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Frequency of *FLT3/ITD* Mutations in Pakistani Acute Myeloid Leukemia Patients

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Abstract.- Acute myeloid leukemia (AML) is characterized by an increase in the number of myeloid cells in the marrow and an arrest in their maturation. *FLT3* gene, a member of the class III receptor tyrosine kinase family, plays an important role in stem cell survival, and the development of dendritic and natural killer cells. Internal tandem duplication (ITD) mutations result from the duplication and tandem insertion of a portion of the juxtamembrane region (exons 14 to 15) of the *FLT3* wild-type gene and occur in 20 to 30% of patients. These mutations are associated with worse prognosis. The aim of this project was to study the spectrum of these mutations in Pakistani AML patients and correlate them with clinical findings. Blood samples of 100 AML patients from different hospitals of Lahore were included in this study. For screening of mutations, a 329 base pair fragment covering *FLT3* exons 14 and 15 of known wild type and mutant-positive samples were PCR amplified. FLT3/ITD mutations were found in 17% AML patients. These mutations did not correlate significantly with age or sex, though the incidence was higher in female patients. These mutations were significantly associated with high WBC count and high blast percentage. The overall incidence of FLT3/ITD mutations was lower in Pakistani patients but was within the globally reported ranges. Like the developed countries of the world, AML patients in Pakistan should also be screened for the presence of these mutations so that proper treatment strategies could be adopted.

Keywords: Acute myeloid leukemia, FMS tyrosine kinase 3, FLT3/ITD mutations,

INTRODUCTION

Acute myeloid leukemia (AML) is type of blood cancer which is characterized by a rapid increase in the number of immature myeloid cells in peripheral blood and bone (Löwenberg *et al.,* 1999). This over production of myeloid blasts may decrease the overall efficiency of the haematopoietic system resulting in anemia, with or without leukocytosis. A number of factors may be responsible for developing AML, including other blood diseases, chemicals, radiation, and inheritance.

During normal haematopoiesis, a myeloblast gradually matures into a functional white blood cell. In AML, however, a myeloblast stops at its immature state and does not differentiate. This happens because of the accumulated genetic changes in the leukemic cells. In 40 to 50% of AML patients, standard karyotyping techniques cannot detect any genetic changes (Grimwade *et al.,* 2001; Farag *et al.,* 2006). In recent years, many specific gene mutations have been detected in AML patients (Döhner and Döhner, 2008).

FMS-like tyrosine kinase 3 (FLT3) is a member of class III tyrosine kinase (TK) receptors, which comprises five immunoglobulin-like loops in the extracellular region, a transmembrane region, an intracellular juxta-membrane (JM) domain, two tyrosine kinase (TK) domains interrupted by a kinase insert and a C-terminal tail (Agnes *et al.,* 1994). The gene has an important role in the proliferation and differentiation of primitive hemopoietic progenitor cells (Lyman, 1995). FLT3 is located at chromosome 13q12 and consists of 24 exons (Rosnet *et al.,* 1991; Abu-Duhier *et al.,* 2001).

The internal tandem duplication (ITD) of the *FLT3* gene, at present, is the most significant prognosis determining factor in normal karyotype AML patients. The *FLT3/ITD* mutations result from the duplication and tandem insertion of a portion of the juxtamembrane (JM) region. The duplicated region is always present between exons 14 and 15 of the *FLT3* gene (Nakao *et al.,* 1996). The FLT3/ITD mutations are always in frame and produce functional FLT3 protein. FLT3/ITDs are one of the

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most frequent mutations reported in AML (Gale, 2003)), found in 13.2 to 32% of adult AML patients (Rosnet *et al.,* 1991). FLT3/ITD mutations are known to have a worse prognosis (Stirewalt *et al.,* 2006). We studied FLT3/ITD mutations in 100 AML patients from different hospitals of Lahore, Pakistan and correlated them with clinical and laboratory findings.

PATIENTS AND METHODS

Subjects

Blood samples from 100 Pakistani AML patients were included in this study. Written informed consents were taken from all patients. The AML patients were selected according to standard hematological and clinical parameters with the help of consultant hematologists from different hospitals of Lahore *viz.,* Mayo Hospital, INMOL Hospital and Shaukat Khanum Memorial Cancer Hospital from January 2006 to February 2009. Clinical and laboratory findings were obtained on a prescribed form from the hospitals.

