Clinical Significance of FLT-3 Internal Tandem Duplication and D835 Mutations in Acute Myeloid Leukemia Patients from West Indian Population

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Abstract :

Introduction: The FMS-like tyrosine kinase-3 (FLT3), a member of the Platelet-derived growth factor (PDGF-R) subfamily of receptor tyrosine kinases, expressed on early hematopoietic progenitor cells play an essential role in survival and differentiation of stem cell. Majority of acute myeloid leukemia (AML) patients have mutation in this gene. Two types of frequent mutations are present in this gene. Both the types FLT3-ITD and D835 mutations play an important role in prognosis of AML patients. **Methods:** Total 33 patients were enrolled in the study. Blood samples were collected from the subjects, from which the DNA isolation was carried out.For FLT3-ITD mutation, PCR was performed and for D835 mutation PCR-RFLP was performed. DNA segments were amplified using Polymerase Chain Reaction (PCR). Results: FLT-3 ITD mutation was detected in 12% of patients and D835 mutation was detected in 3% of patients. The study revealed significant correlation between ITD and Tdt, while D835 negatively correlated with CD33, HLADR and Tdt. However, there was no substantial correlation of D835 with LDH value. **Results** also revealed that FLT-3 ITD significantly correlated with LDH values in AML patients. The mean value of LDH was 753.45 IU/L in ITD positive patients as compared to ITD negative patients with 338 IU/L mean LDH value, suggesting higher LDH values in ITD positive. **Conclusion:** These Genotypic analysis of FLT-3 mutation results from West Indian population provide important tools for understanding of AML pathogenesis and determination of appropriate therapeutic intervention. Further large number of patient data can also corroborate these significant results.

Key words : FLT-3 mutations, Lactate Dehydrogenase, Acute Myeloid Leukemia

Introduction :

Acute Myeloid Leukemia (AML), characterized by the hindered homeostatic mechanisms of normal hematopoietic stem cells, is a clonal disease of leukemic cells which take refuge within the bone marrow niche.[1] Therapy for patients with AML is guided by the molecular and cytogenetic profile.[2] Mutations in the FMS-like tyrosine kinase 3 (FLT3) genes represent one of the most frequently encountered, and clinically challenging classes of AML mutations. Although approximately 30% of AML patients harbor some form of FLT3 mutation, the clinical significance of these genetic lesions in any given patient varies according to the nature of the mutation and the context in which it occurs.[3, 4] In general, FLT3 mutations are divided into 2 categories: (A) internal tandem duplications (FLT3/ITD mutations) and (B) point mutations (FLT3/TKD/D835 mutations), constituting approximately 25-35 % of adults AML patients.[5]

FLT3, located on chromosome 13q12, plays a significant role in cell survival, proliferation, differentiation and pathogenesis of AML[6] and also several reports suggested that it is associated with resistance to therapy confirming its role in drug resistance in AML.[7, 8] FLT3/ITD is the first studied mutation which results from the duplication and tandem insertion of a portion of the juxtamembrane (JM) region (exons 11 to 12) of the FLT3 gene. There are many reports suggesting that AML patients having FLT3/ITD mutations are associated with poor cure rates and higher relapse rates.[9] Further also the other most common residue implicated in clinical resistance to FLT3 tyrosine kinase inhibitor (TKI) therapy is D835. FLT-3 D835 mutations have been reported to occur in almost 7% of patients with AML, 3% of patients with myelodysplastic syndrome (MDS), and 3% of patients with acute lymphocytic leukemia.[10] FLT-3 ITD mutation is one of the most frequent bad prognosis mutation observed in AML.[11] FLT3/ITD and D835

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mutations are found to be present in AML patients independently. Detection of these two mutations have clinical implications as patients harbouring these mutations generally have a worse prognosis and may benefit from aggressive up-front treatment interventions.Association of FLT3-ITD, with the poor clinical outcome in AML patients have been reported bydifferent study groups. However there are very few Indian studies. (12) Therefore, the current study was aimed to evaluate the prevalence of these two mutations and to further evaluate its clinical significance in AML patients. Genotypic analysis of these mutations from West Indian population would help in the understanding of the AML pathogenesis and would provide an important tool for determination of appropriate therapeutic intervention in AML patients.

