MicroRNA gene methylation landscape in pediatric B-cell precursor acute lymphoblastic leukemia

Radosław Chaber[1,2,A–F], Artur Gurgul[3,C], Jacek Tabarkiewicz[1,4,B,C,E], Grażyna Wróbel[5,B,C], Tomasz Szmatoła[3,C], Igor Jasielczuk[3,B,C], Olga Haus[6,C–E], Monika Lejman[7,C], Blanka Rybka[5,D], Renata Ryczan-Krawczyk[5,C,D], Anna Jaśkowiec[8,B,C], Sylwia Paszek[9,C,D], Natalia Potocka[9,C], Christopher J. Arthur[10,C–E], Wioletta Bal[1,2,D,E], Kornelia Łach[1,B–D], Aneta Kowal[1,B–D], *Izabela Zawlik[1,9,D–F], *Elżbieta Latos-Grażyńska[5,C–E]

[1] Institute of Medical Sciences, Medical College of Rzeszów University, Poland
[2] Clinic of Pediatric Oncology and Hematology, State Hospital 2, Rzeszów, Poland
[3] Center for Experimental and Innovative Medicine, University of Agriculture in Krakow, Poland
[4] Laboratory of Translational Research in Medicine, Centre for Innovative Research in Medical and Natural Sciences, Medical College of Rzeszów University, Poland
[5] Department of Paediatric Bone Marrow Transplantation, Oncology and Hematology, Wroclaw Medical University, Poland
[6] Department of Clinical Genetics, Faculty of Medicine, Collegium Medicum, Nicolaus Copernicus University, Toruń, Poland
[7] Laboratory of Genetic Diagnostics, Medical University of Lublin, Poland
[8] Department of Haematology, Blood Neoplasms and Bone Marrow Transplantation, Wroclaw Medical University, Poland
[9] Laboratory of Molecular Biology, Centre for Innovative Research in Medical and Natural Sciences, Medical College of Rzeszów University, Poland
[10] School of Chemistry, University of Bristol, the United Kingdom

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Elżbieta Latos-Grażyńska
E-mail: elzbieta.latos-grazynska@umed.wroc.pl

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None declared

*Izabela Zawlik and Elżbieta Latos-Grażyńska contributed equally to this work.

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Abstract

Background. Aberrant DNA methylation is an important mechanism by which the normal patterns of microRNA expression are disrupted in human cancers including B-cell precursor acute lymphoblastic leukemia (BCP ALL), the most common pediatric malignancy.

Objectives. To characterize the methylation profile landscape of microRNA genes in BCP ALL patients.

Materials and methods. We employed Infinium® MethylationEPIC BeadChip Arrays to measure the methylation of microRNA genes from bone marrow samples of children with BCP ALL (n = 38) and controls without neoplasms (n = 4).

Results. This analysis revealed differential methylation of the microRNA genes in the pediatric BCP ALL when compared to the control. A subcluster amongst BCP ALL patients with TCF3-PBX1 genetic subtype was also observed. No other differences were observed in association with age, gender or risk group. Several interesting leukemia-related phenotypes are enriched by the genes with hyper- and hypomethylated sites located in promoters as well as gene bodies. The top 3 miRNA genes, promoters of which were the most statistically significantly hypermethylated in BCP ALL were *MIR1273G*, *MIR1304* and *MIR663*, and the top 3 hypomethylated were *MIR4442*, *MIR155* and *MIR3909*.

Conclusions. In this study, a different microRNA genes methylation landscape was shown in pediatric BCP ALL compared to children without neoplasms. A visible subcluster among BCP ALL samples consisted of individuals with TCF3-PBX1 genetic subtype. No other differences were observed in association with age, gender or risk group. Several interesting leukemia-connected phenotypes were found, associated with genes with hyper- and hypomethylated sites located on promoters as well as gene bodies.

Key words: children, microRNA, methylation, BCP ALL

Background

B-cell precursor acute lymphoblastic leukemia (BCP ALL) is the most common malignancy in children.[1] Understanding the molecular and genetic pathways that affect the development and clinical course of BCP ALL is a key to improving the treatment outcomes and is, therefore, an important focus of current research into BCP ALL.

Normal hematopoietic cell development is highly controlled epigenetic regulation of genes via DNA methylation, the chemical modification of histones, and the expression of noncoding RNAs. Each of these epigenetic factors can become dysregulated during leukemic transformation. The DNA methylation is by far the most well-characterized epigenetic modification and is involved in the regulation of gene expression, the maintenance of genome stability, and cellular differentiation.[2] The methylation of cytosine residues in CpG dinucleotides plays a pivotal role in the establishment of cellular identity by influencing gene expression.[3]

MicroRNAs (miRNAs) are short, noncoding RNA molecules that regulate gene expression by forming complexes with their mRNA counterparts in order to cause translational repression, either by mRNA degradation or cleavage by deadenylation.[4,5] Furthermore, miRNAs are key regulators of hematopoiesis and are also involved in leukemogenesis.[6,7] To date, at least 32 dysregulated miRNAs are known to be associated with ALL prognosis.[8] Interestingly, aberrant DNA methylation seems to be a major mechanism by which the normal patterns of miRNA expression are disrupted in human cancers,[9] including ALL.[10] Many tumor suppressor miRNAs appear to be downregulated by DNA hypermethylation, and various oncogenic miRNAs (onco-miRNAs) are known to be upregulated via DNA hypomethylation.[9]

We have previously shown significant differences in genomic methylation profiles in the bone marrow of BCP ALL and healthy control patients.[11] Nevertheless, not enough attention was given to the methylation of miRNA genes, which is an important factor affecting oncogenic processes.

Objectives

In this study, we attempted to perform the analysis of methylation profiles of miRNA genes in BCP ALL patients in order to enhance the results of the previous analysis and to shed some light on a potential role of epigenetic regulation of miRNA expression in pediatric leukemia. We have used genome-wide methylation data obtained in our previous work[11] and performed an in-depth analysis of the methylation differences in functional elements of miRNA genes in healthy and leukemic bone marrow samples.

Materials and methods

Patients and samples

The approval of the ethics committee for this study was obtained from the Institutional Review Board of the Medical University of Lodz, Poland (approval No. RNN/226/11/KE). Informed consent was obtained from the parents/legal guardians of all participating children. Forty-two samples of bone marrow were obtained in 2015–2016 from 38 patients (male/female 21/17; median age 5.0 years, age range 1.5–17.0 years) with pediatric BCP ALL at the time of diagnosis. The control samples of bone marrow were collected from children in whom other types of cancer and other genetic diseases had been previously excluded. The patients were stratified into prognostic groups according to the ALL IC-BFM 2009 protocol.[12] This stratification is based on the initial clinical features including patient age, white blood cells count at time of diagnosis, presence of specific genetic aberrations, the response to steroids at day 8 of therapy, the cytomorphological response in bone marrow at day 15 and 33, and the minimal residual disease level at day 15. Finally, 5 patients were included into the high-risk group. Various genetic aberrations associated with ALL were detected among most of the patients. Most frequently, hyperdiploidy (>50 chromosomes) (13 patients) and t(12;21) with fusion ETV6-RUNX1 (7 patients) were revealed. Subsequently, t(1;19) with fusion *TCF3-PBX1* (3 patients), hyper/hypotriploidy (3 patients) and IGH rearrangement (3 patients) were detected. Seven patients had other genetic aberrations, which were different than those mentioned above. In 2 cases, the normal karyotype was confirmed.

