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CLINICAL STUDY

Determination of CEBPA mutations by next generation sequencing in pediatric acute leukemia

Akin DF[1], Oner DA[2], Kurekci E[2], Akar N[3]

Nigde Ömer Halisdemir University, Faculty of Medicine, Medical Biology, Nigde, Turkey.
dilarabali@ohu.edu.tr

ABSTRACT
OBJECTIVES: The CCAAT/enhancer-binding protein-alpha (CEBPA) is lineage-specific transcription factor in the hematopoietic system. In this study, we aimed on the clinical features and the prognostic significance associated with CEBPA mutations in 30 pediatric patients with acute leukemia.
METHODS: In addition, the association between found variants and mutations of Ten-Eleven-Translocation 2 (TET2), Kirsten rat sarcoma viral oncogene homolog (KRAS), and Casitas B-cell lymphoma (CBL), FLT3 (Fms-Related Tyrosine Kinase), JAK2 (Januse Kinase-2) and Nucleophosmin 1 (NPM1) were analyzed, which are important prognostic risk factors for pediatric acute leukemia patients. The entire *CEBPA* coding region was screened using the NGS method.
RESULTS: *CEBPA* mutations were detected in 16 (53.3 %) of 30 patients. In total, ten distinct of nucleotide changes were identified in 30 patients, including 6 novel and 4 known mutations by sequencing the entire CEBPA gene. We found 6 frame shift mutations, 1 missense mutation, 3 synonymous variants. The most common mutation was the c.487del G resulting p.Glu163Ser in 5 cases. Three patients carried *CEBPA* double mutations.
CONCLUSION: The detected variants in this article seemed to be the first screening results of genes studied by NGS in pediatric acute leukemia patients. Our results also showed some degree of association between FLT3-ITD, TET2, KRAS, CBL and CEBPA mutations *(Tab. 4, Fig. 1, Ref. 24).* Text in PDF *www.elis.sk.*
KEY WORDS: CEBPA, pediatric acute leukemia, next generation sequencing, molecular marker, mutation.

Introduction

Clinical and genetic prognostic markers are important in the classification of leukemia patients. Aberrant chromosomal translocations and gene mutations frequently occur in the transcriptional factor that lead to uncontrolled proliferation of lymphoid and myeloid progenitors (1–4). The CEBPA gene is a member of the leucine zipper family of the transcription factor family that is essential for the differentiation of myeloid cells (5). The CEBPA gene located on chromosome 19 q13.1 encodes the basic leucin zipper (bZIP) family of the transcription factors (6). It is expressed at high levels during myeloid cell differentiation and binds to the promoters of multiple specific genes at different levels of myeloid linage maturation (2). In AML patients, it was reported that two types of mutations in CEBPA gene exist; N terminal and C terminal (2, 5, 7–11). The N terminal mutations are located between the major translational start site and the second ATG further downstream lead to premature stop of translation of the wild type p 42 CEBPA protein, while preserving translation of a short p30 isoform that suppresses the function of the full length protein. Mutations in the C terminal are usually in frame and deletions that affect homo-hetero-dimerization and DNA binding (6–8). *CEBPA* mutations are seen in approximately 4.5–6 % of pediatric Acute Myeloid Leukemia (AML) patients. In addition, *CEBPA* mutations are found in 5–14 % of adult patients with AML (5, 11–13). There is no study, which investigates *CEBPA* mutations in Turkish pediatric acute leukemia patients. Therefore, in our study, we investigated entire *CEBPA* coding region in 30 patients with Turkish pediatric acute leukemia by using NGS technique and we assessed the frequency of *CEBPA* mutations and its clinico-hematologic correlation, as well as the cooperating mutations, including *FLT3*, *TET2*, *CBL*, *KRAS*, *JAK2* and *NPM1* mutations.

Patients and study design

Subjects

The study population consisted of 30 patients aged between 1 and 15 years,who were admitted to Losante Children's and Adult Hospital with the diagnosis of pediatric acute leukemia. Seventeen patients were diagnosed as AML, 4 as mixed leukemia, 9 as ALL. The study was carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The Ankara University, School of Medicine Ethics Committee approved the study protocol (project No.03-107-13/2013) and an informed consent was provided by the patients' parents.

[1]Nigde Ömer Halisdemir University, Faculty of Medicine, Medical Biology, Nigde, Turkey, [2]LÖSANTE Children's and Adult Hospital, Ankara, Turkey, and [3]TOBB-ETU Hospital, Ankara, Turkey

Address for correspondence: D.F. Akin, Nigde Ömer Halisdemir University, Faculty of Medicine, Medical Biology, Nigde, Turkey.
Phone: +90.5363026816

Tab. 1. Primers used for amplification of CEBPA gene.

