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Study of FLT3 Gene mutations in Acute Myeloid Leukemia

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Review Article

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ABSTRACT

The prognostic relevance of FLT3 D835/I836 mutations (FLT3-TKD) in cytogenetically normal acute myeloid leukemia (CN-AML) remains to be established. The constitutive activation of FLT3 receptor resulting in the worse disease-free survival in atleast some studies have been resulted by alteration of D835. D835 mutations have been reported to occur in 7% of patients with AML, 3% of patients with myelodysplastic syndrome (MDS), and 3% of patients with acute lymphocytic leukemia. By performing PCR and RFLP in 10AML tumor samples and 10 samples of healthy individuals of a similar age range resulted in detection of polymorphisms in the FLT3 gene mutation at 835 position. In the given study group only one case showed the homo mutant condition. D835Y (point mutation) accounts for ~ 8-12% of all AML patients with 113 bp.The significance of the samples were not determined because of the small sample size. In this study we have demonstrated that polymorphisms (SNP's) may play a role in cancer progression. Further studies are needed for significant results.

INTRODUCTION

FLT3 (Fms-like Tyrosine Kinase-3), also known as FLK2 (Fetal Liver Kinase-2) and STK1 (human Stem Cell Kinase-1) was originally isolated as a hematopoietic progenitor cell-specific kinase, and belongs to the Class-III RTK (Receptor Tyrosine Kinase) family to which c-Fms, c-Kit, and the PDGFR (Platelet Derived Growth Factor Receptor) also belong [1]. Generally FLT3 is expression if restricted to haemopoietic progenitor cells in the bone marrow, thymus and lymph nodes, but is also found on other tissues such as placenta, brain, cerebellum and gonads [2]. The expression of FLT3 is aberrant at high levels in a spectrum of hematologic malignancies which includes 70-100% of AML, (Acute Myelogenous Leukemia), B-precursor cell ALL (Acute Lymphoblastic Leukemia), a fraction of T-Cell ALL, and CML (Chronic Myelogenous Leukemia) in lymphoid blast crisis. In Human FLT3 is aboot 160 kDa. To stimulate the proliferation of stem cells, progenitor cells, dendritic cells and natural killer cells, the ligand for FLT3 is expressed by marrow stromal cells and other cells, synergizing other growth factors [3]. Receptor dimerization, autophosphorylation and subsequent phosphorylation are the resulatants of the interaction of FLT3. Other cytokines such as Kit ligand influence this interaction [4] **(Figure 1).**

The most frequent somatic alterations in AML are FLT3 mutations, which occurs in 1/3rd of the patients approxiemately [5]. There are two types of FLT3 mutations: The Internal Tandem Duplication (ITD) of juxta-membrane domain Point mutation (D835Y) in tyrosine kinase domain [6].

MATERIALS AND METHODS

Study Population and Sample Collection

Acute myeloid leukemia patients were assessed on the basis of clinical examinations as well as pathological examinations. The Study is a case-control study conducted in South India [7]. A total of 10 AML patients and 10 age matched healthy controls were enrolled in the study [8-10].

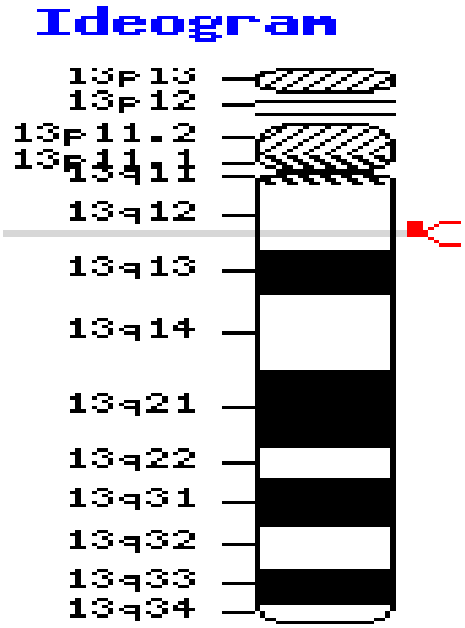


Figure 1. FLT3 gene location.

MATERIALS AND PROTOCOLS

Genomic DNA Isolation from Blood

Materials required

1. Autoclaved eppendorff
2. Autoclaved micropipettes
3. Autoclaved micro tips
4. Autoclaved distilled water
5. Eppendorff stand

Reagents preparation

RBC LYSIS BUFFER/TKM 1 (100ml)

- Tris HCl (10 mM) - 0.121 g
- EDTA (2 mM) - 0.074 g
- KCl (10 mM) - 0.074 g
- $MgCl_2$ (10 mM) - 0.2032 g

In a few ml of autoclaved distilled water tris is first dissolved in few ml of autoclaved distilled water and the pH is adjusted to 7.6. By using 0.1% HCl. Then EDTA is dissolved followed by other chemicals [11-15].