Samples and DNA methylation profiling

The DNA samples were analyzed with the Infinium® MethylationEPIC BeadChip approach (Illumina, San Diego, USA), according to the manufacturer's protocol. It allowed for the analysis of 850,000 methylation sites per sample. These sites include those within known CpG islands and outside CpG islands, as well as non-CpG methylated sites identified in human stem cells and differentially methylated (DM) sites identified in tumors compared to normal samples and across several tissue types. The assay also includes probes for 7084 CpG sites associated with miRNA genes, out of which 4188 probes were retained for further analysis following quality filtering and extensive annotation analysis. These probes are associated with 1008 different miRNA genes, covering 52.5% of the current version of miRBase (v. 22).[13]

Data quality control and statistical analysis

Our previous study describes the steps taken for data quality control and the identification of DM sites.[11] In brief, after initial normalization and removal of batch effects, the ratio of methylated (C) to unmethylated (T) DNA (also called the methylation beta-value (β)) was calculated for each CpG site. A β-value of 0 represents a completely unmethylated CpG site and a β-value of 1 represents a fully methylated CpG site. The differential methylation for individual probes between groups was calculated using the Chip Analysis Methylation Pipeline (ChAMP). The champ.DMP() function of ChAMP package pipeline[14] was applied. It uses the limma package[15] to calculate the p-value by a linear model. The DMP-determined t-test p-values were corrected for multiple testing using the Benjamini–Hochberg procedure.[16] Adjusted p-values (adjPs) <0.05 were considered statistically significant.

The differential methylation analysis was performed with several discrete goals: 1) the identification of general differences between all BCP ALL patients and controls, and 2) the identification of DM sites between standard/intermediate and high-risk group of patients. Additional comparisons have been made within BCP ALL patients group to detect sites associated with confounding factors, such as age (≤6 years compared to >6 years) or gender (males compared to females). The differential methylation analysis was also performed between patients with different chromosomal aberrations characteristic of leukemia (ETV6-RUNX1 (n = 7), TCF3-PBX1 (n = 3), IGH (n = 3), hyperdiploidy (n = 13), and triploidy (n = 3)), using pairwise comparisons between specific cytogenetic subtype and all other leukemia patients with known cytogenetic status (n = 8). Cytogenetic diagnosis was performed as previously described.[11]

Functional gene annotation and target genes prediction

The miRNA genes associated with specific DM sites were analyzed in terms of their disease phenotypes annotated in the available databases. The phenotype enrichment analysis was performed using the WEB-based GEne SeT AnaLysis (WebGestalt) toolkit (www.webgestalt.org),[17] exploiting information from the Human Phenotype Ontology (www.hpo.jax.org)[18] and PharmGKB (www.pharmgkb.org)[19] databases. The WebGestalt analysis was performed using all annotated human genes (genome option), and it was limited to identifying enriched phenotypes with the Fisher's exact test false discovery rate (FDR)-adjP <0.01 and with at least 4 genes within phenotype categories. The prediction of miRNA target genes was done with the use of miRDB tool. Only genes with a prediction score ≥95 (implying the highest confidence of gene as a target) were analyzed.[20]

Results

Differences in miRNA genes methylation profiles between BCP ALL and control samples

General assay performance was evaluated based on control probes. The performance was satisfactory across all studied samples, with all samples passing filtering criteria implemented in the BeadArray Controls Reporter (Illumina) software. After initial filtering, β-values for 800,619 probes were retained and normalized. Subsequently, batch effects were identified and removed by evaluation of components of variation and by the singular value decomposition (SWD) method.[21] Among all the analyzed probes, 4188 probes associated with 1008 different miRNA genes were identified and analyzed (data available on reasonable request).

Comparison of methylation levels between BCP ALL and control samples resulted in the detection of 578 DM probes. Out of 578 probes, 377 were annotated to promoters of 242 different miRNA genes. Promoter sites were defined as: TSS200 (0–200 nt upstream of transcription start site (TSS)) and TSS1500 (200–1500 nt upstream of TSS). The remaining 201 DM sites were annotated as located within the gene bodies of 74 different miRNAs.

The principal component analysis based on all DM probes reveals visible differences in the miRNA genes methylation profiles between BCP ALL and control samples, with greater variation observed among leukemic samples (Fig. 1). Unsupervised hierarchical clustering (based on Euclidean distance and the DM probes β-values) showed the clear separation of the leukemic and non-leukemic methylation profiles into 2 distinct clusters (Fig. 2). A visible subcluster among BCP ALL samples was created by 3 cases of TCF3-PBX1 genetic subtype (Fig. 2), suggesting that it possesses a distinct miRNA genes methylation profile. No clustering of samples with age, gender or risk

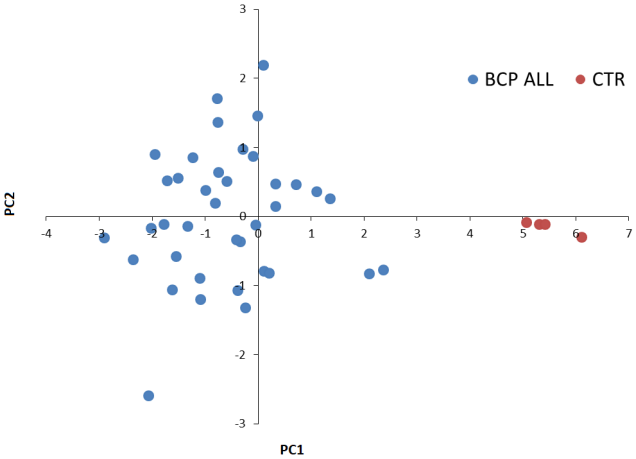

Fig. 1. Principal component analysis using methylation β-values of differentially methylated and miRNA genes-associated probes

BCP ALL – B-cell precursor acute lymphoblastic leukemia; CTR – controls.

