Original Article

Detection and Biological Characteristic of FLT3 Gene Mutations in Children with Acute Leukemia

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Abstract

Introduction: FLT3 ITD and D835 mutations occur in high frequency in AML and to a lower rate in ALL patients with poor prognosis.

Methods: ITD and D835 mutations were studied in 100 diagnosed acute leukemia patients including 27 AML and 73 ALL with various FAB classifications by PCR and PCR-RFLP, respectively. Subsequently, PCR products of positive samples were confirmed by sequencing analyses.

Results: ITD mutations occurred in 10% of all pediatric acute leukemia, including AML and ALL. 25.9% of AML patients harbor a mutation in the ITD in various subtypes. The frequency of ITD mutations was 4% in ALL. Various insertions of nucleotides in ITD were observed, similar to those described in the literature previously.

Conclusion: These preliminary data suggest that flt3-ITD mutations may play an important role in leukemogenesis in a proportion of children, particularly in the case of AML.

Keywords: Acute leukemia, Flt3-ITD, PCR-RFLP

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Introduction

FLT3 receptor (FMS-like tyrosine kinase-3 receptor) is a member of extracellular receptors on hematopoietic precursors and belongs to the class III tyrosine kinase receptor family. The FLT3 receptor gene encodes a 993-amino acid protein.[1,2] This gene is located on the short arm of chromosome 13 and is expressed in bone marrow, thymus and lymph nodes.[3] The FLT3-ligand (FL) is expressed on most cell lineages. In normal situation, FL cannot promote the independent growth of these cells (IL-3), but in case of mutations such as internal tandem duplications (ITD) or point mutations (e.g., D835), the mutant flt3 enables cells to proliferate independently of growth factors like IL3.[4-6] Despite the remarkable diversity in the number of repetitive amino acid sequences (30 to 50 repeats), there is no difference between the biologic function of FLT3-ITD alleles investigated in cell culture studies or animal models.[7,8] Contribution between FLT3 mutations and gene rearrangements may be necessary to develop the AML phenotype. Overexpression of the FLT3 receptor takes place in about 70%-100% of acute myeloid leukemia (AML) as well as the majority of acute lymphoblastic leukemia (ALL) cases.[9–12] The FLT3 receptor gene mutations are the most frequent genetic defects observed in AML patients, and among them ITD mutations and point mutation at location of ASP835 (D835) are the most known.[13–16]

ITD mutations in exons 11 and 12 and intron 11, first discovered in 1996 by Nakao et al, occur in the juxtamembrane domain (JM). D835 point mutations also occur in exon 20 of the FLT3 receptor gene, in the position of aspartic acid 835. Substitution of ASP-836Tyr is the most frequent case.[17] Previous data showed acquired mutations in FLT3 receptor gene occur in approximately 30% of adult AML patients, among which ITD mutations and point mutations in ASP835 are responsible for 24% and 6% of AML patients, respectively. It is known that the frequency of FLT3 receptor mutations increases along with aging. ITD mutations occur in ALL at a lower rate than AML - approximately 1–5%. Regarding the frequencies of mutations in other genes, FLT3 receptor gene is the most frequent mutant gene in AML patients.[15,18,19] Due to the significant prognostic value of FLT3 receptor gene mutations in leukemic patients, particularly AML, many researchers have focused on developing FLT3 inhibitor drugs.[20,21] Since remarkable studies have not been performed on these mutations in Iran, screening molecular diagnostic methods to detect these mutations in children with acute leukemia patients have turned into important concerns.

Materials and methods

Patient samples

In this study, FLT3 receptor gene mutations were assessed in 100 children suffering from AML (27 cases) and ALL (73 cases). Blood samples were collected from Ali-Asghar hospital and

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flowcytometry center of Iranian Blood Transfusion Organization (IBTO), Tehran. Samples were classified according to the French American British morphology (FAB) and immunophenotypic investigations by flowcytometry (BD, Biosciences, USA). The Medical Ethics Committee of the Tehran University of Medical Science (TUMS) approved the study and written informed consent was obtained from all participating patients.

