RESEARCH ARTICLE

Molecular Characterization of FLT3 Mutations in Acute Leukemia Patients

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Abstract

Fms-like tyrosine kinase 3 (FLT3) performs a vital role in the pathogenesis of hematopoietic malignancies. Therefore in recent times, the focus of several studies was on use of FLT3 as a prognostic marker. The present study investigated the molecular characterization and incidence of FLT3 mutations in acute leukemia patients in Pakistan. A total of 55 patients were studied, of which 25 were suffering from acute lymphoblastic leukemia (ALL) and 30 were suffering from acute myeloid leukemia (AML). The polymerase chain reaction demonstrated FLT3/ITD mutations in 1 (4%) of 25 ALL patients, a male with the L2 subtype. In AML cases the rate was 4 (13.3%) of 30, three males and one female. The AML-M4 subtype was found in three and the AML M2 subtype in the other. In the AML cases, a statistically significant (p=0.009) relationship was found between WBC (109/L) and FLT3/ITD positivity. However, no significant relationship was found with other clinical parameters (p>0.05). In acute myeloid leukemia (AML) FLT3/ITD$^+$ mutation was more prevalent in elderly patients 31-40 age groups, 21-30 and 51-60 age groups respectively. In acute lymphoblastic leukemia (ALL) statistically no significant relationship was found between clinical features and FLT3/ITD positivity (p>0.05). However, in acute lymphoblastic leukemia (ALL) FLT3/ITD$^+$ mutation was more commonly found in age groups of 21-30.

Keywords: Acute leukemia - FLT3/ITD - FAB subtypes

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Introduction

The fms-like tyrosine kinase 3 (FLT3) is primarily expressed on hematopoietic progenitor cells in the bone marrow lymph nodes and thymus and is the part of the class III receptor tyrosine kinase (Peng et al., 2008). The FLT3 governs a significant role in the pathogenesis of hematopoietic malignancies (Stirewalt and Radich, 2003). The FLT3 protein is highly expressed almost 100% in most patients with acute myeloid leukemia (AML) and in up to 50% of malignant blasts in patients with acute lymphoblastic leukemia (ALL) (Turner et al., 1996). Incidence rate of internal tandem duplication (ITD) mutations of the FLT3 gene has been described in about 20% of the patients suffering from adult AML (Abu-Duhier et al., 2000) and a lower incidence rate (5%-16.5%) has been found in patients suffering from childhood leukemia (Meshinchi et al., 2001). Recently, it was reported that FLT3 mutations were commonly present in two subtypes of acute lymphoblastic leukemia (ALL) (Taketani et al., 2004). These include infant ALL with translocations involving the mixed lineage leukemia (MLL) gene and in hyperdiploid ALL, where incidence of FLT3 mutations was 18.2% (Taketani et al., 2004) and in 21.5%-25% respectively (Armstrong et al., 2004). FLT3/ITD mutations crowd in exon 11 and 12 of the human FLT3 gene. This gene codes for the juxtamembrane domain of the FLT3 protein on chromosome 13q12. The FLT3/ITD mutation normally consists of an in frame inserted sequence analogous to the part of protein between amino acids (AA) 575 and 613 of the FLT3 protein (Kiyoi et al., 1998). Ligand independent autophosphorylation of FLT3 receptor and consequently, tyrosine kinase activation occurs in FLT3 mutations. As a result of this, the pathways that govern the differentiation, proliferation and survival are activated and the process of apoptosis has become resisted (Karabacak et al., 2010). High leukocyte count was associated with FLT3/ITD mutation in AML

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patients. The presence of FLT3 abnormality appears to be associated with an unfavorable clinical response (Kiyoi et al., 1999). The prevalence and prognostic significance of FLT3/Mut have not been well defined in childhood acute promyelocytic leukemia (APL). In this regard one of the studies provides further evidence for testing APL patients for FLT3/Mut and the potential role of FLT3 inhibitors in this disease (Kutney et al., 2012). FLT3 is frequently mutated in AML. Although number of FLT3 tyrosine kinase inhibitors have been developed but resistance that arises during the course of treatment caused by secondary mutations within the mutated gene itself has limited the potential benefit of (TKI) tyrosine kinase inhibitors. In an effort to predict secondary mutations, saturation mutagenesis studies of FLT3/ITD were carried out (Williams et al., 2012a: 2012b).

