

Detection of FLT3-ITD Mutation in Twenty Child with Acute Myeloid Leukemia in One Iraqi Teaching Hospital

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Summary:

Background: Pediatric acute myeloid leukemia (AML) has a poor prognosis, and novel therapies are needed. The FLT3 tyrosine kinase inhibitors represents a promising target in pediatric AML.

Objectives: This study was done to estimate the frequency of FLT3- ITD mutation in childhood acute myeloid leukemia using conventional PCR & correlate this mutation with the clinical presentation and response to induction therapy.

Patients, Materials & Methods: Twenty children with AML, and 16 children with reactive bone marrow as negative control were enrolled in this study. Those patients were attending Child Welfare Teaching Hospital in Baghdad from March 2010 to July 2011. For each patient hematological investigations including complete blood picture, and bone marrow aspiration were done. FLT3-ITD mutation was detected by conventional PCR technology using specific primers. Complete hematological remission achievement after induction chemotherapy was assessed by clinical examinations and full laboratory investigations.

Results: Out of 20 AML children who participated in this study, 2 (10%) had FLT3-ITD mutation. The mean age of patients who had the mutation was higher than those without the mutation; and the mutated patients were males, ($P > 0.05$). The FLT3-ITD mutation showed no correlation to clinical presentation. The peripheral blood & bone marrow blast cell percent were not significantly higher in mutated patients as compared to non mutated patients. Regarding its relation to FAB classification, the FLT3-ITD mutation was only detected in M3 (1/20) and M3v (1/20), and no mutation was found in other subtypes (M1, M2, M5). Furthermore, mutated patients showed lower response to induction therapy as compared to non mutated patients.

Conclusions: This is a novel study in one Iraqi teaching hospital to detect FLT3-ITD mutation by using conventional PCR in children with AML. This mutation was detected in 10% of those children, and since they were male, older age group, and presented with higher peripheral blood & bone marrow blast cell percent thus we may propose that it may be used as a marker for the aggressiveness of the disease and can be used to modulate the treatment strategy for those patients.

Keywords: Childhood leukemia, acute myeloid leukemia, FMS-related tyrosine kinase gene, internal tandem duplication mutation, polymerase chain reaction, prognosis.

Introduction:

Genetic technologies are powerful ancillary tools for diagnosing, classifying and managing acute leukemia. (1) The FMS-related tyrosine kinase-3 (FLT3) is a receptor tyrosine kinase expressed in early hematopoietic progenitors that plays an important role in hematopoietic development. (2) Interaction with its ligand (FL) results in receptor dimerization, autophosphorylation and the subsequent phosphorylation of cytoplasmic substrates that are involved in signalling pathways regulating the proliferation of pluripotent stem cells, early progenitor cells and immature lymphocytes. (3) FLT3 is constitutively activated either by an internal tandem duplication (ITD) or by a point mutation (PM). (4) Mutations

in FLT3 in AML occur in approximately 25-35% in adults, (5) and its frequency in pediatric AML appears to be somewhat lower than in adults with AML, occurring in about 10% to 15% of pediatric patients. (6, 7) Identification of FLT3 mutation in AML may indicate a need to reassess and modify standard treatment options. (4) Furthermore, the majority of retrospective data indicate that FLT3 mutations are an independent variable that confer a poor prognosis in AML particularly in pediatric AML. (8)

Patients, Materials and Methods:

This prospective study was conducted on 36 subjects including 20 children with AML and 16 children with reactive bone marrow served as negative control for the mutation. Patients were selected randomly in relation to age and sex. All patients were diagnosed as de novo AML and 18 out of 20 patients were newly diagnosed, whereas 2 patients were in relapse. All the

