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Screening of *FLT3* Gene Mutations (*FLT3-ITD* and *D835*) in Turkish Childhood Acute Leukemia Patients

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Abstract

Objective: Important role of FMS-related tyrosine kinase (*FLT3*) in development of hematopoietic and immune system has been known. Studies have shown that *FLT3* mutations play critical role at the pathogenesis acute myeloid leukemia (AML). *FLT3*- internal tandem duplications (*FLT3-ITD)* reported as a significant prognostic marker for pediatric AML. But it is unclear for acute lymphoblastic leukemia (ALL), very few data are presently available in ALL. Studies investigating *FLT3* mutations in Turkish pediatric acute leukemia are very few. Also *FLT3-D835* mutation status in pediatric leukemia has not been explored very well. Therefore, our aim in this study was to screen and analyze *FLT3* mutations in Turkish pediatric acute leukemia patients.

Materials and Methods: Study population was consisted of 27 pediatric patients for *FLT3-ITD* mutation and of 183 pediatric patients for *FLT3-D835* mutation who were diagnosed with acute leukemia. *FLT3-ITD* mutation screening performed by using fluorescence HRM (High Resolution Melting) analysis in real-time Polymerase Chain Reaction (PCR) method. *FLT3-D835* variation was detected with Restriction Fragment Length Polymorphism (RFLP) method.

Results: We detected *FLT3-ITD* mutation in 33.3% of our study group (n=27). *FLT3-D835* screening showed 7.6% of patients were carried heterozygote genotype. Statistical analysis showed that *FLT3-D835* heterozygote mutation was correlated with diagnosis and risk groups. But same correlation was not identified for *FLT3-ITD* mutated samples. Also we found no correlation between *FLT3* mutations and clinical characteristics of patients.

Conclusion: We observed high percentage of *FLT3* mutations in Turkish pediatric acute leukemia patients compare to previous studies. Our results show the importance of *FLT3* mutations in pediatric acute leukemia patients as an important prognostic and diagnostic marker.

Keywords: Childhood acute leukemia; *FLT3-ITD*; *FLT3-D835*; Tyrosine kinase

Abbreviations: AML: Acute Myeloid Leukemia; ALL: Acute Lymphoblastic Leukemia; HRM: High Resolution Melting; PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism

Introduction

The FMS-related tyrosine kinase (*FLT3*) is a receptor tyrosine kinase which has an important role proliferation, survival and differentiation of hematopoietic progenitor cells [1,2]. *FLT3* is one of the class III tyrosine kinase receptors which consist of five immunoglobulin-like domains in extracellular region, an intracellular juxtamembrane (JM) domain, two kinase domains with kinase insert and a C-terminal tail [3]. *FLT3* gene is located at chromosome 13 (13q12) and has 24 exons [4]. High expression of *FLT3* has been shown in acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL) and in the blast crisis of chronic myeloid leukemia (CML) [5]. Rosnet et al. [6] has also shown that *FLT3* is expressed on leukemic blast in AML and B-ALL cases. Two types of *FLT3* mutation have been described in leukemia: *FLT3* internal tandem duplications (*FLT3-ITD*) and tyrosine kinase domain (TKD) mutation. A tandemly duplicated sequence was located in JM domain of *FLT3* coded by the exons 14 and 15. TKD mutation of *FLT3* comprises mutations in the second tyrosine kinase domain especially at codon *D835*. All these mutations generate changes in the amino acid sequences and cause constitutive activation of *FLT3* kinase activity [7]. *FLT3-ITD* mutation prevalence for pediatric AML has been described in 11-33% and identified as a significant independent prognostic factor for poor outcome [8-12]. *FLT3-ITD* allelic ratio (mutant-wild type ratio is greater than 0.4) was also found important prognostic factor for relapse in pediatric AML [13]. It has been suggested that *FLT3-ITD* detection should be considered and performed as a routine test at the diagnosis of AML and management of the therapy [9]. Other type of *FLT3* mutations has been seen at a lower frequency compare to *FLT3-ITD* mutations in acute leukemia patients. There are very few studies which investigate *FLT3* mutations in Turkish pediatric leukemia patients [14,15]. Therefore in our study, we aimed to screen *FLT3-ITD* and *TKD* mutations by HRM and PCR analysis and finding possible genetic markers for molecular leukemia.