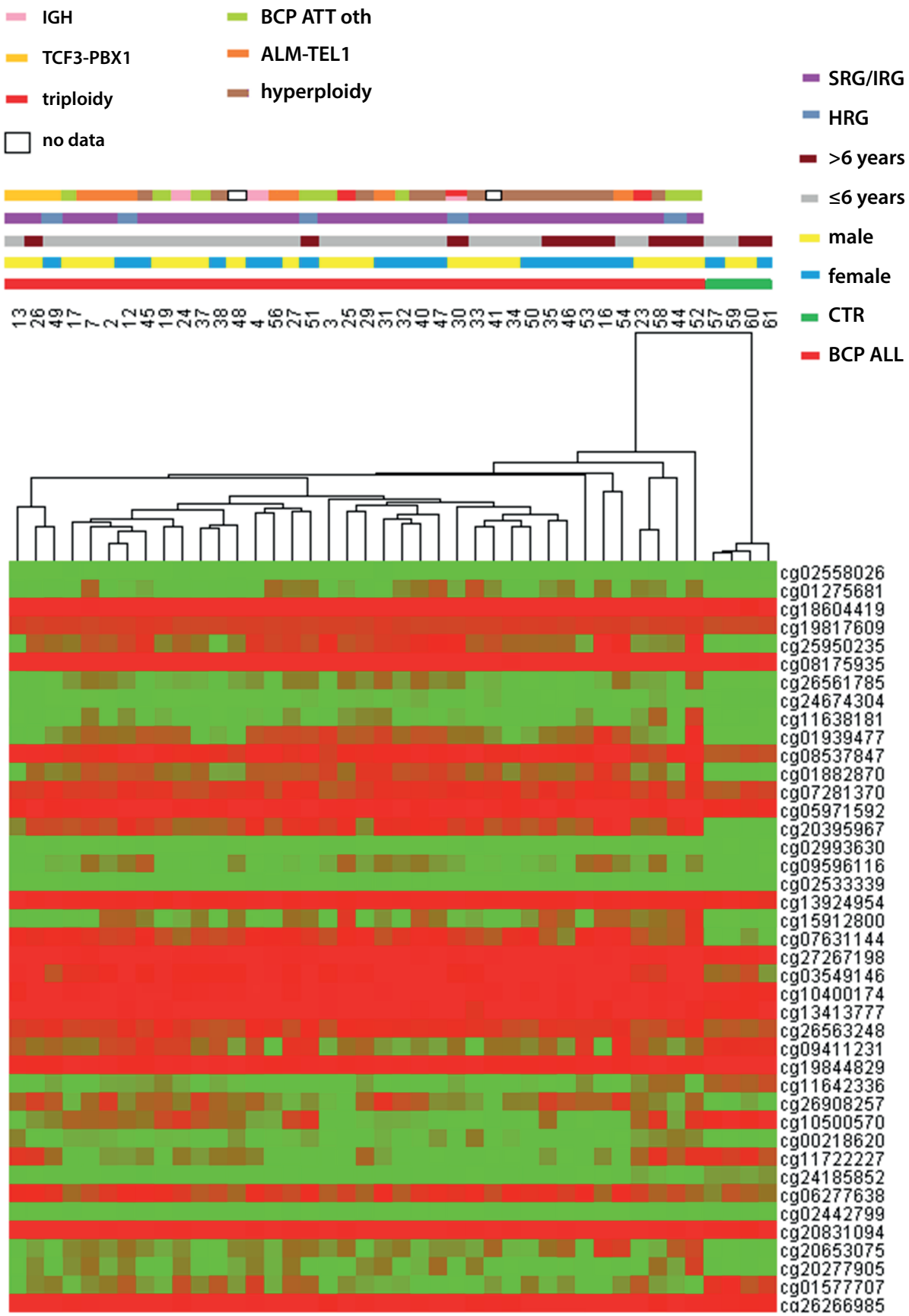

Fig. 2. Hierarchical clustering of the studied B-cell precursor acute lymphoblastic leukemia (BCP ALL) and control samples methylation profiles based on all differentially methylated miRNA genes-associated sites. Only a random subset of probes is shown on the heatmap. The colored bars in upper section classify samples according to disease state, gender, age, risk group, and genetic subtype

CTR – controls.

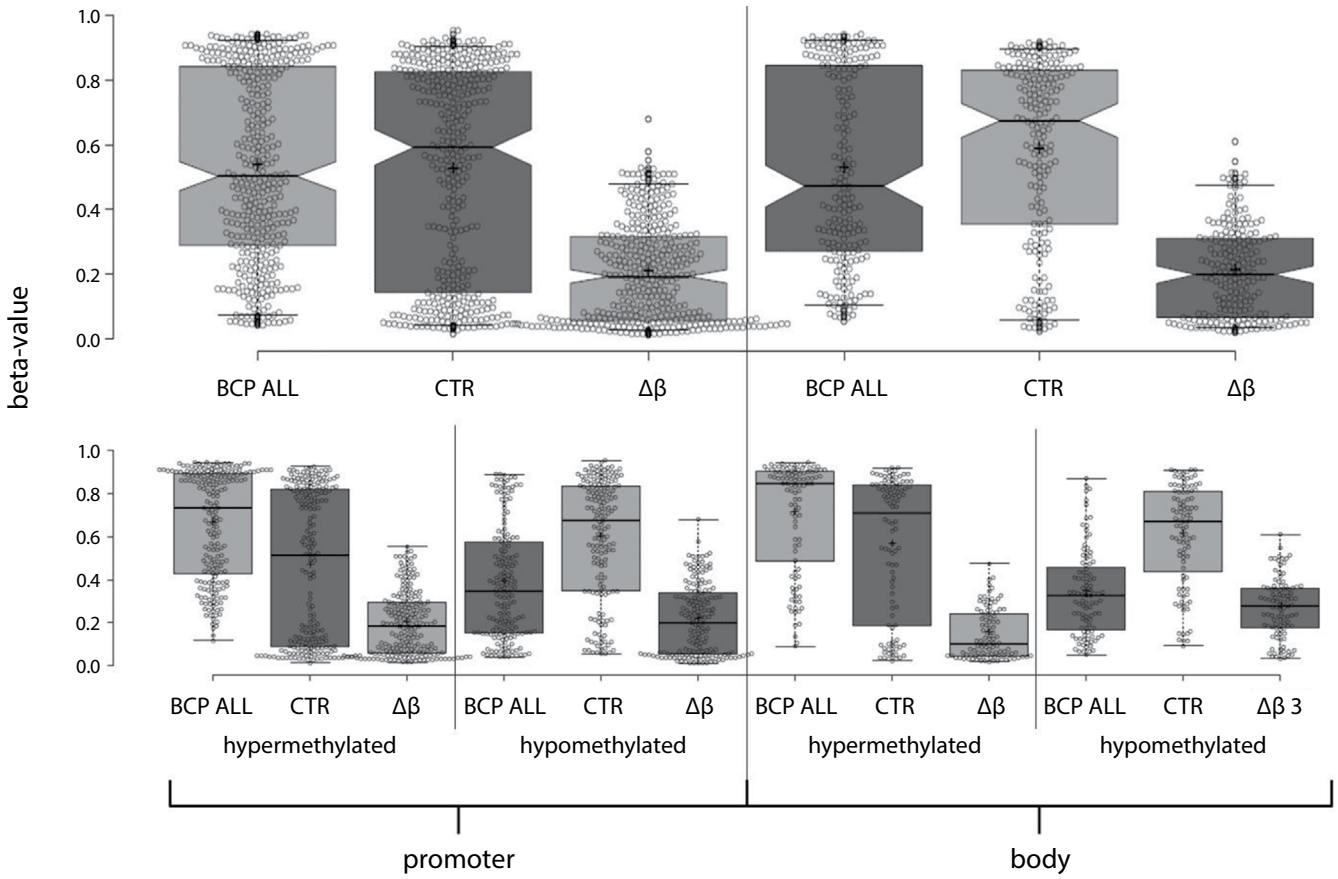

Fig. 3. Boxplot of differentially methylated probes methylation β-values (methylation levels) and delta-beta (Δβ) values with respect to study group and probes location in promoter and gene body

BCP ALL – B-cell precursor acute lymphoblastic leukemia; CTR – controls; Δβ – difference in methylation level between groups.

