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The Frequency of FLT3 Mutation in Fifty Five Iraqi Adult Patients with Acute Myeloid Leukemia

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Abstract

Background Mutations within the *FLT*3 gene, which code for the class-III-receptor kinase FLT3, ranked within the most frequent recurrent known genetic markers in acute myelocytic leukemia (AML). Internal tandem duplication (ITD) mutations in the juxtamembrane domain of FLT3 gene occur in 20-25% of AML.

Objectives This study designed to detect the frequency of FLT3-ITD mutation in adult AML patients, and to correlate the prevalence of this mutation with the clinical presentation of the patients and their response to induction therapy.

Methods The study comprised 55 AML patients and 33 healthy controls. For each patient, complete blood picture, blood film, bone marrow aspiration and biopsy was done. FLT3-ITD mutation was detected by conventional Polymerase Chain Reaction technology. Complete hematological remission achievement after induction chemotherapy was assessed by clinical examination and full laboratory investigations.

Results Out of 55 AML patients 8 (14.54%) had FLT3-ITD mutation and all of them presented as *de novo* AML. Moreover, 6 (75%) out of 8 mutated patients were newly diagnosed whereas 2 out of 8 were in relapse and were not on any therapy. The mean age of patients who had the mutation was lower than those without the mutation; also the majority of patients with mutation were male. The mean WBC count in mutated patients was not significantly higher than non-mutated patients. Higher bone marrow blast cell percent was found in mutated patients. FLT3-ITD mutation was mostly detected in M3 (37.5%) followed by M2 (25%), and lastly in M1 and M4 subtypes (12.5% for both subtypes) of FAB classification. Four out of 8 mutated patients failed to response to induction therapy although they were with good compliance to drug and 1/8 died throughout the induction therapy.

Conclusion Since FLT3-ITD mutation was associated with higher WBC count, significantly higher bone marrow blast cell percent and low rate of response to induction therapy; therefore it had been considered one of poor prognostic factor. It is a factor in defining risk stratification of AML patients.

Keywords AML, FLT3-ITD mutation, conventional PCR, FAB sub-types

Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous disease with accumulation of acquired genetic alterations in hematopoietic progenitor cells that disturb normal mechanisms of cell growth, proliferation and differentiation [(1)]. Its heterogeneity results from a complex network of cytogenetic aberrations and molecular mutations [(2)].

Limited prognostic and predictive ability of traditional morphological, immunophenotypic, and cytogenetic tests has driven research to define more subtle nucleotide-level alterations that not only shed light on pathogenesis but also serve as tumor markers and, in some

cases, impart valuable prognostic information. As a consequence, genetic characterization of all AML patients at presentation is nowadays regarded as mandatory to determine treatment choices [3].

About half of all adult patients with acute myelogenous leukemia (AML) lack any detectable cytogenetic abnormalities and thus display a normal Karyotype [4], and those patients are considered as an intermediate risk group and their clinical outcome is quite variable. So, additional markers with prognostic importance are required in order to detect clinically relevant subgroups in AML patients with normal karyotype [5].

FLT3 is a cell surface tyrosine kinase receptor with important roles in hematopoietic stem/progenitor cell survival and proliferation[6]. The human FLT3 gene is over 1,000 kilobases in length and is composed of 24 exons located on chromosome 13 (13q12) [7].

It is one of the most mutated genes in leukemia, and the internal tandem duplication (FLT3-ITD) which occur in the juxtamembrane domain-coding sequence of the FLT3 gene, is found in approximately 20-25% of cases of adult AML [8]. Additionally, point mutations which may occur in codons 835 and 836 in the tyrosine kinase domain (TKD mutations) can be detected in 7% of AML patients [9]. Clinical and experimental evidence both indicate that FLT3 is a proto-oncogene with the capacity to enhance survival and proliferation of leukemia blast cells [10].

FLT3-ITD has been identified in all FAB subtypes, with the highest frequency in the M3 subtype [11].

Studies had demonstrated very high levels of FLT3 mRNA and protein in adult and pediatric AML patients, without FLT3 mutations, and this over expression may have an unfavorable prognostic impact on overall survival [12].

FLT3 ITDs regarded as an adverse prognostic factor in AML, and because of its importance, inhibitors of FLT3 signaling have been developed and are in clinical trials in AML [13]. The mutant to wild-type allelic ratio AR (FLT3/AR) often increased at relapse and a retrospective analysis of minimal residual disease (MRD) status for AML patients indicated an increased tendency for relapse in those with FLT3-ITD positive [14].

