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ARAŞTIRMA / RESEARCH

Changes in the expression and methylation levels of the MMP-2, MMP-9, TIMP-1, TIMP-2 and VEGF-A genes in children with acute lymphoblastic leukemia

Akut lenfoblastik lösemili çocuklarda MMP-2, MMP-9, TIMP-1, TIMP-2 ve VEGF-A genlerinin ekspresyon ve metilasyon seviyelerindeki değişiklikler

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Abstract

Purpose: The aim of this article is to show the expression and methylation levels of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and vascular endothelial growth factor (VEGF)genes in patients diagnosed with childhood acute lymphoblastic leukemia (ALL) and their relationship with clinical findings.

Materials and Methods: In this study, we determined the expression level and methylation percentage of MMP2, MMP9, TIMP1, TIMP2 and VEGF-A genes between patients with childhood ALL and healthy children. Expression and methylation analyzes were performed by RT-PCR. The results of MMP2, MMP9, TIMP1, TIMP2, VEGF-A and other clinical parameters were compared between the two groups.

Results: The expression levels of MMP2 (2.55-fold), MMP9 (5.06-fold), TIMP1 (2.91-fold) and VEGF-A (4.76-fold) was significantly higher in the patients, but the same results were not found for TIMP-2. The methylation levels of MMP2 (29.74%), MMP9 (30.98%), TIMP2 (11.91%) and VEGF-A (6.55%) were significantly higher in the patients than in the controls.

Conclusion: MMP2, MMP9 and VEGF-A genes have high expression and methylation levels. These results make this complex structure intriguing in childhood ALL for further studies and reinforce the emphasis that it should be investigated.

Keywords:. Acute lymphoblastic leukemia, MMP, TIMP, VEGF-A.

Öz

Amaç: Bu makalenin amacı, çocukluk çağı akut lenfoblastik lösemi (ALL) tanısı almış hastalarda matriks metaloproteinazlar (MMP'ler), doku inhibitörleri metaloproteinazlar (TIMP'ler) ve vasküler endotelyal büyüme faktörünün (VEGF) genlerinin ekspresyon ve metilasyon düzeylerini ve bunların klinik bulgularla ilişkisini göstermektir.

Gereç ve Yöntem: Bu çalışmada, çocukluk çağı ALL hastaları ile sağlıklı çocuklar arasında MMP2, MMP9, TIMP1, TIMP2 ve VEGF-A genlerinin ekspresyon düzeyini ve metilasyon yüzdesini belirledik. Ekspresyon ve metilasyon analizleri RT-PCR ile yapıldı. MMP2, MMP9, TIMP1, TIMP2, VEGF-A ve diğer klinik parametrelerin sonuçları iki grup arasında karşılaştırıldı.

Bulgular: Hastalarda MMP2 (2.55-kat), MMP9 (5.06-kat), TIMP1 (2.91-kat) ve VEGF-A (4.76-kat) ekspresyon seviyeleri anlamlı olarak yüksekti, ancak aynı sonuçlar TIMP-2 için geçerli değildi. Hastalarda MMP2 (% 29.74), MMP9 (% 30.98), TIMP2 (% 11.91) ve VEGF-A (% 6.55) metilasyon düzeyleri kontrollere göre anlamlı derecede yüksekti.

Sonuç: Verilerimiz MMP2, MMP9 ve VEGF-A genlerinin yüksek ekspresyon ve metilasyon seviyelerine sahip olduğunu gösterdi. Bu sonuçlar, çocukluk çağı ALL'de bu karmaşık yapıyı daha ileri çalışmalar için ilgi çekici hale getirmekte ve araştırılması gerektiği vurgusunu pekiştirmektedir.

Anahtar kelimeler: Akut lenfoblastik lösemi, MMP, TIMP, VEGF-A.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a malignant disorder of the bone marrow in which a lymphoid progenitor cell becomes genetically altered. It is the most common hematologic malignancy of childhood and represents approximately 25% of cancer diagnoses among children. There are few identified factors associated with an increased risk of developing ALL such as genetic, parental and environmental factors; however, the etiology of the disease remains largely unknown[1].

