

Screening of C-kit gene Mutation in Acute Myeloid Leukaemia in Northern India

Hussain SR¹, Raza ST¹, Babu SG², Singh P³, Naqvi H¹, Mahdi F¹

Abstract

Background: Acute Myeloid Leukaemia (AML) is a cancer of blood-forming cells in bone marrow. C-kit gene is a Receptor Tyrosine Kinase class III (RTK) that is expressed by early hematopoietic progenitor cells and plays an important role in hematopoietic stem cell proliferation, differentiation and survival. It is known that c-kit is a proto-oncogene and the activating c-kit mutations are likely to contribute in the development of leukaemia in humans. Exon 11 of c-Kit gene is the frequent site for mutations in different kinds of tumours.

Methods: In order to determine the frequency and prevalence of exon 11 mutations in 51 AML cases, we have done polymerase chain reaction-single-strand conformational polymorphism followed by direct DNA sequencing.

Results: The c-kit mutations in exon 11 were detected in 15.68% (8/51) in AML cases. We have detected totally ten missense mutations in eight AML cases those include Lys550Asn, Tyr568Ser, Ile571Leu, Tyr578Pro, Trp582Ser and Arg588Met and novel missense mutations at codons Ile563Lys and Val569Leu. Mutations at codons Ile571Leu and Trp582Ser was found in two independent cases.

Conclusion: The presence of c-kit mutations in our study adds to investigative spectrum of AML cases. Since the c-kit mutations are seen in other malignancies, mutations in exon 11 of the c-kit gene might be involve in pathogenesis and represent useful predictive genetic marker in AML. Further studies in larger group of cases possibly will be required to determine the prognostic implications and to investigate how these mutations are co-related to the progression and pathogenesis of AML.

Keywords: Proto-Oncogene proteins c-kit; Acute Myeloid Leukaemia; Mutation; Polymerase Chain Reaction; Single-Stranded Conformational Polymorphism

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Introduction

Leukemia is a heterogeneous disease in which hematopoietic progenitor cells acquire genetic lesions that lead to a block in differentiation, increased self-renewal, and unregulated proliferation. Leukemia is the 12th most common class of neoplastic disease and the 11th most common cause of cancer-related death [1]. A number of observations suggest the role for c-kit, another member of type III RTK family which is important for the development of a range of cells including haematopoietic cells in leukemogenesis [2]. It is known that c-kit is a proto-oncogene and activating c-kit mutations are likely to contribute in the development of leukaemia in humans [3-5]. Class III RTKs share sequence homology and have an

overall similar structure with five immunoglobulin-like repeats in the extracellular domain, a single Trans Membrane domain (TM), a Juxta Membrane domain (JM), two intracellular Tyrosine Kinase domains (TK1 and TK2) divided by a Kinase Insert domain (KI), and a C-terminal domain [6]. The genomic locus encoding the c-kit gene receptor has 21 exons, ranging 100-300bp [7].

The c-kit gene mutations in exon 11 are reported in gastrointestinal stromal tumours [8], AML [9, 10], human germ cell tumours [11] and adenoid cystic tissue [12]. High expression of c-kit in 60% -80% of AML has been reported [13] and missense mutation of c-kit has been identified in 33.35 -45% of AML [14]. Prognostic impact of c-Kit mutations in core