Materials and Methods

Patient samples

Study population was consisted of 27 patients for *FLT3-ITD* mutation and of 183 patients for *FLT3-D835* mutation their age's between 1 and 15 years who were admitted to Losante Pediatric Hematology-Oncology Hospital, Ankara, Turkey with the diagnosis of acute leukemia. Blood samples were taken to the EDTA-containing tubes and DNA was extracted from peripheral blood leukocytes with MagNA Pure automatic DNA isolation instrument (Roche Diagnostics, Manheim, Germany). Informed consents were collected from the patient's parents prior to the study.

Determination of *FLT3* Mutations

For *FLT3-ITD* mutation: *FLT3-ITD* variation was screened with real time PCR (Genes-4u FLT3-ToolsetTM Roche Diagnostics, Gmbh, Manheim, Germany) using fluorescence HRM analysis based on genotype profiles. Different plots were created by selecting negative controls as the base-line. Therefore, fluorescence of the all other samples was diagramed relative to this sample. Fluorescence signals were analyzed and significant differences used as indicators of mutations [16].

For *FLT3-D835* mutation: Amplification of gene was performed by PCR. Primers used in *FLT3-D835* mutation were as follows: forward primer: 5'-CCG CCA GGA ACG TGC TTG-3'; reverse primer: 5'-GCA GCC TCA CAT TGC CCC -3'. Primers used in *FLT3-D835* mutation were designed as previously described by Moreno et al. [17]. At codon 835 an aspartate amino acid is encoded, providing a recognition site for restriction enzyme EcoRV; as such, mutants can be detected *via* the loss of this enzyme restriction site.

Then amplified fragments were digested with appropriate restriction endonucleases (*EcoRV,* Fermentas, Lithuania) and sequencing of different band profiles was performed by sequencer (Beckman-Coulter CEQ 2000 XL DNA Analysis System, USA).

Statistical analysis

Statistical analyses were performed by using The Statistical Package for Social Sciences (SPSS) version 20 software. Correlations between *FLT3* mutations and either diagnosis or risk groups were analyzed by using One way ANOVA test. Mann-Whitney test was applied to determine correlations between *FLT3* mutations and clinical and laboratory characteristics of patients. P values less than 0.05 were referred as statistically significant.

Results

Twenty seven children diagnosed with acute leukemia were included to screening study of *FLT3-ITD* mutation. Screening performed by using fluorescence HRM analysis (Figure 1). Figure 1 displays evaluation of HRM analysis for *FLT3-ITD* mutations.

Detailed clinical laboratory data of 27 children is given at table 1. We determined *FLT3-ITD* mutation in 9 (33.3%) of them. One (3.7%) of the 27 patients carried both *FLT3-ITD* mutation and *FLT3-D835* heterozygote phenotype. Distribution of *FLT3-ITD* positive patients according to their diagnosis were as follows: AML (n=5); B-ALL (n=3); Pre B-ALL (n=1). Median age for ITD positive patients was 8 (range: 2-13) and for ITD negative patients was 5.25 (range: 1.2-10). Percentage of male patients was higher than female patients in both *FLT3-ITD* positive (male: 66.6%; female: 33.3%) and *FLT3-ITD* negative (male: 61.1%; female: 38.8%) samples. Median white blood cell count (WBC) in *FLT3-ITD* positive patients was 12600 $\times 10^9$ L^{-1} and in *FLT3-ITD* negative patients was 12150 $\times 10^9$ L^{-1}. Median Hemoglobin (Hb) level in *FLT3-ITD* positive patients was 9.3 g dL^{-1} and in *FLT3-ITD* negative patients was 8.3 g dL^{-1}. Median platelet (PLT) in *FLT3-ITD* positive patients was 75000 $\times 10^9$ L^{-1} and in *FLT3-ITD* negative patients was 26000 $\times 10^9$ L^{-1}. Median bone marrow blast in *FLT3-ITD* positive patients was 98% and in *FLT3-ITD* negative patients was 100%. Median CD34 in *FLT3-ITD* positive patients was 10% and in *FLT3-ITD* negative patients was 35%. Diagnostic distribution and summary of clinical features of patients are shown at table 2.