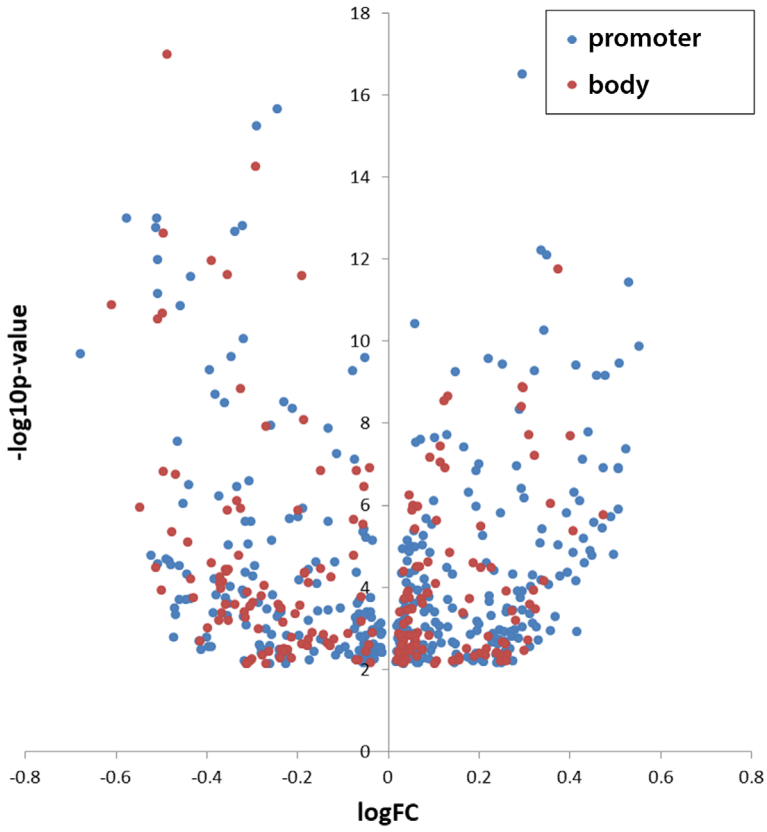

Fig. 4. Volcano plot for sites differentially methylated between B-cell precursor acute lymphoblastic leukemia (BCP ALL) and control samples, with respect to their location in the promoter and gene body of miRNA genes

group was observed, suggesting a lower influence of these factors on global miRNA gene methylation profiles.

The initial analysis of differential methylation of CpG sites between BCP ALL and control samples showed a similar pattern of methylation differences for sites located in promoters and gene bodies of miRNA genes (Fig. 3,4). However, taking into account that the methylation in these regions can have the opposite effect on transcription process, DM CpGs were analyzed separately, depending on their location within the functional element of a given gene.

Differential methylation of miRNA gene promoters in BCP ALL

Changes in methylation profile of miRNA gene promoters in leukemia were studied based on 377 DM (t-test FDR < 0.05) CpG loci between BCP ALL and ccontrols, located within TSS200 and TSS1500 of 242 different miRNA genes (data available on reasonable request). These sites were rarely associated with known CpG islands (17.8% of all) or shores (23.3%), and about half of them (54.9%) were hypermethylated in the BCP ALL group. When comparing the distribution of hyper- and hypomethylated sites across the genome, we found that hypermethylated sites are more commonly situated in CpG islands than hypomethylated (29.5% compared to 3.5%), whereas hypomethylated sites were more common in "open sea" (64.7% compared to 53.8%). The lowest average methylation level was observed for CpGs located within known CpG islands in both the control (17.7%) and BCP ALL samples (43.2%) (Table 1).

A high level of methylation was observed for CpGs located in sites distant from a CpG island ("open sea"; 65.8% in control samples) and was the highest for sites that were hypermethylated in BCP ALL (82.9%) (Table 2).

Table 1. Characteristics of DM miRNA genes promoter-associated sites in B-cell precursor acute lymphoblastic leukemia (BCP ALL), with respect to their location in promoter regions TSS1500 and TSS20

Feature location	TSS1500	TSS200	Promoter
Whole panel			
Number	1934	824	2758
%	70.1	29.9	100.0
All promoter-associated sites			
Number	254	123	377
% of DM	67.4	32.6	100.0
Avg. met. in BCP ALL	0.538	0.520	0.532
Avg. met. in CTR	0.548	0.462	0.520
Hypermethylated in BCP ALL			
Number	134	73	207
% of DM	35.5	19.4	54.9
Avg. met. in BCP ALL	0.673	0.629	0.657
Avg. met. in CTR	0.496	0.393	0.460
Avg. met. difference (Δβ)	0.177	0.235	0.197
Hypomethylated in BPC ALL			
Number	120	50	170
% of DM	31.8	13.3	45.1
Avg. met. in BCP ALL	0.387	0.362	0.380
Avg. met. in CTR	0.605	0.563	0.593
Avg. met. difference (Δβ)	0.218	0.201	0.213

Sites were classified according to their location in TSS1500, TSS200 and jointly (promoter). The number and percentage of probes in each location is given. Additionally, the average methylation (avg. met.) in both study groups and difference in methylation level between them is provided. DM – differentially methylated; CTR – controls.

The average absolute Δβ (average difference in methylation level) was similar for hyper- (0.197) and hypomethylated (0.213) sites.

The DM sites located in promoters were associated with 242 different miRNA genes. The phenotype enrichment analysis showed that the genes were enriched in several cancer-related disease phenotypes, including hematologic neoplasms, carcinoma, B-cell lymphoma, or leukemia itself (Table 3). Moreover, several interesting leukemia-connected phenotypes were found, when genes associated with hyper- and hypomethylated sites were analyzed separately (data available on reasonable request).

The top 3 miRNA genes whose promoters were the most statistically significantly hypermethylated in BCP ALL were *MIR1273G, MIR1304* and *MIR663,* and the top 3 hypomethylated were: *MIR4442, MIR155* and *MIR3909.* The analysis of the most probable target genes (top score from miRDB software) for the miRNAs being a product of those genes allowed for the detection of 44 different coding genes, among which we found e.g., pre-B-cell leukemia transcription factor 2 (*PBX2*) and metastasis associated 1 family member 2 (*MTA2*) genes (Table 4).