Materials and Methods:

Patients: Peripheral blood samples were collected from 33 AML patients in EDTA vaccuttes after signed informed consent.AML cases confirmed histologically and immunohistochemically with no prior treatment were included in the study. The mean age range was 9 to 65 years with median age of 36 years. Out of 33 patients, 17 were male and 16 were female. AML patients were further classified on the basis of FAB classification from M1 to M6 (40% of the patients were M1).

Heamatological and Biochemical Parameters: Routine heamatological and biochemical parameters were carried out by auto analyzer LH-750 (USA).

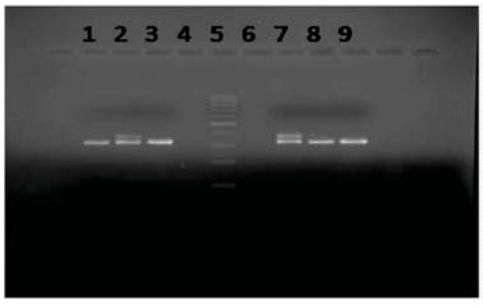
Analysis of FLT3-ITD and D835 Mutations in Acute Myeloid Leukemia Patients: Genomic DNA from peripheral blood was extracted using QIAamp DNA blood Kit using manufacturer protocol (Qiagen, Germany). Further, isolated DNA was amplified by semi quantitative PCR using primers for FLT-3 ITD and D835 mutations (Table 1).

Table 1: Primers used in polymarase chain reaction for FLT-3 ITD and D835 mutations

Targeted gene	Oligonucleotide sequence
FLT-3 ITD	
Forward 5'-	GCAATTTAGGTATGAAAGCCAGC-3'
Reverse 5'-	CTTTCAGCATTTTGACGGCAACC-3'
FLT-3 D835	
Forward 5'-	CCGCCAGGAACGTGCTTG-3'
Reverse 5'-	GCAGCCTCACATTGCCCC-3'

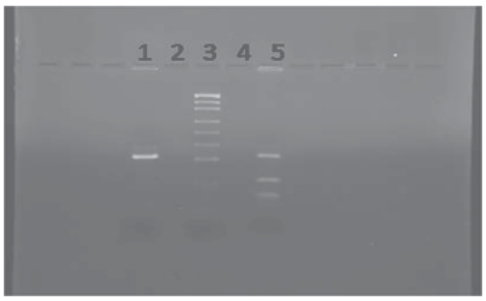
Total 200 ng of DNA was used as template in PCR reaction using Himedia Taqmixture. The cycling conditions for this reaction were 94° C for 3 min, 94° C for 1 min, 62° C for 1 min, 72o C for 1 min and final extension at 72° C for 10 min for 40 cycles. PCR products were then separated on 2% agarose gel stained with ethidium bromide. The gel was analyzed using gel documentation system Alpha Innotech, Inc. Wild type genotype depicted band of 329 bp, while an additional band above 329 bp was observed for ITD mutation. For D835 mutation PCR RFLP was performed. Figure 1 represents the gel image of FLT-3 ITD mutation in AML patients. [13] For D835 mutation, 300 ng of DNA was used as a template in PCR using Himedia Taq mixture. The Cycling conditions were 94° C for 3 min, 94° C for 1 min, 65° C for 1 min, 72° C for 1 min and final extension at 72° C for 10 min for 35 cycles. PCR products were further digested by 0.5 Eco RV endonuclease enzyme (New England Biolabs) at 37°Cfor 3 hours and the digested products were separated on 3.5% agarose stained with ethidium bromide. Figure 2 represents the gel image of FLT-3 D835 mutation in AML patients. Wild type D835 after restriction digestion results into 68bp and 49bp product in the presence of EcoRV restriction enzyme. In heterozygous mutations it produces 3 bands corresponding to 114bp, 68bp and 49bp, while in homozygous mutations, FLT3/D835 allele produces only one band corresponding to 114 bp. [13]

