

Methylation of the MAPK1 Gene as a Potential Diagnostic and Prognostic Biomarker in Childhood B-Acute Lymphoblastic Leukemia

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ABSTRACT

Background: Acute lymphoblastic leukemia is a type of heterogeneous leukemia that is associated with the abnormal proliferation of immature monoclonal B and T lymphoid precursors. Cancer is considered an epigenetic disease due to fundamental changes in the pattern of normal methylation in cancer cells. This study aimed to investigate these changes in 5 candidate genes in childhood B cell-ALL.

Materials and Methods: After blood sampling, DNA extraction, and bisulfite treatment, PCR, and Sanger sequencing were performed for part of the TBX3, SLC38A11, SOX2, KCNQ1, and MAPK1 gene promoter. Finally, the methylation pattern of patients in remission and relapse groups was compared with that of healthy individuals.

Results: In the promoter methylation pattern of MAPK1, a significant difference was observed in the CpG3 site between remission-control and relapse-control groups, and in CpG8, 9, 10 between relapse-control and relapse-remission groups. There was no significant difference in the other 4 genes.

Conclusion: We reported the similarity of the methylation pattern in the CpG 3 site of the MAPK1 gene promoter in the remission and relapse groups, as well as the difference in the methylation pattern of this location in the two mentioned groups with control groups, which can suggest the methylation pattern in this site as a candidate for a prognostic biomarker. We also reported the similarity of methylation pattern in remission and control groups on CpG 8,9,10 site and significant reduction of methylation in the relapse group compared to both control and remission groups, which can introduce them as a candidate for diagnostic biomarkers.

Keywords: Acute lymphoblastic leukemia; Bisulfite sanger sequencing; Epigenetics; MAPK1; Promoter-methylation

INTRODUCTION

The epigenetic control of gene expression might cause different phenotypic characteristics in cells with the same genotype. The deregulations of these mechanisms can have serious effects on the phenotype or viability of cells. Notably, the aberrant expression of oncogenes and tumor suppressor genes following epigenetic changes might pave the

way for the development or progression of cancer and tumorigenesis.

A single cell that has acquired multiple genetic and epigenetic alterations can lead to cancer. Genetic mutations such as point mutations, chromosomal deletions, gene duplication chromosomal translocation ¹, and epigenetics alteration, involving DNA methylation, histone modifications, activator and inhibitory proteins, and regulatory microRNAs

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are known to be associated with altered expression of some genes². In cells, transcription regulation is programmed by epigenetic mechanisms that cause different phenotypes of cells derived from a zygote with the same genotype and, deregulations of these mechanisms can have serious effects on phenotype or even cell survival^{3,4}. In cancer cells, some of these expression changes occur in oncogenes and tumor suppressor genes that can provide the way for other stages of tumorigenesis⁵.

Acute lymphoblastic leukemia (ALL) is the dominant form of childhood leukemia that leads to the formation of leukemic B-cell (B-ALL) and T-cell (T-ALL) lineages in the bone marrow and blood⁶. B-ALL is characterized by its heterogeneity and can affect individuals of all age groups, including both pediatric and adult. The onset of childhood B-ALL commonly occurs between 2 and 5 years of age⁷.

Many cases of B-ALL suffer certain genetic mutations that are considered diagnostic and prognostic biomarkers. These mutations are reviewed in previous studies⁸⁻¹⁰. On the other hand, in some patients, epigenetic modifications of chromatin have been seen. These changes, which can have serious effects on gene expression, phenotype, and cell cycle, are responsible for creating special phenotypes of cancer cells in B-ALL¹¹.

The most widely epigenetic mechanism is DNA methylation which occurs in the CpG islands. CpG island hypermethylation is closely linked to the aberrant silencing of tumor suppressor genes, which can lead to the overactivity of oncogenes^{12,13}. These aberrant DNA methylations have been observed in several types of leukemia and many other cancers. Cancer cells, like normal cells, depend on their special methylation patterns to maintain their unique characteristics. Therefore, these special patterns can be used to improve existing treatments and introduce diagnostic and prognostic biomarkers for ALL and other cancers¹⁴⁻¹⁶.

