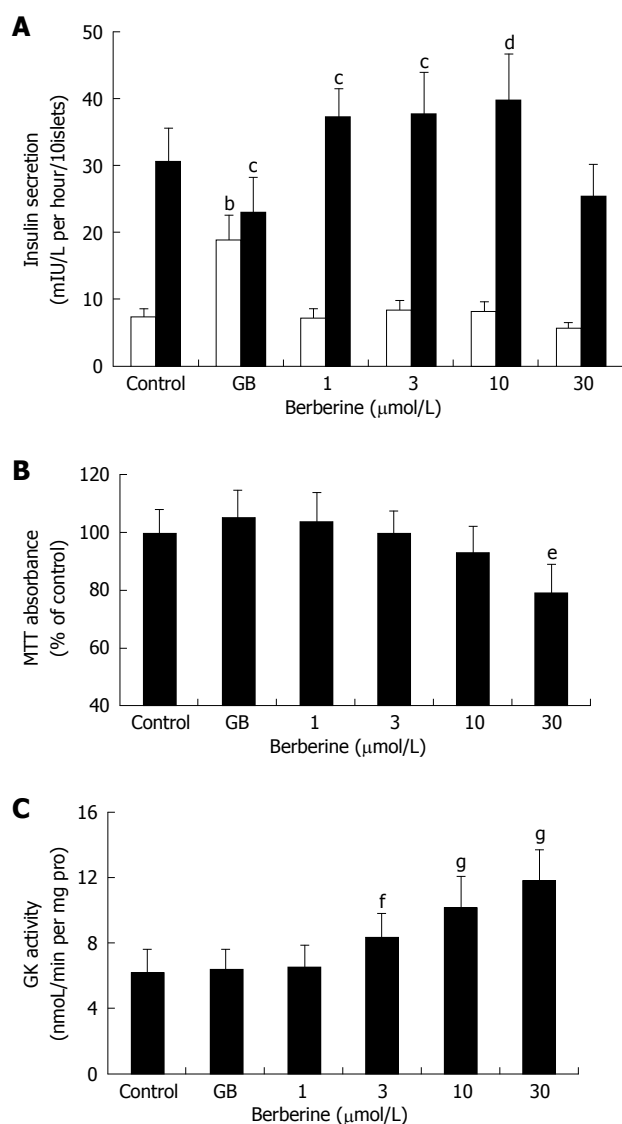


Figure 2 Pharmacological effects of berberine and GB on pancreatic islets. **A:** Effects of berberine and GB on basal (2.8 mmol/L Glucose) and GSIS (16.7 mmol/L Glucose) by pancreatic islets. Control and GB represent islets of untreated and 1 μ mol/L GB treated groups, while 1, 3, 10 and 30 represent berberine groups of indicated concentrations. ^b $P < 0.01$ vs control at 2.8 mmol/L glucose; ^c $P < 0.05$, ^d $P < 0.01$ vs control at 16.7 mmol/L glucose; **B:** Cytotoxicity of berberine and GB on islet cells assessed by MTT assay. Results were expressed as the percentage of absorbance to the control value. Data are presented as mean \pm SD ($n = 6$). ^e $P < 0.01$ vs control. **C:** Effects of berberine and GB on islet GK activity. ^f $P < 0.05$, ^g $P < 0.01$ vs control.

treatment induced a general dose-dependent increase relative to control in *HNF4 α* mRNA expression ($P < 0.05$ or $P < 0.01$). However, no significant difference was observed in islets incubated with either 30 μ mol/L berberine or 1 μ mol/L GB compared with the control (Figure 3).

Immunofluorescence and confocal microscopy

Confocal images showed apparent co-localization of insulin (green) and *HNF4 α* (red) in the islets. As Figure 4 depicts, islets exhibited a normal architecture, featuring predominant distribution of insulin throughout the entire β cell cytoplasm as well as typical nuclear localization of *HNF4 α* in both β cell and peripheral

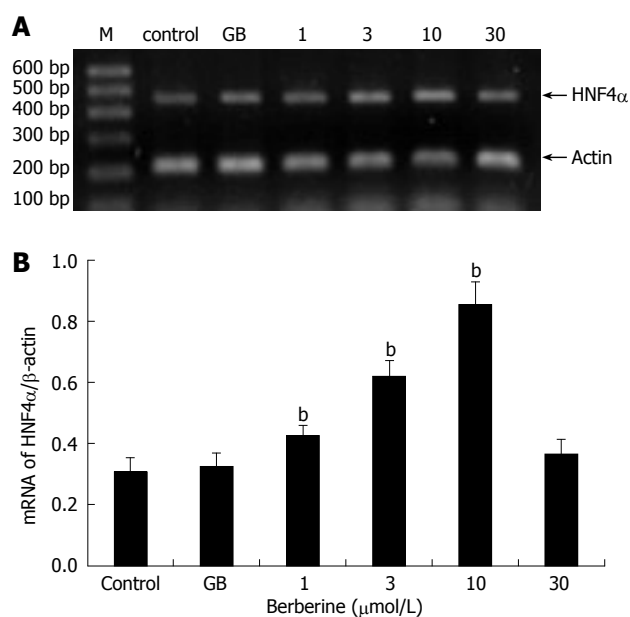


Figure 3 Effects of berberine and GB on *HNF4 α* mRNA expression. **A:** A representative gel electrophoresis profile of DNA fragment; **B:** Semi-quantitative mRNA determination, results were normalized to β -actin and expressed as arbitrary unit. M: DNA Marker, control and GB represent islets of untreated and 1 μ mol/L GB treated groups; 1, 3, 10 and 30 represent berberine groups of indicated concentrations. Data are presented as mean \pm SD ($n = 6$). ^b $P < 0.01$ vs control.

α cell. It was noteworthy that islets of berberine treated groups demonstrated more intense and distinct fluorescence of *HNF4 α* than control group, with the strongest red fluorescence emitted from the 10 μ mol/L berberine treated group. However, no distinguishable change of *HNF4 α* staining was observed in GB group compared with control group.

Effects of berberine and GB on *HNF4 α* protein expression

To further clarify the correlation of berberine's insulinotropic effect with *HNF4 α* expression, we quantified the protein level of *HNF4 α* by Western blot. Similar to the result of immunofluorescence, protein expression of *HNF4 α* also demonstrated a dose-dependent increase in the berberine treated islets ($P < 0.05$ or $P < 0.01$), with maximum expression in 10 μ mol/L berberine group. Still no significant difference was found between GB group and control group (Figure 5).

Effects of berberine and GB on islet GK activity

In the experiment, GK activity was determined in the islet homogenates. As shown in Figure 2C, compared to the control, treatment with 3, 10 and 30 μ mol/L berberine significantly activated islet GK activity ($P < 0.05$ or $P < 0.01$) in a dose-dependent manner, while no enhancement of GK activity was observed in the islets incubated with 1 μ mol/L berberine or 1 μ mol/L GB.

DISCUSSION

The SUs are a family of oral hypoglycemic agents used

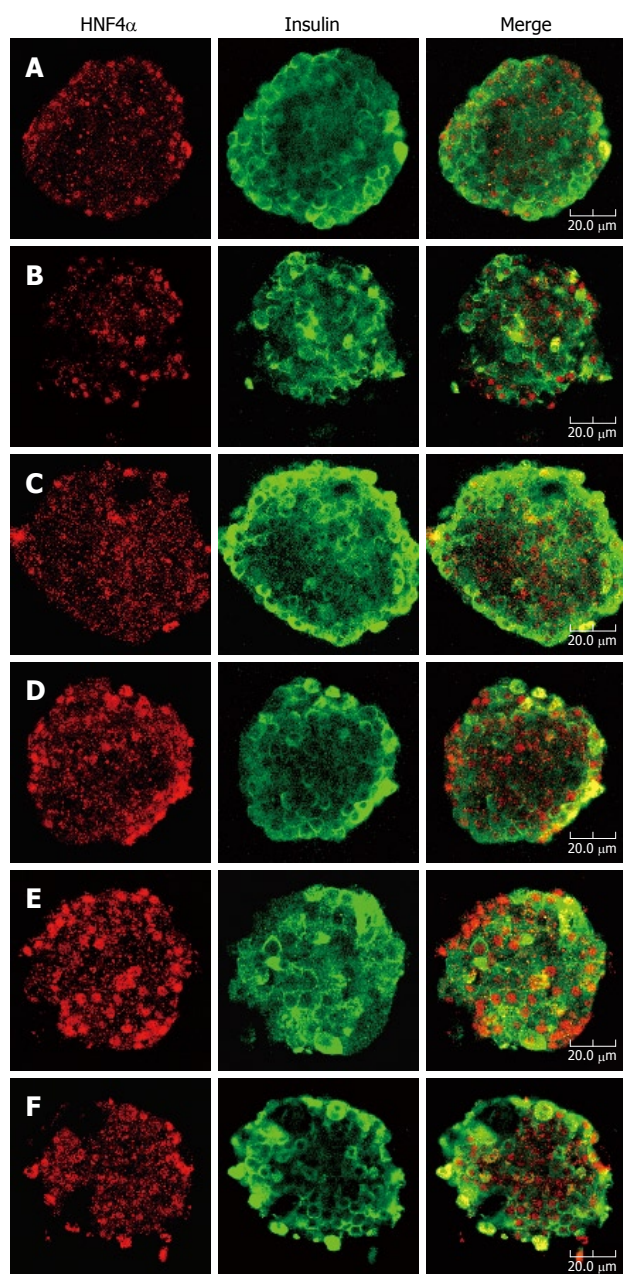


Figure 4 Double immunofluorescence staining for HNF4 α (red) and insulin (green) of rat pancreatic islets. After treatment with indicated concentrations of berberine or GB for 24 h, islets were fixed and stained with anti-HNF4 α and anti-insulin antibodies. Images of islets were taken at the corresponding depth by confocal laser microscopy (x 40). **A:** Control, **B:** 1 μ mol/L GB; **C** to **F:** Represent 1, 3, 10 and 30 μ mol/L berberine. Bar in the figure indicates 20 μ m. Apparent nuclear localization of HNF4 α could be observed in all groups of islets, while insulin fluorescence was diffusely distributed in cytoplasm of β cells. In the islets treated with various concentrations of berberine, the red fluorescence emitted was comparatively intense, suggesting up-regulated expression of HNF4 α , while no obvious difference of HNF4 α staining was found between control and GB treated islets.

extensively for the treatment of type 2 diabetes. They mediate the insulinotropic effect *via* blocking β cell K_{ATP} channels and depolarizing the membrane. Nonetheless, because the potent insulin stimulating property is independent of glucose challenge, they enhance insulin secretion even at basal glucose levels. Therefore, patients receiving SUs therapy are at high risk of hypoglycemia. Moreover, chronic SUs therapy may lead to a selective

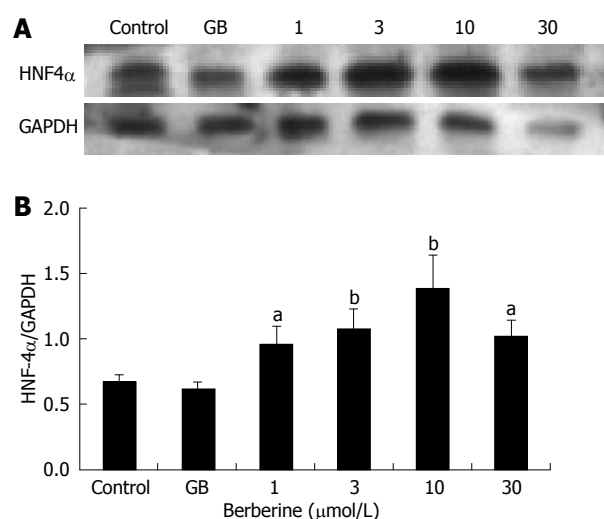


Figure 5 Effects of berberine and GB on protein expression of HNF4 α . **A:** A representative Western blot of HNF4 α ; **B:** Quantification of HNF4 α protein, results were adjusted to GAPDH and expressed as relative density units. Control and GB represent islets of untreated and 1 μ mol/L GB treated groups, 1, 3, 10 and 30 represent berberine groups of indicated concentrations. Data are presented as mean \pm SD ($n = 3$). ^a $P < 0.05$, ^b $P < 0.01$ vs control.

desensitization of pancreatic β cells to SUs^[1,2].

In the present study, the insulinotropic effect of berberine on rat islets was compared with GB, a classical SU derivative. It was demonstrated that 1 μ mol/L GB acutely promoted basal insulin secretion by approximately 250% in isolated islets, while markedly reduced GSIS in the presence of 16.7 mmol/L glucose. This observation is in good agreement with previous reports^[2,23]. The suppressed GSIS might partly result from the reduction of K_{ATP} channel activity induced by chronic exposure to GB and/or the depletion of insulin stores, reflecting the controversy of β -cell desensitization *vs* β -cell exhaustion^[3,24]. In contrast to the action profile of GB, berberine induced no significant changes in basal insulin secretion (2 mmol/L glucose), but increased GSIS at the concentrations of 1–10 μ mol/L. This result is consistent with a previous research by Ko *et al.*^[25], which showed that berberine exerted no stimulatory effect on basal insulin secretion (2 mmol/L glucose), but increased GSIS at the concentration of 5 and 50 μ mol/L in MIN6 cells.

To define the underlying mechanism for the completely different action profile of GB and berberine, we first examined the cytotoxicity caused by the drugs. Our results indicated that only 30 μ mol/L berberine demonstrated statistically significant toxicity on islet cell. Hence, it could not be the distinctions of cell metabolism that account for the different action modalities of the two agents. Accordingly, we concluded berberine probably stimulated insulin secretion *via* a mechanism distinct from SUs! In the following experiments, we determined the gene and protein expressions of HNF4 α , a transcription factor confirmed to play an essential role in GSIS, attempting to explore if HNF4 α a potential target underlying the facilitating effect of berberine on GSIS. Our data conspicuously showed

that both the mRNA and protein expressions increased in a dose-dependent manner, reaching their maximum at 10 $\mu\text{mol/L}$ berberine then declining to the levels parallel those of control. No significant changes were observed in the islets treated with 1 $\mu\text{mol/L}$ GB compared with the control. These results strongly suggest the potential involvement of HNF4 α in berberine's insulinotropic action but not GB-induced insulin secretion. The significant cytotoxicity caused by 30 $\mu\text{mol/L}$ berberine might offer a plausible explanation for the reduction of HNF4 α expression.

The question remains as to how HNF4 α mediate the stimulatory effects of berberine on GSIS in pancreatic islets, as few evidences to date substantiate that HNF4 α regulates GSIS directly in pancreatic islets. For elucidating this, we further investigated the effect of berberine on GK activity in rat islets. It is generally acknowledged that GK plays the crucial role of "glucose sensor" in pancreatic β -cell, due to its specific kinetic properties that include low affinity and positive cooperativity for glucose and a lack of inhibition by its product glucose-6-phosphate. GK regulates GSIS by modulation of the glycolytic pathway and controlling the rate of its subsequent metabolism^[26,27]. It is also deemed that GK is one of the downstream targets of the HNFs transcription regulatory circuit in pancreatic islets^[9]. Therefore, there is a good possibility that berberine exerts the facilitating effect on GSIS through direct action of GK! As expected, our results showed that berberine also elevated GK activity dose-dependently in islets, exhibiting the strongest effect at the concentration of 30 $\mu\text{mol/L}$. Yet, still no significant difference was observed between islets treated with 1 $\mu\text{mol/L}$ GB and the control, which agrees with a previous report indicating that GB exerted no regulatory effect on GK activity in isolated islets^[28]. Thus, our data further support the hypothesis that GK plays a role in the stimulatory effect of berberine on GSIS.

It appears puzzling that discrepancies also existed despite the conspicuous correlation among insulin secretion and HNF4 α expression and GK activity in general. It was demonstrated that 1 $\mu\text{mol/L}$ berberine significantly increased insulin secretion, HNF4 α gene and protein expressions, however, no enhancement of GK activity was observed. In contrast, 30 $\mu\text{mol/L}$ berberine significantly increased GK activity, while both insulin secretion and HNF4 α gene expression were at a normal level. As mentioned above, HNFs form a network, function solely or cooperatively to regulate the expression of multiple target genes that are important in the maintenance of metabolism homeostasis. It is conceivable, therefore, that not only HNF4 α but also other HNFs such as HNF1 α or HNF6 might participate in the modulation of GK activity. However, these need to be clarified further.

Taken together, our results suggest that berberine might exert its insulinotropic effect in isolated rat islets by up-regulating the expression of HNF4 α , which probably acts solely or together with other HNFs to

modulate GK activity, rendering β cell more sensitive to glucose fluctuation and response more effectively to glucose challenge. Interestingly, Ko *et al*^[25] revealed that berberine facilitated GSIS in MIN6 cells partly *via* an enhanced insulin/insulin-like growth factor-1 (IGF-1) signaling cascade, which seems discrepant from the pathway we proposed. However, it is speculated that insulin signaling could interact with HNF-regulated transcription in beta cells, and insulin or IGF-1 act as potential upstream inductive signals regulating the HNFs and their target genes^[9]. This might at least in part provide plausible explanation for the controversy.

In conclusion, our findings indicate that berberine enhances GSIS, rather than basal insulin secretion dose-dependently in isolated rat islets. This might partly be attributable to the up-regulation of HNF4 α expression and GK activity by berberine. It is also suggested that HNF4 α and GK might not participate in GB-induced insulin secretion. Berberine would be a promising insulin secretagogue which works through a mechanism distinct from SUs.

ACKNOWLEDGMENTS

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COMMENTS

Background

Berberine, a main constituent isolated from Chinese herb *Rhizoma coptidis*, is gaining increasing attention, especially for its anti-diabetic properties, including improving insulin resistance, lowering blood glucose and modulating lipid metabolism. Recent researches further reveal that berberine also possesses insulinotropic action, yet, the molecular mechanism remains unclear.

Research frontiers

The transcriptional regulatory circuit of hepatic nuclear factors (HNFs) has recently been identified in pancreatic islets. And numerous evidences suggest an essential role for hepatic nuclear factor 4 alpha (HNF4 α) in the proper secretory function of β cells. This study aims to elucidate if HNF4 α underlies the mechanism of berberine's facilitating effect on insulin secretion.

Innovations and breakthroughs

In this study, the authors found that berberine could promote glucose-stimulated insulin secretion (GSIS) rather than basal insulin secretion in primary rat islets. Furthermore, they revealed that berberine might exert the insulinotropic effect through a mechanism involving HNF4 α and glucokinase (GK), which is absolutely distinct from that of the widely used sulphonylureas (SUs).

Applications

The data suggest that berberine might be a promising insulin secretagogue which works *via* a unique mechanism in diabetes treatment.

Terminology

HNF4 α is a transcriptional factor belonging to the hepatocyte nuclear factor family. It has been demonstrated that HNF4 α is expressed mainly in liver and pancreatic islet tissue, regulating the transcription of multiple target genes implicated in glucose metabolism and insulin secretion. GK is known as "glucose sensor", modulating insulin secretion by controlling the rate of glycolysis in pancreatic β cells.

Peer review

It is a simple and elegant study. Authors observed the effect of berberine on insulin secretion by rat pancreatic islets and explored its possible molecular mechanism.

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RAPID COMMUNICATION

Comparison of the chloride channel activator lubiprostone and the oral laxative Polyethylene Glycol 3350 on mucosal barrier repair in ischemic-injured porcine intestine

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lubiprostone stimulates recovery of barrier function in ischemic intestinal tissues whereas the PEG laxative had deleterious effects on mucosal repair. These results suggest that, unlike osmotic laxatives, lubiprostone stimulates repair of the injured intestinal barrier.

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Key words: Intestinal ischemia; Barrier function; Permeability; Laxative; Polyethylene Glycol

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Abstract

AIM: To investigate the effects of lubiprostone and Polyethylene Glycol 3350 (PEG) on mucosal barrier repair in ischemic-injured porcine intestine.

METHODS: Ileum from 6 piglets (approximately 15 kg body weight) was subjected to ischemic conditions by occluding the local mesenteric circulation for 45 min *in vivo*. Ileal tissues from each pig were then harvested and mounted in Ussing chambers and bathed in oxygenated Ringer's solution *in vitro*. Intestinal barrier function was assessed by measuring transepithelial electrical resistance (TER) and mucosal-to-serosal fluxes of ³H-mannitol and ¹⁴C-inulin. Statistical analyses of data collected over a 120-min time course included 2-way ANOVA for the effects of time and treatment on indices of barrier function.

RESULTS: Application of 1 μmol/L lubiprostone to the mucosal surface of ischemic-injured ileum *in vitro* induced significant elevations in TER compared to non-treated tissue. Lubiprostone also reduced mucosal-to-serosal fluxes of ³H-mannitol and ¹⁴C-inulin. Alternatively, application of a polyethylene laxative (PEG, 20 mmol/L) to the mucosal surface of ischemic tissues significantly increased flux of ³H-mannitol and ¹⁴C-inulin.

CONCLUSION: This experiment demonstrates that

INTRODUCTION

Ischemic intestinal disorders including intestinal volvulus, thromboembolic disease, and low flow states associated with shock, have a high mortality rate due to the rapid onset of sepsis and multiple organ failure^[1-3]. Intestinal ischemic lesions are characterized by sloughing of the apical villus epithelium and rapid breakdown of mucosal barrier function^[2,4,5], accompanied by increased intestinal permeability and subsequent bacterial translocation, sepsis, and multiple organ dysfunction syndrome (MODS)^[6-8]. Rapid restoration of the compromised intestinal barrier is critical for patient survival. However, limited treatment options are available that target mucosal barrier repair^[9].

Lubiprostone, an FDA-approved laxative (Amitiza, Sucampo Pharmaceuticals, Inc.) was previously shown to stimulate rapid repair of intestinal barrier function in ischemic-injured porcine ileum^[10]. Lubiprostone activates CIC-2 Cl⁻ channels resulting in luminal Cl⁻ secretion and water movement responsible for its laxative properties^[11-15]. CIC-2 Cl⁻ channel activation

by lubiprostone is also the predominant mechanism by which this compound stimulates repair of the tight junctions and mucosal barrier repair in ischemic tissues^[10]. The mechanism for this action may relate to co-localization of ClC-2 with tight junction proteins such as occludin^[16,17]. Other commercially available laxative agents such as high molecular weight Polyethylene Glycol (PEG 3350) also induce fluid movement into the lumen *via* different mechanisms. Polyethylene laxatives are composed of high molecular weight PEG which triggers an osmotic gradient in the lumen serving to draw ions and water from the paracellular space. In addition to its laxative effects, PEG agents have been shown to confer mucosal protective effects in various gastrointestinal injury animal models including 2, 4, 6-trinitrobenzene sulphonic acid (TNBS)-induced colitis^[18] and bacterial translocation and sepsis induced by surgical stress^[19].

Given the potential alternative beneficial roles of these two oral laxatives in intestinal injury and repair, the objective of this study was to compare the effects of lubiprostone and PEG 3350 on repair of mucosal barrier function in ischemic-injured intestine.

MATERIALS AND METHODS

Compounds

Lubiprostone was obtained from R-Tech Ueno (Sanda, Japan). PEG 3350 (Miralax, Schering-Plough Health Care Products, Inc. Kenilworth NJ) was obtained from the North Carolina State University, College of Veterinary Medicine pharmacy. ³H-mannitol and ¹⁴C-inulin were obtained from Sigma Chemical (St. Louis, MO).

Experimental animal surgeries

All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Six to eight-week-old Yorkshire crossbred pigs of either sex were housed individually, and maintained on a commercial pelleted feed. Pigs were fasted for 24 h prior to experimental surgery. General anesthesia was induced with xylazine (1.5 mg/kg, IM), ketamine (11 mg/kg, IM), and 5% isoflurane vaporized in 1000 mL/L O₂ and was maintained with 2% isoflurane delivered *via* an endotracheal tube. Pigs were placed on a heating pad and ventilated with 1000 mL/L O₂ using a volume-limited, time-cycled ventilator (Hallowell, Pittsfield, MA). Lactated Ringers solution was administered iv at a maintenance rate of 15 mL/kg per hour. The ileum was approached *via* a ventral midline incision. Ileal segments were delineated by ligating the intestine at 10-cm intervals, and subjected to ischemia by occluding the local mesenteric blood supply for 45 min.

Using chamber studies

Following the 45-min ischemic period, tissues were harvested from the pig and the mucosa was stripped from the seromuscular layer in oxygenated (950 mL/L O₂/50 mL/L CO₂) Ringer's solution (mmol/L: Na⁺,

154; K⁺, 6.3; Cl⁻, 137; HCO₃⁻, 24; pH 7.4) containing 5 μmol/L indomethacin to prevent endogenous prostaglandin production during the stripping procedure. Tissues were then mounted in 1.14 cm² aperture Ussing chambers, as described in previous studies. For Ussing chamber experiments, ileal tissues from one pig were mounted on multiple Ussing chambers and subjected to different *in vitro* treatments. Data means are representative of 6 Ussing chamber experiments (*n* = 6 animals). Tissues were bathed on the serosal and mucosal sides with 10 mL Ringer's solution. The serosal bathing solution contained 10 mmol/L glucose, and was osmotically balanced on the mucosal side with 10 mmol/L mannitol. Bathing solutions were oxygenated (950 mL/L O₂/50 mL/L CO₂) and circulated in water-jacketed reservoirs. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Transepithelial electrical resistance (TER) (Ω.cm²) was calculated from the spontaneous PD and short-circuit current (*I*_{sc}). If the spontaneous PD was between -1.0 and 1.0 mV, tissues were current-clamped at ± 100 μA for 5 s and the PD recorded. *I*_{sc} and PD were recorded at 15-min intervals over a 120-min experiment.

Experimental treatments

After tissues were mounted on Ussing chambers, tissues were allowed to acclimate for 30 min to achieve stable baseline measurements after which experimental treatments were added. Lubiprostone (1 μmol/L) or PEG 3350 (20 mmol/L) were added to the mucosal side of tissues and TER and *I*_{sc} were measured at 15-min intervals over a 120-min recovery period. The PEG 3350 dose was selected as it is the recommended oral dosage for laxative properties and thus would approximate the luminal concentrations attained *in vivo*.

Mucosal-to-serosal fluxes of radiolabeled paracellular probes

To assess mucosal permeability after experimental treatments, 0.2 μCi/mL ³H-labeled mannitol (180 kDa) and 0.2 μCi/mL, ¹⁴C-labeled inulin (5000 kDa) were added to the mucosal side of tissues mounted in Ussing chambers. After a 15-min equilibration period, standards were taken from the mucosal side of each chamber and a 60-min flux period was established by taking 0.5 mL samples from the serosal compartment. The presence of ³H and ¹⁴C was established by measuring β-emission in a liquid-scintillation counter (LKB Wallac, Model 1219 Rack Beta, Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA). Unidirectional mannitol fluxes from mucosa-to-serosa were determined using standard equations.

Histological examination

Tissues were taken at 0 and 120 min for routine histological evaluation. Tissues were sectioned (5 μm)

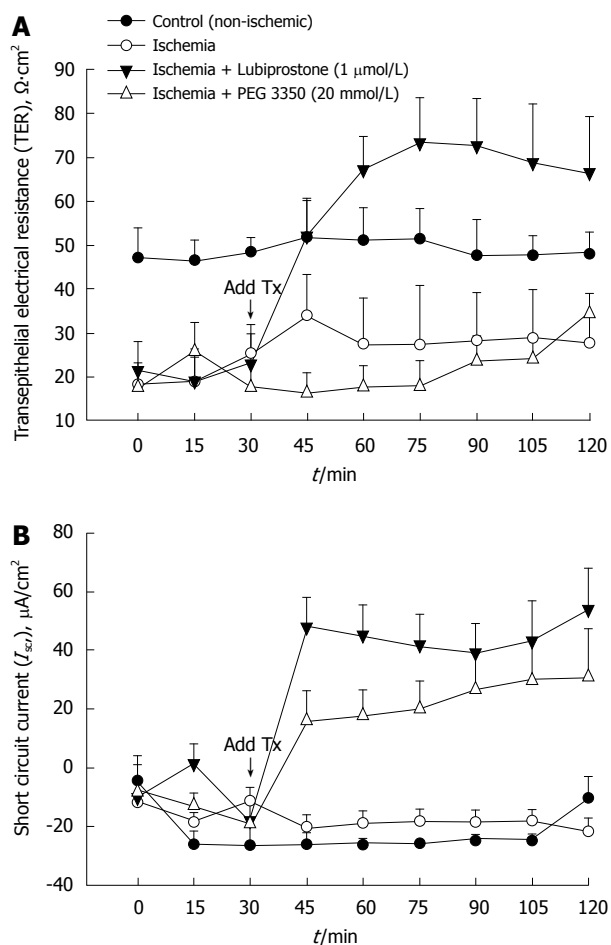


Figure 1 TER (A) and short circuit current (I_{sc}) (B) in ischemic porcine ileum treated with lubiprostone and PEG 3350.

and stained with hematoxylin and eosin. For each tissue, 3 sections were evaluated. Four well-oriented villi and crypts were identified in each section. Villus length was obtained using a micrometer in the eye piece of a light microscope.

Statistical analysis

All data were reported as mean \pm SE. TER and I_{sc} data were analyzed by using an ANOVA for repeated measures. Radiolabeled flux data was analyzed by using a standard one-way ANOVA (Sigmastat, Jandel Scientific, San Rafael, CA). A Tukey's test was used to determine differences between treatments following ANOVA.

RESULTS

TER and I_{sc} in ischemia-injured porcine ileal mucosa treated with lubiprostone or PEG 3350

Porcine ileum was subjected to 45 min of acute mesenteric ischemia and mounted on Ussing chambers for measurement of TER and I_{sc} over a 120-min recovery period. Ischemic-injured mucosa had significantly lower starting TER values (by approximately 40%) compared with non-ischemic control tissue (Figure 1), indicating significant impairment of intestinal barrier function induced by ischemia. Application of 1 $\mu\text{mol/L}$

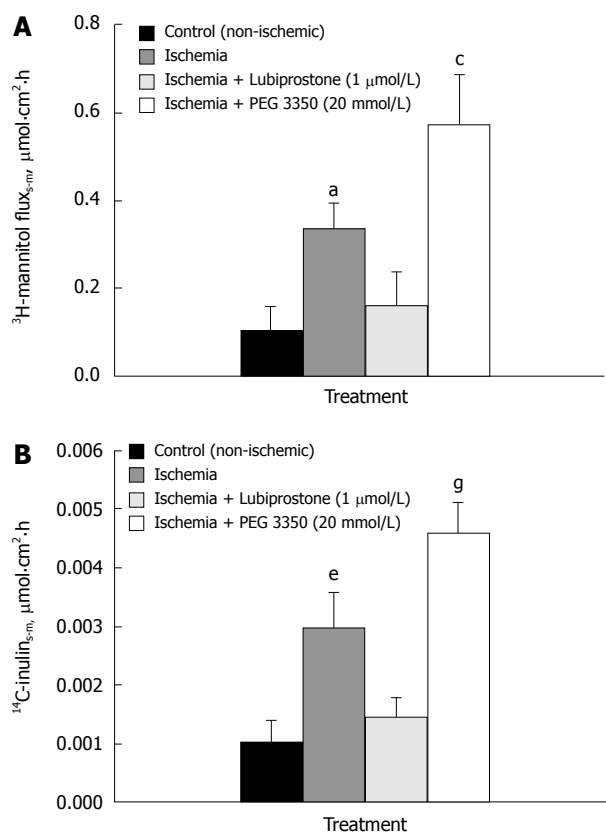


Figure 2 A: Mucosal-to-serosal flux of ^3H -mannitol in porcine ileum. ^a $P < 0.05$ ischemia vs control, ischemia/lubiprostone; ^c $P < 0.05$ ischemia + PEG3350 vs control, ischemia, ischemia + lubiprostone; B: Mucosal-to-serosal flux of ^{14}C -inulin in porcine ileum. ^e $P < 0.05$ ischemia vs control, ischemia/lubiprostone; ^g $P < 0.05$ ischemia + PEG3350 vs control, ischemia, ischemia/lubiprostone.

lubiprostone to the mucosal side of ischemic-injured mucosa induced rapid elevations in TER that attained non-ischemic control tissues within 15 min of its addition and TER continued to increase 45 min post-treatment. Lubiprostone stimulated rapid elevations in I_{sc} (an index of electrogenic ion transport) that peaked at 15 min post-treatment (peak $\Delta I_{sc} = 66 \mu\text{A}/\text{cm}^2$) and remained elevated throughout the remainder of the experiment. Mucosal addition of 20 mmol/L PEG 3350 stimulated a transient increase in TER measured 15 min after treatment; however, TER returned to ischemic control levels within 30 min post-treatment. PEG 3350 stimulated significant elevations in I_{sc} (peak $\Delta I_{sc} = 35 \mu\text{A}/\text{cm}^2$) compared with ischemic control tissues. In non-ischemic ileal tissues, lubiprostone and PEG 3350 induced similar elevations in TER ($\Delta\text{TER} = 30\% \pm 7\%$ and $36\% \pm 8\%$ in lubiprostone and PEG 3350-treated tissues) (data not shown).

Mucosal-to-serosal flux of paracellular probes

Mucosal-to-serosal flux of both medium molecular weight (^3H -mannitol, 180 kDa) and large molecular weight (^{14}C -inulin, 5000 kDa) paracellular probes in ileal tissues mounted in Ussing chambers were conducted as an alternative measurement of mucosal permeability. In line with TER responses, ischemic tissues had greater ($P < 0.01$) serosal-to-mucosal fluxes of both ^3H -mannitol and ^{14}C -inulin (Figure 2). Lubiprostone treatment significantly

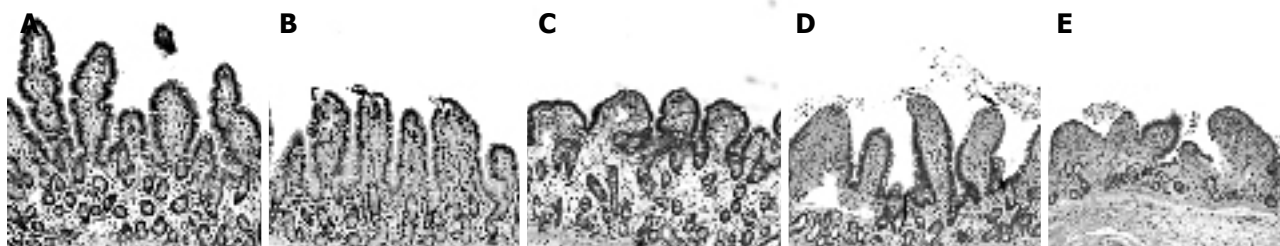


Figure 3 Light microscopic evaluation of ischemic intestinal tissues. **A:** Control (non-ischemic); **B:** Time 0 post-ischemia; **C:** Time 120-min post ischemia; **D:** Lubiprostone-time 120 min post-ischemia; **E:** -PEG 3350-time 120-min post-ischemia.

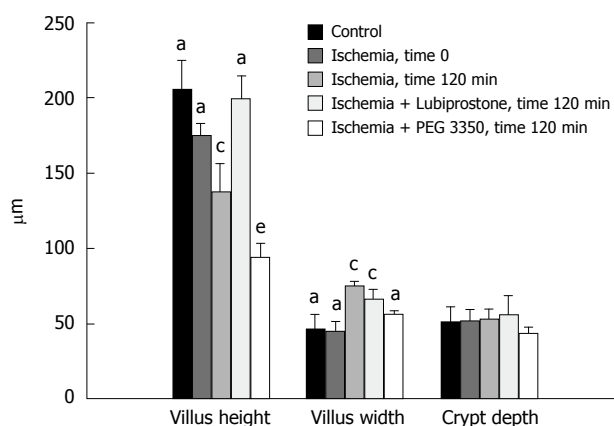


Figure 4 Morphometric evaluations of ischemic intestinal tissues. Villus height: ^a $P < 0.05$ control, ischemia time 0 min, lubiprostone vs ischemia time 120 min, PEG 3350; ^b $P < 0.05$ ischemia time 120 min vs all other groups; ^c $P < 0.05$ PEG 3350 vs all other groups; Villus width: ^a $P < 0.05$ control, ischemia time 0 min vs ischemia time 120 min, lubiprostone; ^c $P < 0.05$ ischemia time 120 min, lubiprostone vs all other groups.

decreased the fluxes of ^3H -mannitol and ^{14}C -inulin ($P < 0.05$). Alternatively, ischemic tissues treated with PEG 3350 displayed significantly increased fluxes of permeability markers compared with all other treatments ($P < 0.05$).

Histological evaluation of ischemic-injured tissues

Control (non-ischemic) tissues treated with lubiprostone or PEG 3350 had no identifiable histopathological findings compared with non-treated control tissues (not shown). Ileal tissues subjected to ischemia displayed classic histological ischemic lesions characterized by sloughing of the surface epithelium of the apical villus (Figure 3). The epithelial layer was completely restituted after 60 min of mounting tissues on Ussing chambers (not shown); this effect has been demonstrated in our previous studies^[4,5]. At 120 min post-ischemia on Ussing chambers, villus contraction was evident demonstrated by villi that were reduced in height and increased in width compared with non-ischemic control tissues (Figure 4). Laxative agents had a significant effect on intestinal villous length in ischemic-injured tissues in this study. Lubiprostone-treated tissues had greater villous lengths compared with ischemic-injured controls when measured at 120 min post-ischemia. In contrast, PEG 3350-treated ischemic tissues had significantly reduced villous height compared with ischemic-injured control tissues.

DISCUSSION

Results from the present study demonstrate marked differences in the ability of two oral laxatives, lubiprostone and PEG 3350, to stimulate repair of intestinal barrier function in ischemic-injured porcine ileum. Lubiprostone stimulated rapid repair of mucosal barrier function in ischemic ileal mucosa as defined by rapid elevations in TER and reductions in the mucosal-to-serosal flux of ^3H -mannitol and ^{14}C -inulin. PEG 3350 failed to induce significant changes in TER and had a detrimental influence on mucosal barrier repair evidenced by enhanced mucosal permeability to ^3H -mannitol and ^{14}C -inulin in ischemic-injured tissues treated with PEG 3350.

Oral lubiprostone and PEG 3350 both have laxative properties *via* different mechanisms. Lubiprostone activates ClC-2 Cl^- secretion promoting Na^+ and water movement into the lumen^[20,21], whereas PEG 3350 induces luminal osmotic effects drawing electrolytes and water into the lumen. This is the reason that both compounds induced significant increases in short circuit current in the Ussing chambers, an indirect measure of ionic movement across the mucosa. Alternatively, there was no change in short circuit current in untreated tissues. Lubiprostone also stimulated rapid repair of mucosal barrier function in ischemic ileal tissues. Previous studies showed that lubiprostone's influence on intestinal permeability is due to its ability to activate $\text{ClC-2 channels}^{[10]}$. Although exact signaling events triggered by ClC-2 activation that lead to intestinal repair are not well understood, lubiprostone treatment was shown to trigger rapid recruitment of the tight junction protein occludin to the apical intercellular space, an event critical for the re-establishment of mucosal electrical resistance. In the present study, lubiprostone increased baseline TER in non-injured control tissues, suggesting that lubiprostone's effect is not restricted to injured mucosa.

To our knowledge, the influence of PEG 3350 on intestinal mucosal repair has not been directly investigated. However, PEG compounds have been shown to be protective against different forms of intestinal injury. Videla *et al.*^[18] demonstrated that oral PEG 4000 was protective against TNBS-induced colitis in rats. In a study by Wu *et al.*^[19], luminal administration of a high molecular weight PEG prevented increases in intestinal permeability induced by *P. aeruginosa* in Caco-2 monolayers and prevented lethal sepsis *in vivo*.

induced by *P. aeruginosa* following surgical stress. In the latter study, PEG 4000 treatment resulted in increased mucosal hydrophobicity and reductions in baseline mucosal permeability. The reduction in permeability seen with PEG is likely attributable to its osmotic effects which may draw water from the paracellular space resulting in collapse of the tight junctions. Madara JL demonstrated that mucosal osmotic loads of 600 mosM induced rapid elevations in TER in guinea pig jejunum in Ussing chambers, an effect mediated by decreased cation selectivity of the tight junctions and alterations in the cytoskeleton^[22]. In the present study, PEG 3350 induced increases in TER (by 36% \pm 8%) in control (non-injured) ileal tissues. However, in the present study PEG 3350 was ineffective in improving TER in ischemic tissues and further increased mucosal permeability in ischemic-injured tissues. This suggests intact barrier function is required for PEG to stimulate increases in TER. In ischemic tissues, it is likely that PEG 3350 would freely traverse the damaged epithelium and equilibrate with the serosal compartment failing to produce an osmotic gradient in tissues.

Villous contraction in response to ischemic injury is a protective mechanism that aids in reducing the surface area of the denuded basement membrane, allowing epithelial cells adjacent to the injury to migrate and cover the epithelial defect and restore epithelial continuity^[23-26]. In the present study, lubiprostone-treated ischemic ileal tissues had significantly greater villous lengths compared with other ischemic tissues. It is unclear whether this response was due to lubiprostone's ability to inhibit villous contraction or stimulate villous lengthening during repair. Lubiprostone could have had a direct action on the principal contractile cells within the villus: myofibroblasts. These cells are arranged in linked chains of cells adjacent to the central lacteal and subjacent to the epithelial basement membrane. More studies, including a detailed time course of lubiprostone's effects on villous architecture during recovery of ischemic injury, are required to determine this and lubiprostone's overall relevance to intestinal barrier repair. In contrast to lubiprostone, PEG 3350-treated ileal tissues had significantly shorter villi compared with ischemic controls measured 120 min post-ischemic injury. This may represent ongoing injury in these tissues supported by increased paracellular permeability induced by PEG 3350.

Overall, this study demonstrates that lubiprostone stimulates recovery of mucosal barrier function in ischemic intestinal tissues, whereas the PEG laxative enhanced intestinal permeability. These results suggest that, unlike osmotic laxatives, lubiprostone stimulates repair of the injured intestinal barrier.

COMMENTS

Background

A number of important intestinal diseases, including ischemia/reperfusion injury, are characterized by damage to the epithelium lining the gut. Mechanisms are in place to rapidly repair epithelial defects, including epithelial migration (restitution). More recently, studies have shown the importance of the interepithelial tight junctions in recovery of the epithelial barrier. Studies

have shown that prostaglandins and prostones increase the rate of epithelial recovery via re-assembly of tight junctions.

Research frontiers

The prostone lubiprostone, a new medication on the market indicated for treatment of chronic constipation and irritable bowel syndrome has its effect on chloride channels (ClC-2) within tight junctions. These channels are involved in secretion of chloride in the intestine, but to a far lesser extent than the chloride channel that is genetically disrupted in patients with cystic fibrosis (CFTR). Recent studies have shown that ClC-2, when activated by prostaglandins or prostones such as lubiprostone, also play an important role in re-assembly of tight junctions, resulting in increases in the speed of epithelial repair.

Innovations and breakthroughs

Stimulation of one of the minor chloride channels, ClC-2, is an innovative way to induce low level secretion into the gut, thereby serving as a laxative. This is in contrast to traditional laxatives such as Polyethylene Glycol 3350 (PEG), which result in increased fluid in the intestinal lumen because of its osmotic properties. Other effects of ClC-2 activation by lubiprostone have recently been discovered, particularly the ability to increase mucosal repair. A comparison of lubiprostone and PEG showed that only lubiprostone facilitated the repair of the mucosa. This increased repair could not be seen at the histological level. This is consistent with prior studies showing that enhanced repair is noted at the level of tight junctions.

Applications

When treating constipation, the choice of medication includes laxatives such as PEG and the ClC-2 activator lubiprostone. The present study suggests that use of lubiprostone will also hasten the recovery of injured gut mucosa in patients with more severe intestinal disease. Further basic science research followed by clinical trials will be needed to determine the validity of these findings.

Terminology

The term ClC-2 is used to describe a chloride channel in the gut epithelium that is localized to interepithelial tight junctions. The term prostone refers to a new group of compounds which are distinct from prostaglandins and specifically activate ClC-2.

Peer review

The present study was performed using porcine tissues; it demonstrates that lubiprostone stimulates recovery of barrier function in ischemic intestinal tissues whereas the PEG laxative had deleterious effects on mucosal repair. This is an interesting study and well written manuscript.

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RAPID COMMUNICATION

Management of recurrent rectal cancer: A population based study in greater Amsterdam

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recurrence. The majority (54%) of these patients were treated with radio- and/or chemotherapy without surgery (median survival 15 mo). Twenty-seven percent of these patients only received best supportive care (median survival 6 mo), while 16% underwent surgery for their recurrence. Survival was best in the latter group (median survival 32 mo).

CONCLUSION: Although treatment options and survival are limited in case of recurrent rectal cancer after radical local resection obtained with TME, patients can benefit from additional treatment, especially if a radical resection is feasible.

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Key words: Rectal cancer; Total mesorectal excision; Local recurrence; Relative survival

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Abstract

AIM: To analyze, retrospectively in a population-based study, the management and survival of patients with recurrent rectal cancer initially treated with a macroscopically radical resection obtained with total mesorectal excision (TME).

METHODS: All rectal carcinomas diagnosed during 1998 to 2000 and initially treated with a macroscopically radical resection (632 patients) were selected from the Amsterdam Cancer Registry. For patients with recurrent disease, information on treatment of the recurrence was collected from the medical records.

RESULTS: Local recurrence with or without clinically apparent distant dissemination occurred in 62 patients (10%). Thirty-two patients had an isolated local recurrence. Ten of these 32 patients (31%) underwent radical re-resection and experienced the highest survival (three quarters survived for at least 3 years). Eight patients (25%) underwent non-radical surgery (median survival 24 mo), seven patients (22%) were treated with radio- and/or chemotherapy without surgery (median survival 15 mo) and seven patients (22%) only received best supportive care (median survival 5 mo). Distant dissemination occurred in 124 patients (20%) of whom 30 patients also had a local

INTRODUCTION

Colorectal cancer is the second most common cancer in the Western world and approximately one third of these tumours are located in the rectum or rectosigmoid^[1]. Annually, over 3000 patients are registered with a newly diagnosed rectal or rectosigmoid carcinoma in the Netherlands^[2,3]. In these patients, locally recurrent disease is a major concern and is often accompanied with intractable pain and severely disabling complications which are difficult to treat^[4-6]. It has a tremendous impact on quality of life^[7] and frequently induces an awful last period of a patient's life. Therefore, the focus in rectal cancer research has been on the prevention of locally recurrent disease, which resulted in the introduction of preoperative radiotherapy and total mesorectal excision (TME)^[8-12].

