The Role of FLT3-ITD and CCAAT-Enhancer-Binding Protein α Mutations on Prognosis of Acute Lymphoblastic Leukaemia in Turkish Patients

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ABSTRACT

Background: *Acute lymphoblastic leukaemia (ALL) is the most common malignancy in childhood. Although some prognostic factors have been defined to date, the estimation of prognosis is currently not perfect. Previous studies had shown an association of FLT3 with poor prognosis and CCAAT-enhancer-binding protein α (CEBPA) mutation with the development of acute myeloid leukaemia (AML). Here, we aimed to evaluate the prognostic value of FLT3-ITD and CEBPA mutations in ALL.*

Methods: *Sixty-one patients with ALL were included in the study. The patients were divided into three risk groups according to BFM risk classification. All of the patients were examined for FLT3-ITD mutations and 45 of them for CEBPA mutations. Mutation positive and negative patients were compared in terms of their risk groups, translocations and cell lineage. The clinical courses of the patients were appraised.*

Results: *FLT3-ITD mutation was detected in 3 of the 61 patients, and CEBPA mutations were detected in 11 of the 45 patients. The incidence of established prognostic indicators including BFM risk classification, t(9; 22); BCR-ABL, t(1; 19); E2A-PBX1, t(12; 21); TEL-AML1, t(4; 11); MLL-AF4 were similar between FLT3-ITD and CEBPA positive and negative patients. A patient with an FLT3-ITD mutation was very susceptible to pancytopenia after maintenance treatment and two other patients with FLT3-ITD mutations were more prone to febrile neutropenia.*

Conclusion: *Our results suggested that CEBPA or FLT3-ITD mutations might not be related to ALL prognosis in the sampled Turkish patients. However, FLT3-ITD mutation might have an influence on the response of bone marrow to chemotherapy.*

Keywords: Acute lymphoblastic leukaemia, CCAAT enhancer binding protein α, FLT3, mutation, prognosis

INTRODUCTION

Although the genetic background of oncogenesis is not fully understood, the deterioration in cell differentiation is a crucial component for the development of cancer (1). Acute lymphoblastic leukaemia (ALL), which comprises B-cell lineage and T-cell lineage subgroup cancers is the most common malignancy in childhood cancers (2). The risk classification of the disease may lead to different outcome estimates.

Numerous prognostic factors related to the decision of treatment regimens had been determined in pediatric ALL. However, there were disagreements among physicians about the use of these prognostic factors for ALL (3). Due to these differences, it may be difficult to compare the results of different clinical studies. Thus, new reliable risk classification and prognostic factors are required.

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Although some prognostic factors were determined for ALL such as age, gender, white blood count (WBC) on the diagnosis of ALL, the response to treatment, DNA index, t(9: 22), and t(4: 11), there is a need for more specific markers (3).

FMS-like tyrosine kinase-3 (FLT3) is a class 3 tyrosine kinase family receptor that is expressed in hematopoietic cells related to lymphocyte development. These receptors play important roles in haematopoiesis, especially on lymphoid lineage (4). Internal tandem duplication (ITD) of *FLT3* gene led to the disruption of FLT3 auto inhibition. Previous studies had suggested that FLT3 mutations and their expression might be associated with poor prognosis for acute myeloid leukaemia (AML), and the over-expression of FLT3 receptor protein had been shown in AML and B-cell acute lymphoblastic leukaemia (B-ALL). It had been considered that FLT3 inhibitor therapy might benefit patients since the over-expression of the *FLT3* gene had been shown in leukaemia (4, 5). Therefore, showing FLT3 mutations may be beneficial for both the treatment and the prognosis of acute leukaemia. However, in ALL the association between FLT3 and prognosis of the disease is unclear and further investigations are required.

CCAAT enhancer binding protein α (CEBPA), a transcription factor, is expressed in granulocytes, monocytes and eosinophils. When CEBPA is bound to DNA, it activates the transcription of two N-terminal trans-activation domains (6). The mutations of this transcription factor might be related to the prognosis of some malignancies such as AML and ALL. CEBPA expression was reduced or inhibited during heterozygous or homozygous mutation for some polymorphisms of the *CEBPA* gene. The reduction or inhibition of CEBPA expression was related to the development of AML. However, the prognostic role of CEBPA in AML is controversial (6–8). On the other hand, CEBPA mutation is suggested to be related to the survival of patients with ALL (9). However, only a few studies had been conducted for the evaluation of the pathogenetic and/or prognostic role of the CEBPA mutation in ALL (6, 7, 9–11).

