Identification of novel point mutations in c-kit gene from Leukemia cases: a study from Lucknow, Uttar Pradesh, India

Identificación de nuevas mutaciones puntuales en el gen c-kit en casos de Leucemia: un estudio realizado en Lucknow, Uttar Pradesh, India

Syed Rizwan Hussain[1], Amna Siddiqui[3], Javier Vargas-Medrano[2], Hena Naqvi[1], Jonathon Mohl[2], Farzana Mahdi[1] and Fahim Ahmad[2,4]

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Abstract

The c-kit gene is a receptor tyrosine kinase (RTK) class III that is expressed in early hematopoietic progenitor cells. Aberrantly activated RTK and related downstream signaling partners have been reported as key elements in the molecular pathogenesis of several malignancies. Within the c-kit gene exon-11 is the most frequent site for mutations in different kinds of tumours. Mutations in c-kit gene may enhance or interfere with the ability of c-kit receptor to initiate the intracellular pathways resulting in cell proliferation. Therefore, we aimed to screen the mutations in c-kit gene at exon-8 and -11 in malignant Leukemias. Ninety Leukemia cases were studied and analyzed by mutation-specific PCR-SSCP followed by DNA sequencing. Twenty point mutations were detected in eight AML (acute myeloid Leukemia) cases within exon-11 which includes *Tyr568Ser, Ile571Thr, Thr574Pro, Gln575His, Tyr578Pro, Asp579His, His580Gln, Arg586Thr, Asn587Asp* and *Arg588Met.* The substitutions *Lys550Asn, Ile571Leu and Trp582Ser* were observed in two independent cases and four novel point mutations at codons *Ile563Lys, Val569Leu, Tyr570Ser,* and *Pro577Ser*. Further, six point mutations were detected at exon-8 in six cases (four AML and two CML cases), comprising three novel mutations *Asn423Asp, Gln448Thr, and Gln448His*. The point mutations *Thr417Asp*, *Tyr418Phe,* and *Leu421His* were observed, but were detected only in three cases. These observations suggest that mutations in c-kit gene might represent a useful molecular genetic marker in Leukemia and incidence of mutation at exon-8 and -11 is high and might be involve in pathogenesis of AML.

Palabras clave: c-kit, exon-8 and -11, Leukemia, mutation, SSCP-PAGE.

Resumen

El gen c-kit, que codifica para un receptor tirosina quinasa (RTK) de clase III, se expresa en las primeras células progenitoras hematopoyéticas. La activación de este RTK y su vía de señalización se encuentran involucradas en la patogénesis molecular de varias enfermedades. La mutación del gen c-kit en el exón 11 es una de las mutaciones más frecuentemente reportadas en diferente tipos de tumores. Mutaciones en c-kit podrían incrementar o interferir con la habilidad del receptor c-kit para iniciar la activación de cascadas de señalización intracelulares responsables en la proliferación celular. Por estas razones, estudiamos las mutaciones del gen c-kit en el exon 8 y 11 en casos con Leucemias. Noventa casos de Leucemia en la India fueron estudiados mediante PCR SSCP, seguida por secuenciación de DNA. Veinte mutaciones puntuales fueron detectadas en el exon 11 en tan solo ocho de los casos con AML (leucemia mieloide aguda), entre las que encontraron las mutaciones *Tyr568Ser, Ile571Thr, Thr574Pro, Gln575His, Tyr578Pro, Asp579His, His580Gln, Arg586Thr, Asn587Asp* y *Arg588Met.* Las sustituciones *Lys550Asn, Ile571Leu y Trp582Ser* fueron observadas en tan solo dos casos. Además, cuatro nuevas mutaciones para los codones *Ile563Lys, Val569Leu, Tyr570Ser,* y *Pro577Ser* se observaron en este estudio. En el exon 8, seis mutaciones puntuales fueron observadas y en seis de los casos (cuatro en AML y dos en CML) encontramos tres nuevas mutaciones *Asn423Asp, Gln448Thr* y *Gln448His*. Sin embargo, las mutaciones puntuales *Thr417Asp, Tyr418Phe* y *Leu421His* fueron observadas en varias ocasiones, pero en tan solo tres de los casos estudiados. Estas observaciones sugieren que las mutaciones en c-kit podrían representar un marcador genético para Leucemia. La incidencia en la mutación del exon 8 y 11 es elevada y podría estar relacionada con la patogénesis de la AML.