Bisulfite Sanger sequencing is used as a gold standard for the study of CpG islands due to its exceptional ability to track changes in a single nucleotide (C/T)^{17,18}. After sequencing, the presence of a cytosine or thymine peak indicates 100% methylation or lack of methylation in both alleles,

respectively. The presence of two peaks (C, T) with the same height indicates cytosine methylation in one of the parental alleles, which means 50% methylation in the read position, and another condition, including two peaks with different heights, means more or less than 50% cytosine methylation that can be observed in cancer cells due to heterogeneous methylation.

In this study, we detect DNA methylation of the promoter region of five genes in childhood B-ALL patients without any known genetic mutation to understand the aberrant pattern of DNA methylation in this cancer. Many of the earlier methylation studies were performed on a single gene and fewer studies have been performed on the methylation pattern of several genes simultaneously. Therefore, we have nominated five genes T-Box Transcription Factor 3 (TBX3), Solute Carrier Family 38 Member 11 (SLC38A11), SRY-Box Transcription Factor 2 (SOX2), Potassium Voltage-Gated Channel Subfamily Q Member 1 (KCNQ1), Mitogen-activated protein kinase 1 (MAPK1) based on the gene expression profiles available in Gene Expression Omnibus^{19,20}, and the methylation pattern of these genes was analyzed in 30 patients in two 15-individual groups, compared to 15 healthy individuals.

MATERIALS AND METHODS

Subjects

In this study, with ethical approval and parental consent, 3 ml peripheral blood samples were collected from 95 children with newly diagnosed B-cell ALL, at the time of first diagnosis (New case), without known genetic mutations, and before treatment (Table 1). Flow cytometric immunophenotyping for the diagnosis of B-ALL, based on bone marrow samples containing 85% blasts in this sample and analysis of CD19, CD34, CD20, CD22, CD117, CD45, and HLA DR. The samples are stored in an institutional genetics laboratory, and diagnoses were performed at an affiliated children's hospital according to current WHO guidelines²¹. Sampling was taken before any treatment protocol (due to the possible effect of drugs on the methylation pattern). Finally, 3 ml peripheral blood samples were taken from 15 children without a

history of hematological disorders and collected in the same lab as above.

In the continuation of the diagnostic process and patient follow-up, 50 cases out of 95 sampled patients had known genetic mutations, which were excluded at this stage. Of the remaining 45 patients, 28 cases relapsed, and 17 cases were cured during the chemotherapy phase. Fifteen patients (two cured and 13 relapsed) were excluded from the study due to the death, lack of consent of the child's parents to continue cooperation, or unwillingness to publish data, and to match the age, sex, and number of two groups. Finally, three groups of 15 people, including remission, relapse, and control, entered the study. Patients were categorized into remission or relapse groups based on their clinical follow-up and diagnostic reports provided by the affiliated children's hospital, following the WHO guidelines for ALL. Complete remission (CR) was characterized by a complete hematological and marrow recovery, while relapse was defined by the reappearance of leukemic blasts in the bone marrow or peripheral blood.

Expression profiling by microarray was obtained from NCBI in Gene Expression Omnibus with <https://www.ncbi.nlm.nih.gov/geo> address.

Two datasets with GSE2628 and GSE76340 accession numbers were analyzed by R software for the determination of differentially expressed genes (DEGs) between cancer and normal cells (19, 20). In this method, binary logarithm ($\log_2 n$) was used for data compression and boxplot was used for data normalization along with principal component analysis for quality control assessment. Finally, with a threshold of 0.05 for adjusted p-value and <-1 and >1 for $\log FC$, five genes were nominated.

DNA extraction and bisulfite genomic sequencing

Mononuclear peripheral blood cells (PBMC) were isolated by Ficoll centrifugation, and DNA was extracted using PrimePrep Genomic DNA Isolation Kit (GeNet Bio, Cat. No. K-2000). NanoDrop (ThermoScientific, Wilmington, DE, USA) was used for DNA quantification and stored at -20°C . Primers were designed using the methprimer2 online tool, available: <http://www.urogene.org/methprimer2/>. Practical phases were stopped in this stage and the patient's follow-up started.