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children were collected from The Children Welfare Teaching Hospital in Baghdad, and they were referred from different governorates of Iraq. From each patient & control subject peripheral blood samples were collected in 2 EDTA tubes, one for analysis of hematological parameters by automated hematology analyzer & the other for DNA analysis which was kept in deep freeze (-70°C) until the day of analysis. Peripheral blood and bone marrow aspirate smears were examined by two hematology consultants for diagnosis of AML & their sub-classification according to FAB classification. (9) Detection of FLT3-ITD Mutation: High molecular weight DNA was extracted according to the kit protocol (Promega) following the instruction manual. (10) All samples were analyzed for FLT3-ITD mutation on chromosome 13, exon 11 using conventional PCR in the Microbiology Department/ Al-Nahrain Medical College. The use of exon 11 specific primers allowed covering the whole juxtamembrane and the first part of tyrosine kinase-1 domain where most of the reported mutations are located. (11) Fifty to 100 ng of DNA (5 µl) was amplified in a 50 µl reaction mixture containing 1.5 mM MgCl₂, 50 mM KCl, 200 µM for each deoxyribonucleotide triphosphate (dNTP), 2.5 units Taq polymerase, 40 picomol of each primer which have the following sequences (Forward Primer 11F: 5'-CAATTTAGGTATGAAAGCC-3', Reverse Primer 12R: 5'-CAAACCTCTAAATTTTCTCT-3'). A positive reaction was assessed in duplicate and a negative control was included in each reaction. PCR amplification was performed using PCR Thermal cycler (Eppendorf Master cycler, France). Amplification process consisted of 40 cycles of 30 sec at 94°C for denaturation, 45 sec at 50°C for annealing, 1 minute at 72°C for extension and 1 cycle of 7 minutes at 72°C for the final extension. (11) Twenty µl of the PCR product was electrophoresed on 2.5% agarose gel (Promega), using 100bp DNA ladder (Promega) as molecular weight marker and was stained with ethidium bromide (Promega). Follow up of patients: The initial response to induction chemotherapy was assessed in each patient whether there is complete hematological remission (CR), treatment failure, or early death. (12) Statistical analysis: Statistical analysis was done using SPSS version 16 & Microsoft Office Excel 2007. Numerical data were expressed as mean ± SD whereas nominal data were expressed as frequency. Analysis of numeric variables was done using student t-test, whereas analysis of nominal data was done using frequency and Chi-square. P-value < 0.05 was considered significant.

Results:

This prospective study involved 20 AML children (18 newly diagnosed and 2 relapsed cases) along with 16 children with reactive bone marrow who were age and sex matched (P-value > 0.05), (Table 1). Furthermore, equal number of males and females patients enrolled in this study with a male to female ratio 1:1, whereas control children had male to female ratio 2:2:1, (Table 1).

Table(1): Demographic features of children enrolled in the study.

Characteristics	Control N(16)	Patients N(20)	P- value
Age/year Mean ± SD	6.4875 ± 4.64	6.8250 ± 3.78	0.817 T -Test
Gender			
Male	11	10	0.257 Chi-Square
Female	5	10	