Primer set	Forward	Tm °C	Reverse	Tm °C	Amplicon Length (bp)
CEBPA set 1	5'- GCCATGCCGGGAGAACT 3'	62	5'-CCCGGGTAGTCAAAGTCG-3'	59	357
CEBPA set 2	5'- CCTTCAACGACGAGTTCCTG-3'	61	5'-CGGCTGGTAAGGGAAGAGG-3'	62	335
CEBPA set 3	5'-GAGGAGGATGAAGCCAAGC-3'	60	5'-CTCGTTGCTGTTCTTGTCCA-3'	60	357
CEBPA set 4	5'-TGGCAGCGCCGTCAAG-3'	65	5'- CCAGGGCGGTCCCACA-3'	65	357

Tm = Melting temperature, bp = Base pair

Cytogenetic techniques

Bone marrow samples were collected with Heparin-containing tubes, and chromosome analysis was performed using G-banding. After the slide preparation, G-banding using Giemsa-staining was carried out according to the standard procedures. On each slide, 20 metaphases were analyzed by a light microscope (Nikon, JAPAN). Karyotypes were described according to International Standing Committee on Human Cytogenetic Nomenclature (ISCN) (14).

Fluorescence in situ hybridization (FISH) was performed on interphase nuclei and metaphase chromosomes of bone marrow cells using dual-color/dual-fusion probes for translocationsof inv (16;16), t(9;22), t(15;17), chromosome 19, and dual-color/deletion probe for del 7q, labeled in green and red spectra according to the manufacturer's protocol provided by Cytocell, UK. Counterstaining was performed with 40,6-dia midino-2-phenylindole (DAPI). At least 100 nuclei were analyzed under the Fluorescence microscope, and image capture was performed using Nikon Eclipse 80i equipped with a CCD-camera (CoolCube1), appropriate filters and Isis software (MetaSystems).

DNA isolation and Next Generation Sequencing

Blood samples were collected with EDTA-containing tubes and DNA was extracted from peripheral blood and bone marrow leukocytes with Mag-NA Pure automatic DNA isolation instrument (Roche Diagnostics, Manheim, Germany).

We used NGS to study the entire CEBPA gene region that was presented by 4 overlapping amplicons. Primer sequences lists are shown in Table 1. NGS sample preparation and process stages were generated, previously described by Grossmann et al (8).

NGS was carried out using 454 GS Junior System Instrument Roche Applied Science. Data were analyzed using the GS Amplicon Variant Analyzer software version 2.3. (Roche Applied Science). We used it for determination of variances; filters were adjusted to show variances in more than 1 % of bidirectional reads per amplicon in at least one patient. Figure 1 summarizes the location of the amplicons on the CEBPA protein.

Detection of FLT3, TET2, CBL, KRAS, JAK2 and NPM1 mutations

FLT3 mutation was analyzed by Real Time PCR on Light Cycler 480 II instrument (Roche Diagnostics, Gmbh, Mannheim, Germany). The results were analyzed with the High -Resolution Melting (HRM) method using genotype profiles. Different plots were created by selecting negative controls as the base-line. Therefore, fluorescence of all the other samples was diagramed relatively to this sample. Fluorescence signals were analyzed, and significant differences were used as indicators of the mutations (15–18).

Tab. 2. Patient characteristics according to CEBPA mutation status.

Characteristic	No *CEBPA* mutation	*CEBPA* mutation	p value
Gender			
Male (n = 21)	11	10	0,33
Female (n = 9)	3	6	
Diagnosis			
AML (n = 17)	7	10	0,94
ALL (n = 9)	4	5	
Biphenotypic Acute Leukemia (n=4)	2	2	
Risk Groups			
High Risk (n = 19)	11	8	0,20
Median Risk (n = 2)	1	1	
Standard Risk (n = 9)	2	7	
Status			
Remission (n = 17)	8	9	0,07
Relapse (n = 6)	0	5	
Ex (n = 7)	3	4	
Genetic Syndrome			
Down Syndrome (n = 4)	–	4	0,52
Fragile X Syndrome (n = 1)	–	1	
Cytogenetic/ Genetics			
Normal Karyotype (n = 19)	10	9	1
t(15;17) (n = 1)	1		
t(9;22) (n = 1)	–	1	
t(4;11) (n = 1)	1		
t(8;21) (n = 1)	1		
t(16;16) (n = 1)	–	1	
FLT3 mutations (n = 8)	5	3	
JAK2 mutations (n = 2)	–	2	
TET2 mutations (n = 5)	–	5	
CBL mutations (n = 1)	–	1	
KRAS mutations (n = 4)	–	4	
NPM1 mutations (n = 1)	1	–	