After DNA extraction, extracted DNA was treated with sodium bisulfite using EpiTect Fast Bisulfite Kit (Qiagen), according to the manufacturer's recommendations. The PCR reaction was conducted in a 50 μL mixture containing 25 μL PCR Master Mix-#K0171, 2 μL each primer, 2 μL Bisulfite-treated DNA as a template, 19 μL nuclease-free water. The cycling and temperature conditions were as follows: Initial denaturation at 97°C for 5 min, followed by 40 cycles of 1 min at 95°C , 30 s at annealing temperature for any sequence, 1 min at 72°C with 5 min at 72°C for final extension and hold at 20°C for 4 min. Annealing temperatures were used at 50, 52, 51, 52, and 50°C for TBX3, SLC38A11, SOX2, KCNQ1, and MAPK1 sequences, respectively. The PCR products were purified in the PyroMark Q24 System (Biotage AB, Uppsala, Sweden), according to the manufacturer's protocol, and the average methylation levels in amplified regions were analyzed with Sanger sequencing. The Tfsitescan (<http://www.ifti.org/cgi-bin/ifti/Tfsitescan>) was examined to determine if a particular transcription factor (TF) could be linked to a significant CpG site.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 9.5.1. The average of Cytosine methylation in each site of each sample was calculated according to the $[C/(C+T)] \times 100$ formula²². Non-parametric, Mann-Whitney, and Pearson correlation tests were used for the analysis of the average methylation levels and correlation analysis in each group. For all calculations, the significance threshold was considered with $P < 0.05$.

Table 1: Baseline clinical and laboratory features of B-ALL patients and healthy controls

	Remission (n=15)	Relapse (n=15)	Control (n=15)
Sex, n			
Male	8	6	7
Female	7	9	8
Age (months), Median (Range)	25 (15-35)	22 (15-35)	22 (8-35)
WBC Count (10⁹/L)	13	9	N/A
<50	2	6	
>50			
BM Blast at diagnosis	12	7	
<20	3	3	N/A
20-50	0	3	
50-80	0	2	
>80			

Abbreviations: WBC, white blood cell count; BM, bone marrow; N/A, not applicable

RESULT

We investigated the CpG island methylation status of 5 candidate genes in 30 children with B-ALL in two groups of remission and relapse, and compared them with 15 healthy controls, using bisulfite Sanger sequencing. Candidate genes TBX3, SLC38A11, SOX2, KCNQ1, and MAPK1, were selected based on the selection of differential gene expression profiles on the data sets available in Gene Expression Omnibus, using R software. In the gene methylation pattern study, in 4 out of the 5 candidate genes, there was no significant difference between the studied groups. Only in one gene, MAPK1, a significant aberrant methylation was a significant aberrant methylation detected. For the MAPK1 methylation study, a CpG island upstream of the gene with a length of 168 nucleotides and 15 CpG sites was examined. The first CpG site was 656 nucleotides away from the adenine, the initiating codon of the MAPK1 gene. The primers were designed to cover the entire area and replicate a piece with a length of 355 nucleotides (Figure 1a). Sequencing was performed with forward primer and all CpG sites were visible on chromatogram analysis. The presence of adenine instead of Guanine shows the antisense strand on the graph (Figure 1b).

In the comparison between remission and control groups, two CpG sites out of the 15 sites showed a lack of methylation in both groups, and there was no significant difference for the other 12 positions. However, our study showed a significant difference in the CpG 3 site (Table 2), with a p value of 0.0309. (Figure 1c left) There was also a significant difference in the same position between relapse and control groups (Table 3), with a value of 0.006 (Figure 1c right).

Table 2: Comparison of methylation percentage in part of the MAPK1 gene promoter in remission and control groups

CpG Site	The relative distance from the ATG in the initiation codon	Methylation status						P
		Remission (2n=15)			Control (2n=15)			
		MM	MU	UU	MM	MU	UU	
CpG1	-636	4	9	2	6	8	1	0.402
CpG2	-619	0	0	15	0	0	15	N. A
CpG3	-602	4	4	7	10	3	2	0.0309
CpG4	-599	7	6	2	7	7	1	0.771
CpG5	-593	0	0	15	0	0	15	N. A
CpG6	-583	10	4	1	10	4	1	0.705
CpG7	-581	7	5	3	8	5	2	0.904
CpG8	-578	11	3	1	12	2	1	0.703
CpG9	-575	9	5	1	10	5	0	0.299
CpG10	-557	8	5	2	8	6	1	0.780
CpG11	-547	6	7	2	8	6	1	0.416
CpG12	-534	10	2	3	11	3	1	0.558
CpG13	-523	8	5	2	10	3	2	0.498
CpG14	-519	11	1	3	12	1	2	0.267
CpG15	-515	11	1	3	12	3	0	0.511