There are many reports on the treatment of

recurrent rectal cancer^[4,6,13-16]. However, these reports present mainly results from randomised clinical trials or specialised institutes, which are known to be biased^[17]. There are only a few population-based reports on the treatment of locally recurrent rectal cancer disease^[13,18], although they are probably the best reflection of daily practice.

In 1996, TME was introduced in Greater Amsterdam, the region of the Comprehensive Cancer Centre Amsterdam (CCCA). Its introduction was facilitated by the CCCA. Surgeons were supervised by teacher-surgeons in order to qualify as TME-surgeon and a documentation project was started to investigate the influence of TME-surgery on the incidence of local recurrences and survival^[19]. From 1998 on, all patients in Greater Amsterdam are treated with TME in case of rectal resection.

The aim of the present study was to analyze, retrospectively in a population-based setting, the management and survival of patients with recurrent rectal cancer, initially treated with macroscopically radical local resection obtained with TME.

MATERIALS AND METHODS

Cancer registry data

All primary rectal carcinomas (rectosigmoid excluded) diagnosed in patients with residence in Greater Amsterdam, the region of the CCCA, between January 1, 1998 and December 31, 2000, and who underwent a macroscopically radical resection obtained with TME in the absence of distant dissemination, were selected from the Amsterdam Cancer Registry of the CCCA. The Amsterdam Cancer Registry is a regional, population-based cancer registry with complete regional coverage. Non-epithelial cancers, carcinoids and cases with preceding invasive cancers were excluded. The population of the region amounted to 2.8 million inhabitants on December 31, 2000, approximately 17% of the total population of the Netherlands.

The information for the cancer registry is routinely extracted from detailed hospital and outpatient clinic records by registration clerks. Apart from demographic data, data are collected on morphological classification, stage of the tumour and primary treatment of the patients. The TNM system for classification of malignant tumours is prospectively registered to classify all rectal carcinomas. Stage grouping in this study was performed according to the 6th edition of the TNM-classification^[20], based on the available information after surgery (pTNM).

Of the selected cases, a supplementary data set was extracted from the medical records. This data set included the occurrence and the date of local recurrence or distant dissemination. Local recurrence was defined as cancer recurrence within the lower pelvis. Additional treatment of recurrence, the presence of microscopic or macroscopic residual disease after salvage surgery for recurrent disease, the date of salvage surgery and the

Table 1 Initially applied radiotherapy in surgically treated, primary rectal carcinoma patients according to pTNM-stage in Greater Amsterdam, the Netherlands, 1998 to 2000

Stage of disease	Number of cases (% of total)	Radiotherapy, number of patients (%)		
		No RT	Postoperative RT	Preoperative RT
I	209 (33)	115 (55)	1 (0)	93 (45)
II A	180 (28)	72 (40)	26 (14)	82 (46)
II B	20 (3)	2 (10)	6 (30)	12 (60)
III A	32 (5)	8 (25)	11 (34)	13 (41)
III B	113 (18)	26 (23)	32 (28)	55 (49)
III C	72 (11)	13 (18)	23 (32)	36 (50)
Unknown	6 (1)	2 (33)	-	4 (67)
Total	632	238 (38)	99 (16)	295 (47)

RT: Radiotherapy.

cause of death were also collected. Cases were generally followed for five, but at least three years after the date of initial surgery.

Vital status

The vital status was updated by active follow-up in the hospitals, by linking files with deceased persons to the cancer registry and by linkage to the electronic death registry of the Central Office for Genealogy in September, 2003 and February, 2005, as described earlier^[21]. Completeness of follow-up of the vital status is estimated to be over 99.5%.

Statistical analysis

$P < 0.05$ was considered statistically significant. All statistical analyses were performed using a two-sided 5% level of significance.

Survival probabilities were estimated using the Kaplan-Meier method^[22]. Multivariate analyses using the Cox proportional-hazard method were performed to calculate the hazard ratio (HR) for death after recurrent disease^[23]. Cox regression and Kaplan-Meier survival curves were calculated with STATA (Stata Corporation, College Station, TX, USA).

RESULTS

Initial treatment and incidence of local recurrence

A total of 632 patients diagnosed with primary rectal carcinoma in the absence of clinically manifest distant dissemination between 1998 and 2000 underwent a macroscopically radical local resection obtained with TME. Characteristics of the initial treatment of the primary tumour in these patients are given in Table 1. Local recurrence within five years after diagnosis occurred in 62 patients (10%), including 30 cases with distant dissemination (6%). Of these 30 patients, 24 patients had synchronous local and distant recurrence, while 6 patients developed distant dissemination after the local recurrence.

Treatment of local recurrence

There were 32 out of 62 patients (52%) without signs

Table 2 Secondary treatment of local recurrence (in the absence of distant dissemination) according to treatment with radiotherapy and stage at initial diagnosis (after a macroscopically radical resection obtained with TME)

Secondary treatment	Stage and treatment with radiotherapy at initial diagnosis									Total <i>n</i> (%)
	Stage I			Stage II			Stage III			
	No RT	Post RT	Pre RT	No RT	Post RT	Pre RT	No RT	Post RT	Pre RT	
Radical surgery ¹ (± radiotherapy and/or chemotherapy)	3	-	1	3	-	1	1	-	1	10 (3)
Non-radical surgery ¹ (± radiotherapy and/or chemotherapy)	-	-	-	2	1	2	-	3	-	8 (2)
Radiotherapy and/orchemotherapy without surgery	2	-	-	1	1	-	1	2	-	7 (2)
Best supportive care	1	-	-	-	1	3	1	-	1	7 (2)
Total	6	-	1	6	3	6	3	5	2	32

¹Radical surgery was defined as surgery without microscopically residual disease; all other surgery cases were classified as non-radical. No RT: No radiotherapy; Post RT: Postoperative radiotherapy; Pre RT: Preoperative radiotherapy.

Table 3 Multivariate analysis of potentially prognostic factors for improved survival after treatment of patients with a locally recurrent rectal carcinoma in Greater Amsterdam (cases with distant dissemination and/or macroscopic residual disease at time of initial treatment are excluded)

Parameter	Number of cases	HR (95% CI)
Sex		
Male (reference) <i>vs</i> female	33/29	1.9 (0.9-3.7)
Radiotherapy at initial treatment		
No radiotherapy	23	1.0
Preoperative radiotherapy	23	1.2 (0.6-2.5)
Postoperative radiotherapy	16	0.9 (0.4-1.8)
Distant dissemination at time of local recurrence		
Absent (reference) <i>vs</i> present	32/30	0.8 (0.4-1.6)
Surgical treatment of locally recurrent disease		
No surgery	42	1.0
Radical surgery	10	0.1 (0.0-0.3)
Non-radical surgery	10	0.5 (0.2-1.3)

HR > 1: Worse prognosis; HR < 1: Better prognosis.

of distant dissemination at the time of diagnosis of recurrent disease. Median survival after recurrence in the absence of distant dissemination was 25 mo. Ten of these 32 patients underwent a microscopically radical resection of their recurrence (Table 2). As is depicted in Figure 1, radical surgery resulted in a significantly better survival than non-radical surgery, radio- and/or chemotherapy without surgery or best supportive care (log-rank test radical surgery *vs* other treatments: $P < 0.001$). About three quarters of the patients who underwent a radical resection survived for at least three years. Median survival after non-radical surgery (8 patients) was 24 mo, 7 mo after radio- and/or chemotherapy without surgery (7 patients) and was 5 mo in case of best supportive care only (7 patients).

In 30 patients (48%), distant dissemination was present at the time of diagnosis of local recurrent disease. Median survival after local recurrence in the presence of distant dissemination was 10 mo. None of these patients underwent curative surgery, two patients underwent non-radical surgical resection, 14 patients were treated with radio- and/or chemotherapy without surgery (median survival 14 mo) and 14 patients received best supportive care only (median survival 9 mo).

Table 4 Treatment of distant dissemination in patients initially treated with a macroscopically radical local resection obtained with TME in the absence of distant metastasis

Treatment	No local recurrence		Local recurrence ¹		Total	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Surgery ¹ (± radiotherapy and/or chemotherapy)	18	19	2	7	20	16
Radiotherapy and/or chemotherapy	54	57	13	43	67	54
Other	1	1	2	7	3	2
Best supportive care	21	22	13	43	34	27
Total	94		30		124	

¹Synchronous with distant metastasis or prior to distant metastasis.

Prognostic factors for survival after recurrence

Several factors were analysed to identify prognostic factors for improved survival after local recurrence. The results of the multivariate analysis are shown in Table 3. Surgery for recurrent disease (radical and non-radical) was a prognostic factor for improved survival, while radiotherapy applied during the initial treatment did not influence survival after local recurrence (Figure 2).

Distant dissemination

Distant dissemination within five years after diagnosis occurred in 124 patients (20%). The majority of patients (54%) with distant dissemination were treated with radio- and/or chemotherapy (Table 4). The median survival after distant dissemination was 15 mo.

Twenty patients (16%) underwent surgery for their recurrence, including liver resections in eight patients, lung resections in five patients, and other surgical procedures in seven patients. Median survival after surgery was 32 mo, while median survival after radiotherapy and/or chemotherapy without surgery was 15 mo and 6 mo if best supportive care was applied (Figure 3). Patients with distant dissemination who were treated surgically experienced the highest survival (log-rank test surgery *vs* other treatments: $P < 0.001$).

DISCUSSION

This is the first population-based study concerning

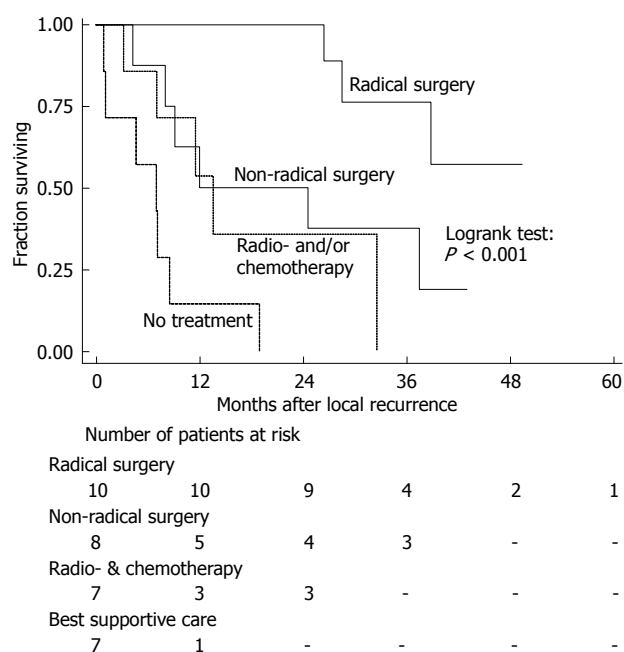


Figure 1 Crude survival after isolated local recurrence in rectal cancer patients initially treated with a macroscopically radical local resection obtained with TME in Greater Amsterdam according to treatment for recurrence. Radical surgery is defined as surgery without macroscopically or microscopically residual disease. Non-radical surgery is defined as surgery with macroscopically or microscopically residual disease.

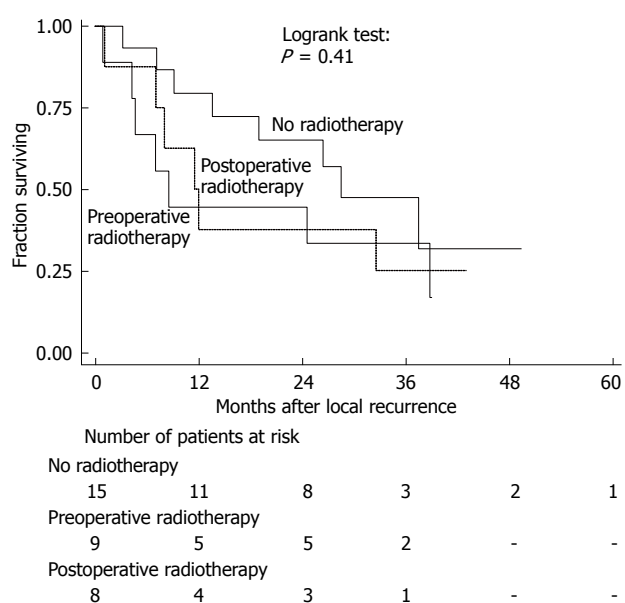


Figure 2 Crude survival after isolated local recurrence in rectal cancer patients initially treated with a macroscopically radical local resection obtained with TME in Greater Amsterdam according to radiotherapeutic treatment of the primary tumour.

recurrent rectal cancer treatment after the introduction of TME. All patients in this study were initially diagnosed between 1998 and 2000 in Greater Amsterdam and treated by macroscopically radical local resection obtained with TME. A local recurrence occurred in 62 of the 632 patients (10%), while distant dissemination was found in 124 patients (20%).

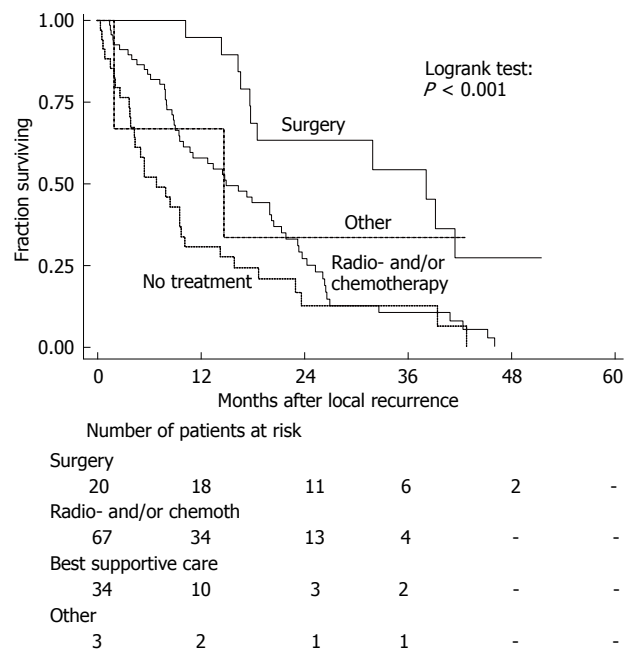


Figure 3 Crude survival after distant dissemination in rectal cancer patients initially treated with a macroscopically radical local resection obtained with TME in Greater Amsterdam according to treatment for distant dissemination.

Treatment of patients with isolated recurrent disease

Of the 32 patients with an isolated local recurrence, 31% were treated by a radical resection. These patients experienced a significantly better survival compared to patients who underwent a non-radical resection for their recurrence. As has been shown previously, radical resection of locally recurrent disease can achieve long-term survival^[4,13-15,24] and should, therefore, be aimed at, even if extended resection (*e.g.* abdominosacral resection or exenteration)^[16,25,26] or flap-reconstruction^[27] is required.

Survival in patients treated with non-radical surgery and patients treated with radiotherapy and/or chemotherapy without surgery was comparable, but was significantly worse in patients not treated with surgery, radiotherapy or chemotherapy. Although no information concerning the extent of recurrent disease was available in this study, treatment has probably been more aggressive in case of limited disease and, therefore, selection bias may have played an important role in the outcome of the various treatment modalities.

Treatment of patients with distant dissemination

The median survival after distant dissemination was 15 mo for patients diagnosed between 1998 to 2000. In a previous study, we have described that patients diagnosed in 1988 between 1991 in Greater Amsterdam only survived 9 mo after distant dissemination (log-rank test: $P = 0.004$)^[19]. The majority of patients diagnosed between 1998-2000 (54%) with distant dissemination were treated with radiotherapy and/or chemotherapy without surgery, while 16% were treated with surgical resection and 27% received only best supportive care. Survival was significantly better in the group of patients

treated with surgery compared to other groups. This is probably due to the limited spread of disease in these patients (selection bias). As no treatment data were available for the patients diagnosed between 1988 to 1991 in Greater Amsterdam, it is unclear which treatment modality has contributed to the increase in the median survival.

CONCLUSION

In this population-based study, treatment options and survival were limited in patients with recurrent rectal cancer after macroscopically radical local resection obtained with TME. Approximately one third of the patients only received best supportive care with a subsequent poor survival. On the other hand, in one third of the patients with an isolated local recurrence, radical resection was feasible with a favourable survival. We conclude that a locally recurrent rectal cancer without distant dissemination does not automatically lead to a hopeless situation^[28]. However, survival after local recurrence in combination with distant dissemination remains extremely poor.

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COMMENTS

Background

Colorectal cancer is a common cancer in the Western world and rectal cancer has been subject to significant treatment changes over the last decade. Despite this change, recurrent rectal cancer treatment remains a frustrating and ongoing process.

Research frontiers

In the recent decade, the main changes in rectal cancer treatment have been the application of preoperative radiotherapy and the introduction of total mesorectal excision (TME) which led to a significant decrease in local recurrence percentages. In the current article, the influence the introduction of TME on recurrent rectal cancer treatment is evaluated in a population based cohort.

Applications

The current results show that recurrent rectal cancer treatment is in some cases worthwhile, especially if a radical resection is feasible. However, avoiding a local recurrence remains an important aspect in future rectal cancer treatment.

Terminology

TME is a surgical technique initiated by B. Heald during which the entire mesorectum is excised using the so-called "holy" plane. This provides the opportunity to remove the entire rectum with possibly infiltrated lymph nodes.

Peer review

This is an interesting study which was well organized. It demonstrated that although treatment options and survival are limited in case of recurrent rectal cancer after radical local resection obtained with TME, patients can benefit from additional treatment, especially if a radical resection is feasible.

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RAPID COMMUNICATION

Prospective evaluation of laparoscopic Roux en Y gastric bypass in patients with clinically severe obesity

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Abstract

AIM: To evaluate and present our experience with laparoscopic Roux en Y gastric bypass (RYGB) in a selected patient population.

METHODS: A cohort of 130 patients with a body mass index (BMI) between 35 and 50 kg/m² were evaluated in relation to postoperative morbidity, weight loss and resolution of co-morbidities for a period of 4 years following laparoscopic RYGB.

RESULTS: Early morbidity was 10.0%, including 1 patient with peritonitis who was admitted to Intensive Care Unit (ICU) and 1 conversion to open RYGB early in the series. There was no early or late mortality. Maximum weight loss was achieved at 12 mo postoperatively, with mean BMI 30 kg/m², mean percentage of excess weight loss (EWL%) 66.4% and mean percentage of initial weight loss (IWL%) 34.3% throughout the follow-up period. The majority of preexisting comorbidities were resolved after weight loss and no major metabolic disturbances or nutritional deficiencies were observed.

CONCLUSION: Laparoscopic RYGB appears to be a safe and effective procedure for patients with BMI 35-50 kg/m² with results that are comparable to previously published data mostly from the USA but from Europe as well.

INTRODUCTION

Obesity has reached epidemic proportions in society today^[1] and it is associated with high rates of morbidity and mortality due to the presence of severe comorbidities, many of which show complete resolution after weight loss^[2]. According to published data, surgery is the only reliable way of achieving both adequate and long-term weight loss in the morbidly obese population^[3]. Roux en Y gastric bypass (RYGB), which was first described by Mason and Ito at the University of Iowa in 1967, is now considered the gold standard of bariatric procedures^[4]. In 1994, Wittgrove *et al*^[5] first introduced a totally laparoscopic approach to this procedure, which is now a routine practice for the majority of bariatric centers in United States and this trend seems to be spreading throughout Europe as well. However, there are few published studies to date regarding the implementation of this procedure in Europe^[6].

The aim of this study, therefore, is to report the experience of a single centre in Greece with the laparoscopic RYGB.

MATERIALS AND METHODS

Since 1994, 1095 surgical procedures for clinically severe obesity have been performed at our institution. The type of procedure performed is based on specific selection criteria according to an algorithm developed

Table 1 Patients' preoperative characteristics

Period of study	September 2003 to June 2008
Number of patients	130
Male:Female	21:109
Age (yr)	37 ± 9.4 (18-55)
Weight (kg)	125 ± 13.4 (95-170)
Excess weight (kg)	65 ± 9.7 (39-103)
% EBW	208 ± 14.5 (160.9-253.7)
BMI (kg/m ²)	46 ± 3.0 (37.5-50)

Values are mean ± SD (range).

by our center, whereby patients with body mass index (BMI) over 50 kg/m² undergo biliopancreatic diversion with RYGB (BPD-RYGB), as modified in our center^[7], while patients with BMI < 50 kg/m² undergo RYGB with long limb (RYGB-LL) or sleeve gastrectomy (SG) depending on comorbidities and eating habits. Sweet consumers and patients with diabetes mellitus undergo RYGB-LL whereas volume consumers may undergo SG. Since October 2003 in patients with BMI < 50 kg/m² all procedures have been performed laparoscopically, and the present study refers to 130 of these patients who underwent RYGB-LL as their primary procedure.

All patients participated in a multistage educational and informational program to accomplish full disclosure preoperatively and to engage them in compliance with bariatric management postoperatively.

A multidisciplinary team including the surgeon, an endocrinologist, a cardiologist, a pneumonologist, a psychiatrist and a nutritionist-dietitian evaluate all patients preoperatively and postoperatively to assess and optimize their physical condition. Table 1 shows the patients' preoperative characteristics and Table 2 shows the patients' preoperative comorbidities.

At the operating room, during induction in anesthesia, a single dose of antibiotics (cephalosporin second generation 1.5 g and metronidazole 500 mg) was given and continued for 48 h postoperatively. Thromboembolic prophylaxis using low molecular weight heparin at a dose adapted to patients' weight was started the day before operation and continued till the discharge. Complementary measures consisting of air compression boots and specially designed mattress were used.

Surgical technique

The main characteristics of the RYGB-LL are a gastric pouch of 15 ± 5 mL, a biliopancreatic limb of 50 cm, an alimentary limb of 150 cm and the remainder of small intestine as the common limb. We prefer to pass the alimentary limb antecolic and antegastric and to create a gastrojejunostomy using a 25 mm circular stapler performing a circular end to end anastomosis (CEEA) by passing the anvil transorally. The gastrojejunostomy is reinforced with interrupted non-absorbable sutures. The proximal end of Roux limb is closed with application of linear stapler. A side to side jejunojunctionostomy is performed using a linear stapler and the mesenteric defects are closed with non-absorbable running sutures.

Table 2 Preoperative comorbidities

Comorbidities	No. of patients (%)
Hypertension	21 (16.3)
COPD	22 (17.0)
Sleep apnea	13 (10.0)
Osteoarthritis	21 (16.3)
Depression	11 (8.5)
Diabetes Mellitus	24 (18.6)
OGTT	8 (6.2)
Hypercholesterolemia	64 (49.6)
Hypertriglyceridemia	36 (27.9)

COPD: Chronic obstructive pulmonary disease.

Cholecystectomy is always added to the main procedure. Furthermore, because fatty liver and non-alcoholic steatohepatitis are common, liver biopsy is routinely performed to assess preoperatively liver histopathology and will be used as a baseline for comparison if a problem in hepatic function arises in the future.

Postoperative management

Patients were mobilized in the evening of surgery. Pain management consisted of parecoxib 40 mg intravenously every 12 h and meperidine 50 mg intramuscularly as needed for 3 d.

On the fourth postoperative day, after an uneventful upper gastrointestinal evaluation, a clear liquid diet was begun with gradual progression to full liquids and blenderized food. High protein supplements were administered to all patients until 4 wk postoperatively when per os feeding was advanced by the nutritionist.

All patients received a daily multivitamin and mineral supplement and 1000 mg of calcium. An iron supplement was also prescribed for premenopausal women at a dose of 80 mg/d. After the 6th postoperative month, vitamin B₁₂ supplementation was given intramuscularly at a dose of 1000-3000 µg depending upon the measured values.

Follow-up visits were scheduled at 1, 3, 6 and 12 mo postoperatively and annually thereafter. Each follow-up visit included complete medical and nutritional evaluation, with laboratory workup and evaluation by other specialists as needed.

Statistical analysis

The data presented are expressed as mean ± SD, unless otherwise stated. During the study comparisons of the observed values at various time periods were performed using ANOVA (one way analysis of variance). If there were any statistic significant differences, the Tukey post-test was used to determine the time points that contribute to this significance.

RESULTS

From October 2003 to June 2008, 130 patients with BMI 35-50 kg/m² underwent laparoscopic RYGB-LL as their primary bariatric operation. In addition,

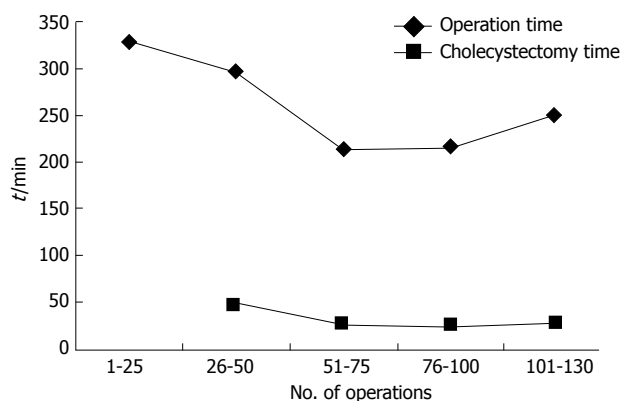


Figure 1 Operation time for the first 130 patients. Values in graph lines are mean values.

cholecystectomy was performed in all but nine patients who had previously undergone this procedure elsewhere and other abdominal procedures were performed in five patients. The mean total operative time was 260 ± 87.4 min (range 120-270) and the mean operative time for cholecystectomy was 18 ± 17.2 min (range 15-70). The operative time decreased significantly over time, especially after the first 50 cases ($P < 0.05$) (Figure 1). Conversion to open RYGB was necessary in one patient (0.8%) early in the series.

The mean length of hospital stay was 9 d (range 8-29) primarily due to standard hospital protocol in Greece. The mean follow-up time was 24.3 mo (range 1-48). The number of patients who successfully completed follow-up was 110 of 112 patients (98.2%) at 12 mo, 85 of 90 patients (94.4%) at 24 mo, 51 of 55 patients (92.7%) at 36 mo and 16 of 18 patients (88.9%) at 48 mo. Four patients were lost to follow-up. Thus far there has been no need for revision surgery.

Mortality

There was no early or late mortality.

Complications

Intraoperative period: One conversion to open RYGB was necessary early in the series, due to inability to construct the jejuno-jejuno anastomosis.

Early postoperative period (< 30 d): In the early postoperative period, as shown in Table 3, there were three gastrointestinal haemorrhages, one of which was intraluminal, probably from the jejunojejunal anastomosis, presenting as small obstruction and requiring reoperation, while the others were intrabdominal and treated conservatively. In addition, there were two enterocutaneous fistulas, caused by attachment of small intestine loop to mesh after simultaneous repair of incisional hernias, one episode of ileus due to volvulus and one case of peritonitis, all of which required surgical intervention. The last patient required admission to the Intensive Care Unit (ICU) after reoperation for peritonitis caused by leakage of the alimentary limb stump on the 6th postoperative day. The

Table 3 Incidence of postoperative complications and management

Time	Complications	Treatment	% of patients
Early < 30 d			
	3 gastrointestinal hemorrhages	Conservative (2)	2.4
		Operation (1)	
	2 enterocutaneous fistulas	Operation	1.6
	1 peritonitis	Operation, ICU admission	0.8
	1 ileus due to volvulus	Operation	0.8
	1 portal vein thrombosis	Anticoagulants	0.8
	1 wound infection of port site	Antibiotics	0.8
	2 orthopedic injuries	Immobilization	1.6
	1 atelectasis	Antibiotics peros	0.8
	No incisional hernia		0.0
	1 anastomotic leak	Conservative	0.8
Intermediate 30-90 d			
	1 incisional hernia	No operation	0.8
Late > 90 d			
	5 incisional hernias	Operation (4)	3.9
	4 episodes of ileus	Operation (1)	3.1
		Conservative (3)	
	1 suicide attempt		0.8
	1 gastrojejunal anastomosis stenosis	Endoscopic dilatation	0.8

latter was caused by herniation of a small bowel limb to port site. The patient was finally discharged 168 d after surgery following a complicated and difficult postoperative course, including acute compartment syndrome requiring reoperation and development of acute renal insufficiency requiring dialysis. There was also one episode of portal vein thrombosis treated with anticoagulant medication, one wound infection of port site treated with antibiotics, one lung atelectasis, two orthopaedic injuries due to inappropriate patient positioning and securing intraoperatively. Finally, there was one anastomotic leakage from gastrojejunal anastomosis, which presented on the 6th postoperative day with severe sepsis without hemodynamic instability and was treated successfully conservatively with drainage under computer tomography (CT) guidance and total parenteral nutrition. The total early postoperative morbidity was 10.0% (13 patients).

Intermediate postoperative period (30-90 d): During the intermediate postoperative period there was one incisional hernia with no other major complications.

Late postoperative period (> 90 d): During the late postoperative period there were 5 incisional hernias, 4 of which were treated surgically, 4 episodes of small bowel obstruction, one of which was due to adhesions requiring surgical intervention, one episode of gastrojejunal anastomosal stenosis, which was treated with endoscopic dilation and one suicide attempt 5 mo after surgery due to undetected severe pre-existing depression.

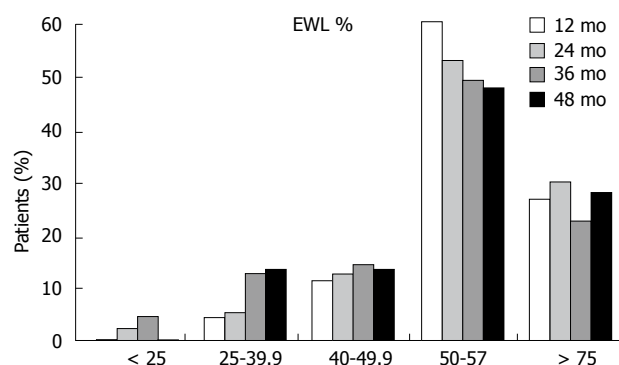


Figure 2 Percentage of EWL during follow-up.

Weight loss

The results of weight loss expressed as actual weight, BMI, percentage of excess weight loss (EWL%) and percentage of initial weight loss (IWL%) at 12, 24, 36 and 48 mo, respectively, are shown in Table 4. Maximum weight loss was observed at 12 mo, with mean BMI 30 kg/m², mean EWL 66.4% and mean IWL 34.3% which remained stable throughout the follow-up period. Ninety-two (83.6%) of 110 patients achieved an EWL of at least 50% at 12 mo which was maintained throughout the follow-up period (Figure 2). An EWL of 50% is considered successful.

Comorbidities

Comorbidities were present in 117 (90.7%) patients preoperatively with an average of 3 ± 2.6 (range 0-10) per patient. The majority of these showed significant improvement during the 48 mo follow-up. Table 5 presents the postoperative results of relevant comorbidities.

Hypertension was present in 21 patients (16.3%) preoperatively, 16 of whom (76.2%) were on medication. After the 2nd postoperative year, only one patient (4.8%) remained on antihypertensive medication.

Preoperatively 24 patients had diabetes mellitus (blood glucose > 125 mg/dL), 14 of whom were on oral hypoglycemic agents while 2 were being treated with insulin. An additional 8 patients had impaired oral glucose tolerance test (OGTT) preoperatively. Postoperatively, diabetes mellitus was completely resolved in 22 patients (91.7%) by the 12 mo follow-up. Only one patient remained on insulin therapy throughout the study period.

Hypercholesterolemia (blood cholesterol > 200 mg/dL) was present in 64 patients preoperatively. Mean cholesterol levels were 230 ± 28.8 mg/dL preoperatively and had decreased to 179 ± 42 mg/dL by the 12 mo follow-up and remained at these levels for the rest of the follow-up period. Among the 52 of 64 patients who completed the 12 mo follow-up, 14 (26.9%) still had elevated blood cholesterol levels > 200 mg/dL. By the 36th month following surgery, only 6 of the 24 patients (25.0%) still had cholesterol levels > 200 mg/dL.

There were 34 patients with hypertriglyceridemia (TGs > 160 mg/dL) preoperatively, with mean levels

Table 4 Weight loss during follow-up

	0 mo	12 mo	24 mo	36 mo	48 mo
Patients	130	110	85	51	16
Weight (kg)	125	81.6	81.4	84.1	79.2
BMI	46	30	31	32	31
IWL %		34.2	34.3	32.2	31.4
EWL %		66.2	65.9	61.5	61.1
Pts > 50% EWL		92	68	35	12

Values for weight, BMI, EWL, IWL are mean values.

Table 5 Postoperative resolution of comorbidities

Comorbidities	No. of patients	Follow-up period (mo)	Resolved (%)	Improved (%)	Without change (%)
Hypertension	21	24	84		16
COPD	22	12		100	
Sleep apnea	13	12		100	
Osteoarthritis	21	12		100	
Depression	11	24		89	11
Diabetes Mellitus	24	12	91.7		8.3
OGTT	8	12	100		
Hypercholesterolemia	64	36	94.4		5.6
Hypertriglyceridemia	36	12	100		

225 ± 64.8 mg/dL. Postoperatively, mean TGs levels dropped to 96 ± 33.2 mg/dL and 96 ± 37.7 mg/dL at 12 and 24 mo respectively and none of the patients had hypertriglyceridemia.

Metabolic complications

Anaemia: Anaemia, as defined by haemoglobin level < 13.5 mg/dL in men and < 12.5 mg/dL in women, was observed at mean rate of 20.1%. More specifically, a low haemoglobin level was seen in 14.8%, 20.2%, 22% and 26.7% of patients at 12, 24, 36 and 48 mo, respectively. Regarding other parameters associated with anaemia including iron (< 35 µg/dL), vitamin B12 (< 200 pg/mL) and ferritin (< 9 ng/mL), the mean rates of postoperative deficiency were 6.1%, 18.7% and 10.0%, respectively. No deficiency of folic acid was observed following surgery and none of the above deficiencies were clinically severe. All patients were treated successfully with oral supplementation.

Hypoalbuminemia: Hypoalbuminemia did not occur at any time during the postoperative follow-up. The mean levels of albumin were > 4 g/dL at all follow-up evaluation and no patient presented with albumin levels below the minimum normal level.

DISCUSSION

In this study, 130 patients underwent laparoscopic RYGB-LL with an acceptable total early complication rate (minor and major) 10.0%, a low conversion rate (0.8%) and no mortality.

The mean hospital stay was quite high (9 d) but this is due to the existing health care system in Greece, which

lacks the services of early postoperative care for patients from distant places.

Maximum weight loss in our series was achieved at 12 mo, with a mean EWL of 66.4%, which was maintained through out the study period. However, 14% of patients did not achieve adequate weight loss (EWL < 50%) and the reason for these failures has not been clarified yet causing problems for the surgeons and the patients.

In addition to adequate weight loss, improvement of pre-existing comorbidities is one of the most important criteria for evaluating success of a bariatric procedure. In our study, as in others^[1,2,6,8-10], that was achieved, as shown in Table 2. Diabetes mellitus was completely resolved in the majority of the patients, finding supported by other reports, which also proposed mechanisms that this can be achieved^[11,12]. Hypercholesterolemia was resolved in the majority of patients and hypertriglyceridemia in all patients, results similar to those described for malabsorptive procedures^[13,14]. Regarding metabolic complications the most significant were vitamin B₁₂ and iron deficiency. However there was no clinical impact and all the patients were treated conservatively with intramuscular injections of vitamin B₁₂ and oral iron supplements as necessary.

The surgical complications rate in our series was comparable to that described by others^[6,15-20] and included primarily gastrointestinal haemorrhages, enterocutaneous fistulas and obstructive ileus. We had no internal hernias, which was probably due to the antecolic position of the Roux limb^[21]. Furthermore, the low incidence of anastomotic gastrointestinal stricture (0.8%) may be attributed to the use of 25 mm circular stapler. The prevalence of wound infection and anastomotic leakage was similar to that reported by others^[15,17,18].

The laparoscopic approach to bariatric surgery is technically challenging, but with experience it can be mastered. The learning curve is steep and long operating times are required^[22]. As shown in Figure 2, our operating time decreased significantly as our experience accumulated, which was supported by Wittgrove *et al*^[5]. Since practice makes perfection, we could also manage to improve our port-site closure technique and after the first 5 unfortunate incisional hernias there was no new case recorded. Furthermore, our 2 cases of enterocutaneous fistulas were attributed to simultaneous mesh placement and eventually this approach was abandoned.

Our results suggest that laparoscopic RYGB-LL as performed at our institution provides adequate weight loss and resolution or improvement of comorbidities up to 4 years. Recently, it has been increasingly recognised that RYGB may have an effect on appetite and eating behaviour, which, in turn, may improve long-term results. These effects may be related to changes in the circulating levels of the gastrointestinal hormones known to influence appetite including PYY^[7,23-28], and this may play a role in the overall success of the procedure.

CONCLUSION

In our experience laparoscopic Roux-en-Y gastric bypass

appears to be a safe and effective surgical procedure for patients with BMI 35-50 kg/m² with results which are comparable to previously published data primarily from the USA, but some from Europe as well. It is associated with minor postoperative pain, early patient mobilization, low incidence of perioperative complications, improvement or complete resolution of preexisting comorbidities and weight loss results that are comparable to those seen following the open procedure. For all these reasons we consider laparoscopic RYGB-LL to be a procedure of choice for the treatment of clinically severe obesity in properly selected patients.

COMMENTS

Background

Obesity, has reached epidemic proportions in society today and it is associated with high rates of morbidity and mortality due to the presence of severe comorbidities. Surgery is the only reliable way of achieving adequate and long-term weight loss in the morbidly obese population. Nowadays, Roux en Y gastric bypass (RYGB) is considered as the gold standard of bariatric procedures. In the present study, the experience of a single centre in Greece with the laparoscopic RYGB is reported.

Research frontiers

Laparoscopic RYGB-LL as performed at authors' institution provides adequate weight loss and resolution or improvement of comorbidities up to 4 years. Apparently, RYGB seems to have an effect on appetite and eating behaviour, which, in turn, may improve long term results. These effects are related to changes in the circulating levels of the gastrointestinal hormones known to influence appetite including ghrelin, PYY and GLP-1. Further in depth research upon this topic is required in order to elucidate the precise mechanisms involved in weight loss.

Innovations and breakthroughs

According to authors' experience, laparoscopic Roux-en-Y gastric bypass appears to be a safe and effective surgical procedure for patients with BMI 35-50 kg/m². The vast majority of their complications occurred early at the learning curve. The authors were able to minimize their complication rate and further improve operation and shorten hospitalization.

Applications

Laparoscopic RYGB-LL is a safe and efficacious bariatric procedure as it is associated with minor postoperative pain, early patient mobilization, low incidence of perioperative complications, improvement or complete resolution of preexisting comorbidities and durable weight loss. For these reasons, it should be a procedure of choice for the treatment of clinically severe obesity in properly selected patients.

Peer review

A single institutional series of 130 patients with laparoscopic gastric bypass is presented. Although not a huge series the results are excellent and the series is from a European country where lap gastric bypass is less common. The manuscript is likely of interest.

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S- Editor Li DL L- Editor Ma JY E- Editor Yin DH



RAPID COMMUNICATION

Is ineffective esophageal motility associated with gastropharyngeal reflux disease?

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Abstract

AIM: To evaluate the association between ineffective esophageal motility (IEM) and gastropharyngeal reflux disease (GPRD) in patients who underwent ambulatory 24-h dual-probe pH monitoring for the evaluation of supraesophageal symptoms.

METHODS: A total of 632 patients who underwent endoscopy, esophageal manometry and ambulatory 24-h dual-pH monitoring due to supraesophageal symptoms (*e.g.* globus, hoarseness, or cough) were enrolled. Of them, we selected the patients who had normal esophageal motility and IEM. The endoscopy and ambulatory pH monitoring findings were compared between the two groups.

RESULTS: A total of 264 patients with normal esophageal motility and 195 patients with the diagnosis of IEM were included in this study. There was no difference in the frequency of reflux esophagitis and hiatal hernia between the two groups. All the variables showing gastroesophageal reflux and gastropharyngeal reflux were not different between the two groups. The frequency of GERD and GPRD, as defined by ambulatory pH monitoring, was not different between

the two groups.

CONCLUSION: There was no association between IEM and GPRD as well as between IEM and GERD. IEM alone cannot be considered as a definitive marker for reflux disease.

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Key words: Ineffective esophageal motility; Gastroesophageal reflux disease; Gastropharyngeal reflux disease

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is characterized by increased exposure of the esophageal mucosa to the gastric contents. This is mainly due to a various combinations of an increased number of gastroesophageal reflux episodes and abnormally prolonged clearance of the refluxed material^[1,2]. The mechanisms for efficient clearance are effective peristalsis, the volume of saliva and gravity.

Ineffective esophageal motility (IEM) is the most recently described esophageal motility abnormality. IEM is defined as contractions with an amplitude of less than 30 mmHg and/or with a rate of nontransmission to the distal esophagus in number of 30% or more of water swallows^[3,4]. IEM is associated with an increased acid clearance times in the distal esophagus^[3]. Increased acid exposure in these patients is associated with the development of erosive esophagitis and GERD-

associated respiratory symptoms^[5,6].

Gastropharyngeal reflux, also called laryngopharyngeal reflux, is a term used to describe esophageal acid reflux into the laryngeal and pharyngeal areas. It causes supraesophageal manifestations (*e.g.* globus, chronic cough, hoarseness, asthma, chronic sinusitis, or other pulmonary or otorhinolaryngologic diseases). Currently, the best way to demonstrate gastropharyngeal reflux is ambulatory 24-h dual probe pH monitoring^[7].

It might be hypothesized that patients with IEM would be unable to clear refluxed acid; this would lead to a prolonged esophageal dwell time of the refluxed acid and then the refluxed acid would reach to a higher level. As a result, it would be presumed that patients with IEM have more gastropharyngeal reflux than those patients with normal esophageal motility.

Therefore, the aim of this study was to evaluate the association between IEM and gastropharyngeal reflux in a large series of patients who underwent ambulatory 24-h dual-probe pH monitoring for the evaluation of supraesophageal symptoms.

MATERIALS AND METHODS

Study population

We retrospectively analyzed the medical records and the findings from endoscopy, esophageal manometry and ambulatory 24-h pH monitoring of an unselected group of consecutive patients who were referred to our motility laboratory from July, 2003 to December, 2006. A total of 632 patients received all three examinations due to supraesophageal symptoms (*e.g.* globus, hoarseness or cough). Of them, we selected the patients who had normal esophageal motility and a diagnosis of IEM. We did not enroll those patients who had a history of gastric surgery, a diagnosis of scleroderma or those who were on anti-reflux medications at the time of the study.

This study was reviewed and approved by the Institutional Review Board of Pusan National University Hospital.

Assessment by endoscopy

The presence or absence of reflux esophagitis, hiatal hernia and endoscopically suspected esophageal metaplasia (ESEM) were determined by two endoscopists (G.H. Kim, G.A. Song).

Reflux esophagitis: If esophagitis was present, it was graded according to the Los Angeles classification^[8].

Hiatal hernia: Hiatal hernia was defined as a circular extension of the gastric mucosa above the diaphragmatic hiatus greater than 2 cm in the axial length.

Endoscopically suspected esophageal metaplasia: The presence or absence of endoscopically suspected esophageal metaplasia (ESEM) was examined in the lower portion of the esophagus, including the esophagogastric junction, during inflation of the esophagus before inserting the endoscope into the

stomach. The esophagogastric junction was defined as the oral side end of the fold, which exists continuously from the gastric lumen^[9], as well as the end of the anal side of the fine longitudinal vessel, because the veins in the lower part of the esophagus were distributed uniformly, running parallel and longitudinally in the lamina propria^[10,11]. The squamo-columnar junction was defined by a clear change in the color of the mucosa. ESEM was defined as the area between the squamo-columnar junction and the esophagogastric junction.

Esophageal manometry

All antisecretory and prokinetic medications were discontinued at least 7 d before testing. Esophageal manometry was performed, after an overnight fast, with using an eight-lumen catheter (Synetics Medical Co., Stockholm, Sweden) with side holes 3 cm, 4 cm, 5 cm, 6 cm, 8 cm, 13 cm, 18 cm, and 23 cm from the catheter tip and a water-perfused, low-compliance perfusion system (Synetics Medical Co., Stockholm, Sweden), according to a standard protocol. Briefly, the manometry protocol included the following: First, a station pull-through was performed through the lower esophageal sphincter (LES) to determine the end-expiratory resting pressure, the LES length and the location relative to the nares. The catheter was then positioned with the most distal side-hole 2 cm below the upper margin of the LES. Ten 5-mL water swallows were given to evaluate peristalsis; only the esophageal body contractions, measured at 3 cm, 8 cm and 13 cm above the LES, were recorded for data analysis. The catheter was then pulled through the upper esophageal sphincter (UES) in the same manner (station pull-through) to determine the resting UES pressure, the length and the location relative to the nares. Patients were identified as having IEM when the total sum of the low amplitude peristaltic contractions (the distal amplitude measured at 3 or 8 cm above the LES was < 30 mmHg) and the nontransmitted peristaltic contractions (dropouts at either 3 cm or 8 cm above the LES) was equal or greater than 30% of the total number of swallows used for the esophageal body study^[4].