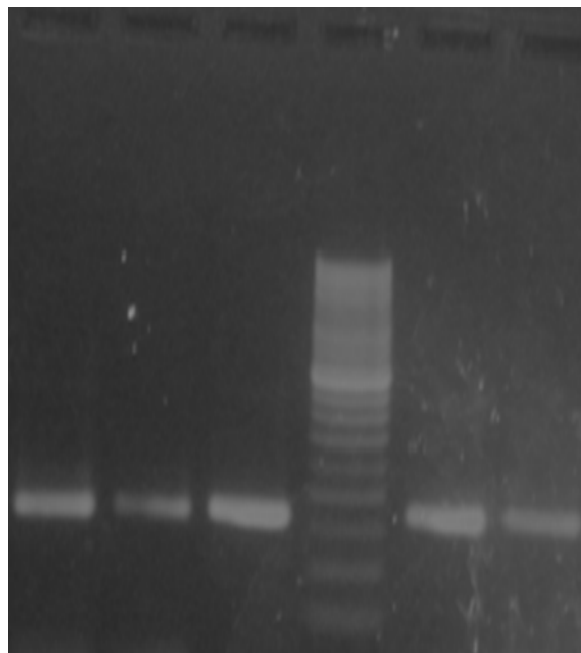


Figure 1. Sizing of 257bp long PCR products of c-kit exon 11 on 2 % agarose in AML cases. 50bp Ladder in lane 4th and cases in 1, 2, 3, 5 and 6th lane.

binding factor in AML is reported in Korean population [15], however, many studies screened the c-kit mutations only in a proportion of c-kit coding sequence and others were limited to small number case-studies. The activation sphere of the receptor has resulted in the constitutive c-kit kinase activity and c-kit receptors harbouring such mutations when introduced into mammalian cells downstream signalling pathways lead to the factor independent growth in vitro and leukemogenesis in vivo [4,16]. No study so far has reported the frequency and prevalence of mutations in exon 11 of c-kit gene in AML in Northern India. In our study, we have screened the mutation status of exon 11 of c-kit gene in leukemia and further explored whether the c-kit gene mutations were valuable as predictive genetic marker in AML cases.

Materials and Methods

Subject

The study group included 51 cases of AML. Ethical approval was obtained from the institutional ethical committee of Era's Lucknow Medical College and Hospital, Lucknow, Uttar Pradesh, India. Clinical data was recorded. The blood or bone marrow samples were stained by Leishman stain method and the cases were classified, according to the French American British (FAB) criteria [17]. All the 51 AML

cases were categorised as, M0 (n =8), M1 (n = 8), M2 (n = 8), M3 (n = 8), M4 (n = 8), M5 (n = 6) and M6 (n = 5). Out of 51 AML cases 27 were male and 24 cases were female with age ranging from 2 years to 65 years. The mean age of cases was 30.3 years and SD \pm 1.00 (mean age of male cases was 23.0 years, SD \pm 3.25 and mean age of female cases was 40 years, SD \pm 5.30). The median WBC count of cases was 40000 cells/ μ l/cumm (ranging from 15000 to 74000 cells/ μ l/cumm) and the median count of blast cells was found 70% (ranging from >40% to >80%).

DNA Extraction

Specimens were collected from 51 routinely-processed unstained bone marrow slides and blood diagnosed as AML, from Haematology unit of Department of Pathology, Era's Lucknow Medical College and Hospital as well as other hospitals of Lucknow, Northern India and stored in -20° C. Genomic DNA was extraction by using a commercially available DNA extraction kit (Medox, India).

Polymerase Chain Reaction analysis

Polymerase Chain Reaction (PCR) was performed with 25 μ l PCR reaction mixture containing 200ng of template DNA, 10 pmol of each primer, 10mmol/L

CODON	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591
Wild Type	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F
Case9	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	■	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	■	E	F	P	R	N	R	L	S	F
Case10	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	■	L	S	F
Case11	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	■	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F
Case12	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	■	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F
Case13	■	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F
Case20	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	■	Y	I	D	P	T	Q	L	P	■	D	Q	K	W	E	F	P	R	N	R	L	S	F
Case23	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	Q	K	■	E	F	P	R	N	R	L	S	F
Case27	K	P	M	Y	E	V	Q	W	K	V	V	E	E	■	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F

Figure 2. Amino acid sequences of the Exon 11 of c-kit gene. The sequence starts at codon 550 and ends at 591. The wild-type sequence is shown above. Point mutations are shown in ■.