Mutation detection

Mononuclear cells were purified by Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) centrifugation and their DNA was then extracted by the standard method. To detect ITD mutations, Polymerase chain reaction (PCR) amplification of genomic DNA was carried out using primers; Forward (5'-GCA ATT TAG GTA TGA AAG CCA GC-3') and Reverse (5'-CTT TTT TGA CGG CAA CCT CAG CA-3').

Activating loop mutations were determined by PCR amplification with primers; 20 F (5'-GCA GCC TCA CAT TGC CCC-3') and 20 R (5'-CCG CCA GGA ACG TGC TTG-3'), followed by EcoRV digestion (Fermentas Co, Canada) (19, 24). Furthermore, the positive cases of ITD were confirmed by sequencing technique. PCR products were purified (AccuPrep®PCR Purification Kit, BioNEER, Korea) and then sequenced directly (ABI 3130 DNA analyzer, Applied Biosystems, USA). Chromas software was used to interpret graphs and mutations in form of insertion/deletion were found.

Results

Frequency of mutations in leukemia samples

ITD mutations were detected in 10 out of 100 (10%) leukemic samples with a prevalence of 25.9% in AML and 4.1% in ALL specimens (Table 1) (Figure 1).

The point mutation of D835 was detected in two out of 100 (2%) AML and ALL blood samples with a prevalence of 3.7% and 1.3%, respectively (Table 1, Figure 2). Interestingly, one case of M3 had both ITD and D835 mutations.

Table 1. Correlation of FLT3 mutations with the FAB subtypes in 100 children with acute leukemia.

Subtype of leukemia	Total (100)	No mutation	FLT3-ITD	FLT3-D835
AML:ALL	27:73	20(74%):69(94.5%)	7(25.9%):3(4.1%)	1(3.7%):1(1.36%)
M0	2	2	0	0
M1	5	4	1	0
M2	5	3	2	0
M3	3	1	2	1
M4	4	3	1	0
M5	4	3	1	0
M6	3	3	0	0
M7	1	1	0	0
Early pre B-cell	39	36	3	0
Pre B-cell	14	13	0	1
B-cell	5	5	0	0
T-cell	15	15	0	0

AML = acute myeloid leukemia; FLT3 = fms-like tyrosine kinase; ITD = internal tandem duplication.

Figure 1. In the image, 328bp bands are related to exons 11 and 12 and intron 11 of FLT3 gene. Mutant samples belong to different subtypes of the FAB classification including AML-M3 (column 2), Early pre-B cell ALL (columns 6–8), AML-M2 (column 9) and AML-M1 (column 13). In all cases, the lower band (328bp) depicts normal clones or wild type gene and the higher band (> 328 bp) indicates mutant clones of leukemic cells.
M = marker, N = negative control.

Figure 2. This image shows PCR-RFLP (ECOR1) products of exon 20 for FLT3 gene. All columns, except 5 and 13, show 2 bands (68bp and 46bp) which indicate wild type gene in which ECOR1 enzyme with the restriction site (5'-…ATC…-3') yields two bands after digestion. Point mutation at D835 changes this restriction site and leads to an 114bp mutant band in addition to 68bp and 46bp wild type bands in heterozygous samples, as in samples 5 and 13. M = marker , N = negative control.

Table 2. Characterization of leukemic patients (AML and ALL) with FLT3-ITD and FLT3-D835 mutations.

Subtype of leukemia	Age	Sex	Cytogenetics	FLT3-ITD	FLT3-D835
M1	15	Male	Unknown	+	-
M2	9	Female	+8,t (8;21)	+	-
M2	12	Male	Normal/46XX	+	-
M3	4	Male	t(15;17)	+	+
M3	7	Male	t(15;17)	+	-
M4	10	Male	Inv(16)	+	-
M5	10	Female	Normal/46XX	+	-
Early pre B-cell	2	Female	Not done	+	-
Early Pre B-cell	2	Female	Normal/46XX	+	-
Early pre B-cell	13	Male	+8,+21	+	-
Pre B-cell	17	Female	Hyperdiploidy, +21	-	+

AML = acute myeloid leukemia; FLT3 = fms-like tyrosine kinase; ITD = internal tandem duplication.

