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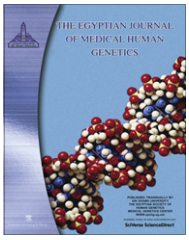

ORIGINAL ARTICLE

A study of KIT activating mutations in acute myeloid leukemia M0 subtype in north India

Syed Rizwan Hussain [a,*], Hena Naqvi [a], Pradyumn Singh [c], Sunil G. Babu [b], Farzana Mahdi [a]

[a] *Department of Biotechnology, Era's Lucknow Medical College and Hospital, Lucknow 226 003, India*
[b] *Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow 226 025, India*
[c] *Department of Pathology, Christian Medical College, Vellore 632 002, India*

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Abstract Acute Myeloid Leukemia (AML)-M0 is a cancer of blood-forming cells in the bone marrow. KIT gene is a receptor tyrosine kinase class III that is expressed on by early hematopoietic progenitor cells and plays an important role in hematopoietic stem cell proliferation, differentiation and survival. Mutations of KIT receptor tyrosine kinase are involved in the constitutive activation and development of human hematologic malignancies. We have designed this study aiming to identify and determine the frequency and prevalence of mutations in North Indian patients suffering from AML-M0. To perceive the KIT gene mutations, we have carried out PCR–SSCP followed by direct DNA sequencing in 50 AML-M0 cases. We have found eight cases (24.2%) with *t*(8;21) having 12 point mutations whereas three cases (17.6%) with inv(16) having four point mutations. The point mutation detected at exon 9 in five cases is Asp496Val. Eight different point mutations were identified at exon 11 in seven AML-M0 cases that include Lys550Asn, Tyr568Ser, Ile571Leu, Tyr578Pro, Trp582Ser and Arg588Met. Point mutations at codons Ile571Leu and Trp582Ser was found in two independent cases. Three point mutations were found in exon 17 (Leu813Pro, Lys818Arg, Val825Ala) in three AML-M0 cases. The results underline that the KIT gene appears to be most frequently mutated target in AML-M0 cases. These observations suggest

* Corresponding author. Tel.: +91 522 2408122/2408123.
E-mail address: rizwan_59@rediffmail.com (S.R. Hussain).

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that mutations in exon 11 of the KIT gene might be useful molecular genetic markers in AML-M0 and these mutations might be related to progression and clinical pathogenesis.

1. Introduction

KIT, a proto-oncogene is a member of the type III receptor tyrosine kinase (RTK) family [1] and plays a crucial role in normal haematopoiesis and acute myeloid leukemia (AML) [2,3]. The activation sphere of the receptor has resulted in the constitutive KIT kinase activity and KIT receptors harboring such mutations when introduced into mammalian cells downstream signaling pathways lead to factor-independent growth in vitro and leukemogenesis in vivo [4,5]. The genomic locus encoding the KIT gene receptor has 21 exons, ranging 100–300 bp [6] among various factors, mutations for cell differentiation and proliferation are considered to be effective factors in the development of AML [7]. Leukemia is classified based on the presence of specific cytogenetic abnormalities as well as the French–American–British (FAB) classification of the leukemic cells [8]. AML 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 [9]. In 1991 the FAB group published a proposal to designate minimally differentiated acute myeloid leukemia as AML-M0. By definition, the diagnosis of AML-M0 requires less than 3% MPO/ and/or SBB/blasts, expression of myeloid-associated markers, and lack of B/T-lineage-associated antigens [10]. Abnormalities in genes that are involved in signal transduction pathways are common in AML. Mutations in the KIT and FLT3 RTK have been described frequently in AML [11]. It is evident from several studies that there is a high expression of KIT in AML cases (60–80%) [12,13]. KIT point mutations have been reported in 33.35–45% of AML cases together with inv(16) and 12.8–46.8% of AML M2 along with *t*(8;21) [14–16]. Intriguingly, KIT mutations have been preferentially associated with AML exhibiting either an inv(16) or a *t*(8;21) karyotype, i.e. the core binding factor leukemias [14]. Point mutations of KIT have been found in approximately 5% of AML samples [17]. KIT mutation provides the myeloid blasts with the extra hit by conferring proliferation and anti-apoptotic activity, as the chimeric transcription factor impairs normal differentiation but has a limited effect on cellular proliferation [18]. Although, many studies screened KIT mutations but only in a proportion of KIT coding sequence and others were limited by small case number. Additional chromosomal abnormalities are frequently associated with *t*(8;21) and trisomy 4 in particular has freshly been suggested to constitute a individual subtype of *t*(8;21) AML [19]. Findings of studies investigating the molecular consequences of trisomy 4 focus on the dosage effect resulting from the duplication of a mutated KIT allele [20]. Until now, no study has reported the frequency and prevalence of mutations in exon 9, 11 and 17 of KIT gene in AML-M0 cases in India. In this study we have screened the mutation status of KIT gene in AML-M0 and further explored whether the KIT gene mutations frequently occurs in AML-M0. Due to insufficient studies of the mutations in North India, the diagnosis and frequency of these mutations in AML-M0 patients are an important concern.

