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ARAŞTIRMA / RESEARCH

Plasma expression and methylation levels of vascular endothelial growth factor (VEGF-C) and basic fibroblast growth factor (bFGF) in children with acute lymphoblastic leukemia in Çukurova Region, Turkey

Çukurova Bölgesinde akut lenfoblastik lösemili çocuklarda vasküler endotelyal büyüme faktörü (VEGF-C) ve temel fibroblast büyüme faktörü (bFGF) plazma ekspresyonu ve metilasyon seviyeleri

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Abstract

Purpose: The aim of this study was to determine the expression and methylation levels of vascular endothelial growth factor-C (VEGF-C) and basic fibroblast growth factor (bFGF) in children with acute lymphoblastic leukemia (ALL).

Materials and Methods: We detected mRNA expression profiles and methylation levels of VEGF-C and bFGF genes in the peripheral blood samples of 20 patients with newly diagnosed ALL and 30 healthy controls by real-time quantitative PCR.

Results: The expression level of bFGF was significantly increased (4.23-fold) in ALL patients as compared with controls. Moreover, VEGF-C were significantly decreased (3.41-fold) in ALL patients as compared with controls. The methylation of the promoter region of VEGF-C (6.88%) and bFGF (16.64%) genes was higher in ALL patients than in healthy controls.

Conclusion: Long-term changes in VEGF-C and bFGF at different time intervals as a result of years of follow-up of patients may show stronger associations with disease risk. Further studies are required to establish strong links with both VEGF-C and bFGF, and ALL risk factors.

Keywords: Acute lymphoblastic leukemia, basic fibroblast growth factor, gene expression, methylation, vascular endothelial growth factor

Öz

Amaç: Bu çalışmanın amacı akut lenfoblastik lösemili (ALL) çocuklarda vasküler endotel büyüme faktörü-C (VEGF-C) ve temel fibroblast büyüme faktörünün (bFGF) ekspresyon ve metilasyon düzeylerini belirlemekti.

Gereç ve Yöntem: Gerçek zamanlı kantitatif PCR ile yeni tanı almış olan 20 hasta ve 30 sağlıklı kontrolün periferik kan örneklerinde VEGF-C ve bFGF genlerinin mRNA ekspresyon profilleri ve metilasyon seviyelerini tespit ettik.

Bulgular: bFGF ekspresyon seviyesi ALL hastalarında kontrollere kıyasla anlamlı derecede artmıştı (4.23 kat). Ayrıca, VEGF-C ALL hastalarında kontrollere kıyasla anlamlı derecede azalmıştı (3.41 kat). VEGF-C (%6.88) ve bFGF (%16.64) genlerinin promotor bölgelerinin metilasyon oranı ALL hastalarında sağlıklı kontroller göre daha yüksekti.

Sonuç: Hastaların yıllar süren takibi sonucunda VEGF-C ve bFGF'de farklı zaman aralıklarında uzun süreli değişiklikler hastalık riski ile daha güçlü ilişkiler gösterebilir. Hem VEGF-C hem de bFGF ve ALL risk faktörleri ile güçlü bağlantılar kurmak için daha ileri çalışmalara ihtiyaç vardır.

Anahtar kelimeler: Akut lenfoblastik lösemi, temel fibroblast büyüme faktörü, gen ekspresyonu, metilasyon, vasküler endotel büyüme faktörü

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INTRODUCTION

Angiogenesis and lymphangiogenesis are important steps in tumor progression. Several regulatory molecules have been reported to be involved in the angiogenic process (1). The two most potent and specific regulators are VEGF-C and bFGF that are commonly found in malignant tumors.[1,2] Not only in solid tumors but also in different leukemias the expression of these regulators carries an adverse prognosis.[3,4] It has been suggested that angiogenesis plays a role in the pathophysiology of HM as well as[5,6] and higher levels of angiogenic factors in adult ALL patients are predictive of longer survival.[7] The angiogenic genes VEGF-C and bFGF have been studied and evaluation of their prognostic impact in childhood ALL has been reported in several studies, though with controversial results. Lymph-angiogenesis during postnatal development is mainly modulated by the VEGF-C. Tumor cell-derived VEGF-C plays a causal role in lymph-angiogenesis and lymphatic metastasis.[1,4,5,8] Overexpression of VEGF-C has been detected in a variety of cancers. In addition, VEGF-C has been reported to enhance resistance to chemotherapy in the leukemic cells.[9] These results suggest that VEGF-C is an important anticancer target. Another major factor is bFGF which plays an important role in the regulation of cell survival, cell division, angiogenesis, cell differentiation, and cell migration. It is known that bFGF acts synergistically with VEGF-C in the angiogenesis. However, it is not known whether the putative effect of such factors on ALL. In children with ALL, elevated levels of urine bFGF have been associated with increased density of bone marrow vessels[10], but no studies have so far addressed the question of expression and methylation of bFGF. The clinical importance of the VEGF-C and bFGF levels in leukemia is still unclear and few studies have been conducted on childhood ALL. In the present study, we examined the correlation between VEGF-C and bFGF genes expression and methylation status in children with ALL.