Figure 1: Agarose gel representing FLT-3 ITD amplified product



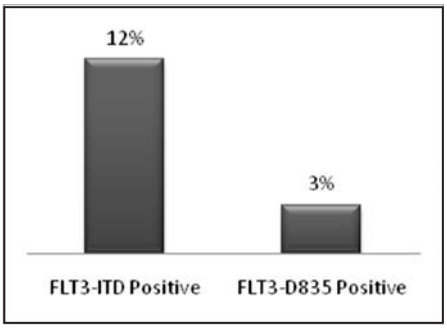
Lane 1, 3, 8 and 9: FLT-3 ITD negative sample,
Lane 2 and 7: FLT-3 ITD Positive sample,
Lane 4 and 6: blank,
Lane 5: 100 base pair ladder.

Figure 2: Representative pattern of FLT-3 D835 mutation



Lane 1: homozygous mutation,
Lane 3: PUC19 Ladder,
Lane 5: heterozygous mutation
Lane 2 and 4: blank.

Figure 3: Frequency of FLT-3 ITD and D835 positive patients in AML patients



Statistical Analysis:

Statistical analysis was performed using SPSS computer software version 15. Student independent 't' test was performed to analyze the significance between different groups. Receiver's operating characteristic (ROC) curve analysis was performed to analyze the discriminatory efficacy between different molecules under study. Spearman's correlation analysis was performed to analyze the correlation between markers.

Results:

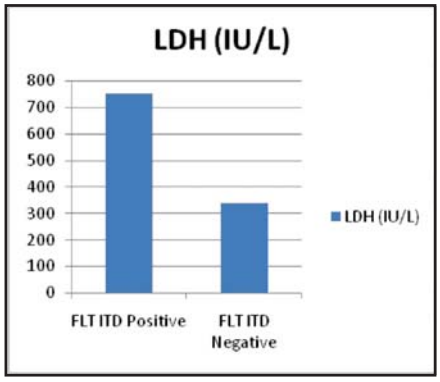
Frequency of FLT-3 ITD and D835 mutations in Acute Myeloid Leukemia: As depicted in Figure3, FLT-3 ITD mutation was observed in 12% of patients while 3% of the patients showed FLT-3 D835 mutation. Positivity of FLT-3 ITD mutation were observed in patients having age range 9-45 years, while one patient of 38 years was positive for D835 mutation.FLT3-ITD mutations were most common with the FAB subtype M1 in 2 patients (50%) and M5 in 2 patients (50%) while patient having D835 mutation belonged to M5 subtype, There was no significant correlation of white blood counts (WBC) and cretanine levels with FLT-3 ITD and D835 mutations. (Table 2).

Table 2: Biochemical parameters in FLT-3 ITD and FLT-3 D835 mutations positive and negative AML patients

Parameters	No Mutation N=28	FLT-3 ITD Positive N=4	FLT-3 D835 Positive N=1
WBC (Mean)	39.9 X 10^3/cmm	39 X 10^3/cmm p=0.976	5.2 X 10^3/cmm
Cretanine (Mean)	0.82 (mg/dl)	0.48 (mg/dl) p=0.101	0.49 (mg/dl)
M1	44%	50%	0
M2	20%	0	0
M4	20%	0	0
M5	12%	50%	100%
M6	4%	0	0

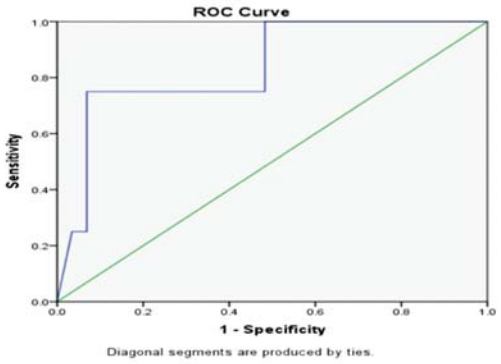
Correlation of FLT-3 ITD Mutations with Lactate Dehydrogenase Activity in Acute Myeloid Leukemia Patients: Figure 4 shows the value of LDH activity in FLT-3 ITD positive and negative patients. It was observed that FLT-3 ITD positivity in AML significantly correlated (p=0.029) with higher values of LDH. The mean value of LDH was 753.5 IU/L in ITD positive patients as compared to ITD negative patients with 338.0IU/L mean LDH value, suggesting higher LDH values in ITD positive patients.