Ambulatory 24-h dual-probe pH monitoring

Ambulatory 24-h dual-probe pH monitoring was performed immediately after esophageal manometry with using a single-use monocrystalline antimony dual-site pH probe (Zinetics 24, Medtronic Inc., Minneapolis, USA) with the electrodes placed at the tip and 15 cm proximal to the tip. A cutaneous reference electrode placed on the upper chest was also used. All the electrodes were calibrated in buffer solutions of pH 7 initially and then pH 1. The pH catheter was introduced transnasally into the stomach and it was withdrawn back into the esophagus until the electrodes were 5 cm above the proximal margin of the LES. The subjects were encouraged to eat regular meals with restriction for the intake of drink or food with a pH below 4. All the subjects recorded their meal times (start and end), body position (supine and upright) and any symptoms

in a diary. The data were collected using a portable data logger (Ditrigger Mark III, Synetics Medical Co., Stockholm, Sweden) with a sampling rate of 4 seconds, and the data was then transferred to a computer for analysis using "Polygram for Windows" (Release 2.04, Synetics Medical Co., Stockholm, Sweden). For both sites, a decrease in pH below 4, which was not induced by eating or drinking, was considered the beginning of a reflux episode, and the following rise to pH above 4 was considered the end of such an episode. To be accepted as a gastropharyngeal reflux event, the decrease at the proximal probe had to be abrupt and simultaneous with the decrease at the distal probe, or it was preceded by a decrease in pH of a similar or larger magnitude at the distal probe. Thus, acid episodes induced by oral intake, aero-digestive tract residue and secretions, proximal probe movement or loss of mucosal contact in which the proximal pH decline may precede the esophageal pH drop were not included as gastropharyngeal reflux episodes.

The variables assessed for gastroesophageal reflux at the distal probe were the total percentage of time the pH was < 4, the percentage of time the pH was < 4 in the supine and upright positions, the number of episodes the pH was < 4, the number of episodes the pH was < 4 for ≥ 5 min, the duration of the longest episode the pH was < 4 and the DeMeester composite score^[12].

The variables assessed for gastropharyngeal reflux at the proximal probe were the total percentage of time the pH was < 4, the percentage of time the pH was < 4 in the supine and upright positions, and the number of episodes the pH was < 4.

For the diagnosis of GERD at the distal probe, two different aspects were analyzed^[13,14]: (1) the total reflux time: the total proportion of the recorded time with pH < 4; a value of > 4% was considered abnormal; (2) the number of reflux episodes: the total number of pH episodes with pH < 4 during the recording; a value of > 35 episodes was considered abnormal.

For the diagnosis of gastropharyngeal reflux disease (GPRD) at the proximal probe, we considered more than 0.1% for the total time, 0.2% for the upright time and 0% for the supine time of pH < 4 to be pathological. For the number of reflux episodes, more than 4 reflux episodes were considered pathological^[15,16].

Statistical analysis

The data are expressed as mean \pm SE unless otherwise noted. The student *t*-test was used to assess the statistical significance of age, the body mass index, the pressure and length of the LES and the parameters of ambulatory pH monitoring between the two groups. The differences in gender, alcohol intake, smoking, typical reflux symptoms, indications for pH monitoring, reflux esophagitis, hiatal hernia, ESEM, GERD and GPRD, as defined by the ambulatory pH monitoring between the two groups were assessed using the χ^2 test. A *P* < 0.05 was considered statistically significant. Statistical calculations were performed using the SPSS version 12.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Table 1 Patient profiles and the endoscopic findings in the patients with normal esophageal motility and ineffective esophageal motility *n* (%)

	Normal (<i>n</i> = 264)	IEM (<i>n</i> = 195)	<i>P</i> value
Age (yr, mean \pm SD)	50.8 \pm 11.1	51.1 \pm 12.0	0.782
Gender (men/women)	99/165	87/108	0.125
BMI (kg/m ² , mean \pm SD)	23.6 \pm 2.7	23.2 \pm 2.7	0.393
Alcohol intake	58 (22.0)	31 (15.9)	0.104
Smoking	43 (16.3)	19 (9.7)	0.043
Heartburn/acid regurgitation ¹	128 (48.5)	104 (53.3)	0.304
Indication for pH monitoring			0.542
Globus	118 (44.7)	88 (45.1)	
Hoarseness	63 (23.9)	35 (17.9)	
Cough	27 (10.2)	24 (12.3)	
Sore throat	30 (11.4)	28 (14.4)	
Others ²	26 (9.8)	20 (10.3)	
Reflux esophagitis ³	30 (11.4)	30 (15.4)	0.206
A	22	16	
B	7	11	
C	1	2	
D	0	1	
Hiatal hernia	17 (6.4)	10 (5.1)	0.555
Endoscopically suspected esophageal metaplasia	23 (8.7)	11 (5.6)	0.214

¹More than 2 d per wk; ²Other indications were halitosis, throat clearing and laryngeal pathology such as vocal polyp; ³Los Angeles classification grade. IEM: Ineffective esophageal motility.

RESULTS

A total of 264 patients with normal esophageal motility and 195 patients with the diagnosis of IEM were included in this study. Age, gender, the body mass index, typical reflux symptoms and indications for pH monitoring were not different between the two groups. There was no difference in the frequency of reflux esophagitis and hiatal hernia between the two groups (Table 1).

The LES pressure was lower in the patients with IEM than in those patients with normal esophageal motility. All the variables showing gastroesophageal reflux at the distal probe were not different between the two groups. There was no difference in all the variables showing gastropharyngeal reflux at the proximal probe between the two groups (Table 2).

The frequency of GERD and GPRD, as defined by ambulatory pH monitoring was not different between the two groups (Table 3, Figure 1).

DISCUSSION

Esophageal acid clearance consists of two processes, first is rapid removal of most of the intraluminal refluxate, which is achieved by gravity and primary or secondary peristalsis (volume clearance), and this is followed by a slow neutralization of the acidified mucosa by the swallowed saliva (chemical clearance). Previous analysis of the relationship between peristaltic dysfunction and the efficacy of esophageal emptying, with using concurrent manometry and fluoroscopy, illustrated that absent or incomplete peristaltic contractions invariably

Table 2 Results of the lower esophageal sphincter and ambulatory 24-h dual probe pH monitoring in the patients with normal esophageal motility and ineffective esophageal motility

	Normal (<i>n</i> = 264)	IEM (<i>n</i> = 195)	<i>P</i> value
Lower esophageal sphincter			
Pressure	21.4 ± 0.5	18.6 ± 0.5	< 0.001
Length	3.3 ± 0.1	3.3 ± 0.1	0.921
Proximal probe			
Time pH < 4 (total) (%)	0.4 ± 0.1	0.7 ± 0.2	0.225
Time pH < 4 (upright) (%)	0.7 ± 0.1	1.0 ± 0.2	0.230
Time pH < 4 (supine) (%)	0.1 ± 0.0	0.6 ± 0.4	0.214
No. of reflux episodes	8.7 ± 1.0	12.2 ± 2.6	0.219
Distal probe			
Time pH < 4 (total) (%)	2.9 ± 0.2	3.5 ± 0.3	0.185
Time pH < 4 (upright) (%)	4.8 ± 0.7	5.0 ± 0.5	0.827
Time pH < 4 (supine) (%)	1.6 ± 0.3	1.9 ± 0.4	0.564
No. of reflux episodes	44.1 ± 2.5	47.8 ± 4.0	0.430
No. of reflux episodes ≥ 5 min	1.4 ± 0.2	1.9 ± 0.3	0.116
Longest reflux episode (min)	8.6 ± 0.8	10.2 ± 1.0	0.199
DeMeester composite score	12.1 ± 0.9	14.1 ± 1.3	0.217

IEM: Ineffective esophageal motility.

Table 3 Distribution of the patients with gastroesophageal reflux disease and/or gastropharyngeal reflux disease, as defined by ambulatory pH monitoring, in the patients with normal esophageal motility and ineffective esophageal motility *n* (%)

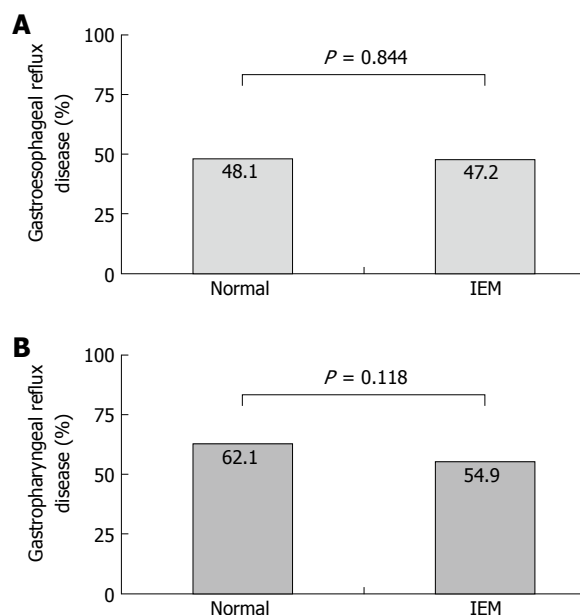
	Normal (<i>n</i> = 264)	IEM (<i>n</i> = 195)
GERD and GPRD	108 (40.9)	74 (37.9)
GPRD only	56 (21.2)	33 (16.9)
GERD only	19 (7.2)	18 (9.2)
Normal	81 (30.7)	70 (35.9)

GERD: Gastroesophageal reflux disease; GPRD: Gastropharyngeal reflux disease; IEM: Ineffective esophageal motility.

resulted in little or no volume clearance and ineffective esophageal propulsion of a bolus occurs when the amplitude of the peristaltic waves is below 30 mmHg^[17]. Thus, peristaltic dysfunction could potentially prolong esophageal acid clearance by delaying the first phase, that of esophageal emptying.

GERD motility abnormalities are part of the nonspecific motor disorders that have been described many years ago^[18], and IEM has been found in 20%-50% of the patients with GERD^[19]. In addition, there have been some studies suggesting a link between IEM and delayed esophageal acid clearance^[3,5,20]. When GERD patients underwent pH monitoring, there were significantly more recumbent and upright reflux episodes and delayed acid clearance in the patients with IEM than in those patients without IEM^[3,20]. A greater frequency of IEM was found in patients with respiratory presentations of GERD (chronic cough, asthma and laryngitis) and identification of IEM was particularly useful for patients with supraesophageal GERD^[5].

In present study, we selected the patients who had normal esophageal motility and IEM among the patients who received the endoscopy, esophageal manometry and ambulatory pH monitoring due to supraesophageal symptoms. We then analyzed the

**Figure 1** Frequency of gastroesophageal reflux disease (A) and gastropharyngeal reflux disease (B), as defined by ambulatory pH monitoring, in the patients with normal esophageal motility and ineffective esophageal motility (IEM).

degree of gastroesophageal and gastropharyngeal reflux in both group. Our results indicated that IEM was not associated with GPRD as well as GERD, as defined by ambulatory pH monitoring. In addition, all the variables for gastropharyngeal reflux and gastroesophageal reflux were not higher in the patients with IEM than those with normal esophageal motility. These findings are consistent with the previous studies^[21,22] showing that there was no association between esophageal dysmotility and abnormal acid reflux in patients with supraesophageal GERD symptoms. We also examined the degree of gastroesophageal and gastropharyngeal reflux according to the severity of IEM, but there was no association (data not shown), which was similar to the previous report^[23] showing that the severity of IEM was not different in erosive and in nonerosive GERD patients. These results suggest that IEM alone is unlikely to be the major determinant of abnormal esophageal acid exposure.

Although many studies have assessed the link between IEM and esophagitis, this issue remains controversial. Most of the previous studies restricted the enrolled subjects to the GERD patients. IEM was associated with reflux esophagitis in some studies of patients with confirmed GERD^[6,24]. However, other studies showed that the presence of reflux esophagitis was similar between the patients with IEM and those patients with normal esophageal peristalsis^[20] and there was no difference in the severity of IEM when comparing the erosive and non-erosive GERD patients^[23]. In our present study, we included the patients who had normal esophageal motility and IEM over a defined period, providing that the ambulatory study had been done in the absence of anti-secretory therapy, thereby insuring the presence of a control group with normal esophageal acid exposure. Our result showed that reflux esophagitis was not associated with IEM.

There were some merits of this study when

comparing it with the previous studies. First, in contrast to previous reports^[5,21,22] that focused on an association between IEM and supraesophageal reflux disease, our study limited the enrolled subjects to patients with normal esophageal motility and those with IEM to maximize the effect of IEM on GPRD. Second, in the current analysis, we defined GERD and GPRD according to the strict criteria of ambulatory 24-h dual-probe pH monitoring, which is the best available test for diagnosing GPRD, as well as GERD^[7]. Third, because all the patients in the current study underwent upper endoscopy, we were able to classify them according to the presence or absence of esophagitis and hiatal hernia.

There were some limitations in this study. First, the ambulatory pH monitoring is not 100% accurate and it has a sensitivity as low as 70% in patients with esophagitis, and the sensitivity is substantially lower in patients with nonerosive disease^[25], so that some of our patients may have been misclassified. Yet, we included a large number of cases (459 cases), so this limitation was probably lessened. Second, a great deal of controversy exists about the location of the proximal probe. Recording the pH in the hypopharynx is technically difficult. Acid exposure in the hypopharynx can easily be missed because of the relatively large space within the hypopharynx^[15]. On the contrary, placement of the proximal probe in or below the upper esophageal sphincter allows for more permanent contact with the mucosa during the 24-h period and this results in fewer artifacts^[15,16]. We used a dual-site pH probe with electrodes placed at the tip and 15 cm proximal to the tip, and we could not choose the exact location of the proximal probe. Yet in most cases (75.4%, 346/459), the proximal probe was located in the UES. So, for the diagnosis of GPRD, we used the criteria proposed by Smit *et al*^[15,16].

Why is IEM not associated with GPRD as well as GERD? Conventional manometry may be unable to evaluate the “true effectiveness” of esophageal peristalsis^[26,27]. In addition, the refluxed acid is neutralized by both the esophageal submucosal secretions and the swallowed salivary secretions, so it becomes non-acid reflux material. Therefore, even though this non-acid refluxate in the upper level actually increased in the patients with IEM, the proximal pH probe cannot detect it. To solve this problem, a prospective study using a combined multichannel intraluminal impedance and pH measurement, which are able to detect both acid and non-acid reflux, as well as the proximal extent of the refluxate, will be needed.

In conclusion, by analyzing a large cohort of patients who had normal esophageal motility and IEM, we demonstrated that there was no correlation between IEM and GPRD, as well as between IEM and GERD, as defined by ambulatory pH monitoring. Although we do not completely exclude that such an association may be possible, IEM alone cannot be considered a definitive marker for reflux (gastroesophageal or gastropharyngeal).

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COMMENTS

Background

Ineffective esophageal motility (IEM) is associated with an increased acid clearance times in the distal esophagus. Gastropharyngeal reflux causes supraesophageal manifestations such as globus, chronic cough, hoarseness, asthma, chronic sinusitis, or other otorhinolaryngologic diseases. It might be hypothesized that patients with IEM would be unable to clear refluxed acid; this would lead to a prolonged esophageal dwell time of the refluxed acid and then the refluxed acid would reach to a higher level. As a result, it would be presumed that patients with IEM have more gastropharyngeal reflux than those patients with normal esophageal motility.

Research frontiers

The research front in this area is focused on evaluating the association of IEM and gastropharyngeal reflux disease (GPRD), as well as gastroesophageal reflux disease (GERD). Although many studies have assessed the link between IEM and esophagitis, this issue remains controversial. Most of the previous studies restricted the enrolled subjects to GERD patients. IEM was associated with reflux esophagitis in some studies of patients with confirmed GERD. However, other studies showed that the presence of reflux esophagitis was similar between the patients with IEM and those patients with normal esophageal peristalsis. This study showed no association between IEM and GPRD, as well as between IEM and GERD in a large series of patients who underwent ambulatory 24-hour dual-probe pH monitoring, for the evaluation of supraesophageal symptoms.

Innovations and breakthroughs

There are few reports on the association between IEM and GPRD. Most previous studies are symptom-based and lack objective tests such as ambulatory 24-h dual-probe pH monitoring. This study is the largest study to evaluate the association of IEM and GPRD in patients who underwent ambulatory 24-h dual-probe pH monitoring for the evaluation of supraesophageal symptoms.

Applications

IEM is not associated with GPRD, as well as GERD. Further studies using a combined multichannel intraluminal impedance and pH measurement, which are able to detect both acid and non-acid reflux, as well as the proximal extent of the refluxate, will be needed.

Terminology

Ineffective esophageal motility (IEM) is defined as contractions with an amplitude of less than 30 mmHg and/or with a rate of nontransmission to the distal esophagus in number of 30% or more of water swallows. Esophageal acid reflux into the laryngeal and pharyngeal areas causes extraesophageal manifestations such as chronic cough, hoarseness, asthma, globus sensation, chronic sinusitis, or other otorhinolaryngologic diseases. This condition is called as gastropharyngeal reflux disease (GPRD).

Peer review

This is an interesting study since physicians who perform esophageal manometry frequently find IEM. This study is well structured and definitions of esophagitis, GERD and GPRD are adequate since they were based on endoscopy and 24-h dual esophageal pH monitoring.

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RAPID COMMUNICATION

Decreased phagocytic activity of Kupffer cells in a rat nonalcoholic steatohepatitis model

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Author contributions: Tsujimoto T and Fukui H designed the research project; Tsujimoto T, Kawaratani H, Kitazawa T and Kitade M contributed to the immunohistochemical staining; Tsujimoto T, Hirai T and Ohishi H contributed to contrast enhanced ultrasonography (CEUS) examination; Tsujimoto T, Kawaratani H, Yoshiji H and Uemura M analysed the data; and Tsujimoto T wrote the paper.

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control group and the CDAA group.

CONCLUSION: CEUS examination using Levovist® demonstrated reduced contrast effect and phagocytic activity in the liver parenchymal phase, although the Kupffer cell numbers were unchanged, indicating reduced phagocytic function of the Kupffer cells in the rat NASH model. We believe that CEUS examination using Levovist® is a useful screening modality, which can detect NASH in fatty liver patients.

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Key words: Nonalcoholic steatohepatitis; Kupffer cells; Contrast enhanced ultrasonography; Levovist; Ultrasound contrast agent; Phagocytic activity

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Tsujimoto T, Kawaratani H, Kitazawa T, Hirai T, Ohishi H, Kitade M, Yoshiji H, Uemura M, Fukui H. Decreased phagocytic activity of Kupffer cells in a rat nonalcoholic steatohepatitis model. *World J Gastroenterol* 2008; 14(39): 6036-6043 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6036.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6036>

Abstract

AIM: To investigate Kupffer cell dynamics and phagocytic activity, using a rat nonalcoholic steatohepatitis (NASH) model.

METHODS: Male F344 rats were fed either a control diet or a choline-deficient L-amino acid-defined (CDAA) diet, followed by contrast enhanced ultrasonography (CEUS) using Levovist®. The uptake of latex beads by the Kupffer cells was determined by fluorescent microscopy. The status of the Kupffer cells was compared between the two groups, using the immunohistochemical staining technique.

RESULTS: After 4 or more wk of the CDAA diet, CEUS examination revealed a decrease in the signal intensity, 20 min after intravenous Levovist®. Fluorescent microscopic examination showed that the uptake of latex beads by the Kupffer cells was reduced at week 1 and 2 in the study group, compared with the controls, with no further reduction after 3 wk. Immunohistochemical staining revealed no significant difference in the Kupffer cell counts between the

INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is characterized by hepatic steatosis, inflammation and fibrosis, with increased risk of developing cirrhosis and hepatocellular carcinoma (HCC)^[1-3]. The progression from simple steatosis to cirrhosis has been attributed to inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), oxidative stress and endotoxins, in combination with fatty degeneration due to insulin resistance^[4]. At present, histopathological examination of liver biopsy tissue is the only way to definitively diagnose NASH^[5-8].

The diagnosis of NASH is important in clinical practice, since this condition can progress to cirrhosis and HCC. When patients with NASH undergo contrast enhanced ultrasonography (CEUS) using Levovist

[®] (galactose-palmitic acid ultrasound contrast agent), a reduced contrast effect is seen in the liver parenchymal phase^[9]. The activity of Kupffer cells and hepatic sinusoids can be evaluated using the contrast effect in the liver parenchymal phase during CEUS examination with Levovist[®]. The findings strongly implicate Kupffer cells in the pathogenesis of NASH. Reduced function or uneven distribution of Kupffer cells in the liver may play a part in the development of NASH, although this hypothesis remains conjectural at the present time.

In this study, we investigated the contrast effect in the liver parenchymal phase of CEUS using Levovist[®], and assessed the Kupffer cell dynamics and phagocytic activity.

MATERIALS AND METHODS

Animals, NASH model induction

Six-week-old male F344 rats weighing 180–200 g were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). The animals were housed in stainless steel mesh cages under controlled conditions of temperature (23 ± 3°C) and relative humidity (50% ± 20%), with 10 to 15 air changes per hour, and light illumination for 12 h a day. The animals were allowed access to tap water *ad libitum* throughout the duration of the study. A choline-deficient L-amino acid-defined (CDAA), and a choline-supplemented L-amino acid-defined (CSAA) diet were purchased from CLEA Japan Inc. (Tokyo, Japan). The details of both diets are described elsewhere^[10]. The study group was fed a CDAA diet for 8 wk in order to produce NASH. All procedures were approved by the institutional animal care committee and conducted in accordance with Nara Medical University Guidelines for the Care and Use of Laboratory Animals.

Serum alanine aminotransferase (ALT)

Serum samples from CSAA-fed rats and CDAA-fed rats, killed at 1, 4 and 8 wk were used to measure the serum ALT levels. The levels of serum ALT were determined using a 7170 Clinical Analyzer (Hitachi High-Technologies, Tokyo, Japan).

Histological examination

The liver tissues were fixed in 10% formalin, and the first section was stained with hematoxylin and eosin for histological examination. Another section was stained with Azan and Sirius red to detect fibrosis. Histological grading and staging were performed using a modified scoring system based on the classification of either Matteoni *et al.*^[5] or Brunt *et al.*^[6]. Matteoni *et al.*^[5] proposed NAFLD types 1–4 based on long-term outcome studies; Brunt *et al.*^[6] proposed a system of grading and staging for NASH that follows methods of separate assessment of necroinflammatory lesions (grading) and fibrosis (staging), accepted in other forms of non-biliary chronic liver diseases.

Levovist[®] CEUS studies

We compared the contrast effects in the CEUS liver

parenchymal phase before administering the CDAA diet (control group, $n = 5$), and after 1, 4, and 8 wk of the CDAA diet (1-, 4-, and 8-wk groups, $n = 5$ for each group). The studies were performed with the Logiq 7 ultrasonographic system (GE Healthcare, Tokyo, Japan), using a 7L probe (3–7 MHz), and employing the Coded Harmonic Angio mode. Levovist[®] (Schering AG, Berlin, Germany) was diluted to 300 mg/mL, and injected into the animal's tail vein at a dose of 0.1 mL/100 g body weight. Following confirmation that the contrast had entered the right kidney, we scanned the liver, using 1-s intermittent transmission scans at 5, 10, 15, and 20 min, with a different section for each scan. The fluorescent intensity in the region of interest (ROI) in each image was calculated by the equipment software using the time intensity curve (TIC).

Observation of FITC-latex beads phagocytosis by Kupffer cells *in vivo*

An injection of 2×10^{10} /kg 1 μ m fluorescent latex beads (Polyscience, Warrington, PA, USA) was given into the animal's tail vein before CDAA (control group, $n = 5$), and after 1, 2, 3, 4, and 8 wk of CDAA (1-, 2-, 3-, 4- and 8-wk groups, $n = 5$ for each group). Two hours after the injection, the animals were killed and 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer saline (PBS) was perfused into the portal vein. After fixation with the same fixative for 60 min, the liver specimens were sliced into 5- μ m-thick sections with a cryostat microtome CM 1510 (Leica Microsystems, Wetzlar, Germany). The uptake of latex beads by the Kupffer cells was determined using fluorescent microscopy Leica DM IRB (Leica Microsystems), and the fluorescent intensities were analyzed and compared using IP LabTM software (BD Biosciences, Rockville, MD, USA).

Isolation and culture of Kupffer cells

The Kupffer cells were harvested from the liver using the isolation buffers described by Seglen^[11]. The liver was perfused *in situ* with Ca²⁺-free minimum essential medium (Sigma, St. Louis, MI, USA) followed by 0.3% pronase (Roche Diagnostics Corp., Indianapolis, IN, USA), and 0.05% type IV collagenase (Sigma) in Dulbecco's modified eagle's medium/F-12 (Sigma) at a rate of 10 mL/min through the portal vein. The liver was carefully removed and minced with scissors. The minced liver was incubated in a shaker water bath with 0.035% pronase and 62.5 U/mL DNase (Sigma) in Dulbecco's modified eagle's medium/F-12 at 37°C for 20 min, and was then filtered through gauze; the parenchymal cells were removed by low-speed centrifugation. The resultant supernatant was laid on top of 4 separate densities (1.035, 1.045, 1.058, and 1.085) of arabinogalactan solution (Sigma) in one test tube and centrifuged at 400 r/min for 45 min at 37°C using a Beckman SW41-Ti rotor (Beckman Instruments, Fullerton, CA, USA). The third and fourth layers from the top were recovered and washed twice with Ca²⁺-free Hanks balanced salt solution (Sigma). The final cell pallet was subsequently suspended in RPMI 1640 medium,

and cultured in a culture flask at 37°C in humidified atmosphere containing 50 mL/L CO₂ and air for 2 h.

The purity of the isolated Kupffer cells was over 98%, as determined by the uptake of 1 µm latex beads^[12], and the viability was over 95% in the trypan-blue-dye exclusion test.

The Kupffer cells were seeded in 12-well plastic plates and incubated in RPMI 1640 medium at a concentration of 5×10^5 cells/mL. The dishes were washed with Hanks balanced salt solution to remove the unattached cells. The Kupffer cells were cultured in RPMI 1640 supplemented with 50 µg/mL streptomycin and 50 µg/mL ampicillin (Nakalai tesque, Kyoto, Japan).

Observation of FITC-latex beads phagocytosis by Kupffer cells in vitro

Fluorescent latex beads (1×10^7 1 µm) were placed in each well, and culture was performed at 37°C for 2 h under 50 mL/L CO₂ on plastic dishes that were washed three times with Hanks balanced salt solution to remove un-phagocytosed latex beads. The uptake of latex beads by the Kupffer cells was determined using fluorescent microscopy, and the fluorescent intensities were analyzed and compared using IP LabTM software.

Kupffer cells immunohistochemical staining

The Kupffer cell dynamics were analyzed by comparing the cell counts of each group, using immunohistochemical staining with anti-rat macrophage/dendritic cell monoclonal antibody (RM-4: Trans Genic Inc., Kobe, Japan), a Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA), and DAB peroxidase substrate solution (Vector Laboratories), with counterstaining by Hematoxylin Mayer. The stained areas were analyzed and compared using NIH-image software (Version 1.61; U. S. National Institute of Health, Bethesda, MD, USA).

Statistical analysis

P-values were calculated, assuming equal sample variance, using the paired *t*-test, and Statview software (version 5.0; SAS Institute, Cary, NC, USA), considering *P* < 0.05 as statistically significant. The values are mentioned as mean ± SD.

RESULTS

Serum ALT

The mean serum ALT level in the control rats was 41.7 ± 7.4 IU/L, compared to 524.6 ± 101.7 IU/L, 267.9 ± 47.5 IU/L and 251.4 ± 81.6 IU/L in the 1-, 4-, and 8-wk, respectively in the CDAA-fed rats. All NASH groups showed statistically significant elevations, compared with the control animals (*P* < 0.01) (Figure 1).

Changes in liver histology

Histological examination of the liver of 1-wk CDAA-fed rats revealed inflammation and fat deposits, but no fibrosis, and was considered as Matteoni's type 2.

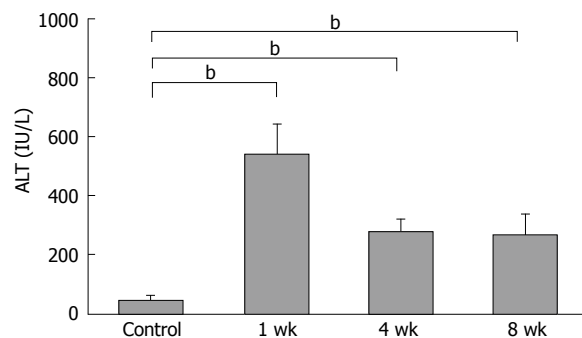


Figure 1 Serum ALT levels were elevated significantly in 1-wk CDAA-fed rats, and decreased gradually in the 4 and 8-wk CDAA-fed rats, although, both were elevated significantly compared with the control animals (*n* = 5), ^b*P* < 0.01.

The 4-wk CDAA-fed rats had more inflammation, fat deposits, and fibrosis, which was equivalent to Matteoni's type 3 and Brunt's NASH classification of grade 2/stage 2. The histological findings in the 8-wk CDAA-fed rats were equivalent to Matteoni's type 4 and Brunt's grade 2/stage 3. In the 4- and 8-wk groups, Sirius red staining revealed abundant collagen (Figure 2).

Levovist® CEUS examination

Assessment of changes in fluorescent intensity up to 20 min (-25.5 ± 6.4 dB, -37.5 ± 7.5 dB, -55.2 ± 3.9 dB, -59.3 ± 5.6 dB in the control, 1-, 4-, and 8-wk CDAA-fed rats) after administration of Levovist®, with the values at 5 min (-30.2 ± 4.4 dB, -31.8 ± 1.8 dB, -38.8 ± 4.1 dB, -39.7 ± 6.2 dB in the control, 1-, 4-, and 8-wk CDAA-fed rats, respectively) considered as the standard, showed that the fluorescent intensity in the control group tended to rise from 10 min onwards, and remained elevated. In the 1-wk group, the contrast effect remained fairly constant from 5 min to 20 min. In the 4- and 8-wk groups, the contrast effect was decreased significantly at 20 min (Figure 3A). When the fluorescent intensity was quantified using TIC, the fluorescent intensity at 20 min tended to be lower in the 1-wk group and significantly lower in the 4- and 8-wk groups compared with the control group (Figure 3B).

Phagocytosis of FITC-latex beads by Kupffer cells in vivo

Fluorescent microscopic examination showed that the latex bead uptake per Kupffer cell *in vivo* was lower in the 1-wk group compared to the controls, with no further reduction after 4 and 8 wk (Figure 4A and B). To avoid the influence of Kupffer cell cross-sections, and to count the Kupffer cells more accurately, we observed the images again at lower magnification. In comparison with the control group, the uptake was reduced to approximately 50% in the 1-wk group, and to 30% in the 2-wk group, with no further decrease after 3 wk (Figure 4C).

Phagocytosis of FITC-latex beads by Kupffer cells in vitro

Similar to the *in vivo* findings, the fluorescent microscopic examination showed that the *in vitro* latex bead uptake

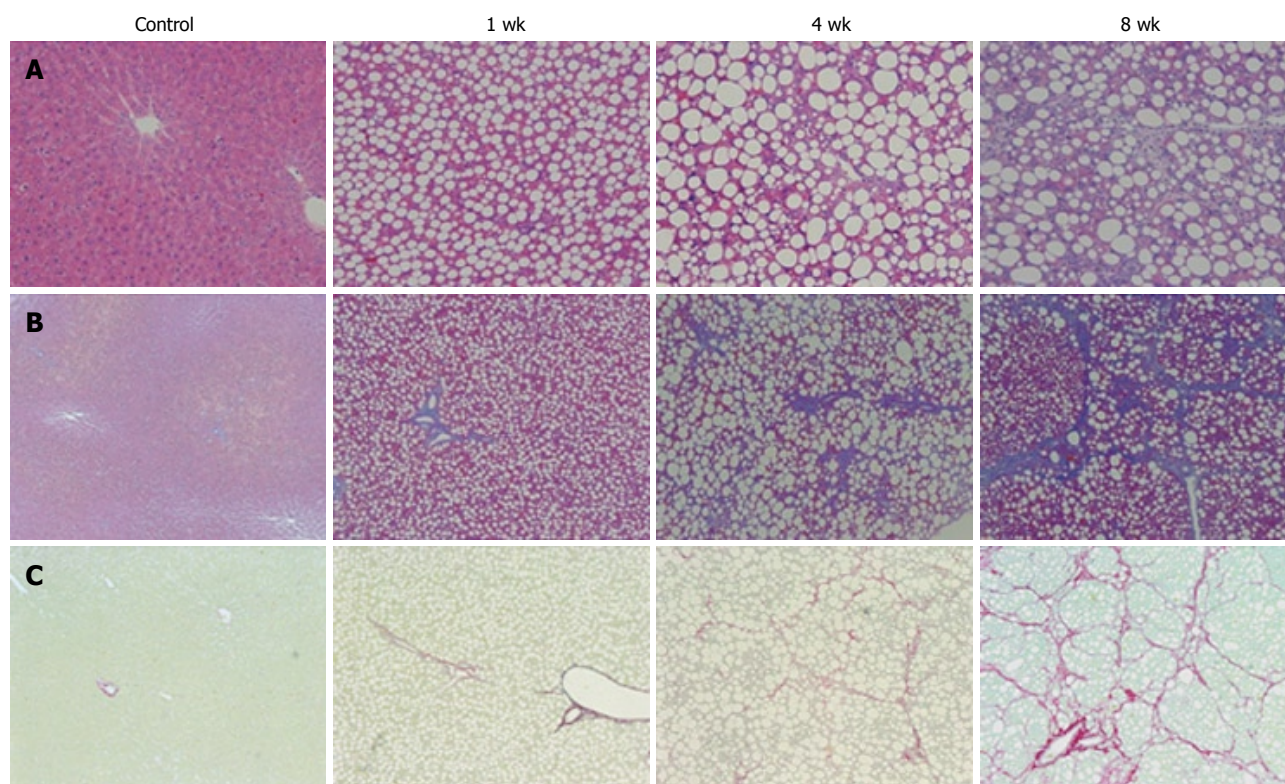


Figure 2 Histological analysis of the liver sections. **A:** Hematoxylin and eosin stain (x 200); **B:** Azan stain (x 100); **C:** Sirius red stain (x 100). Histological examination of the liver tissue of 1-wk CDAA-fed rats showed inflammation and fat deposits, but no fibrosis, corresponding to Matteoni's type 2. The 4-wk CDAA-fed rats had more inflammation, fat deposits, and fibrosis, which was equivalent to Matteoni's type 3 and grade 2/stage 2 of Brunt's NASH classification. The histological findings in the 8-wk CDAA-fed rats were equivalent to Matteoni's type 4 and Brunt's grade 2/stage 3. In the 4 and 8-wk groups, Sirius red staining revealed abundant collagen ($n = 5$).

per Kupffer cell was lower in the 1-wk group than in the controls, with no further reduction after 4 and 8 wk. To avoid the influence of Kupffer cell cross-sections, and to count the Kupffer cells more accurately, we observed the images again at lower magnification. In comparison with the control group, the uptakes were reduced to approximately 60% in the 1-wk group, and to 30% in the 2-wk group, with no further decrease after 3 wk (Figure 5).

Kupffer cells immunohistochemical staining

Immunohistochemical staining showed no significant difference in the number of stained cells per field between the control group and any of the CDAA groups. Quantitative analysis using NIH Image™ also showed no significant difference between the groups (Figure 6).

Relationship of Kupffer cell phagocytic activity with Kupffer cell count and liver histology

There was no correlation between the changes in the contrast effect, and the Kupffer cell count or the degree of fat deposition.

DISCUSSION

Ultrasonographic examinations have been performed to assist in the diagnosis of abdominal diseases since the 1970s, and continue to be widely used in clinical

practice because of the ease of use and low level of invasiveness^[13]. Until recently, ultrasonography enhanced by CO₂ microbubbles, delivered through an intra-arterial catheter (developed in the 1980s), was the only CEUS technique available^[14,15]. The intravenous ultrasonographic contrast agent Levovist®, available for clinical use in Japan since September 1999, facilitates the hepatic blood flow imaging with an inherently low level of invasiveness^[16-19]. Levovist® CEUS provides information on both the blood flow and the parenchyma through the characteristics of the contrast agent^[20,21]. Microbubbles injected *via* the intravenous route travel through the blood vessels, producing vascular images, and the gas is finally eliminated through the lungs^[22]. However, some kinds of microbubbles accumulate in the organs such as the liver^[23] and the spleen^[24], which allows delayed phase imaging^[25]. Recently, Levovist® CEUS has been reported to be a useful screening modality for NASH^[9]. The reduced contrast effect in the liver parenchyma has been attributed to sinusoidal or Kupffer cell dysfunction, although this finding remains to be established^[26].

In the present study, we performed Levovist® CEUS examination using a rat NASH model induced by the CDAA diet. We also assessed Kupffer cell dynamics and phagocytic activity, and confirmed that the contrast effect is reduced in the hepatic parenchymal phase. The liver histology progressed from steatosis and inflammation to marked fibrosis during 8 wk of CDAA

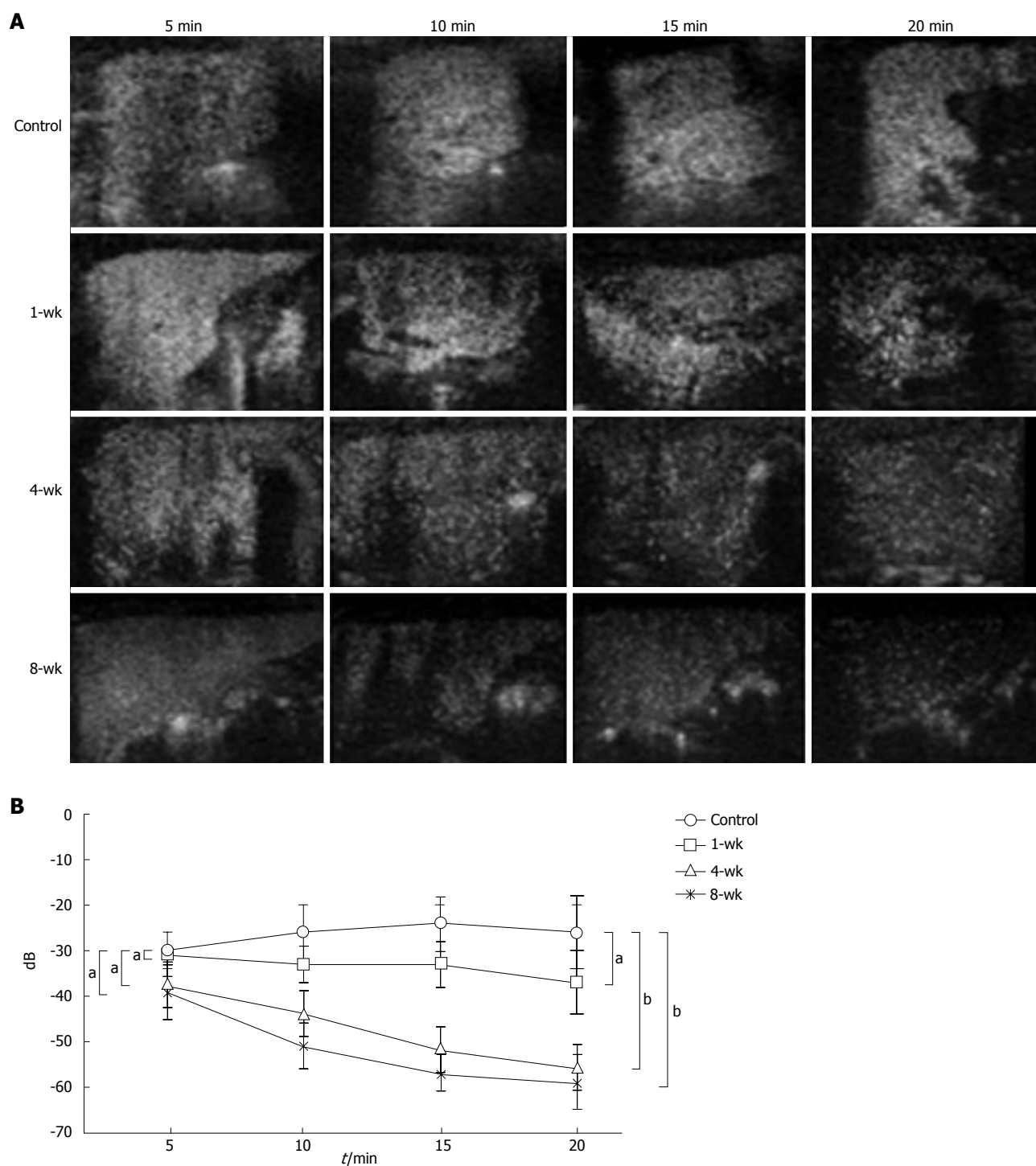


Figure 3 Results of Levovist® CEUS in each group. **A:** Examination of changes in the fluorescent intensity up to 20 min after administration of Levovist®, with the values at 5 min considered as the standard, showed that the fluorescent intensity in the control group tended to rise from 10 min onwards, and remained elevated. In the 1-wk group, the contrast effect remained fairly constant from 5 min to 20 min. After 4 or more wk of the CDAA diet, CEUS examination revealed a decrease in the signal intensity, 20 min after intravenous Levovist®; **B:** Changes in the fluorescent intensity in Levovist® contrast enhanced ultrasonograms in each group. In the control group, the fluorescent intensity increased significantly at 20 min compared with the findings at 5 min, whereas a significant chronological decrease was seen in the NASH groups ($n = 5$). ^a $P > 0.05$, ^b $P < 0.01$.

feeding. The changes in fluorescent intensity up to 20 min after the administration of Levovist® revealed distinct differences depending on the duration of the CDAA diet. In the early phase, the effect of Levovist® in the hepatic sinusoids and the blood stream was strong, whereas at 20 min the effect of the contrast taken up by the Kupffer cells was apparent. When the

values obtained at 5 min were taken as the standard, the contrast effect at 20 min was low even in the 1-wk CDAA-fed group. The contrast effect at 20 min was significantly lower in the 4-wk and 8-wk CDAA-fed groups compared with the findings in the control group. The decrease in the phagocytic activity of Kupffer cells in the early phase of rat steatohepatitis was further

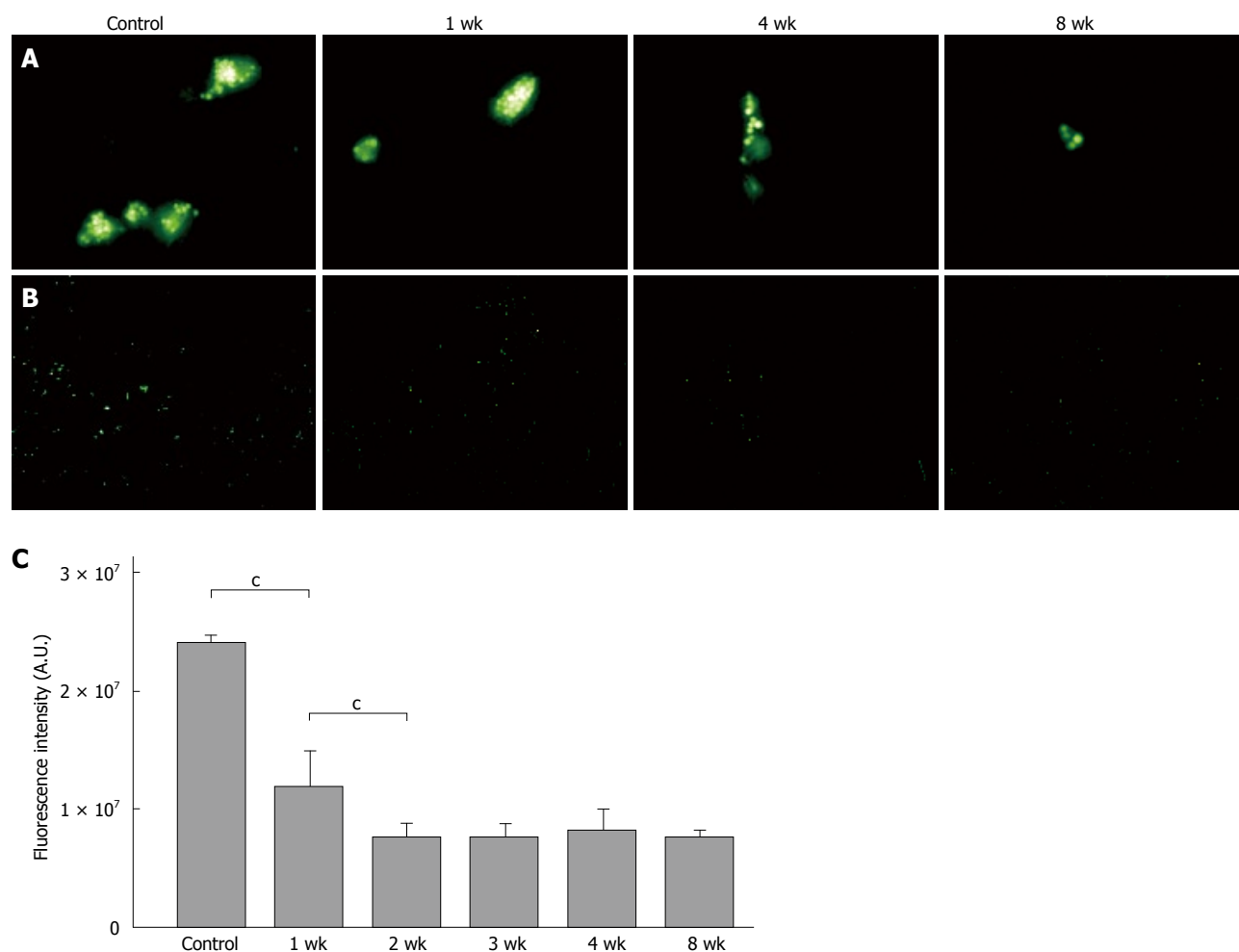


Figure 4 **A:** Fluorescence micrographs (x 1000). In the control group, there was no uptake of multiple latex beads (phagocytic activity) by the triangular shaped Kupffer cells, whereas the latex bead uptake was reduced in the NASH groups; **B:** Phagocytosis of FITC-latex beads by Kupffer cells *in vivo* (x 100). Decreased latex bead uptake was seen in the NASH groups compared with the control group; **C:** When compared with the control group, fluorescence was reduced to approximately 50% in the 1-wk group, and to 30% in the 2-wk group, but there was no further decrease after 3 wk ($n = 5$). $^cP < 0.001$.