The FLT3/ITD mutation is a poor prognostic factor, which occurs frequently in AML, associated with higher incidence of early disease relapse and shorter overall survival (Govedarvic et al., 2012). Patients with acute myeloid leukemia (AML) and FLT3/ITD have poor prognosis if treated with chemotherapy only (Brunet et al., 2012). FLT3/TKD mutations are common in children with AML-M3 (Ruan et al., 2011). Some of the recent studies report that CD123 Immunohistochemical expression in AML is associated with underlying FLT3-ITD and NPM1 Mutations (Rollins-Raval et al., 2012).

So far, no data on incidence of FLT3/ITD mutation in acute leukemia patients from Pakistan has been reported. Present study is the first of its kind from this region to report the incidence of FLT3 mutations in a cohort (55) of acute leukaemia patients. In addition to the characterization of incidence of FLT3 mutation, the present study is aimed to develop a PCR based molecular assay for detection of FLT3 mutation in acute leukemia patients. This study will also lead to identification of difference in clinical features from patients with and without this mutation by statistical analysis and in determining the co-relation of WBCs count with FLT3 mutation.

Materials and Methods

Patients Samples

Fifty five patients of acute leukemia (25 ALL, 30 AML) were examined for the presence of FLT3 mutation. Samples were collected from INMOL and other hospitals from Lahore city, Pakistan. Clinical and laboratory features (age, sex, subtype, Hgb, WBCs and PLTs count) of each patient were recorded at the time of collection. Informed consent was taken from all the patients who participated in the study. The study was approved by the local ethical committee of the University of Lahore.

Laboratory Methods

For the detection of FLT3 mutation, DNA was extracted from peripheral blood samples according to alkaline lysis DNA isolation protocol (Grimberg et al., 1989).

PCR Amplification of FLT3 Gene

The PCR amplification of FLT3 gene done by using gene specific primers described previously (Kiyoi et al., 1999). Different reaction parameters (Mg^+ conc, Taq DNA polymerase conc, dNTPs and primers) for PCR assay were optimized. Optimized PCR was used to detect FLT3 mutation in acute leukemia patients. Following cyclic conditions were used for FLT3 gene amplification by PCR. Denaturation at 95^0C for 5 min, Annealing at 50^0C for 45 sec, Extension at 72^0C for 50 sec, Final extension at 72^0C for 7 min and Hold at 22^0C for 5 min.

Agarose Gel Electrophoresis

All FLT3 amplificons were subjected to Gel Electrophoresis for detection of FLT3/ITD mutation. Amplified products were visualized directly under gel documentation system (GDS) (Vilber Lourmat, France), in which the gel was directly exposed to UV light with a wavelength of 230 nm (Sambrook and Russel, 2000).

Statistical analysis

All statistical analysis were done using SPSS version 16.020 (statistical package for social sciences) software. Pearson Chi-Square test was used to assess the data of the patients and one-way ANOVA test was used. The level of significance was set to a p value <0.05.

Results

Characteristics and clinical features of acute leukemia patients

The present study investigated the molecular characterization of FLT3 mutation in acute leukemia patients. A total of 55 patients were studied, of which 25 were suffering from acute lymphoblastic leukemia (ALL). In ALL 19 were males and 6 were females in ratio 76:24 and had been following different FAB subtypes. 17 were L1 and 8 were L2 in frequency, and 30 were suffering from acute myeloid leukemia (AML). In AML 22 were males and 8 were females in ratio 73.3:26.6 and had been following different subtype, 4 from M1, 10 from M2, 4 from M3, 8 from M4, 2 from M5 and 2 from M7 were in frequency respectively, as mentioned in Table 1.

FLT3/ITD mutation status in acute leukemia patients

Both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) were classified as $FLT3/ITD^+$ and $FLT3/ITD^+$ that was based on FLT3 mutation status. The mean age of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients were 17.96±12.44 and 28.03±10.75 respectively. FLT3/ITD mutation was detected by RT-PCR using primers specific for FLT3/ITD gene as shown in Figure 1.

FLT3/ITD mutation in ALL and AML patients according to clinical features

In ALL, no statistically significant ($p>0.05$) relationship was found between clinical features and FLT3/ITD mutation. In AML patients, statistically significant ($p=0.009$) relationship was found between high WBC (10^9/L) count and FLT3/ITD mutation. No significant ($p>0.05$) relationship was found between other clinical parameters and FLT3/ITD mutation (see Table 2).