N.A: Not Applicable, M: Methylated allele, U: Unmethylated allele

Table 3: Comparison of methylation percentage in part of the MAPK1 gene promoter in the relapse and control groups

CpG Site	The relative distance from the ATG in the initiation codon	Methylation status						P
		Relapse (2n=15)			Control (2n=15)			
		MM	MU	UU	MM	MU	UU	
CpG1	-636	4	3	8	6	8	1	0.069
CpG2	-619	0	0	15	0	0	15	N. A
CpG3	-602	3	2	10	10	3	2	0.006
CpG4	-599	8	6	1	7	7	1	0.651
CpG5	-593	0	0	15	0	0	15	N. A
CpG6	-583	10	3	2	10	4	1	0.869
CpG7	-581	2	9	4	8	5	2	0.065
CpG8	-578	2	0	13	12	2	1	<0.0001
CpG9	-575	5	4	6	10	5	0	0.004
CpG10	-557	3	4	8	8	6	1	0.001
CpG11	-547	6	5	4	8	6	1	0.262
CpG12	-534	9	1	5	11	3	1	0.286
CpG13	-523	8	3	4	10	3	2	0.370
CpG14	-519	7	6	2	12	1	2	0.166
CpG15	-515	10	2	3	12	3	0	0.223

N.A: Not Applicable, M: Methylated allele, U: Unmethylated allele

The results of the study showed that this site, which is inserted in the 226-227 position of the sequence, is a binding site for a transcription factor called GCF (CG-rich sequence DNA-Binding Factor) (Fig. 1d).

GCF, which binds to this location, is a DNA and RNA-binding transcription factor.