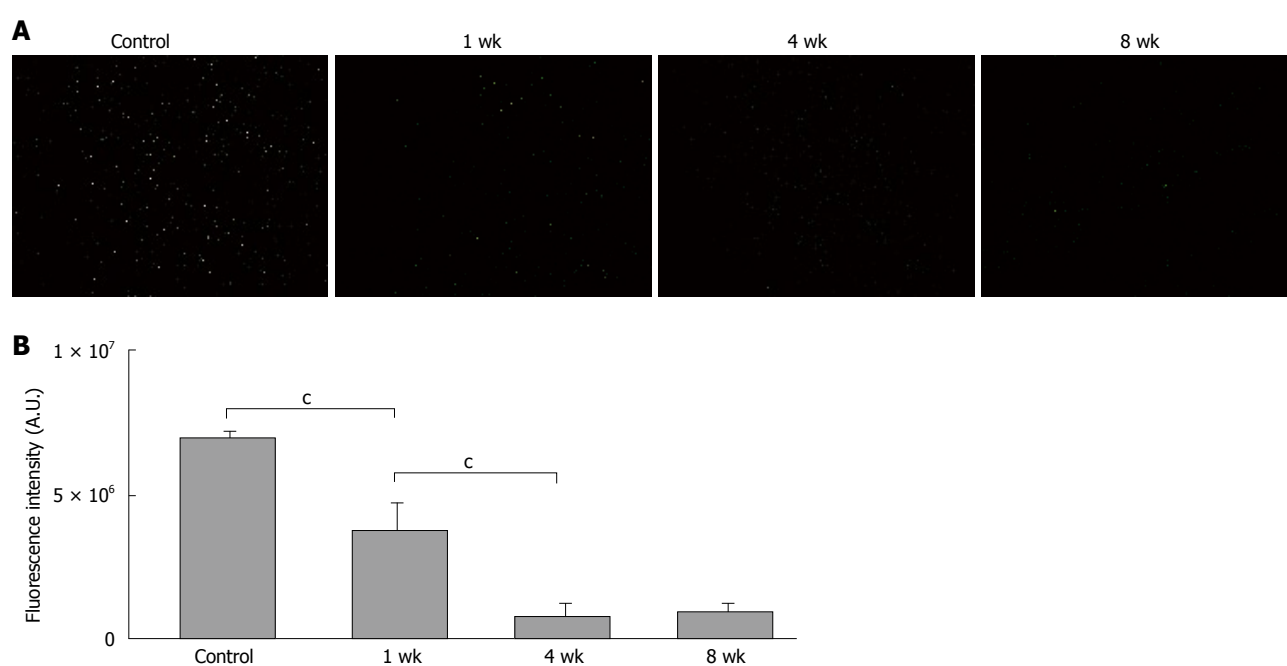


Figure 5 **A:** Phagocytosis of FITC-latex beads by the Kupffer cells *in vitro* (x 100). There was reduced latex bead uptake in the NASH groups compared with the control group; **B:** When compared with the control group, fluorescence was reduced to approximately 60% in the 1-wk group, and to 30% in the 2-wk group, but no further decrease was observed after 4 wk ($n = 5$). $^cP < 0.001$.

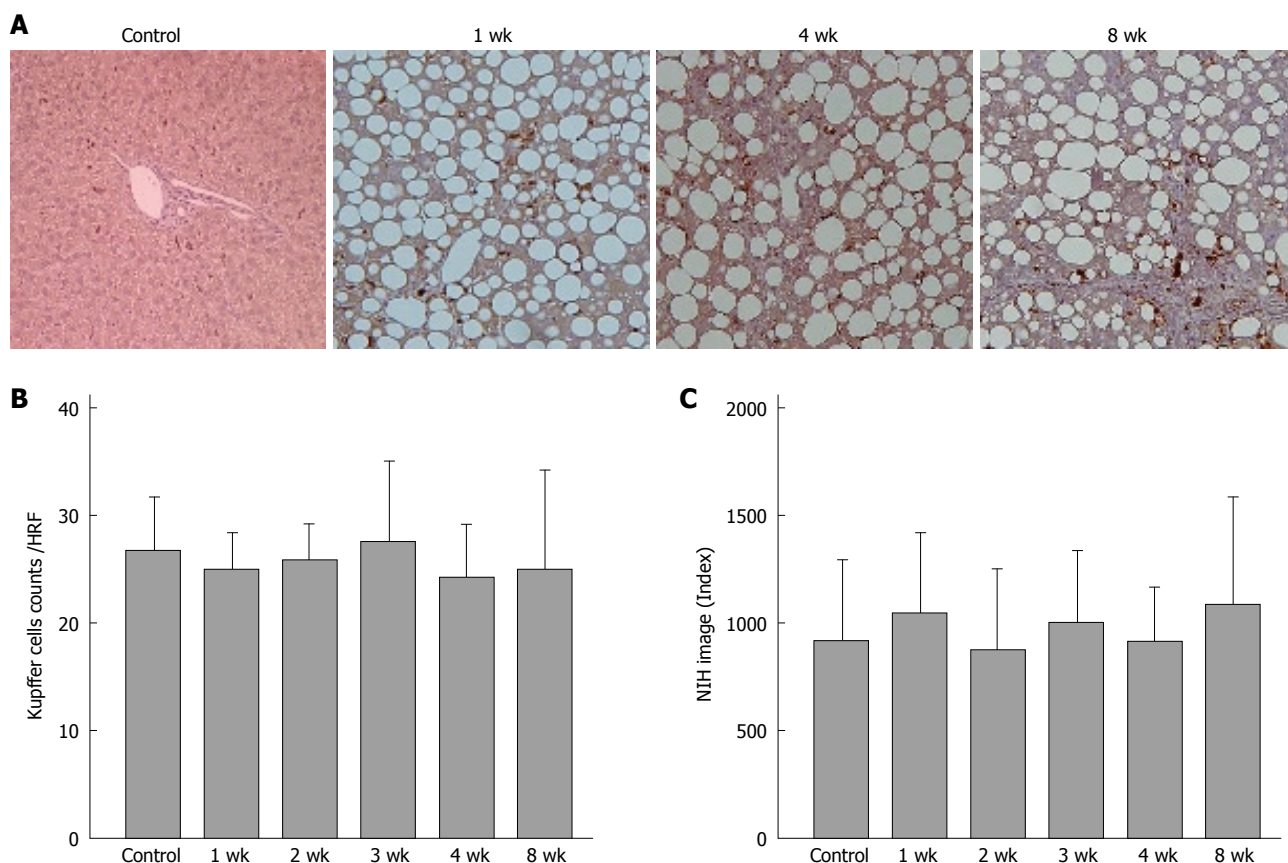


Figure 6 A: Kupffer cell immunohistochemical staining (x 200). Brown-stained cells are positive; B: There were no significant differences between the different groups in the number of stained cell per field; C: No significant differences were found between the groups in the quantitative analyses ($n = 5$).

confirmed by the latex bead uptake test. In the present *in vitro* study, we demonstrated that isolated Kupffer cells from CDAA-fed rats had reduced phagocytic activity. The marked decrease in the phagocytic activity in the presence of normal Kupffer cell counts suggests the presence of Kupffer cell functional abnormalities in our NASH model. We propose that persistent inflammation leads to reduced phagocytic activity of the Kupffer cells. Although the cause of the marked decrease in the phagocytic activity is unclear^[27], it may be attributable to intestinal bacterial endotoxins, which are believed to play a key role in the choline deficiency model of liver injury^[28]. Further studies are necessary to exclude the possibility of hypoperfusion of the sinusoids, and to confirm Kupffer cell abnormality in steatohepatitis.

In the present study, we used the CDAA model, because the changes induced in the liver are reproducible and similar to those observed in NASH. The development of NASH in patients receiving total parenteral nutrition has been attributed to choline deficiency^[29]. Furthermore, alcoholic liver disease may be associated with hepatic choline deficiency and hepatic steatosis, abnormalities that are also observed in rats fed a CDAA diet^[27]. Although, our steatohepatitis model lacks obesity and insulin resistance, two major characteristics of human NASH, a possible association between Kupffer cells, with inflammation and fibrosis may resemble the findings in humans. Further studies on cytokine production by the Kupffer cells in the present

NASH model may reveal Kupffer cell dysfunction and add new insight to the hepatic consequences of human NASH.

In conclusion, ultrasound examination by Levovist[®] confirmed the presence of a reduced contrast effect in the liver parenchymal phase in the rat NASH model. We believe that CEUS examination using Levovist[®] is a useful screening modality which can detect NASH in patients with fatty liver.

COMMENTS

Background

The diagnosis of nonalcoholic steatohepatitis (NASH) is important in clinical practice since this condition can progress to hepatic cirrhosis and hepatocellular carcinoma (HCC). At present, histopathological examination of liver biopsy tissue is the only way to definitively diagnose NASH. When NASH patients undergo contrast enhanced ultrasonography (CEUS) using Levovist[®], reduced contrast effect is seen in the liver parenchymal phase.

Research frontiers

Levovist[®] CEUS provides useful information on both the blood flow and the liver parenchyma, based on the characteristics of the contrast agent. Recently, Levovist[®] CEUS is being increasingly used as a screening modality for NASH. The reduced contrast effect in the liver parenchyma has been attributed to sinusoidal or Kupffer cell dysfunction, but this finding remains to be established. Therefore, the present workers performed Levovist[®] CEUS in a rat NASH model, induced by a choline-deficient L-amino acid-defined (CDAA) diet, and examined the Kupffer cell dynamics and phagocytic activity. The results obtained confirmed that the contrast effect is reduced in the liver parenchymal phase in the rat NASH model.

Innovations and breakthroughs

The present study demonstrated the contrast effect in the liver parenchymal

phase of CEUS using Levovist®. In addition, Kupffer cell dynamics and phagocytic activity were assessed in the rat NASH model. The results confirmed the effectiveness of CEUS in diagnosing NASH.

Applications

The authors believe that the CEUS with Levovist® is a useful screening modality, which can detect NASH in patients with fatty liver.

Terminology

Levovist (Schering AG, Berlin, Germany) is a galactose-palmitic acid ultrasound contrast agent, currently in use in European and Asian countries.

Peer review

The manuscript includes well designed figures, and the results clearly show a reduction in KC function, which was not attributed to changes in KC number, during the course of CDAA feeding. The hypothesis is simple and clear.

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RAPID COMMUNICATION

Torque teno virus: Its prevalence and isotypes in North India

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moderately present in Indian patients, with G1 to be the major genotype in North India. The pathogenicity and etiological role of TTV in different diseases is still a question mark and warrant further studies.

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Key words: Torque teno virus; Genotype; Restriction fragment length polymorphism; Torque teno; Hepato-cellular carcinoma

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Abstract

AIM: To investigate the prevalence and genotype distribution of Torque teno virus (TTV) in patients with different liver diseases and chronic renal failure treated at a referral hospital in North India.

METHODS: Whereas prevalence of TTV was based on amplification of conserved region of ORF2 of TTV genome, the genotyping of TTV was carried out using restriction fragment length polymorphism (RFLP) procedure on the N22 region of ORF1.

RESULTS: TTV-DNA was detected in 137 of 513 (26.7%) patients with liver diseases and 38 of 65 (58.5%) patients with chronic renal failure. TTV was also detected in 27% of healthy controls. The sequence analysis of the PCR product from 10 randomly selected cases failed to show a significant sequence divergence when compared with that of the TRM1 isolate of TTV genotype 1. The results of genotyping in 55 randomly selected patients showed the presence of genotype 1 (G1) in 53 (96.4%) and genotype 2 (G2) in 2 cases (3.6%), respectively. Other genotypes were not identified in this patient subgroup, suggesting that G1 is predominant in this area. The results of genotyping by RFLP were also supported by phylogenetic tree analysis, where G1 was found to be the major genotype.

CONCLUSION: These results indicate that TTV is

INTRODUCTION

Torque teno virus (TTV), formerly known as transfusion-transmitted virus, is a small, non-enveloped, icosahedral, single-stranded, circular-DNA virus^[1] approximately 30 nm in diameter. TTV contains a 3.6-3.9 kb DNA genome of minus polarity^[2-4] and belongs to a novel virus family, *Circoviridae*^[3]. At least 40 TTV genotypes from 5 major phylogenetic groups have been identified^[5]. They display over 30% nucleotide diversity^[6]. TTVs are ubiquitous in nature and have been demonstrated in high proportion of serum samples from healthy individuals where they persist overtime^[3,7-9]. A tissue culture system that supports efficient replication of TTV is not available^[10], and this has delayed the study of both TTV genome replication and TTV gene expression. Antibodies reacting with two proteins encoded by the 2.8-kb mRNA have been detected in TTV-infected individuals^[11,12]. The first is the large protein encoded by TTV ORF1 (the ORF1 protein), which is predicted to be 736 amino acids in length and initiates from a methionine at nucleotide (nt) 581 (O1AUG). The second protein is encoded by the 2.8-kb mRNA in ORF2, initiating at a methionine in the position of nt 354 (O2AUG) and extending for 117 amino acids^[13].

TTV belongs to the group of *Anelloviruses* that are widely diverse. Indeed, based on their heterogeneity,

Anelloviruses are currently classified into two species, each subdivided into numerous genotypes. Thus, TTV, the first anellovirus species identified^[1], is currently subdivided into approximately 40 genotypes, which cluster in five clearly distinct phylogenetic groups designated from 1 to 5^[1,6,10,14]. TTV is transmitted parenterally through transfusion with blood or blood products, but the natural route of its transmission is still unknown^[15,16].

TTV is found in the plasma of > 80% of the human population worldwide. Co-infection of single individuals with multiple TTV isolates is frequent^[17]. The epidemiology and pathogenic potential of TTV is poorly understood. In several studies, however, the viral genome has been detected at comparable prevalence rates in the blood of healthy persons and patients and this led to the hypothesis that TTV might be essentially non-pathogenic in nature^[18].

TTV can be transmitted by parenteral route, although its role in causing post-transfusion hepatitis has not been established. The majority of individuals who become TTV-DNA-positive after blood transfusion usually have normal ALT and do not develop chronic hepatitis, although TTV viremia frequently persists for several years. Patients who develop chronic hepatitis are invariably coinfecting with HBV or HCV and chronic hepatitis is closely correlated with HBV or HCV infection. This raises the possibility that TTV is merely an innocent bystander rather than a primary hepatitis virus^[19].

Although TTV appears to be widespread in the general population of several geographical regions, its prevalence in many areas is still unknown. The reports on status of TTV available from India are very preliminary and therefore there is a need of extensive studies to understand the endemicity, epidemiology and etiological potential of TTV infection in various diseases. Also, very little is known about the genotyping of TTV strains circulating in this country. Thus, the present study was undertaken to elucidate the prevalence and detect genotypes distribution of TTV in patients with liver and renal diseases in North India.

MATERIALS AND METHODS

Patients and blood samples

Five hundred and seventy eight adult patients of both sexes were included. There were 126 patients with acute viral hepatitis (AVH, age range: 21-48 years), 111 patients with chronic viral hepatitis (CVH, age range: 19-48 years), 132 patients with liver cirrhosis (CIR, age range: 34-57 years), 51 patients with fulminant hepatic failure (FHF, age range: 28-46 years), 93 patients with hepatocellular carcinoma (HCC, age range: 24-71 years) and 65 patients with chronic renal failure (CRF, age range: 20-74 years)^[19]. All these patients attended either the Outpatient Department or were admitted to the Liver and Renal Units of All India Institute of Medical Sciences, New Delhi, from June 2001 to March 2008. They were evaluated clinically and biochemically and their sera were tested for various markers and

parameters. The diagnosis of different types of diseases was based on accepted clinical, biochemical and histological criteria as outlined elsewhere^[20].

AVH was diagnosed when patients exhibited overt jaundice and/or increased alanine aminotransferase levels (at least 3 times above the normal value) documented at least twice at a 1-wk interval without any history of pre-existing liver disease. None of the patients had a past history of alcohol intake or were using any drug or had clinical or serological evidence of autoimmune diseases or biliary infection. The patients with CVH and liver cirrhosis were diagnosed based on histopathological criteria established by the International Study Group on Chronic Hepatitis^[21]. All of them had persistent elevation of transaminases (at least twice the upper limit of the normal range) for more than six months and histologic evidence of chronic hepatitis on liver biopsy at the beginning of follow-up. FHF was diagnosed if the patients developed hepatic encephalopathy within 4 wk from the onset of acute hepatitis, as outlined elsewhere^[20]. The diagnosis of HCC was based on histological criteria. CRF was diagnosed using criteria as detailed elsewhere^[22]. One hundred age- and sex-matched healthy subjects were used as controls.

From each of the above patients, 6-10 mL of venous blood was drawn and aliquoted in plain tubes without anticoagulant. Serum was separated after centrifugation and then stored at -70°C until further analysis. Repeated freezing and thawing of serum was avoided as far as possible. These serum samples were used to analyze liver function tests and routine hematogram.

Detection of TTV-DNA by PCR

Viral DNA was extracted from 200 µL of sera stored at -20°C using QiAmp Mini Elute viral spin kit (Qiagen, Germany) and following the manufacturer's instructions. The DNA was eluted in 50 µL of elution buffer supplied with the kit. TTV-DNA (conserved region of ORF2) was detected by nested PCR using primers NS1 (sense) 5'-GGGTGCCGAAGGTGAGTTTAC-3' (175-195), NS2 (anti-sense) 5'-GCGGGGCACGAA-GCACAGAAG-3' (474-494), NS3 (sense) 5'-AGTTTACACACCGAAGTCAAG-3' (189-209) and NS4 (anti-sense) 5'-AGCACAGAAGCAAGATGATTA-3' (463-483) as described by Biagini *et al*^[23], 1999 (Accession No. AB008394). Briefly, 10 µL of DNA was used for the amplification in a 50 µL reaction mixture containing 10 × PCR buffer, 25 pmol/µL of each primer, 10 mmol/L of each dNTPs, and 1.5 U Taq polymerase (Qiagen). Each of the 35 cycles of the 1st round and 25 cycles for the 2nd round of amplification consisted in an initial denaturation step at 95°C for 5 min and a cycling denaturation at 94°C for 30 s, annealing at 60°C for 45 s, extension at 74°C for 45 s, with a final extension for 3 min at 74°C.

Genotyping of TTV was done by amplifying the N22 region using specific primers as described by Okamoto *et al*^[4], 1999. Briefly, a first round of amplification was performed with sense primer NG059 5'-ACAGAC-

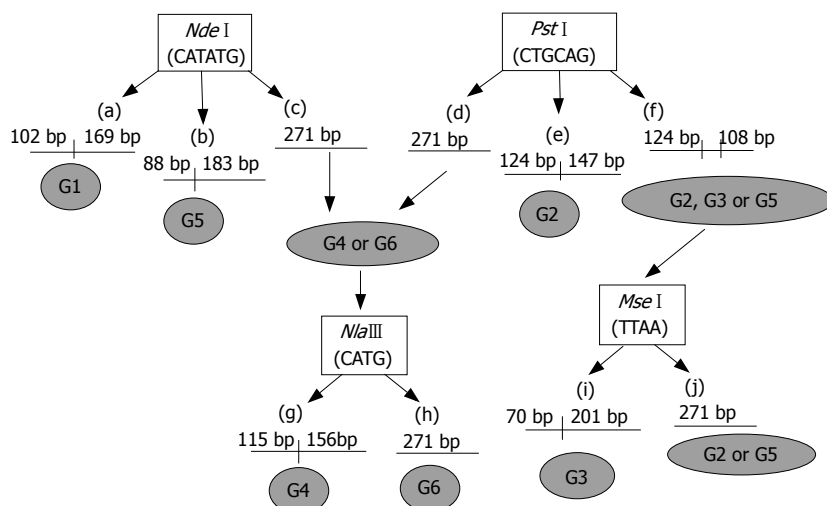


Figure 1 Strategy for RFLP analysis (Tanaka *et al*^[24], 1998).

AGAGGAGAAGGCAACATG-3' and anti-sense primer NG063 5'-CTGGCATTTCATTTCCAAAGTT-3' for 10 min at 95°C (initial denaturation) followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 74°C for 1 min with a final extension at 74°C for 5 min. The second round of PCR was performed using sense primer NG061 5'-GGCAACATGYTRTGGATAGACTGG-3' and anti-sense primer NG063 5'-CTGGCATTTCATTTCCAAAGTT-3' following same conditions as used for first round amplification. Three microliters of the PCR product were electrophoresed on 2% agarose gel and stained by ethidium bromide to observe as 295 bp product of ORF2 region and 271 bp product of N22 region.

Sequence analysis

PCR products of ORF2 were recovered from 2% agarose gels after staining with ethidium bromide and visualized under UV and were purified with QIAquick Gel Extraction Kit, Qiagen, Germany. They were sequenced in both directions using an automated DNA sequencer at M/s Lab India. The same set of primers was used for both sequencing and amplification by PCR.

Restriction fragment length polymorphism (RFLP)

Restriction digestion was carried out overnight using 10 µL of the second round PCR product of the N22 region and 10 × enzyme buffer according to the manufacturer's instructions. Reactions were carried out with 20 U each of *Nde* I, *Pst* I (New England Biolabs, MA, USA), *Nla* III (MBI Fermentas, Canada) at 37°C. Similarly, 20 U of *Mse* I (MBI Fermentas, Canada) were used for digestion at 65°C. The digested PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The RFLP pattern was then evaluated under UV light^[24]. The procedure using restriction digestion enzymes for genotyping by RFLP is shown schematically in Figure 1. Briefly, restriction digestion pattern with *Nde* I producing two fragments

of 102 bp and 169 bp shows the presence of genotype 1 (G1). Production of fragments of 88 bp and 183 bp with *Nde* I shows the presence of genotype 5 (G5). Similarly, a digestion pattern with *Pst* I producing two fragments of 124 bp and 147 bp size shows the presence of genotype 2 (G2). Restriction digestion with *Nla* III produces two fragments of 115 bp and 156 bp if genotype 4 (G4) is present. Digestion pattern of *Mse* I, producing two fragments of 70 bp and 201 bp length, shows the presence of genotype 3 (G3). Genotype 6 (G6) does not contain site of any of the above restriction enzymes.

Phylogenetic analysis

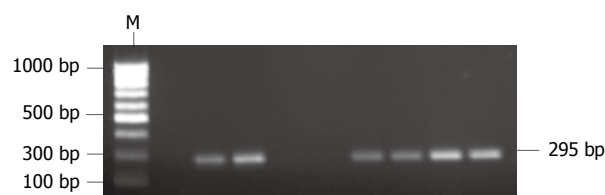
DNA sequences derived from TTV ORF2 positive samples were compared to an online database for the best possible match using the BLAST (Basic Local Alignment Search Tool) program of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and CLUSTALX program^[25]. Phylogenetic tree was constructed using the neighbor-joining method in CLUSTALX program and PHYLIP version 3.5^[26]. The data set was bootstrap re-sampled 1000 times to ascertain support for major branches of the tree.

RESULTS

Presence of TTV in sera samples from healthy persons and patients with different liver and renal diseases was detected using PCR amplifying the conserved region of ORF2 (107 to 715 nt) (Accession No. AB008394)^[23]. TTV-DNA was detected in 175 of 578 patients, giving an overall prevalence of 30.3% (Table 1). Similarly, it was detected in 27 of 100 healthy persons (prevalence of 27%). Typical TTV amplicons of 295 bp are shown in Figure 2. The break-up of TTV prevalence in different disease groups shown its presence in 29 of the 126 (23.0%) patients with AVH, 26 of 111 (23.4%) with CVH, 46 of 132 (34.8%) with CIR, 15 of 51 (29.4%) with FHF, 21 of 93 (22.6%) with HCC and 38 of 65

Table 1 Prevalence of TTV-DNA in different liver & renal disease groups

Disease group	Number of samples	TTV-DNA positivity <i>n</i> (%)
Acute viral hepatitis (AVH)	126	29 (23.0)
Chronic viral hepatitis (CVH)	111	26 (23.4)
Cirrhosis (CIR)	132	46 (34.8)
Fulminant hepatic failure (FHF)	51	15 (29.4)
Hepatocellular carcinoma (HCC)	93	21 (22.6)
Chronic renal failure (CRF)	65	38 (58.5)
Healthy controls	100	27 (27.0)

**Figure 2** TTV detection by PCR (ORF2: nt 107-712). Amplification of ORF-2 region of TTV-DNA using NS1, NS2, NS3 and NS4 primers. (Biagini *et al.*^[23], 1999. Accession No.: AB008394)**Table 2** Relation between TTV infection & ALT level in different liver disease groups *n* (%)

Diseases group	TTV-DNA + samples	ALT level (IU/L)			
		50-200	201-400	401-600	> 600
AVH	29	17 (59)	8 (28)	0	4 (14)
CVH	26	18 (69)	6 (23)	0	2 (8)
CIR	46	35 (76)	19 (41)	0	2 (4)
FHF	15	2 (13)	6 (40)	1 (7)	6 (40)
HCC	21	8 (38)	5 (24)	3 (14)	5 (24)

Percent value was computed in comparison to total number positive for TTV-DNA.

Table 3 TTV genotype analysis by RFLP *n* (%)

Restriction enzymes used	Number of cases treated	Digestion noticed	Possible TTV genotypes	Genotypes prevalence
<i>Nde</i> I	55	53	G1	53 (96.4)
<i>Pst</i> I	55	2	G2	2 (3.6)
<i>Mse</i> I	55	0	-	0
<i>Nla</i> III	55	0	-	0

Each sample was treated with four restriction enzymes (*Nde* I, *Pst* I, *Mse* I, *Nla* III) to determine genotyping of TTV following criteria mentioned in material and methods
 The DNA remaining undigested with REs indicated absence of corresponding genotypes
 There was no effect of *Mse* I and *Nla* III on TTV-DNA on digestion with these enzymes

(58.5%) with CRF.

The relation of TTV-DNA with ALT level in sera in these disease groups is shown in Table 2. The majority of cases with liver diseases had low levels of ALT, suggesting a very benign role of TTV in causing liver cell necrosis. The raised level of ALT up to the level of more than 600 IU was detected only in FHF cases, where TTV infection may not be the major cause.

The PCR products from 10 sera were processed for DNA sequencing. The sequence of TTV-DNA from each case was compared to the corresponding region of ORF2 from the TRM1 isolate of genotype 1 (Accession No. AB026345), gathered from the published literature. The full length sequence of amplicons compared to the published sequence of this same region is shown in Figure 3. The results of sequencing indicate the substitution of C by T or Y at nt position 632, A by C at 640 position, substitution of A by G at position 643, T by C at position 696 and at 770 C is being substituted by T or Y. Significant sequence divergence involving more than 2 bp was not observed. Sequencing of additional cases is underway.

Characterization of the TTV genotypes prevalent in the current study population was conducted in 55 cases

by RFLP analysis of N22 nucleotide sequence belonging to ORF1. Digestion of the N22 amplicon with *Nde* I produced fragments of 169 and 102 bp, corresponding to genotype 1, in 53 of 55 cases. Digestion with *Pst* I produced fragments of 124 and 147 bp, corresponding to genotype 2, in 2 of 55 cases. The digestion with other restriction enzymes, *Nla* III and *Mse* I, could not produce any fragments, thus indicating the absence of other genotypes. The results of genotype distribution are shown in Table 3 and Figure 4.

Genotypes of TTV in these cases were also confirmed by phylogenetic analysis of DNA sequences obtained from above. The results are shown in Figure 5. This analysis shows the maximum alignment of the sample sequence with TRM1 isolate of genotype 1 indicating a major presence of TRM1 isolate genotypes 1 and supporting the results of RFLP analysis. From these results, it is clear that genotype 1 is predominantly prevalent in all these cases with TTV infection.

DISCUSSION

TTV was assumed to be one of the possible agents

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TRM1 CCGAGGGCGGGTGCCGAAGGTGAGTTTACACACCGAAGTCAAGGGGCAATTCGGGCT---
5f -----GGGCA-TTCGGGCT---
4f -----GGCA-TTCGGGCT---
2f -----GAGTCCTAGGCAATTCGGGCT---
9f -----AATTCGGGCT---
7f -----GCAATTCGGGCT---
6f -----CATTCGGGCT---
3f -----CATTCGGGCT---
10f -----AATTCGGGCT---
8f -----
1f -----TCGCTTTTACGTGATGTTAG

TRM1 CGGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
5f CGGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGG-AGGCATTA
4f CGGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
2f CGGGACTGG-CCGGGCTATGGGCTAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
9f CGGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
7f CGGGACAGGGCCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
6f CGGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
3f CGGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
10f CGGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
8f -GGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATTGGCAGGCATTA
1f CCGGACTGG-CCGGGCTATGGGCAAGGCTCTGAAAAAGCATGTTTATCCCCAGGCACTA
    **** *

TRM1 CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
5f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
4f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
2f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
9f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
7f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
6f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
3f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
10f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
8f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
1f CAGAAAGAAAAGGGCGCTGTCACTGTGTGTTGTGCGACCAACAAAGAAGGCTTGCAAACT
    **** *

TRM1 ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
5f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACATTCTCTTAACCTGGCAATGGTA
4f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
2f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
9f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
7f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
6f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
3f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
10f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
8f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
1f ACTAATAGTAATGTGGACCCACCTCGCAATGATCAACAGTACCTTAACCTGGCAATGGTA
    **** *

TRM1 CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
5f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
4f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
2f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
9f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
7f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
6f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
3f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
10f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
8f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
1f CTCAAGTGTACTTAGCTCCCACGCTGCTATGTGCGGGTGTCGCGCTCATT
    **** *

TRM1 TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCAAAACCCACCCCTCCCGGTCCCCAGC
5f TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCA-----
4f TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCA-----
2f TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCA-----
9f TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCA-----
7f TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCA-----
6f TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCA-----
3f TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCA-----
10f TAATCATCTTGCTTCTGTGCTTCGT-GCCCCGCA-----

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Figure 3 Sequence analysis of amplicons and their comparison with TRM1 isolates of Genotype 1 (ORF2: nt 107-712).

causing non A-G hepatitis. Its characterization and significance in causing hepatitis became more interesting with the findings by Okamoto *et al*^[27], showing level of TTV-DNA in liver tissue to be 10-100 times higher than those in serum. A similar report by Nishizawa *et al*^[1], who reported the appearance of TTV-DNA in the sera of patients with post-transfusion hepatitis of unknown etiology and displayed a close correlation with ALT levels, triggered an additional interest in this virus for

its potential relationship with hepatitis. In India, viral hepatitis is a common disease in all parts of the country, with an established endemicity of all known hepatitis viruses. Previous studies from India have reported the existence of a group of viruses other than A-G hepatitis viruses, causing liver diseases^[28]. This remains an enigma to investigate and characterize the responsible agent for non A-G hepatitis in this country. TTV attracted our attention to study this virus for its prevalence and

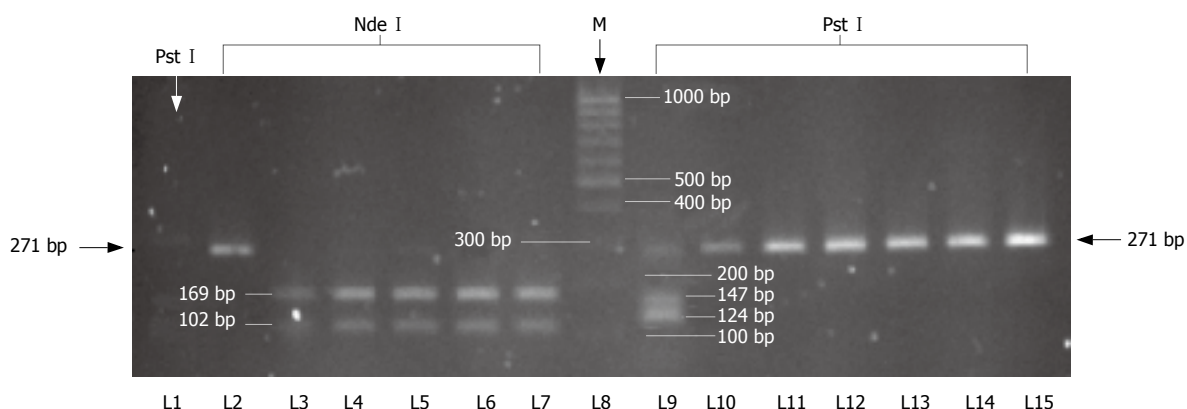


Figure 4 RFLP pattern of N22 region of TTV-DNA. L1-15: Lane 1 to lane 15. L1 is Pst I undigested product (271 bp), L2 is Nde I undigested product (271 bp), L3-7 is Nde I digested product resulting in 102 bp & 169 bp fragments, L8 is marker, L9 is Pst I digested product resulting in fragments of 124 bp & 147 bp size, L10-15 is Pst I undigested product (271 bp).

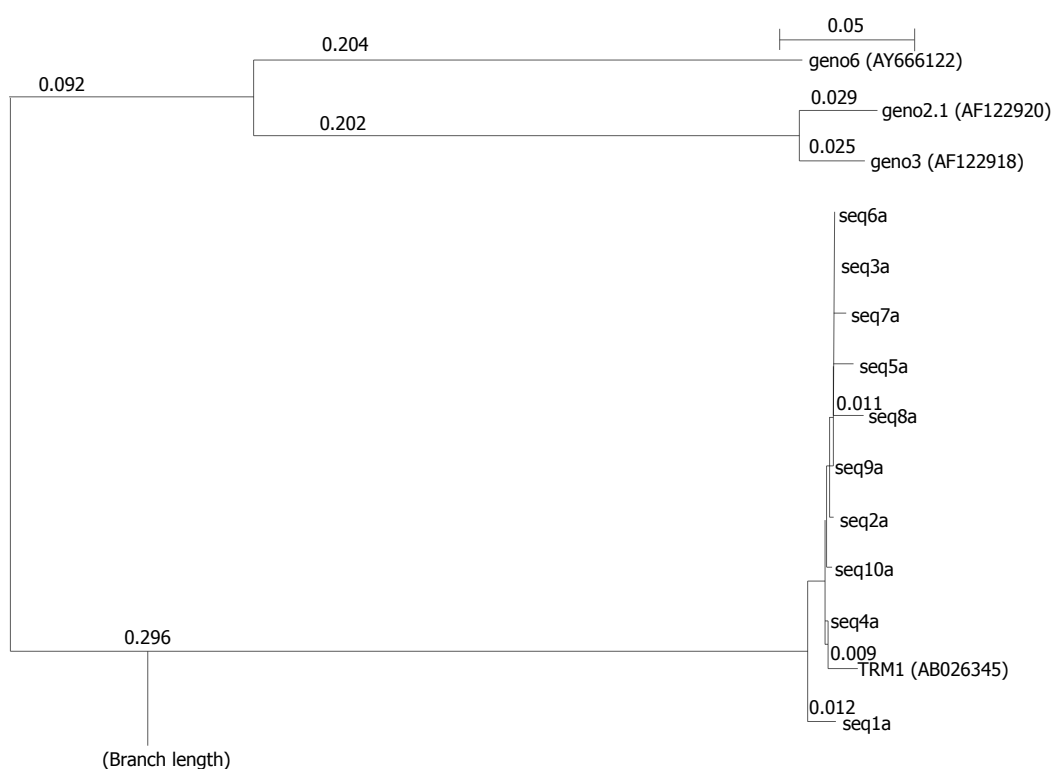


Figure 5 Phylogenetic tree analysis of amplicons with known TTV genotypes.

molecular form in Indian populations to understand its possible role in causing hepatitis as well as other blood transmitted diseases.

The prevalence of TTV infections in India has not been well documented. Only a few reports are available^[19,29] that demonstrate the presence of TTV in patients population randomly selected from various studies. In this study, we found the presence of TTV-DNA in 22 to 35% of patients with different liver diseases. In CRF, on the contrary, its prevalence was relatively higher, reaching the level of 58.5%. A low prevalence of TTV in liver diseases, comparable to that reported in healthy persons (27%) supports our earlier preliminary report demonstrating a little role of TTV in liver diseases^[19]. TTV infection has been found to be common in humans with prevalence which may exceed

90%. Its prevalence in healthy populations in India is lower than those previously reported for Turkish (51.6%), Japanese (92.0%) and Polish (78%) blood donors^[30].

Our findings indicate that TTV prevalence in all tested groups was comparable to that among healthy controls, with the exception of a higher value in chronic renal failure patients, thus indicating that TTV infection in both the healthy population and liver disease patients is not very frequent in the northern part of India. Moreover, presence of TTV in liver diseases does not necessarily indicate its role in the etiology of liver damage. This is supported by the low level of ALT detected in all liver disease groups carrying TTV infection. Several other studies have similarly suggested that TTV does not seem to cause disease, simply acting as a by-stander virus. High prevalence in CRF patients

may be attributed to repeated blood transfusion or procedural transmission in these patients, due to the fact that TTV is frequently transmitted *via* parenteral routes.

The results of the sequence analysis in these cases has shown a point mutation at a fixed position of TTV genome, corresponding to ORF2. A nucleotide sequence heterogeneity is not very frequent, but is premature to conclude from these data about the impact of the genomic variation on its pathogenicity or change in etiological potency. It was interesting to find here that the heterogeneity in nucleotide sequence was at the same position in all amplicons studied. This suggests the likelihood of a single isotype circulating in all of the patients studied.

In this study, the genotyping of TTV was based on a RFLP procedure involving a set of four restriction enzymes targeting the N22 nucleotide region. The results indicate that most isolates assessed in our study belong to genotype 1, with only a minor contribution of genotype 2. Since there are very few reports available from our country, it is not possible to support or contradict other observations and therefore this report should be taken as accepted at this stage. While comparing TTV genotypes circulating in India with those reported from other countries, there are reports available showing evidence for the existence of 4 major genotypes, G1, G2, G3 and G4 with most belonging to G1 and G2. There are three major genotypes, i.e. G1, G2, G3, that are prevalent worldwide. Different countries have reported different genotypes in their population. G2 was reported from Western Anatolia, where appears to be very common^[31]. Similarly, G3 & G4 genotypes were reported from Turkey, whereas G3 is detected primarily from Europe^[32]. Asian countries have reported predominantly G4 genotype^[24]. All these findings indicate diversity in the prevalence of TTV genotypes in different countries of the world. The reason(s) why G1 is predominant in India is difficult to explain and needs further investigations. The predominance of G1 was also supported by phylogenetic tree analysis of the amplicons studied in our patients' population.

In conclusion, our results indicate a low prevalence of TTV infections in North India. This also demonstrates that the prevailing genotype in North India is G1 with minor prevalence of G2 genotype. The presence of strains belonging to G1 reveals the limited genetic diversity in TTV genome circulating in this country.

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COMMENTS

Background

Our results indicate that Torque teno virus (TTV) infection is moderately present

in North India. Analysis of different genotypes of TTV demonstrate that G1 is the predominant genotype prevailing in North India. The presence of strains belonging mostly to G1 reveals that there is a limited genetic diversity in the TTV genome circulating in this part of country.

Research frontiers

There are only preliminary informations available on the status of TTV in India. There is a need to study and understand the endemicity, epidemiology and etiological potential of TTV infection in various diseases. The present study was planned to determine the prevalence of TTV in North India and which was the prevailing genotype(s).

Innovations breakthrough

The prevalence of TTV infection was observed in various categories of liver and renal diseases. In particular, the rate of TTV infection in liver diseases was comparable to that of a normal healthy populations, although it was significantly higher in chronic renal failure patients. In all these cases genotype 1 (G1) was the predominant genotype with a minor contribution of genotype 2 (G2).

Applications

The results of prevalence and genotyping of TTV infection in Indian patients' will be helpful in understanding the role of TTV in causing various diseases.

Peer review

Authors have studied TTV infection and found that TTV infection has minor role in causation of liver diseases. Its significant prevalence in renal diseases needs further investigations.

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RAPID COMMUNICATION

Overall expression of *beta-catenin* outperforms its nuclear accumulation in predicting outcomes of colorectal cancers

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Abstract

AIM: To examine the expression of *beta-catenin* in colorectal cancer and look for association with other clinico-pathological parameters.

METHODS: Tumor samples from 163 cases of colorectal cancer (CRC) who had undergone primary colectomy between May, 1998 and November, 2002 with complete follow-up data for either 5 years or until death were recruited for a *beta-catenin* immunohistochemical study. The percentage of immunoreacted tumor cells was defined as overall staining density (OSD) and percentage of cells having nuclear localization was counted as nuclear staining density (NSD). Univariate exploration used log-rank test and multivariate survival analysis used Cox's hazard regression model.

RESULTS: *Beta-catenin* immunoreactivity was detected in 161 samples (98.8%), of which 131 cases had nuclear staining. High OSD ($\geq 75\%$), detected in 123 cases (75.5%), was significantly associated with earlier clinical staging ($P < 0.01$), lower nodal status ($P = 0.02$), non-metastatic status ($P < 0.01$) and

better differentiation ($P = 0.02$). Multivariate analysis found that high OSD was independently associated with better survival [Cox's hazard ratio 0.51, 95% confidence interval (CI) 0.31-0.83]. Although high NSD ($\geq 75\%$) was correlated with high pre-operative serum CEA ($P = 0.03$), well differentiation ($P < 0.01$), and increased staining intensity ($P < 0.01$), the parameter was not significantly associated with survival.

CONCLUSION: Unlike previous reports, the study did not find a predictive value of nuclear *beta-catenin* in CRC. Instead, the overall expression of *beta-catenin* in CRC showed an association with better differentiation and earlier staging. Moreover, the parameter also independently predicted superior survival.

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Key words: Colorectal carcinoma; *beta-catenin*; *CTNNB1*; Wnt-signaling pathway; Prognosis

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INTRODUCTION

According to the recent World Cancer Report^[1], colorectal cancer (CRC) ranks as the third most common malignancy, following breast and lung cancers. The incidence of CRC in developing countries, including Thailand, has been increasing in recent years^[2]. The increasing adoption of Western life style habits in these countries is believed to be responsible for the growing magnitude of the CRC problem. Although surgical therapy is the mainstay treatment for early stage CRC, there is an expanding role for multidisciplinary treatment. As adjuvant treatments are considered based on an individual patient risk, prognosticating factors

are essential for risk stratification. Well accepted factors influencing outcome in CRC patients are tumor invasion, nodal status, lymphovascular invasion and serum carcinoembryonic antigen level^[3,4]. Various histological parameters and molecular markers have been investigated and some have shown promising results in CRC, such as microvascular density^[5] and microsatellite instability^[6]. However, none of those biological factors have yet been integrated into the treatment algorithm.

Beta-catenin, a central molecule of the Wnt-signaling system, expresses in epithelial cells as two main forms; membrane localization and nuclear accumulation. Membrane localization can be detected in normal cells and tumor lineage, whereas nuclear beta-catenin is exclusively detected in immature cells and tumor cells^[7]. Evidence of somatic mutations and nuclear accumulation of beta-catenin in various pediatric cancers signifies a role of the Wnt-signaling pathway in their tumorigenesis^[8,9]. Accumulation of beta-catenin is a result of defects in its degradation process, which usually takes place in the cytoplasm by an interaction between *beta-catenin* and a complex of *APC*, *AXIN* and *GSK-3-beta*. Stabilized beta-catenin translocates into the nucleus where the protein acts as a transactivation factor and promotes tumor growth. In CRC, the molecular pathology involving members of the pathway has been elucidated^[10]. Inactivation of APC, the tumor suppressor gene which regulates intracellular level of beta-catenin, is one of the earliest events observed in CRC development. Loss of APC function leads to pathologically increased cytoplasmic beta-catenin, which can be translocated into the nucleus or to the cell membranes^[11]. At cell membranes, beta-catenin forms a complex with E-cadherin which plays a role as an adhesion molecule. Furthermore, Wnt-signaling may contribute to the process of colonic epithelial cells differentiation^[12].

Recent studies have focused on the clinical meaning of nuclear beta-catenin accumulation in CRC and demonstrated its diagnostic as well as prognostic significance^[13-19]. A high density of beta-catenin nuclear accumulation was associated with higher mortality in selected groups of CRC patients^[14,18,19]. However, data from different series have been inconsistent. We conducted a study of beta-catenin immunohistochemistry in our clinical CRC series. With awareness that beta-catenin does not have only a growth promoting role, we did not limit our analysis to nuclear accumulation only, but also examined overall staining density against outcomes. The study failed to find evidence that nuclear beta-catenin accumulation could be a risk factor for unfavorable outcome in CRC. However, the study found a strong correlation between overall beta-catenin expression and better survival probability.

MATERIALS AND METHODS

CRC patients

Archival tumor samples from 163 non-consecutive

patients with histologically proven colonic adenocarcinoma who underwent primary resection at Songklanagarind Hospital, a tertiary teaching hospital in southern Thailand, between May, 1998 and November, 2002, were examined for this immunohistochemical study. CRC patients who were treated elsewhere, received a non-curative excision so that accurate pathological staging could not be determined, or were lost to follow-up after surgical treatment were not included in this study. All patients were evaluated for at least 5 years after surgery or until death. Survival status was evaluated by the institutional Tumor Registry Unit on December, 2007.

Before the surgical treatment, all patients had routine pre-operative investigations, including chest-X-rays, blood chemistry including serum albumin and carcinoembryonic antigen (CEA), and an evaluation for liver metastasis by ultrasonography and/or computerized tomography. Primary tumor staging followed the sixth edition of TNM staging system of the American Joint Committee on Cancer (AJCC)^[20]. Adjuvant chemotherapy was given to stage III, colonic cancer patients, and adjuvant chemo-radiation was reserved for stages II and III rectal cancer cases. In stage IV CRC patients, chemoradiation therapy for advanced disease was considered for patients who were in status 0-2, according to the Eastern Cooperative Oncology Group. Follow-up visits were scheduled at 1-mo intervals during the first year after surgery, every 3-mo period during the second year, and every 6 mo, thereafter. Access to pathological samples and clinical records was approved by the institutional research ethics committee.

For analysis, age of the patients was stratified to less than 65 years and 65 years or more. Pre-operative serum CEA level was stratified at 5 ng/mL.

Beta-catenin immunohistochemistry

Hematoxylin and eosin stained slides were re-examined by a gastrointestinal pathologist and selected for the beta-catenin immunohistochemical study. From formalin-fixed paraffin embedded tissue, sections of 3 micrometer thickness were cut, deparaffinized, and rehydrated. Beta-catenin monoclonal antibody (1:500, Abcam Plc., UK) was used as the primary antibody. The staining protocol followed the manufacturers' instructions of Dako EnVision + System (Dako). Briefly, antigen retrieval was performed in a microwave oven using Tris-EDTA buffer. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide containing sodium azide. Slides were incubated with non-immune serum for 30 minutes and were then incubated with the primary antibody for 120 minutes in a moist chamber, followed by a 30 minute incubation with peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins. Color was then developed by the liquid 3,3'-diaminobenzidine chromogen solution. Light counterstaining was done with hematoxylin. All immunohistological staining in this study was performed by one technician.