In this study, we aimed to investigate whether FLT3-ITD or CEBPA mutations are related with the established prognostic factors in children with ALL.

SUBJECTS AND METHODS
The study included 61 children (44 males, 17 females) with ALL, who were diagnosed and treated between February 2009 and March 2014. The age, gender, white blood cell count (WBC), haemoglobin value and platelet count at diagnosis, treatment protocol, and risk groups of the patients were recorded. The patients were classified as standard risk group (SRG), medium risk group (MRG), and high-risk group (HRG) according to the Berlin-Frankfurt-Munster (BFM) risk classification. The standard risk group included patients aged 1–6 years who had white blood cell counts of less than 20 000/mm^3 at diagnosis, good response to prednisone and no-T-ALL. The high-risk group included those with a poor prednisone response, and/or evidence of t(9; 22); BCR/ABL, and/or evidence of t(4; 11); MLL/AF4. Patients with no HRG or SRG criteria were accepted as MRG. The flow cytometry and oncogene fusion transcript screening for t(9; 22); BCR-ABL, t(1; 19); E2A-PBX1, t(12; 21); TEL-AML1, and t(4; 11); MLL-AF4 were recorded. The FLT3-ITD and FLT3-D835 mutations were studied in all the patients. CEBPA mutations were studied in 45 of the patients. The study's protocol was approved by the local Ethics Committee.

The cells from the diagnostic bone marrow samples were cultured for 24 hours according to the standard cytogenetic protocols. The chromosomes were banded using a GTG technique and karyotyping was performed. All the patients were confirmed by conventional cytogenetic and reverse transcriptase polymerase chain reaction (RT-PCR) for t(9; 22); BCR-ABL, t(1; 19); E2A-PBX1, t(12; 21); TEL-AML1 and t(4; 11); MLL-AF4 fusion transcript.

DNA extractions and genotyping
Genomic DNA was extracted from the bone marrow cells using a DNA isolation kit (High Pure PCR Template Preparation-Roche, Mannheim, Germany) according to the manufacturer's instructions. The DNA concentration was determined using a Nano-Drop spectrophotometer (Thermo Scientific, USA), and the samples were stored at –20°C until the polymerase chain reaction (PCR) was performed.

The screening for CEBPA mutations were performed using a high resolution melting curve based on LightCycler 480 assay (Roche Diagnostics, Mannheim, Germany).

The analysis for the FLT3/D835 mutations were carried out by LightCycler based high resolution melting curve analyses with the forward primer 5´-AGAGACCTGGCCGCC-AGGA-3´; reverse primer 5´-CAACACAAAATAGCCGTATAA-3´ and hybridization probes, 835V GGATTGGCTCGAGATATCATG–FL, and Red LC640–TGATTCCAA-CTATGTTGTCAGGGGCAATGT-PH.

The PCR reaction was performed in a 20 μL reaction volume contained with 5 μL DNA, 1 μL (0.2 μM) each of forward and reverse primer, 1 μL hybridization probes, 1, 6 μL (3 mM) MgCl$_2$ and 2 μL LightCycler-FastStart DNA Master HybProbe Mix (Roche Diagnostics, Mannheim, Germany).

The amplification was performed with 45 cycles using 55°C annealing temperature. The final melting curve analysis was started at 40°C up to 95°C with a slope of 0.2°C/s and continuous detection with channel 640. The data were analysed using the LightCycler 480 software (Roche Diagnostics, USA) and the second derivative maximum method by high resolution melting curve analysis mode.

The detection of FLT3/ITD, polymerase chain reaction was performed using specific primers 11F, 5′-GCAATTTAGGTATGAAAGCCAGC-3′; 12R, 5′-CTTCAGCATTTTGA-CGGCAACC-3′ and probes 20F, 5′-CCGCCAGGAACGTGCTTG-3′; 20R, 5′-GCAGCCT-CACATTGCCCC-3′. The amplification was performed in a 20 μL reaction volume with 5 μL DNA, 0.3 μM each of primers and probes, 3 mM MgCl$_2$, and 5 μL LightCycler-FastStart Master Mix (Roche Diagnostics, Mannheim, Germany). Denaturing, annealing, and extension steps were performed at 95°C for 5 seconds, 64°C for 10 seconds, 72°C for 15 seconds, respectively, for 45 cycles. The final melting curve analysis was started at 40°C up to 95°C with a slope of 0.2°C/s.