Methods

This prospective study was conducted on 88 subjects including 55adult patients with AML and 33 healthy controls, who were selected randomly in relation to age and sex. Fifty patients were diagnosed as *de novo* AML and 5 patients were presented as secondary AML after other hematological diseases. Thirty six patients were newly diagnosed, and 19 patients were relapse. Forty nine out of them attending Baghdad Teaching hospital, and other patients were taken from Al-Kadhimiya Teaching hospital and Al-Yarmook teaching hospital. From each patient and control subject 2 ml peripheral blood sample divided in 2 EDTA tubes was collected one for analysis of hematological parameters by automated coulter and the other for DNA analysis in the Microbiology Department, Al-Nahrain Medical College. The Tubes were kept in deep freeze (-70^{o}C) until the day of analysis. Peripheral blood and bone marrow aspirate smears of the patients were examined by two hematology consultants for diagnosis of AML and their sub classification according to FAB classification.

Detection of FT3- ITD Mutation

High molecular weight DNA was extracted according to the kit protocol (Promega) following instruction manual [15]. All samples were analyzed for FLT3 mutation in exon 11using PCR method. 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 [16].

Fifty to 100 nanogram of DNA(5 µl)was amplified in a 50 µl reaction mixture containing 1.5 mM MgCl, 50 mM KCl, 200 µM each deoxy ribonucleotide triphosphate (dNTP), 2.5 units Taq polymerase, 40 picomol of each primer 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}C for extension and 1 cycle of 7 minutes at 72^{o}C for the final extension. [16]

Twenty µl of the PCR product was electrophoresed on 2.5% agarose gel (Promega), using 100bp DNA ladder (Promega) as molecular weight marker and stained with ethedium bromide (Promega).

Follow up of patients: Patients had received induction chemotherapy consisting of doxorubicine (Adriamycin) 25 mg/m2 I.V. for 3 days and cytosine arabinoside (Ara-C); 100mg/m2 I.V. infusion over 24 h for 7 days, (The 3 and 7 protocols).

The initial response to induction chemotherapy was assessed in each patient whether there is complete hematological remission (CR), treatment failure, or early death. Complete remission was defined as apparent recovery of hematopoiesis with< 5% blast cells on aspirate and near normal peripheral blood counts(hemoglobin >10.0g/dl; neutrophil counts>1.5 x10^{9}/l) [17].

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 one way ANOVA or t-test, whereas analysis of nominal data was done using Chi-squire. P-value < 0.05 was considered significant.

Results

This study was conducted on 55 AML patients along with 33 healthy control subjects who were cases age and sex matched (p value > 0.05). Most of the patients enrolled in this study were males (54.5%), with a male to female ratio 1.2:1 (Table 1).

Table 1. Characteristics of persons involved in the study

Characteristics		Control	AML Patients	P-value
Age/Year		37.03±9.78	39.81±19.24	1.000
Gender	Male	23	30	0.160
	Female	10	25	
Total		33	55	

In the current study by using conventional PCR, the amplified DNA product of the wild type from the patients and healthy control was about 133 bp band while the mutated type showed additional band > 133bp, (Figure 1). The presence of any PCR fragment longer than the wild-type allele was considered positive for FLT3-ITD.

FLT3-ITD mutations were found in 8 patients (14.54%). All the eight mutated cases were *de novo*-AML cases and no mutation was detected in the five secondary AML cases, (p-value 0.501). Furthermore 6 out of 36 newly diagnosed cases (16%) were mutated whereas 2(10.5%) of the relapsed cases had FLT3-ITD mutation, (p value 0.333) as shown in table 2.

Patients with *FLT3-ITD* mutation were younger than non mutated patients, (31.12±14.06; 41.29±19.73, respectively), but of no significance (p-value 0.169), (Table 2).

Out of 55 patients 54.5% were males and higher percentage of them had the mutation, (p-value 0.209).

Regarding the distribution of FLT3-ITD mutation within the FAB subtypes; the mutation was higher in patients with M3

followed by M2, and lastly by M1 and M4, (p value 0.169), (Table 2). Moreover, most of the patients presented with fever 90.9% followed by pallor 70.5% with no specific relation to FLT3-ITD mutation, (p value 0.386), (Table 2).

Table 2. FLT3-ITD Mutation relation to clinical presentation

Clinical Presentation		FLT3-ITD -ve	FLT3-ITD +ve	Total	%	P-value
Type of AML	*De novo*	42	8	50	89	0. 501
	Secondary	5	0	5	11	
Gender	Male	24	6	30	54.5	0.209
	Female	23	2	25	45.5	
Age/Year		41.29±19.73	31.12±14.06	55	100	0.169
FAB subtype	M1	21	1	22 (40%)	12.5	0.153
	M2	12	2	14 (25.45)	25	-
	M3	3	3	6 (10.9%)	37.5	-
	M3v	2	1	3 (5.5%)		
	M4	5	1	6 (10.9%)	12.5	-
	M5	3	0	3 (5. 5%)	0	-
	M6	1	0	1 (1.8%)	0	-
Lymphadenopathy		16	4	20	36.4	0.386
Splenomegaly		31	4	35	63.6	0.386
Hepatomegaly		22	4	26	47	0.867
Pallor		43	7	50	90.9	0.717
Fever		34	7	41	74.5	0.363
Weight loss		11	1	12	21.8	0.490
Total		47	8	55	100	-