FLT3/ITD mutation status in ALL and AML patients according to FAB subtype

Of ALL patients, 19 patients were males and 6 were females while for acute myeloid leukemia (AML) patients, 22 were males and 8 were females. The FLT3/ITD mutation was found in 1 (4%) of 25 ALL patients. The patient positive for FLT3/ITD mutation was male and had L2 subtype. In acute myeloid leukemia (AML) the FLT3/ITD mutation was found in 4 (13.3%) of 30 acute myeloid leukemia (AML) patients. Of the patients positive for FLT3/ITD, three patients were males and one patient was female. AML-M4 subtype was found in three patients while one patient was AML M2 subtype as shown in Figure 2a and 2b respectively.

Table 1. Characteristics and Clinical Features of ALL and AML Patients

Characteristics	ALL n=25		AML n=30					
Male	19 (76%)		22 (77.33%)					
Female	6 (24%)		8 (26.66%)					
Age (mean)	17.96		28.03					
Subtypes	L1	L2	M1	M2	M3	M4	M5	M7
(FAB)	17	8	4	10	4	8	2	2
n (%)	68	32	13.3	33.3	13.3	26.6	6.6	6.6

Table 2. Clinical and Laboratory Characteristics of ALL and AML Patients According to FLT3/ITD Mutation Status

ALL & AML	Groups	Mean±SD of ALL	Mean±SD of AML	Sig (ALL)	Sig (AML)
Age	FLT3/ITD+	26	35.00±6.16	0.521	0.168
	FLT3/ITD-	17.62±2.57	26.96±10.98		
Sex	FLT3/ITD+	1	1.25±0.50	0.544	0.93
	FLT3/ITD-	1.29±0.46	1.26±0.45		
Subtype	FLT3/ITD+	2	4.50±1.00	0.149	0.27
	FLT3/ITD-	1.29±0.46	5.46±1.65		
WBCs 10^9/L	FLT3/ITD+	10.2	41.72±45.50	0.288	0.009*
	FLT3/ITD-	5.94±0.78	9.22±16.61		
PLTs 10^9/L	FLT3/ITD+	41	3.19±297.48	0.31	0.29
	FLT3/ITD-	2.31±36.72	1.68±256.38		
Hgb g/dl	FLT3/ITD+	9.5	9.38±3.15	0.546	0.75
	FLT3/ITD-	10.74±0.40	9.83±0.86		

*Significant (P<0.05)

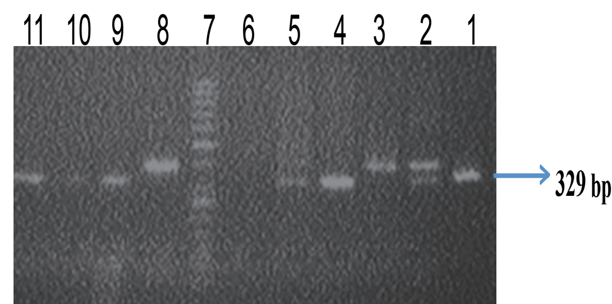

Figure 1. FLT3/ITD Mutation Screening by Gel Electrophoresis. Lane 1 shows wild type FLT3 gene of AML patient, Lane 2 shows mutated FLT3 gene of AML patient, Lane 3 shows mutated FLT3 gene of AML patient, Lane 4 shows wild type FLT3 gene of AML patient, Lane 5 shows mutated FLT3 gene of AML patient, Lane 6 shows negative control, Lane 7 shows 50bp marker, Lane 8 shows mutated FLT3 gene of ALL patient, Lane 9 shows wild type FLT3 gene of ALL patient, Lane 10 shows negative control, Lane 11shows wild type FLT3 gene of ALL patient.

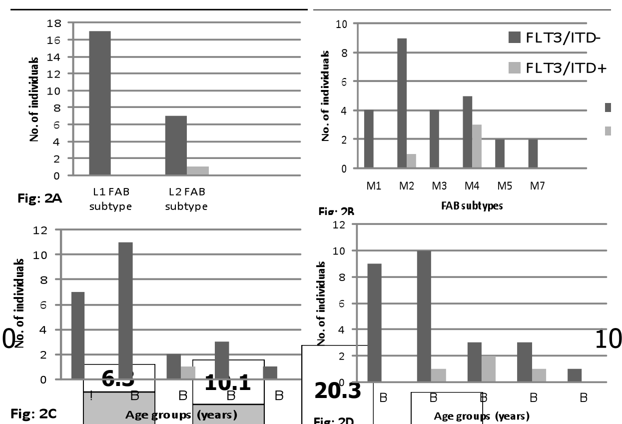

Figure 2. A) FLT3/ITD Mutati on in Accordance to FAB Subtype in AMI patients, B) FLT3/ITD Mutati on Accordance to FAB Subtype in AML Patients ents, C) FLT3/ITD Mutati on in Differrent Age Groups of ents, D) FLT3/ITD Mutati on in Different Age Groups of AML Patients.