Screening for *FLT3-D835* mutation was performed on 183 children who were diagnosed with either acute lymphoblastic or myeloid leukemia. RFLP method was applied for this screening (Figure 2). Experiment results showed that 14 of 183 children (7.6%) were carried heterozygote genotype. Sequencing of the gene of heterozygous patients revealed a heterozygous "C/A" variation at c.32705. Nine of 14 heterozygote patients (64.2%) were diagnosed with Pre B-ALL. Two of 14 heterozygote patients (14.2%) were diagnosed with B-ALL. Distribution based on their diagnosis of the remaining heterozygote patients (three out of 14) were T-ALL, ALL and JMML.

Experiment results showed significant correlation between *FLT3-D835* heterozygote mutation and diagnosis (p=0.003) and also risk groups (p=0.045). There was no correlation between *FLT3-ITD* mutation and either diagnosis or risk groups. We explored the possibility of correlation between *FLT3* mutations and clinical characteristics of patients (age, WBC, PLT, Hb, CD34 and BM blast) and we found no correlation.

Discussion

Significance of *FLT3* in normal lymphohematopoietic stem/progenitor cell function has been known. Hence *FLT3* mutations play critical role in pathogenesis of leukemia. *FLT3-ITD* mutation is one of the most common mutations in adult AML, occurring approximately 20-27% of patients [9,18-21]. In pediatric AML patients, frequency of *FLT3-ITD* mutation is lower (5.3-22.2%) than adult patients [20]. There is no too many studies that investigate *FLT3-D835* mutations in pediatric acute leukemia patients. Liang et al. [22] investigated *FLT3-D835* mutation in pediatric AML patients and found 3.3% frequency of this mutation. Nasiri et al. [23] studied *FLT3-D835* mutation in 100 pediatric acute leukemia patients and found frequency of this mutation as 3.7% in AML and 1.3% in ALL patients. Studies performed in Saudi pediatric ALL patients and Serbian pediatric AML patients showed frequency of *FLT3-D835* mutation as 2.1% and 9.5%, respectively [24,25]. To the best of our knowledge, there are only two studies that were investigated *FLT3* mutations in Turkish pediatric acute leukemia patients [14,15]. Ozbek et al. [14] reported *FLT3-ITD* mutation rate as 4% and they observed *FLT3-D835* point mutation heterozygosity in only 1 patient (2.0%). Karabacak et al. [15] found *FLT3-ITD* mutation in 7.5% in ALL patients and 22.5% in AML patients. They didn't identify *FLT3/TKD* mutation in any of their patients. In our study, we found *FLT3-ITD* mutation in 55.5% of AML diagnosed patients and in 22.2% of ALL diagnosed patients. *FLT3-D835* heterozygosity was observed 7.6% of our study group. Therefore, our results showed higher *FLT3-ITD* and *FLT3-D835* mutation frequencies than Ozbek et al. [14] and Karabacak et al. [15] Beside Ozbek et al. [14] used PCR method to

Characteristics	*FLT3-ITD* Positive patients	*FLT3-ITD* Negative patients
No patients	9	18
Diagnosis, no. (%)		
AML	5 (55.5)	4 (22.2)
Pre B-ALL	1 (11.1)	8 (44.4)
B-ALL	3 (33.3)	2 (11.1)
T-ALL	0	2 (11.1)
JMML	0	1 (5.5)
ALL	0	1 (5.5)
Median age	8 (2-13)	5.25 (1.2-10)
Sex, no. (%)		
Male	6 (66.6)	11 (61.1)
Female	3 (33.3)	7 (38.8)
Median WBC count (× 10^9 L^{-1})	12600	12150
Median Hb (g dL^{-1})	9.3	8.3
Median PLT (× 10^9 L^{-1})	75000	26000
Median BM Blast (%)	98	100
Median CD34 (%)	10	35
T(9;22)		
Positive	1	3
Negative	8	15