Genomic PCR assay for FLT3/ITD

For screening of mutations, a 329 base pair fragment covering FLT3 exons 14 and 15 was amplified by polymerase chain reaction (PCR). DNA was isolated using guanidine - thiocyanate/silica gel powder method (Melferrari *et al.,* 2002), and was diluted to a 30ng working concentration with distilled water. The PCR mixture (20 µl) contained 1 X PCR buffer (Fermentas), 1.0mM $MgCl_2$, 0.2mM dNTPs, 0.5µM each of the following primers, 0.5 units Taq polymerase (Fermentas) and 30ng DNA.

Forward (11F) 5'-GCAATTTAGGTATGAAAGCCAGC-3' and
Reverse (12R) 5'-CTTTCAGCATTTTGACGGCAACC-3' primers,

Thermal cycling conditions for PCR were: initial denaturation at 95°C for 2 min followed by 35 cycles, each of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and then a final extension at 72°C for 5 min. A known wild type and mutant-positive sample were also amplified along with the unknown samples. The PCR products were run on 2% agarose gel.

Statistical analysis

For the calculation of P values, Chi square test was performed on online software SISA, while t test was performed using Microsoft Excel.

RESULTS

The cohort

A total of 100 blood samples collected from AML patients (9-68 years of age) from different hospitals of Lahore, Pakistan were included in this study. Out of these 100 patients, 56 were males and 44 were females. Median values and range of age, WBC count, platelet count and % blasts for males and females along with FAB (French-American-British) types are shown in Table I. There was no difference in the median values for WBC count (P = .89), platelet count (P = .26) and % blasts (P = .32) of the male and female patients. Median age of male patients was significantly higher than that of female patients (P = .008). There was no patient with either M0 or M7 FAB type. FAB type M1 had the maximum number of patients followed by M2 and M4. Relatively smaller number of patients was seen in M3, M5 and M6. Percentages of FLT3/ITD was higher in female than in male patients but this was not statistically significant (P = .18).

The mutations

Wild type samples produced a single PCR band at 329bp. Any sample having an additional band of larger size was considered as mutant. A representative gel is shown in Figure 1.

Out of 100 AML patients, 17 had one or more FLT3/ITD mutations. The size of the duplicated region varied from 15bp to 93bp (Table II). Out of the 17 FLT3/ITD positive patients, 16 (94%) had a single mutation and 1 (6%) had two mutations. No patient had more than two mutations (Table II).

Median age of FLT3/ITD wild type and mutant-positive patients was 37 and 31years, respectively. Three age groups were made as are shown in Table III. Although the percentage of FLT3/ITD mutants was higher in first age group (up to 25 years) but this was not statistically significant (P = .50). The number and percentage of FLT3/ITD wild type and mutant-positive patients in each FAB type were calculated. FLT3/ITD mutations were not

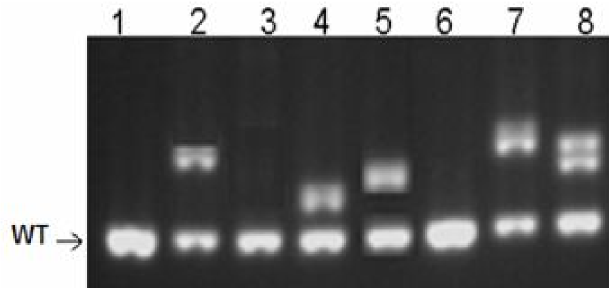

Fig. 1. A representative agarose gel showing wild-type and FLT3/ITD mutant bands. Lanes 1 and 2 contain the negative and positive controls, respectively. Lanes 3-8 show PCR products from six different AML patients. Lanes 3 and 6 show only wild-type bands, while lanes 4, 5, 7 and 8 show wild-type plus mutant bands. Lane 8 has two mutant bands which predict the possible presence of two duplication mutations in this sample.

Table I.- Age and haematological characteristics (medians and ranges) of patients, diagnosed for AML and included in the present study.