2. Subjects and methods

2.1. Specimen collection

The study group included 50 cases of AML-M0. Ethical approval was obtained from the institutional ethical committee of Era's Lucknow Medical College and Hospital, Lucknow, Uttar Pradesh, India. The bone marrow samples were stained by Leishman stain method and cases were classified, according to the French American British (FAB) criteria [10]. The study was carried out during June 2006 to May 2011.

2.2. Molecular studies

Specimens collected were 50 bone marrow slides diagnosed as leukemia from the Department of Pathology at Era's Lucknow Medical College and Hospital and other hospitals and pathology centers in Lucknow. Extraction of genomic DNA was done by using a commercially available DNA extraction kit (Medox, India) and the DNA was stored at −20 °C.

2.2.1. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed with 20 μl PCR reaction mixture containing 250 ng of template DNA, 20 pmol of each primer, 10 mmol/L of each mix dNTPs, 1× reaction buffer and 1 unit of Taq polymerase enzyme (Fermentas, Germany) in an MJ Mini Thermocycler (Bio-Rad, UK). The cycling conditions included preliminary denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, followed by annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. A final extension was given at 72 °C for 10 min using the primers for Exon 9 Forward 5′-GGC TTT TGT TTT CTT CCC TTT -3′ and Reverse 5′- GAA GTC TTG CCC ACA TCG TT -3′ [Designed by JustBio.com software], Exon 11 Forward 5′-ATT ATT AAA AGG TGA TCT ATT TTT C-3′ and Reverse 5′-ACT GTT ATG TGT ACC CAA AAA G-3′, Exon 17 Forward 5′-TTC ACT CTT TAC AAG TTA AAA TG-3′ and Reverse 5′-GGA CTG TCA AGC AGA GAA TG-3′ [21].

2.2.2. Mutational screening of KIT exons

Single-strand conformational polymorphism (SSCP) analysis was performed according to Orita et al. [22] with little modifications. Samples were denatured at 95 °C for 6 min with denaturing dye and immediately transferred to ice. Fifteen microliter of amplified PCR product was loaded along with 15 μl of denaturing dye on 10% polyacrylamide gel. Gel was run in pre-cooled 1× TBE buffer (Tris–Borate EDTA). The gel tank was placed in a cold room at 4 °C and run for 12 h at 150 V. 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 by comparison with DNA product from normal controls run in adjacent lanes (Fig. 1).