Cell lysis buffer/Tkm2 (100 ml)

- Tris (10 mM) -0.121 g
- KCl (10 mM) -0.074 g
- $MgCl_2$ (10 mM) -1.203 g
- EDTA (2 mM) -0.074 g
- NaCl (0.4 M) -0.464 g

Tris is first dissolved in few ml of autoclaved distilled water and the pH is adjusted 7.6 with HCl. Then EDTA is dissolved followed by other chemicals and the volume is made up to 100 ml with distilled water [16-20].

10% SDS (10 ml)

- 1 g of SDS is dissolved in 10 ml of autoclaved distilled water [21].

0.6 M of NaCl (25 ml)

- 8.765g of Nacl is dissolved in 25 ml of autoclaved distilled water [22].

TE buffer (25 ml)

- Tris (10 mM) - 0.030 g
- EDTA (1 mM) - 0.009 g

Tris is dissolved in few ml of autoclaved distilled water, after adjusting the pH, EDTA is dissolved, and the volume is made up to 25 ml [23-25].

70 % Ethanol

- Dissolve 7 ml of absolute Ethanol in 10 ml of distilled water [26].

Principle

RBC lysis buffer and triton X 100 is used to remove the RBC' S

- Since RBC has no charge on their plasma membrane, non- ionic detergent called, Triton X 100 removes them out. KCl and $MgCl_2$ in TKM1 helps in lysis of the RBC cell membrane and EDTA acts as a divalent ion chelator (it contains di-sodium atom). Hence, it helps in de-activating the metallozymes as DNAses. Tris acts as a buffering agent maintaining the pH at 7.6 for the proper
- Function of the lysis buffer. In addition, it helps in solubility of the ions so that they do not precipitate out [27-30].
- Centrifugation at 10000 rpm for 5 min, after incubation with RBC lysis buffer step separates out the lysed RBCs in the supernatant and intact lymphocytes precipitate out as pale colored pellet [31-35].

TKM2 and 10% SDS are used to lyse the lymphocytes

- TKM2 or Cell lysis buffer has a higher concentration of $MgCl_2$, KCl and NaCl to lyse both the cell and the nuclear membrane. KCl also acts as solubilizer of proteins. NaCl acts as extractor of RNA and used in salting out of proteins. SDS acts as anionic detergent and both acts on anionic lymphocytic cell membranes and help in their lysis deactivate the negatively charged proteins [36-40].
- 6 M NaCl is added to precipitate the proteins by salting out method [41].
- Centrifugation at 10000 rpm for 5 min helps in the precipitation of the aggregates of the proteins and cell debris as pellet and the supernatant contains the DNA strands in a solubilized form [42-45].
- Addition of the supernatant to cold absolute ethanol dehydrates the DNA and extracted as visible strands [46].
- The visible DNA threads are precipitated as pellet, washed using 70% alcohol, and again precipitated to remove agents like $MgCl_2$, EDTA, KCl, NaCl that can inhibit Taq Polymerase during PCR of these samples [47-50].

Procedure

- Take 300 µl of blood sample in eppendorff [51].
- Add 600 µl of TKM1 and 1 drop of 100% Triton X 100 to it, mix well, and incubate for 5 min.
- Centrifuge at 10000 rpm for 5 min, and then discard the supernatant.
- To the pellet add 800 µl of TKM1 and repeat the steps 2 and 3 until a white pellet is obtained.
- To the pale pellet, add 280 µl of TKM2 and 80 µl of 10% SDS and incubate for 30 minutes.
- Add 80 µl of 6 M NaCl and mix well by tapping for 5 min.
- Centrifuge at 10000 rpm for 5 min.
- Transfer the supernatant carefully to 600 µl of cold absolute Ethanol.
- Centrifuge at 10000 rpm for 5 min.
- Discard the supernatant, add 300 µl of 70% Ethanol to the DNA pellet.
- Centrifuge at 10000 rpm for 5 min and air dry the pellet.
- To the dried pellet add 50 µl of TE buffer for hydration of DNA and preserve at freezing temperature.