Differential methylation of miRNA gene bodies in BCP ALL

In total, 201 DM sites located in the gene bodies of 74 different miRNA genes between BCP ALL and control samples were detected (data available on reasonable request). As expected, the DM sites were mainly located outside known CpG islands (80.1%). No sites that were hypomethylated and located within known CpG islands were detected in BCP ALL. Most of the gene body-associated sites were characterized by a high level of methylation in both groups (45–85% on average in control samples). Only sites positioned within CpG islands were characterized by lower methylation level (15.1% in controls (CTR)) (Table 5).

Sites were classified according to their location in open sea, shore, shelf, and island of known CpG islands. The number and percentage of probes in each location is given. Additionally, the average methylation in both study groups and the difference in methylation level between them is provided.

Out of the gene body-associated sites DM between BCP ALL and control groups, 102 (50.7%) were hypermethylated in BCP ALL. The average difference in methylation level (average absolute Δβ) between BCP ALL and control samples was higher for hypo- (0.27) than hypermethylated (0.14) sites. The hypermethylated sites were associated with 51 different miRNA genes, out of which 3 most significantly DM were *MIR1273H, MIR5096* and *MIR5095.* The sites hypomethylated in BCP ALL were connected with bodies of 45 different miRNA genes. Among the top 3 genes with hypomethylated bodies, we found *MIR548Q, MIR3163* and *MIR181A1HG* (the host gene). The analysis of the top 3 hyper- and hypomethylated miRNAs target

Table 2. Characteristics of differentially methylated miRNA genes promoter-associated sites in B-cell precursor acute lymphoblastic leukemia (BCP ALL), with respect to the location in known CpG islands

Feature CGI context	Open sea	Shore	Island	Shelf	All
Whole panel					
Number	1692	483	414	169	2758
%	61.3	17.5	15.0	6.1	100.0
All DM promoter-associated sites					
Number	203	88	67	19	377
% of DM	53.8	23.3	17.8	5.0	100.0
Avg. met. in BCP ALL	0.593	0.482	0.432	0.466	0.532
Avg. met. in CTR	0.658	0.451	0.177	0.568	0.520
Hypermethylated in BCP ALL					
Number	93	48	61	5	207
% of DM	24.7	12.7	16.2	1.3	54.9
Avg. met. in BCP ALL	0.829	0.580	0.448	0.751	0.657
Avg. met. in CTR	0.701	0.336	0.161	0.637	0.460
Avg. met. difference (Δβ)	0.128	0.244	0.287	0.114	0.197
Hypomethylated in BPC ALL					
Number	110	40	6	14	170
% of DM	29.2	10.6	1.6	3.7	45.1
Avg. met. in BCP ALL	0.393	0.365	0.269	0.363	0.380
Avg. met. in CTR	0.614	0.589	0.339	0.543	0.593
Avg. met. difference (Δβ)	0.221	0.224	0.070	0.180	0.213

Avg. met. – average methylation; DM – differentially methylated; CTR – controls; CGI – Common Gateway Interface.

Table 3. Selected disease phenotypes enriched by miRNA genes with promoter CpGs differentially methylated between B-cell precursor acute lymphoblastic leukemia (BCP ALL) and control samples

Disease	FDR*	miRNA gene
Hematologic neoplasms	7.51E-74	*MIR194-2, MIR708, MIR518F, MIR431, MIR574, MIR873, MIR379, MIR485, MIR516B2, MIR589, MIR760, MIR495, MIR516B1, MIR520C, MIR518D, MIR625, MIR367, MIR889, MIR432, MIR548A1, MIR627, MIR548C, MIR885, MIR519D, MIR339, MIR409, MIR517C, MIR579, MIR636, MIR512-2, MIR135A2, MIR520A, MIR654, MIR330, MIR7-3, MIR525, MIR496, MIR876, MIR30B, MIR153-2, MIR124-1, MIR487A, MIR526A2, MIR561, MIR203, MIR186, MIR665, MIR653, MIR101-2, MIR570, MIR34B, MIR154, MIR527, MIR618, MIR518A1, MIR874*
Neoplasms	4.34E-23	*MIR429, MIR708, MIR146B, MIR25, MIR574, MIR155HG, MIR375, MIR192, MIR625, MIR7-1, MIR199B, MIR143, MIR9-1, MIR27A, MIR125B1, MIRLET7A1, MIR155, MIR215, MIR206, MIR9-3, MIR10A, MIR196B, MIR196A1, MIR200B, MIR145, MIR124-1, MIR138-1, MIR30A, MIR181A1, MIR494, MIR148A, MIR122, MIR203, MIR128-2, MIR137, MIR34B, MIR423*
Carcinoma, small cell	3.13E-14	*MIR708, MIR146B, MIR25, MIR574, MIR330, MIR124-3, MIR30B, MIR375, MIR196A1, MIR200B, MIR145, MIR124-1, MIR138-1, MIR30A, MIR101-2, MIR124-2, MIR34B, MIR423*
Cancer or viral infections	1.05E-11	*MIR429, MIRLET7A1, MIR155, MIR146B, MIR206, MIR215, MIR155HG, MIR10A, MIR196B, MIR375, MIR196A1, MIR200B, MIR145, MIR192, MIR124-1, MIR199B, MIR143, MIR30A, MIR148A, MIR122, MIR203, MIR9-1, MIR27A, MIR34B, MIR125B1, MIR423*
Lymphoma, large-cell, diffuse	4.02E-07	*MIR4505, MIR155, MIR4422, MIR4531, MIR155HG, MIR4485, MIR4439, MIR4442, MIR4517, MIR4462, MIR125B1*
Carcinoma	3.85E-05	*MIRLET7A1, MIR155, MIR146B, MIR143, MIR138-1, MIR30A, MIR122, MIR203, MIR375, MIR200B, MIR145, MIR124-1*
Precursor cell lymphoblastic leukemia-lymphoma	0.0002	*MIR1973, MIR196B, MIR1976, MIR128-2, MIR5197, MIR125B1*
Leukemia	0.0041	*MIR155, MIR181A1, MIR142, MIR10A, MIR196B, MIR128-2, MIR125B1, MIR150*
Lymphoma, B-cell	0.0087	*MIR196B, MIR155, MIR125B1, MIR155HG, MIR127*

* Fisher's exact test false discovery rate (FDR), as implemented in WebGestalt software.

genes showed 483 potential targets (data available on reasonable request), among which we found few having annotation to leukemia phenotype in PharmGKB database (e.g. *TRIM72, SPI1, MARVELD3, TOPORS, SSBP2, GAB2*). Altogether, the miRNA genes with DM sites in gene body significantly enriched 6 disease phenotypes, including hematologic neoplasms, lymphoma, carcinoma, or viral infections (Table 6).