Regarding the relation of FLT3-ITD mutation to hematological parameters of the patients enrolled in the study, the mean WBC count in mutated patients was 58.44±50.29 $X10^9$/L which was higher than non mutated patients 38.24±31.24, (p value 0.703), whereas platelet count and hematocrit were lower in patients with mutation, (56.75±59.27; 24.62±5.78, respectively) than in patients without mutation (62.70±44.24; 26.80±5.60, respectively), (P-value 0.316), (Table 3).

Table 3. FLT3-ITD mutation and hematological parameters of patients with AML

Hematological Indices	FLT3-ITD -	FLT3-ITD +	P-value
WBC count $X10^9$/L	38.24±31.24	58.44±50.29	0.303
Platelet count $X10^9$/L	62.70±44.24	56.75±59.27	0.703
Hematocrit %	26.80±5.60	24.62±5.78	0.316
Periphral blood blast %	63.22±27.35	74.75±17.46	0.257
Bone marrow blast %	70.11±24.25	86.12±8.21	**0.003***
Total	47	8	----

Table 3 show that the mean peripheral blood blast cell percent (74.75±17.46; 63.22±27.35, respectively) was higher in FLT3-ITD positive cases than FLT3-ITD negative cases, but it did

not reach level of significance, (P value 0.257), (Table 3). The mean bone marrow blast cells percent in mutated patients (86.12±8.21) was significantly higher than non mutated patients, (70.11±24.25) (P-value 0.003), (Table 3).

Furthermore, 4 out 8 mutated cases showed failure of response to induction therapy, however; this correlation was insignificant, (p-value 0.53), (Table 4).

Table 4. FLT3-ITD and the response to induction therapy

Response to induction therapy	FLT-ITD -ve	FLT-ITD +ve	N	%	P-value
Remission	19	3	22	40	0.53
Failure	26	4	30	54.5	-
Death	2	1	3	5.5	-
Total	47	8	55	100	-