FLT3/ITD mutation in ALL and AML patients of different age groups

No statistically significant (p>0.05) relationship was found between age and FLT3/ITD mutation in ALL and AML patients. FLT3/ITD+ mutation was more commonly found in elderly patients in both ALL and AML as shown in Figure 2c and 2d respectively.

Discussion

In our study, FLT3/ITD mutation was found in 5 (9.09%) of 55 patients with acute leukemia. We found that FLT3/ITD mutation was positive in 1(4%) of ALL patients and in 4 (13.3%) of AML patients. In total, FLT3/ITD mutation rate was found to be nearly similar to the other reported studies. A study was conducted on pediatric patients (87 AML patients and 60 ALL patients), in which RT-PCR method was used as a method of choice to find out the frequency of FLT3/ITD mutations. The incidence of this mutation was found in the rates of 13.3% in AML and 3.3% in ALL. The occurrence of tandem duplication of FLT3 gene in childhood AML was lower than that in adult AML reported so far (Xu et al., 1999).

Our studies have also shown statistically significant (p=0.009) relationship between FLT3/ITD mutation and high WBCs count in AML. While no statistically significant (p>0.05) relationship was found between FLT3/ITD mutation and WBCs count in ALL. Our analysis is similar to some of the studies reported earlier. Thiede et al. (2002) reported that, FLT3/ITD mutation was more often demonstrated to be associated with high WBCs count. Another study, conducted on adult leukemia patients revealed that the ITD mutation was found to be related to high WBCs count and significant relationship (p<0.001) was found between WBCs count and FLT3/ITD mutation. The most significant effect of an ITD has been reported to be its association with increased leukocyte count (Kottaridis et al., 2001).

In addition, there are other parameters of our study which are different from some of the previously reported FLT3/ITD mutation studies. In one of these studies

conducted on 979 AML patients, the incidence of FLT3/ITD mutation with respect to FAB subtype was reported to be significantly lower in patients with AML-M2 and AML-M6 morphologies than in patients with AML-M5a and AML-M5b (Thiede et al., 2002). Another study carried over adult leukemia patients described that the presence of FLT3/ITD mutation was not associated to gender or age. Mutation was present in all FAB subtypes except M0 and M7. FLT3/ITD is significantly (p=0.004) more common in M3 (kottaridis et al., 2001). The rate of FLT3 mutation has been reported to be significantly higher in M3 and M5 (Wang et al., 2004), different from our present study revealed FLT3/ITD gene mutation was found only in AML-M2 and AML-M4 subtypes, and does not show any significant relationship with FLT3/ITD mutation (p>0.05).

Our studies report ITD mutation was found in 13.3% of AML patients, of which 12.3% have been found in AML-M4 and 1% in AML-M2 subtype. Zakar et al, (2010) reported the percentage of ITD mutation to be 18% of AML patients, which is little higher than the percentage reported from our studies. 16% of these have been found in the M3 subclass and the remainder found in both M2 and M4 subtypes. Their data showed that approximately one third of AML patients had mutation in tyrosine kinase receptor. While their data did not support their significance as prognostic factor in AML patients. In conclusion, although substantial amount of knowledge is available in literature on FLT3/ITD mutations in Western and other populations, but the information from Pakistan is lacking and this is the first study of this kind from this region, reporting the incidence of FLT3 mutation in acute leukemia patients. The incidence rate of FLT3/ITD mutation in acute leukemia patients was 9.09%, more commonly found in males in comparison to females and is more frequently exhibited by elderly patients in both ALL and AML. There was no significant relation found between FAB subtypes and these mutations. Our studies reported these mutations to be more commonly found in M4 and M2 subtypes of AML and L2 subtype of ALL, which are different from previously reported studies. In future simultaneous study of patients for FLT3 mutations and NPM1 aberrancies could be carried out from population of this region which would guide us in correlating these abnormalities with cytogenetics and immunophenotypic characteristics.

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