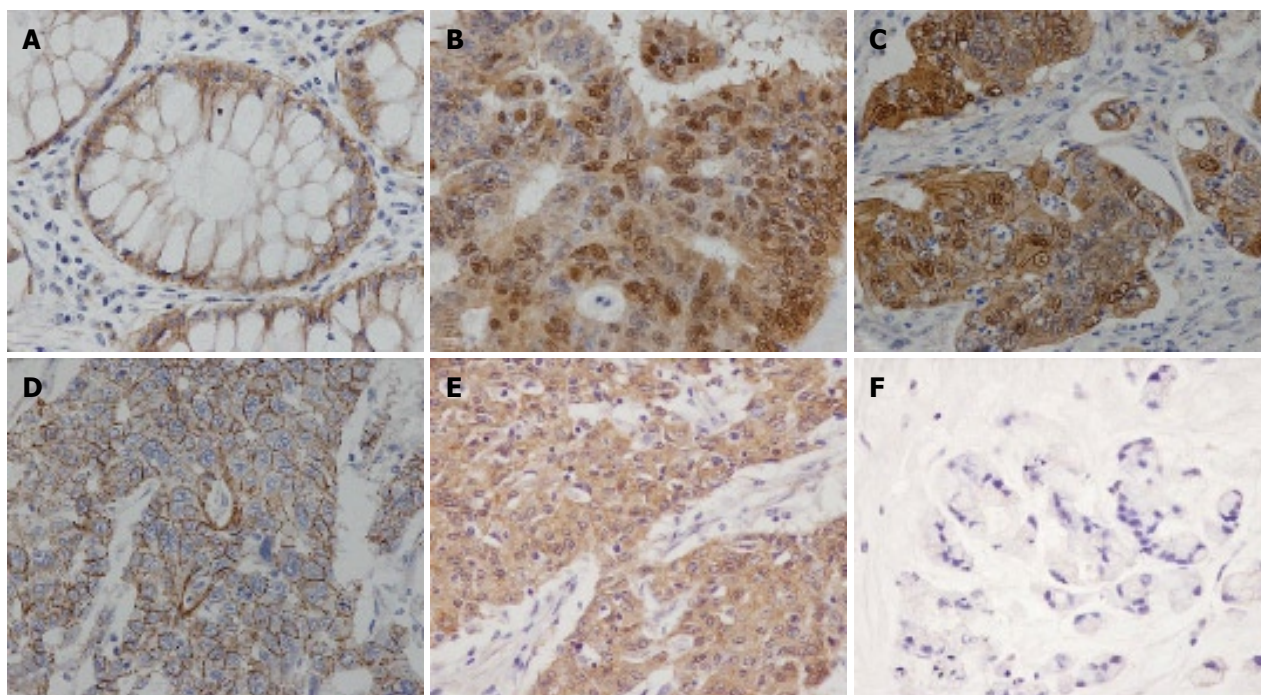


Figure 1 Immunohistochemistry study of beta-catenin in CRC. **A:** Membranous staining pattern in normal colonic mucosa; **B:** Nuclear staining pattern and high OSD in a case with well differentiation histology; **C:** Nuclear and membranous staining pattern; **D:** Membranous staining pattern; **E:** Cytoplasmic staining pattern; **F:** Very weak staining intensity in a case of poorly differentiated CRC. (40 × magnification).

Staining intensity of beta-catenin was graded as weak, moderate, strong and very strong intensity. Intracellular localization patterns of beta-catenin were categorized according to the dominant pattern as nuclear staining, nuclear together with membrane staining, cytoplasm staining and membrane staining.

Beta-catenin nuclear staining density (NSD) was defined as the number of tumor cells with nuclear staining per 100 cells examined. Overall staining density (OSD) meant number of beta-catenin immunoreacted cell per 100 tumor cells examined. Tumors were defined as having high NSD when NSD was 75% or more, and OSD at 75% or more indicated high OSD. The pathologists responsible for histological slide examination were blinded to the clinical outcomes before data analysis.

Statistical analysis

Death from cancer was assigned as failure in the overall survival analysis. Local recurrence and distant metastasis were counted as failure in disease-free survival, which were analyzed only for cases in AJCC stage I-III. Univariate analysis used Chi-square test to evaluate any association between parameters and Log rank test for survival analysis. Multivariate survival analysis used a multiple Cox's regression model. Parameters with *P*-value less than 0.3 from the Log rank test were included for analysis. Those with a *P*-value ≥ 0.05 in Cox's regression were excluded until every parameter in the model was independently associated with survival. A *P*-value of less than 0.05 was considered statistically significant.

Calculations used the R-program (Foundation for Statistical Computing, Vienna, Austria).

RESULTS

The clinical characteristics and pathological descriptions of the 163 patients are shown in Table 1. The mean age of the patients was 61.6 years (range 20-88 years). Median pre-operative serum CEA was 4 ng/mL (range 0.5-1215 ng/mL). Eleven of the primary tumors (6.7%) were at the cecum, 13 (8.0%) at the ascending colon, 5 (3.0%) at the hepatic flexure colon, 11 (6.7%) at the transverse colon, 2 (1.2%) at the splenic flexure colon, 3 (1.8%) at the descending colon, 37 (22.7%) at the sigmoid colon and 81 (49.7%) at the rectum.

Staining characteristics of beta-catenin

Beta-catenin immunoreactivity was detected in 161 cases (98.8%). Among these, 131 cases (80.3% of all cases) were positive for nuclear beta-catenin staining while the remaining cases showed limited immunoreactivity within the cell membranes and/or cytoplasm. Among cases with positive nuclear beta-catenin, 40 (30.5%) were also positive for membrane immunoreactivity. Concerning staining intensity, 74 cases (45.4%) displayed very strong immunoreactivity, 35 (21.5%) showed strong intensity, 27 (16.6%) had moderate intensity, and 25 (15.5%) cases exhibited weak staining (Figure 1). Interestingly, nuclear beta-catenin was detected in a significantly higher proportion in cases with strong or very strong intensity (99.1%), compared to cases with moderate or weak immunoreactivity (44.23%). Cases with positive nuclear staining had a higher incidence of lymph node metastasis (50.3%) when compared to cases with only membranes or cytoplasm staining (33.3%), however, the difference was not statistically significant (Chi-square *P*-value = 0.09).

Table 1 Demographic data of the study population and association of studied parameters and overall staining density, *n* (%)

Parameter	Cases	Overall staining density		<i>P</i>
		Low	High	
Sex				
Male	80 (49.3)	18 (45.0)	62 (50.8)	0.52
Female	83 (50.6)	22 (55.0)	60 (49.2)	
Age (yr)				
< 65	87 (53.4)	21 (52.5)	66 (53.7)	0.90
≥ 65	76 (46.6)	19 (47.5)	57 (47.3)	
CEA				
< 5 ng/mL	83 (50.9)	25 (62.5)	58 (47.1)	0.09
≥ 5 ng/mL	80 (49.1)	15 (37.5)	65 (52.9)	
AJCC staging				
Stage 1	20 (12.3)	4 (10.0)	16 (13.0)	0.004
Stage 2	61 (37.4)	11 (27.5)	50 (40.7)	
Stage 3	63 (38.7)	14 (35.0)	49 (39.8)	
Stage 4	19 (11.7)	11 (27.5)	8 (6.5)	
Tumor size				
T 0-1	25 (15.3)	5 (12.5)	20 (16.3)	0.57
T 2-3	138 (84.7)	35 (87.5)	103 (83.7)	
Nodal status				
N0	86 (52.8)	17 (42.5)	69 (56.1)	0.02
N1	39 (23.9)	7 (17.5)	32 (26.0)	
N2	38 (23.3)	16 (40.0)	22 (17.8)	
Metastatic status				
M0	144 (88.3)	29 (72.5)	115 (93.5)	< 0.001
M1	19 (11.7)	11 (27.5)	8 (6.5)	
Differentiation				
Well	83 (50.9)	19 (47.5)	64 (52.0)	0.02
Moderate	60 (36.8)	11 (27.5)	49 (39.8)	
Poor	20 (12.3)	10 (25.0)	10 (8.1)	
Staining localization				
Nuclear	122 (50.3)	19 (47.5)	63 (51.2)	0.68
Non-nuclear	31 (49.6)	21 (52.5)	60 (48.8)	
Staining intensity				
Weak to moderate	54 (33.1)	18 (45.0)	34 (28.1)	0.05
Strong to very strong	109 (66.9)	22 (55.0)	87 (71.9)	
NSD				
Low (< 75%)	101 (47.2)	33 (82.5)	44 (35.8)	< 0.001
High (≥ 75%)	62 (52.8)	7 (17.5)	9 (64.2)	

Overall staining density ranged from 5% to 100% with an average value of 86%. One hundred and twenty-three cases (75.5%) were rated as high OSD. On univariate analysis, high OSD was significantly associated with earlier AJCC staging, lower nodal status, non-metastatic status and better differentiation status (Table 1).

Among the 131 cases in which the nuclear staining appeared positive, nuclear accumulation density ranged from 1% to 99 % with an average value of 65.6%. Eighty-six cases (52.8%) had nuclear accumulation density at 75% or more and were counted as high NSD. High NSD had significantly positive correlation with pre-operative serum CEA ($P = 0.03$), well differentiation ($P < 0.001$), and staining intensity ($P < 0.001$). In addition, cases with NSD at 25% or more had significantly higher incidence of nodal metastasis at the operation ($P = 0.01$).

Survival analysis

There were no missing data in this study. The mean

Table 2 Time-points of 5-yr overall survival and disease free survival according to each parameter and *P*-value of univariate survival analysis with log-rank test

Parameter	5-yr DFS (%)	5-yr OS (%)	Log-rank <i>P</i>
Overall	54	57.7	-
Sex			0.77
Male	50.6	56.3	
Female	56.9	58.5	
Age (yr)			0.3
< 65	55.7	59.7	
≥ 65	52.1	55.3	
CEA			0.09
< 5 ng/mL	59.7	65.1	
≥ 5 ng/mL	48.2	50.0	
AJCC staging			< 0.001
Stage 1	68.6	75.0	
Stage 2	81.2	86.9	
Stage 3	39.2	41.3	
Stage 4	0	0	
Tumor size			0.38
T 0-1	61.9	72.0	
T 2-3	52.4	55.1	
Nodal status			< 0.001
N0	73.1	79.1	
N1	42.6	46.1	
N2	21	21.1	
Metastatic status			< 0.001
M0	61.1	65.2	
M1	0	0	
Location			0.03
Colon	61.6	65.8	
Rectum	46.1	49.4	
Differentiation			0.06
Well	61.9	62.0	
Moderate	49.4	49.5	
Poor	34.2	34.3	
Staining localization			0.09
Nuclear	69.2	73.3	
Non-nuclear	50.5	54.1	
Staining intensity			0.61
Weak to moderate	60.9	65.4	
Strong to very strong	50.7	54.1	
OSD			< 0.001
Low (< 75%)	34.7	63.4	
High (≥ 75%)	60.5	40.0	
NSD			0.59
Low (< 75%)	51.1	55.8	
High (≥ 75%)	56.5	59.3	

OSD: Overall staining density; NAD: Nuclear staining density; OS: Overall survival; DFS: Disease free survival.

follow-up period was 56.2 mo. Overall survival and disease-free survival at 5-years were 57.7% and 54.0%, respectively. Univariate analysis found that AJCC staging, nodal status, metastatic status and tumor location were among the clinical parameters that had significant association with both overall survival and disease-free survival. Tumor differentiation was correlated with disease-free survival and the overall survival only at borderline significance (Log rank *P*-values 0.05 and 0.06, respectively) (Table 2). CEA at 5 ng/mL or more predicted significantly poorer outcome only in the AJCC stage I subgroup (Log rank *P*-value 0.045).

Five-year overall survival in patients with high NSD (59.3%) was not different from cases with low density (55.8%) when compared by Log-rank analysis ($P = 0.59$).

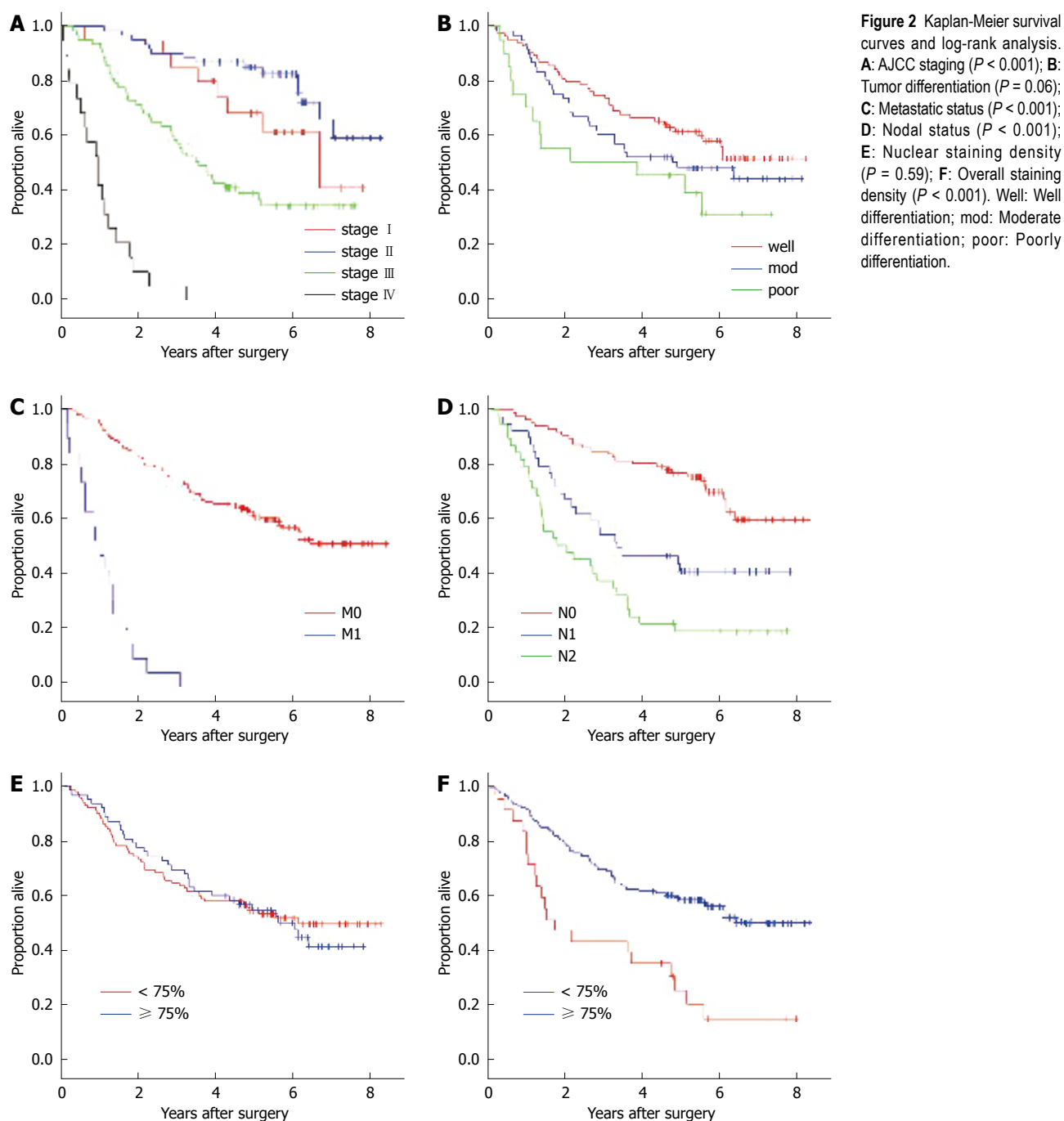


Table 3 Intensity-adjusted 5-yr overall survival and disease-free survival by overall staining density and nuclear staining density

Parameter	Intensity-adjusted 5-yr survival	
	5-yr DFS (%)	5-yr OS (%)
OSD		
Low (< 75%)	34.9	38.2
High ($\geq 75\%$)	73.2	77.8
NSD		
Low (< 75%)	55.3	58.4
High ($\geq 75\%$)	68.7	75.7

OS: Overall survival; DFS: Disease free survival; OSD: Overall staining density; NSD: Nuclear staining density.

On the other hand, OSD showed a significant influence on both overall ($P < 0.001$) and disease-free survival

probability ($P < 0.001$) (Figure 2). Two and five-year overall survival rates in cases with high OSD (84.6% and 60.5%, respectively) were obviously higher than low OSD (50.0% and 34.7%, respectively). Adjusting for the staining intensity resulted in a greater difference of 5-year overall survival probability between the high OSD (77.8%) and low OSD group (38.2%) (Table 3).

With Cox's hazard multivariate analysis, the final model demonstrated that perioperative parameters that were independently associated with survival probability in colorectal cancer were nodal status, metastatic status, and OSD (Table 4). When subgroup analysis was performed for cases without metastasis at the time of diagnosis, nodal status (hazard ratio 2.0, $P < 0.001$), OSD (hazard ratio 0.36, $P < 0.001$), serum CEA at 5 ng/mL or more (hazard ratio 1.8, $P < 0.001$) and differentiation status (hazard ratio 1.4, $P < 0.02$) were significantly

Table 4 Final Cox's regression model (P -value of the model < 0.001), Log likelihood = -347.75

Parameter	Hazard ratio	95% CI	P
Nodal status	1.87	1.43-2.44	< 0.001
Metastatic status	7.50	3.89-14.43	< 0.001
OSD	0.51	0.31-0.83	0.007

OSD: Overall staining density.

associated with disease-free survival. Within the same non-metastatic subgroup, factors that significantly fit the final regression model analyzing overall survival were nodal status (hazard ratio 2.3, $P < 0.001$), OSD (hazard ratio 0.4, $P < 0.001$), and age more than 65 years (hazard ratio 1.6, $P < 0.04$).

Excluding OSD from the model, the Cox's hazard analysis showed that high NSD had a hazard ratio of 2.04 [95% confidence interval (CI) 1.0-4.2], compared with NSD less than 50, when adjusted for tumor stage, differentiation, nodal and metastatic status.

DISCUSSION

An outcome prognosticating factor is an essential component for risk categorization in utilizing the risk-based therapy concept. Defining an individual at-risk of unfavorable prognosis helps in selecting patients who are most likely to benefit from intensive adjuvant treatment. Earlier successful examples of risk-based therapy in biological factors have been integrated into the management scheme are neuroblastoma^[21], breast cancer^[22] and gastrointestinal stromal tumors^[23]. In CRC, although various biological outcome predictors have been discovered, those parameters have not yet been adopted into a standard treatment protocol of CRC.

The Wnt-signaling pathway plays several roles in humans; a physiological role in normal development and a pathological role in tumorigenesis^[7]. A common denominator of the pathway activation is the intracellular amount and localization of beta-catenin, the central molecule. The carcinogenic process of CRC could be linked to the Wnt-signaling cascade through the loss of APC function, which has been attributed to up to 60% of sporadic tumors^[24]. Because APC is essential for clearance of excessive intracellular beta-catenin, accumulation of beta-catenin occurs as a result of APC down-regulation. Positive nuclear accumulation has been reported between 21%-100% in sporadic CRC^[15-17,19], depending on the characteristics of the patients in the series, the staining protocol and their histological parameter. In our study, tumor cells with nuclear staining was detected in at least 80% of the cases, however, only 66% of these cases had high NSD. In a series of 136 patients with CRC, Cheah and colleagues identified a negative survival influence of nuclear beta-catenin^[15]. In their study, the nuclear beta-catenin was covariate with tumor stage; however, it could be a strong prognosticator when combined

with another factor p27b12. Two publications by Wong and colleagues reported that progression of nuclear beta-catenin immunohistochemistry scores was correlated with advances in the malignant potential of colonic neoplasms^[13,14]. The studies also showed survival differences between cases with high and low expression density of nuclear beta-catenin. Outcome prognosticating ability of non-membranous beta-catenin was also supported by another two other separate works from Europe^[16,17]. Lines of epidemiological evidence suggested that nuclear accumulation of beta-catenin could be employed as a factor identifying cases at risk of treatment failure. However, consensus histological criteria had not been established at the time of these studies and most of them focused only on nuclear localization.

Our study aimed to evaluate the roles of beta-catenin in our clinical series of CRC patients in whom the therapeutic process was homogeneously performed by the same multidisciplinary team and the follow-up period was long enough to give reliable data. Apart from nodal status, pre-operative CEA and chronological age predicted disease-free survival and overall survival non-metastatic CRC in the series. Although NSD showed no significant survival function, hazard analysis demonstrated that the factor was associated with mortality risk when other factors were adjusted. Moreover, high NSD was linked to lymph node metastasis and high serum CEA, a finding consistent with previous reports which found that nuclear-accumulated beta-catenin predicted an unfavorable outcome. However, in our series, the influence of nuclear beta-catenin was not independent from other major prognosticators. On the other hand, when we analyzed the overall expression of beta-catenin in term of OSD, it was found that the factor was strongly associated with survival advantage. This conflicting data could not be simply explained by using the mainstream theory of the Wnt-signaling cascade. A positive correlation between NSD, OSD and staining intensity was one of the interesting findings in our study. If NSD is regarded as a localization parameter of beta-catenin and OSD represents an overall expression level of the protein, it appears that the two parameters are co-variant and OSD has much stronger influence on survival.

Besides the growth promoting function, alternative roles of beta-catenin should not be overlooked. Beta-catenin performs necessary physiologic tasks in cellular differentiation and cell-to-cell adhesion^[25]. Our study detected a significant correlation between beta-catenin expression and tumor differentiation. Poorly differentiated tumors harbored a significantly smaller proportion of high NSD and OSD. This association was also shown in previous series, although the survival correlation appeared in an opposite direction^[15]. A recent study from our group demonstrated that beta-catenin promotes differentiation in another malignancy, neuroblastoma^[26]. If these things are taken together, it might be hypothesized that beta-catenin plays a role in

maintaining a good differentiation status in cancer cells. Furthermore, the physiologic function of beta-catenin at the cell membranes is related to cell-to-cell adhesion, which possibly prevents metastasis. A study by Lugli^[18] and colleagues found that membranous beta-catenin co-localized with E-cadherin and loss of membranous or cytoplasmic beta-catenin characterized a higher stage disease. In our series, loss of OSD was evident in advanced stage CRC, and there was also a significantly higher OSD proportion in early nodal status. The physiologic functions of beta-catenin in tumor cell differentiation and adhesion may explain our findings. However, to conclude that beta-catenin expression provides a protective role in CRC, further functional genetics research needs to be performed.

In summary, expression and localization of beta-catenin immunohistochemistry in a series of CRC were analyzed. The study did not find a prognosticating role of the nuclear localization; however, overall expression of beta-catenin was found as a strong and independent predictor of favorable outcome.

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COMMENTS

Background

The authors examined the expression of beta-catenin in colorectal cancer and looked for association with other clinicopathological parameters, and outcomes.

Research frontiers

Beta-catenin immunohistochemistry was performed in 163 cases of colorectal cancer in whom the outcome data was clearly available.

Innovations and breakthroughs

The study found certain data that was not in-line with previous reports. Instead of nuclear beta-catenin that was associated with survival, the overall staining density of this protein showed strong and independent correlation with overall survival and disease-free survival. Moreover, the parameter (overall staining density) also had positive association with tumor differentiation.

Applications

The data suggested that beta-catenin may have an alternative role in colorectal cancer that was associated with differentiation of tumor cells.

Terminology

Beta-catenin nuclear staining density (NSD) was defined as the number of tumor cells with nuclear staining per 100 cells examined. Overall staining density (OSD) meant number of beta-catenin immunoreacted cell per 100 tumor cells examined. Tumors were defined as having high NSD when NSD was 75% or more, and OSD at 75% or more indicated high OSD.

Peer review

This is a well analyzed paper and provides a new insight into the importance of beta catenin staining as a prognostic marker in colorectal cancer.

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RAPID COMMUNICATION

Therapeutic effect of traditional Chinese medicine on coagulation disorder and accompanying intractable jaundice in hepatitis B virus-related liver cirrhosis patients

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Abstract

AIM: To observe the therapeutic effects of new traditional Chinese medicine (TCM) therapy on coagulation disorder and accompanying intractable jaundice in HBV-related liver cirrhosis patients.

METHODS: Using stratified random sampling according to fibrinogen (Fib) levels, 145 liver cirrhosis patients due to hepatitis B complicated by coagulation disorder were treated. Of them, 70 in research group were treated with TCM by "nourishing yin, cooling blood and invigorating blood circulation" and Western medicine, 75 in control group were treated with conventional Western medicine. The indexes of liver function, coagulation function and bleeding events were observed and compared.

RESULTS: The prothrombin time (PT) was shorter and the fibrinogen (Fib) level was higher in the research group than in the control group (Fib = 1.6-2.0 g/L, 1.1-1.5 g/L, and ≤ 1.0 g/L). The total bilirubin (TBIL) level was significantly lower in the research group than in the control group, except for the subgroup of FIB ≤ 1.0 g/L.

CONCLUSION: TCM therapy can improve coagulation function and decrease TBIL.

INTRODUCTION

About 25% of hepatitis B virus (HBV)-infected patients would die of severe chronic liver diseases such as liver cirrhosis and liver failure^[1]. Coagulation disorder is prevalent in patients with chronic liver disease which is usually detected in laboratory tests and characterized by prolonged prothrombin time (PT), decreased fibrinogen (Fib, coagulation factor I) level and thrombocytopenia^[2]. For the lack of blood products (plasma and coagulation factor) and high medical expenditure, economical and effective treatment modalities for coagulation disorder are demanded. Moreover, intractable jaundice accompanying coagulation disorder in HBV-related liver cirrhosis patients is also a puzzle and there is no effective treatment for it. We have proved in our prophase researches that coagulation function indexes are significantly related to total bilirubin (TBIL). Therefore, this study was to observe the therapeutic effects of traditional Chinese medicine (TCM) on coagulation disorder and accompanying intractable jaundice in HBV-related liver cirrhosis patients.

MATERIALS AND METHODS

Inclusion criteria

Patients with HBV-related liver cirrhosis^[3,4], patients with

coagulation disorder (PT > 14.5 s, fib < 2.0 g/L with or without platelets < 100×10^9 /L before admission), patients with no bleeding events (such as epistaxis, gum bleeding, hematemesis and hematochezia before admission), and those at the age of 20-75 years, were included in the study.

Exclusion criteria

Patients with coagulation disorders accompanying liver cirrhosis due to different reasons (such as parasitic infection, autoimmune liver disease, intrahepatic cholestasis, alcoholic liver disease, drug-induced liver disease, fatty liver disease, liver hereditary diseases and liver vascular diseases), patients with other hepatovirus superinfection, haemolysis, disseminated intravascular coagulation (DIC), complications of severe diseases (such as cardio-cerebrovascular disease, hematological disease, respiratory disease, urinary disease and psychosis), and those with pregnancy and lactation, patients with poor compliance, incomplete clinical data, hospitalization time < 14 d, were excluded from the study.

All patients were given their informed consent before therapy.

Information about patients

All the 145 patients with HBV-related liver cirrhosis accompanying coagulation disorder were randomly chosen according to their Fib levels from the Third Affiliated Hospital of Sun Yat-Sen University from January 2002 to February 2008. The data were collected and analyzed retrospectively. The 145 patients were assigned to three subgroups

Subgroup A: Sixty patients (Fib = 1.6-2.0 g/L) were divided into research group and control group ($n = 30$). There were 20 males and 10 females in the research group, their average age was 49.83 ± 12.32 years and the average hospitalization time was 35.73 ± 24.20 d. There were 21 males and 9 females in the control group, their average age was 44.67 ± 10.34 years and the average hospitalization time was 36.83 ± 18.15 d.

Subgroup B: Sixty patients (Fib = 1.1-1.5 g/L) were divided into research group and control group ($n = 30$). There were 20 males and 10 females in the research group, their average age was 50.27 ± 11.71 years and the average hospitalization time was 37.10 ± 19.94 d. There were 21 males and 9 females in the control group, their average age was 49.60 ± 10.45 years and the average hospitalization time was 30.37 ± 16.81 d.

Subgroup C: Twenty-five patients (Fib ≤ 1.0 g/L) were divided into research group ($n = 10$) and control group ($n = 15$). There were 7 males and 3 females in the research group, their average age was 40.80 ± 8.92 years and the average hospitalization time was 41.70 ± 27.57 d. There were 10 males and 5 females in the control group, their average age was 43.20 ± 10.17 years and the average hospitalization time was 54.93 ± 37.10 d.

Methods

Control group: Patients in the control group were treated with conventional Western medicine supplemented with coagulation factors and platelets. Artificial liver system therapy and liver transplantation were not performed.

Research group: Patients in the research group were treated with TCM by nourishing yin, cooling blood and invigorating blood circulation (basic prescription: Yiwei Decoction and Dahuang Zhechong Pills: shashen 15 g, maidong 15 g, shengdi 30 g, yuzhu 15 g, dahuang 6-30 g, huangqin 12 g, gancao 6 g, taoren 9 g, xingren 12 g, shaoyao 12 g, shuizhi 6 g, tubiechong 6 g), in combination with conventional Western medicine. The prescription was modified if symptoms changed.

The herbal decoction was taken half an hour after each meal, one dose a day for 2-3 wk according to the severity of liver cirrhosis.

Observation indexes

Observations included serological index, coagulation function (PT, Fib and PLT), liver function [alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB) and TBIL], bleeding events and other complications, death rate and side effects during the treatment.

Statistic analysis

Statistical analysis was performed using Spss11.5. Data were expressed as mean \pm SD and analyzed by *t*-test. Numeration data were analyzed by chi square test. $P < 0.05$ (two-sided test) was considered statistically significant.

RESULTS

Analysis of comparability

Fib level was used as the standard for all the patients who were divided into three subgroups. Chi square test and *t*-test showed that the general conditions of patients in the research and control groups were similar (Table 1). The serological indexes of coagulation function, liver function and the severity of liver cirrhosis were similar in the two groups before treatment (Tables 2-4). Improvement in coagulation function of the three subgroups was comparable.

Analysis of data

PT, Fib and TBIL levels were significantly higher in subgroups (Fib = 1.6-2.0 g/L and Fib = 1.1-1.5 g/L) of the research group than those in subgroup of the control group after treatment. There was no significant difference in ALB and PLT between the groups (Tables 5 and 6).

PT and Fib levels were significantly higher in subgroups (Fib ≤ 1.0 g/L) of the research group were significantly higher than those in subgroups of the control group after treatment. There was no significant difference in ALB, PLT and TBIL between the two groups (Table 7).

Bleeding events occurred in 3 patients of the

Table 1 Balance test for general information

		Cases (<i>n</i>)	Sex (male/female)	Age (mean \pm SD)	Days of hospitalization (mean \pm SD)
Fib (1.6-2.0 g/L) level	Research group	30	20/10	49.83 \pm 12.32	35.73 \pm 24.20
	Control group	30	21/9	44.67 \pm 10.34	36.83 \pm 18.15
	<i>P</i> value		0.781	0.084	0.843
Fib (1.1-1.5 g/L) level	Research group	30	20/10	50.27 \pm 11.71	37.10 \pm 19.94
	Control group	30	19/11	49.60 \pm 10.45	30.37 \pm 16.81
	<i>P</i> value		0.787	0.817	0.128
Fib (\leq 1.0 g/L) level	Research group	10	7/3	46.80 \pm 8.92	41.70 \pm 27.57
	Control group	15	10/5	43.20 \pm 10.71	54.93 \pm 37.10
	<i>P</i> value		1.000	0.389	0.346

Anyone in the three subgroups divided by the standard Fib level, age, sex and days of hospitalization was balanced between research and control groups before treatment.

Table 2 Balance test for indexes of coagulation function before treatment (mean \pm SD)

		Fib (g/L)	PT (s)	PLT (10^9 /L)
Fib (1.6-2.0 g/L) level	Research group	1.68 \pm 0.18	20.52 \pm 3.12	69.07 \pm 32.57
	Control group	1.72 \pm 0.15	21.01 \pm 3.31	83.60 \pm 56.41
	<i>P</i> value	0.279	0.563	0.227
Fib (1.1-1.5 g/L) level	Research group	1.25 \pm 0.13	22.27 \pm 2.18	59.87 \pm 29.90
	Control group	1.29 \pm 0.11	22.60 \pm 5.75	71.67 \pm 24.24
	<i>P</i> value	0.138	0.765	0.099
Fib (\leq 1.0 g/L) level	Research group	0.77 \pm 0.19	26.59 \pm 5.39	62.80 \pm 33.19
	Control group	0.79 \pm 0.18	31.49 \pm 8.68	73.27 \pm 57.34
	<i>P</i> value	0.861	0.127	0.608

Anyone in the three subgroups divided by the standard Fib level and the indexes of coagulation function was balanced between research and control groups before treatment.

Table 3 Balance test for indexes of liver function before treatment (mean \pm SD)

		ALT (U/L)	AST (U/L)	TBIL (μ mol/L)	ALB (g/L)
Fib (1.6-2.0 g/L) level	Research group	180.90 \pm 255.59	163.17 \pm 176.13	104.51 \pm 65.26	32.79 \pm 4.69
	Control group	241.87 \pm 349.01	190.87 \pm 191.11	126.97 \pm 61.69	33.47 \pm 3.72
	<i>P</i> value	0.089	0.562	0.176	0.537
Fib (1.1-1.5 g/L) level	Research group	118.57 \pm 121.99	144.37 \pm 113.62	129.71 \pm 95.70	31.74 \pm 4.69
	Control group	234.07 \pm 392.07	232.87 \pm 265.04	169.95 \pm 156.22	29.59 \pm 5.54
	<i>P</i> value	0.129	0.098	0.234	0.111
Fib (\leq 1.0 g/L) level	Research group	74.40 \pm 30.89	92.40 \pm 78.44	198.40 \pm 123.37	29.60 \pm 3.82
	Control group	181.67 \pm 283.59	142.80 \pm 128.34	245.57 \pm 193.69	30.15 \pm 6.61
	<i>P</i> value	0.249	0.280	0.503	0.817

Anyone in the three subgroups divided by the standard of Fib level and the indexes of liver function was balanced between research and control groups before treatment.

Table 4 Balance test for related clinical materials before treatment

		Combined with ascites liquid	Combined with hepatic encephalopathy	Combined with infection	Combined with liver cancer
Fib (1.6-2.0 g/L) level	Research group	10	0	8	5
	Control group	15	3	15	2
	<i>P</i> value	0.190	0.236	0.063	0.421
Fib (1.1-1.5 g/L) level	Research group	17	1	10	6
	Control group	20	0	13	2
	<i>P</i> value	0.426	1.000	0.426	0.255
Fib (\leq 1.0 g/L) level	Research group	8	1	4	0
	Control group	12	1	5	1
	<i>P</i> value	1.000	1.000	0.734	1.000

Anyone in the three subgroups divided by standard Fib level, and clinical materials such as complications was balanced between research and control groups before treatment.

Table 5 Fib (1.6-2.0 g/L) level and serum index before and after treatment (mean \pm SD)

		Fib (g/L)	PT (s)	PLT (10^9 /L)	TBIL (μ mol/L)	ALB (g/L)
Research group 30 cases	Before treatment	1.68 \pm 0.18	20.52 \pm 3.12	69.07 \pm 32.57	104.51 \pm 65.26	32.79 \pm 4.69
	After treatment	1.95 \pm 0.43	17.66 \pm 2.38	80.10 \pm 42.12	34.44 \pm 17.10	36.32 \pm 3.98
Control group 30 cases	Before treatment	1.72 \pm 0.15	21.01 \pm 3.31	83.60 \pm 56.41	126.97 \pm 61.69	33.47 \pm 3.72
	After treatment	1.64 \pm 0.44	19.07 \pm 7.13	67.63 \pm 42.65	113.60 \pm 163.86	35.69 \pm 5.21
P value		< 0.0001	< 0.0001	0.259	0.008	0.604

PT, Fib and TBIL were significantly higher in the research group than in the control group after treatment.

Table 6 Fib (1.1-1.5 g/L) level before and after treatment (mean \pm SD)

		Fib (g/L)	PT (s)	PLT (10^9 /L)	TBIL (μ mol/L)	ALB (g/L)
Research group 30 cases	Before treatment	1.25 \pm 0.13	22.27 \pm 2.18	59.87 \pm 29.90	129.71 \pm 95.70	31.74 \pm 4.69
	After treatment	1.72 \pm 0.33	18.45 \pm 2.11	59.50 \pm 27.86	46.75 \pm 19.83	36.46 \pm 3.83
Control group 30 cases	Before treatment	1.29 \pm 0.11	22.60 \pm 5.75	71.67 \pm 24.24	169.95 \pm 156.22	29.59 \pm 5.54
	After treatment	1.29 \pm 0.41	20.56 \pm 9.99	68.37 \pm 27.20	130.95 \pm 180.92	35.51 \pm 4.75
P value		< 0.0001	0.032	0.217	0.014	0.399

PT, Fib and TBIL were significantly higher in the research group than in the control group after treatment.

Table 7 Fib (\leq 1.0 g/L) level before and after treatment (mean \pm SD)

		Fib (g/L)	PT (s)	PLT (10^9 /L)	TBIL (μ mol/L)	ALB (g/L)
Research group 10 cases	Before treatment	0.77 \pm 0.19	26.59 \pm 5.39	62.80 \pm 33.19	198.40 \pm 123.37	29.60 \pm 3.82
	After treatment	1.29 \pm 0.35	23.29 \pm 5.35	54.80 \pm 37.42	77.85 \pm 39.21	35.31 \pm 5.07
Control group 10 cases	Before treatment	0.79 \pm 0.18	31.49 \pm 8.68	73.27 \pm 57.34	245.57 \pm 193.69	30.15 \pm 6.61
	After treatment	0.90 \pm 0.36	39.08 \pm 22.78	70.93 \pm 54.06	173.47 \pm 149.30	35.86 \pm 5.71
P value		0.013	0.043	0.421	0.061	0.807

PT and Fib were significantly higher in the research group than in the control group after treatment.

research group and in 19 patients of the control group ($P < 0.0001$).

DISCUSSION

Fib, which has coagulation function, is a kind of protein that is synthesized in the liver. Fib, the most important coagulation factor in human body, is transformed into fibrin in the coagulation process. Fib decrease is a sensitive change in chronic hepatitis patients, which means that the biological enzyme is declined and the coagulation function is abnormal^[5]. Fib can also be used to diagnose DIC caused by liver diseases. It was reported that Fib contents are closely related with the damage degree of hepatocytes, the severity and prognosis of liver cirrhosis^[6-10]. Therefore, Fib was chosen as a criterion in this research.

TCM believes that the original etiological factor for HBV infection is “damp-heat”, which belongs to the category of warm pathogens. By analyzing and differentiating the development of an epidemic febrile disease and by studying conditions of the four systems (Wei, Qi, Ying, Xue) of patients with coagulation disorder, Yingfen syndrome and Xuefen syndrome are diagnosed. As one of the febrile disease characteristics, warm pathogen can injure yin easily, meanwhile “cooling the blood and invigorating blood circulation” is the traditional therapeutic method for Xuefen syndrome.

Therefore, we chose TCM to treat liver cirrhosis accompanying coagulation disorder by nourishing yin, cooling the blood and invigorating blood circulation.

This study showed that TCM therapy for liver cirrhosis could improve PT and Fib, and reduce occurrence of bleeding events by improving microcirculation, increasing blood and oxygen supply to the liver, thus promoting regeneration and restoration of hepatocytes. It was reported that this new TCM therapy has anti-thrombosis effects by relieving the microangium spasm and hypercoagulable state in the liver^[11-15]. Heat-clearing and blood-cooling drugs can stimulate pituitary-adrenal axis, enhance stress capability, dredge microcirculation, protect vessel wall, and maintain the balance between coagulation and anti-substance^[14], suggesting that such drugs can promote cell proliferation and speed up cell cycle progression. This new TCM therapy can alleviate hepatocellular immune injury caused by HBV infection and degenerative necrosis of hepatocytes. It was reported that blood circulation promoting therapy can inhibit cellular and humoral immunity. Herbal medicine for cooling the blood and invigorating blood circulation can alleviate immune injury by inhibiting autoimmune effect and γ -globulin^[12,14,15], and damaged hepatocytes and vascular endothelial cells caused by endotoxemia and inflammatory factors. Studies showed that nourishing yin, cooling the blood and invigorating blood circulation can antagonize apoptosis of vascular endothelial cells

induced by endotoxin^[16-18]. The reason why PLT does not ameliorate is that coagulation disorder in patients with HBV-related liver cirrhosis is usually accompanied with hypersplenism and PLT is severely destroyed and phagocytosed by the spleen. In addition, PLT does not come from liver but from bone marrow megakaryocytes.

This study also showed that the new TCM therapy could significantly decrease TBIL. TCM believes that jaundice would not regress easily if only the blood circulation is fluent. Since the pathogenesis of jaundice is blood stasis which is one of the pathogenic factors for coagulation disorder in liver cirrhosis patients, the new TCM therapy can achieve the purpose of treating different diseases with the same method. On the one hand, it can improve hepatocyte function by exerting its anti-thrombosis microcirculation effect and by improving the blood circulation of liver. On the other hand, it can promote biliary excretion by inhibiting immunologic reaction, alleviating inflammation of intrahepatic bile ducts, which can improve the coagulation disorder in liver cirrhosis patients and decrease jaundice. The reason why TBIL can be decreased only when Fib > 1.0 g/L, may be due to the impaired liver function, a short course of treatment and a relative small sample.

In conclusion, this new TCM therapy can improve coagulation function indexes, such as PT and Fib in patients with HBV-related liver cirrhosis and reduce bleeding events which can also decrease TBIL.

COMMENTS

Background

Hepatitis B virus (HBV) infection is prevalent all over the world and 2000 million people have been infected with HBV, 350 million of them are chronic HBV carriers and 25% of HBV-infected individuals will die of chronic severe liver diseases. Coagulation disorder is an important clinical feature of chronic liver disease characterized by prolonged PT, decreased Fib and thrombocytopenia. HBV infection usually leads to bleeding, anaemia, decreased granulocytes, thrombosis and even multiple organ failure, etc. Plasma infusion can improve coagulation disorder. We performed this study to find a new traditional Chinese medicine (TCM) therapy for coagulation disorder in patients with HBV-related liver cirrhosis.

Research frontiers

Conventional treatment modalities for coagulation disorders in Western medicine are to improve liver function, avoid using drugs which can affect platelet function and aggravate coagulation disorder, and supply vitamin K, coagulation factors and platelets. Due to the disadvantages of blood products, such as limited supply, allergic reaction and virus infection during infusion, the third generation recombinant coagulation factor VIIa (rhVIIa) is a highlight and has been used in clinical practice. In the field of TCM, promoting blood circulation to remove blood stasis for coagulation disorder can increase fibrinogen (Fib), improve prothrombin time (PT) and eliminate complications.

Innovations and breakthroughs

Fib level was used as a criterion to observe the new TCM therapeutic effects on coagulation disorder in patients with HBV-related liver cirrhosis. Early treatment of coagulation disorder by nourishing yin, cooling the blood and invigorating blood circulation before occurrence of bleeding events can reduce bleeding events and prevent disseminated intravascular coagulation (DIC). Meanwhile, this TCM therapy could improve coagulation function and decrease total bilirubin (TBIL).

Applications

The present study confirmed that the TCM therapy by nourishing yin, cooling

the blood and invigorating blood circulation focusing on the pathogenic factors and pathogenesis of coagulation disorders in patients with HBV-related liver cirrhosis, could improve coagulation function, decrease TBIL. Therefore, it can be used in the treatment of chronic liver diseases.

Peer review

Deterioration of coagulation function is a serious problem in liver cirrhosis patients. This new TCM therapy is encouraging and interesting with satisfactory therapeutic effects on serum prothrombin, fibrinogen and TBil in patients with HBV-related liver cirrhosis.

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Drug utilization of clarithromycin for gastrointestinal disease treatment

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Abstract

AIM: To evaluate the patterns of use of clarithromycin for gastrointestinal disease treatment and promote its rational use.

METHODS: Using a structured pro forma, we conducted a two-month survey of the electronic prescriptions containing immediate-release (IR) or sustained-release (SR) product of clarithromycin for outpatients with gastrointestinal diseases in a 2200-bed general hospital. Suitability of the prescription was audited retrospectively.

RESULTS: One hundred and sixty-four prescriptions of SR product and 110 prescriptions of IR product were prescribed for gastrointestinal disease treatment. Among prescriptions for anti-*Helicobacter pylori* (*H. pylori*) therapy, triple therapy take the dominant position (91.8%), followed by quadruple therapy (4.3%) and dual therapy (3.9%). Amoxicillin was the most frequently co-prescribed antibiotic.

Furazolidone and levofloxacin are used more widely than metronidazole or tinidazole. Clarithromycin SR was administered at inappropriate time points in all prescriptions. Fifty percent of all prescriptions of clarithromycin SR, and 6.4% of prescriptions of clarithromycin IR, were prescribed at inappropriate dosing intervals. Surprisingly, discordance between diagnoses and indications was observed in all prescriptions of clarithromycin SR which has not been approved for treating *H. pylori* infection although off-label use for this purpose was reported in literature. On the contrary, only one prescription (0.9%) of clarithromycin IR was prescribed for unapproved indication (i.e. gastro-oesophageal reflux disease). 1.4% of prescriptions for chronic gastritis or peptic ulcer treatment were irrational in that clarithromycin was not co-prescribed with gastric acid inhibitors. Clinical significant CYP3A based drug interactions with clarithromycin were identified.

CONCLUSION: There is a great scope to improve the quality of clarithromycin prescribing in patients with gastrointestinal disease, especially with regard to administration schedule, concordance between indications and diagnoses and management of drug interactions.