Table 1: Clinical characteristic and mutational status of 27 patients included *FLT3* mutation screening

Case No	SEX	AGE (year)	WBC count (× 10^9 L^{-1})	Hb (g dL^{-1})	PLT COUNT × 10^9 L^{-1}	BM Blast (%)	CD 34 (%)	T(9;22)	*FLT3-ITD*	*FLT3-D835*	Diagnosis	Risk Group
1	E	11	37 900	10	110 000	NA	32	Negative	Positive	Negative	AML-M1	HR
2	E	3	7 500	8.7	120 000	96	5.24	Negative	Positive	Negative	AML-M1	HR
3	K	9	22000	9.9	73 000	100	3.37	Negative	Positive	Negative	AML-M4	HR
4	E	3.5	12600	8	35 000	20	10	Negative	Positive	Negative	AML-M4	HR
5	E	10	22 200	6.8	10 000	77	68	Positive	Negative	Negative	AML	HR
6	E	5.5	15 800	6.7	20 1000	56	0,22	Negative	Negative	Negative	AML-M3	HR
7	K	7.5	30 900	5.5	12 000	100	38	Negative	Negative	Negative	AML-MDS	HR
8	E	12	28 100	7.1	75 000	100	3,2	Negative	Positive	Negative	AML-M2	SR
9	K	5.5	8 340	7	5 000	48	24	Negative	Negative	Negative	AML-M2	SR
10	E	5.5	154000	10	29 00	100	56,13	Negative	Negative	Heterozygote	Pre B ALL	HR
11	E	2	144000	6.4	7000	96	49	Negative	Negative	Heterozygote	Pre B ALL	HR
12	E	3.5	2 810	10.1	223 000	74	41,78	Negative	Positive	Negative	My+ PreB-ALL	HR
13	E	2.5	22500	9.5	56 000	100	92,2	Negative	Negative	Heterozygote	Pre B ALL	MR
14	K	9	96000	8.3	8000	100	3	Negative	Negative	Heterozygote	Pre B ALL	MR
15	E	5	3300	8.3	75 000	100	NA	Negative	Negative	Heterozygote	Pre B ALL	SR
16	K	3.5	5400	9.8	19 000	100	94	Positive	Negative	Heterozygote	Pre B ALL	SR
17	E	2.5	7600	10.9	39 000	100	30.41	Negative	Negative	Heterozygote	Pre B ALL+Down snd	SR
18	E	2.5	8 500	10	69 000	78	4	Positive	Negative	Negative	Pre B ALL	SR
19	K	2	5 300	4.7	8 000	100	53.07	Positive	Positive	Negative	B-ALL	HR
20	K	8	12 000	10.5	115 000	18	8	Negative	Positive	Heterozygote	B-ALL	HR
21	E	13	225 000	5.04	24 400	100	64.74	Negative	Positive	Negative	B-ALL	HR
22	E	5	6 400	9.4	129 000	100	27	Negative	Negative	Negative	B-ALL	SR
23	E	7.5	2610	4.9	180 000	100	83.3	Negative	Negative	Heterozygote	B-ALL	MR
24	E	9	491000	5.5	137 000	100	35	Negative	Negative	Heterozygote	ALL	HR
25	K	14 months	89000	5.5	26 000	14	40	Negative	Negative	Heterozygote	JMML	HR
26	K	4	2300		7000	100	0	Negative	Negative	Heterozygote	T ALL	MR
27	K	6.5	7100	9.8	36 000	65	2	Negative	Negative	Negative	T cell leukemia/ lymphoma	HR