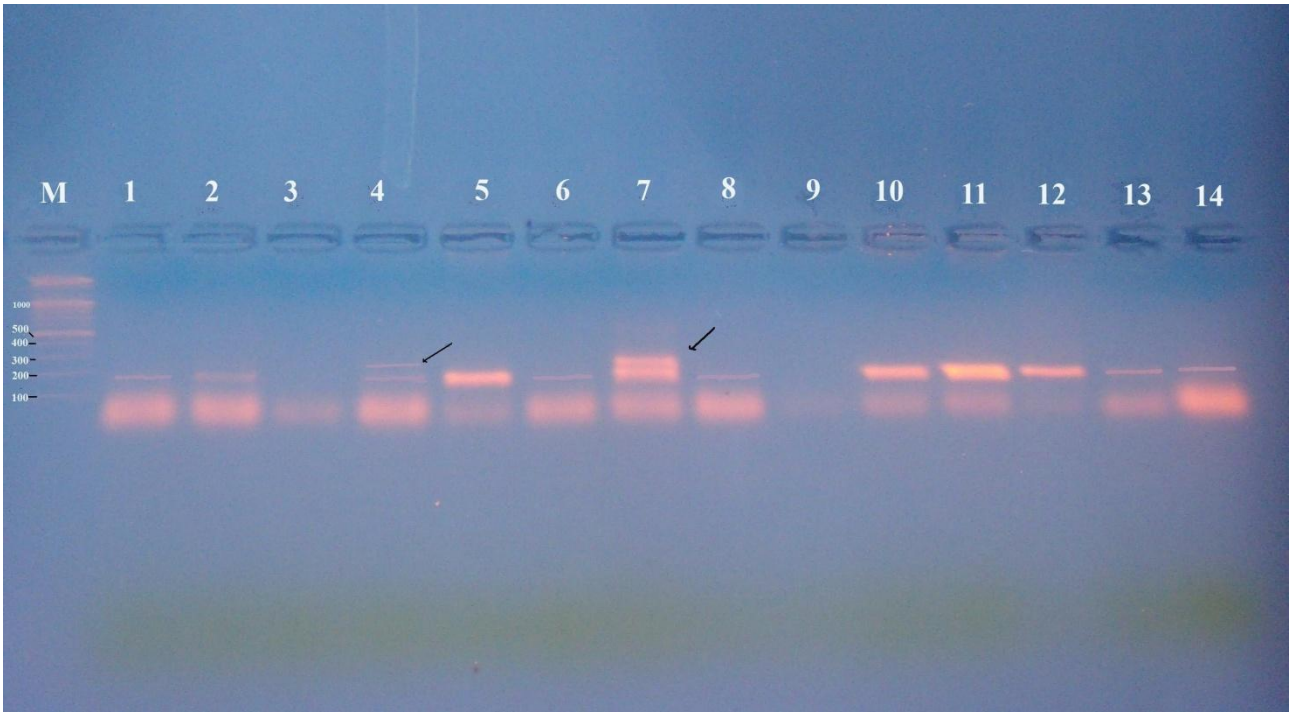

Figure 1. Detection of FLT3-ITD mutation using PCR in adult AML patients. Lane 1: Amplified product from healthy control. Lanes 2,5,6,8,10-14 amplified product from patients wild type (about 133 bp). Lanes 4 and 7 amplified products from patients show extra mutated band (> 133bp arrows) of FLT3-ITD. Lane 9 negative control (no template). M: Molecular weight marker. Electrophoresis was carried in 2.5% agarose gel at (4V/cm) for 60 min.

Discussion

Acute myeloid leukemia in general has a poor prognosis [18]. It is infrequent, yet highly malignant neoplasm responsible for a large number of cancer-related deaths. In Iraq leukemia ranks the 4th cancer among the commonest ten cancers according to Iraqi Cancer Registry 2005. It constitutes 6.4% of all cancers with an annual incidence of 3.34 per 100000 populations. [19] In this study 54.5% of the patients were males with a male to female ratio1.2:1 which was in accordance with the last statistic reported by the Iraqi ministry of health 2010 [20] and other Iraqi study [21].

In this study FLT3 gene mutation was detected in 8 (14.54%) of AML cases, which was lower

than Schnittger et al study [22] which had reported that the incidence of the mutation was 18% and this may be explained by the difference in PCR circumstances and difference in the primer set which was applied.This result was consistent with a study done by Emami et al [23] and Vahid et al [24] who reported that FLT3-ITD frequency was 16%. Also Gari et al [25] had reported that the incidence of the mutation was 11.6%.

Since the incidence of FLT3-ITD in adult patients with AML in different studies done in Arab countries and Iran was lower than that discovered in other parts of the world [26-28], thus we may propose that this difference may be due to ethnic and geographical differences.

Furthermore the present study found that FLT3-ITD mutation in females was lower than in males and this was in agreement with other studies applied [23]. On the other hand Gari et al [25] had reported higher frequency of the mutation in females; this difference may be due to larger sample size, and different method of screening, using conformation sensitive gel electrophoresis.

This study showed that patients with FLT3-ITD mutation were non significantly younger than patients without mutation, this result was in agreement to other studies.[22] Also, the mean WBC count at the time of diagnosis of those patients with FLT3-ITD was non significantly higher than that in patients without this mutation ,which may be due to that FLT3-ITD mutation can cause constitutive activation of the receptor tyrosine kinase leading to autonomous cytokine independent cellular proliferation, leading to leukocytosis [29]. This result was consistent with the result of other studies [21,30]. Moreover, the mean blast cells percent in bone marrow in patients with FLT3-ITD mutation was significantly higher than in patients without this mutation which was similarly found by Thiede et al [31]. FLT3 expression may play a role in the survival or proliferation of leukemic blasts, and FL (FLT3 Ligand) induced dose-dependent proliferation of leukemic blasts [32].

FLT3-ITD mutation was not exclusively correlated with any certain FAB sub-type, however it occur mostly in M3 sub-type, which was similarly reported by other studies [33,34]. Similar to Kiyo et al [30], the current study found that hepatosplenomegaly or lymphadeno-pathy, pallor; fever and weight loss was not affected by the presence of this mutation.

The present study found that FLT3-ITD mutation was detected only in *de novo* AML cases and not in secondary AML cases, this was in concordance with Hayakawa et al [34] study which stated that there was higher frequency of this mutation in de novo AML cases as compared to secondary and therapy related AML. Also higher frequency of mutation was found in newly diagnosed cases as compared to relapsed cases, which may be explained by the fact that those patients with relapse had received chemotherapy which may have altered over all pathophysiology. Similarly other study found that the mutation which was detected at the time of diagnosis had disappeared on relapse [35]. Also this study showed that FLT3-ITD mutation had no influence on the remission rate in mutated cases as compared to non mutated cases. This result was assisted by Thiede et al who had reported that FLT3-ITD mutation had only inferior disease free survival in AML patients with this mutation [31].

Conclusion

FLT3-ITD mutation was detected in 14.54% of AML patients and since it was associated with higher WBC count, significantly higher bone marrow blast cell percent and low rate of response to induction therapy, therefore; it had been considered one of poor prognostic factor. It is a factor in defining risk stratification of AML patients.

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