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Key words: Clarithromycin; Drug utilization; Prescriptions; *Helicobacter pylori*; Gastrointestinal diseases; Drug administration schedule; Drug interactions; Polypharmacy

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INTRODUCTION

Clarithromycin is a semi-synthetic macrolide antibiotic

that inhibits bacterial protein synthesis. It is more acid-stable, better absorbed, and is widely used as a component of anti-*Helicobacter pylori* (*H. pylori*) regimens^[1,2]. The oral clarithromycin formulations available on the market include immediate-release (IR) clarithromycin and sustained-release (SR) clarithromycin. The two formulations have different administration schedule, clinical indications and therapeutic cost. The SR clarithromycin has obvious advantages over the IR product when they are prescribed for the same indications. These advantages are as follows: (1) higher antimicrobial activity in that clarithromycin is a time-dependent antibiotic; (2) better tolerability, fewer gastrointestinal adverse reactions and reports of abnormal taste^[3,4]; (3) bioequivalence between the SR (1000 mg *qd*) and IR (500 mg *bid*)^[4]; and (4) enhanced medication compliance due to its convenience.

The patterns of combination use of clarithromycin for *H. pylori* infection have not been reported in literature. Meanwhile, many patients with *H. pylori* infection also suffer from other diseases and hence may receive polytherapy regimens, which may exert complex, significant drug interactions^[5]. Up to now, drug utilization of clarithromycin for gastrointestinal disease treatment has not been available. Targeting inappropriate prescribing is one means of trying to reduce drug costs and promote rational use of drug. The aim of this two-month drug utilization study was to assess the extent and appropriateness of clarithromycin by examining prescribing practice for outpatients in a general hospital.

MATERIALS AND METHODS

The setting of this study is a 2200-bed general hospital in Zhejiang Province, China. The pharmacy has two products of clarithromycin [Klaci® (clarithromycin IR tablets, Abbott S.P.A.) and Nuobang® (clarithromycin SR tablets, Jiangsu Hengrui Medicine Co., Ltd, China)]. Each tablet of BIAXIN® contains 250 mg of clarithromycin. Each tablet of Nuobang® contains 500 mg of clarithromycin. Prescribing information for these products along with BIAXIN® XL Filmtab® (clarithromycin SR tablets, Abbott S.P.A.) were referenced^[6]. Relevant literature was identified by performing Pubmed searches until the end of 2007. A structured pro forma was used to perform a survey of electronic prescriptions containing IR or SR product for outpatients with alimentary disease covering the period from December 2007 to January 2008. The pro forma included details of the patient's age and sex, indication for clarithromycin therapy, the type of clarithromycin product prescribed, the dose and duration of therapy and details of other prescribed medications. Suitability of the prescription was audited retrospectively.

Differences between patient groups were tested for statistical significance using χ^2 analysis. A *P*-value < 0.05 was considered significant.

RESULTS

Over a two-month period clarithromycin SR was

Table 1 Details of prescriptions of the two clarithromycin products

	Clarithromycin	
	Sustained-release	Immediate release
Total number of prescriptions	949	197
Number of prescriptions for alimentary disease	164	110
Mean age (range) yr	45.9 (18-82)	
Male:Female	137:137	
Number of prescriptions for anti- <i>H. pylori</i> therapy	159	96
Triple therapy	151	83
PPI/Clarithromycin/Amoxicillin	43	58
PPI/Clarithromycin/Furazolidone	44	21
PPI/Clarithromycin/Levofloxacin	63	1
PPI/Clarithromycin/Metronidazole	1	1
PPI/Clarithromycin/Tinidazole	0	2
Quadruple therapy	5	6
PPI/Bismuth/Amoxicillin/Clarithromycin	0	2
PPI/Bismuth/Furazolidone/Clarithromycin	1	3
PPI/Bismuth/Levofloxacin/Clarithromycin	4	1
Dual therapy	3	7
Clarithromycin/PPI	2	7
Clarithromycin/Ranitidine bismuth citrate	1	0

PPI: Proton pump inhibitor.

prescribed for 949 patients whereas clarithromycin IR product was prescribed for 197 patients (Table1). With respect to use for alimentary disease treatment by gastroenterologists, 164 patients (17.3%) were on SR product compared to 110 patients (55.8%) on IR product (*P* < 0.05). The mean age of these patients was 45.9 years (range: 18-82 years). The number of male patients was equal to that of female patients. Among prescriptions for anti-*H. pylori* therapy, triple therapy take the dominant position (91.8%), followed by quadruple therapy (4.3%) and dual therapy (3.9%).

Administration schedule

Post-meal dosing of clarithromycin was specified in all investigated electronic prescriptions. According to the prescribing information, Nuobang® should be taken with food whereas Klaci® may be given irrespective of food intake. Thus, Nuobang® in all prescriptions was administered at inappropriate time.

Eighty-two prescriptions of Nuobang® (50%) were prescribed twice daily, which was inconsistent with the once-daily dosing method according to its prescribing information. Klaci® was given twice daily according to all prescriptions, which met the requirements for triple therapy. However, dual therapy requires clarithromycin IR 500 mg to be given three times daily^[6]. Thus, 7 prescriptions of dual therapy containing clarithromycin IR tablet (500 mg *bid*) and PPI were identified as irrational.

Diagnoses of patients on clarithromycin-based therapy

For patients with alimentary disease receiving clarithromycin-based therapy, diagnoses were summarized in Table 2. The diagnoses were various. Combining the results of upper gastrointestinal

Table 2 Diagnoses of patients on clarithromycin-based therapy

Diagnoses	Clarithromycin	
	SR	IR
Chronic gastritis	90	87
Peptic ulcer	40	5
Duodenal ulcer	9	5
Gastro-oesophageal reflux disease	5	1
Chronic gastritis, <i>H pylori</i> infection	4	3
Gastric ulcer	1	4
Chronic gastritis, Gastric ulcer	2	1
Gastro-oesophageal reflux disease, Gastric ulcer	1	
<i>H pylori</i> infection	2	
Chronic gastritis, Peptic ulcer	1	3
Peptic ulcer, <i>H pylori</i> infection		1
Mesenteric lymphadenitis	7	
Abdominal pain of unknown origin	2	

SR: Sustained-release; IR: Immediate release.

Table 3 Concomitant drugs used in clarithromycin-based triple therapy

Comedicated drugs	Clarithromycin	
	SR	IR
PPI		
Omeprazole	17	6
Lansoprazole	68	6
Pantoprazole	30	66
Esomeprazole magnesium	15	11
Rebeprazole	23	21
Antibiotics		
Amoxicillin	38	63
Furazolidone	47	24
Levofloxacin	67	2
Metronidazole	1	1
Tinidazole	0	13
Amoxicillin/clavulanate potassium	5	0

SR: Sustained-release; IR: Immediate release.

Table 4 CYP3A based clinical significant drug interactions with clarithromycin

Concurrent medications primarily metabolized by CYP3A	Clarithromycin		References
	SR	IR	
Alprazolam and zolpidem	1		[6,7]
Midazolam	1		[6,8]
Amlodipine	1	2	[9]
Levoamlodipine		1	
Nifedipine		1	[10]
Carbamazepine		1	[11]
Nifedipine, clopidogrel and atorvastatin		1	[10,12-14]
Amlodipine and ergoloid mesylate sustained release capsules		1	[9,15]
Prednisone		1	[16]

SR: Sustained-release; IR: Immediate release.

endoscopy or ¹³C-urea breath test, concordance between diagnoses and indications were examined. Except for patients with mesenteric lymphadenitis or abdominal pain of unknown origin, 90.6% of other patients test positive for *H pylori* infection prior to initiation of anti-*H pylori* regimen. Surprisingly, discordance between diagnoses and indications was observed in all

prescriptions of clarithromycin SR. On the contrary, only one prescription (0.9%) of clarithromycin IR was prescribed for unapproved indication (i.e. gastro-oesophageal reflux disease).

Drug interactions

Concomitant PPIs and anti-*H pylori* agents used in clarithromycin-based triple therapy were listed in Table 3. The PPIs included omeprazole, lansoprazole, pantoprazole, esomeprazole magnesium and rebeprazole. The antibiotics co-prescribed with clarithromycin included amoxicillin, furazolidone, levofloxacin, metronidazole, tinidazole and amoxicillin/clavulanate potassium. Amoxicillin was the most frequently co-prescribed antibiotic. Furazolidone and levofloxacin were used more widely than metronidazole or tinidazole. The CYP3A dependent clinical significant drug interactions with clarithromycin in this survey were summarized in Table 4.

Clarithromycin plays its role of anti-*H pylori* only under the circumstance of pH more than 4.0 and thus it usually needs concomitant use of anti-gastric-secretion drugs. However, 4 prescriptions for chronic gastritis or peptic ulcer treatment did not contain gastric acid inhibitors, and thus were judged as irrational.

DISCUSSION

Administration schedule

Food has no significant effects on pharmacokinetics of IR clarithromycin and thus the product may be given irrespective of food intake. With regard to Nuobang®, administration under fasting conditions is associated with approximately 30% lower area under the plasma concentration-time curve (*AUC*) for clarithromycin relative to administration with food. Therefore, it should be taken with food to maximize bioavailability. Physicians and pharmacists should pay attention to this biopharmaceutical requirement and strengthen patient education.

Compared to the triple therapy, the dual therapy has a lower eradication rate of *H pylori*. Moreover, regimens which contain clarithromycin as the single antibiotic are more likely to be associated with the development of clarithromycin resistance among patients who fail therapy. When the IR clarithromycin tablet is combined with PPI as dual therapy, the dose needs to be tailored to 500 mg three times daily^[6].

The SR clarithromycin has obvious advantages over the IR product when they are prescribed for the same indications^[3,4]. However, the novelty of the SR product and its administration of only once a day would decrease the benefit for patients and their compliance if given twice daily as detected in most prescriptions.

Concordance between indications and diagnoses

Chronic gastritis is an inflammation of the lining of the stomach that occurs gradually and persists for a prolonged time. It can be classified based on the underlying etiologic agent (e.g. *H pylori*, bile reflux,

nonsteroidal anti-inflammatory drugs, autoimmunity, allergic response) and the histopathological pattern. Diagnosis of chronic gastritis is broad and discordant with indications described in the package insert of clarithromycin. It should be further specified if patients test positive for *H. pylori* (i.e. *H. pylori*-associated chronic gastritis instead of chronic gastritis) and the rationale also applies to diagnoses of gastric ulcer, peptic ulcer and duodenal ulcer.

Gastro-oesophageal reflux disease (GERD) is an unapproved indication for IR or SR clarithromycin. Although a significant proportion of patients with GERD have *H. pylori* infection, it is unclear whether or not *H. pylori* should be treated. Eradication therapy is currently not recommended for most of GERD patients with *H. pylori* infection^[1,2]. Relief of abdominal pain of unknown origin was also an unapproved indication for clarithromycin-based therapy. Seven prescriptions for mesenteric lymphadenitis treatment included monotherapy with clarithromycin ($n = 1$), dual therapy with clarithromycin-levofloxacin ($n = 3$), clarithromycin-cefdinir ($n = 1$), clarithromycin-amoxicillin/clavulanate potassium ($n = 2$). Given the predominance of *Y. enterocolitica* in mesenteric lymphadenitis infection, initial oral antibiotic selection from third-generation cephalosporins, broad spectrum penicillins, fluoroquinolones and doxycycline may be considered. Recently, association of mesenteric lymphadenitis with mycoplasma was revealed by Tao *et al*^[17]. Among 108 patients with mesenteric lymphadenitis in that study, 36 patients (33%) were Mycoplasma-IgM positive. The switch to macrolide azithromycin provided a benefit for patients with an unsatisfactory response to third-generation cephalosporins or broad spectrum penicillins. In our survey, follow-up indicated that the combination of macrolide clarithromycin with levofloxacin, amoxicillin/clavulanate potassium or cefdinir showed satisfactory results in patients suffering from mesenteric lymphadenitis.

Clarithromycin IR based triple therapy or dual therapy is indicated for the treatment of patients with *H. pylori* infection. However, the efficacy and safety of clarithromycin SR treatment for *H. pylori* infection have not been established, as indicated in the prescribing information for Biaxin XL Filmtab®. There have been three studies on clinical efficacy of clarithromycin SR-based triple therapy to cure *H. pylori* infection. Coelho *et al*^[18] observed that the combination of lansoprazole 30 mg, clarithromycin SR 500 mg and furazolidone 400 mg, once daily for 7 d, was inexpensive, safe and an effective alternative for anti-*H. pylori* therapy in family members of gastric cancer patients. Chu *et al*^[19] proved that one-week once-daily course of lansoprazole 30 mg, clarithromycin SR 500 mg and metronidazole 800 mg was a safe, well-tolerated, easy to comply with, and efficacious treatment for *H. pylori* infection. A randomized controlled trial study by Liou *et al*^[20] provided the direct evidence that clarithromycin SR 1000 mg once daily can be used as an alternative to clarithromycin IR 500 mg twice daily for the treatment

of *H. pylori*-associated peptic ulcer disease. In that study, 161 patients with *H. pylori*-associated peptic ulcer were randomized to receive one-week triple therapy with either clarithromycin SR 1000 mg once daily or clarithromycin IR 500 mg twice daily combination with amoxicillin 1000 mg twice daily and esomeprazole 40 mg once daily. The eradication rates were comparable in the two groups. Further clinical trials with a larger sample size are required to establish the efficacy and safety of clarithromycin SR. Effective communication between patients and gastroenterologists are rather necessary prior to initiation of off-label use of clarithromycin SR.

Drug interactions

The combination of clarithromycin with omeprazole has a synergic effect. The C_{max} , AUC_{0-24} , and $T_{1/2}$ derived from omeprazole increased by 30%, 89%, and 34%, respectively by the concomitant administration. The mean 24-h gastric pH value was 5.2 when omeprazole was administered alone and 5.7 when co-administered with clarithromycin^[6]. On the other hand, by concomitant administration of omeprazole, clarithromycin concentrations in the gastric tissue and mucus increased (e.g. clarithromycin mucus concentrations 2 h after application increased by about 9-fold)^[21]. Simultaneous administration of lansoprazole, amoxicillin and clarithromycin increases the serum concentrations of lansoprazole and the active 14-OH-clarithromycin metabolite significantly^[22]. Compared to treatment with esomeprazole alone, the mean steady state AUC and C_{max} of esomeprazole increased by 70% and 18%, respectively, during triple therapy (esomeprazole magnesium 40 mg *qd*, clarithromycin 500 mg *bid* and amoxicillin 1000 mg *bid* for 7 days)^[23]. The AUC and C_{max} of rabeprazole and 14-hydroxylclarithromycin (active metabolite of clarithromycin) increased, although the AUC and C_{max} for clarithromycin were not different following combined administration consisting of rabeprazole, amoxicillin and clarithromycin compared to values following single administration^[24]. Although there is no significant pharmacokinetic interaction between clarithromycin and pantoprazole, clarithromycin has a better effect in *H. pylori* treatment when pantoprazole is used concomitantly^[25].

Clarithromycin is a potent inhibitor of CYP3A4 and P-gp. Concomitant administration of clarithromycin and any of the following CYP3A4 substrates is contraindicated: cisapride, pimozide, astemizole, terfenadine, and ergotamine or dihydroergotamine, as described in standard information sources. In this survey, such prescriptions were not found. Coadministration of clarithromycin and a drug primarily metabolized by CYP3A may be associated with elevations in drug concentrations that could increase or prolong both the therapeutic and adverse effects of the concomitant drug. Dosage adjustments may be considered, and when possible, plasma concentrations of drugs primarily metabolized by CYP3A should be monitored closely in patients concurrently receiving clarithromycin.

Triazolobenzodiazepines (e.g. triazolam

and alprazolam) and related benzodiazepines (e.g. midazolam) have been observed of CYP3A based drug interactions with erythromycin products and/or with clarithromycin in postmarketing experience. For example, intestinal and hepatic CYP3A inhibition by clarithromycin can significantly reduce the clearance of midazolam, resulting in an increase in the AUC of midazolam by 8-fold following oral dose in the elderly^[8]. Zolpidem is extensively metabolized, mainly by CYP3A4^[7]. Thus, a prescription containing alprazolam, zolpidem and clarithromycin has a high risk for excessive sedation (Table 4). Pharmacotherapy monitoring and dosage adjustment for these sedative drugs should be implemented accordingly.

Clarithromycin may increase the levels/effects of amlodipine^[9]. Levoamlodipine is an eutomer of amlodipine and the first enantiomerically pure dihydropyridine calcium channel blockers. Levoamlodipine is also mainly metabolized by CYP3A4, so its levels/effects may also be affected by clarithromycin. A case of vasodilatory shock possibly resulting from a clarithromycin-nifedipine interaction was reported by Gerónimo-Pardo *et al.*^[10]. A potentially significant pharmacokinetic interaction between clarithromycin and carbamazepine was identified in two patients with long-standing epilepsy who were given omeprazole/clarithromycin therapy for *H pylori* gastritis^[9]. Serum carbamazepine levels were augmented by clarithromycin and returned to the therapeutic range following cessation of clarithromycin therapy. Empirically in such cases carbamazepine dose need to be tailored by 30% to 50%.

Clarithromycin did have a significant effect on atorvastatin pharmacokinetic parameters. When coadministered, clarithromycin raised atorvastatin AUC by 82% and C_{max} by 56%. Hence, clarithromycin should be avoided in patients taking atorvastatin and similarly metabolized HMG-CoA inhibitors^[12]. Sipe *et al.*^[13] reported a case of rhabdomyolysis causing AV blockade due to possible atorvastatin, esomeprazole, and clarithromycin interaction. The antiplatelet effects of the prodrug clopidogrel can be reduced by concomitant administration of erythromycin or troleandomycin. The proposed mechanism is inhibition of CYP3A4 activity, which is responsible for the conversion of clopidogrel to its active metabolite. Clarithromycin also inhibits CYP3A4 activity and is also expected to affect clopidogrel metabolism^[14]. Until more information is available, monitoring for altered efficacy of clopidogrel may be advisable if clarithromycin is co-administered with clopidogrel.

In a combination of clarithromycin, nifedipine, clopidogrel and atorvastatin (Table 4), at least 4 clinical significant pharmacokinetic interactions are involved, e.g. clarithromycin-nifedipine, clarithromycin-clopidogrel, clarithromycin-atorvastatin and atorvastatin-clopidogrel^[20]. Such a prescription with high risk of adverse drug interactions is irrational. Considering the short course of clarithromycin therapy, close monitoring and proper dose adjustment may be more practicable

than to switch to alternatives not mainly metabolized by CYP3A4.

Clarithromycin may increase levels of ergoloid mesylate by inhibiting CYP3A4 metabolism, resulting in toxicity (ischemia, vasospasm) and the combined use is contraindicated^[15]. So the combination therapy of clarithromycin with amlodipine and ergoloid mesylate at conventional dosage is irrational. A case of mania due to prednisone-clarithromycin interaction was reported by Finkenbine *et al.*^[16], suggesting that pharmacotherapy monitoring should be performed during the concurrent therapy.

Triple therapy with a PPI, clarithromycin and either amoxicillin or metronidazole is the first-line treatment regimen to eradicate *H pylori* infection^[1,2,27]. Significant differences are observed in the prevalence of metronidazole resistance between developed and developing countries^[28-30]. High levels of resistance to metronidazole mainly relates to the wide application in parasite infection, dental infection and gynecological diseases in developing countries. Antimicrobial susceptibility tests performed in Zhejiang Province of China indicated that the antibiotic resistance rate increased perceptibly during the period of 2003-2007^[31,32]. Among six antibiotics (metronidazole, amoxicillin, gentamycin, levofloxacin, furazolidone and clarithromycin), the rate of resistance to metronidazole (99.32%) appeared to be the highest and the levofloxacin resistance rate (0.51%) was the lowest. Amoxicillin rarely induces resistance^[33]. Fluoroquinolones are active against *H pylori* in vitro and have a synergistic effect with PPIs^[34]. Strains resistant to furazolidone are rare. Furthermore, there is no cross-resistance to metronidazole and furazolidone is effective in populations with a high prevalence of metronidazole resistance^[35]. The resistance status may explain the pattern of antibiotic use in this hospital, i.e. furazolidone and levofloxacin are used more widely than are metronidazole or tinidazole. Guo *et al.*^[28] reported that *H pylori* eradication rates were significantly different in patients receiving OAC (omeprazole/amoxicillin/clarithromycin) and OFC (omeprazole/furazolidone/clarithromycin) compared to those receiving OMC (omeprazole/metronidazole/clarithromycin). The eradication rate for *H pylori* infection was 90.3%, 90.9% and 70.9% in OAC, OFC and OMC groups, respectively. Based on these results, one-week of triple therapy with OAC or OFC were recommended for Chinese patients with duodenal ulcers and chronic gastritis. Since furazolidone is cheap and the *H pylori* eradication rate is high, OFC regimen is recommended to be one of choices for *H pylori* eradication.

PPI-based double combinations were clearly inferior to triple regimens, which is in accordance with the evidence-based data and they are not recommended in the first-line treatment. However, concurrent therapy of ranitidine bismuth citrate and clarithromycin have a similar efficacy compared to the triple regimens^[36-38]. Thus, the prescription of a combination of clarithromycin and ranitidine bismuth citrate in our survey is rational. A 7 d

quadruple therapy based on PPI, bismuth, tetracycline and metronidazole is the more frequently accepted^[1,2]. Our survey found that some patients received quadruple therapy regimens containing PPI, bismuth, clarithromycin, and one of antibiotics including amoxicillin, furazolidone or levofloxacin.

In conclusions, a retrospective utilization study of clarithromycin for gastrointestinal disease treatment was conducted. There is a great scope to improve the quality of clarithromycin prescribing, especially with regard to administration schedule, concordance between indications and diagnoses and management of drug interactions.

COMMENTS

Background

The oral clarithromycin formulations available on the market include immediate-release (IR) clarithromycin and sustained-release (SR) clarithromycin. Due to difference in pharmaceutical forms, the IR and SR formulations have different administration schedule, clinical indications and therapeutic cost. Meanwhile, the patterns of combination use of clarithromycin for *Helicobacter pylori* (*H. pylori*) infection have not been reported in literature. Many patients with *H. pylori* infection also suffer from other diseases and hence they may receive polytherapy regimens, which may exert complex, significant drug interactions if clarithromycin is used. In order to promote its rational use in gastrointestinal disease treatment, it is essential to assess the extent and appropriateness of clarithromycin by examining prescribing practice.

Research frontiers

Drug utilization studies can provide useful information to improve the appropriate and effective use of pharmaceuticals in populations. In recent years many such studies have been performed to monitor prescribing patterns and assess adherence to standard therapeutic guidelines in clinical practice.

Innovations and breakthroughs

This is the first drug utilization study of clarithromycin for gastrointestinal disease treatment and the article critically compares the prescribing patterns of clarithromycin with different pharmaceutical forms.

Applications

The significance of this article is: (1) it provides insights into the aspects of drug use and prescribing pattern; (2) it helps to improve the quality of clarithromycin prescribing, especially with regard to administration schedule, concordance between indications and diagnoses and management of drug interactions; (3) it helps doctors to attach equal importance to other medicines for gastrointestinal disease treatment and finally promote rational drug use in clinical practice.

Terminology

Drug utilization: The study to describe the extent, nature and determinants of drug exposure, consider clinical appropriateness and cost effectiveness, and facilitate rational use of drugs in populations. Drug administration schedule: time schedule for administration of a drug in order to achieve optimum effectiveness and convenience. Drug interactions: The action of a drug that may affect the activity, metabolism, or toxicity of another drug. Polypharmacy: The use of multiple drugs administered to the same patient, most commonly seen in elderly patients.

Peer review

This retrospective utilization study is well designed. It is of particular interest to the practical medicine and can improve the quality of clarithromycin prescribing in patients with gastrointestinal disease.

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RAPID COMMUNICATION

Distinct expression patterns in hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma

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Abstract

AIM: To identify biomarkers indicating virus-specific hepatocarcinogenic process, differential mRNA expression in 32 patients with hepatitis B virus (HBV)/hepatitis C virus (HCV)-associated hepatocellular carcinoma (HCC) were investigated by means of cDNA microarrays comprising of 886 genes.

METHODS: Thirty two HCC patients were divided into two groups based on viral markers: hepatitis B virus positive and HCV positive. The expression profiles of 32 pairs of specimens (tumorous and surrounding non-tumorous liver tissues), consisting of 886 genes were analyzed.

RESULTS: Seven up-regulated genes in HBV-associated HCC comprised genes involved in protein synthesis (*RPS5*), cytoskeletal organization (*KRT8*), apoptosis related genes (*CFLAR*), transport (*ATP5F1*), cell membrane receptor related genes (*IGFBP2*), signal transduction or transcription related genes (*MAP3K5*), and metastasis-related genes (*MMP9*). The up-regulated

genes in HCV-infected group included 4 genes: *VIM* (cell structure), *ACTB* (cell structure), *GAPD* (glycolysis) and *CD58* (cell adhesion). The expression patterns of the 11 genes, identified by cDNA microarray, were confirmed by quantitative RT-PCR in 32 specimens.

CONCLUSION: The patterns of all identified genes were classified based on the viral factor involved in HBV- and HCV-associated HCC. Our results strongly suggest that the pattern of gene expression in HCC is closely associated with the etiologic factor. The present study indicates that HBV and HCV cause hepatocarcinogenesis by different mechanisms, and provide novel tools for the diagnosis and treatment of HBV- and HCV-associated HCC.

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Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus-infected; cDNA microarray; Expression profiling

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Lee CF, Ling ZQ, Zhao T, Lee KR. Distinct expression patterns in hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma. *World J Gastroenterol* 2008; 14(39): 6072-6077 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6072.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6072>

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide^[1]. The major risk factors for HCC are chronic hepatitis resulting from infection with HBV and HCV, and exposure to various exogenous carcinogens, including aflatoxin B1^[2]. Several studies have shown that the incidence of HCC has increased substantially in East Asia, including China, Korea and Japan^[3,4]. More than 350 million people worldwide are known to be chronic carriers of HBV^[5]. Moreover, the incidence of HCC is increasing in many countries in

parallel with the increase in chronic HCV infection^[1,2]. Therefore, clarification of the genetic portrait of hepatocarcinogenesis caused by HBV or HCV infection may provide clues to help reduce the incidence of HCC, and establish effective treatments for HCC. However, the molecular nature of this association is poorly understood.

The phenotypic diversity of cancer is accompanied with a corresponding diversity in the gene expression patterns^[6-10]. Honda *et al.*^[11] showed the presence of different gene expression profiles in the liver lesions of chronic hepatitis caused by HBV and HCV, and suggested that the molecular mechanisms responsible for the pathogenesis of HCC differ between HBV and HCV infections. In the present study, we investigated the gene expression patterns of 32 HCC samples, using cDNA microarrays containing 886 clones in order to gain additional insight into hepatocarcinogenesis or cancer progression related to HBV and HCV infections. The aim of the present study was to characterize the gene expression associated with HCC, with a view to better understand the molecular pathophysiology, which may lead to better methods of detection, diagnosis, and classification of HCC.

MATERIALS AND METHODS

Patient material

The Institutional Review Board on Medical Ethics, Zhejiang Provincial People Hospital (China), approved the method of tissue collection. The present study was conducted in the department of surgery, Zhejiang Provincial People Hospital, on 32 patients who underwent hepatectomy for sporadic HCC without preoperative radio- or chemotherapy. All of tissue samples were immediately frozen in liquid nitrogen, and stored at -80°C until use. A total of 32 HCC samples from 15 lymph node negative and 17 lymph node positive cases were used (Table 1).

Laser microdissection

Eight μm -thick sections of the frozen tissue were cut at -20°C and stained with HE. Under microscopic observation, parts of cancer cells nests in the invasive and intraductal components were microdissected, using the LM100 laser capture microdissection system (Arcturus Engineering, Mountain View, CA, USA). A 15 μm -diameter beam was used to capture the tumor cells and the corresponding non-cancerous liver tissues. The cell nests were transferred to a LCM transfer film (CapSure TF-100S transfer film carrier, 5 mm-diameter optical-grade transparent plastic; Arcturus Engineering).

RNA preparation and T7-based RNA amplification

Total RNA was isolated from the dissected specimens using Trizol reagent (Gibco BRL) and a modified acidic guanidinium phenol-chloroform method, following the manufacturer's recommendations. Total RNA was treated with DNase I for removal of genomic DNA, and the mRNA was purified using a poly(A) purification kit (Oligotex, Qiagen), according to the manufacturer's

Table 1 Clinical data of patients with hepatocellular carcinoma

Case	Sex	Age	Hepatitis virus	Differentiated grade	TNM score
1	M	54	HBV	WD	T1N0M0
2	M	60	HCV	WD	T1N0M0
3	F	61	HBV	WD	T2N0M0
4	M	62	HBV	WD	T2N0M0
5	M	58	HBV	WD	T1N0M0
6	F	56	HCV	MD	T3N0M0
7	F	44	HBV	WD	T2N0M0
8	M	49	HCV	WD	T1N0M0
9	M	58	HBV	WD	T2N0M0
10	M	67	HCV	PD	T3N0M0
11	M	69	HBV	WD	T2N0M0
12	F	63	HCV	WD	T1N0M0
13	M	48	HCV	MD	T2N0M0
14	F	63	HBV	WD	T1N0M0
15	M	49	HCV	MD	T1N0M0
16	F	51	HBV	PD	T3N1M0
17	M	65	HCV	MD	T3N1M0
18	F	58	HBV	PD	T4N1M1
19	M	60	HBV	MD	T2N1M0
20	F	56	HCV	PD	T3N1M1
21	M	42	HCV	PD	T3N1M0
22	M	55	HBV	PD	T4N1M1
23	M	66	HBV	MD	T3N1M0
24	F	70	HCV	WD	T2N1M0
25	M	58	HBV	PD	T4N1M1
26	M	53	HCV	PD	T3N1M0
27	M	61	HBV	PD	T4N1M0
28	F	65	HBV	MD	T3N1M0
29	M	59	HCV	MD	T3N1M1
30	M	50	HBV	PD	T3N1M0
31	F	63	HCV	PD	T4N1M1
32	M	66	HCV	PD	T3N1M0

M: Male; F: Female; HBV: Hepatitis B virus infection; HCV: Hepatitis C virus infection; WD: Well differentiated HCC; MD: Moderately differentiated HCC; PD: Poorly differentiated HCC.

instructions. The quality of mRNA was assessed by $A_{260/280}$ ratios and the contamination of genomic DNA was checked using the PCR method. cDNA was synthesized with T7-oligo (dT) primer (Ambion) and Superscript II enzyme (Gibco BRL), as described in the instruction manual. cDNA was purified by cDNA clean-up column (DNA clear™ kit, Ambion). cRNA was generated by T7 MEGAscript™ kit (MEGAscript *in vitro* Transcription Kit, Ambion, AUSTIN, Tex), per the manufacturer's recommendations. Column purification of cRNA was performed with RNeasy kit (Qiagen), according to the manufacturer's protocol. The concentration and quality of cRNA were analyzed by GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia biotech).

Microarray hybridization and scanning

Human Cancer Chip version 4.0 (IntelliGene, TaKaRa) was used for these studies. This array was spotted on a glass slide with 886 cDNA fragments of human genes, which are composed of 588 human identified genes related to cancer, and 298 cDNA fragments prescreened by differential display method between cancer tissue and normal tissue. Three μg of cRNA from the tumor and

Table 2 Primers used to amplify cDNA at various genes in real time RT-PCR

Target gene	Objective	Forward primer sequence (5'-3')	Reverse primer sequence (3'-5')	Genebank accession no./Amplicon size
RP55	qRT-PCR	GTATGCCGCCAAACGCTTC	CGCCTGTGAGCAGGTGTAT	NM_001009, 152 bp
KRT8	qRT-PCR	GGAGGCATCACCAGCTTAC	GGTTGGCAATATCCTCGTACTGT	NM_002273, 637 bp
CFLAR	qRT-PCR	GACAGAGCTTCTTCGAGACAC	GCTCGGGCATAACAGGCAAAT	AF009616, 116 bp
ATP5F1	qRT-PCR	ACTGGGCTTATCTGTACGCT	GCAAAGTCIGCAACAAAGGGA	NM_001688, 131 bp
IGFBP2	qRT-PCR	GACAATGGCGATGACCACTCA	GCTCCTTCATACCCGACTTGA	NM_000597, 121 bp
MAP3K5	qRT-PCR	AAAAAGGCATTGAATCTGAGCC	GCTTGAATGACTCTCATGTGGTC	NM_005923, 233 bp
MMP9	qRT-PCR	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC	NM_004994, 139 bp
VIM	qRT-PCR	AGTCCACTGAGTACCGGAGAC	CATTTACGCATCTGGCGTTC	AK093924, 98 bp
ACTB	qRT-PCR	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTACAGCACGAT	NM_001101, 250 bp
GAPD	qRT-PCR	CAACTGGTCGTGGACAACCAT	GCACGGACACTCACAATGTTT	AC002389, 260 bp
CD58	qRT-PCR	CTCATGGGATTGTCCTATGGAGC	GCTTGGGATACAGGTTGTCAAA	NM_001779, 154 bp

the matched normal tissue were labeled with Cy3-dUTP and Cy5-dUTP respectively (Amersham Pharmacia Biotech, Buckinghamshire, England), using a labeling kit (RNA Fluorescence Labeling Core kit, TaKaRa), according to the manufacturer's instructions. The labeled probe was purified by centrifugation in a spin column (Centrisep, Princeton Separations, Adelphia, NJ). Two separate probes were combined, and 2 μ L of 5 \times competitor containing Cot I (Gibco BRL), poly dA (Amersham Pharmacia Biotech), and tRNA (TaKaRa) were added. After addition of 50 μ L of 100% ethanol and 2 μ L of 3 mol/L sodium acetate (pH 5.2), the mixture was cooled at -80°C for 30 min, followed by centrifugation at 15 000 g for 10 min, and pelleted down. For final probe preparation, the pellet was washed in 500 μ L of 70% ethanol twice, and eluted in 10 μ L hybridization buffer (6 \times SSC, 0.2% SDS, 5 \times Denhardt's solution, 0.1 mg/mL salmon sperm solution). The probe were denatured by heating for 2 min at 95°C, cooled at room temperature, and centrifuged at 15 000 g for 10 min (20-26°C). Supernatants were placed on the array and covered with a 22-mm \times 22-mm glass coverslip. The coverslip was sealed with a glue, and the probes were incubated overnight at 65°C for 16 h in a custom-made slide chamber with humidity maintained by underlying moist papers. After hybridization, the slides were washed in 2 \times SSC with 0.1% SDS, 1 \times SSC, and 0.05 \times SSC, sequentially for 1 min each, and then spin dried. Hybridized arrays were scanned using a confocal laser-scanning microscope (Affymetrix 428 array scanner, Santa Clara, CA). Image analysis and quantification were performed with ImaGene 4.2 software (BioDiscovery), according to the manufacture's instructions.

Data processing

Each spot was defined by manual positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined, and a local background, outside of 3 pixel buffer range from the circle was computed for each spot. Net signal was determined by subtraction of the local background from the average intensity of each spot. Signal intensities between the two fluorescent images were normalized by the intensities of the house-keeping genes provided on the arrays. The fluorescence intensities of Cy5 (non-tumor) and Cy3 (tumor) for each target

spot were adjusted so that the mean Cy3: Cy5 ratios of 32 housekeeping gene spots were equal to one. Because data derived from low signal intensities are less reliable, we first determined the cutoff values for signal intensities on each slide so that all of the filtered genes had greater S:N (signal to noise) ratios of Cy3 or Cy5 than three, and we excluded genes for further analysis when both Cy3 and Cy5 dyes gave signal intensities lower than the cutoff. To estimate the range of expression ratio within which the expression change could be considered as fluctuation in non-cancerous cells, we compared expression profiles of non-cancerous cells from 6 patients. Because 90% of expression ratios in non-cancerous cells fell within the range of 1.726 and 0.503, we categorized genes into three groups according to their expression ratios (Cy3: Cy5): up-regulated (ratio, 2.0); down-regulated (ratio 0.5); and unchanged expression (ratios, between 0.5 and 2.0); provided that signal counts of T (Cy3) and R (Cy5) were > 500. Genes with Cy3: Cy5 ratios > 2.0 or < 0.5 in more than 75% of the cases examined were defined as commonly up- or down-regulated genes, respectively.

Real-time reverse transcription PCR

LightCycler (Roche Diagnostics) technology was applied to confirm the data obtained by cDNA microarray. The primer sequences of 11 genes were obtained from the GDB Human Genome Database (<http://www.gdb.org/gdb/>) (Table 2). We used the same RNA from the dissected cells for the microarray analysis. First-strand cDNA was obtained by reverse transcription using a commercially available kit (first strand synthesis kit, Amersham). For each PCR, 2 μ L (20 ng) first strand cDNA template, 50 pmol of each primer, 2.4 μ L (3 mmol) MgCl₂, and 2 μ L 10 \times SYBR Green I (Roche Laboratories) were mixed in 20 μ L of PCR mixture. The running protocol was programmed as follows. In the first step, initial denaturation, reaction mixture was incubated for 10 min at 95°C. In the second step, DNA was amplified for 45 cycles at 95°C for 10 s, specific annealing temperature (the primer sequences dependent) for 0-10 s, and elongation at 72°C for some seconds [amplicon (bp)/25 s]. Finally, the temperature was raised gradually (0.2°C/s) from the annealing temperature to 95°C for the melting curve analysis. Twelve μ L of PCR product were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide.

The amount of gene expression was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Human GAPDH kit (GmbH Heidelberg, Heidelberg, Germany). The qRT-PCR analysis was carried out in triplicate for each cDNA sample, and the median values were used for the three experiments. Up- and down-regulation were defined as the median value > 2.0 and < 0.5 , respectively.

Statistical analysis

Statistical analysis among mean values was performed on the association of lymph node metastasis with expression levels by applying non-parametric Kruskal-Wallis and Mann-Whitney *U* tests. Statistical significance was defined as a *P*-value of < 0.05 . Differential expression between the groups of HBV-infected and HCV-infected HCC was considered significant, with $P < 0.05$.

RESULTS

Quality analysis of total RNA after LCM and cRNA after T7-based amplification

About 20 slides were prepared from each sample, and the target cells were captured with at least 1000 cells per slide. Consequently, we captured a total of approximately 25000-30000 tumor cells and normal cells for RNA extraction. The quality of total RNA extracted after LCM was assessed by A_{260}/A_{280} and electrophoresis. To be considered for microarray analysis, the RNA samples were required to pass quality control criteria, with integrity of 28S and 18S, and A_{260}/A_{280} greater than 2.0. Products of cDNA synthesis and cRNA were also checked by A_{260}/A_{280} and electrophoresis. The results showed that A_{260}/A_{280} of all the RNA samples met the quality control criteria for sample preparation. Clear image appearance of 28S and 18S of ribosomal RNA was seen under the electropherogram for each total RNA sample, which had to be intact and without degradation. RNA was subjected to two rounds of T7-based RNA amplification after removal of DNA contamination by RNase-free Dnase I treatment as described in the methods section. All RNA samples were successfully amplified by an estimated 250-fold, using T7 RNA polymerase. cDNA synthesis and cRNA showed satisfactory quality control criteria, with 1.5 kb $<$ cDNA $<$ 5.0 kb; 1.0 kb $<$ cRNA $<$ 4.5 kb; and A_{260}/A_{280} ratio of cDNA and cRNA greater than 2, respectively.

Identification of genes related to HBV-positive or HCV-positive status

After reverse transcription, each cDNA probe was labeled with Cy3- or Cy5-conjugated dyes and hybridized to microarray cDNAs with 886 genes. We compared the expression profiles of cancer cells and the corresponding normal cells in each case. A representative scatter plot of microarray analysis of carcinoma cells and non-cancerous tissue in case 20 (HCV-infected HCC) is shown in Figure 1. Up-regulated, down-regulated and unchanged genes are indicated by red, green and blue spots, respectively. We first arranged the relative expression of each gene (Cy3/Cy5 intensity ratio) into one of four categories:

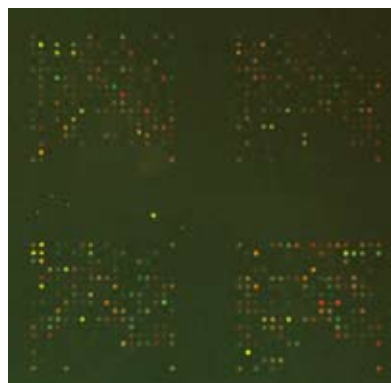


Figure 1 A representative of cDNA microarray expression pattern obtained from case 20. Up-, down-regulated and unchanged genes were indicated by red, green and blue spots, respectively.

up-regulated (ratio > 2.0), down-regulated (ratio < 0.5), unchanged (ratio between 0.5 and 2.0), and not expressed (or slight expression but under the cutoff level for detection).

To identify the genes related to HBV-positive and HCV-positive status, 32 patients were divided into two groups: HBV-associated HCC group in which HBV was positive in 17 patients, and HCV-associated HCC group in which HCV was positive in 15 patients (Table 1). When comparing gene expression profiles in the two groups, there were 7 genes that were commonly up-regulated, and expressed more than 2.09-fold in the HBV-infected group compared with in the HCV-infected group. On the other hand, 4 down-regulated genes in HBV-infected group correlated significantly with the HCV-infected group. Table 3 shows the list of differentially expressed genes and their respective category based on the GO (Gene Ontology) system and TreeView. The up-regulated genes in HBV-infected group were involved in protein synthesis (RPS5), cytoskeletal organization (KRT8), apoptosis related genes (CFLAR), transport (ATP5F1), cell membrane receptor related genes (IGFBP2), signal transduction or transcription related genes (MAP3K5), and metastasis-related genes (MMP9). The up-regulated genes in HCV-infected group included genes such as VIM (cell structure), ACTB (cell structure), GAPD (glycolysis) and CD58 (cell adhesion).

Verification of the genes related to HBV- and HCV-associated HCC using quantitative RT-PCR

To more quantitatively examine our data on hepatitis virus infection in HCC, we selected 7 up-regulated genes from the HBV-infected group, and 4 up-regulated genes from the HCV-infected group. The expression level of the selected genes was confirmed by quantitative RTPCR analysis in 32 patients. We used cDNA synthesized from 32 pair samples without amplification as template for real-time semiquantitative reverse transcription PCR. The results demonstrated that the samples obtained by means of T7-based amplification appropriately reflected the status of the original RNA in a proportional manner. The results of the DNA microarray were reproduced by reverse transcriptase PCR.

DISCUSSION

Genome-wide gene expression analysis of human cancer

Table 3 The 11 genes for which expression levels differed between HBV- and HCV-associated HCC

Gene name	Symbol ¹	Accession ²	Fold change ³	HBV:HCV ⁴
7 genes up-regulated in HBV-associated HCC				
Ribosomal protein S5	RPS5	NM_001009	6.35	2.38
Keratin 8	KRT8	NM_002273	5.68	3.19
CASP8 and FADD-like apoptosis regulator	CFLAR	Y14039	2.86	2.09
ATP synthase, H+transporting, mitochondrial F0 complex, subunit b, isoform 1	ATP5F1	X60221	4.11	3.52
Insulin-like growth factor binding protein 2 (36 kDa)	IGFBP2	NM_000597	3.37	2.49
Mitogen-activated protein kinase kinase kinase 5	MAP3K5	NM_005923	3.76	2.33
Matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	MMP9	NM_004994	7.43	3.74
4 genes up-regulated in HCV-associated HCC				
Vimentin	VIM	NM_003380	8.61	0.28
Actin-β	ACTB	X00351	4.13	0.37
Glyceraldehyde-3-phosphate_dehydrogenase	GAPD	NM_002046	5.27	0.29
CD58 antigen, (lymphocyte function-associated antigen 3)	CD58	NM_001779	4.68	0.31

¹Symbol in LocusLink database; ²GeneBank accession number; ³Fold change, ratio of mean expression values in HBV- or HCV-associated cases (cancer cells vs non-cancerous cells); ⁴HBV:HCV ratio of mean expression values (HBV positive cases to HCV positive cases).

may provide important clues in understanding HCC oncogenesis and may lead to improvement in predicting its clinical behavior^[12]. Using cDNA microarray, we examined the difference in gene expression profiles between normal liver tissues and HCC cells, as well as between HBV positive and HCV-associated HCC. The data from cDNA microarray are consistent with RT-PCR data from HCC tissues and the corresponding non-tumor tissues. These expression profiles may be useful in elucidating the molecular carcinogenesis of HCC, especially HBV- and HCV-associated HCC.

In the present study, we attempted to establish a link between gene expression and the viral status of HCC. Comparative analysis of HBV- and HCV-associated HCC revealed that 11 genes, for which the expression levels differed between HBV- and HCV-associated HCC. Ribosomal-related genes such as RPS5 (RPL family genes) were up-regulated in HBV-associated HCC compared to HCV-associated HCC, suggesting the activation of protein translation in HBV-infected HCC. This observation is consistent with a previous report that major classes of genes encoding ribosomal proteins were up-regulated by the HBX protein^[13]. Cytoskeletal organization, such as KRT8 was shown to be up-regulated in HBV-associated HCC, as well as genes such as ACTB in HCV-associated HCC. Our results support the hypothesis that the deregulation of genes encoding proteins associated with cytoskeleton play a role in liver carcinogenesis^[14]. These findings also indicate that the pathway for liver carcinogenesis in the cytoskeleton may be different in HBV- and HCV-associated HCC. Cell adhesion genes such as CD58 were found to be up-regulated in HCV-associated HCC, but have not been reported to be related with human HBV-associated HCC. Xu *et al*^[15] showed that several signal transduction related genes, including MAPK family genes were up-regulated in HBV-associated HCC. Up-regulation of MAPK has also been suggested as a common pathway for hepatocarcinogenesis caused by HBV and HCV infections^[16]. In the present study, MAP3K5 was up-regulated in HBV-associated HCC compared with the non-tumorous liver tissue. However, MAP3K5 was down-regulated

in HCV-associated HCC compared with the non-tumorous liver. Thus, additional studies are necessary to clarify the contribution of the MAPK pathway to each type of HCC. MMP9, which may promote metastasis, was up-regulated in HBV-associated HCC compared with HCV-associated HCC. Other genes such as IGFBP2, ATP5F1, VIM and GAPD, which are expressed differently in HBV- and HCV-associated HCC, were newly identified, although the findings of up-regulation of genes such as IGFBP2 and ATP5F1 in the HBV-associated HCC, and the up-regulation of genes such as VIM and GAPD in the HCV-associated HCC, were in agreement with previous observations^[17]. It has been suggested that liver carcinogenesis induced by HBV and HCV, in addition to common genetic and epigenetic alterations, may involve distinct pathways^[18]. Our expression profiles suggest that hepatitis viruses affect the expression of dozens of genes in HCC in a type-specific manner, thus invoking slightly different mechanisms of carcinogenesis. We believe that the results obtained in the present study will help our understanding of the molecular mechanisms underlying the pathogenesis of HBV- and HCV-associated HCC. The identification of genes defining virus type-specific expression profiles may contribute to our ability to develop virus type-dependent treatment regimens.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common fatal cancers worldwide. The major risk factors for HCC are chronic hepatitis resulting from infection with hepatitis B virus (HBV) and hepatocellular carcinoma (HCC), and exposure to various exogenous carcinogens, including aflatoxin B1. It has been reported that the incidence of HCC is increasing in several countries in parallel with the increase in chronic HBV and HCV infections. Therefore, clarification of the genetic portraits of hepatocarcinogenesis caused by HBV and HCV infection may provide clues to reducing the incidence of HCC, and establishing effective treatments for each type of HCC. However, the molecular nature of this association is poorly understood.