Detection of DNA in the Isolated Samples Using 0.8% of Agarose Gel by Electrophoresis

Materials required

- Horizontal electrophoresis unit

- Gel plate
- Combs
- Adhesive tapes
- 10T micropipette and autoclaved tips

Reagent preparation

10x TAE Buffer (100) ml

- Solution A: dissolve 19.36 g of Tris in 50 ml of water.
- Solution B: dissolve 1.86 g of EDTA in 10 ml of water.
- Solution C: add 8 ml of B to solution A and add 4.36 ml of acetic acid. Then make up the volume to 100 ml with water.

1X TAE Buffer

- Dissolve 30 ml of 10X TAE Buffer in 270 ml of water to make 1:10 dilution.
- 0.8% Agarose: dissolve 0.2 g of Agarose in 25 ml of 1X TAE Buffer.
- 1% Ethidium bromide solution: dissolve 0.1 g of Ethidium bromidein 10 ml water.
- Gel loading solution and dye used is 6X concentrate is obtained readymade.

Principle

An electric field is developed across the Agarose gel with incorporated DNA samples. The DNA being negatively charged migrates towards anode. The Ethidium bromide acts as an interchelating agent and incorporates itself into the DNA strands. Since Ethidium bromide fluoresce under UV rays, hence site of fluorescence in the gel detects presence of DNA [52-55].

Bromophenol blue is used as loading dye to track the movement of the sample. It is mixed well

with the sample. In addition, glycerol increases the density of the mixture, so that they reside down at the bottom of the well and are diffused in the gel [56].

Procedure

- Close the open sides of the gel plate using adhesive taps.
- Place the combs.
- Add 10 µl of Ethidium bromide solution to the cold molten Agarose and pour it in the gel plate. Keeps it resting for casting of the gel for 15-20 min.
- Remove the taps and comb carefully.
- Pour the 1X TAE Buffer in the unit tank and place the gel placing the wells at cathode end.
- Mix 1.5 µl of the loading concentrate with 4.5 µl of the DNA sample on a piece of parafilm.
- Add 5.0 µl of the mixture into the well.
- Connect the wires and set the volts at 80.
- Run the gel at 80 V for 20–30 min.
- Observe the gel under UV in a transilluminator

Polymerase Chain Reaction of the Specific Exons Using a Thermo cycler

Principle

Polymerase chain reaction invitro was designed first by Karry Mullis in 1983 [57]. It follows the process of DNA replication using temperature variations with a help of a thermocycler [58]. This process include five major steps, at specific accurate temperature for each step for exact specificity of the amplification or duplication of the specific DNA sequence or gene out of the whole genomic DNA sequence [59]. This is possible by using specific complementary forward and reverse primers that specified the region of duplication [60]. The enzyme used for the amplification is generally consists of 3' end to 5' end extension and 5' end to 3' end exonuclease activity. The enzyme used is called Taq polymerase, which is extracted from thermostable bacteria Thermus aquaticus, generally found in hot springs. This makes the enzyme thermo stable and it can work actively at higher temperatures at which the double strand remains denatured after the denaturation step at 94°C. Denaturation of the double strands occurs by melting of the interstand hydrogen bonds in between the bases [61-65]. Denaturation is required for annealing of the primers, which

specify the region of amplification and initiate binding of the polymerase enzyme and replication of the template or given strand. The enzyme has an extension rate of 2–4 kb per min. It is industrially or mass-produced by cloning in *E. coli* cells. It requires Mg2+ ion as its cofactor. Hence, addition of $MgCl_2$ is a crucial factor [66-68].

Mg_2+ ion also effects enzyme fidelity, primer annealing, i.e., attachment of primers to specific site etc. For the optimal activity of the enzyme specific pH is required which is maintained by KCl buffer at 8.3 [69]. KCl also helps in primer annealing. Primer specificity and annealing are the prime factors for accurate and successful amplification. Primers should be complementary to the sequence of interest. The primers should be of 18-30 nucleotides [70]. The GC content of the primers is crucial as the annealing temperature depends on them. The GC content should be up to 40%-60% [71]. The annealing temperature should be 5°C lesser than the melting temperature of the primer. The melting temperature (Tm) is calculated based on GC content using Thien and Wallence equation –Tm=20C(A+T)+40C(G+C). The Tm of the forward and reverse primers for a single sequence set should be similar or near by [71]. For the formation of new strands, dNTPs are provided as dNTPs mix, which contains equal concentration of each of the four dNTPs as 200 µM. The dNTPs mix added should be 10 µM per 50µl of reaction mix [72-75].