Table 4. Top score target genes for miRNA whose genes are most statistically significantly hyper- and hypomethylated in B-cell precursor acute lymphoblastic leukemia (BCP ALL)

miRNA	Target rank	Target score	Gene symbol	Gene description
Hypermethylated				
MIR1273g	1	97	*SLC2A1*	solute carrier family 2 (facilitated glucose transporter), member 1
	2	96	*CDH8*	cadherin 8, type 2
	3	96	*NLK*	nemo-like kinase
	4	95	*TAF5*	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa
	5	95	*SLC30A5*	solute carrier family 30 (zinc transporter), member 5
MIR1304	1	98	*FBXO45*	F-box protein 45
	2	98	*AKR1B1*	aldo-keto reductase family 1, member B1 (aldose reductase)
	3	97	*PFKFB2*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2
	4	97	*CAPRIN2*	caprin family member 2
	5	96	*ACBD3*	acyl-CoA binding domain containing 3
	6	96	*PRR9*	proline rich 9
	7	96	*USP47*	ubiquitin-specific peptidase 47
	8	96	*B4GALT6*	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6
	9	95	*MKX*	mohawk homeobox
	10	95	*KIAA1324*	KIAA1324
	11	95	*FAM122B*	family with sequence similarity 122B
MIR663	1	100	*ABO*	ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase)
	2	99	*ESPN*	Espin
	3	98	*YIF1B*	Yip1 interacting factor homolog B (*Saccharomyces cerevisiae*)
	4	98	*NFIX*	nuclear factor I/X (CCAAT-binding transcription factor)
	5	97	*DPP9*	dipeptidyl-peptidase 9
	6	96	*SPTBN4*	spectrin, beta, non-erythrocytic 4
	7	95	*GRIN2D*	glutamate receptor, ionotropic, N-methyl D-aspartate 2D
Hypomethylated				
MIR4442	1	100	*MTA2*	metastasis associated 1 family, member 2
	2	95	*PSME4*	proteasome (prosome, macropain) activator subunit 4
MIR155	1	98	*WEE1*	WEE1 G2 checkpoint kinase
	2	98	*IRF2BP2*	interferon regulatory factor 2, binding protein 2
	3	98	*HIVEP2*	human immunodeficiency virus type I enhancer binding protein 2
	4	98	*JARID2*	jumonji, AT rich interactive domain 2
	5	98	*ZNF652*	zinc finger protein 652
	6	98	*BACH1*	BTB and CNC homology 1, basic leucine zipper transcription factor 1
	7	97	*TP53INP1*	tumor protein p53 inducible nuclear protein 1
	8	97	*TM9SF3*	transmembrane 9 superfamily member 3
	9	96	*FAR1*	fatty acyl CoA reductase 1
	10	96	*GABRA1*	gamma-aminobutyric acid (GABA) A receptor, alpha 1
	11	96	*JADE1*	jade family PHD finger 1
	12	96	*RCN2*	reticulocalbin 2, EF-hand calcium-binding domain
	13	95	*SOCS5*	suppressor of cytokine signaling 5
	14	95	*ZIC3*	Zic family member 3
MIR3909	1	98	*PBX2*	pre-B-cell leukemia homeobox 2
	2	97	*ARIH1*	ariadne RBR E3 ubiquitin protein ligase 1
	3	97	*SLITRK4*	SLIT and NTRK-like family, member 4
	4	95	*SH3D19*	SH3 domain containing 19
	5	95	*CASKIN2*	CASK interacting protein 2

Table 5. Characteristics of differentially methylated miRNA gene bodies-associated sites in B-cell precursor acute lymphoblastic leukemia (BCP ALL) with respect to the location in known CpG islands

Feature CGI location	Open sea	Shore	Island	Shelf	All
Whole panel					
Number	1161	101	106	58	1426
%	81.4	7.1	7.4	4.1	100.0
All DM gene body-associated sites					
Number	161	18	17	5	201
% of DM	80.1	9.0	8.5	2.5	100.0
Avg. met. in BCP ALL	0.542	0.493	0.343	0.654	0.523
Avg. met. in CTR	0.636	0.479	0.151	0.698	0.583
Hypermethylated in BCP ALL					
Number	71	11	17	3	102
% of DM	35.3	5.5	8.5	1.5	50.7
Avg. met. in BCP ALL	0.796	0.664	0.343	0.669	0.703
Avg. met. in CTR	0.670	0.452	0.151	0.595	0.558
Met. difference	0.126	0.212	0.192	0.074	0.145
Hypomethylated in BPC ALL					
Number	90	7	0	2	99
% of DM	44.8	3.5	0.0	1.0	49.3
Avg. met. in BCP ALL	0.341	0.224	–	0.633	0.338
Avg. met. in CTR	0.609	0.522	–	0.854	0.608
Met. difference	0.268	0.298	–	0.221	0.270

Avg. met. – average methylation; DM – differentially methylated; CTR – controls; CGI – Common Gateway Interface.

Table 6. Disease phenotypes enriched by miRNA genes with differentially methylated sites located in gene bodies in B-cell precursor acute lymphoblastic leukemia (BCP ALL)

Disease	FDR*	miRNA gene
Hematologic neoplasms	2.10e-11	*MIR301B, MIR487A, MIR548A2, MIR518C, MIR187, MIR425, MIR433, MIR589, MIR34B, MIR124-1*
Lymphoma, large-cell, diffuse	1.95e-07	*MIR548O2, MIR548AC, MIR548AE2, MIR548H5, MIR548AJ2, MIR155HG, MIR548AI*
Neoplasms	3.90e-07	*MIR17HG, MIR494, MIR155HG, MIR9-3, MIR137, MIR128-2, MIR375, MIR34B, MIR146A, MIR124-1*
Carcinoma, small cell	5.58e-05	*MIR124-2MIR375, MIR34B, MIR124-1, MIR124-3*
Cancer or viral infections	0.0020	*MIR17HGMIR375, MIR34B, MIR155HG, MIR124-1, MIR146A*
Neoplasm of unspecified nature of digestive system	0.0031	*MIR375MIR34B, MIR124-1, MIR146A*

* Fisher's exact test false discovery rate (FDR), as implemented in WebGestalt software.

miRNA genes differentially methylated in specific leukemia genetic subtypes

To detect miRNA genes potentially associated with separate leukemia genetic subtypes (*ETV6-RUNX1, TCF3-PBX1, IGH*, hyperdiploidy, triploidy), patients with a specific subtype were compared against all the other patients with known cytogenetic status. This analysis allowed us to detect 21 DM sites in patients with *ETV6-RUNX1*, corresponding to promoters and gene bodies of 12 different miRNA genes (Table 7). Only 7 of these sites were located in promoters and were mainly (71.4%) hypomethylated with an average absolute Δβ of 0.27. Nearly 66.7% of DM sites in *ETV6-RUNX1* cases were located in gene bodies and were predominantly associated with hypomethylation (64.2% of sites). For *TCF3-PBX1* subtype, we found 56 DM sites associated with 37 miRNA genes (Table 7). Most of the sites were located in promoters (n = 38, 67.8%). Moreover, most of them (89.5% of sites) were hypomethylated in *TCF3-PBX1* cases. The DM sites (between TCF3-PBX1 cases and other leukemia subtypes) located in gene bodies (n = 18) also showed high Δβ values (around 0.39) and were predominately (61.1%) hypomethylated. In patients with *IGH* rearrangements, only 3 probes (2 in promoters and 1 in gene body) associated with 3 different miRNA genes were hypomethylated with a low average Δβ of 0.18.