AML = Acute Myeloid Leukemia, ALL = Acute Lymphoblastic Leukemia, Ex = Exitus

Hot-spot exons of TET2, KRAS and CBL genes were screened using the NGS method. All coding exons of *TET2* (exons 3 and 11) were presented by 27 amplicons. Besides, two primer pairs were amplified, known as mutational hotspot regions, to describe the RING finger domain and linker sequence for CBL (exons 8 and 9) and KRAS (exons 2 and 3). The analyses were performed as previously described by Kohlmann et al (15). The twelfth of the exons of the JAK2 and NPM-1 genes was amplified by polymerase chain reaction (PCR). PCR products were sequenced using the Beckman DNA Sequencer System. (Beckman Coulter, USA).

Tab. 3. Characterization of CEBPA mutations in pediatric acute leukemia.

Mutation No	Type of mutations	No of patients	Nucleotide change	Amino-acid change	Localization	Mutation Type	Rs number	Clinical Significance
1	Nucleotide change	3	g.5800G>T c.690 G>T	p.Thr230Thr	Between TAD2-DNA binding domain	Synonymous variant	Rs 34529039	Benign
2	Duplication	3	g.5308_5311dupCTAC c.198_201dupCTAC	p.Ile68LeufsTer41	TAD-1 domain	Frameshift mutation in regulatory region	Rs 137852731	Pathogenic for AML
3	Insertion	1	g.5327_5328insC c.217_218insC	p.Phe73SerfsTer35	N-terminal domain	Frameshift mutation	Rs 137852733	Pathogenic for AML
4	Nucleotide change	1	g.5683C>T c.573C>T	p.His191His	Between TAD2-DNA binding domain	Synonymous variant	Rs 192240793	Benign
5	Nucleotide change	3	g.5444 C>T c.336 C>T	p.Pro112Pro	Between TAD1-TAD2 domain	Synonymous variant	Novel	NA
6	Deletion	1	g.5492_5493delC c.382 del C	p.Pro128ProsTer31	Between TAD1-TAD2 domain	Frameshift mutation	Novel	NA
7	Deletion	5	g. 5597 c.487 del G	p.Glu163Ser	Between TAD1-TAD2 domain	Frameshift mutation	Novel	NA
8	Deletion	1	g.5410-5411delC c.300 del C	p.Gly100GlysTer59	TAD-1 domain	Frameshift mutation	Novel	NA
9	Insertion	1	g.1107-1113ins TTGACC c.955-961 ins TTGACC	p.Ser319TrpsTer13	C-terminal domain	Frameshift mutation	Novel	NA
10	Nucleotide change	1	g.5599 C>A c.489 C>A	p.Glu163Asp	Between TAD1-TAD2 domain	Missense variant	Novel	NA

NA = Not Available, Rs = Reference SNP

Statistics

Statistical analyses were performed with SPSS 15.0 (SPSS IBM, USA). Chi-square test, independent sample *t*-test, or Mann–Whitney *U*-test, as appropriate for the type of data being analyzed, were used to assess the statistical significance of the difference between the two groups. p values less than 0.05 were considered statistically significant.

Results

Incidence of CEBPA mutations

Among 30 pediatric acute leukemia patients, *CEPBA* mutations were found in 16 cases (53.3 %). Patient characteristics of the 30 pediatric acute leukemia cases are shown in Table 2. Ten distinct nucleotide changes were identified in 30 patients, including 6 novel and 4 known mutations by sequencing the entire gene as shown in Table 3. The frame shift mutation was the most common mutation subtype that was found in 6 types. The remaining mutation subtypes were 3 synonymous variants and 1 missense mutation.

Three patients carried CEBPA double mutations. The other 13 patients carried a single CEBPA mutation. As showed in Table 3, 6 patients had mutations occurring between TAD-1 domain and TAD2 domain. 4 patients had mutations in TAD1 domain, 2 patients had the combination of mutations between TAD2 domain and DNA binding domain mutation and a deletion between TAD-1 Domain and TAD-2 Domain. 4 patients had frame shift mutations in TAD1 domain and N-terminal part of protein.