Palabras clave: c-kit, exones 8 y 11, Leucemia, mutación, SSCP PAGE.

[1] Department of Biochemistry, Era's Lucknow Medical College and Hospital, Lucknow, India.
[2] Center of Excellence for Infectious Diseases, Biomedical Sciences Department, Texas Tech University Health Science Center and Paul L. Foster School of Medicine, El Paso, TX, U.S.A. 79905
[3] Department of Anestheology, Texas Tech University, El Paso, TX, U.S.A.
[4] Author for correspondence: fahim.ahmad@ttuhsc.edu.

Introduction

Leukemia is classified based on the presence of specific cytogenetic abnormalities as well as the French-American-British (FAB) classification of the leukemic cells (Rowley, 1973). A mutation on c-kit gene, a member of the receptor tyrosine kinase (RTK) family type III, is the most frequently occurring genetic aberration in acute myeloid leukemia (AML). A number of observations also suggest a role for c-kit that is important for the development of a range of cells including hematopoietic cells in leukaemogenesis (Reilly, 2002).

High expression of c-kit in AML (60%-80% higher than control) has been reported (Reuss-Borst *et al.*, 1994; Cole *et al.*, 1996) and point mutations in c-kit gene have been identified in 33.4-45.0% of AML cases (Higuchi *et al.*, 2002). However, many of these studies looked for mutations in c-kit gen only at coding sequence region. It is known that c-kit is a Leukemia proto-oncogene and activating c-kit mutations are likely to contribute in the development of Leukemia in humans (Smith *et al.*, 2004; Piloto *et al.*, 2006; Gao *et al.*, 2011; Marcucci *et al.*, 2011). The activation sphere of the receptor has resulted in the constitutive c-kit kinase activity and c-kit receptors harboring such mutations when introduced into mammalian cells downstream signaling pathways lead to factor-independent growth *in vitro* and leukemogenesis *in vivo* (Ihle *et al.*, 1995; Gao *et al.*, 2011). The c-kit gene is a member of the class III tyrosine kinase receptor family that includes the platelet-derived growth factor receptors (PDGFRs) (Ullrich *et al.*, 1990; Matthews *et al.*, 1991; Martín-Broto *et al.*, 2010). Class III receptor tyrosine kinases (RTKs) share sequence homology and have an overall similar structure with five immunoglobulin-like repeats in the extracellular domain, a single transmembrane domain (TM), a juxtamembrane domain (JM), two intracellular tyrosine kinase domains (TK1 and TK2) divided by a kinase insert domain (KI), and a C-terminal domain (Yarden and Ullrich, 1988). The genomic locus encoding the c-kit gene receptor has 21 exons, ranging 100-300 base pairs (bp) (Abu-Duhier *et al.*, 2001). The c-kit gene mutations in exon-11 are reported in gastrointestinal stromal tumors, human solid tumors and human germ cell tumors (Qingsheng *et al.*, 1999; Hou *et al.*, 2004; Harri *et al.*, 2005). Until now, no study has reported the frequency and prevalence of mutations in exon-8 and -11 of c-kit gene in Leukemia patients from northern India. In this study we have screened the mutation status of exon-8 and -11 of c-kit gene in malignant Leukemias (Acute Myeloid Leukemia, Acute Lymphoblastic Leukemia, Chronic Myeloid Leukemia and Chronic Lymphocytic Leukemia) and further explored whether the c-kit gene mutations were valuable as malignant markers in Leukemia.

Material and Methods

Subjects. The study group included 90 cases of Leukemia, from the Department of Pathology at Era's Lucknow Medical College and Hospital, and from other hospitals and pathologies situated in and around the city of Lucknow, Uttar Pradesh, in northern India. Ethical approval was obtained from the Institutional Ethical Committee of Era's Lucknow Medical and Hospital, Lucknow, Uttar Pradesh, India. In addition, clinical data was also recorded. The blood or bone marrow samples were stained by Leishman stain method and the cases were classified, according to the FAB criteria (Bennett *et al.*, 1976). From the 90 Leukemia patients, 60 (66.7%) samples were with Acute Myeloid Leukemia (AML), 10 (11.1%) samples with Acute Lymphoblastic Leukemia (ALL), 10 (11.1%) samples with Chronic Myeloid Leukemia (CML) and 10 (11.1%) samples with Chronic Lymphocytic Leukemia (CLL). The demographic profile of patients can be finding at supplementary table 1, as well as for controls (supplementary table 2).