The occurrence of hyperploidy in BCP ALL patients was associated with differential methylation of 34 sites (25 different miRNA genes), the majority of which (61.7%) was located in gene bodies of 14 different genes. The sites located

Table 7. The miRNA genes with differentially methylated sites between specific genetic subtype and all other samples with known cytogenetic status

Promoter		Gene body	
hypo-	hyper-	hypo-	hyper-
eTV6-RUNX1			
MIR320B1	*MIR548F3*	*MIR3163*	*MIR99AHG*
MIR1306	*MIR375*	*MIR548F3*	*MIR548H2*
MIR1205	–	*MIR4529*	–
MIR320B1	–	*MIR548H2*	–
MIR219A2	–	*MIR548H4*	–
–	–	*MIR181A1HG*	–
TCF3-TBX1			
MIR874	*MIR3945*	*MIR548W*	*MIR3134*
MIR5685	*MIR5191*	*MIR5694*	*MIR7853*
MIR369	*MIR497*	*MIR1268A*	*MIR5095*
MIR1470	*MIR1207*	*MIR548N*	*MIR1273E*
MIR410	–	*MIRLET7BHG*	*MIR5096*
MIR140	–	*MIR5096*	*MIR548F3*
MIR4287	–	*MIR548D1*	–
MIR412	–	*MIR1273E*	–
MIR4720	–	*MIR548H4*	–
MIR183	–	*MIR1237*	–
MIR6775	–	–	–
MIR1272	–	–	–
MIR496	–	–	–
MIR5571	–	–	–
MIR6742	–	–	–
MIR135A2	–	–	–
MIR656	–	–	–
MIR4265	–	–	–
MIR3660	–	–	–
IGH			
MIR495	–	*MIR548H4*	–
MIR6790	–	–	–
Hyperploidy			
MIR650	*MIR8089*	*MIR99AHG*	–
MIR7850	*MIR6746*	*MIR1273H*	–
MIR548I4	*MIR922*	*MIR5009*	–
MIR614	–	*MIR663AHG*	–
MIR298	–	*MIR548AU*	–
MIR3666	–	*MIR100HG*	–
MIR299	–	*MIR548AI*	–
MIR1297	–	*MIR548A2*	–
MIR3201	–	*MIR548AY*	–
–	–	*MIR7853*	–
–	–	*MIR548AE2*	–
–	–	*MIR548W*	–
–	–	*MIR548I4*	–
–	–	*MIR6130*	–
Triploidy			
–	–	–	*MIR548H4*

in promoters showed slightly higher absolute Δβ (0.27) than those located in gene bodies (0.21). Both promoter and gene body-associated sites predominantly showed hypermethylation in patients with hyperploidy, with 71.4% of promoter sites hypomethylated and all sites (100%) located in gene bodies hypomethylated in hyperploidy cases.

Only a single probe, associated with the body of *MIR548H4* gene, was DM (hypermethylated) in samples obtained from patients with triploidy (Table 7).

Discussion

Aberrant expression of some miRNA genes may be a contributing factor in the oncogenesis of many cancers,[22,23] including acute leukemias.[24–27] Epigenetic modifications, such as methylation of miRNA genes, regulate the expression of genes.[28] Therefore, altered miRNA gene methylation may be regarded as a causal factor for leukemogenesis and it can determine the clinical course of acute leukemia as well.

In this study, 377 sites located in the promoters of 242 different miRNA genes were DM in BCP ALL compared to control samples. Many of these miRNAs participate in cell cycle and differentiation control (Table 4). This research was focused on determining the methylation landscape of miRNA genes only; therefore, the expression level of the examined miRNA remains unknown. Despite the lack of evidence from this study to link the particular miRNA methylation level with its expression, the results obtained by others may indicate such a relationship.[29–34] Stumpel et al.[30] identified 11 miRNAs that were downregulated in t(4;11)-positive infant ALL, as a consequence of CpG hypermethylation. Seven of those miRNAs were reactivated after the exposure to the demethylating agent, zebularine. In our study, 4 out of these 7 genes (*MIR200B*, *MIR429*, *MIR10A* and *MIR432*) were hypermethylated as in the previously mentioned study.[30] Their impact on the development and clinical course of leukemia may be significant. For example, the zinc finger E-box binding homeobox 2 (*ZEB2*) gene is the best-known, validated target gene of the miR-200b/a-429 cluster.[31] Homeobox A3 (*HOXA3*) gene has been described as a potential *MIR10A* targeted gene.[32] For *MIR432*, more than 100-fold downregulated expression was observed in t(4;11)-positive infant ALL, as compared with normal bone marrow.[30] The *MIR432* is located within the large *MIR127* cluster which is silenced in various malignancies by CpG island hypermethylation and aberrant histone modifications.[33]

Schotte et al.[34] showed the reduced methylation level at CpG islands in the promoter regions of *MIR196B*, yet it was limited to MLL-rearranged BCP ALL cases. It corresponded to an upregulation of *MIR196B*, suggesting an epigenetic origin for its overexpression. This is in line with the results of our study, where *MIR196B* gene is hypomethylated in all BCP ALL cases.

Table 8. The comparison between the methylation status of selected miRNAs and their expression according to available references

miRNAs	Expression	Reference	β-value BCP ALL	β-value controls	p-value	Methylation
MIR125B	high	Swellam et al.[54]	0.618	0.109	<0.001	high
MIR203	low	Swellam et al.[54]	0.335	0.066	0.003	high
MIR181A	low	Nabhan et al.[55]	0.857	0.777	<0.001	high

The methylation beta-value (β) – the ratio of the methylated (C) to unmethylated (T) signals. The p-value was calculated for the differential methylation between BCP ALL and control individuals. BCP ALL – B-cell precursor acute lymphoblastic leukemia.

The results of other studies[27] revealed that the genes of at least 5 miRNAs (*MIR326*, *-200c*, *-125B*, *-203*, and *-181A*) have a significantly different expression in BCP ALL cases compared to healthy controls. According to our study, the gene promoters of 3 out of the abovementioned miRNA (*MIR125B*, *-203* and *-181A*) are DM in BCP ALL patients. The comparison between their methylation status and their expression (according to the available references) was shown in Table 8. The expression of *MIR125B* gene, opposite to *MIR203* and *MIR181A*, was higher in BCP ALL patients, although its promoter was hypermethylated.