The sequence variants in the DNA, either homozygous or heterozygous, gave rise to heteroduplexes that were easily detected by high-resolution melting curve analysis from earlier melting products.

Statistical analyses
The numerical variables were presented as means ± standard deviations. The comparison of the groups having abnormally distributed numerical data was performed with Mann–Whitney U test or Kruskal–Wallis test. The Chi-square test was used to compare the categorical variables between the independent groups. A p-value less than 0.05 was accepted as statistically significant.

RESULTS
A total of 61 children (44 males, 17 females) with ALL were evaluated. The mean age of the patients at diagnosis was 6.1 ± 3.8 years. In the complete blood count analysis at diagnosis: the white blood cell count (WBC) was 34 400 ± 60 208/mm^3, the haemoglobin was 8.7 ± 2.2 g/dL, and the platelet count was 109 559 ± 97 080/mm^3. The characteristics of patients in each risk group are summarized in Table 1.

Thirty-four patients had been treated according to TR ALL BFM 2000 treatment protocol and 27 patients had been treated according to ALLIC BFM 2009 treatment protocol.

FLT3-ITD mutation was detected in 3 (4.9%) of all the patients and CEBPA mutation in 11 (24.4%) patients. The characteristics of mutation-positive patients are summarized in Table 2. FLT3-D835 mutation was not detected in any patient. There was no significant difference between the BFM risk groups in terms of the presence of FLT3-ITD and CEBPA mutations ($p = 0.33$ and $p = 0.86$, respectively). There were no differences between the FLT3-ITD positive and negative groups in terms of the positivity or negativity of t(9; 22); BCR-ABL1, t(1; 19); E2A-PBX1, t(12; 21); ETV6-RUNX1 and t(4; 11); MLL-AF4 translocations. Similarly, these translocations were observed in similar frequency among CEBPA mutation-positive and negative patients. In respect of B cell or T cell distribution, the positive–negative FLT3 mutation groups and positive-negative CEBPA mutation groups, were found to be similar ($p = 0.71$, $p = 0.52$). There were no differences between the mutation positivity when patients were divided into two age subgroups (1–6 years age group and > 6 years

Table 1: Characteristics of risk groups at diagnosis (mean ± standard deviation or number)

	SRG (n = 17)	MRG (n = 37)	HRG (n = 7)	p-Value
Diagnosis age	3.5 ± 1.6	6.6 ± 3.8	9.5 ± 4.7	< 0.001[a], 0.003[b], 0.0001[c], 0.12[d]
Gender (M/F)	12/5	25/12	7/–	0.21*
Positive FLT3-ITD	–	2	1	0.33*
Positive CEBPA	3	6	2	0.86*
WBC, /mm^3	6 622 ± 3 686	46 093 ± 70 572	49 747 ± 52 876	0.01*
Haemoglobin, g/dL	9.1 ± 1.8	8.2 ± 2.4	10.9 ± 2.2	0.16*
Platelet count, /mm^3	93 707 ± 99 970	113 866 ± 100 297	1 127 585 ± 70 709	0.60*

[a]Between three groups, [b]Between SRG and MRG, [c]Between SRG and HRG, [d]Between MRG and HRG.
SRG = standard risk group; MRG = medium risk group, HRG: high-risk group.

Table 2:   Characteristics of FLT3-ITD and CEBPA mutations positive patients

	Diagnosis age, years	WBC (/mm³) at diagnosis	t(12,21)	t(1,19)	t(9,22)	t(4,11)	Cell lineage	BFM risk group
Patients with FLT3-ITD mutation								
Patient 1	13.5	158 000	−	−	−	−	B cell	HRG
Patient 2	5	3980	−	−	−	−	T cell	MRG
Patient 3	7.5	30 100	+	−	−	−	B cell	MRG
Patients with CEBPA mutation								
Patient 4	8	5790	−	−	−	−	B cell	MRG
Patient 5	14.5	50 700	−	−	−	−	T cell	HRG
Patient 6	7	7550	−	−	−	−	T cell	MRG
Patient 7	3.5	6950	−	+	−	−	B cell	SRG
Patient 8	4	11 900	−	−	−	−	T cell	MRG
Patient 9	1.5	3980	−	−	−	−	B cell	SRG
Patient 10	1	14 100	−	−	−	−	B cell	MRG
Patient 11	3	76 800	+	−	−	−	B cell	MRG
Patient 12	5	12 400	−	−	−	−	B cell	SRG
Patient 13	7	33 200	−	−	−	−	B cell	MRG
Patient 14	12.5	57 850	−	−	−	−	B cell	HRG