Along with the above reagents, one major component added is water, which provides the reaction medium and required for DNA synthesis. It occupies the major volume. The procedure includes denaturation, primer annealing, extension and renaturation for a single round of replication [76-78]. These steps are orderly repeated to obtain number of replication required by variation of temperatures [79]. The specific temperature of each step should not coincide with other. The initial denaturation generally occurs at 94°C for about 5 min for denaturation of the whole genomic DNA [80].

Materials

PCR mixture of 50 L for each tube

Nuclease free water	-	36.5 µl
Taq buffer (10x)	-	5 µl
Mgcl2	-	3.5 µl
dNTP's mix	-	1 µl
Primers:		
Forward	-	1 µl
Reverse	-	1 µl
Taq DNA polymerase	-	1 µl
Template DNA	-	1 µl

Primer sequence

F: 5'- gcagcctcacattgcccc3'

R: 5'- ccgccaggaacgtgcttg3'

PCR programme

Initial denaturation	-94°C for 5 min
Denaturation	- 94°c for 0.45 s
Annealing	- 64°C for 0.35 s, 35 cycles
Extension	-72°C for 0.45 s

Restriction Fragment Length Polymorphism

After PCR amplification, PCR products were digested with specific restriction enzyme to the target site of the mutation on the sequence. Restriction end nucleases cut amplified sequences at a limited number of specific nucleotide sequences generating fragments of different length. These fragments separate on electrophoresis into distinct bands depending on the size of the fragment, which can be determine using appropriate marker [81-85].

In the present study The PCR product of 113 bp was digested with ecoRv enzyme at 37°C overnight. EcoRV can recognize the normal sequence and digest the PCR product into 2 fragments with 68 bp and 46 bp. The sample with mutation at 835 position sequence will not be recognized by this enzyme, and 113 bp product remains intact. These results will be analyzed on the 3% agarose gel [86].

Composition for RFLP

D.D.Water	-	5.5 l

PCR product	-	12 l
10x buffers	-	2 l
EcoRV enzyme	-	0.5 l
Incubation	-	37°C overnight

RESULTS AND DISCUSSION

The polymorphisms in the FLT 3 gene mutation at 835 positions were detected by performing PCR amplification and RFLP analysis in 10 AML tumor samples and in blood samples of equal number of healthy individuals of a similar age range. The demographic details of the cases and controls were collected and recorded in **Table 1.**

Table 1. The polymorphisms in the FLT 3 gene mutation at 835 positions.

CONTROLS : (n=10)		CASES : (n=10)	
AGE	: 22-40 years	AGE	: 30-50 years
SEX	: F-2; M-8	SEX	: F- 3; M- 7

Genotyping

The DNA isolated from the cases and the healthy blood samples were verified using 0.8% Agarose gel electrophoresis. The purified DNA is indicated as sharp distinct orange band under UV transilluminator due to the presence of ethidium bromide in the Agarose gel. The presence of smeary bands or streak may indicate RNA or protein contamination and also degradation of the DNA [87-90] **(Figure 2).**

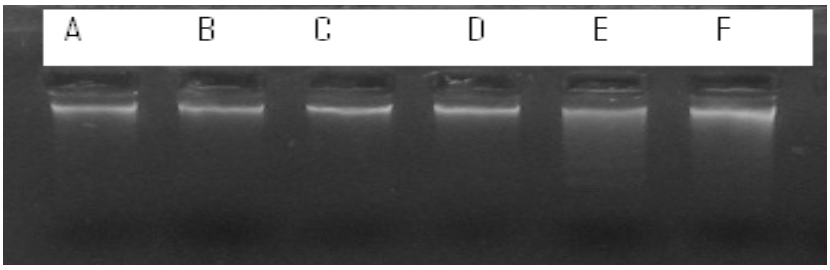
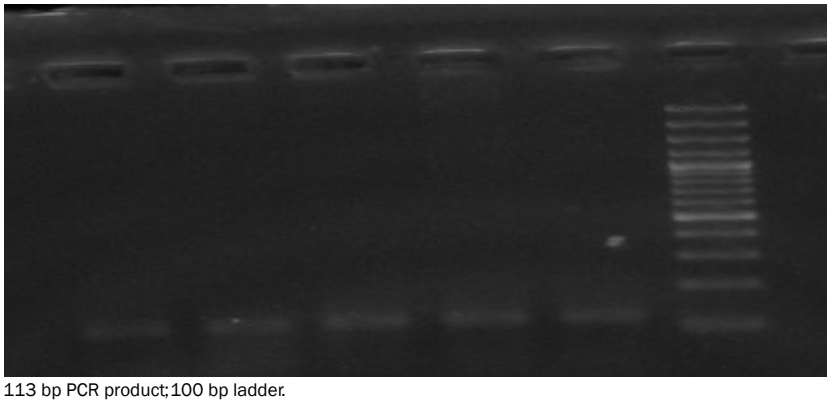


Figure 2. Genomic DNA On 0.8% Agarose gel electrophoresis.