MATERIALS AND METHODS

Twenty (13 female and 7 male) children with newly diagnosed ALL who were referred to Çukurova University, Medical Faculty Hospitals, Department of Pediatric Oncology. This study was performed according to the principles of the Declaration of Helsinki. The ethical trial of this study was approved by the local ethics committee of Çukurova University (document number:04.04.2013-18/8). Children between the ages of 3-11 years old, who were diagnosed with ALL by evaluating according to immunophenotypes and FAB classification by physicians, without any other accompanying disease (congenital abnormalities, metabolic disease, acute-chronic infection, cardiac-thyroid-lung diseases, renal failure and liver diseases), were included in the study.

Immunophenotypes and other criteria (age range, sex, remission status, WBC) of the patients were determined (Table I). The control group consisted of 30 healthy volunteers who were age, sex-matched to the patients. They were collected from the Department of Child Health and Diseases, Çukurova University, Medical Faculty Hospitals. Control group has composed the absence of any HM as well as personal or family history of ALL. Information of informed consent was obtained from the parents of the participants to take part in our research.

Procedure

RNA isolation and RT-PCR array

Five ml of peripheral blood samples were collected from the subjects and patients into vacutainers containing EDT. β-actin gene (ACTB) served as positive control. RNA isolation was performed by using high pure RNA isolation kit (PureLink RNA Mini Kit, Invitrogen). The RNA quality was measured with a spectrophotometer. RNAs were directly translated into cDNAs (RT² First Strand Kit, SABiosciences). The 2 (μg/μl) of RNA were used for cDNA synthesis. The same amount of RNA was used for each sample. All steps were done according to the manufacturer's protocol for the Roche Light Cycler 480 System. The PCR amplification was done under the following conditions: initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by a final extension at 60°C to 95°C with temperature transition rate of 1°C/s. PCR primers were as follows: forward primer ACTB, 5'-GCTGTGCTACGTCGCCCTG-3'; reverse primer ACTB, 5'-GGAGGAGCTGGAAGCAGCC-3'; forward primer VEGF-C, 5'-GATCTGGAGGAGCAGTTAGG-3'; reverse primer VEGF-C, 5'-GAGTTGAGGTTGGCCTGTTC-3'; forward primer bFGF, 5'-GGTCCTGTTTTGGATCCA-3'; reverse primer bFGF, 5'-AGAGAGAGGAGTTGTGTC-3'. The interplay

between VEGF-C and bFGF gene expression levels was determined by using the data analyser template provided by Superarray (http://www.sabiosciences.com/pcrarraydataanalysis.php). To analysis the PCR-array data, MS-Excel sheet was downloaded from the manufacturer's website (http://www.sabiosciences.com/pcrarraydataanalysis.php). The website also allowed online analysis. RT-PCR analysis results were evaluated and compared to the outcome of the gene expression values.

DNA isolation and methylation analysis

Genomic DNA was isolated with a Genomic DNA Purification Kit (Qiagen, Germany) from peripheral blood samples of patients and controls. The DNA quality was measured with a spectrophotometer. We investigated the methylation status of the promoter regions of ACTB, VEFG-C, and bFGF. The restriction digestions were performed using the EpiTect Methyl II DNA Restriction Kit provide by (Qiagen, Germany) which was specially designed for our study.

The thermal cycler was programmed according to the manufacturer's instructions. Cycling conditions involved 10 min at 95°C; 3 cycles of 99°C for 30s and 72°C for 1 min; 40 cycles of 97°C for 15s and 72°C for 1 min. After the cycling program has completed, export and/or copy/paste the CT values from the instrument software to a blank Microsoft Excel spreadsheet (http://www.sabiosciences.com/dna_methylation_data_analysis.php/) according to the manufacturer's instructions for the real-time PCR instrument. Samples were analyzed as recommended by the manufacturer. In most cases, the minimum level of hypermethylation considered to be positive can be set at 10 to 20%, and the minimum level of high intermediately methylated DNA considered to be positive can be set at >60% according to the manufacturer instructions.

Statistical analysis

All analyses were performed using SPSS 16.0 statistical software package (IBM SPSS Statistics). Categorical variables were expressed as numbers and percentages, whereas continuous variables were summarized as mean and standard deviation (SD) and as median and minimum–maximum where appropriate. Independent-samples ttest was used to compare normal distribution data between groups. The Mann-Whitney U test was used for the comparison of the groups that showed non-normally distributed data. $p<0.05$ was considered statistically significant.