The novel 487 deletion of G variant was observed in 5 patients (16.6 %) and 3 (10 %) patients presented a novel 336 C>T substitution. The 198_201 duplication of CTAC was detected 3 (10 %) patients. Three (10 %) of 30 patients had the 690 G>T synonymous variant. Patient 28 had a 573C>T synonymous variant. Patient 29 had a 955_961 insertion of TTGACC in DNA binding domain of CEBPA protein. Patient 10 had combined variants of 300 delG and 487 delG.

Cooperating mutations

Patients with *CEPBA* mutations were also analyzed for mutations of *FLT3, NPM1* and *JAK2* Exon 12. In the 30 pediatric acute leukemia patients examined, *FTL3* mutations were detected in two of the sixteen patients with *CEBPA* mutations and 5 of the 14 patients without *CEBPA* mutations had *FLT3* mutations. Two patients had both *JAK2* and *CEBPA* mutations. One patient had *NPM1* mutation without *CEBPA* mutations.

Clinic and hematologic correlation with CEBPA mutations

Karyotype analysis was normal in 19 (63 %) of the 30 patients. Trisomy 14, Trisomy 19, Trisomy 22, monosomy 7, monosomy 14, inv (16;16), t(9;22), t(15;17), t(4;11) were found using chromosome banding and FISH analyses. We screened all the patients during treatment or relapse phases.

Tab. 4. CEBPA mutations in sixteen children with acute leukemia.

Patient No	Age/ Gender	Diagnosis	Risk Group	Cytogenetics-Molecular abnormalities	CEBPA Mutations	FLT3		TET2	CBL	KRAS	JAK2
						ITD	TKD				
1	5,5/ M	Biphenotypic (Mixed Phenotypic) Acute Leukemia	HR	Ex-46 ,XY	c.690 G>T p.Thr230Thr	–	–	–	–	-	c.1641+179_1641+ 183delTCTTA-intronic
2	2/M	Biphenotypic (Mixed Phenotypic) Acute Leukemia	HR	Ex-46 ,XY	c.690 G>T p.Thr230Thr c.487 del G p.Glu163Ser	–	–	–	-	-	
3	12/M	AML-M2	SR	46 ,XY	c.690 G>T c.487 del G	–	–	–	-	-	
4	11/M	AML-M1	HR	Ex-46 ,XY	c.198_201dupCTAC p.Ile68LeufsTer41	+	–	–	-	-	
5	4/M	Pre B-ALL	SR	46,XY	c.336 C>T p.Pro112Pro	–	–	–	-	-	
6	7/M	AML-M4	HR	47, XY,+ 22[12] Inv (16;16), Fragile X syndrome	c.198_201dupCTAC p.Ile68LeufsTer41	–	–	p.Pro363Leu p.Gly614Gly p.Val218Met c.*128-7564G>A p.Lys1678Glu	-	-	
7	6/F	AML-NA	SR	46,XY,t(15;17)	c.487 delG p.Glu163Ser	–	–		-	-	
8	13/M	AML-M4	HR	46, XY, Ms,Ts 14,	c.336 C>T p.Pro112Pro	–	–	p.Ile1762Val		-	
9	4/M	AML-M4	HR	46, XY	c.382 del C	–	–	–	-	-	
10	5,5/F	AML-M2	SR	46, XX	c.300 del C c.487 delG p.Glu163Ser	–	–	p.Gly614Gly	-	p.Ile20Thr	
11	8/F	AML-M5	SR	46, XX	c.198_201dupCTAC p.Ile68LeufsTer41	–	–	p.Gly614Gly p.Ser217Ser	-	p.Ile20Thr	
20	6,5/F	AML-M5	HR	46, XX	c.487 del G p.Glu163Ser c.336 C>T	–	–	–	-	-	c.1641+179_1641+ 183delTCTTA-intronic
22	3/F	AML-NA	HR	Ex, 47, XX (+21) c 21 der(14) (14q1,2→q 3,2ii 1q21→q43),der(19)(19qdel) →p13.3: : 11q13)11qdel [9]. t(9,22)	c.217_218insC	+	+	p.Pro363Leu p.Pro761Leu p.Ser217Ser	c.714-72237T>C	p.Ile20Thr	
28	3/M	Pre B-ALL	MR	47,XY(+21)	c.573C>T	–	–	–	-	-	
29	2,5/M	Pre B-ALL	SR	47,XY(+21)	c.955-961 ins TT-GACC	–	+	–	-	-	
30	3/F	Pre B-ALL	SR	47,XX(+21)	c.489 C>A	–	–	–	-	-	