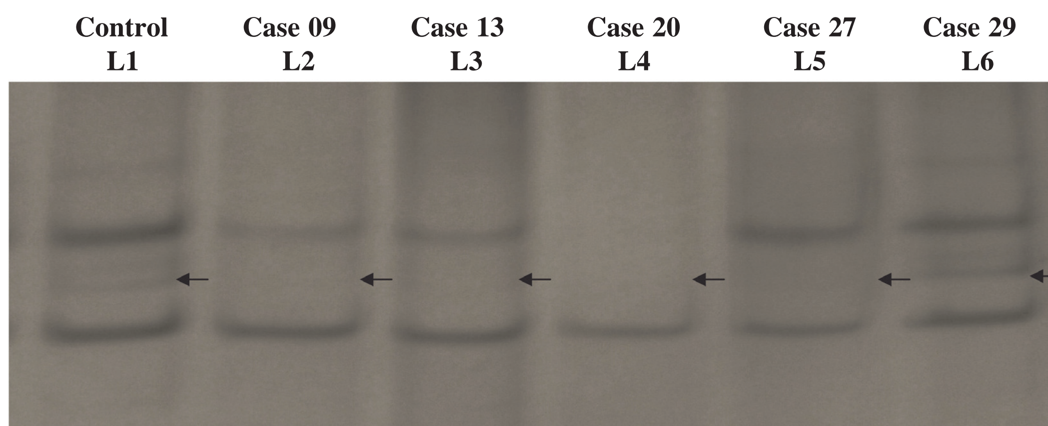
Sample collection and DNA extraction. Specimen was collected from 90 routinely-processed unstained bone marrow slides and blood diagnosed as Leukemia. Patients were from the Department of Pathology at Era's Lucknow Medical College and Hospital, and from other hospitals and pathologies situated in and around the city of Lucknow, Uttar Pradesh, India. Finally, samples were stored at -20°C. Genomic DNA was extracted according to Moskaluk *et al.* (1997) with little modifications.

Polymerase Chain Reaction and Single-Strand Conformational Polymorphism (PCR SSCP). Polymerase Chain Reaction (PCR) was performed in a 25 µl of 1X PCR reaction containing 200 ng of template DNA, 10 pmol of each primer (forward and reverse primers), 10 mmol/L of dNTPs and 0.3 units of Taq DNA polymerase (Fermentas, Germany). Forward and reverse primers for exon-8 were 5'-GGCCATTTCTGTTTTCCTGT-3' and 5'-TCTGCTCAGTTCCTGGACAA-3' respectively. Both were designed and customized by entering the sequence from exon-8 into the JustBio.com server. Forward and reverse primers for exon-11 5'-ATTATTAAAAGGTGATCTATTTTTC-3' and 5'-ACTGTTATGTGTACCCAAAAAG-3' respectively, were proposed by Qingsheng Tian *et al*. (1999).

Amplifications were done using a MJ Mini Thermocycler (Bio-Rad, UK). The cycling conditions were adjusted from the procedure proposed by Tian *et al.* (1999). Briefly, denaturation was at 94°C for 40 seconds, followed by annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, repeated for 30 cycles followed by a final extension step at 72°C for 8 minutes. Single-strand conformational polymorphism (SSCP) analysis was performed according to Orita *et al*. (1989) with few modifications. Samples were denatured at 94°C for 8 minutes and immediately snap-cooled. Fifty µl of amplified PCR product were loaded along with 20 µl of stop dye in a 10% polyacrylamide gel. The gel was run in pre-cooled 2X buffer at 4°C, for 12 hours at 150 volts. The DNA in the gel was stained after separation by electrophoresis using a silver stain. Electrophoresis mobility shift in single stranded or double stranded DNA from patients was detected and compared with DNA from wild-type controls (Fig. 1).

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

Figure 1. SSCP-PAGE analysis showing electrophoresis mobility shift on native page. DNA control was loaded in lane 1 and DNA from cases in 2, 3, 4, 5, and 6th lane (no shift in case 29 was observed, however, there were shifts in cases: 09, 13, 20, and 27).



