Original Article

Detection of FLT3-ITDMutation in Twenty ChildwithAcuteMyeloidLeukemia in One Iraqi Teaching Hospital

Ethar k. Dhahir* **MBChB, PhD. Hematology.**
Subh S. Al-Mudallel** **MBChB, FICS Hematology.**
Maysaa A. Dhahi*** **BS, PhD Microbiology.**

Summary:

Background: Pediatric acute myeloid leukemia (AML) has a poor prognosis, and novel therapies are needed. The FLT3 tyrosine kinase inhibitorsrepresents 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 patientswere males, (P> 0.05).The FLT3-ITD mutation showed no correlation to clinical presentation. The peripheral blood & bone marrow blast cell percent werenon significantly higherin mutated patients as compared to non mutated patients. Regardingits 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 inductiontherapy as compared to non mutated patients.

Conclusions: This is a noval 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 thosechildren , 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 duplicationmutation,polymerase chain reaction,prognosis.

J Fac Med Baghdad
2012; Vol. 54, No.2
Received Oct.2011
Accepted May 2012

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 tyrosinekinase expressed in early hematopoietic progenitors that plays animportant role in hematopoietic development.(2) Interaction with its ligand (FL) results in receptordimerization, autophosphorylation and the subsequentphosphorylation of cytoplasmic substrates that are involvedin signalling pathways regulating the proliferation ofpluripotent stem cells, early progenitor cells and immaturelymphocytes.(3) FLT3 is constitutively activated either by an internal tandem duplication (ITD) or by a point mutation (PM).(4)Mutations in FLT3 in AMLoccur in approximately 25-35% inadults, (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 FLT3mutation in AML may indicate a need to reassess and modify standard treatmentoptions.(4)Furthermore, the majority of retrospective data indicate that FLT3 mutations are an independent variable that confer a poor prognosis in AML particularly in pediatricAML.(8)

*Al-Karama Teaching Hospital/Wasit.
** Department of Pathology and Forensic Medicine, College of Medicine, Al-NahrainUnversity.
*** Department of Microbiology College of Medicine, Al-NahrainUnversity.

Patients, Materials and Methods:

This prospective study was conducted on 36 subjects including 20childrenwith 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 18out of 20 patients were newly diagnosed, whereas 2 patients were in relapse. All the

children were collected fromThe Children Welfare Teaching Hospital in Baghdad,and they were referred from different governorates of Iraq.From each patient & control subject peripheral blood sampleswere 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^{o}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 FT3- 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 11using 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 mMMgCl, 50 mMKCl, 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 12 R: 5>-CAAACTCTAAATTTTCTCT-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^{o}C for denaturation,45 sec at 50^{o}C for annealing , 1 minute at 72^{o}Cfor extention and 1 cycle of7 minutes at 72 ^{o}C for the final extention. (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 ethedium bromide(Promega) .Follow up of patients:The initial response to induction chemotherapy was assessed in each patientwhether 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±SDwhereas nominal data were expressed as frequency. Analysis of numeric variables was done using studentt-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 with16 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 ratio2.2:1, (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 Female	11 5	10 10	0.257 Chi-Square

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,(Figure1).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 x10^{9}/L)which was significantly lower than non mutated patients (42.32±53.80 x10^{9}/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 x10^{9}/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 –veN(18)	FLT3-ITD+ve N(2)	N	%	P-value
Gender Male Female	 8 10	 2 0	 10 10	 50 50	0.136
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 X109/L Mean±SD	42.32±53.804	11.55±10.394	0.046*
Platelet count X109/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
Periphral 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)