M = Male, F = Female, HR = High Risk, MR = Medium Risk, SR = Standard Risk, AML = Acute Myeloid Leukemia, ALL = Acute Lymphoblastic Leukemia, Pre B-ALL = Precursor B- Acute Lymphoblastic Leukemia, NA = Not Available, Ex = Exitus, Del = Deletion, Ins = Insertion, Dup = Duplication

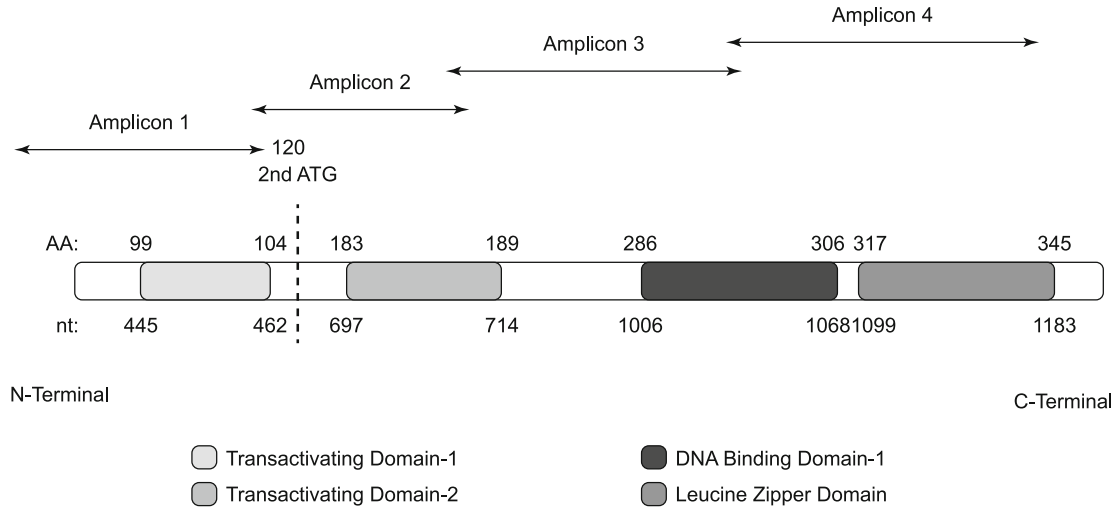

Fig. 1. Location of the amplicons on the CEBPA protein.

All relapse patients (Patient No 1–4, 9, 20) had *CEBPA* mutations. The one patient with t(15;17)/*PML-RARα*, 1 patient with t(9;22) *BCR-ABL*, and 1 with inv(16)/ *CBFβ-MYH11* had *CEBPA* mutations.

Four patients including *CEBPA* mutations did not have cytogenetic and molecular aberrations. The presence of *CEPBA* mutations with no correlation with prognostic classification were divided into "high", "moderate" and "standard" risk groups of acute leukemia. There was no difference in FAB classification between *CEBPA* wild type and *CEBPA* mutant type. Four patients (Patients No 22, 28–30), who had Down syndrome had *CEPBA* mutations. Two mutations (198_201dupCTAC and 217_218insC) reported in the previous study were described as pathogenic for AML. Ten (33.3 %) patients died during the treatment or relapse. The clinical and laboratory features of the study group were summarized in Table 4. No statistical significant differences were detected in the two groups.

Discussion

Acute leukemia is a heterogeneous disorder of hematopoietic stem cells, characterized by multiple genetic events, which have an impact on proliferation and differentiation. Some of the genetic and epigenetic alterations play a major role in leukemogenesis; gene mutations, deletions, translocations, and DNA methylation. Compared with adult leukemia, there were fewer studies of gene mutations in pediatric leukemia patients. We previously reported the frequencies of *TET2*, *CBL*, and *KRAS* mutations by using NGS in pediatric AML patients and the frequencies of FLT3-ITD and FLT3-TKD mutations in acute leukemia patients (18).

Although *CEBPA* mutations have been studied for many years in AML, there were no data about its prevalence and prognostic significance in Turkish patients with AML or ALL. In this study, we investigated *CEBPA* aberrations in pediatric acute leukemia patients to determine their frequency and prognostic impact. In previous studies, several common patterns of *CEBPA* mutations have been reported in AML. In the N-terminus, small out-of-frame insertions or deletions occurred resulting in a premature stop codon, which inhibits transcription of the p42 product (4, 5, 6, 9–13, 20).