of each mix dNTPs, 1X reaction buffer and 0.3 units of Taq polymerase enzyme (Fermentas, Germany) in an MJ Mini Thermocycler (Bio-Rad, UK). As per the cycling conditions denaturation at 94°C for 30 seconds, followed by annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, repeated for 35 cycles followed by a final extension step at 72°C for 10 minutes using the primers [11], forward 5'- ATTATTAAGGTTGATCTATTTTC- 3' and reverse 5'- ACTGTTATGTGTACCCAAAAG- 3'. Resultantly, obtained 257 bp long amplicons and tested out on 2% agarose gel electrophoresis (Figure1).

Single-Strand Conformational Polymorphism analysis

Single-Strand Conformational Polymorphism (SSCP) analysis was performed according to Orita et al. [18], with little modifications. Samples were denatured at 94°C for 5 minutes with denaturing dye and immediately snap cooled. Equal amount of amplified PCR product (15µl) was loaded along with denaturing dye on 8% polyacrylamide gel. Gel was run in pre-cooled 1X TBE (Tris Borate EDTA) buffer. The gel tank was placed in a cold room at 4°C and run for 15 hours at 140V. DNA on the gel was stained after electrophoresis with silver stain. Electrophoresis mobility shift in single-stranded or double stranded DNA product from patients was detected while comparing with the DNA product from normal controls run in adjacent lanes.

Sequencing

Amplicons was sequenced using an automated sequencer, ABI 3730XL DNA Analyzer (Applied Biosystems, Foster city, California, USA) and analyzed using FinchTV Software. Mutations were reconfirmed by sequencing amplicons in both directions and in independent second samples. Sequence was analysed using the BioEdit and BLAST

(National Center for Biotechnology Information) software.

Results

Out of 51 acute myeloid leukaemia cases, 33 samples (64.70%) showed a position shift in native SSCP-PAGE. In our study, 15.68% (8/51) AML cases showed c-kit exon 11 mutations. We have detected total 10 mutations in 8 AML cases that include Lys550Asn, Tyr568Ser, Ile571Leu, Tyr578Pro, Trp582Ser and Arg588Met and novel missense mutations at codons Ile563Lys and Val569Leu in M0, M1, M2 and M4 subtypes of AML. Mutations at codons Ile571Leu and Trp582Ser were obtained in two independent cases and the majority of mutations detected in our study were found in AML cases with high count of WBC and blast cells (Figure2-3 and Table 1).

Discussion

This is the first study to report mutations in exon 11 region of the c-kit gene in AML cases in Northern India. Previous molecular studies have showed a number of mutations in exon 11 in diverse types of tumour in different ethnic groups. Mutations in exons 8, 9, 13 and 17 of the c-kit gene are less frequently detected than in exon 11 in dissimilar kind of neoplasia [15, 19,20]. In Gastrointestinal Stromal Tumours (GISTs) 65–92% of them are reported to harbour KIT-activating mutations, the majority of which are localized in the juxtamembrane region involving exon 11 [19, 21]. C-kit exon 11 missense mutations is the most common one seen in GISTs and it appears as deletion, missense mutation and duplication of 3' end [22].

Novel exon 11 mutations Glu562Val, His580Tyr, Phe584Leu and phe591Leu has been reported by Lizette V et al [12]. The majority of exon 11 mutations are clustered within the classic hotspot region of the 50 end involving codons 550–560;