Haematological parameters	Total (n=100)	Male (n=56)	Female (n=44)
Age (Years)	36 (9-68)	38.5 (16-68)	30 (9-62)
WBC count (x10^9 /L)	30 (1.2-196)	26 (0.8-193)	32 (1.2-196)
Platelet count (x10^9 /L)	64 (7-322)	55 (7-231)	72 (12-322)
Blasts (%)	48 (15-98)	48 (15-98)	43.5 (18-91)
FAB Types*			
M0 (acute myeloblastic leukemia – minimally differentiated)	0	0	0
M1(acute myeloblastic leukemia – without maturation)	33	20	13
M2 (acute myeloblastic leukemia – with granulocytic maturation)	26	13	13
M3 (Acute promyelocytic leukemia)	4	2	2
M4 (Acute myelomonocytic leukemia)	13	7	6
M5 (Acute monocytic/ monoblastic leukemia)	9	3	6
M6 (Acute erythroleukemia)	5	3	2
M7 (Acute megakaryoblastic leukemia)	0	0	0
Unknown	10	8	2

*The reported incidence (%) for various morphological categories is as follows: M0, 1-3; M1, 15-20; M2, 25-30; M3, 5-10; M4, 25-30; M5, 2-10, M6, 3-5; M7, 3-12.
Normal values for WBC count, 4.5-10.5 x 10^9 per litre, Platelet count, 150-400 x 10^9 per litre; Blasts, <5%.

found to be significantly associated with any of the FAB types (Table III). WBC count, platelet count and %blasts were arranged in different groups. Median WBC count (x10^9/L) in FLT3/ITD wild type and mutant-positive patients was 24 and 57, respectively. A significantly higher incidence of FLT3/ITD mutations was seen in patients with high WBC count (*P* = .02). Median platelet counts (x10^9/L) of FLT3/ITD wild type and mutant-positive patients were 62 and 82, respectively. No significant association of the mutations with platelet count was seen in these samples (*P* = .30). Median values of percent blasts of FLT3/ITD wild type and mutant-positive patients were 43 and 68, respectively. The mutations were found to be significantly associated with high blast percentage (*P* = .03) (Table III).

DISCUSSION

Many adult AML cases are characterized by the presence of a number of chromosomal abnormalities and in many instances these abnormalities closely correlate with specific clinical characteristics. The karyotype analysis for diagnosis, therefore, provides a basic framework for deciding treatment strategies. However, approximately 40–50% of the adult AML patients do not have such chromosome aberrations and are classified as an intermediate risk group. Such patients lack markers that can be used for classification and prognostic assessment. Even within this group, a spectrum of responses to treatment and outcomes exist. It has now been widely studied that numerous genetic alterations exist within these cytogenetically normal AML patients and that these molecular abnormalities are associated with prognostic significance. Recently, it has been shown that FLT3-ITD mutations have bad while NPM1 mutations have good prognostic effect. Therefore, mutation screening, along with clinical, morphological, immunophenotypic and cytogenetic features, are of great importance in determining the optimal therapy for AML patients. In addition, new treatment strategies like FLT3 inhibitors are under trial and will soon be available for therapeutic use. It is therefore important to determine the FLT3 mutation status in all new NK-AML patients.

Table II.- FLT3/ITD mutants with their relevant duplicated size.

S.N.	Patient identity	Sex	Age	WBC count ($X10^9$/l)	Platelet count ($X10^9$/l)	% blasts	FAB	Size of insert (bp)
1	1002	M	22	2	24	21	1	63
2	1010	M	22	85	53	90	2	30
3	1015	F	23	68	41	37	4	81
4	1021	F	9	49	30	56	2	15
5	1026	F	35	17	80	29	2	93
6	1029	F	50	57	121	33	4	48
7	1030	M	35	140	53	92	4	63
8	1037	F	15	146	84	73	5	15, 93
9	1039	F	31	97	97	24	2	93
10	1052	M	17	79	--	64	2	63
11	1058	F	36	124	127	61	5	21
12	1071	M	53	108	178	68	-	54
13	1079	F	42	11	214	84	1	75
14	1081	F	27	114	123	83	2	69
15	1088	F	18	92	69	91	1	78
16	1093	M	38	58	66	75	-	51
17	1096	M	49	193	108	76	5	45

FLT3/ITD mutations can also be used as molecular markers in such patients as these mutations occur at higher frequency in CN-AML.

Majority of the patients in current study were below 50 years. There could be several reasons behind it. One of the reasons could be the lack of education and medical facilities especially regarding cancer treatment in Pakistan. Older patients who already have lost the hope for life do not like to bear the pain of chemotherapy. Also they believe more in witch doctors than in medical doctors. Younger patients on the other hand, have stronger hope for life and want to get rid of the disease.

The male to female ratio in Pakistani AML patient was 1.3:1.0 (56% male, 45% female) which was in accordance with US National Cancer Institute statistics about Asian population (Miller *et al.,* 2008).