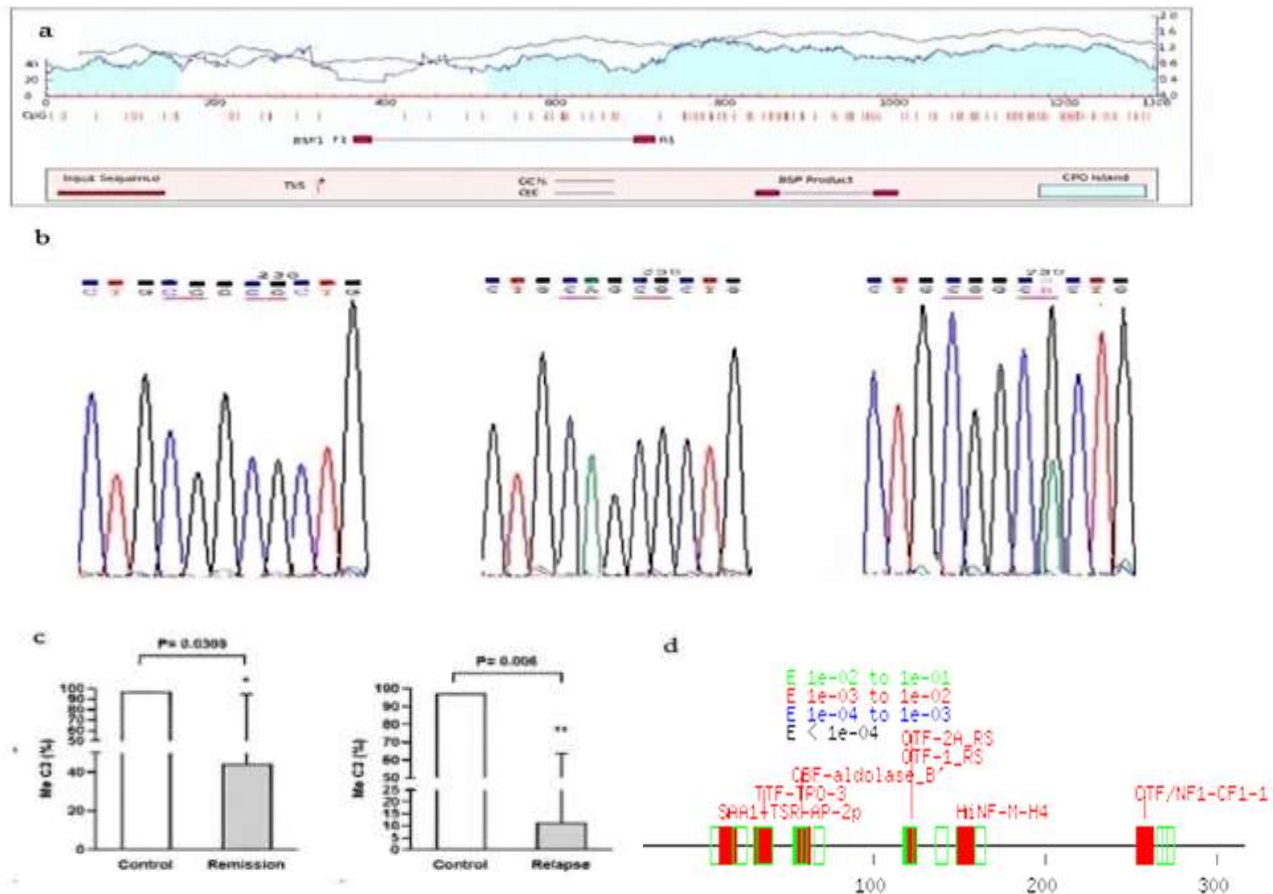


Figure 1. The replicated area of MAPK1 (a) includes a 168-nucleotide CpG island and 15 CpG sites, with a 355-nucleotide piece replicated by Methprimer2 primers. (b) From left to right respectively, Chromatogram of CpG 3 and 4 sites in one sample of the control, The presence of CG on two sites indicates methylated cytosine in the sense strand, which has not changed during the bisulfite reaction. remission: The presence of CA instead of CG on CpG 3 and non-changed CG on CpG 4 indicate non-methylated cytosine, which has been changed during the bisulfite reaction, and methylated cytosine in the sense strand, respectively, and relapse groups: The presence of CG on CpG3 indicates methylated cytosine, and CR instead of CG on CpG4 indicates methylation on one of the parent's alleles in some cells due to acquired mutations. Areas with a colored line represent a CpG site. (c) Left: Comparison of CpG3 site between control & remission, and right: control & relapse. There is a significant difference in the CpG 3 site with, $P = 0.0309$ and $P = 0.006$ between control & remission and control & relapse, respectively. (d) Checking the binding of a special transcription factor at each site. The binding of GCF to CpG3 and AP-2-alpha, AP-2-gamma, and AP-2 to CpG 9, 10 (<http://www.ifti.org/ifti-imaps/im1594106402.gif>)

The comparison of the relapse and control groups showed a significant difference in the CpG 8,9,10 sites (Table 3), with the P-value <0.0001, 0.004, and 0.001, respectively, in addition to the CpG 3 site. Analysis of methylation status between two groups

of remission and relapse (Table 4) showed a significant difference in the same location with P-value <0.0001, 0.042, and 0.012, respectively, but not on the CpG3 site (Figure. 2).

Table 4: Comparison of methylation percentage in part of the MAPK1 gene promoter in remission and relapse groups

CpG Site	The relative distance from the ATG in the initiation codon	Methylation status						P
		Remission (2n=15)			Control (2n=15)			
		MM	MU	UU	MM	MU	UU	
CpG1	-636	4	9	2	4	3	8	0.222
CpG2	-619	0	0	15	0	0	15	N. A
CpG3	-602	4	4	7	3	2	10	0.2733
CpG4	-599	7	6	2	8	6	1	0.801
CpG5	-593	0	0	15	0	0	15	N. A
CpG6	-583	10	4	1	10	3	2	0.859
CpG7	-581	7	5	2	2	9	4	0.089
CpG8	-578	11	3	2	2	0	13	< 0.0001
CpG9	-575	9	5	1	5	4	6	
CpG10	-557	8	5	2	3	2	10	0.012
CpG11	-547	6	7	2	6	5	2	0.692
CpG12	-534	10	2	3	9	1	5	0.616
CpG13	-523	8	5	2	8	3	4	0.717
CpG14	-519	11	1	3	7	6	2	0.267
CpG15	-515	11	1	3	10	2	3	0.749