FLT3-ITD frequency: Using conventional PCR, the amplified DNA product of the wild type from the patients and healthy controls was approximately 133 bp band whereas the mutated type showed additional band > 133bp (approximately 180 bp), (Figure 1). Although equal number of males and females were included in the study, the FLT3-ITD mutation was found in males only, (P-value 0.136). Also the mean age ± SD of mutated cases (7.75 ± 4.59) was nonsignificantly higher than non mutated cases (6.72 ± 3.83), (P-value 0.726), (Table 2). All the control children enrolled in this study showed wild type FLT3 gene, (Figure 1). Most of the patients presented as M2 (35%), followed by M3 (30%), then M3v (15%). Furthermore, the mutation was detected only in M3 and M3v, (Table 3). Moreover, most of the patients presented with pallor 90%, fever 70% followed by bleeding 55% with no specific relation to FLT3-ITD mutation, (Table 3). Regarding the relation of FLT3-ITD mutation to hematological parameters of the patients, the mean WBC count in mutated patients was (11.55 ± 10.39 × 10⁹/L) which was significantly lower than non mutated patients (42.32 ± 53.80 × 10⁹/L), (P-value 0.046), whereas mean platelet count in mutated patients was lower than mean platelet count in patients without mutation [19.00 ± 15.55 & 25.67 ± 27.40 × 10⁹/L, (mean ± SD)], respectively, (P-value 0.743). Similarly the mean hematocrit was lower in patients with mutation as compared to patients without mutation [18.00 ± 4.24 & 21.73 ± 7.17 %, (mean ± SD)], respectively, (P-value 0.486), (Table 4). The mean peripheral blood blast cell percent was higher in FLT3-ITD positive cases than FLT3-ITD negative cases [50.50 ± 31.82 & 35.78 ± 27.88 %, (mean ± SD)], respectively, (P-value 0.491); also the mean bone marrow blast percent was higher in FLT3-ITD positive cases as compared to FLT3-ITD negative cases [64 ± 19.79 & 56.94 ± 19.76, (mean ± SD)], respectively, (P-value 0.638), (Table 4). Both mutated cases were newly diagnosed de novo-AML cases, (P-value 0.531). Furthermore, one child with FLT3-ITD mutation (50%) had achieved complete hematological remission whereas, the other did not (P-value 0.619), whereas 15/18 (83%) patients without the mutation achieved complete hematological remission, (Table 5).

Table (2): The demographic features of pediatric AML patients in relation to FLT3-ITD Mutation

Demographic features	FLT3-ITD -ve N(18)	FLT3-ITD+ve N(2)	N	%	P-value
Gender					
Male	8	2	10	50	0.136
Female	10	0	10	50	
Age /Year Mean±SD	6.72±3.83	7.75±4.59	20	100	0.726

Table(3): Clinical presentation of AML patients in relation to FLT3-ITD mutation

Clinical presentation	FLT3-ITD -ve N(18)	FLT3-ITD+ve N(2)	N	%
FAB subtype M1	2	0	2	10
M2	7	0	7	35
M3	5	1	6	30
M3v	2	1	3	15
M5	2	0	2	10
Lymphadenopathy	5	0	5	25
Splenomegaly	9	1	10	50
Hepatomegaly	7	1	8	40
Bleeding	9	2	11	55
Fever	14	0	14	70
Pallor	17	1	18	90
Weight loss	2	0	2	10

Table(4): Hematological parameters of pediatric AML patients in relation to FLT3-ITD mutation

Characteristics	FLT3-ITD-ve	FLT3-ITD +ve	P-value
WBC count X10 ⁹ /L Mean±SD	42.32±53.804	11.55±10.394	0.046*
Platelet count X10 ⁹ /L Mean±SD	25.67±27.405	19.00±15.556	0.743
Hematocrit % Mean±SD	21.7389±7.17612	18.00±4.24264	0.486
Peripheral blood blast % Mean±SD	35.78±27.885	50.50±31.820	0.491
Bone marrow blast % Mean±SD	56.94±19.76	64±19.79	0.638

* Significant

Table (5): Comparison between type of AML and response to induction therapy in relation to FLT3-ITD mutation:

Response to induction	FLT3-ITD-ve /new N(16)	FLT3-ITD-ve /relapse N(2)	FLT3-ITD+ve /new N(2)	FLT3-ITD +ve / relapse N(0)	N (%)
Remission	15	0	1	0	16 (80)
Failure	1	2	1	0	4 (20)