CEBPA mutations were detected in 30 pediatric acute leukemia cases (53.3 %), with 9 cases combination of mutations and 7 cases single mutations. As the frequency of *CEBPA* mutations in this study was quite high, the number of patients was not enough to assess the clinical association and mutations. We found no statistical differences in the clinical parameters. The combined mutations (*CEBPA, FLT3*, *NPM1*) have been detected in adult AML, their frequencies varied considerably, ranging between 25 and 35 % (21). Mutations in *CEBPA* have been described in approximately 5–14 % of adult patients with AML (4, 5, 22–24). The frequency of *CEBPA* mutations have been reported to be lower in pediatric AML when compared to the adult AML (5, 19, 20). The study by Hollink et al showed *CEBPA* mutations in 20 out of the 252 (7.9 %), including 14 double mutant and 6 single mutant cases in the pediatric AML patients (5). Fröhling et al showed that frequency of CEBPA was 15 % in young adults with AML. They reported that mutations of *CEBPA* predicted a favorable prognosis and improved risk stratification in AML patients with normal cytogenesis (10).

We identified 10 mutations in *CEBPA* by NGS. Six novel mutations were detected in the present study. The remaining 4 types of *CEBPA* mutations have been reported in the previous studies. We used an amplicon- based sequencing method to find possible new genetic markers for leukemia diagnosis. The c.690G>T, c.198_201dupCTAC, C.217_218insC and c.573C>T variants had been reported in previous studies, whereas we detected 6 *novel* variants in CEBPA gene in this present study. We detected three types of variants in *CEBPA*; frame-shift, missense and synonymous (Tab. 3). Totally ten variants were identified involving N-terminal, TADs and C-terminal domains. The most frequent type of variants

in *CEBPA* in pediatric acute leukemia patients was 487 deletion of G, which led to a glutamin-serin deletion between the TAD1 and TAD2 domain in CEBPA protein.

In our study, we detected that frame-shift mutations (68.75 %) resulted in a premature terminal of the full length 42–kd protein of *CEBPA*, which is shown in Table 3. The 198_201 duplication of CTAC and 217_218 insertion of C was previously reported as pathogenic mutations for AML. The four of sixteen patients had the pathogenic mutations and one patient had benign mutations, which were previously reported. Two types of mutations including c.198_201dupCTAC and c.300delC occurred in TAD1 domain. These two types of mutations could be predicted to loss of the transactivation activity of CEBPA protein. The one type mutation (c.955_961 delTTGAC) in the C terminal domain was caused by truncated the b-ZIP domain of CEBPA protein. In our study as well as in previous studies, the patients with frame shift mutations in encoding regions were associated with favorable clinical outcome (11, 23)

The only patient (patient no 22) in our study group had combination mutations of *CEBPA*, *FLT3*, *TET2*, *CBL* and *KRAS* genes (Tab. 4). This patient was a three year old girl diagnosed with AML and classified as a high risk group and 18 months later she was diagnosed with a relapse and died during the treatment. The combination of mutations cases were very few. The patients with combination of mutations died in the reported studies either during treatment or after relapse (11, 13). We screened 6 patients at relapse: All the relapse-screened patients carried *CEBPA* mutations (Tab. 4). Fröhling et al. detected both types of *FLT3* mutations and found no correlation with prognostic influence among AML patients with *CEBPA* mutations (10). In this study, we found 3 patients, who carried both *CEBPA* and *FLT3* mutations and two of them died during treatment.

The 5-base deletion in the intronic region (1641+179_1641+183delTCTTA-intronic) of JAK2 gene was first reported in a Down syndrome patient associated with B-cell precursor ALL was detected with *CEBPA* synonymous variant in pediatric biphenotypic acute leukemia and AML patients in this present study. In our study, we found no significant correlation between *CEPBA* mutations and other gene mutations and clinical parameters. In addition, four patients with *CEBPA* mutations did not have cytogenetic and molecular aberrations.

This study is the first report of the frequency of *CEBPA* mutations and its correlation with other genes mutations in Turkey. We think that c.487del G and c.198_201dupCTAC could be important prognostic markers for pediatric acute leukemia patients at relapse. There may be biologic differences between adults and children, which may require another study on a larger group of patients to validate the prognostic significance of *CEBPA* mutations in pediatric acute leukemia.

Conclusion

CEBPA may be potential genetic markers for pediatric acute leukemia diagnosis. However, these results need to be confirmed by further studies on a larger number of patients.

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