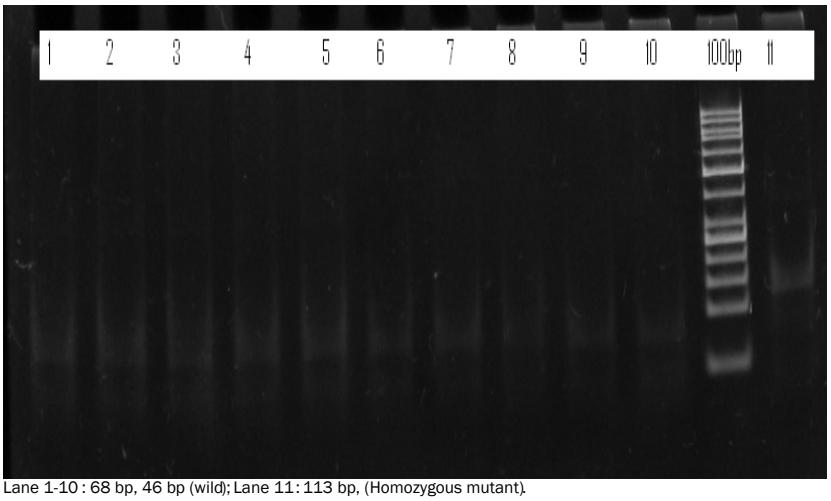
The PCR reaction mixture used of volume of 50 µl containing 50 µg of genomic DNA, 10X PCR buffer, 3 µM $MgCl_2$, 200 µM dNTPs mix, 0.5 µM each primer and one unit of Taq DNA polymerase. The PCR amplified products were verified using 2% Agarose gel using ethidium bromide staining along with a 100 bp ladder/marker DNA. The amplified product of exon 18 region corresponds to size of 113 bp [91-95] **(Figure 3).**



113 bp PCR product;100 bp ladder.

Figure 3. PCR products on 2% agarose gel electrophoresis.

To detect the presence of polymorphisms in the specified region RFLP analysis was performed. 12 µl of the PCR products were digested with 5U of enzyme and incubated at 37 overnight. Samples were run in 3% agarose gel at 70 v for 40 min [96-98]. The enzyme will recognize the normal sequence of D835 and cleave the 113 bp PCR products into 68bp, and 46 bp [99-100]. The variant sequence would be intact, i.e., 113 bp after the restriction digestion. The variants of the exonic region described as follows. After RFLP the samples with 113 BP products were considered as the homozygous mutant [101]. The heterozygosity at the gene locus was revealed by 3 bands corresponding the 113 bp, 68 bp and 46 bp [102,103]. The presence of 2 band at 68 bp, 46 bp indicates the homozygous mutant condition. Among the 10 cases 1 male cases have shown the presence of homozygous allele (10%), Among the healthy smoker controls all of them showed the homozygous wild condition **(Figure 4 and Table 2).**



Lane 1-10 : 68 bp, 46 bp (wild); Lane 11: 113 bp, (Homozygous mutant).

Figure 4. RFLP Products on PAGE.

Table 2. Presence of polymorphisms in the specified region RFLP analysis.

CONTROLS: n=10	CASES : n=10
Wild type (HOMO) : 2 females, 8 males	Wild type (Homo) : 3 female, 6 male
Mutant type(Homo) : 0	Mutant type(Homo) : 1 male

CONCLUSION

In the present investigation FLT3 gene 835 positions has been screened for the D835 mutation. In this , D835 activation loop domain mutation, a wealth of reports has emerged suggesting that FLT3 mutation is the single most common molecular of genetic abnormality in acute myeloid leukemia with direct clinical impact on the disease outcome .In the given study group only one case showed the homo mutant condition. D835Y (point mutation) accounts for ~ 8-12% of all AML patients. In the present group under investigation, a similar frequency was observed. The significance of the samples were not determined because of the small sample size. In this study we have demonstrated that polymorphisms (SNP's) may play a role in cancer progression. Further studies are needed for significant results. Detecting SNP's in genes like FLT3, which involve in the cancer development would be useful for the early detection and monitoring of disease recurrence. This study would assist the future studies in evaluating the role of FLT3 gene in AML cancer.

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