SRG = standard risk group. MRG = medium risk group, HRG = high risk group; CEBPA = CCAAT-enhancer-binding protein α.

group) ($p = 0.42$). Similarly, there were no differences between the positive and negative mutations when the patients were divided into two groups according to their ages (1–6 years age group and > 6 years age group) ($p = 0.67$). One patient had developed a relapse. This patient did not have FLT3-ITD or CEBPA mutations. Bone marrow transplantation was applied to a patient with CEBPA mutation in the high-risk group. No significant differences were found when the B cell ALL and the T cell ALL patients were compared in terms of WBC, platelet count and haemoglobin values at diagnosis, ($p = 0.27$, $p = 0.37$, and $p = 0.27$, respectively).

Pancytopenia developed in a patient with medium risk having an FLT3-ITD mutation after maintenance treatment. Thus, 25% of the total dose of methotrexate and 6-mercaptopurine was given to this patient. On the other hand, two other patients with FLT3-ITD mutations were frequently hospitalized due to febrile neutropenia. No patients died.

DISCUSSION
Recently, the ALL patients have had better survival rates because of effective treatment regimens and improved healthcare following chemotherapy. In order to increase the survival rates of high-risk patients, reliable markers for risk estimation and better treatment modalities are required.

Internal tandem duplication of FLT3 in juxtamembrane domain is a mutation detected frequently in AML but is rarely seen in ALL (12). Previous studies had found increased expression of FLT3 RNA and FLT3-ITD proteins in the bone marrow of AML and ALL patients compared with the healthy subjects. FLT3-ITD mutation is associated with the over-expression of FLT3-ITD protein. FLT3 expression might be related to the survival or proliferation of leukemic blast cells (4, 12). FLT3-ITD mutation is frequently observed in AML patients and it is related to poor prognosis in AML. The prognostic role of FLT3-ITD mutation in ALL is unclear and this mutation is rarely found in ALL (12, 13). Thus, the role of FLT3-ITD mutation in ALL is controversial and further investigations are required. In addition, detecting the relationship between ALL prognosis and FLT3 mutation may indicate the beneficial effects of kinase inhibitors in ALL management.

In this study, FLT3-ITD mutation distribution was found comparable among the three ALL risk groups and not related with other known prognostic factors such as WBC and age at diagnosis and translocations (9, 22), (4, 11), or (12, 21). Therefore, our results showed that FLT3-ITD mutation might not be related to ALL prognosis in children. Because of the limited number of patients, our results need to be confirmed by further studies with more patients. Two of ours FLT3-ITD mutation positive patients developed neutropenia and pancytopenia developed in another patient with FLT3-ITD mutation. The explanation of this predisposition is unclear. However, FLT3-ITD mutation may aggravate leukopenia as a side effect of chemotherapy.

In the proliferation and differentiation of normal lymphocytes and lymphoid malignancies, the functions of the *CEBP* genes remain largely unknown. CEBP mutations are accepted to be associated with a relatively good overall prognosis (14). In a study done by Park *et al*, CEBPA mutated patients had better prognoses than CEBPA wild-type patients in AML (8). On the other hand, loss of CEBP expression is thought to be concomitant with B-cell differentiation (14). *CEBP* genes are stated to be novel oncogenes in B cell precursor ALL (14). Thus, *CEBP* genes may be related to the pathogenesis and prognosis of ALL. However, the data about the prognostic role of the CEBPA mutation in ALL are limited.

Our present study revealed that CEBPA mutation was not related with other known favourable or unfavourable prognostic factor in ALL such as WBC at diagnosis (9, 22), (4, 11), (12, 21) translocations, age at diagnosis and B cell or T cell subgroups. These results suggest that CEBPA may not be related to ALL prognosis.

The main limitations of this study were the small number of patients and the absence of a control group.

CONCULSION
In conclusion, we did not detect any relationship between mutation positive and negative patients with FLT3-ITD or CEBPA mutations in terms of the known prognostic factors and clinical course of the patients. However, FLT3-ITD mutation may affect the response to chemotherapy.

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