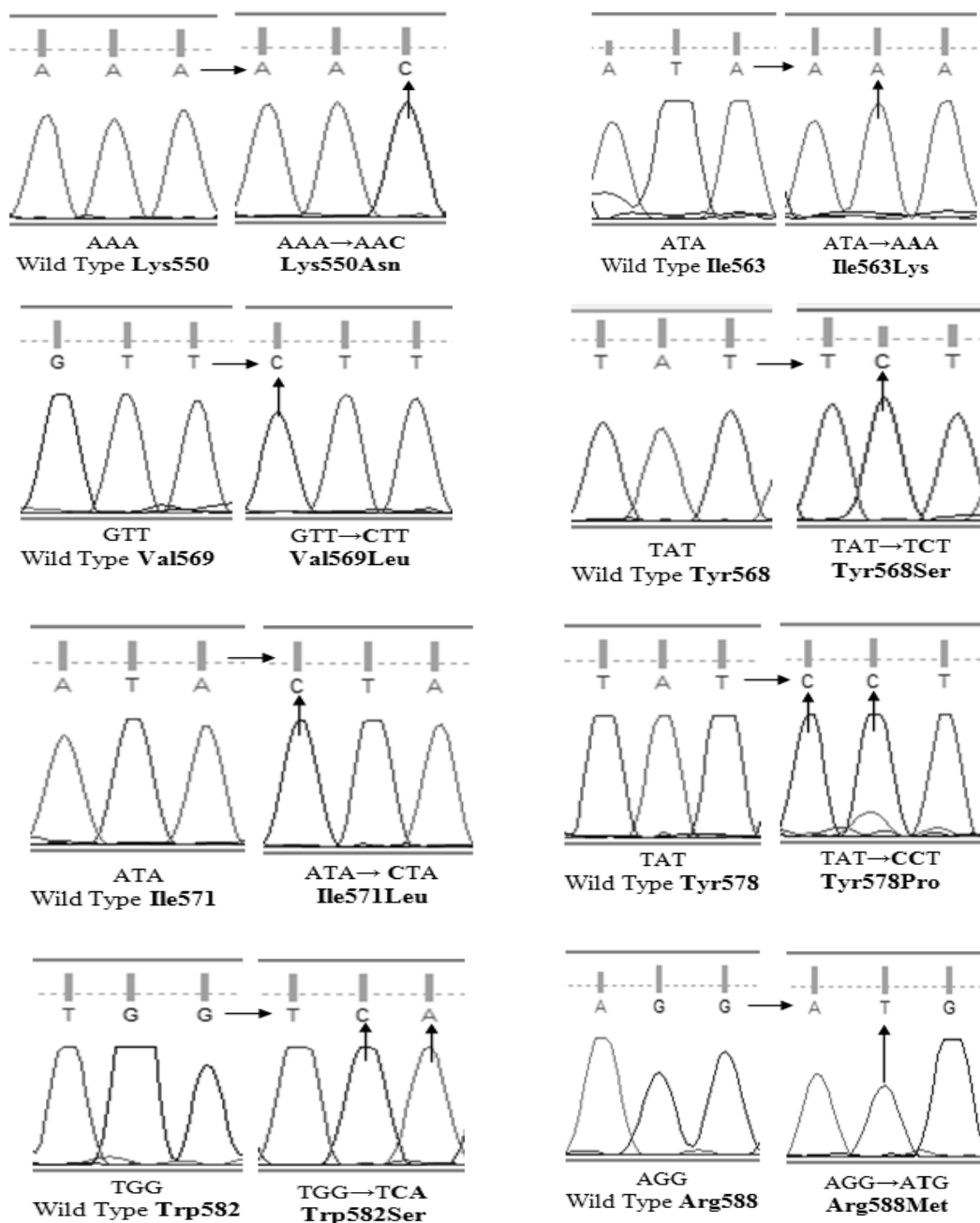


Figure 3. C-kit gene exon 11 point (missense) mutations A→C, T→A, G→C, A→C, A→C, T→C, A→C, G→C, G→A and G→T (resulting in the amino-acid substitution Lys550Asn, Ile563Lys, Val569Leu, Tyr568Ser, Ile571Thr, Tyr578Pro, Trp582Ser, Arg588Met)

however, another hotspot at the 30 end involving codons 576–590 have been described by Antonescu et al. 2003 [23]. These include frame deletions of one to several codons (typically involving codons 557–560); missense mutations and internal tandem duplications (typically involving the 30 end). We have reported total 8 different missense mutations in