2.2.3. KIT mutational analysis

Amplicons were sequenced using an automated sequencer, ABI 3730XL DNA Analyzer (Applied Biosystems, Foster city,

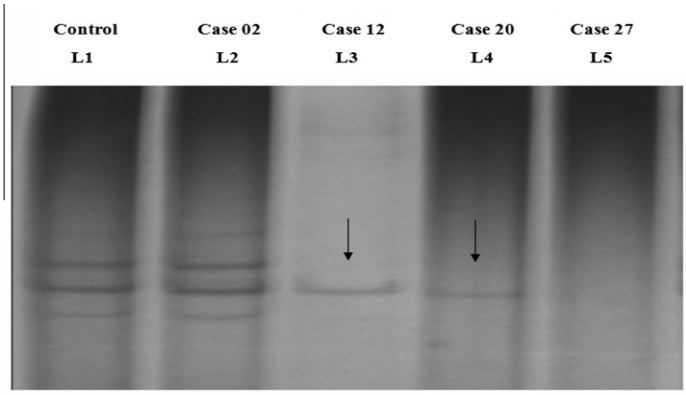

Figure 1 SSCP–PAGE showing electrophoresis mobility shift on native page. Control in Lane 1 and cases in 2, 3, 4 and 5th lane (no shift in case 02; shift in case 12, 20 and no result in case 27).

California, USA). Mutations were reconfirmed by sequencing amplicons in both directions and in independent second samples. Sequence was analyzed using the FinchTV, BioEdit and BLAST (National Center for Biotechnology Information) software.

3. Results

The study comprised of bone marrow slides from 27 males and 23 females with age ranging from 5 years to 60 years. The mean age of onset of AML was 30.3 years and SD ± 1.00 (mean age of male cases was 23.0 years, SD ± 3.25 and mean age of female cases was 40 years, SD ± 5.30) years. The median WBC count in the cases was 40,000 cells/μl/cumm (ranging from 15,000 to 74,000 cells/μl/cumm) and the median count of blast cells was 70% (ranging from >40% to >80%). Out of 50 AML cases, 33 cases carried *t*(8;21) and 17 cases had inv(16). (Table 1).

3.1. Mutational analysis of exon 9

Out of 50 AML cases, 21 samples displayed a shift in position in native SSCP–PAGE. These were directly sequenced by an automated sequencer. Five point mutations were detected in five AML-M0 cases (Table 2 and Table 3).

3.2. Mutational analysis of exon 11

Out of 50 AML cases, 33 samples displayed a shift in position in native SSCP–PAGE. These were directly sequenced by an automated sequencer. Eight point mutations were detected in seven AML-M0 cases (Table 2 and Table 3).

3.3. Mutational analysis of exon 17

Out of 50 AML cases, 27 samples displayed a shift in position in native SSCP–PAGE. These were directly sequenced by an automated sequencer. Three point mutations were detected in three AML-M0 cases (Table 2 and Table 3).

4. Discussion

This study is the first to report mutations in the KIT gene in AML-M0 cases in north India. Previous molecular studies have revealed several mutations in different types of tumors in different ethnic groups. Mutations in exons 9, 13 and 17 of the KIT gene are less frequently detected than in exon 11 in different types of neoplasia [23–25]. In gastrointestinal stromal tumors, 65–92% of tumors are reported to harbor KIT-activating mutations, the majority of which are localized in the juxtamembrane region involving exon 11 [23]. The majority of exon 11 mutations is clustered within the classic hotspot region of the 50 end involving codons 550–560; however, a second hotspot at the 30 end involving codons 576–590 has been described by Antonescu et al. [26]. These include frame deletions of one to several codons (typically involving codons 557–560); point mutations and internal tandem duplications (typically involving the 30 end).