As is known, angiogenesis is a well-regulated process with a balance between proangiogenic and antiangiogenic factors[2]. Recently, the importance of angiogenesis has been established in hematological malignancies[3]. Some studies demonstrated that the blood vessel content in the bone marrow increases in children with ALL, and this could be due to the angiogenesis of leukemia. Moreover, they showed that angiogenic therapy may increase in the treatment of leukemia[4]. Vascular endothelial growth factor A (VEGF-A) is the most known proangiogenic factor. VEGF-A is active in angiogenesis, vasculogenesis and endothelial cell growth. It has other functions, such as inhibition of apoptosis, inducing endothelial cell proliferation, promoting cell migration[3,5]. VEGF-A is one of the most active factors in the secretion of metalloproteinases (MMPs), especially MMP2 and MMP9, which can hydrolyze collagen IV and V of the vascular basement membrane[6]. MMP2 and MMP9 are well known for their critical roles in the invasion, angiogenesis and metastasis in several cancers, such as lung cancer, breast cancer, cervical cancer and ovarian cancer[7]. The activity of MMPs is regulated by gene transcription, activation of proenzymes and inhibition by different inhibitors. The most important inhibitors of these proteinases are tissue inhibitors of matrix metalloproteinases (TIMPs). TIMP1 is known as a human collagenase inhibitor. It inhibits metalloproteinases that show collagenase activity. TIMP1 and TIMP2 are capable of selectively inhibiting MMP2 and MMP9. These functions include control of cell growth, migration, differentiation, and apoptosis[8]. Their overexpression occurs in many cancers and is correlated with tumor invasiveness and poor prognosis. It is thought that the increased expression of TIMPs is related to the decrease in metastasis and tumor invasion in vivo and in vitro[9]. In the present study, we examined the correlation of MMP2, MMP9, TIMP1, TIMP2, and VEGF-A genes expression and methylation status in children with ALL. The clinical significance of the expression of these genes for leukemia is still unclear and few studies have been conducted on childhood ALL.

MATERIALS AND METHODS

This study was carried out on 26 patients diagnosed with ALL attending the Pediatric Oncology Clinic of Faculty of Medicine, Çukurova University. Newly diagnosed, untreated and uneventful patients, who did not suffer from refractory ALL, who had relapsed or who did not receive hematopoietic stem cell transplantation after diagnosis, were included in the study. Blood samples were obtained at the initial presentation from 26 patients. Also, a control group consisting of 26 individuals who were the same age and gender as the target group and without a family history of cancer were included in the study.

The ethical approval of our study was taken from the Ethics Committee of Çukurova University, Faculty of Medicine (Document number: 09/02/2012-5/3). Informed consent forms were obtained from the parents of the children for our study.

RNA isolation and RT-PCR assay

Blood samples were collected with tubes containing EDTA from the patients and healthy children. The RNA was isolated by RNA isolation kit (PureLink RNA Mini Kit, Invitrogen). RNAs were directly translated into cDNAs by RT[2] First Strand Kit (SABiosciences, Frederick, USA). cDNA concentrations equaled 20 ng / µl in all samples. All steps were done according to the manufacturer's protocol for the Roche Light Cycler 480 System. The interplay between MMP2, MMP9, TIMP1, TIMP2 and VEGF-A gene expression levels were determined by using a data analyzer template provided by Superarray (http://www.sabiosciences.com/pcrarraydataanalysis.php), using glyceraldehyde 3-phosphate dihydrogen (GAPDH) and β-actin (ACTB) as references. The cycle of quantitation (Ct) of each sample was recorded, and the data were analyzed by normalization to β-actin values using the $2^{-\Delta\Delta Ct}$ method.

DNA isolation and methylation analysis

DNA was extracted from peripheral blood samples using a DNA isolation kit (Qiagen, Germany). The

methylation kit, which was especially designed for our study, was used for methylation analysis (EpiTect Methyl II DNA Restriction Kit, Qiagen, Germany). After the cycling program was completed, export and/or copy/paste of the CT values from the instrument software to a blank Microsoft Excel spreadsheet (http://www.sabiosciences.com/dna_methylation_data_analysis.php/) were done according to the manufacturer's instructions for the real-time PCR instrument. In most cases, the minimum level of hypermethylation considered to be positive can be set at 10 to 20%, and the minimum level of intermediately methylated DNA considered to be positive can be set at >60% according to the manufacturer instructions.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS software (Version 25.0, SPSS Inc., Chicago, IL, USA). If continuous variables were normal, they were described as the mean ± standard deviation ($p>0.05$ in Kolmogorov-Smirnov test or Shapiro-Wilk ($n<30$)), and if the continuous variables were not normal, they were described as the median.