Figure 4: Correlation of FLT-3 ITD Mutations with Lactate Dehydrogenase Activity in Acute Myeloid Leukemia Patients



ROC curve is a significant statistical approach to evaluate discriminatory efficacy of the biomolecule. It is meaningful way of discriminating two groups under the study as it simultaneously considers sensitivity and specificity of the method. ROC curve suggested that LDH activity could significantly discriminate FLT3-ITD positive patients from FLT3 ITD negative patients (AUC= 0.841 and p=0.029) (Figure5).

Figure 5: ROC curve analysis of LDH for FLT-3 ITD positive and negative patients



Association of FLT-3 mutations with biological parameters by Spearman's Correlation Analysis: Spearman's Correlation Analysis was performed to correlate FLT-3 ITD mutations with other clinical parameters including Stage, CD33, CD34, Terminal deoxynucleotidyl transferase (Tdt) etc. Significant correlation between FLT-3 ITD mutations and Tdt was documented (p=0.002).Spearman's correlation between CD33 and CD34 expression with FLT-3 ITD mutations revealed no significant correlation. Moreover any significant correlation between disease stage and FL-3 ITD mutations was not observed (Table 3).

Table 3: Spearman's Correlation of FLT-3 ITD Mutations with Biological Parameters in AML patients

Variable	Stage	Cd33	Cd34	Tdt
FLT-3 ITD mutations	r =0.132 p=0.463	r=0.075 p=0.694	r=0.199 p=0.291	**r=0.533*** **p=0.002**

Discussion:

Activating mutations in FLT3 receptor are one of the most frequent genetic alterations reported in AML.[14] Few of these mutations are an independent adverse prognostic factor in AML publicized by several reports.[15] The patients who have a constitutively activating FLT3-ITD mutation have poor initial therapy response, high relapse rate, and low-grade overall survival.[16] The FLT3 molecular diagnostic assay provides a consistent method to simultaneously detect the two different types of FLT3 mutations currently known to be of importance in AML. In the present study, FLT3-ITD and D835 mutations were analyzed in 33 patients with AML by PCR assay. FLT-3 ITD mutation was detected in 12% of patients. FLT-3 D835 mutation was detected in 3% of patients. Our results are in accordance with previous studies. [14-15, 17-20] None of the patients was positive for both FLT3-ITD and D835 mutations. In the present study FLT-3 ITD was found more frequently in M5 and M1 and similar results were observed by Chauhan et al.[14] Further there was no significant association between the FLT3-ITD mutations with white blood cell count. Similarly, there was no correlation between the percentage of blast count in bone marrow with any of the mutation status and these results were similar to Shih et al. [21]

Previous study also observed higher LDH activity in leukemia patients as compared to healthy individuals. (22-24) To the best of our knowledge no significant correlation between high LDH levels and FLT-3 ITD mutation in AML has been reported yet. The present investigation found that most of the AML showed elevated LDH activity. Furthermore, FLT-3 ITD positive patients were observed to have higher LDH activity as compared to those patients who had negative FLT-3 ITD mutations. Thus the results suggested that FLT-3 ITD mutation play important role in AML patients. The present study also observed that Tdt (Terminal deoxynucleotidyl transferase) expression was observed in 50% of samples with FLT-3 ITD mutation and was statistically significant (p=0.002). Results also revealed that the FLT3-ITD but not the FLT3-D835 mutation can be used as a valid marker of minimal residual disease. However, larger studies are required to validate these finding. In conclusion the present study observed positivity of FLT-3 ITD mutation with high levels of LDH activity and Tdt expression in AML patients. These results from genotypic analysis revealed that FLT-3 mutations in AML patients are an important molecular target for precise therapeutics against FLT-3 receptor. It also suggested that FLT-3 ITD mutation study in combination with LDH analysis would have more clinical significance.

Conflict of Interest:

None

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