In our study, when the samples were analyzed on the basis of cytogenetics, there were two major sets: Set I comprised of 33 cases having *t*(8;21) and Set II comprised of 17 cases having inv(16). When these cases were further analyzed for kit mutations at exon 9, 11 and 17, we found that eight cases (24.2%) with *t*(8;21) showed 12 point mutations whereas three cases (17.6%) with inv(16) showed four point mutations. The point mutation detected at exon 9 in five cases is Asp496Val which has already been reported [25] (Table 2 and Table 3). Eight different point mutations were identified at exon 11. The point mutations Lys550Asn and Ile571Leu detected in our study have been previously reported [24,27] and the mutation Ile571Leu is detected in two independent cases. The mutations at codon 582 reported by Tae et al. [28] are Trp582Tyr and Trp582His, whereas by Ying-Yong et al. [29] are Trp582Try and Trp582Gln, but we have detected different substitution at the same codon in which tryptophan is replaced by serine (Trp582Ser) in two independent cases. Mutations at codons Tyr568Asp, Tyr578Phe, and Arg588Phe, Arg588Tyr, Arg588 Lys have been reported by Tae et al. [28], Masahiko et al. [30], Ying-Yong et al. [29]. At Exon 17 we detected three point mutations at codons Leu813Pro, Lys818Arg and Val825Ala [25,31,32]. Noteworthy to quote here, that we have identified point mutations at exon 9, 11 and 17 in eleven cases. The c-kit gene exon 9, 11 and 17 mutations detected during our study, located between codons 450–500 in exon 9, 550–591 in exon 11 and 788–828 in exon 17, endorse previous studies reporting mutations in different populations with neoplasia which has been summarized in Table 2 and Table 3. The majority of mutations detected in our study was found in cases with high count of WBC and Blast cells as shown in Table 1 and might be

Table 1 Clinical and genetic features with cytogenetic findings of 50 AML-M0 cases.

Total	*t*(8;21)					inv(16)				Total
	KIT(+) mutation		KIT(−) mutation		Total	KIT(+) mutation		KIT(−) mutation		
	n	%	*n*	%	*n*	*n*	%	*n*	%	*n*
	8	24.2	25	75.7	33	3	17.6	14	82.3	17
Age (years)										
⩽60	5	62.5	3	37.5	8	1	12.5	7	87.5	8
>60	3	12	22	88	25	2	22.2	7	77.7	9
Sex										
Male	4	23.5	13	76.4	17	1	10	9	90	10
Female	4	25	12	75	16	2	28.5	5	71.4	7
WBC										
$\leqslant 40^{\times} 10^{9}$/L	6	42.8	8	57.1	14	3	33.3	6	66.6	9
$> 40^{\times} 10^{9}$/L	2	10.5	17	89.4	19	0	0	8	100	8
Blast cells										
⩽80	5	41.6	7	58.3	12	1	7.69	12	92.3	13
>80	3	14.2	18	85.7	21	2	50	2	50	4

Table 2 Amino acid sequences of KIT gene point mutations at exon 9 (codon 450–500), 11 (codon 550–591) and 17 (codon 788–828).

Exon 9						
Codon	450	460	470	480	490	500
Wild type	CSASVLPVDV	QTLNSSGPPF	GKLWQSSID	SSAFKHNGTV	ECKAYNDVGK	T
Case 3	CSASVLPVDV	QTLNSSGPPF	GKLWQSSID	SSAFKHNGTV	ECKAYNVVGK	T
Case 12	CSASVLPVDV	QTLNSSGPPF	GKLWQSSID	SSAFKHNGTV	ECKAYNVVGK	T
Case 20	CSASVLPVDV	QTLNSSGPPF	GKLWQSSID	SSAFKHNGTV	ECKAYNVVGK	T
Case 23	CSASVLPVDV	QTLNSSGPPF	GKLWQSSID	SSAFKHNGTV	ECKAYNVVGK	T
Case 27	CSASVLPVDV	QTLNSSGPPF	GKLWQSSID	SSAFKHNGTV	ECKAYNVVGK	T
Exon 11						
Codon	550	560		570	580	590
Wild type	KPMYEVQWKV	VEEINGNNYV		YIDPTQLPYD	HKWEFPRNRL	SF
Case 9	KPMYEVQWKV	VEEINGNNSV		YIDPTQLPYD	HKSEFPRNRL	SF
Case 10	KPMYEVQWKV	VEEINGNNYV		YIDPTQLPYD	HKWEFPRNML	SF
Case 11	KPMYEVQWKV	VEEINGNNYV		YLDPTQLPYD	HKWEFPRNRL	SF
Case 12	KPMYEVQWKV	VEEINGNNYV		YLDPTQLPYD	HKWEFPRNRL	SF
Case 13	NPMYEVQWKV	VEEINGNNYV		YIDPTQLPYD	HKWEFPRNRL	SF
Case 20	KPMYEVQWKV	VEEINGNNYV		**YIDPTQLPPD**	HKWEFPRNRL	SF
Case 23	KPMYEVQWKV	VEEINGNNYV		YIDPTQLPYD	HK SEFPRNRL	SF
Exon 17						
Codon	788	798		808	818	828
Wild type	CIHRDLAARN	ILLTHGRITK		ICDFGLARDI	KNDSNYWKG	N
Case 3	CIHRDLAARN	ILLTHGRITK		ICDFGPARDI	KNDSNYVVKG	N
Case 39	CIHRDLAARN	ILLTHGRITK		ICDFGLARDI	**HNDSNYWVKG**	N
Case 46	CIHRDLAARN	ILLTHGRITK		ICDFGLARDI	KNDSNYVAKG	N