For each continuous variable, normality was checked by Kolmogorov Smirnov and Shapiro-Wilk tests. The categorical variables between the groups were analyzed by using the Chi square test or Fisher Exc. test. Comparisons between groups were applied using Student T test for normally distributed data and Mann Whitney U test were used for the data not normally distributed. The Pearson (for data with normal distribution) or Spearman (for data not showing normal distribution) tests were performed for correlation analysis. Values of $p<0.05$ were considered statistically.

RESULTS

Among 26 children diagnosed with ALL, 42.3% were female, and 57.7% were male, the mean age was 6.94 years. Clinical characteristics of the patients, French–American–British (FAB) classification, immunophenotyping, and stages according to Berlin-Frankfurt-Münster (BFM) protocol, subtypes, survival and event status are shown in Table 1. According to the FAB classification, in it was found that 8 cases (30.8%) were in the L1, and in 18 cases (69.2%) were in the L2 subgroup. According to the prognosis of the BFM group; medium risk group (MRG) and high risk group (HRG) were found to be in 84.6% (22) and in 15.4% (4) of the patients, respectively. The immunophenotypic examination was performed in the patients by Flow cytometry; B-ALL and T-ALL were classified as in 73.1% (19) and 26.9% (7) of the patients, respectively.

During the follow-up of patients, it was found that 4 patients (17.4%) with remission had a recurrence. When recurrent patients were evaluated, according to the FAB classification, 1 of ALL-L1 and in 3 of ALL-L2 had a recurrence. According to the prognosis of the BFM group, two of the patients had MRG and HRG risk. When immunophenotypical distributions were examined, 2 patients diagnosed with B-ALL and 2 patients diagnosed with T-ALL had a recurrence. Recurrence showed CNS involvement in 2 patients and bone marrow involvement in the other 2 patients. A total of 4 patients including 2 recurrent patients with CNS involvement died. According to the FAB classification, four of these patients had an L2 subset. According to the BFM classification, three and one of the patients had MRG and HRG risk, respectively. According to immunophenotypical distribution, it showed that one and three of the patients had B-ALL and T-ALL, respectively. WBC, platelets and hemoglobin values of the patient group are shown in Table-2; the average WBC/mm^3 value of the patients was 52020.76; mean platelet/mm^3 and hemoglobin g/dl values were reported as 77142.31 and 8.44, respectively. Anemia was detected in 92.3%, neutropenia in 34.6% and thrombocytopenia in 80.8% of patients. Furthermore, anemia, neutropenia and thrombocytopenia were found together in 23.1% of the patients. The diagnosis leukocytes, platelets, hemoglobin values of the patients are shown in Table-1.

Expression analysis results of the patient and control group are shown in Table-2 and Figure-1. While MMP2, MMP9, TIMP1 and VEGF-A were statistically significant ($p<0.05$), TIMP2 was not ($p>0.05$). It was analyzed whether there was a correlation between expression results and age, WBC, hemoglobin, platelet, and CD cell surface antigens in the patient group. As a conclusion, there was a posistive correlation between MMP9 and CD10 ($p<0.05$; $r=0.470$), and a negative correlation between MMP9 and CD20 ($p<0.05$; $r=-0.637$) and WBC ($p<0.05$; $r=-0.443$). Also, there was a negative correlation between TIMP1 and platelet ($p<0.05$; $r=-0.589$); and o positive correlation between VEGF-A and CD10 ($p<0.05$; $r=0.443$).

Table-1. Patient and control group demographic and clinical data.