Table 2: Clinical characteristic and mutational status of 27 patients included *FLT3* mutation screening
WBC: White blood cell; Hb: Hemoglobin; PLT: platelet; BM: Bone Morrow; WT: Wild Type; HR: High Risk; ST: Standard Risk; MR: Moderate Risk, NA: Not Available

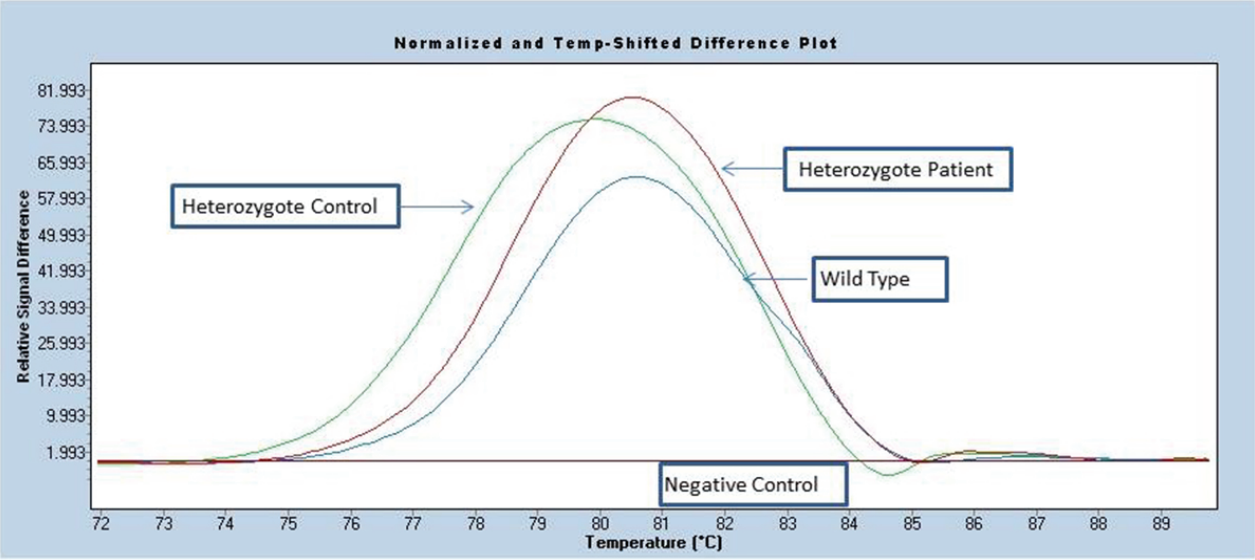

Figure 1: HRM assay for *FLT-ITD* mutation

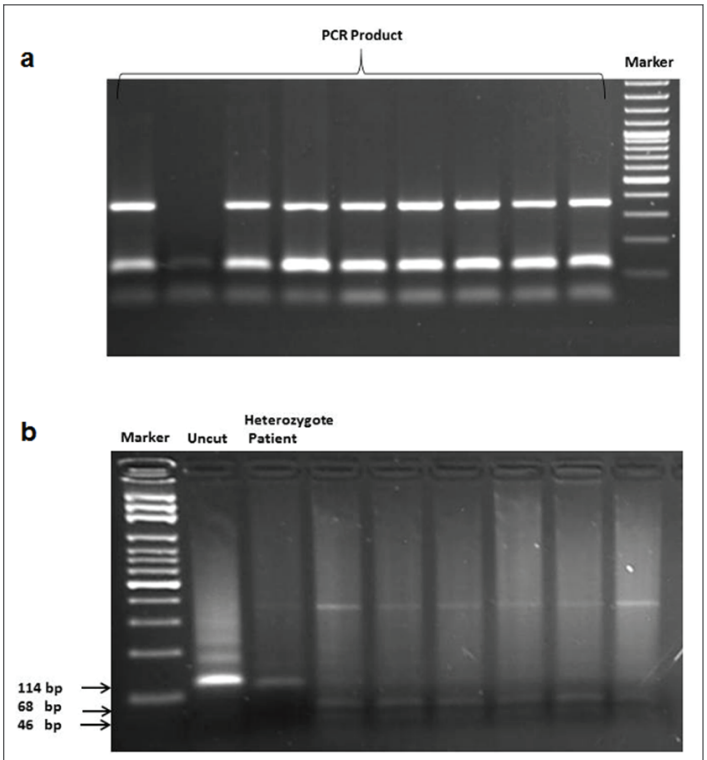

Figure 2: a) PCR amplification of *FLT3-D835* region. **b)** Digestion products of amplified fragments for detection of *FLT3-D835* mutation.

screen *FLT3-ITD* mutation. But we prefer to use HRM analysis to identify *FLT3-ITD* mutation. HRM analysis is more sensitive method compare to PCR [26]. Our high *FLT3-ITD* mutation rate was possibly due to our sensitive analysis method. Additionally our results show differences and significance compare to Ozbek and Karabacak studies in terms of study group composed of not only AML patients and also ALL patients and we observed 7.6% *FLT3-D835* heterozygosity in our leukemia group.

One patient in our study group showed *FLT3-ITD* mutation and *FLT3-D835* point mutation heterozygosity together. This patient is eight year old girl diagnosed with B-ALL and classified as a high risk group. She has 12000 ($\times 10^9$ L^{-1}) WBC count, 10.5 (g dL^{-1}) Hb, 115000 ($\times 10^9$ L^{-1}) PLT count, 18% BM blast and 8% CD34. Cytogenetic tests showed trisomy 8 in her bone morrow samples. She entered first remission at 28th day of her chemotherapy treatment. But 18 months later she diagnosed with relapse. Reported double mutation cases have been very few [14,17,27]. These studies performed on AML patients. Double mutation carrying patients died in reported studies either during treatment or after relapse [17]. To the best of our knowledge, this is the first case with double *FLT3* mutation, trisomy 8 and ALL. Beside *FLT3* mutations, trisomy 8 may play role of this patient's poor prognosis [28]. Therefore double *FLT3* mutations in ALL could be associated with bad prognosis and relapse like AML patients.