10 AML cases. In our study the missense mutation Lys550Asn found in one AML-M2 case and Ile571Leu found in two AML-M4 cases that has been previously reported [20, 24], and the missense mutation Ile571Leu is detected in two independent cases. The mutations at codon 582 reported by Tae et al. 2004 [25], are Trp582Tyr and Trp582His, whereas

Table 1. Clinical data and summary of c-kit exon 11 missense mutations detected in AML and comparison with kit mutation reported in major studies

Case No.	Clinical History	Substitution	Type AML	Mutations (our study)	Reported Mutations	
	WBC Count	Blast Cells/ μ l/cu mm				
9	50,000	>80	TAT \rightarrow TCT TGG \rightarrow TCA	M2	Tyr568Ser Trp582Ser	Tyr568Asp [26] Trp582Tyr, Trp582His, Trp582Gln [11]
10	25,000	>60	AGG \rightarrow ATG	M0	Arg588Met	Arg588Phe, Arg588Tyr, Arg588Lys [8, 25]
11	30,000	<50	ATA \rightarrow CTA	M4	Ile571Leu	Ile571Leu [24]
12	35,000	>40	ATA \rightarrow CTA	M4	Ile571Leu	Ile571Leu [24]
13	15,000	>40	AAA \rightarrow AAC	M2	Lys550Asn	Lys550Asn [25]
20	60,000	>80	GTT \rightarrow CTT TAT \rightarrow CCT	M1	Val569Leu Tyr578Pro	Novel mutation Tyr578Phe [25]
23	18,000	<50	TGG \rightarrow TCA	M0	Trp582Ser	Trp582Tyr, Trp582His, Trp582Gln [11, 25]
27	74,000	>60	ATA \rightarrow AAA	M1	Ile563Lys	Novel mutation

by Ying-Yong et al. 2004 [8], are Trp582Try and Trp582Gln, but we have detected different substitution at the same codon in which tryptophan is replaced by serine Trp582Ser found in two independent AML-M0, M2 cases. Mutations at codons Tyr568Asp, Tyr578Phe, and Arg588Phe, Arg588Tyr, Arg588Lys have been reported by Tae et al. 2004 [25], Masahiko et al. 1999 [26], Ying-Yong et al. 2004 [8], but we have detected different substitutions at Tyr568Ser found in one AML-M2 case, Tyr578Pro found in one AML- M1 case and Arg588Met found in one AML-M0 case, which has not been previously reported.

Moreover, we have detected two novel missense mutations at codons Ile563Lys found in one AML-M1 case and Val569Leu found in one AML-M1 case, which has not been reported in any types of neoplasia. The c-kit gene exon 11 mutations detected in our study, positioned between codons 550-591 obviously endorse previous studies reporting mutations in different populations with neoplasia and the majority of mutations detected in our study were found in AML cases with high count of WBC and Blast cells as shown in (Table 1) and might be associated with disease development and the prognosis.

In summary, this study for the first time report the presence of c-kit gene mutations in AML cases in Northern India. These mutations in exon 11 might be involved in the KIT over-expression in AML cases as these mutations are located in juxtamembrane

domain. Since the c-kit mutations are seen in other malignancies, it appears that mutations in exon 11 of the c-kit gene might be involved in pathogenesis and represent valuable predictive genetic marker in case of AML. Further studies with a larger group of cases will be required to determine the prognostic implications and to investigate how these mutations are co-related to the progression and pathogenesis of AML.

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Conflict of Interest

The authors have no conflict of interest in this article.

Authors' Contribution

SRH, STR conceived, designed the study, interpreted the results, draft the manuscript and carried out the data analyses. SGB, PS, HN contributed to data gathering, participated in writing and revising the manuscript, while FM revised and approved the final manuscript. All authors read and approved the final manuscript.

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