FLT3/ITD mutations were seen in 17% AML patients. In one of the Pakistani patients, an insertion rather than the duplication of 15bp was observed. In literature these mutations have been reported from 13% (Abu-Duhier *et al.,* 2000) to 38% in adult AML patients (Kuchenbauer *et al.,* 2005).

FLT3/ITD mutations are not associated with any specific age group although they occur at a very low frequency in patient younger than 10 years of age (Kondo *et al*., 1999). No statistically significant association of the mutations with different age groups was seen although the incidence was higher in younger patients.

FLT3/ITD mutations are seen in all FAB types. Although significant association of the mutation with any FAB type has not been observed, but high frequencies have been reported in M3 and M5, and low frequencies in M6 and M7 (Thiede *et al.,* 2002; Kottaridis *et al.,* 2001; Noguera *et al.,* 2002; Schnittger *et al.,* 2002). In the current study, no significant association of the mutation with any of the FAB types was observed.

Several studies have reported that FLT3/ITD mutations are associated with high WBC counts (Stirewalt *et al.,* 2006; Noguera *et al.,* 2002; Thiede *et al.,* 2002). Because FLT3/ITD has been shown to cause constitutive activation of the receptor tyrosine kinase, leading to autonomous, cytokine-independent cellular proliferation (Fenski *et al*., 2000; Rombouts *et al.,* 2001), it may be a causative factor for high WBC count. FLT3/ITD mutations were found to be associated with high WBC count in current study (P = .02). Although the incidence of the mutation was higher in patients with high platelet count, this was not significant (P = 0.3). Association of the mutation with platelet count has not been established in the literature. FLT3/ITDs are

Table III.- Distribution of mutations according to patient characteristics.

Mutation type	Total	FLT3/ITD Wild type (%)	FLT3/ITD mutant (%)	P	% FLT3/ITD mutant
Sex					
Female	44	34 (41)	10 (59)	0.18	23
Male	56	46 (49)	7 (41)		13
Age (Years)					
Upto 25	32	25 (30)	7 (41)	0.50	22
26-50	55	46 (55)	9 (53)		16
>50	13	12 (14)	1 (6)		8
FAB types					
M1	35	30 (36)	5 (47)	0.59	14
M2	25	20 (24)	5 (18)	0.64	20
M3	4	4 (5)	0 (0)	0.21	0
M4	13	10 (12)	3 (18)	0.53	23
M5	9	6 (7)	3 (12)	0.17	33
M6	5	5 (6)	0 (0)	0.16	0
Unknown	9	8 (10)	1 (6)	-	17
WBC count (x10^9/L)					
<10	32	31 (37)	1 (6)	.02	3%
11-50	26	21 (25)	5 (25)		19%
51-100	23	17 (20)	6 (35)		26%
> 100	13	8 (10	5 (29)		38%
Unknown	6	6 (7)	0		0%
Platelet count (x10^9/L					
01-50	33	30 (36)	3 (18)	0.30	9%
51-100	34	27 (33)	7 (41)		21 %
>100	26	20 (24)	6 (35)		23%
Unknown	7	6 (7)	1 (6)	-	14
Blasts (%)					
01-50	51	46 (55)	5 (29)	0.03	10%
51-100	44	32 (39)	12 (71)		27%
Unknown	5	5 (6)	0 (0)	-	0%

associated with higher blast percentage (Stirewalt *et al.,* 2006; Kottaridis *et al.,* 2001; Thiede *et al.,* 2002). Significantly high blast percentages were seen in patients harboring these mutations in the current study (*P* = .02)

The number of *FLT3*/ITDs in an individual patient may vary, and up to 5 different mutants of varying size and relative level have been reported in a proportion of patients (Kottaridis *et al.,* 2001; Thiede *et al.,* 2002; Schnittger *et al.,* 2002). Two different mutants of varying size and level were seen in one patient (1% of total cohort, 6% of total mutants). No patient had more than two mutants. There is no significant correlation between FLT3/ITD mutant number and clinical outcome (Gale *et al.,* 2008).

Approximately 80% of AML patients below 55 years of age achieve complete remission (CR) following intensive induction therapy. However, 5 years survival is only just over 40% because most patients relapse and die of their disease. It is important to identify molecular markers that can be used to detect residual disease or predict relapse at an earlier stage. Ideally a candidate marker for minimal residual disease (MRD) should frequently be present in a specific disease, it should be easy to assay and stable, *i.e.* always present at relapse. In AML, the identification of MRD has been limited

by the lack of suitable specific molecular markers and the diversity of those that do exist (Kottaridis *et al.,* 2002).

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