Top score target genes for miRNA whose gene promoter sites are most statistically significantly hyper- and hypomethylated in BCP ALL are listed in Table 4. Some of these genes are known to be associated with carcinogenesis. For example, *MIR1273* expression is increased in the pancreas of mouse model of pancreatic cancer.[35] The *MIR1304* is a tumor suppressor and HO-1 is its direct target in non-small cell lung cancer.[36] The downregulation of *MIR1304* is related to early stage breast cancer.[37] The overexpression of *MIR663* significantly suppressed the proliferation and invasion of glioblastoma cells in vitro as well as in vivo.[38] The *MIR663* may act as an oncogene in nasopharyngeal carcinoma.[39] Interestingly, in our study on childhood BCP ALL, the promoter of *MIR663* was hypermethylated in pediatric acute myeloid leukemia (AML), with significantly lower expression compared to normal bone marrow.[40]

The *MIR4442* was amidst the genes with the top hypomethylated promoters. Although its role in leukemogenesis is unclear, its predicted targets include GTPase activating Rap/RanGAP domain-like 3 and zinc finger protein 765.[41] The next gene with hypomethylated promoter is *MIR155*, which plays a complex role in AML.[42] The levels of MIR155 significantly influence the set of genes involved in biologic processes related to antiapoptotic, proliferative, and inflammatory activities.[43] The increased expression of *MIR155* causes the downregulation of SPI1 and CEBPB, and consequently may block myeloid differentiation in AML.[44] Moreover, the expression of HSA21-encoded *MIR155* is altered in B cells of Down syndrome individuals and may play a role in Down syndrome-associated leukemia.[45] There is also some evidence linking the significantly upregulated *MIR155* expression level to the high levels of minimal residual disease and poor prognosis in ALL patients.[46] According to the results from other study,[47] *MIR155* was upregulated in the HCV-4 associated ALL group. Hence, the increased *MIR155* level may be related to acute leukemia development. Our results, where *MIR155* promoter was hypomethylated, are consistent with this observation.

To sum up, there was a slight predominance of differentially hypermethylated (207/377) over hypomethylated (170/377) promoter-associated sites in miRNA genes in BCP ALL patients. However, the potential impact of miRNAs genes methylation level on their expression has to be established in future studies.

There were also 201 DM gene body associated sites with nearly equal (102:99) distribution between their hyper- and hypomethylated status. They corresponded to 74 different miRNA genes. The function of gene body methylation is not well understood. Typically, DNA promoter methylation is believed to be a marker of transcriptional repression. However, the DNA methylation within the gene body appears to serve a different function than DNA methylation within the promoter region. While there is some evidence that intragenic DNA methylation is related to transcriptional repression,[48] the bulk of evidence suggests that it is associated with gene activation.[49] Characteristics of DM in BCP ALL miRNA gene bodies-associated sites are presented in Table 6. Interestingly, a few of the 483 potential target genes of the most hyper- and hypomethylated miRNAs are annotated to leukemia phenotype.

Among different genetic subtypes, only 1 subtype (3 patients) with the translocation t(1;19) generating the *TCF3-PBX1* fusion gene, had a distinct miRNA gene methylation profile. For this subtype there were 56 DM sites found, associated with 37 miRNA genes. Most of the sites were located in promoters and were hypomethylated. According to the previously published data, several miRNAs are downregulated or upregulated in *TCF3/PBX1*-positive ALL.[50–52]

Herein, we have shown that the miRNA genes of pediatric BCP ALL patients are DM compared to controls. This epigenetic dysregulation seems to play an important role in controlling miRNA expression, therefore affecting the clinical course of BCP ALL. To date, several miRNAs have been observed to have altered expression in patient cohorts compared to healthy individuals, while several studies have identified specific miRNAs that can be used as biomarkers to diagnose ALL, classify it into subgroups and predict the prognosis.[27,53–55] Moreover, hypermethylated genes can be targeted by hypomethylating agents, such as cytosine analogs, azacitidine or decitabine, which may open up new potential treatment options for this type of leukemia.[56,57]

Limitations

In this study, we presented a methylation profile of genes for miRNAs in the bone marrow of BCP ALL and healthy subjects; however, due to major constraints, we were not able to determine the expression of DM miRNA genes. Thus, we had to refer to data regarding their expression level from other studies. The second limitation is using only one method for determining DNA methylation level. Additionally, for some of the analyzed miRNAs (i.e., the ones encoded in introns of mRNA genes) we were unable to determine if the observed methylation level of DNA is associated with the regulation of the miRNA or its host mRNA gene expression. However, this does not affect the main goal of this study, which was to select miRNA genes with the most altered methylation as targets for future studies. The last limitation is the small control group. This was due to the limited availability of the bone marrow samples from healthy children. The bone marrow aspiration is an invasive procedure, so performing it without clear medical indications would be highly unethical. Because of that, we were not able to obtain bone marrow samples from completely healthy children. On the other hand, only individuals with any cancer and genetic disease were ruled out, as well as with the normal bone marrow smears, were included in the control group.

Conclusions

In this study, a different were genes methylation landscape was shown in pediatric BCP ALL compared to children without neoplasms. A visible subcluster among BCP ALL samples consisted of individuals with TCF3-PBX1 genetic subtype. No other differences were observed in association with age, gender or risk group. Several interesting leukemia-connected phenotypes were found to be enriched in genes associated with hyper- and hypomethylated sites located on promoters as well as gene bodies.

ORCID iDs

Radosław Chaber https://orcid.org/0000-0002-6862-9142
Artur Gurgul https://orcid.org/0000-0001-5979-144X
Jacek Tabarkiewicz https://orcid.org/0000-0002-1264-2882
Grażyna Wróbel https://orcid.org/0000-0003-4374-6083
Tomasz Szmatoła https://orcid.org/0000-0003-1588-4198
Igor Jasielczuk https://orcid.org/0000-0001-8583-3850
Olga Haus https://orcid.org/0000-0002-5206-0553
Monika Lejman https://orcid.org/0000-0002-8760-0775
Blanka Rybka https://orcid.org/0000-0002-4216-0673
Renata Ryczan-Krawczyk https://orcid.org/0000-0002-4936-4951
Anna Jaśkowiec https://orcid.org/0000-0002-5966-3402
Sylwia Paszek https://orcid.org/0000-0002-7865-2661
Natalia Potocka https://orcid.org/0000-0002-5505-3922
Christopher J. Arthur https://orcid.org/0000-0001-9647-1509
Wioletta Bal https://orcid.org/0000-0003-4992-5322
Kornelia Łach https://orcid.org/0000-0001-8339-1374
Aneta Kowal https://orcid.org/0000-0002-5341-4851
Izabela Zawlik https://orcid.org/0000-0001-7992-9100
Elżbieta Latos-Grażyńska https://orcid.org/0000-0002-7154-3702

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