Research frontier

The aim of the present study was to identify any useful biomarkers indicating virus-specific hepatocarcinogenic process. The differential mRNA expression in 32 patients with HBV/HCV-associated HCC was investigated by means of

cDNA microarrays comprising of 886 genes.

Innovations and breakthroughs

It has been suggested that liver carcinogenesis induced by HBV and HCV, in addition to common genetic and epigenetic alterations, may involve distinct pathways. The results of the present study suggest that hepatitis viruses affect the expression of dozens of genes in HCC in a type-specific manner, thus invoking slightly different mechanisms of carcinogenesis. Genome-wide gene expression analysis of human cancer may provide important clues to understanding HCC oncogenesis and lead to improvements in predicting its clinical behavior.

Applications

We believe that the results obtained in this study will provide greater understanding of the molecular mechanisms underlying the pathogenesis of HBV- and HCV-associated HCC. The identification of genes defining virus type-specific expression profiles may contribute to our ability to develop virus type-dependent treatment regimens.

Terminology

DNA microarray is a meticulous technology used in molecular biology and in the field of biomedicine. This technique involves an arrayed series of thousands of microscopic spots of DNA oligonucleotides. It may involve a short section of a gene or other DNA elements that are used as probes to hybridize cDNA or cRNA samples (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Peer review

This is a nice study on the changes in the expression patterns in cancer liver tissue associated with two different hepatitis viruses involved in hepatocarcinogenesis. The paper is well written and contains valuable data. The authors, using microarray technology, have compared gene expression between cancerous and non-cancerous liver tissue in both HBV and HCV infected patients. Seven genes were up-regulated in HBV and 4 genes, which were different, were up-regulated in HCV infected patients.

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CASE REPORT

Endoscopic ultrasound-guided choledochoduodenostomy in patients with failed endoscopic retrograde cholangiopancreatography

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the choledochoduodenostomy site. Although further studies and development of devices are mandatory, EUS-guided choledochoduodenostomy appears to be an effective alternative to ERCP in selected cases.

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Key words: Endoscopic ultrasonography-guided fine needle aspiration; Biliary drainage; Endoscopic retrograde cholangiopancreatography

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Abstract

Endoscopic ultrasonography (EUS)-guided biliary drainage was performed for treatment of patients who have obstructive jaundice in cases of failed endoscopic retrograde cholangiopancreatography (ERCP). In the present study, we introduced the feasibility and outcome of EUS-guided choledochoduodenostomy in four patients who failed in ERCP. We performed the procedure in 2 papilla of Vater, including one resectable case, and 2 cases of cancer of the head of pancreas. Using a curved linear array echoendoscope, a 19 G needle or a needle knife was punctured transduodenally into the bile duct under EUS visualization. Using a biliary catheter for dilation, or papillary balloon dilator, a 7-Fr plastic stent was inserted through the choledochoduodenostomy site into the extrahepatic bile duct. In 3 (75%) of 4 cases, an indwelling plastic stent was placed, and in one case in which the stent could not be advanced into the bile duct, a naso-biliary drainage tube was placed instead. In all cases, the obstructive jaundice rapidly improved after the procedure. Focal peritonitis and bleeding not requiring blood transfusion was seen in one case. In this case, pancreatoduodenectomy was performed and the surgical findings revealed severe adhesion around

INTRODUCTION

Endoscopic transpapillary biliary stenting is the most common procedure for biliary drainage in patients with obstructive jaundice. However, there are patients who failed to achieve bile duct access because of failed biliary cannulation or an inaccessible papilla due to severe duodenal stenosis caused by tumor invasion. Percutaneous transhepatic biliary drainage (PTBD) or surgical intervention is required in such cases. Both methods have a higher morbidity and mortality than endoscopic methods^[1-5]. Recently, endoscopic ultrasonography (EUS)-guided choledochoduodenostomy has been reported as an alternative biliary drainage technique^[6-10]. The aim of the study is to evaluate the potential role of EUS-guided choledochoduodenostomy in the biliary drainage.

CASE REPORT

This series includes all procedures performed at our institution between June 2005 and January 2008. At first,

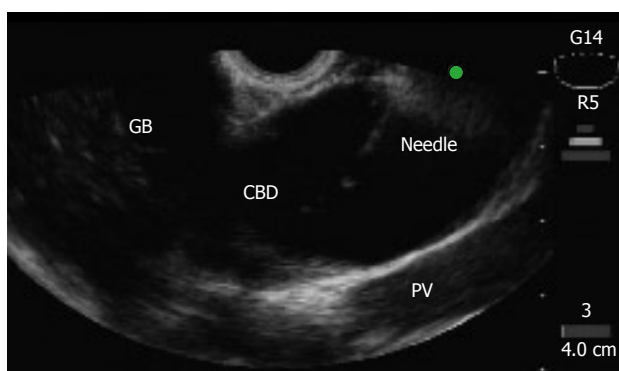


Figure 1 Convex echoendoscope clearly depicts the extrahepatic bile duct (green) (patient 3). GB: Gallbladder; CBD: Common bile duct; PV: Portal vein.



Figure 2 After EUS-guided puncture, contrast medium is injected into the bile duct for cholangiogram, a 0.035-inch guidewire was inserted into the outer sheath (patient 3).

we attempted endoscopic retrograde cholangiography (ERCP) in all patients. If standard ERCP techniques failed, precut sphincterotomy was used for biliary cannulation. The EUS-guided choledochoduodenostomy was performed only in case of failed biliary cannulation or inaccessible papilla due to severe duodenal stenosis caused by tumor invasion. Using an echoendoscope with a curved linear array transducer, and a 3.7-mm accessory channel with an elevator (GF-UCT2000-OL5, Olympus Medical Systems Co. Ltd, Tokyo, Japan), the extrahepatic bile duct was visualized at the level of the duodenal bulb (Figure 1). A 19-gauge needle (EchoTip, Wilson-Cook, Winston-Salem, NC) without electrocoagulation, or a needle knife (Zimmon papillotomomy knife, Wilson-Cook) with electrocoagulation (EndoCut ICC200, ERBE ELEKTROMEDIZIN GmbH, Tübingen, Germany) was inserted transduodenally into the bile duct under EUS visualization. After the central needle is removed, bile is aspirated and contrast medium is injected into the bile duct for cholangiography, and 450 cm long, a 0.035-inch guide wire is inserted into the outer sheath (Figure 2). If necessary, a biliary catheter for dilation, or papillary balloon dilator is used for dilation of duodenocholedochostomy site. Finally, a 7-Fr biliary plastic stent (FLEXIMA, Boston Scientific Japan, Tokyo, Japan) was inserted through the choledochoduodenostomy site into the extrahepatic bile

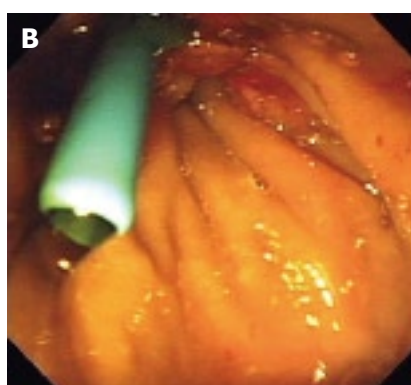
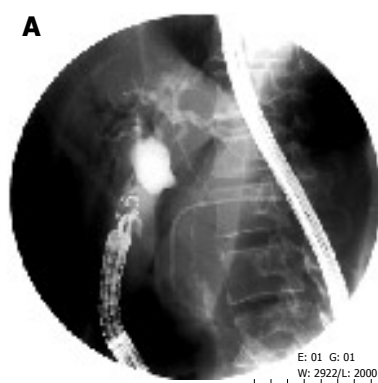


Figure 3 **A:** Choledochoduodenostomy was accomplished with a 7-Fr plastic stent in the apex of the duodenal bulb (patient 3); **B:** The stent was visible in the duodenal bulb (patient 3).

duct (Figure 3).

After written informed consent was obtained from patients, all endoscopic procedures were performed with the patients under conscious sedation with intravenous flunitrazepam (5-10 mg). As for antibiotics, 1 g cefotiam hydrochloride was administered by intravenous drip infusion once on the test day and twice on the following day. This study was approved by the institutional review board of our institution.

Patient 1

An 80-year-old man was admitted for treatment of obstructive jaundice. Tumor invasion to the duodenal wall at the circumference of the major papilla was detected when the procedure was impossible. Biopsy specimens from the major duodenal papilla revealed adenocarcinoma of the papilla of Vater. Using a Zimmon needle knife, EUS-guide choledochoduodenostomy was performed. After dilation by a Soehendra dilator catheter (SBDC-7 and SBDC-9, Wilson-Cook), an indwelling 7-Fr plastic stent was placed across the choledochoduodenostomy site into the extrahepatic bile duct without any complications. The obstructive jaundice rapidly improved after insertion of the biliary stent. The stent did not occlude, and the patient died of pneumonia 3 mo after the procedure.

Patient 2

A 71-year-old man admitted for abdominal pain and obstructive jaundice. Computer tomography (CT)



Figure 4 Choledochoduodenostomy was accomplished using a 5-Fr naso-biliary tube (patient 2).

showed a huge papilla of Vater tumor with a dilated bile duct and mild pancreatitis. Biopsy specimens from the major duodenal papilla revealed adenocarcinoma of the papilla of Vater. Imaging revealed that the tumor was resectable. However, since the biliary cannulation for biliary decompression was impossible because the tumor occupied the duodenal lumen, EUS-guided choledochoduodenostomy was performed. After puncture using a Zimmon needle knife, dilation of the choledochoduodenostomy site by a Soehendra dilator catheter was performed. Subsequently, we attempted to insert a 7-Fr plastic stent from the first portion of duodenum into the extrahepatic bile duct, but stent insertion was impossible because the tip of the stent became impacted in the bile duct site of choledochoduodenostomy. Although balloon dilation was performed across the choledochoduodenostomy site, a 7-Fr stent could not be inserted, therefore, a 5-Fr naso-biliary drainage tube was inserted into the left intrahepatic bile duct (Figure 4). Although the obstructive jaundice rapidly improved after the procedure, there was bleeding not requiring blood transfusion and smoldering focal peritonitis of choledochoduodenostomy site. No evidence of intra-abdominal bile leak was found by several cholangiography procedures via the naso-biliary drainage tube. Pancreatoduodenectomy 16 d after the procedure revealed severe adhesion around the choledochoduodenostomy site although choledochoduodenostomy was completed (Figure 5A). Histological examination revealed mild inflammatory cell infiltrate adjacent to the sinus tract in the duodenal and bile duct walls (Figure 5B-D). The patient is presently healthy 13 mo after surgery.

Patient 3

A 69-year-old man with a history of chronic pancreatitis and placement of pancreatic stent for the treatment of abdominal pain, was admitted with obstructive jaundice. ERCP was impossible because the duodenoscope could not pass through a severe stricture. EUS-FNA specimens from pancreas mass showed adenocarcinoma. Using a 19-gauge needle (EchoTip, Wilson-Cook), EUS-guided choledochoduodenostomy was performed. After dilation by a Soehendra dilator catheter, a 7-Fr plastic

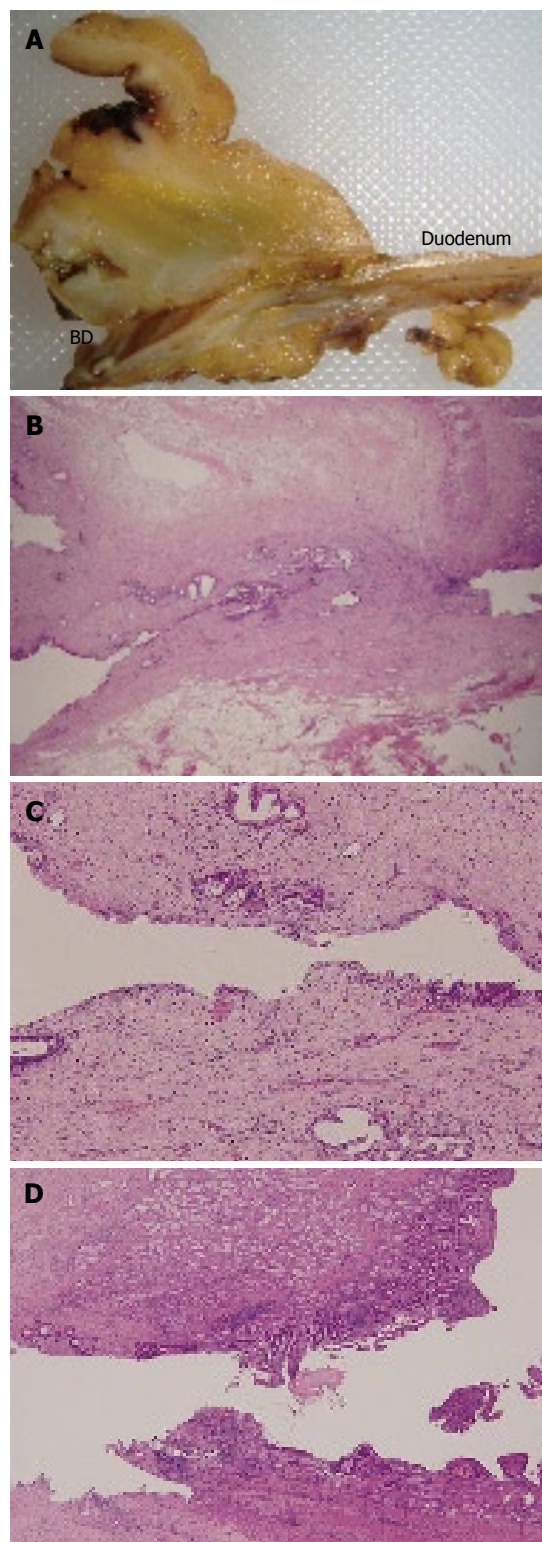


Figure 5 A: Macroscopic view of completed choledochoduodenostomy (patient 2); B: Surgical specimens showing the completed choledochoduodenostomy with mild inflammatory cell infiltrate adjacent to the sinus tract in the duodenal and bile duct walls (HE, $\times 20$, patient 2); C: Magnification of bile duct site (HE, $\times 100$, patient 2); D: Magnification of bile duct site (HE, $\times 100$, patient 2). BD: Bile duct.

stent was placed into the extrahepatic bile duct without any complications. The obstructive jaundice rapidly improved after insertion of the biliary stent. Since acute cholangitis occurred 2 wk after the procedure due to stent clogging, we replaced the plastic stent

by a self-expandable metallic stent (SEMS) using a guide wire plus snare forceps technique because the choledochoduodenal fistula seemed to be incomplete. Actually, after placement of the first 10-Fr uncovered SEMS (Niti-S, Teung Medical Co. Ltd. Seoul, Korea), contrast medium flowed out of the bile duct to the intra-abdominal space. A second 10-Fr covered SEMS (Combi-S, Teung Medical Co. Ltd.), therefore, was placed in the first SEMS. No complications occurred and the patient had no symptoms 3 mo later at follow-up.

Patient 4

An 86-year-old woman with unresectable cancer of the head of the pancreas who had undergone biliary stenting 1 mo previously, was admitted with obstructive jaundice. At initial ERCP, an indwelling 10-Fr plastic stent (FLEXIMA, Boston Scientific Japan, Tokyo, Japan) was placed. ERCP was impossible because the duodenoscope could not pass through the tight stricture caused by cancer invasion. Then, EUS-guided choledochoduodenostomy was performed. Using a 19-gauge needle (EchoTip, Wilson-Cook), EUS-guided choledochoduodenostomy was performed. After dilation by a Soehendra dilator catheter, a 7-Fr plastic stent was placed into the extrahepatic bile duct without any complications. The obstructive jaundice rapidly improved after insertion of the biliary stent. No complications occurred and the patients had no related symptoms till she died 1 mo later.

DISCUSSION

Endoscopic biliary stent placement is the most well-established method for the treatment of obstructive jaundice^[11]. When ERCP fails, usually, PTBD is chosen as an alternative method for treating biliary decompression^[12]. Recently, EUS-guided biliary drainage using either direct access or a rendezvous technique, has attracted attention as an alternative procedure to ERCP or PTCS^[6-10,13-17]. Until now, of these EUS-guided biliary drainage procedures, EUS-guided choledochoduodenostomy has been performed in 17 cases consisting of 11 pancreatic cancer, 4 papilla of Vater cancer, 1 bile duct cancer, and 1 bile duct stone^[6-10].

The methodology and devices of EUS-guided choledochoduodenostomy are not yet fully established. Therefore, there are several important factors during the procedure to ensure technical success. First, as Yamao *et al*^[9] mentioned, the scope position and puncture site are very important. Theoretically, the scope pushing position at which the tip of the convex transducer is directed at the hepatic hilum, is promising because the access route to the bile duct is shorter from the duodenal bulb to the bile duct and the echoendoscope is stable when several devices are advanced into the bile duct through the working channel^[9]. However, when there is duodenal stenosis due to tumor invasion, anatomically abnormal situation after surgery, the same scope position may not be always possible. Second, the

type of puncture needle also may be one of the most important factors. Several reports describe that various types of needle knife are used for puncture in all but two cases (88%, 15/17)^[6-10]. Although a needle knife could make a larger hole compared to a fine-needle aspiration (FNA) needle which needs dilators or balloon dilation for the subsequent procedure, a larger hole may lead to possible intra-abdominal bile leak. In the present series, we used both a FNA needle and a needle knife with electrical coagulation. Unfortunately, one case using a needle knife failed in stent insertion despite making a comparatively large hole and also using a dilator and balloon. The main reason for this may be that the tip of the convex transducer was directed not at the hepatic hilum but the distal bile duct. In this case, the direction of the guidewire was changed from distal bile duct to hepatic hilum to enable technical success. Therefore, it may be possible that the choledochoduodenostomy site became kinked and the stent could not torque adequately because of the instabilization of the scope. These data suggest that technical success of EUS-guided choledochoduodenostomy may depend not on the choice of needle, but mainly the scope position and direction of puncture.

Several investigators used various diameter plastic stents^[6,7,9,10,18] or a SEMS^[8]. Although the stent diameter depends on the echoendoscope used, large bore plastic stent may be better for long patency, similar to the conventional transpapillary plastic stent. Recently, Yamao *et al*^[9] has reported that the mean stent patency of 7- 8.5-Fr plastic stents for EUS-guided choledochoduodenostomy was 211.8 d. Since there are few data on SEMS in this procedure, the usefulness of SEMS should be evaluated from the aspect of cost effectiveness including the issue of the use of uncovered or covered SEMS.

Previous data revealed that the procedure was successful in all but 2 cases (a total success rate of 88%) and once stents were placed, all patients had successful resolution of biliary decompression. In the present study, in all patients, the obstructive jaundice rapidly improved after insertion of the biliary stent. These data suggest that this procedure may be as effective as conventional transpapillary biliary drainage once the indwelling stent is placed.

Surprisingly, no serious procedure-related complications were found in several reports although there was 1 case of mild focal bile peritonitis and 3 of pneumoperitoneum^[6-10]. In the current study, we encountered 1 focal peritonitis with bleeding without severe complications. However, we must be cautious in evaluating the safety of the procedure because previous published data may be biased towards successful cases.

In conclusion, we report four cases of EUS-guided choledochoduodenostomy for the treatment of obstructive jaundice. This procedure was performed successfully, without severe complications and with highly effective biliary drainage. Although further studies and development of devices are necessary, EUS-guided choledochoduodenostomy can be an effective alternative to ERCP.

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A unique case of collagenous colitis presenting as protein-losing enteropathy successfully treated with prednisolone

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INTRODUCTION

Since the first report in 1976^[1], collagenous colitis has been found to be associated with a variety of conditions, including use of non-steroidal anti-inflammatory drugs^[2] and proton pump inhibitors^[3]. This condition is characterized by chronic watery diarrhea and abnormal deposition of collagen beneath the colonic epithelium^[4]. Severe hypoproteinemia due to enteric protein loss is rare unless accompanied by small bowel malabsorption syndrome, such as celiac sprue. To the best of our knowledge, only 1 case of collagenous colitis associated with protein-losing enteropathy (PLE) in the absence of small bowel disease has been reported^[5]. We report herein another case of collagenous colitis associated with PLE without small bowel disease, in which PLE was diagnosed by 99mTc-human albumin (HSA) scintigraphy.

CASE REPORT

A 76-year-old woman with a 5-mo history of recurrent diarrhea and generalized edema was admitted to our hospital. Repeated colonoscopies and gastrointestinal scope at a previous hospital had shown no abnormal changes in the gastrointestinal mucosa. The patient had been taking 180 mg of loxoprofen sodium and 30 mg of lansoprazole every day for the past 3 years, for osteoarthritis of the knees, but had otherwise been healthy with no prior history of gastrointestinal disease. Family history was unremarkable.

On admission, weight was 53 kg and height was 144 cm. She was afebrile, with a blood pressure of 128/84 mmHg and a heart rate of 82 beats/min. The right lower abdominal quadrant was slightly painful on palpation, the face was swollen, and pretibial pitting edema was also present. No superficial lymph nodes

Abstract

A 76-year-old woman with a 5-mo history of recurrent diarrhea and generalized edema was admitted to our hospital. Colonoscopy revealed edematous mucosa, and histopathological examination was compatible with collagenous colitis. Protein leakage from the colon, particularly in the ascending portion, was identified on 99mTc-human serum albumin scintigraphy. Collagenous colitis associated with protein-losing enteropathy (PLE) without small bowel disease was diagnosed. Prednisolone treatment ameliorated diarrhea and hypoproteinemia. Collagenous colitis should be included in the differential diagnosis of chronic diarrhea with hypoproteinemia for appropriate management.

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Key words: Collagenous colitis; Protein-losing enteropathy; Prednisolone; 99mTc-human serum albumin scintigraphy; chronic diarrhea

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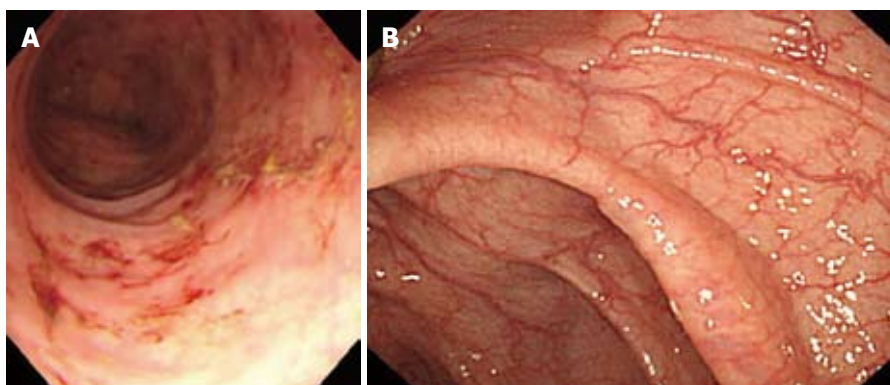


Figure 1 Colonoscopy shows an edematous mucosa, diminished vascular transparency throughout the colon on admission. Friability of mucosa and multiple erythema were observed (A). Endoscopic findings 1 mo after treatment showed normal mucosa (B).

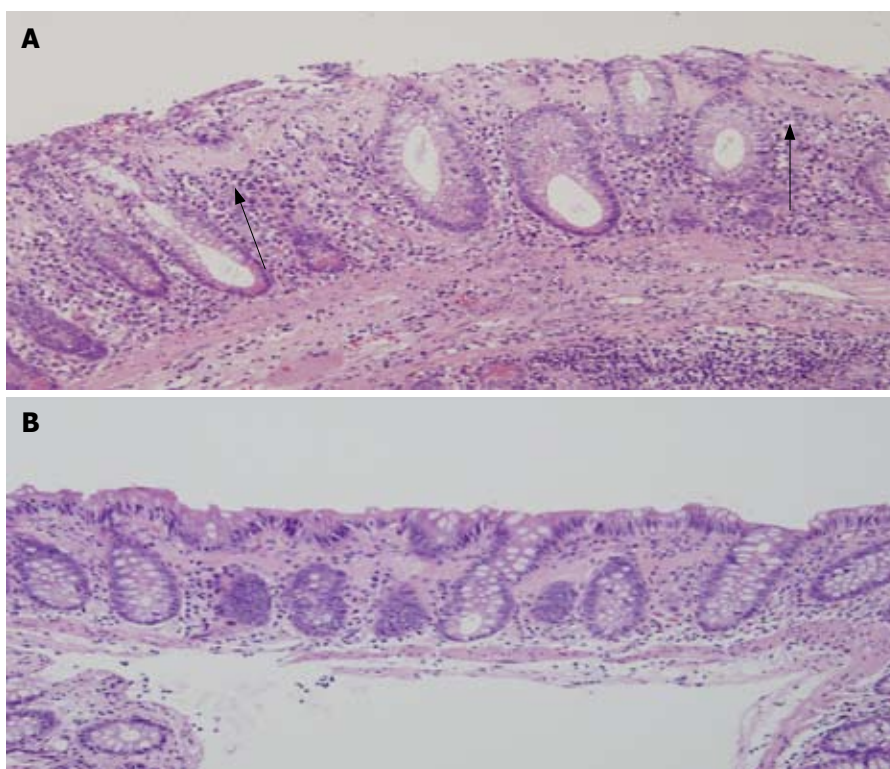


Figure 2 Histology of the biopsied specimen demonstrates subepithelial from eosinophilic band-like deposit (arrows), with increased lymphocytes and plasma cells. Sloughing of surface epithelium is also shown (A). Epithelial detachment and inflammatory cells decreased, although the collagen band beneath the mucosa was not reduced (B).

were palpable. Laboratory investigations revealed: white blood cells, 7960/mm³; C-reactive protein, 0.31 mg/dL; total protein, 4.8 g/dL; plasma albumin, 2.8 mg/dL; no abnormalities in liver, renal, thyroid and adrenal function tests; negative serology for rheumatoid factor; normal results of urinalysis and no proteinuria. Moreover, no steatorrhea was identified, indicating that malabsorption syndrome was unlikely.

Endoscopic examination of the lower intestinal tracts showed edematous mucosa, diminished vascular transparency and multiple areas of erythema (Figure 1A). Multiple biopsies revealed changes consistent with collagenous colitis, showing prominent subepithelial eosinophilic band-like deposits with increased lymphocytes and plasma cells (Figure 2A). The distribution of the disease was whole colon and the findings were worse in the right compared to the left colon. The thickness of the collagen layer was 80-100 μ m in the right and 40-50 μ m in the left. There were no areas of cryptitis, crypt abscesses and no area of superimposed infection inflammatory bowel

disease. Sloughing of surface epithelium was also identified. Endoscopic examination and biopsies of other portion of the gastrointestinal tract showed no obvious abnormalities in the stomach and the small intestine, and there was no evidence of celiac sprue. The duodenum showed normal villi and no obvious lymphocyte infiltration. Small bowel barium study showed no abnormalities. On 99mTc HSA scintigraphy, protein leakage was detected throughout the whole colon, particularly in the ascending portion (Figure 3A). No leakage was apparent from the stomach or small intestine. Neither chest/abdominal computed tomography (CT) nor ⁶⁷Ga scintigraphy showed any evidence of malignancy. On the basis of these findings, collagenous colitis associated with PLE was diagnosed.

After diagnosis, loxoprofen sodium and lansoprazole were discontinued. However, general condition remained unimproved. Administration of 1.8 g polycarbophil calcium, 2 g natural aluminum silicate, 2 g bifidobacterium, 20 mg scopolamine butylbromide, 4 mg loperamide hydrochloride and 2 g albumin tannate

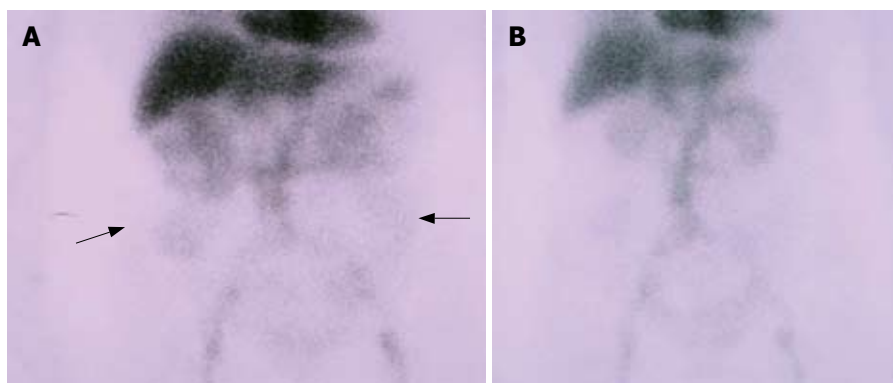


Figure 3 99mTc-human serum albumin scintigraphy shows leakage of the tracer in the large bowel (arrows) on admission (A) but no accumulation 1 mo after starting oral prednisolone (B).

likewise did not improve symptoms, and serum protein and albumin levels remained low despite administration of human serum albumin. Frequency of diarrhea increased and abdominal pain became extremely severe, and the condition of the patient deteriorated. We decided to start prednisolone therapy after hypovolemic shock developed. Soon after initiating prednisolone treatment at 30 mg/d, abdominal pain and diarrhea dramatically improved along with general condition. Total plasma protein and plasma albumin levels gradually increased to 5.4 g/dL and 3.4 g/dL, respectively, by 1 mo after starting prednisolone therapy. Follow-up endoscopy showed normal-appearing colonic mucosa (Figure 1B), and multiple biopsies showed decreased epithelial detachment and inflammatory cells, although the collagen band beneath the mucosa was not reduced (Figure 2B). Additional 99mTc HSA scintigraphy showed no accumulation of tracer in the large bowel, indicating remission of PLE (Figure 3B).

DISCUSSION

This patient presented with chronic diarrhea and anasarca with severe hypoproteinemia, and colonoscopic examination revealed collagenous colitis. We considered that hypoproteinemia was enteric in origin, as no proteinuria, liver disease, cardiac disease or other inflammatory disease was present. Severe enteric protein loss causing hypoproteinemia is reportedly rare in collagenous colitis unless accompanied by small bowel malabsorption syndrome. In the present case, coexistence of malabsorption syndrome was unlikely given the absence of steatorrhea, which is characteristic of malabsorption syndrome. Furthermore, histopathology of the duodenum revealed no evidence of celiac sprue.

The cause of hypoproteinemia was thus considered to be excessive enteric protein loss without malabsorption syndrome. Small bowel barium radiography showed no abnormality such as Crohn's disease or malignant lymphoma, or other diseases that could cause PLE. Furthermore, 99mTc HSA scintigraphy revealed no accumulation of tracer in the small bowel. Considering that no obvious small bowel disease was present, collagenous colitis itself might have been the cause of PLE and 99mTc HSA scintigraphy is

useful in this case^[6,7].

Although the mechanisms of enteric protein loss in patients with collagenous colitis have not been clarified, abnormalities of the surface epithelium^[8], superficial capillaries^[9] and pericryptal fibroblasts^[10] have been hypothesized. Long-term use of loxoprofen sodium and lansoprazole may be associated in the pathogenesis of severity of collagenous colitis in this patient. In the present case, histological examination revealed marked reductions in epithelial detachment together with clinical improvement after prednisolone therapy, suggesting that abnormalities of surface epithelium play an important role in the development of PLE. The endoscopic findings of collagenous colitis are thought to be normal and nonspecific^[11]. In the present case, however, endoscopic examination revealed edematous mucosa, diminished vascular transparency and multiple red spots, and histological examination revealed sloughing of surface epithelium, suggesting this was a severe case of collagenous colitis. This may be one reason why the patient developed PLE.

No established treatments have yet been defined for PLE associated with collagenous colitis^[12]. Corticosteroids are occasionally used for the treatment of collagenous colitis and some studies have shown that prednisolone treatment is ineffective^[13] and budesonide is effective for collagenous colitis without PLE^[14,15]. As oral budesonide is unavailable in Japan, prednisolone treatment was selected because prednisolone has been reported to ameliorate PLE caused by other diseases through the reduction of submucosal edema and inflammation in the gastrointestinal tract. In the present case, oral prednisolone was effective for refractory diarrhea and exudative enteropathy. Corticosteroid was provided at a dose of 30 mg/d, and general status of the patient immediately improved in parallel with a decrease in the frequency of diarrhea and an increase in serum total protein.

Abnormal protein leakage on 99mTc HSA scintigraphy was diminished and biopsy specimens from the colon after treatment histopathologically reflected the efficacy of this therapy in terms of reduced epithelial sloughing and inflammatory cells, although the collagen band under the mucosa was largely unchanged. These results suggest that treatment with prednisolone may be effective for PLE associated with collagenous colitis.

In summary, we have reported a patient with collagenous colitis who presented with PLE. Prednisolone treatment ameliorated diarrhea and hypoproteinemia. Collagenous colitis should be included in the differential diagnosis of chronic diarrhea with hypoproteinemia to achieve appropriate management.

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Pneumatosis cystoides intestinalis following alpha-glucosidase inhibitor treatment: A case report and review of the literature

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Abstract

A 69-year-old man was diagnosed as having myasthenia gravis (MG) in September 2004, and treated with thymectomy and prednisolone. He was then diagnosed as having steroid-induced diabetes mellitus, and received sulfonylurea (SU) therapy in May 2005. An alpha-glucosidase inhibitor (α GI) was added in March 2006, resulting in good glycemic control. He experienced symptoms of abdominal distention, increased flatus, and constipation in October 2007, and was admitted into our hospital in late November with hematochezia. Plain abdominal radiography revealed small linear radiolucent clusters in the wall of the colon. Computed tomography (CT) showed intramural air in the sigmoid colon. Colonoscopy revealed multiple smooth surfaced hemispherical protrusions in the sigmoid colon. The diagnosis of pneumatosis cystoides intestinalis (PCI) was made on the basis of these findings. As the α GI voglibose was suspected as the cause of this patient's PCI, treatment was conservative, ceasing voglibose, with fasting and fluid supplementation. The patient progressed well, and was discharged 2 wk later. Recently, several reports of PCI associated with α GI therapy have been published, predominantly in Japan where

α GIs are commonly used. If the use of α GIs becomes more widespread, we can expect more reports of this condition on a global scale. The possibility of PCI should be considered in diabetic patients complaining of gastrointestinal symptoms, and the gastrointestinal tract should be thoroughly investigated in these patients.

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Key words: Alpha-glucosidase inhibitor; Colonoscopy; Diabetes mellitus; Pneumatosis cystoides intestinalis; Voglibose

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Tsujimoto T, Shioyama E, Moriya K, Kawaratani H, Shirai Y, Toyohara M, Mitoro A, Yamao J, Fujii H, Fukui H. Pneumatosis cystoides intestinalis following alpha-glucosidase inhibitor treatment: A case report and review of the literature. *World J Gastroenterol* 2008; 14(39): 6087-6092 Available from: URL: <http://www.wjgnet.com/1007-9327/14/6087.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6087>

INTRODUCTION

Pneumatosis cystoides intestinalis (PCI) is a rare condition in which multiple submucosal or subserosal pneumocysts develop in the submucosa or in subserosa of the colon^[1,2]. The etiological mechanisms are unclear, although PCI has been reported to develop in association with raised intra-abdominal pressure due to ileus surgery^[3-5], colonoscopy^[6], pulmonary diseases such as chronic bronchitis and emphysema^[7], trichloroethylene exposure^[8], connective tissue disorders^[9,10], the use of immunosuppressants^[11], and ingestion of carbohydrates such as lactulose^[12] and sorbitol^[13]. Recently, the development of PCI during treatment with alpha-glucosidase inhibitors (α GIs), a new class of anti-diabetic agents, has been reported^[14,15]. Our literature search yielded only 13 cases of PCI associated with α GI therapy^[14-26]. Herein, we present a case depicting α GI as the probable cause of PCI, along with a review of the literature.



Figure 1 Plain radiography of the abdomen on admission revealing small linear and round radiolucent clusters in the wall of the colon (black arrows).



Figure 2 Computed tomography (CT) scanning of the abdomen on admission revealing intramural gas in the sigmoid colon (white arrows).

CASE REPORT

A 69-year-old man was diagnosed as having severe myasthenia gravis (MG) in September 2004, and treated with prednisolone (5 mg/d) from October of that year. He underwent thymectomy in March 2005. Hyperglycemia was detected in May 2005, leading to the diagnosis of steroid-induced diabetes mellitus, and sulfonylurea (SU) therapy was commenced immediately. As his blood sugar could not be controlled, α GI was prescribed in March 2006, resulting in good glycemic control. He claimed to have experienced abdominal distension, increased flatus and constipation, and noticed small amounts of bright rectal bleeding as early as mid-October 2007, but did nothing about it. The amount of rectal bleeding increased in late November that year, and he was referred to our hospital for investigation and treatment.

Laboratory investigations revealed no abnormalities in white blood cell (WBC) count, hemoglobin (Hb), or C-reactive protein, and HbA1c was slightly elevated to 6.0%. Plain abdominal radiography revealed small linear radiolucent gas collections along the wall of the colon (Figure 1). Unenhanced computed tomography (CT) of the abdomen showed intramural air in the sigmoid colon, and free gas in the peritoneal cavity around the sigmoid colon (Figure 2). Colonoscopy revealed multiple smooth surfaced small hemispherical protrusions in the sigmoid colon, and endoscopic ultrasonography (EUS) demonstrated highly echogenic submucosal lesions with

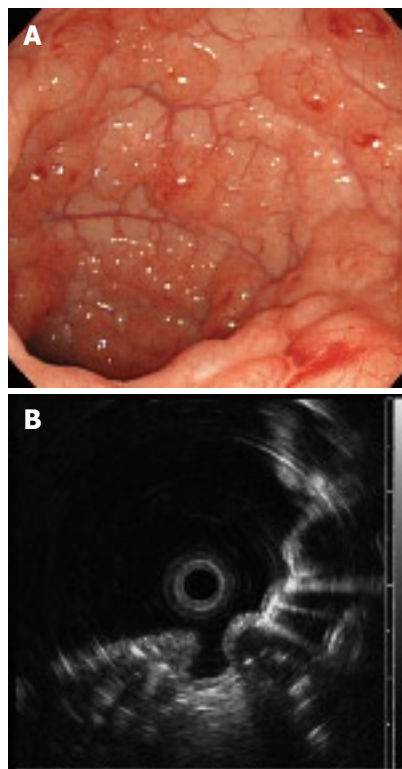


Figure 3 Colonoscopy on admission showing multiple round and smooth-surfaced elevated lesions like submucosal tumors in the sigmoid colon (A) and endoscopic ultrasonography (EUS) revealing hyperechoic lesions and acoustic shadows in the submucosal layer (B).

acoustic shadows (Figure 3). The diagnosis of PCI was made on the basis of these findings.

As voglibose was suspected to be the cause of this patient's PCI, conservative treatment was administered, including ceasing the voglibose, along with fasting and fluid supplementation. The patient progressed well and plain abdominal radiography 2 wk later showed that the linear collections of gas along the wall of the colon were disappeared, and consequently the patient was discharged. Colonoscopy 3 mo later showed complete resorption of the pneumocystis throughout the sigmoid colon, leaving white scars. EUS confirmed disappearance of the submucosal acoustic shadows, indicating the resolution of PCI.

DISCUSSION

PCI, first reported by Du Vernoi^[27] in 1730, is a rare condition in which multiple submucosal or subserosal pneumocystis develop in the submucosa or subserosa of the colon. It was previously thought to occur most frequently in the ileum, but with the recent increase in the number of barium enemas and colonoscopies performed, PCI now reportedly affects the colon more commonly.

There are some recent reports on PCI associated with α GI therapy^[14-26]. The mechanism is thought to involve intestinal gas production through fermentation by the intestinal flora of carbohydrates whose absorption is inhibited by α GI. Along with peristaltic hypofunction

Table 1 A summary of previously reported cases of pneumatosis cystoides intestinalis (PCI) after an alpha-glucosidase inhibitor (α GI) treatment

Case No.	Author Reference number Yr	Age Sex	Chief complaint	The α GI agent Quantity of α GI Dosage period of α GI prior to PCI onset	Disease other than diabetes mellitus	Concomitant drug	Prescription of α GI after PCI onset	Treatment Outcome Duration to the outcome
1	Hayakawa <i>et al</i> 14 1999	64 F	Abdominal distention	Voglibose 0.6 mg/day 1 mo	Unknown	Insulin	Discontinuation	Conservative treatment Healing 4 d
2	Azami 15 2000	87 F	Abdominal distention Appetite loss	Acarbose 150 mg/day 1 yr	Hypothyroidism	SU	Discontinuation	Conservative treatment Healing 5 d
3	Maeda <i>et al</i> 16 2002	55 F	Abdominal distention	Acarbose 300 mg/day 42 d	Pemphigus vulgaris	Insuline steroid Immunosupressant	Continuation	Conservative treatment Healing 141 d
4	Tachibana <i>et al</i> 17 2002	73 F	Abdominal distention	Acarbose 150 mg/day 8 yr	Henoch-Schonlein purpura nephritis	SU Steroid	Discontinuation	Conservative treatment Healing 28 d
5	Yanaru <i>et al</i> 18 2002	61 M	Abdominal distention Constipation Hematochezia	Voglibose 0.6 mg/day 5 yr	Unknown	SU	Discontinuation	Conservative treatment Healing 28 d
6	Matsuda <i>et al</i> 19 2004	62 M	Abdominal pain	Voglibose Unknown Unknown	Lung cancer	Morphine sulfate	Unknown	Operation Remission 16 d
7	Nagahara <i>et al</i> 20 2006	66 M	Left abdominal pain	Acarbose Unknown 11 yr	Unknown	Unknown	Discontinuation	Conservative treatment Healing 21 d
8	Hisamoto <i>et al</i> 21 2006	56 M	No abdominal symptoms	Voglibose 0.6 mg/day 7 d	Interstitial pneumonia	Steroid	Discontinuation	Conservative treatment Healing 7 d
9	Furio <i>et al</i> 22 2006	64 F	Abdominal pain Diarrhea Tenesmus Weight loss	Acarbose Unknown 3 yr	Unknown	Insulin	Discontinuation	Conservative treatment Healing 15 d
10	Miyagawa <i>et al</i> 23 2006	65 M	Abdominal pain Diarrhea	Voglibose 0.6 mg/day 6 years	Gastric cancer	SU	Continuation→Discontinuation	Conservative treatment Healing 120 d
11	Yasuoka <i>et al</i> 24 2007	75 M	Abdominal distention	Voglibose 0.6 mg/day 10 yr	Lung cancer Rectal carcinoid	SU	Discontinuation	Conservative treatment Healing 20 d
12	Maeda <i>et al</i> 25 2007	72 F	Rt lower abdominal pain	Voglibose 0.9 mg/day 3 yr	Minimal change disease	Insulin Steroid Immunosupressant	Discontinuation	Conservative treatment Healing 7 d
13	Saito <i>et al</i> 26 2007	53 F	Abdominal distention Nausea	Voglibose 0.6 mg/day 1 mo	Dermatomyositis	Steroid Immunosupressant	Discontinuation	Conservative treatment Healing 21 d
14	Our case 2008	69 M	Abdominal distention Hematochezia	Voglibose 0.6 mg/day 1 yr 8 mo	Myasthenia gravis	SU Steroid	Discontinuation	Conservative treatment Healing 14 d

associated with diabetes mellitus, this leads to raised intraluminal pressure, allowing the gas-producing bacteria to invade the colonic mucosa through breaks in the mucosal integrity, forming pneumocysts^[14,15].