N.A: Not Applicable, M: Methylated allele, U: Unmethylated allele

The results of the investigation for the possibility of linking a specific transcription factor to these sites, which were in the 247-248, 250-251, and 271-272 positions of the sequence, showed a binding site for three transcription factors. It was determined that the position of 249-276 is a binding site for AP-2-alpha, AP-2-gamma, and AP-2 (Figure 1). The activating enhancer-binding protein 2 (AP-2) family can form hetero- or homo-dimers and bind to GC-

rich DNA sequences within regulatory regions of their target genes, mediating both activation and inhibition of gene transcription.

In the analysis of age and gender in three groups, only a significant correlation was observed in the CpG 8 site in the control group with a P-value=0.019, which was not representative of a clear result.

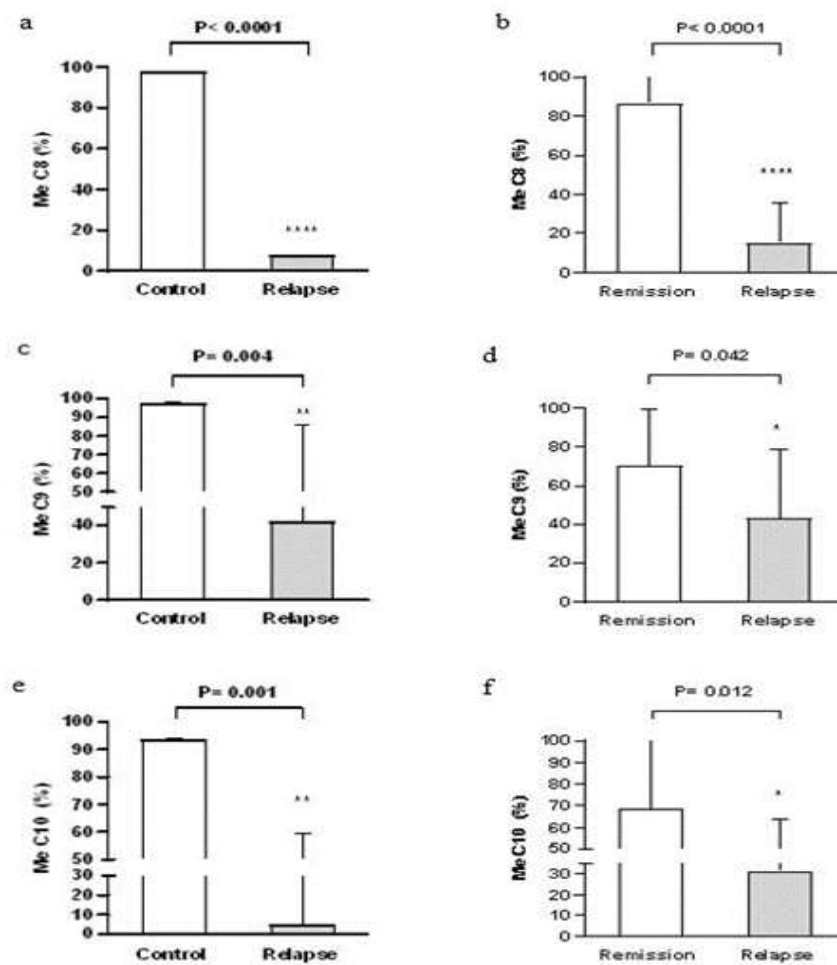


Figure 2. a, b, c, and d, Comparison of CpG 8,9,10 sites between control, relapse, and remission

DISCUSSION

The nature of the B-ALL disease is heterogeneous, while in approximately one-third of the cases, patients lack the genetic defect and have a normal karyotype²³. Although the treatment of this malignancy has improved tremendously in recent years, it still has a worse prognosis in adult patients than in patients²⁴. In children, despite effective treatment of this malignancy in %80 of cases, %20 of patients show resistance to treatment, which is still high^{25, 26}. Consequently, the identification of novel molecular markers is crucial for improving the diagnostic accuracy and understanding the heterogeneity of B-ALL across all age groups.