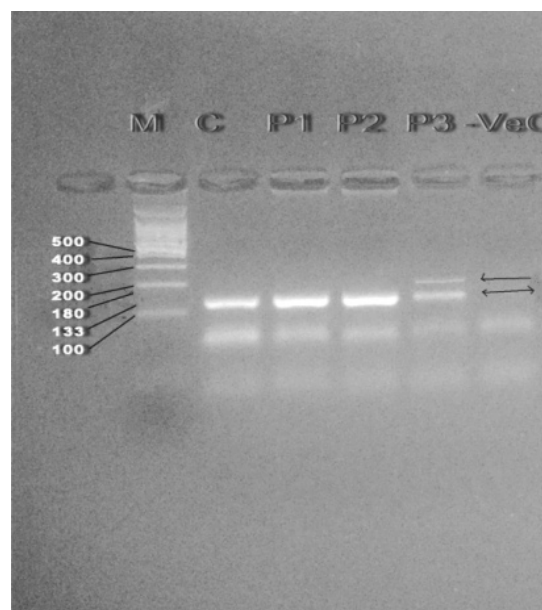


Figure (1): Detection of FLT3-ITD mutation using PCR. Lane C: Amplified product from healthy control .Lanes P1, P2: amplified product from patients wild type (app.133bp ,double head arrow).Lane P3: amplified products from patients show extra mutated band (app180 bp arrow) of FLT3-ITD. Lane -VeC: negative control(no template). M:Molecular weight marker (DNA ladder).Electrophoresis was carried in 2.5% agarose gel at (4V/cm) for 60 min.

Discussion:

The incidence of childhood leukemia had doubled over the last 15 years especially in the southern of Iraq ,andtheir rates were higher than that found in nearby countries, in the European Union or in the United States.(13) In Iraq leukemia ranks the 1st cancer among the commonest ten childhood cancers according to Iraqi Cancer Registry 2008 with an incidence 32.59 % .(14) AML constitutes 16.35% of all types of leukemia in all age groups.(14) Moreover, 85% of Iraqi children with myeloid leukemia fall within the age group 5-14 years .In this study, the male to female ratio was 1:1 which was similar to that reported by the Iraqi ministry of health last statistics in 2009.(15) FLT3-ITD mutation was detected in 2 out of 20 pediatric AML patients (10%), this result was in agreement with many studies (6,7,8), and was in line with Zakeret al., study from Iran who had reported that the frequency of this mutation in pediatric AML patients was 7.7%(16) , whereas an Egyptian study (11) reported a higher FLT3-ITD mutation frequency of 20.3% ;this variation in the incidence of the mutation may be due to environmental factors. This study showed that patients with FLT3-ITD mutation were not significantly older than patients without mutation ,and both mutated patients were male, this result was in agreement to other studies,(6,8,11,16) and since age and sex is a known prognostic factor in pediatric AML, we may propose that the detection of FLT3-ITD mutation in male and older pediatric group may add an adverse risk

factor to the disease. FLT3-ITD mutations were detected in M3 (M3&M3v) subtype patients, this was in agreement to other studies. (6,11,16,17,18) On the other hand Soheil et al., study (8) reported that FAB- M1 had the highest frequency among other subtypes and this difference may be explained by contrariety in PCR circumstances and the use of different primer set for the detection of the mutation. The current study found that hepatosplenomegaly or lymphadenopathy, pallor, fever and weight loss was not affected by the presence of this mutation. This was similarly reported by Kiyoi et al. (19) The mean WBC count at the time of diagnosis of those patients with FLT3-ITD was significantly lower than that in patients without this mutation, which is expected since FLT3-ITD mutation was detected in M3 & M3v which usually present with lower WBC count particularly M3 subtype compared to other subtypes. (20) Furthermore, the mean blast cells percent in peripheral blood and bone marrow in patients with FLT3-ITD mutation was non significantly higher than in patients without this mutation which was in agreement to many studies. (8,11) Piacibello W. et al had found that FLT3 expression may play a role in the survival or proliferation of leukaemic blasts, and that FL (FLT3 Ligand) may induce dose-dependent proliferation of leukaemic blasts. (21) Those patients with FLT3-ITD had lower remission rate 50% as compared to non mutated cases (83%), this result was confirmed by other studies. (8,11)

Conclusions:

The frequency of FLT3-ITD mutation in pediatric AML in this Iraqi teaching hospital was comparable to the incidence worldwide. This mutation was found in high risk patients male, older age group who had high peripheral blood & bone marrow blast percent and did not respond well to induction therapy. Thus, further studies should be applied to evaluate the role of FLT3 inhibitors in the induction therapy for those patients.

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