associated with disease development and prognosis. Basically, these mutations are huddled in the same region as other known KIT 16 mutations and most probably code for a constitutively activated protein. It is worth mentioning here that our study is the first to report the presence of KIT gene mutations in AML-M0 cases in north India. These findings point toward an important mutational part of KIT exon 8, 9 and 17 mutations in the pathogenesis of AML-M0 and provide the source for the KIT gene that might represent useful molecular genetic marker in AML-M0.

5. Conclusion

AML-M0 is heterogeneous with respect to morphology, cytochemistry, immunophenotyping, cytogenetics, and molecular genetics. At present, we still lack a specific marker or a set of biological and/or clinical features to single out this FAB subtype as a distinct disease entity. This study is first to report the presence of KIT gene mutations in AML-M0 cases in north India. These mutations in exon 11 may be involved in KIT over-expression in AML-M0 cases as these mutations

Table 3 Summary of KIT gene point mutations at exon 9, 11 and 17 in AML-M0 cases.

Case no.	Exons	Substitution	Mutations (our study)	Reported mutations	
3	Exon 9	GAT→G**TT**	Asp**496**Val	Asp**496**Val	[25]
	Exon 17	CTA→C**CA**	Leu**813**Pro	Leu**813**Pro	[25]
9	Exon 11	TAT→T**CT**	Tyr**568**Ser	Tyr**568**Asp	[30]
		TGG →T**CA**	Trp**582**Ser	Trp**582**Tyr, Trp**582**His, Trp**582**Gln	[21,28]
10	Exon 11	AGG→A**TG**	Arg**588**Met	Arg**588**Phe, Arg**588**Tyr, Arg**588**Lys	[28,29]
11	Exon 11	ATA→**CTA**	Ile**571**Leu	Ile**571**Leu	[27]
12	Exon 9	GAT→G**TT**	Asp**496**Val	Asp**496**Val	[25]
	Exon 11	ATA→**CTA**	Ile**571**Leu	Ile**571**Leu	[27]
13	Exon 11	AAA→AA**C**	Lys**550**Asn	Lys**550**Asn	[24]
20	Exon 9	GAT→G**TT**	Asp**496**Val	Asp**496**Val	[25]
	Exon 11	TAT→**CCT**	Tyr**578**Pro	Tyr**578**Phe	[28]
23	Exon 9	GAT→G**TT**	Asp**496**Val	Asp**496**Val	[25]
	Exon 11	TGG →T**CA**	Trp**582**Ser	Trp**582**Tyr, Trp**582**His, Trp**582**Gln	[21,28]
27	Exon 9	GAT→G**TT**	Asp**496**Val	Asp**496**Val	[25]
39	Exon 17	AAG→A**GG**	Lys**818**Arg	Lys**818**Arg	[31]
46	Exon 17	GTT→G**CT**	Val**825**Ala	Val**825**Ala	[32]

are located in Juxtamembrane domain. These observations suggest that mutations in exon 11 of the KIT gene might be useful molecular genetic markers in AML-M0 and these mutations might be related to progression and clinical pathogenesis.

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