Results

Out of 90 Leukemia cases 51 (56.7%) were male and 39 (43.3%) were female with age ranging from 2-65 years. The mean age of cases is 38.25 years with a SD ± 6.21 (mean age of male cases was 38.60 years, SD ± 6.27 and mean age of female cases was 37.79 years, SD ± 6.15). The cases were classified according to the FAB criteria (Moskaluk *et al.*, 1997) as acute myeloid leukemia (AML) (*n*=60), ALL (*n*=10), CML (*n*=10), CLL (*n*=10). The details of clinical feature and demographic profile are shown in supplementary tables 1 and 2. Out of 90 Leukemia cases, 80 samples were found to have mutations by a shift in DNA position on SSCP-PAGE with respect to DNA from healthy donors (Fig. 1). A total of 17 point mutations for c-kit gene at exon-11 were found in this investigation and only in eight cases with AML (Table 1, Fig. 2 and 5). In addition, six point mutations for c-kit gene at exon-8 for six AML and CML cases were detected by our experiments (Table 2, Fig. 3 and 4). After comparison to previous reported findings, as is shown in tables 3 and 4, c-kit point mutations at exon-11 for codons Ile563Lys, Val569Leu, Tyr570Ser, and Pro577Ser, and at exon-8 for codons Asn423Asp, Gln448Thr, and Gln448His are describe here for the first time.

Table 1. c-kit gene point mutations at exon-11 in Leukemia cases.

Case	Leukemia Type	Nucleotide	Codon
09	AML	TAT → TCT	Tyr**568**Ser
		TGG → TCA	Trp**582**Ser
		AGG → ATG	Arg**588**Met
11	AML	ATA → CTA	Ile**571**Leu
12	AML	ATA → CTA	Ile**571**Leu
13	AML	AAA → AAC	Lys**550**Asn
17	AML	AAA → AAC	Lys**550**Asn
20	AML	TAC → TCC	Tyr**570**Ser
		CCT → TCC	Pro**577**Ser
		TAT → CCT	Tyr**578**Pro
		GAT → CAT	Asp**579**His
		CAC → CAA	His**580**Gln
		TGG → TCA	Trp**582**Ser
		AAC → GAC	Asn**587**Asp
23	AML	GTT → CTT	Val**569**Leu
27	AML	ATA → AAA	Ile**563**Lys
		ATA → ACA	Ile**571**Thr
		ACA → CCA	Thr**574**Pro
		CAA → CAC	Gln**575**His
		AGA → ACA	Arg**586**Thr

After our findings, where we found point mutations around the protein, it was important to address where in the protein these mutation where located in order to determine the possible implication(s) of these mutations in protein function. Therefore, we analyzed the protein sequence (reference number for c-kit protein is P10721) using the UnitPro Knowledge Base server. Mutations for exon-11 are located between positions 546-976 bp and are in a cytoplasmic domain. Mutation Tyr568Ser is located in a metal binding site, specifically, a magnesium binding site. In addition, the Tyr residue at this position is normally autophosphorylated by autocatalysis (Price *et al.*, 1997; Mol *et al.*, 2003; Sun *et al.*, 2009; Zadjali *et al.*, 2011). Moreover, mutations Val569Leu and Tyr570Ser are located in a domain that interacts with phosphotyrosine-binding proteins, and residue Tyr570 is

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. Seventeen point mutations in c-kit gene at exon-11 are highlighted in grey colour. Case number is indicated at the left column.

CODON	550 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 591
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
Case 9	K P M Y E V Q W K V V E E I N G N N S V Y I D P T Q L P Y D H K W E F P R N M L S F
Case 11	K P M Y E V Q W K V V E E I N G N N Y V Y L D P T Q L P Y D H K W E F P R N R L S F
Case 12	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 H H K W E F P R N R L S F
Case 13	N 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
Case 17	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 P D H K W E F P R N R L S F
Case 20	K P M Y E V Q W K V V E E I N G N N Y V S I D P T Q L S Y D Q K S E F P R D R L S F
Case 23	K P M Y E V Q W K V V E E I N G N N Y L Y I D P T Q L P Y D H K W E F P R N R L S F
Case 27	K P M Y E V Q W K V V E E K N G N N Y V Y T D P P H L P Y D H K W E F P T N R L S F

normally autophosphorylated by autocatalysis. All point mutations in exon-8 are located in an Ig-like C2-type 5 domain which is located in position 413-507 bp and is part of the extracellular portion of the protein (residues 26-527). According to this information it seems most probably, that mutations in exon-11 will produce the worse alterations to the normal function of c-kit, because these mutations are located in places for autophosphorylation and magnesium binding sites. However, mutations at exon-8 may be affected ligand binding.