Table 1. Characteristics of participants

Characteristics	Patients	Control
Number of patients	20	30
Sex (male/female)	7/13	14/16
Median age (month)*	76.8 ± 41.7	85.3 ± 53.2
WBC (mm^3)*	49905.2 ± 88523.3	-
Immunophenotypical classification number (FAB classification number) B-ALL (L1/L2) T- ALL (L1/L2)	 15 (4/11) 5 (2/3)	-

*: Mean value ± standard deviation

Table 2. Expression results of ACTB, VEGF-C and bFGF genes.

Gene Symbol	AVG ΔC_t		$2^{-} \Delta C_t$		Fold Change*	p-Value**	Fold Up or Down Regulation
	ALL group	Control group	ALL group	Control group			
ACTB	0.00	0.00	1.000000	1.00000	1.00	0.000	1.00
VEGF-C	13.52	11.75	0.000085	0.00029	0.29	0.000	-3.41
bFGF	10.72	12.80	0.000593	0.00014	4.23	0.000	4.23

*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.

Table 3. The methylation percentage and the expression levels of VEGF-C and bFGF genes.

	Gene methylation levels (%)		Gene expression levels
Gene symbol	The patient group	Control group	The patient group
VEGF-C	6.88	0.77	3.41-fold (down)
bFGF	16.64	0.85	4.23-fold (up)

RESULTS

A total of 7 male and 13 female patients were recruited for the present study, with a mean age of 6 years. The initial diagnosis of ALL and its subtypes were determined according to the French-American-British classification[11]. Immunophenotypical examination by flow cytometry revealed[12]; ALL patients were classified and summarized in Table-1. Compared with VEGF-C and bGF expression levels of ALL patients and controls; bFGF gene expression increased 4.23-fold ($p<0.05$) and VEGF-C gene expression decreased 3.41-fold in the patient group ($p<0.05$). Gene expression results were given in Table 2 and Figure 1. Real-time methylation analysis of VEGF-C and bFGF genes was carried out. The methylation percentage of VEGF-C (6.88%) and bFGF (16.64%) genes was significantly higher in patients ($p<0.05$). Quantitation of methylation values was performed and results were given in Table-III. In both of the VEGF-C and bFGF gene regions, there were 4 patients whose methylation value was more than 20% and all were B-ALL. There were only 3 patients with more than 60% methylation in the bFGF gene region, all of which were T-ALL.

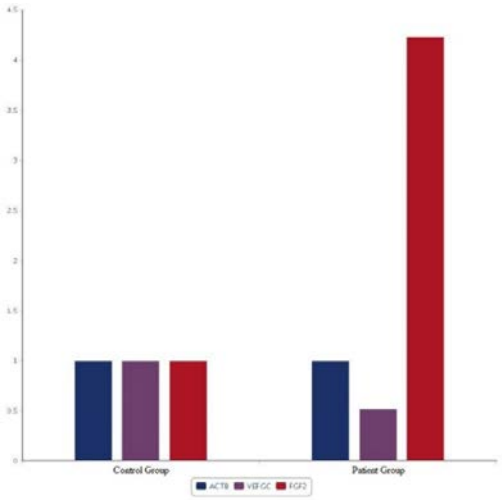

Figure 1. The Expression Profile of ACTB, VEGF-C and bFGF Genes in Patient and Control Groups.

DISCUSSION

The role of angiogenic factors in childhood ALL is unclear. Until today, the importance of these factors on leukemia, especially childhood ALL has received limited attention. Speculations should be proved by extensive and correlated studies of leukemia, in which, angiogenesis in the bone marrow and growth factors in the blood at the time of diagnosis, and in remission. Therefore, this study examined the expression profiles and the methylation levels of the promoter region of the VEGF-C and bFGF genes in childhood ALL patients. The VEGF-C and bFGF expression levels were statistically different; the bFGF gene expression increased 4.23-fold ($p<0.05$) and VEGF-C gene expression decreased 3.41-fold ($p<0.05$) in newly diagnosed patients compared to controls.

Expression of VEGF-C and bFGF genes in leukemia and their plasma concentrations serve as predictors of poor prognosis. Because experiments with transgenic mouse show that expression of VEGF-C is associated with the development of lymphangiogenesis in normal tissues.[13,14] VEGF-C associated lymphangiogenesis also contributes to tumor metastasis and VEGF-C has potent angiogenic effects in vivo.[15] In some studies on children and adults with different hematological malignancies, the VEGF-C expression level was found to be high.[16,17] Interestingly, we found conflicting unexpected results with regard to reports in the literature concerning the VEGF-C values, which at the time-diagnosis of childhood ALL were 3.41-fold lower than concentrations in controls and why is decreasing? The reason may be that angiogenic factors present in pediatric ALL are different from the mediators in the other HM. In a study of adult AML patients, similar to our findings, Aliparasti et al. confirmed decreased of VEGF-C expression value.[18] Although there are studies observing that confirmation of increased expression of VEGF-C in childhood ALL[9,19] or the expression value of VEGF-

C did not change in pediatric ALL patients with and without relapse[20], the number of clinical studies to demonstrate especially the effect of VEGF-C on childhood ALL is still very limited.