Variable	Patient Group	Control Group	P value
Age (month)	83.38 ± 54.87	73.61 ± 41.21	0.20
Gender (girl/boy)	11/15	16/10	0.172
Subtype			
B-cell ALL	19/26	-	-
T-cell ALL	7/26	-	-
Risk category BFM			
MRG	22/26	-	-
HRG	4/26	-	-
FAB classification			
L1	8/26	-	-
L2	18/26	-	-
WBC* (mm^3)	52020.8 ± 94734.6	8762.0 ± 4338.2	0.0000
Platelets* (mm^3)	77142.3 ± 73442.7	253450.5 ± 60822.5	0.0000
Hemoglobin* (g/dL)	8.44 ± 2.3	12.54 ± 3.7	0.001
CD10**	16/26	-	-
CD19**	18/26	-	-
CD22**	19/26	-	-
CD34**	16/26	-	-
Anemia	24/26	-	-
Neutropenia	9/26	-	-
Thrombocytopenia	21/26	-	-
Event Status			
Remission	19/26	-	-
No Remission	3/26	-	-
Relapse	4/26	-	-
Survival			
Lives	17/26	-	-
Exitus	4/26	-	-
Lost to follow-up	5/26	-	-

BFM: Berlin-Frankfurt-Münster classification; CD: Cluster of Differentiation; FAB: French-American-British classification; WBC: White blood cell; *Mean value ± standard deviation; **positive; $p<0.05$

Table-2. Expression results of MMP-2, MMP-9, TIMP-1, TIMP-2 and VEGF-A genes.

Gene Symbol	AVG ΔCt		2^-ΔCt		Fold Change*	P Value**	Fold Up-or Down Regulation
	Patient group	Control group	Patient group	Control group	Patient/ Control		Patient/ Control
ACTB	0.00	0.00	1.000000	1.000000	1.00	nan	1.00
MMP-2	11.31	12.66	0.000394	0.000154	2.55	0.000	2.55
MMP-9	5.05	7.39	0.030113	0.005948	5.06	0.000	5.06
TIMP-1	6.49	8.03	0.011099	0.003817	2.91	0.000	2.91
TIMP-2	12.24	12.69	0.000207	0.000151	1.37	>0.05	1.37
VEGF-A	8.18	10.43	0.003440	0.000723	4.76	0.000	4.76

*Fold-Change (2^(-Delta Delta CT)) is the normalized gene expression (2^(- Delta CT)) in the Test Sample divided the normalized gene expression (2^(- Delta CT)) in the Control Sample. Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicates a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change. **The p values are calculated based on a Student's t-test of the replicate 2^(- Delta CT) values for each gene in the control group and treatment groups, and p values less than 0.05 are indicated in red. The p-value calculation used is based on parametric, unpaired, two-sample equal variance, two-tailed distribution – a method widely accepted in scientific literature.

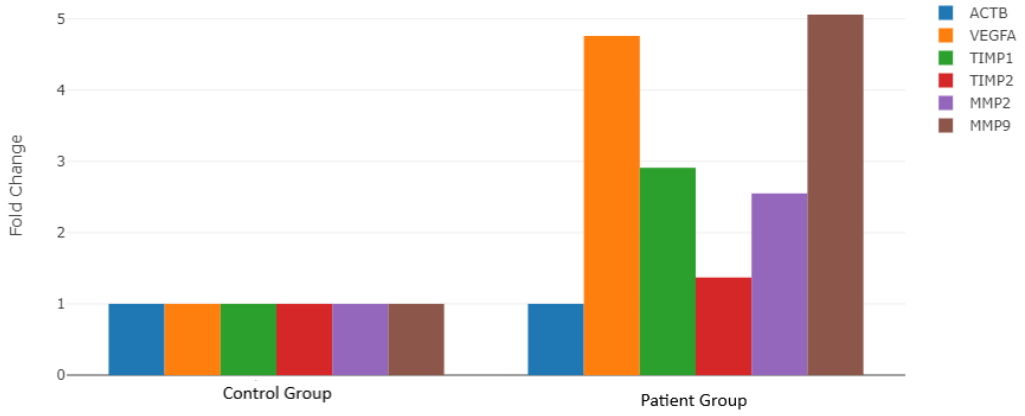

Figure-1. The expression profile of ACTB, MMP-2, MMP-9, TIMP-1, TIMP-2 and VEGF-A genes in patient and control groups.

The methylation analysis results of the patient and control groups are shown in Table-3. We could not measure the methylation level since there was no TIMP1 methylation panel. It was analyzed whether there was a correlation between age, WBC, platelet, hemoglobin, CD surface antigen values and methylation percentage in the patient group. There was no statistically significant result for MMP2 and TIMP2 ($p > 0.05$). There was only a statistically negative correlation between MMP 9 and CD22 ($p<0.05$; $r=-0.477$), and between VEGF-A and WBC value ($p<0.05$; $r=-0.405$).