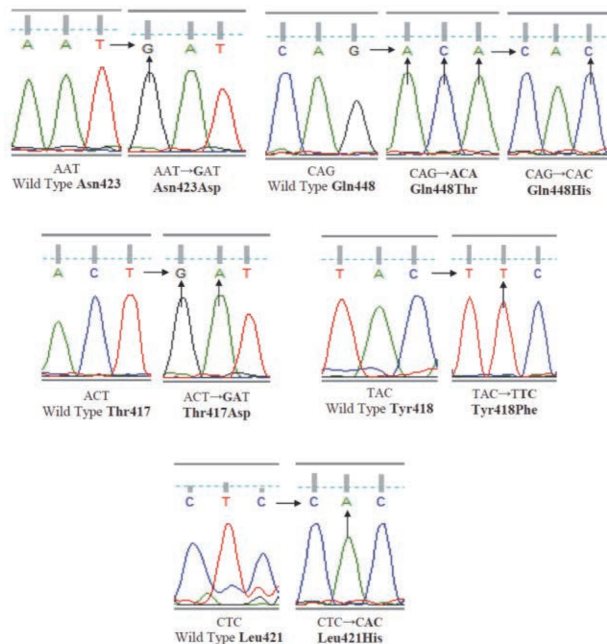
Table 2. c-kit gene point mutations at exon-8 in Leukemia cases.

Case	Leukemia Type	Nucleotide	Codon
05	AML	AAT → GAT	Asn423Asp
33	AML	CAG → CAC	Gln448His
56	AML	ACT → GAT	Thr417Asp
59	AML	CAG → ACA	Gln448Thr
60	CML	TAC → TTC	Tyr418Phe
81	CML	CTC → CAC	Leu421His

Figure 3. Amino acid sequences of the exon-8 of c-kit gene. The sequence starts at codon 412 and ends at 448. The wild-type sequence is shown above. Six point mutations in c-kit gene at exon-8 are shown in gray colour. Case number is indicated at the left column.

CODON	412 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 448
Wild Type	K P E I L T Y D R L V N G M L Q C V A A G F P E P T I D W Y F C P G T E Q
Case 5	K P E I L T Y D R L V D G M L Q C V A A G F P E P T I D W Y F C P G T E Q
Case 33	K P E I L T Y D R L V N G M L Q C V A A G F P E P T I D W Y F C P G T E H
Case 56	K P E I L D Y D R L V N G M L Q C V A A G F P E P T I D W Y F C P G T E Q
Case 59	K P E I L T Y D R L V N G M L Q C V A A G F P E P T I D W Y F C P G T E T
Case 60	K P E I L T F D R L V N G M L Q C V A A G F P E P T I D W Y F C P G T E Q
Case 81	K P E I L T Y D R H V N G M L Q C V A A G F P E P T I D W Y F C P G T E Q