Today, cancers, including ALL, are also considered epigenetic diseases²⁷. The results of previous studies showed evidence of the importance of epigenetic alterations along with heterogeneity in the onset, progression, relapse, and prognosis in pediatric B-ALL^{28,29}. Since epigenetic modifications of chromatin play an important role in gene transcriptional and translation regulation, it can be responsible for special phenotypes of cancer cells.

In this study, we examined and compared the methylation pattern of part of the 5-candidate gene promoter (TBX3, SLC38A11, SOX2, KCNQ1, MAPK1) in three groups. Blood samples were taken from the participating children at the beginning of the B-ALL diagnosis, before starting treatment. After monitoring response to treatment, the children were divided into two groups of 15 as remission and relapse status, and compared with the 15 healthy controls. We have reported a significant difference in the methylation pattern on some CpG sites of MAPK1, between groups. MAPK1 is on the 22q11.2 and encodes a protein kinase from a family of the same name. This family of kinases is the common point of several biochemical signals and is involved in a wide range of cellular processes such as differentiation, transcription regulation, and evolution.

The upregulation or downregulation of MAPK1 expression in the MAPK/ERK signaling pathway has been reported in many tumors, including thyroid, colon, prostate, breast, gastric, pancreas, cervical cancer and bladder³⁰⁻³⁷. In this study, we have shown the similarity of the methylation pattern in the CpG

3 site of the MAPK1 gene promoter in the remission and relapse groups, as well as the difference in the methylation pattern of this location in the two mentioned groups with control groups, suggesting that the methylation status of this site may be associated with the disease state in B-ALL patients.

On the other hand, in examining these sites in terms of binding a specific transcription factor to these locations, we observed that the CpG3 site is the binding site of the GCF (GC-rich sequence DNA-Binding Factor). This TF regulates the transcription and expression of many genes, by binding to both DNA and RNA polymerase II. GCF binding to GC-rich sequence, was shown to inhibit the transcriptional activity of epidermal growth factor receptor³⁸, and the reduction of CGF binding to the mentioned areas was found, which caused more expression of Immediate-early response 5 (IER5) as a G2/M checkpoint and suppressor of cell proliferation³⁹.

We also reported the methylation behavior on the CpG 8,9,10 site as the similarity of the remission and control groups and the significant reduction of methylation in the relapse compared to both control and remission groups in this region. Together, we suggested that the significant reduction in methylation at these three loci could potentially identify them as candidates for diagnostic and prognostic biomarkers.

Moreover, our results showed that CpG 9, and 10 are binding sites for AP-2 family transcription factors. The only role associated with the AP-2 family transcription factor and blood cells that we found in previous studies is that the AP-2 families are the crucial proteins for CD4 down- modulation by HIV-1 Nef in Infected Primary CD4+ T Cells⁴⁰.

From another perspective, according to our findings, we propose a hypothesis that there might be an increase in the transcription of MAPK1 during the following two epigenetic processes: 1. Reduction of methylation in these 4 sites, as part of the MAPK1 promoter, and preparation for better GCF, AP-2 α , and γ binding in this area ;2 Histone changes and formation of euchromatin structure after GCF binding, given its oncogenic role, could potentially contribute to progression and relapse in childhood B-ALL.

CONCLUSION

In summary, the observed alterations in the methylation pattern of the MAPK1 CpG sites suggest that these locations may hold potential as candidate diagnostic and prognostic biomarkers for childhood B-ALL. However, given the limited sample size of the current study, to achieve such indicators, it's necessary to examine these findings in other geographical and biological areas, because epigenetic processes depend to a large extent on environmental factors and biological behaviors. We also recommend that this study be conducted in larger groups to confirm these results and provide the necessary clinical validation for these candidate biomarkers.

Moreover, the methylation reduction in these sites may suggest a potential increase in transcription, which could potentially contribute to the progression and relapse of the disease due to the oncogenic role of the mentioned gene. In this case, it should also be noted that without examining the number of mRNA transcripts and the product produced by this gene unit, the proposed performance for the mentioned TFs could not be confirmed. For this reason, we recommend gene expression studies to investigate the effect of this methylation pattern on MAPK1 expression to reach more conclusions that are definitive.

FUNDING

This research received no external funding.

COMPLIANCE WITH ETHICAL STANDARDS

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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