Table-3: Methylation percentage (%) and expression levels of genes in patient and control groups.

	Gene methylation levels (%)		Gene expression levels
Gene symbol	Patient Group	Control Group	Patients Group
MMP-2	29.74	0.44	2.55
MMP-9	30.98	4.19	5.06
TIMP-1	-	-	2.91
TIMP-2	11.91	0.71	1.37
VEGF-A	6.55	0.52	4.76

DISCUSSION

Angiogenic activity has been an interesting subject in hematological malignancies, because leukemia cells grow in the absence of connective tissue stroma and circulate in blood vessels and body fluids. In this case, neovascularization needed for proliferation and growth in leukemic cells and the increase in angiogenesis in the bone marrow of patients with acute leukemia have been more interesting. In the light of these questions, bone marrow biopsy materials obtained from 40 children with ALL were evaluated in terms of angiogenesis and it was found that the microvessel density increased in the bone marrow[4]. Likewise, it was determined that the number of microvessels significantly increased in patients with AML[10]. Although the increase in the number of vessels has provided findings that may be important for progression and invasion, the investigation of many molecules for leukemia angiogenesis provides important data for

understanding the process. MMPs, TIMPs, and VEGFs are some of these molecules.

MMPs and its inhibitors TIMPs are markers of the degradation of the extracellular matrix. They play roles in the breakdown of the basement membrane of the extracellular matrix and blood vessels. This causes leukemia cells to separate from the extracellular matrix in the bone marrow, penetrate the blood vessels and exit the vein[11]. Kuittinen et al. explained that the expression of MMPs was rare in pediatric cases compared to adult ALL, and MMPs' expression in childhood ALL was associated with high-risk disease[12]. While cell surface expression of MMP2 or MMP9 was found to be low in B-ALL and T-ALL, it was found that MMP2 expression on the cell surface at the time of diagnosis was higher in patients with relapsed B-ALL compared to patients with the first remission[13]. It was determined that increasing hypoxia in T-ALL increased the expressions of MMP2 and MMP9, and it resulted in T-ALL with increased invasiveness[14]. A significantly higher percentage of blast cells containing intracytoplasmic MMP9 was observed in patients with newly diagnosed B-ALL and tumoral syndrome[13]. Although it was stated in some studies that a large amount of MMP9 secreted was associated with decreased overall survival[13], there were studies stating that MMPs were not associated with survival[12]. Higher MMP9 expression levels were associated with a lower chance of extramedullary infiltration[8], but were present in data that could not detect the same association[15]. In an animal experiment, it was defined that MMP9 deficiency in the microenvironment of the bone marrow prolonged the survival of B-ALL mice and caused a decrease in B-ALL infestation[16]. It is unclear why high levels of MMP9 secretion by cells are or not associated with poor prognosis or organ infiltration. In our study, we found higher MMP2 and MMP9 expression levels compared to the control group.

Human TIMPs have strong growth-promoting activity. TIMPs form a complex with MMPs and inhibit their proteolytic activity in vitro and in vivo. Overexpression of TIMPs leads to decreased tumor growth and metastasis in various tumors[17,18]. In hematological malignancies, TIMP1 has promoted the differentiation of lymphoma cells, while high TIMP1 serum levels have been associated with advanced myeloma[19]. TIMP1 expression, which was expressed as one of the two candidate proteins, was found to be significantly higher among proteins screened in the bone marrow plasma of children with B-precursor ALL[20]. There was a significant relationship between the high expression of the TIMP1 gene and the event-free survival of ALL patients less than 5 years[8]. It was shown that high TIMP1 expression at the time of diagnosis was a marker showing the tendency of leukemic cells to CNS infiltration[21]. As a result of the detection of TIMP1 increase in bone marrow plasma of AML patients at the time of diagnosis, in vitro studies have shown that TIMP1 modulates the survival, migration and function of leukemic blasts via the CD63 / PI3K / Akt / p21 signal pathway. In the light of these data, TIMP1 was suggested as a potential new therapeutic target in the leukemic bone marrow microenvironment[22]. While TIMP2 gene was found to be expressed higher in T-ALL children[8]; there was a positive correlation between hepatosplenomegaly with MMP2/TIMP1 and MMP2/TIMP2 ratios, and between CNS involvement and MMP2/TIMP2 ratio. Moreover, it was shown that MMP/TIMP mRNA balance was closely related to infiltration of leukemia cells into extramedullary organs. However, MMP9/TIMP1 and MMP9/TIMP2 ratios were not associated with organ involvement[15]. On the contrary, in the study where the amount of MMP2, MMP9, TIMP1 and TIMP2 was low on the surface of B- or T- lymphoblasts; MMP / TIMP protein ratio was not found to be significantly associated with peripheral organ infiltration or prognosis[13]. Although we found the expression level of the TIMP1 gene higher than the control group in our study, we could not find the same result for the TIMP2 gene. The differences between mRNA and methylation levels of MMP and TIMP show the importance of studying all components of the MMP system rather than individual components.