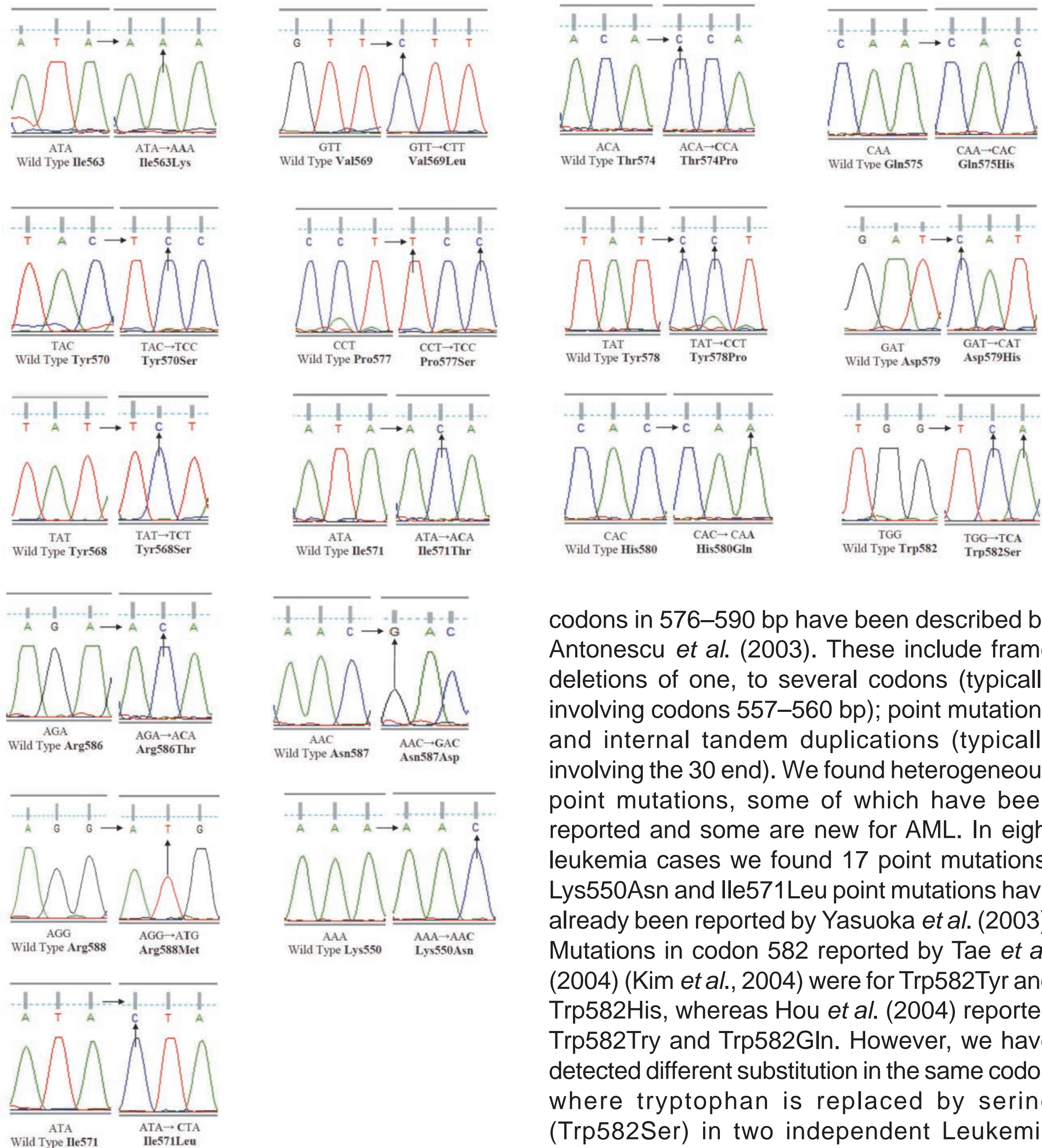
Figure 4. Mutations found during the sequencing analysis of c-kit at exon-8. Point mutations A→G, C→A, A→C, G→A, G→C, A→T, and T→A (resulting in the amino-acid substitutions Asn423Asp, Gln448Thr, Gln448His, Thr417Asp, Tyr418Phe, and Leu421His).



Discussion

To the best of our knowledge, this study is the first done from in and around the city of Lucknow, Uttar Pradesh, northern India. Here we report mutations in exon-8 and exon-11 of c-kit gene in Leukemia patients. Previous molecular studies in Asian populations (Chinese, Korean, and Japanese) have revealed several mutations in exon-11 in various types of tumours (Hou *et al.*, 2004; Choe *et al.*, 2006; Taniguchi *et al.*, 1999; Kim *et al.*, 2004). Mutations in exons-9, -13 and -17 of c-kit gene are less frequently detected than in exon-11. These are considered rare in gastrointestinal stromal tumors with a reported frequency of less than 10%, but are seen more commonly in hematopoietic malignancies and germ cell neoplasms (Lux *et al.*, 2000; Lasota *et al.*, 2000; Lasota *et al.*, 2008). In gastrointestinal stromal tumors, 65–92% of tumors are reported to harbor kit-activating mutations, the majority of which are localized to the juxtamembrane region involving exon-11 (Lasota *et al.*, 1999; Rubin *et al.*, 2001).

Figure 5. Mutations found during the sequencing analysis of c-kit gen at exon-11. Point mutations T→A, G→C, A→C, C→T, T→C, C→A, G→A, A→G, and G→T (resulting in the amino-acid substitution Ile563Lys, Val569Leu, Tyr570Ser, Pro577Ser, Tyr568Ser, Ile571Thr, Thr574Pro, Gln575His, Tyr578Pro, Asp579His, His580Gln, Trp582Ser, Arg586Thr, Asn587Asp, Arg588Met, Lys550Asn, and Ile571Leu).