In our study, we found significant correlation between *FLT3-D835* heterozygote mutation and diagnosis (p=0.003) and also risk groups (p=0.045). But similar correlation was not detected between *FLT3-ITD* mutation and either diagnosis or risk groups.

Ozbek et al. [14] reported no correlation between *FLT3* gene mutations and age, gender, WBC count, blast cell rate or FAB classification in their study group composed of AML patients. Karabacak et al. [15] concluded that there is no significant relationship between laboratory results and *FLT3/ITD* positivity in both ALL and AML patients. But they found association between *FLT3-ITD* positivity and increase in age. We found no correlation between *FLT3* mutations and clinical characteristics of patients (age, WBC, PLT, Hb, CD34 and BM blast) in our study similar with Özbek's and Karabacak's study. But some studies found positive association with high WBC count and high percentage of bone morrow blast cells in *FLT3-ITD* positive samples [9,23].

Our statistical analysis showed significant correlation between *FLT3-D835* heterozygote mutation and diagnosis (p=0.003) and also risk groups (p=0.045). This result showed that *FLT3-D835* mutation can be used as a prognostic marker in ALL and helpful information for the diagnosis. It is known that *FLT3-ITD* mutation is a poor prognostic marker for acute leukemia. But we didn't find similar correlation between *FLT3-ITD* mutation and either diagnosis or risk groups. This result could be due to our small and mixed study group.

Cytogenetic prognostic factors are preferred more in clinical use. But there is a need to enhance analysis and clinical use of molecular factors such as mutational status *FLT3, MLL, NPM1, CEBPA* especially in cases with normal karyotype.

Our study is one of the few studies that analyze *FLT3* mutational status in Turkish pediatric acute leukemia patients. It also points out the importance of *FLT3-D835* mutations in ALL prognosis.

Conflict of Interest

The authors declare that they have no conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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