Our review of the medical literature between 1983 and 2008 yielded 7 cases of PCI associated with α GI

therapy in PubMed [English language; 14, 15, 18, 21, 22, 25, 26], and 6 in Japana Centra Revuo Medicina [Japanese language; 16, 17, 19, 20, 23, 24]. The details of these cases, totaling 14 with the addition of our present case, are shown in Tables 1 and 2. All but 1 of the 14 cases was reported in Japan. About 30% of

Table 2 Imaging findings in previously reported cases of PCI after an α GI treatment

Case No.	Author Reference number Year	Plain radiography of the abdomen	Computed tomography of the abdomen	Barium enema	Colonoscopy
1	Hayakawa <i>et al</i> 14 1999	Distention of the ascending and proximal transverse colon with cystic radiolucencies, indicating intramural gas	Subserosal cystic areas of gas and distention of the involved segments	Translucent areas of gas clustered along the distorted contours of the ascending and transverse colon	ND
2	Azami 15 2000	Noticeable gaseous distension of the small intestine	Noticeable gaseous distention of the small intestine with pockets of small gas bubbles in the submucosal space	No constriction in the sigmoid or lower descending colon	ND
3	Maeda <i>et al</i> 16 2002	Multiple cystic radiolucencies in the abdomen	Pneumatosis intestinalis around the bowel wall and gas within the retroperitoneum	ND	ND
4	Tachibana <i>et al</i> 17 2002	Free gas of the right peritoneal cavity and pneumatosis intestinalis throughout the ascending colon	Free gas below the right diaphragm, and pneumatosis intestinalis throughout the ascending colon	ND	Polypoid lesions in the ascending and transverse colon
5	Yanaru <i>et al</i> 18 2002	Small round radiolucent clusters in the middle abdomen	ND	Numerous submucosal protrusions of sessile of semipedunculated configurations	Numerous submucosal protrusions of sessile of semipedunculated configurations
6	Matsuda <i>et al</i> 19 2004	Noticeable gaseous distention of the colon, and curvilinear radiolucency within the bowel wall	Free gas in the peritoneal cavity, pneumatosis intestinalis throughout the bowel wall	ND	ND
7	Nagahara <i>et al</i> 20 2006	Free air below the diaphragm	Pneumatosis intestinalis throughout the ascending colon	ND	ND
8	Hisamoto <i>et al</i> 21 2006	Free air below the diaphragm, and noticeable gaseous distention of the ascending and transverse colon	Slight dilatation, mesenteric edema, and diffuse pneumatosis intestinalis throughout the ascending colon	Many cystic areas of the ascending colon	Multiple sessile polypoid lesions covered with normal-appearing mucosa in the area from the ascending colon
9	Furio <i>et al</i> 22 2006	ND	The presence of numerous intraparietal cysts, of varying size, diffuse in the varied colic segments, compatible with wall pneumatosis of the colon	ND	Multiple polypoid formations of varying sizes in the sigmoid, descending, ascending and cecum
10	Miyagawa <i>et al</i> 23 2006	Cystic radiolucencies in the colon	ND	Multiple numerous round polypoid lesions from the ascending colon to the sigmoid colon	Numerous round polypoid lesions from the ascending colon to the sigmoid colon
11	Yasuoka <i>et al</i> 24 2007	Noticeable gaseous distension of the small intestine	Free gas in the peritoneal cavity	ND	ND
12	Maeda <i>et al</i> 25 2007	Diffuse air shadows along the intestine suggesting gas accumulation in the bowel wall	Circumferential collections of air adjacent to the bowel lumen that ran parallel to the bowel wall	ND	ND
13	Saito <i>et al</i> 26 2007	Pneumoperitoneum with free air under the diaphragm and curvilinear radiolucency within the bowel wall	Intramural air in the ascending colon, and gas collection in the mesentery	ND	ND
14	Our case 2008	Small linear radiolucent gas collections along the wall of the colon	Intramural air in the sigmoid colon, and free gas in the peritoneal cavity around the sigmoid colon	ND	Multiple smooth surfaced small hemispherical protrusions in the sigmoid colon

ND: Not done.

the Japanese diabetics are prescribed α GIs, which are rarely administered in Western countries where fats account for a larger proportion of the caloric intake than carbohydrates^[28,29]. The preponderance of Japanese reports on α GI-associated PCI is not surprising as the Japanese market accounts for 98% of the total sales of

voglibose, and 34% of those for acarbose.

The mean age of the 14 patients was 65.9 years, while 7 were male and 7 were female. The causative agent was voglibose in 9 cases and acarbose in 5, while none was caused by miglitol. The global market share in 2005 for voglibose and acarbose was in a ratio of

roughly 3:2. Miglitol was not released in Japan until 2006, and accordingly no reports are available on PCI associated with the newest agent. As future cases are reported, we expect that there will be no significant differences in the incidence of PCI between these agents. The mean prescribed dosages were 0.64 mg/d for voglibose and 200 mg/d for acarbose. The interval between commencement of α GI therapy and the onset of PCI varied greatly, ranging from 7 d to 11 years. The most common symptoms were abdominal distention (57%) and abdominal pain (36%), while only 2 cases had hematochezia (14%) as in the case described herein (Table 1).

Different radiological and endoscopic modalities are useful in the diagnosis of PCI. To summarize the imaging findings in the 14 reported cases (Table 2), linear or round radiolucent gas collections were seen along the wall of the colon in plain abdominal radiographs in most cases, and pneumatosis was seen within or along the wall of the colon on abdominal CT scanning. Subserous pneumocystis in particular are liable to rupture, releasing free gas into the peritoneal cavity, making it important to distinguish this condition from bowel perforation^[30]. Multiple rounded protrusions are a common finding in barium enema examinations of patients with PCI. The colonoscopic findings may be similar to multiple polyposis or collections of submucosal tumors, but subserous pneumatosis may go undetected.

With cessation of α GI therapy, conservative treatment could lead to resolution of PCI within 28 d. In the 2 cases where α GI therapy was continued, resolution took more than 120 d. Therefore, ceasing the α GI therapy is the key to successful treatment of PCI. One case underwent emergency surgery due to the presence of free air in the peritoneal cavity, where bowel perforation could not be ruled out^[19]. In our case, there were 2 possible causes, namely, the α GI voglibose and prednisolone. Since our patient claimed to have experienced abdominal distention, increased flatus and constipation prior to the onset of PCI, we considered voglibose the causative agent. We therefore ceased α GI and continued corticosteroid therapy, and kept our patient fasting with fluid supplementation, achieving resolution of PCI after 14 d (Table 1).

The symptoms of PCI include abdominal pain, diarrhea and abdominal distention, none of which is disease specific. Diabetic patients sometimes develop autonomic neuropathy, with gastrointestinal symptoms similar to those of PCI. As α GI therapy is commonly used in Japan, it is difficult to determine whether diabetic patients complaining of gastrointestinal symptoms are suffering only from diabetes mellitus or from PCI. If the clinical picture of diabetes mellitus is consistent with that of PCI, diabetes mellitus can be detected by plain abdominal radiography. The possibility of PCI should be considered in diabetic patients complaining of gastrointestinal symptoms, and appropriate investigations should be performed with this potential diagnosis in mind.

In this report, we presented a case of PCI associated

with α GI therapy, and a review of the literature. Our patient recovered rapidly after conservative treatment, including ceasing of the voglibose, fasting, and fluid supplementation. Recently, several reports on PCI associated with α GI therapy have been published, predominantly from Japan where α GIs are commonly used^[14-21,23,24]. If the use of α GIs becomes more widespread internationally, we can expect more reports of this condition globally. The possibility of PCI should be considered in diabetic patients complaining of gastrointestinal symptoms, and the gastrointestinal tract should be thoroughly investigated in these patients.

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Endoscopic fibrin glue injection for closure of pancreatocutaneous fistula following transgastric endoscopic necrosectomy

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Abstract

Transgastric endoscopic necrosectomy has been recently introduced as the effective and alternative management of infected pancreatic necrosis and pancreatic abscess. However, up to 40% of patients who undergo endoscopic necrosectomy may need an additional percutaneous approach for subsequent peripancreatic fluid collection or non-resolution of pancreatic necrosis. This percutaneous approach may lead to persistent pancreatocutaneous fistula, which remains a serious problem and usually requires prolonged hospitalization, or even open-abdominal surgery. We describe the first case of pancreatocutaneous fistula and concomitant abdominal wall defect following transgastric endoscopic necrosectomy and percutaneous drainage, which were endoscopically closed with fibrin glue injection *via* the necrotic cavity.

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Key words: Fibrin glue; Pancreatocutaneous fistula; Infected pancreatic necrosis; Pancreatic abscess; Endoscopic necrosectomy

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INTRODUCTION

Infected pancreatic necrosis and pancreatic abscess are serious complications of acute pancreatitis, and open necrosectomy is the mainstay of management for these complications^[1]. However, operation-related morbidity and mortality, and longer hospitalization are not uncommon complications^[1,2]. Transgastric endoscopic necrosectomy has been recently introduced as the effective and alternative management for infected pancreatic necrosis and pancreatic abscess^[3-7]. However, up to 40% of patients who undergo endoscopic necrosectomy may need an additional percutaneous approach for subsequent peripancreatic fluid collection or non-resolution of pancreatic necrosis^[8]. This percutaneous approach may lead to persistent pancreatocutaneous fistula, which remains a serious problem and usually requires prolonged hospitalization, or even open-abdominal surgery^[9-12]. We describe the first case of pancreatocutaneous fistula and concomitant abdominal wall defect following transgastric endoscopic necrosectomy and percutaneous drainage, which were endoscopically closed with fibrin glue injection *via* the necrotic cavity.

CASE REPORT

A 63-year-old woman with acute biliary pancreatitis was admitted to our hospital. A computer tomography (CT) scan showed pancreatitis, peripancreatic fluid collection and gallbladder (GB) wall thickening with polypoid lesions. Endoscopic retrograde cholangiopancreatography (ERCP) showed a GB cancer with combined anomalous union of the pancreaticobiliary duct. After interdisciplinary consultation, although the patient suffered from acute biliary pancreatitis with peripancreatic fluid collection,

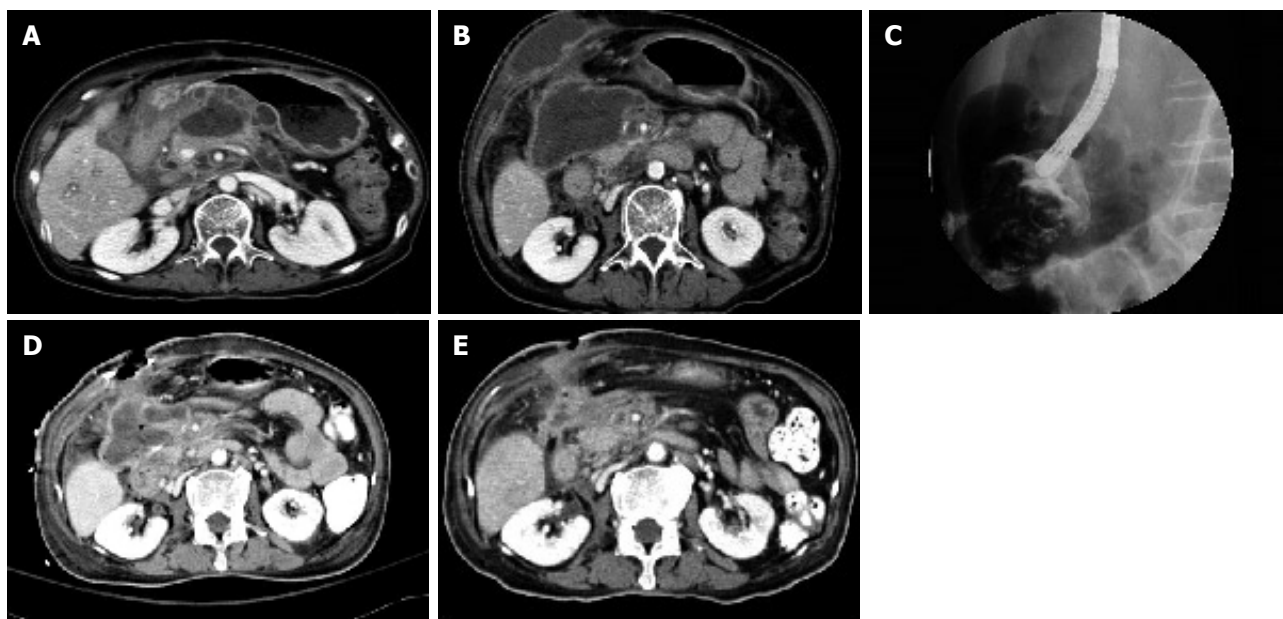


Figure 1 Multiple peripancreatic fluid collections on the body and head portions of the pancreas with a subcutaneous abscess on the right upper quadrant connected to these peripancreatic fluid collections (A, B), fluoroscopy and follow-up CT showing a frank pancreatocutaneous fistula and abdominal wall defects alongside the percutaneous route (C, D), follow-up CT showing nearly resolved infected peripancreatic necrosis, pancreatocutaneous fistula, and abdominal wall defect after endoscopic necrosectomy and repair of the fistula with fibrin glue (E).

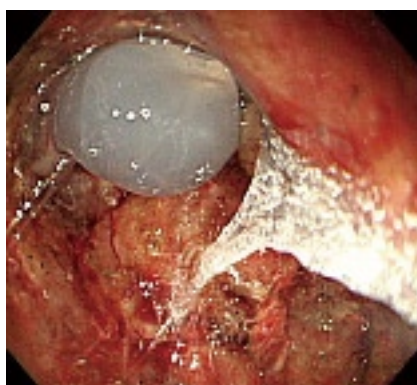


Figure 2 Complete obliteration of the fistula tract after injection of fibrin glue beyond and on the luminal side of the fistula.

we decided to perform an open cholecystectomy. Resected specimen revealed a stage II GB cancer. The patient's postoperative recovery was uneventful.

Two months later, she was readmitted for fever and previous operation wound site bulging, redness, and heat. A CT scan revealed the aggravation of fluid collection in her abdominal wall's right upper quadrant, in addition to peripancreatic fluid collection bulging into the body portion of her stomach (Figure 1A and B). We inserted a percutaneous pig-tail catheter for subcutaneous fluid collection and performed an endoscopic transgastric necrosectomy because the patient refused a surgical intervention.

As our previous reports illustrate^[13,14], a transgastric endoscopic necrosectomy was performed. During endoscopic necrosectomy and saline irrigation with a water-jet scope (GIF-Q 260J; Olympus Optical Co, Tokyo, Japan), skin wound dehiscence at the incision site of open cholecystectomy occurred alongside the percutaneous

route (Figure 1C and D). The patient suffered from discharge (about 100 CC/d) of necrotic materials through the skin wound dehiscence, and extensive skin excoriation. During follow-up endoscopic examination, 50-100 CC of pus with a high concentration of amylase (23220 IU/L) was drained daily through the fistula tract and abdominal wall defect. Subsequent ERCP showed the long common channel of pancreatobiliary duct but no disruption of the main pancreatic duct. Because the surgeon recommended conservative management with additional insertion of a Penrose drain to resolve the wound dehiscence, rather than an operative wound revision, we decided to close the fistula endoscopically. On follow-up endoscopic evaluation, we identified a fistula tract between the necrotic cavity and skin connected to the subcutaneous abscess. The size of fistula was about 1 cm. We endoscopically injected fibrin glue as a sealing material *via* the necrotic cavity (Figure 2). A total of 8 mL of fibrin glue (Tisseel; Baxter, West-lake Village, Calif.) was injected into the fistula tract through a double lumen catheter. The patient showed no post-procedural complications, such as anaphylactic reaction. The day after endoscopic closure of the fistula with fibrin glue injection, no pus was discharged from the pancreatocutaneous fistula. On the fifth day after endoscopic closure of the fistula, the wound's dehiscence was also spontaneously closed, with no additional sutures. A follow-up CT scan also showed near resolution of the infected peripancreatic necrosis and sealing of the pancreatocutaneous fistula and abdominal wall defect (Figure 1E). During a 3-mo follow-up period, no relapse of the pancreatocutaneous fistula occurred.

DISCUSSION

During transgastric endoscopic necrosectomy with

a percutaneous approach, patients may develop a pancreatocutaneous fistula. Although small external fistulae with integrity of pancreatic duct can be spontaneously closed, management of large external fistulae or abdominal wall defects, as in our case, may be cumbersome^[9-11]. During endoscopic necrosectomy, adequate endoscopic irrigation is essential for removal of necrotic tissue^[7]. As in this case, however, saline irrigation with a water-jet scope during endoscopic necrosectomy may be a precipitating factor for frank pancreatocutaneous fistula and wound site dehiscence alongside the percutaneous catheter because the endoscope may block the outflow tract of the cavity during lavage and consequence pressure becomes too high and the wall is perforated^[7]. For closure of this pancreatocutaneous fistula, we selected fibrin glue. Fibrin glue injection has been previously used during endoscopy for wound closure and fistula repair^[15-18]. Fibrin glue is a biologic tissue adhesive based on a combination of fibrinogen and thrombin that forms a cell-free clot, and it has been shown to have tissue-healing properties and to be fully reabsorbed by macrophages and fibroblasts within 2 wk of application^[15,16,19].

High-output (> 500 CC/d) external fistulae have a particularly poor prognosis, and are a potential cause of recurrence compared to low-output (< 200 CC/d) external fistulae^[10,11]. Our patient showed a low-output external fistula and an intact main pancreatic duct on pancreatography. This is why endoscopic fibrin glue injection *via* the necrotic cavity alone is effective for rapid closure of fistula and abdominal wall defect, without further surgical management. Though there are few data about the fistula size, a small size of fistula might have contributed to our complete result and good prognosis. In addition, we did not consider using a hemoclip to close the fistula because its placement *via* the necrotic cavity would have been “in-body” and we were concerned with the difficulty of approximating, and bleeding of the friable necrotic cavity using a hemoclip. After the fistula was closed, no fluid recollection was observed, thus complete removal of necrotic tissue was achieved.

In summary, endoscopic fibrin glue injection *via* necrotic cavity is a safe, less invasive, rapid, and effective technique for closure of pancreatocutaneous fistulae and abdominal wall defects following transgastric necrosectomy with percutaneous drainage. We think that this procedure is proper for low-output (< 200 CC/d), small size (diameter < 1 cm) fistulae, and inner surface should be reached endoscopically. However, some established indications for selecting patients are lack as yet. Therefore, further studies are necessary.

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CASE REPORT

Perforation of the colon by invading recurrent gastrointestinal stromal tumors during sunitinib treatment

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Hur H, Park AR, Jee SB, Jung SE, Kim W, Jeon HM. Perforation of the colon by invading recurrent gastrointestinal stromal tumors during sunitinib treatment. *World J Gastroenterol* 2008; 14(39): 6096-6099 Available from: URL: <http://www.wjg-net.com/1007-9327/14/6096.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.6096>

INTRODUCTION

Sutent (Pfizer, New York, NY) is the malate salt of sunitinib, which is a small molecule that inhibits multiple receptor tyrosine kinases (RTKs)^[1]. This molecular target is the basis for the application of sunitinib in the treatment of gastrointestinal stromal tumors (GISTs), which result from the molecular abnormalities of tyrosine kinases such as KIT^[2]. In addition, the targets of sunitinib involve vascular endothelial growth factor receptors (VEGFR1, VEGFR2 and VEGFR3), platelet-derived growth factor receptors (PDGFR α and PDGFR β) and the like. Although this drug has been approved by the US Food and Drug Administration (FDA) for treatment of GIST patients following progression or resistance to imatinib (Gleevec; Novartis, Switzerland), randomized phase III clinical trials have shown some common adverse effects such as diarrhea, mucositis, abnormal heart function and myelosuppression of imatinib^[3]. However, there has never been a report focused on the relationship between bowel perforation and sunitinib treatment. In the present paper, we describe, for the first time, a case of unexpected colon perforation during sunitinib treatment.

CASE REPORT

Here we report a 70-year-old patient who was initially treated with proximal gastrectomy, distal pancreatectomy, splenectomy and transverse colectomy for a GIST of the stomach that was categorized as high risk in 1993. Eight years later, the GIST relapsed on the gastrohepatic ligament, which led to left hepatectomy

Abstract

The molecular targets of sunitinib are receptor tyrosine kinases (RTKs), and this drug has also been known to exert blocking effects on the activation of KIT, which is similar to the mechanism of action of imatinib. Moreover, sunitinib has an additional anti-angiogenic effect through its inhibition of the vascular endothelial growth factor receptor activation. We report here a 70-year-old patient diagnosed with a recurrent gastrointestinal stromal tumor (GIST), which invaded the transverse colon and led to a perforation during sunitinib treatment. A computed tomography scan and 3-dimensional reconstruction showed necrosis of the recurrent hepatic mass and perforation of the invaded transverse colon. After percutaneous drainage of the intraperitoneal abscess, antibiotic treatment and restricted diet, the condition of the patient improved. The present case is the first to report that sunitinib, which is administered to treat GIST resistant to imatinib, can cause unexpected colon perforation and subsequent peritonitis.

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Key words: Gastrointestinal stromal tumors; Recurrence; Sunitinib; Intestinal perforation

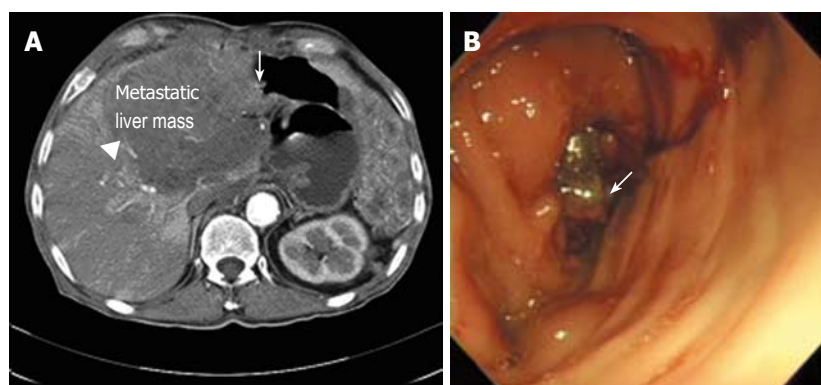


Figure 1 Diagnostic evaluations of recurrent gastrointestinal stromal tumors before starting sunitinib. **A:** In computed tomography, arrowhead and arrow show metastatic liver mass and colon invasion, respectively. **B:** In the colonoscopic finding, arrow shows a protruding mass invading from the external lumen.

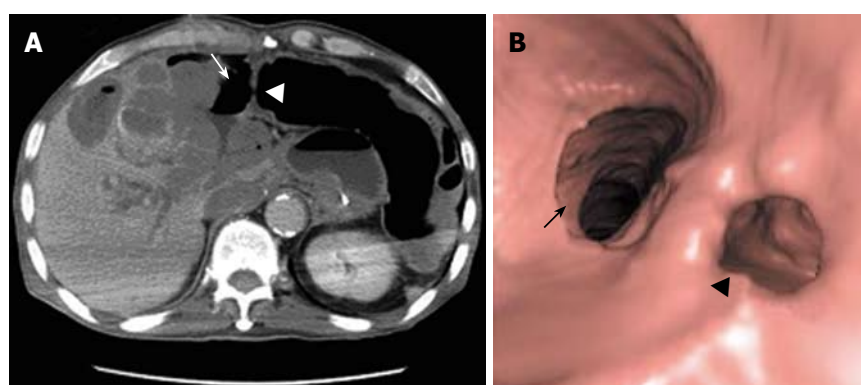


Figure 2 Diagnostic evaluation of perforating colon in present symptomatic patient during sunitinib treatment. **A:** In computed tomography, arrow and arrowhead show intraperitoneal free air and the site of colon perforation, respectively. **B:** In 3-dimensional reconstruction, arrow and arrowhead show the colon lumen and the site of perforation, respectively.

and cholecystectomy to excise the mass curatively. A year later, the tumor recurred in the liver, when imatinib (400 mg/d) was started as the initial treatment. Three years later, a follow-up computed tomography (CT) showed the progression of the hepatic metastasis, which resulted in an escalation in the imatinib dose to 600 mg/d. Unfortunately, two years later in April 2007, the hepatic mass grew so large that it invaded the transverse colon as evaluated by CT and colonoscopy (Figure 1). Consequently, we changed the therapy to oral sunitinib at 50 mg/d with a 4-wk-on and 2-wk-off regimen. He had no combined diseases but a history of smoking with two to thirty packs/year.

In June 2007, the patient was on the second day of the second cycle of sunitinib treatment and complained of diffuse abdominal pain and general prostration upon visiting our emergency room. The patient presented with symptoms and signs of localized peritonitis on the right side of the abdomen. The systolic and diastolic blood pressures were 90 mmHg and 60 mmHg, respectively, pulse rate was 120 per min and respiratory rate was 25 per min. The body temperature was 37.3°C. Laboratory studies were conducted immediately after the patient's arrival at the emergency room. He had anemia (Hb 98 g/L) (normal range: 140-180) and thrombocytopenia ($83 \times 10^9/L$) (normal range: 150-450), but a WBC count was normal ($7.25 \times 10^3/mm^3$) with 84% neutrophils. Other laboratory findings were presented as high serum levels of CRP (293.07 mg/L) (normal range: < 5), ALP (416 IU/L) (normal range: 96-254), slightly increased BUN (34 $\mu\text{mol/L}$) (normal range: 8-23) and creatinine (1.53 $\mu\text{mol/L}$) (normal range: 0.5-1.2), low level of sodium (127 mEq/L) (normal range: 135-148),

potassium (2.8 mEq/L) (normal range: 3.5-5.1) and serum albumin (2.17 g/dL) (normal range: 3.8-5.3), and normal range of GOT (15 IU/L) (normal range: 13-36), GPT (8 IU/L) (normal range: 5-33). A CT scan showed necrosis of the recurrent hepatic mass and perforation of the invaded transverse colon, which led to intraperitoneal air and pus collection (Figure 2A). Three-dimensional reconstruction also revealed colonic perforation (Figure 2B). We could not perform the operation for a correction of colon perforation owing to the poor patient's condition. With percutaneous drainage of the intraperitoneal abscess under guided ultrasonography, the patient's diet was restricted to parenteral nutrition, intravenous fluid administration for correction of dehydration, and both cefoperazone (4 g/d, intravenous) and metronidazole (1500 mg/d, intravenous) were injected for 2 wk. After drainage procedure and conservative treatments, the patient's condition was fortunately improved without operation and laboratory findings 2 wk after treatment were decreased serum CRP level (70.43 mg/L), normal range of platelets ($413 \times 10^9/L$), BUN (11.3 $\mu\text{mol/L}$), creatinine (0.72 $\mu\text{mol/L}$), albumin (3.23 g/dL), sodium (139 mEq/L) and potassium (3.6 mEq/L) (Figure 3). The patient was discharged from the hospital after his condition improved. After completion of the second cycle, the disease was stable according to the response evaluation criteria in the solid tumor group (RECIST) with a 23% decrease in the diameter of the mass.

DISCUSSION

GIST is the most common mesenchymal neoplasm

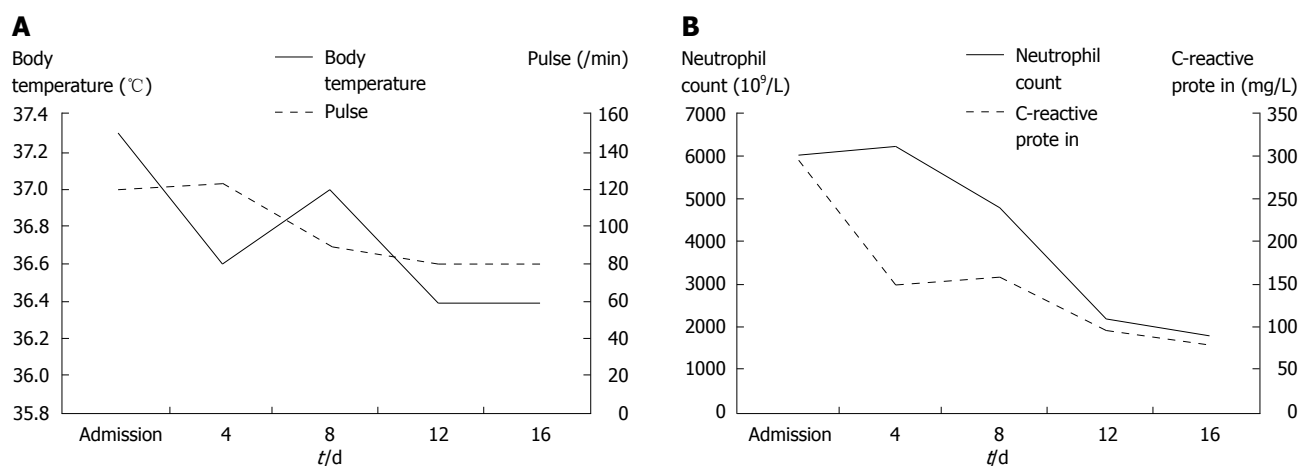


Figure 3 Changes in body temperature and pulse (A) and in neutrophil counts and C-reactive protein (B) of the patient.

of the gastrointestinal tract. Although this tumor has been known as a neoplasm that is highly resistant to conventional chemotherapy, several recent findings of molecular abnormalities could provide a rationale for treatment with targeted therapies^[2]. Recently, applications of several molecularly targeted therapies like imatinib and sunitinib have been proven sufficient for systemic treatment of inoperable or metastatic GIST.

Imatinib is a small molecule tyrosine kinase inhibitor with actions on various transduction molecules such as ABL, BCR-ABL, and KIT^[4]. Some clinical trials have shown that imatinib therapy affects prolonged disease-free survival of advanced GIST patients. Sunitinib has recently received approval from the FDA for patients with GIST who are resistant to imatinib^[3]. In one randomized controlled trial, sunitinib resulted in a significantly longer time of tumor progression than placebo in patients with GIST after failure of imatinib treatment^[5]. Thus, the drug has become a new treatment strategy for these patients, since there is no proven second-line therapy for GIST. The molecular targets of sunitinib are RTKs, and this drug has also been known to have blocking effects on the activation of KIT or PDGFR, which has a similar mechanism of action to imatinib^[1]. Moreover, sunitinib exerts an additional anti-angiogenic effect by inhibiting the activation of VEGFR^[6]. Because of this effect, sunitinib has been considered beneficial for GIST patients who are resistant to imatinib or cannot tolerate it as a second line treatment^[7,8]. A series of clinical studies on sunitinib reported that sunitinib can result in various adverse events, including hypertension, abnormalities of the thyroid and adrenal function, gastrointestinal symptoms such as constipation or diarrhea, and dermatologic abnormalities such as hand-foot syndrome^[5,9]. However, bowel perforation associated with the use of sunitinib has not been reported to date. In this case, physical examination of localized tenderness and 3-D reconstructed CT scan confirmed the colon perforation. Then, the perforation was completely treated by percutaneous drainage without operation. This treatment strategy was possible because there was a small amount of colon content localized in a particular area of

the peritoneum due to the adhesions from previous operations.

Several cases of colon perforation related to anti-cancer therapy have been reported^[10,11], particularly with the VEGFR inhibitor bevacizumab (Avastin; Genentech, South San Francisco, CA), which shares one of the mechanisms of action of sunitinib. A randomized controlled clinical trial showed that bevacizumab, a humanized monoclonal antibody that binds to and neutralizes VEGF, led to gastrointestinal perforation in six of 393 patients (1.5%) who had metastatic colon cancer^[10]. Furthermore, another case of colon perforation was documented after treatment with bevacizumab in a patient with non-small cell lung cancer^[11]. In addition, the mechanism by which bevacizumab causes bowel perforation is attributed, at least in part, to the increased risk of arterial thrombosis that is observed in the use of drugs targeting VEGF^[12,13]. Therefore, this drug is likely to generate rapid tumor degeneration, which in turn leads to colon perforation in patients with bowel metastasis or invasion, due to this inhibitory effect. In addition, the patient's history of smoking might contribute to risk of colon perforation in our case. However, the mechanism of anti-angiogenic agents-induced colon perforation remains to be elucidated.

In conclusion, sunitinib, which is administered to treat GISTs resistant to imatinib, causes unexpected colon perforation and subsequent peritonitis. Hence, careful attention and appropriate clinical evaluation are required for patients presenting with gastrointestinal symptoms during sunitinib treatment.

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CASE REPORT

Concomitant gastric carcinoid and gastrointestinal stromal tumors: A case report

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INTRODUCTION

Gastrointestinal neuroendocrine tumors are derived from the diffuse neuroendocrine system of the gastrointestinal (GI) tract, composed of amine- and acid-producing cells with different hormonal profiles, depending on their site of origin^[1]. Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors arising from interstitial Cajal cells of the wall of the GI tract^[2,3]. GISTs can be distinguished from other mesenchymal tumors by optimal immunostaining for CD117, and a prognostic classification is based on tumor size, mitotic score, and MIB-1 grade^[4]. Gain-of-function mutation of the *c-kit* gene, and immunoreactivity of the c-kit protein (CD117) in many GIST support the idea that GIST is a biologically distinct entity. Both carcinoid tumors and GISTs are malignant or potentially malignant tumors, and are considered to have a specific molecular pathogenesis. Herein we report a gastric carcinoid tumor concomitant with a gastric GIST, and also provide a review of the literature.

CASE REPORT

In October 2005, a 65-year-old Asian female came to our hospital for a routine physical examination. She had no history of peptic ulcer, epigastralgia, abdominal pain, diarrhea, flushing, or palpitations. Esophagogastroduodenoscopy showed an approximately 0.8 cm sessile polypoid lesion, with superficial reddish striation, on the posterior wall of the upper gastric corpus (Figure 1). A biopsy sample was taken and eight specimens were acquired. Histological studies showed a gastric mucosa tumor. The tumor demonstrated uniform ovoid cells with cordal and small nestic patterns within the lamina propria. After immunohistochemical

Abstract

A gastric carcinoid tumor concomitant with gastrointestinal stromal tumor (GIST) is rarely encountered in clinical practice. We report a 65-year-old female who had a 0.8 cm gastric carcinoid tumor on the posterior wall of the upper gastric corpus detected during an esophagogastroduodenoscopy at a routine physical examination, and a concomitant 1.1 cm GIST on the anterior wall of the upper gastric corpus incidentally found during surgery of the gastric carcinoid tumor. Normal serum gastrin level and histological findings suggested that she had a type III gastric carcinoid tumor and a GIST which were categorized a very low risk of malignancy, based on their small size and lack of mitosis. Both tumors were treated successfully by surgical excision. The patient had an uneventful recovery. Neither recurrence nor metastasis was found after a 28-mo follow-up.

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Key words: Gastric carcinoid tumor; Gastrointestinal stromal tumor; Esophagogastroduodenoscopy; Digestive system

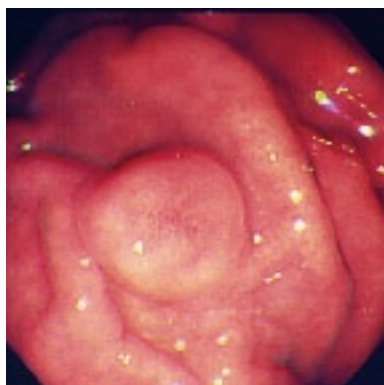


Figure 1 EGD endoscopy showing a protruding polypoid mass with superficial reddish striation on the posterior wall of the gastric upper corpus.

(IHC) staining, the tumor cells were positive for cytokeratin, synaptophysin, and chromogranin-A (Figure 2A-D). The Ki-67 index was $< 1\%$. A gastric carcinoid tumor was diagnosed pathologically, and *Helicobacter*-like microorganisms were also found. A biopsy urease test (CLO test) for *Helicobacter pylori* (*H. pylori*) infection also demonstrated a positive reaction. The serum gastrin level was 34.4 ng/L (normal range, 25-111 ng/L). Owing to the tumor's potentially malignant nature, surgery was performed one week later. During the surgical procedure, in addition to the 0.8 cm carcinoid tumor, a 1.1 cm tumor was found incidentally over the serosal side of the anterior wall of the upper gastric corpus. Both small tumors were locally resected simultaneously and separately. Grossly, a patch of gastric tissue, measuring 3.9 cm \times 2.5 cm \times 0.7 cm, from the posterior wall of the upper corpus disclosed a 0.8 cm \times 0.6 cm \times 0.3 cm carcinoid tumor. The other gastric tissue taken from the anterior wall of the upper gastric corpus, measuring 2.5 cm \times 2.0 cm \times 0.5 cm, demonstrated a 1.1 cm \times 0.7 cm \times 0.3 cm tumor within the muscularis propria of the gastric wall. Histologically, this 1.1 cm tumor displayed swirling bundles of spindle cells with focally palisading areas within the fibrotic stroma. Neither mitotic activity nor tumor necrosis was found. The spindle tumor cells were positive for CD117, CD34, neuron-specific enolase, and S-100 protein, but negative for actin-M851 and glial fibrillary acidic protein after IHC staining (Figure 2E-H). Finally, a gastric carcinoid tumor and a GIST were pathologically diagnosed. The patient had an uneventful recovery, and was discharged one week after surgery. After a 28-mo follow-up, there was no evidence of tumor recurrence or metastasis.

DISCUSSION

Gastric carcinoid tumor concomitant with gastric GIST is clinically rare. To the best of our knowledge, it has not been reported in the English literature. The pathogenesis of gastric carcinoid tumor concomitant with GIST is unclear. It was reported that *H. pylori* are related to the pathogenesis of gastric carcinoma and mucosa-associated lymphoid tumor^[5,6]. We previously reported

a case of a 0.4-cm GIST concomitant with an early gastric cancer in 2005^[7], and between 2003 and 2007, we intermittently detected one case of multiple 0.2-cm to 0.5-cm gastric neuroendocrine tumors and additional 5 cases of 0.4-15 cm gastric GISTs. All except for 2 of the cases yielded positive CLO tests. The present case also showed positive *H. pylori* infection. However, this finding is more like an incidental event rather than a causal association. Whether the concomitant carcinoid tumor with GIST correlates to *H. pylori* infection or not requires more collected cases and further studies.

Gastric carcinoids are classified into three subtypes, all of which originate from gastric enterochromaffin-like cells in the gastric mucosa. The first subtype is combined with chronic atrophic gastritis (type I). The second subtype, Zollinger-Ellison syndrome, is nearly a part of the multiple endocrine neoplasia-1 (MEN-1) syndrome (type II). Clinically, these two subtypes are linked to a hypergastrinemic state. The third sporadic subtype (type III) occurs without hypergastrinemia but takes an aggressive course, with 54%-66% metastasis^[1]. As stated by Shinohara and colleagues, even a 0.5-cm carcinoid tumor can present with metastasis^[8]. On account of neither atrophic gastric mucosa nor elevated serum gastrin level in our case, a small type III carcinoid tumor was favored. The potential for metastasis cannot be ignored and demands close follow-up.

Gastric carcinoids may have different clinical features in different locations of GI tract, including abdominal pain, vomiting, and anemia^[9]. Carcinoid tumor associated with vascular malformation may cause massive gastric bleeding^[10]. Carcinoid syndrome with symptoms of flushing, diarrhea, abdominal pain, cutaneous edema, and bronchoconstriction is uncommon. Due to a small nonfunctional carcinoid, our case never experienced any GI symptom or carcinoid syndrome.

Since 1999, GISTs have been considered to be a group of mesenchymal neoplasms arising from interstitial Cajal cells of the gastrointestinal walls^[2,3]. GISTs are now preferentially defined as tumors with c-kit (CD117) positive mesenchymal spindle cells or epithelioid neoplasms, found primarily in the GI tract, omentum, and mesentery^[11]. The most important manifestation of this tumor is its indolent, slow-growing nature. This tumor is generally found within the deeper stroma and the submucosa, and incidentally during an imaging study and surgery. In our case, a GIST protruding to the serosal side of the gastric wall was found incidentally during a surgical procedure. Histologically, it arose from the muscularis propria of the gastric wall.

Patients with GIST often present with nonspecific symptoms, such as nausea, vomiting, abdominal pain, GI bleeding, and may have metastatic disease. Bleeding is the most common symptom. The tumor size and mitotic score are considered important diagnostic criteria and prognostic predictive indicators^[12]. Our case was asymptomatic and diagnosed as GIST with a very low risk of malignancy based on its small size and lack of mitosis and was positive for CD117 after IHC staining.

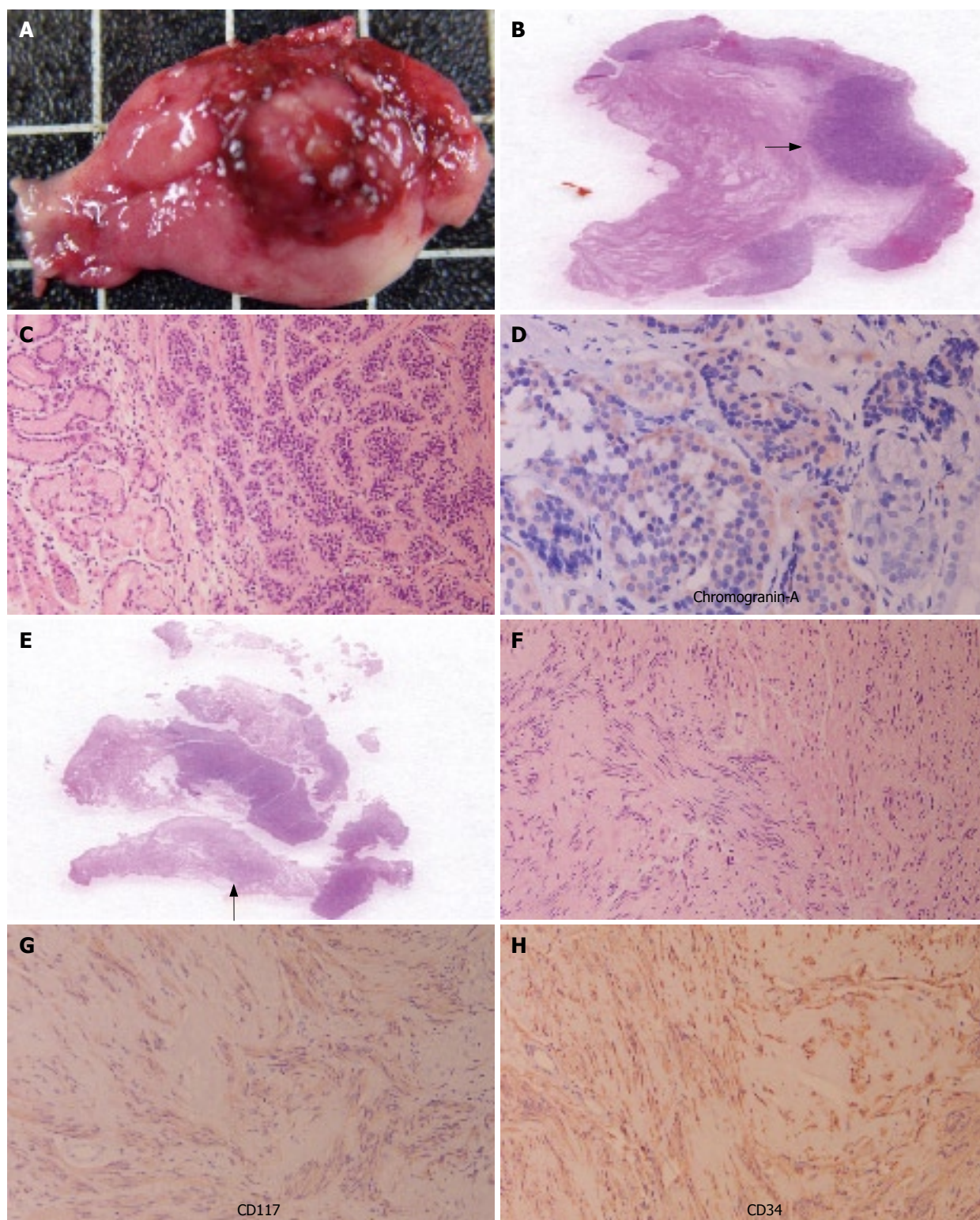


Figure 2 Postoperative wedge-shaped gastric tissue demonstrating a 0.8-cm centrally polypoid mass (A), scanning microscopic view disclosing a crescent ulcerative mass (arrow) involving gastric mucosa and submucosa (B), carcinoid tumor (right half) displaying uniform ovoid cells in chordal and small nestic patterns (hematoxylin and eosin stain, $\times 200$) (C), chromogranin a positively stained tumor cells (IHC staining, $\times 400$) (D), stromal tumor (arrow) within the gastric wall (scanning microscopic view) (E), tumor cells demonstrating whirling and palisading spindle cells interlaced with smooth muscle cells of gastric muscularis propria (hematoxylin and eosin stain, $\times 200$) (F), CD117 (G) and CD34 (H) stained spindle tumor cells.

Treatment modalities for non-metastatic small carcinoid tumors include endoscopic mucosal resection, minimally invasive laparoscopic wedge resection, and surgery^[13,14]. To date, surgery is the mainstay and the only potentially curative therapy for carcinoid tumors.

Treatment modalities for metastatic carcinoid tumors include orthotopic liver transplant, hepatic artery embolization, and somatostatin analog, adjuvant indium-111 octreotide-receptor targeted therapy^[9]. Therapeutic options for GISTs include surgery and

treatment with STI-571 (Gleevec). When inoperative, residual or recurrent tumor exists, STI-571 is the choice^[15,16]. Owing to the treatment of two synchronized small gastric tumors in our case, local resections were performed simultaneously and separately. No evidence of tumor recurrence or metastasis was found after a 28-mo follow-up period.

In conclusion, we report a rare case of small gastric carcinoid tumor concomitant with a small gastric GIST with no clinical symptoms and positive *H pylori* infection. More studies are required for evaluating the relation between *H pylori* infections and tumorigenesis of concomitant gastric carcinoid and gastric GIST. A long term follow-up period of all carcinoids and GISTs is greatly needed, due to their potential for metastasis.

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
 January 24-25, Frankfurt, Germany
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008
 February 14-16, Paris, France
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
 8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
 3rd Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
 Canadian Association of Gastroenterology
 E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
 British Society of Gastroenterology Annual Meeting
 E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
 Asian Pacific Association for the Study of the Liver
 18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
 Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
 OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
 E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
 SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
 9th World Congress of the International Hepato-Pancreato Biliary Association
 Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
 43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
 Falk Symposium 164: Intestinal

Disorders

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008

May 21-22, California, USA
 ASGE Annual Postgraduate Course Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
 E-mail: education@asge.org

June 4-7, Helsinki, Finland
 The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
 Semana de las Enfermedades Digestivas
 E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
 3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
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June 11-13, Stockholm, Sweden
 16th International Congress of the European Association for Endoscopic Surgery
 E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
 Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
 E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
 10th World Congress on Gastrointestinal Cancer
 Imedex and ESMO
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June 25-28, Lodz, Poland
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatologists (IAP)
 E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
 5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
 ILTS 14th Annual International Congress
www.ilt.s.org

September 10-13, Budapest, Hungary
 11th World Congress of the International Society for Diseases of the Esophagus
 E-mail: isde@isde.net

September 13-16, New Delhi, India
 Asia Pacific Digestive Week
 E-mail: apdw@apdw2008.net

APDW 2008
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III FALK GASTRO-CONFERENCE

September 17, Mainz, Germany
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September 18-19, Mainz, Germany
 Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
 Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
 Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
 Third Annual Meeting European Society of Coloproctology
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October 8-11, Istanbul, Turkey
 18th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists
 E-mail: orkun.sahin@serenas.com.tr

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 The Liver Meeting
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November 6-9, Lucerne, Switzerland
 Neurogastroenterology & Motility Joint International Meeting 2008
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 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
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 Digestive Disease Week 2009

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Format

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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