Profile and sequencing

The median age in pediatric acute leukemia was 5.5 ± 1.6 (range: 1–17) years and the male/female (M/F) ratio (M/F) was 1.2. There was no correlation between samples' mutation status and gender and age. A positive correlation with high WBC count (over 20,000/μL in 58% of samples) was demonstrated in FLT3-ITD positive cases ($P < 0.05$). Table 1 represents the profile of leukemic patients, including subtype of leukemia and karyotype with ITD and D835 mutations. Sequencing data on ITD positive samples demonstrated insertion of 27, 27, 47, and 63 nucleotide fragments, which is similar to those previously reported in the literature.

Discussion

Mutations affecting the FLT3 receptor play an important role in pathogenesis of leukemia. Evidence to date suggests that mutation of FLT3 could confer a proliferative advantage, thereby complementing the differentiation block induced by PML-RARA.[25] ITD mutations in the FLT3 gene have been reported to be associated with poor prognosis and resistance to common therapies.[17,35] In this study, ITD and D835 mutations were detected in 10% and 2% of blood samples from children with acute leukemia, respectively, with various distributions in the flt3 gene subtypes of FAB classification. ITD mutations were found in AML patients subtypes M1, M2, M3, M4, M5 and early Pre-B cells ALL. Various other studies have reported irregular occurrence of the mutation among subclasses.[26] Other studies on children suffering from AML have shown that possessing ITD mutations indicates poor prognosis. Approximately 10%–20% of pediatric AML and 1%–5% of pediatric ALL harbor a mutation in FLT3. In the largest study on AML patients, 16% of samples showed FLT3-ITD mutations. Remission of disease occurred in 40% of FLT3-ITD positive patients compared to 73% in FLT3-ITD negative patients. The ITD has been mostly observed within M3 samples while it occurs least commonly within M2.[24,25,27]

Our results showed that FLT3-ITD mutations occurred in 25.9% of AML and 4.1% of ALL patients. Regardless of the number of inserted or repeated nucleotides, insertion of new amino acids at any number could cause conformational changes in protein structure and demolish the inhibitory function of the juxtamembrane region in receptor autophosphorylation. These repeats can take place in exons 11 and 12 or intron 11 of FLT3 gene or may occur in common region between exons 11 and 12.[22,28] The point mutation of exon of the FLT3 gene causes substitution of aspartic acid in position 835 (D835) by other amino acids like tyrosine or valine, leading to promoting the activation loop.[29–31] D835 mutation was detected in 3.7% of AML and 1.3% of ALL patients. Due to the low number of our samples and low frequency of the mutation, we were not able to define any relationship between D835 mutation and FAB classification. Other studies[32–34] have indicated that D835 mutations are found in 7% of children with AML, 2.7% of ALL patients and 3.4% of myelodysplastic syndromes (MDS). Among ALLs, FLT3 mutations were more common in patients with hyperdiploidy. The D835 mutation occurs in the FLT3 gene with a significantly lower rate than ITD mutations, but both mutations were found in AML patients with a high frequency. Ethnicity may strongly influence the frequency of reported mutated gene. In our study one AML patient was found to have both ITD and D835 mutations and sequencing revealed that these two mutations were not located on the same allele.[29–35] The sequencing data showed different insertions of nucleotides in the juxtamembrane region of ITD, similar to previous reports in the literature. It is suggested that insertion of amino acids in the juxtamembrane region causes autocrine stimulation of FLT3 receptor, followed by survival and proliferation of leukemic cells.[22,28] Despite the pathogenic effect of this mutation, it cannot cause acute leukemia by itself and requires other genomic alterations related to cell differentiation. Certain limitations in this study should be considered when interpreting our results. The first limitation is the sample size and the second is lack of information about survival of patients with or without FLT3-ITD. The prognostic impact and definition of appropriate strategies for therapeutic procedures remain to be determined in a wider Iranian population.

In conclusion, our data demonstrated a high frequency of FLT3-ITD mutation in the AML-M3 and may play an important role for leukemogenesis in a proportion of children, particularly with AML.

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