Expression of VEGF and its isoforms (VGEF165, VEGF121) was identified in pediatric ALL cells[23]. Although the cellular viability, growth, proliferation and survival of ALL cells were found to be independent of VEGF, it was reported that transendothelial migration was regulated by VEGF through CNS microvascular endothelial cells[24]. Studies reported that VEGF-A regulates MMP secretion through an autocrine mechanism[25] and that children diagnosed with ALL who have significant VEGF elimination in urine have a poor prognosis[26]. In addition, VEGF has regulated the integrity of the endothelial barrier contributing to extravasation of tumor cells and metastasis[27]. It was emphasized that the autocrine cycle of VEGF, which was shown to

increase the survival of lymphoblasts, might lead to increased secretion of MMP9[28], and a high expression of MMP9 might be associated with resistance to apoptosis[13]. In our study, we found that the expression of VEGF-A in ALL patients was higher than in the control group. The higher VEGF-A expression compared to the methylation rate indicates that the gene cannot be silenced. In addition, this result at the time of first diagnosis shows the importance in leukemia.

DNA methylation plays an important role in genome organization, but its role in cancer is still complicated. Hypermethylation of CpG islands is the most studied type of abnormal methylation among human cancers. The pathogenesis and phenotypic characteristics of leukemia cells indicate that DNA methylation is the result of a combination of genome-wide changes[29]. In a limited number of methylation studies conducted in ALL patients, methylation differences during diagnosis and relapse were measured, and higher methylation levels were found at relapse[30]. Among the genes of interest in this study were CDKN2A, COL6A2, PTPRO and CSMD1[31]. In another study conducted with T-ALL patients, on the contrary, it was observed that promoter region hypomethylation was not observed more frequently in relapse patient[32]. In our study, we observed that the percentage of methylation was higher in the promoter regions of MMP2, MMP9, TIMP2 and VEGF-A genes. However, since there were no methylation studies related to these genes in ALL patients, we could not compare our findings. In this sense, it is the one of the pioneer studies to show the methylation changes in the promoter regions of these genes in childhood ALL patients. The fact that the methylation percentages of MMP2, MMP9 and VEGF-A genes were very high in our patients suggests that the methylation effect on the expression of genes is more complex and must be investigated. It may be speculated that reasons for the difference between gene expression and methylation values in complex diseases such as cancer are the copy number of the gene, mutations in the promoter region, histone modifications and chromatin modifier genes.

In conclusion, in our study, we found results that support the hypothesis that angiogenesis may play a role in the pathogenesis of acute leukemia by showing that the expression and methylation results of MMP2, MMP9 and VEGF-A genes are high at the first diagnosis in children with ALL. Although it is thought that antiangiogenic agents may play an active role in future treatment modalities for this disease, more studies on methylation and expression profiles in ALL will provide more evidence to elucidate disease progression, and relapse. Our study has a few limitations. First of all, the sample size is relatively small. Secondly, some of the patients were lost of follow-up and some died. Therefore, the values determined at the time of the first diagnosis could not be evaluated after the treatment. However, evaluating both expression and methylation values together are a factor that strengthens our study.

Yazar Katkıları: Çalışma konsepti/Tasarımı: Nİ, OD, İB, AT; Veri toplama: Nİ, OD, İB, AT; Veri analizi ve yorumlama: Nİ, OD, İB, AT; Yazı taslağı: Nİ, OD; İçeriğin eleştirel incelenmesi: OD, AT; Son onay ve sorumluluk: Nİ, OD, İB, AT; Teknik ve malzeme desteği: Nİ, OD; Süpervizyon: OD, AT; Fon sağlama (mevcut ise): yok.
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