The different studies focused mainly on bFGF, because bFGF is a key regulator of tumor angiogenesis and lymphangiogenesis and has been indicated for solid tumors and HM.[8,10,21] In a study on mice, the interaction of VEGF-C and bFGF, which are commonly expressed in the tumor microenvironment, was found to increase lymphangiogenesis and metastasis.[22] In childhood ALL, an increased microvessel density histologically in the bone marrow and an elevated urinary bFGF level have been reported.[10] The previous study has shown that bFGF level in relapsed patients was higher than in other risk groups and controls.[23] Serum bFGF level was increased in remission of ALL when compared to the diagnostic values.[24] The increasing bFGF levels were associated with shorter overall and disease-free survival.[4] In the present study, bFGF mRNA levels found four-fold higher in childhood ALL patients compared to controls ($p<0.05$). On the contrary, there was a study showing that the expression value of bFGF did not change in pediatric ALL patients with and without relapse.[19] However, our data have confirmed the results of previous studies which demonstrated the changes of bFGF in hematopoietic malignancy.

DNA methylation is an important epigenetic mechanism of gene regulation. Alterations in methylation status within promoter regions can affect gene expression and hence the phenotype.[25] The methylation of genes promoter regions plays an important role in cancer pathogenesis. Although, VEGF-C and bFGF expression values were studied in various ALL patients, to our knowledge, the methylation patterns of VEGF-C and bFGF have so far not been evaluated in ALL. In some studies in the literature, different gene regions have been evaluated in ALL patients and no study related to VEGF-C and bFGF genes has been found.[26,27] This is the first study to show methylation changes in promoter regions of VEGF-C and bFGF genes in childhood ALL. The methylation levels of VEGF-C (6.88%) and bFGF (16.64%) genes were higher in ALL patients than in controls ($p<0.05$). In light of these results, it could be said that this methylation status suppressed transcription and reduced the expression of VEGF-C gene by 3.41-fold for our research. But, the expression level of bFGF gene increased (4.23-fold) while the methylation level increased (16.6%). There was an inverse relationship between DNA methylation and expression of bFGF gene. This increase in methylation level may not have affected gene expression, but we could not explain this situation. Because, there are anti-cancer studies related to methylation and demethylation of VEGF-C and bFGF in solid tumors.[28-30] Unfortunately, we could not find any studies on methylation status of VEGF-C and bFGF in HM. We know that the epigenetic mechanism is more complex in a complex disease such as cancer, and there are different mechanisms affecting hypomethylation and hypermethylation.[31] Heterochromatic DNA repeats, karyotype instability, activation of tumor-promoting genes by cis or trans effects and heterochromatin-euchromatin interactions are important reasons for abnormal DNA methylation patterns in cancer cells.[32] However, the importance of methylation alterations depends on the genomic region and functions of CpG islands at specific sites have not been completely illuminated.[33] Differences in methylation and expression levels of genes may be as markers for ALL. But it will demonstrate extensive researches to be done in the future. Our study has some limitations. First of all, the small number of patients with new diagnosis of ALL was restrictive. Finding a newly diagnosed patient with no drug therapy was limiting for our study. Secondly, we wanted to evaluate post-treatment value but we could not study this stage because of the high number of patients who we could not communicate with.

This study also highlights that DNA methylation may not explain all gene expression changes, and other regulatory mechanisms of gene expression also come into play. The other epigenetic mechanisms, such as modifications of histones and non-coding RNAs, might have caused an increase in gene expression. The half-life or stability of the mRNA or regulation of fusion genes may have influenced. Inhibition of the action of VEGF-C can be a potential target for therapeutic intervention in childhood ALL. The bFGF expression level was not related to promoter CpG methylation level. Our data and the findings of other authors suggest that the roles of VEGF-C and bFGF genes in ALL have not been clarified for HM and progression of ALL. The results of our study suggest that angiogenic factors are differently implicated in the evolution of childhood ALL and must be better characterized. We hope that our data will be supported in studies of ALL with large sample groups. However, these findings could help provide

new insights into the investigation of target gene/genes for the following up clinically in children diagnosed with ALL. Future studies on the sequence content of genes and epigenetic mechanisms will better explain the regulation of methylation and expression.

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