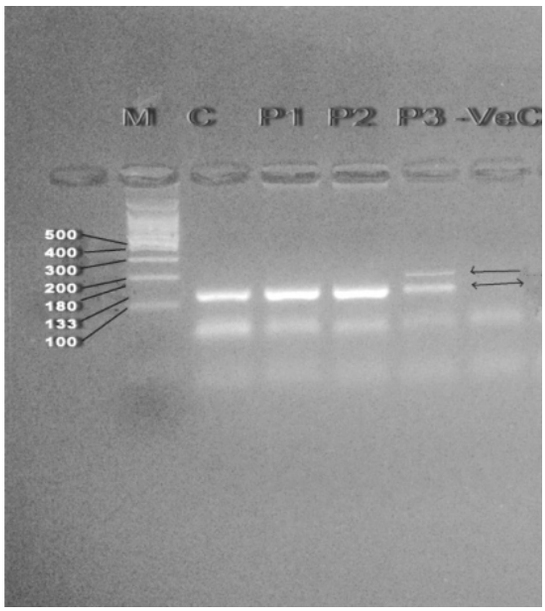

Figure (1): Detection of FLT3-ITD mutation using PCR. Lane C: Amplified product from healthy control .Lanes P1, P2: amplified product from patients wild type (app.133bp ,double head arrow).Lane P3: amplified products from patients show extra mutated band (app180 bp arrow) of FLT3-ITD. Lane –VeC: negative 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 ,andtheirrateswere higher than that found in nearby countries, in the European Union or in the United States.(13) In Iraq leukemiaranks 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 withmyeloid leukemiafall within the age group5-14 years .In this study, themale to female ratio was 1:1which was similar to that reported by the Iraqi ministry of health last statisticsin 2009.(15) FLT3-ITD mutation was detected in 2out 20 pediatric AML patients (10%), this result was in agreement withmany studies (6,7,8), and wasin line withZakeret 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 werenonsignificantly 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 pediatricAML, 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 was detected in M3(M3&M3v) subtype patients, this was in agreement to other studies.(6,11,16,17,18)On the other handSoheilet al., study(8) reported that FAB- M1 had the highest frequency among other subtypes and this difference may be explained by contrarietyin 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 Kiyo 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 &M3vwhich 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 Iraqiteaching 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.

References:

1. Margaret L G, Thomas CS,and Yuri F:Genetic Tests To Evaluate Prognosis and Predict Therapeutic Response in Acute Myeloid Leukemia. Journal of Molecular Diagnostics 2010 Jan;12(1): 3-16.

2. Armstrong S, Mabon M, Silverman L, et al. FLT3 mutations in childhood acute lymphoblastic leukemia. Blood 2004, May;103(9):3544-3546.

3.Carow CE, Levenstein M, Kaufmann SH, et al. Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. Blood1996;87:1089-1096.

4. Donald S:FLT3 Mutations: Biology and Treatment. American Society of Hematology 2006:178-184.

5.Brown P, Soheil M,Mark L, et al. Pediatric AML primary samples with FLT3/ITD mutations are preferentially killed by FLT3 inhibition.Blood2004 Sep 15;104 (6):1841-9 151

6. Chang P, Kang M, Xiao A, et al. FLT3 mutation incidence and timing of origin in a population case series of pediatric leukemia .BMC Cancer 2010; 10:513

7.Stirewalt DL, Radich JP: The role of FLT3 in haematopoieticmalignancies.Nat Rev Cancer 2003; 3(9):650-665.

8.Soheil M, Alonzo T, Stirewalt D,Zwaan M, et al.Clinical implications of FLT3 mutations in pediatric AML. Blood 2006, Aug; 15:3654-3661.

9.Lewis SM andBarbara B J :Classification of hematological neoplasms. In: Dacie and Lewis Practical Haematology 2006, 10th ed. ch.23:618.

[My paper]10.PromegaNotes Number 68, 1998 : 20. Incited by: Promega website:www.promega.com.

11.Yassin D and SidhomI .Internal Tandem Duplication of FLT3 Gene in Egyptian Pediatric Acute Myeloid Leukemia and Acute Lymphoblastic Leukemia.The Journal of Egyptian Nat.Cancer Inst.2003March ;15(1):17-23

12.Cheson BD, Bennett JM, Kopecky KJ, Buchner T, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria,Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J ClinOncol 2003;21:4642-9.

13. Amy H, Riyadh L ,Jenan H, Scott D, et al. Trends in Childhood Leukemia in Basrah, Iraq, 1993–2007.Am J Public Health. Published online ahead of print February 18, 2010: e1–e7.

14. Reports of The Iraqi cancer registry 2008: The Commonest Ten Cancers by Site / IRAQ / 2008.

15. Annual reports of The Iraqi Ministry of Health 2009.

16.ZakerF,SheykhiM,Mohammadi M, et al:Diagnosis and frequency of FLT3 mutations in pediatric and adult Acute leukemic patients with different subtypes.International Journal of Hematology-Oncology and Stem Cell Reseach(IJHOSCR) 2009; 3(4):27.

17.Kondo M, Horibe K, Takahashi Y, et al: Prognostic value of internal tandem duplication of the FLT3 gene in childhood acute myelogenousleukemia.Med.Ped.Oncol.1999 ;33:525-529.

18.Liang D,Shih L,Hung I,et al :Clinical relevance of internal tandem duplication of the FLT3 gene in childhood acute myeloid leukemia.Cancer 2002, 15;94(12): 3292-8.

19.Kiyoi H, Nakaoe T,Nakano Y, et al: prognostic implication of FLT3 and N-RAS gene mutation in Acute myeloid leukemia. Blood 1999;93:3074-3080.

20. McKenna RW, Parkin J, Bloomfield CD, et al. Acute promyelocyticleukaemia: a study of 39 cases with identification of a hyperbasophilicmicrogranular variant. Br J Haematol 1982;50:201–214.

21. Piacibello W, Fubini L, Sanavio F, et al. Effects of human FLT3 ligand on myeloid leukemia cell growth: heterogeneity in response and synergy with other hematopoietic growth factors. Blood1995;86:4105-4114.