The majority of exon-11 mutations are clustered within the classic hotspot region of the codon 5 end involving codons in 550–560 bp, however, a second hot spot at the codon 3 end involving codons in 576–590 bp have been described by Antonescu *et al*. (2003). These include frame deletions of one, to several codons (typically involving codons 557–560 bp); point mutations and internal tandem duplications (typically involving the 30 end). We found heterogeneous point mutations, some of which have been reported and some are new for AML. In eight leukemia cases we found 17 point mutations. Lys550Asn and Ile571Leu point mutations have already been reported by Yasuoka *et al*. (2003). Mutations in codon 582 reported by Tae *et al*. (2004) (Kim *et al*., 2004) were for Trp582Tyr and Trp582His, whereas Hou *et al*. (2004) reported Trp582Try and Trp582Gln. However, we have detected different substitution in the same codon where tryptophan is replaced by serine (Trp582Ser) in two independent Leukemia cases. Mutations at codons Tyr568Asp, Ile571Leu, Thr574Tyr, Gln575Ile, Tyr578Phe, Asp579Gln, Asp579Pro, His580Leu, His580Tyr, His580Pro, Arg586Trp, Arg586Ile, Arg586Phe, Arg586Asp, Asn587Glu, Asn587Pro, Asn587His

and Arg588Phe, Arg588Tyr, Arg588Lys have already been reported (Yasuoka *et al.*, 2003; Hou *et al.*, 2004; Kim *et al.*, 2004; Choe *et al.*, 2006).

As we aimed, we analyzed exon-11 of c-kit gene in order to detect point mutations in patients with Leukemia. In our analysis, we were able to determine new amino acids substitutions derived from the point mutations that we detected in exon-11 and we found: Tyr568Ser, Ile571Thr, Thr574Pro, Gln575His, Tyr578Pro, Asp579His, His580Gln, Arg586Thr, Asn587Asp, and Arg588Met (Table 1). From these mutations, we are reporting here, 4 novel mutations: Ile563Lys, Val569Leu, Tyr570Ser, and Pro577Ser which have never been reported in the literature before (Table 3). All the mutations detected in exon-11, lie between codons 550-591. For a comparison purposes, we arrayed our findings with the ones found in the literature, see Table 3. From this table, it is easy to determine which point mutations are novel for the field.

Table 3. Comparison between mutations detected in our study or described already for c-kit gene at the exon-11.

Mutations	Novel Mutations	Mutation with different substitution		Existing Reported Mutations	References
		Not reported substitution (Our Result)	Reported Substitution		
ATA → AAA	Ile563Lys				
GTT → CTT	Val569Leu				
TAC → TCC	Tyr570Ser				
CCT → TCC	Pro577Ser				
TAT → TCT		Tyr568Ser	Tyr568Asp		Taniguchi *et al.* (1999)
ATA → ACA		Ile571Thr	Ile571Leu		Choe *et al.* (2006)
ACA → CCA		Thr574Pro	Thr574Tyr		Hou *et al.* (2004)
CAA → CAC		Gln575His	Gln575Ile		Hou *et al.* (2004)
TAT → CCT		Tyr578Pro	Tyr578Phe		Kim *et al.* (2004)
GAT → CAT		Asp579His	Asp579Gln Asp579Pro		Kim *et al.* (2004)
CAC → CAA		His580Gln	His580Leu His580Tyr His580Pro		Hou *et al.* (2004) Kim *et al.* (2004)
TGG → TCA		Trp582Ser	Trp582Tyr Trp582His Trp582Gln		Hou *et al.* (2004) Kim et al. (2004)
AGA → ACA		Arg586Thr	Arg586Trp Arg586Ile Arg586Phe Arg586Asp		Hou *et al.* (2004) Kim *et al.* (2004)
AAC → GAC		Asn587Asp	Asn587Glu Asn587Pro Asn587His		Hou *et al.* (2004) Kim *et al.* (2004)
AGG → ATG		Arg588Met	Arg588Phe Arg588Tyr Arg588Lys		Hou *et al.* (2004) Kim *et al.* (2004)
AAA → AAC				Lys550Asn	Taniguchi *et al.* (1999)
ATA → CTA				Ile571Leu	Choe *et al.* (2006)

On the other hand, we analyzed exon-8 of c-kit gene and we were able to find 6 point mutations (Table 2) in Leukemia cases: Thr417Val, Tyr418Arg, and Leu421Gly were previously reported by Taniguchi *et al.* (1999), and Kohl *et al.* (2005). In addition, we also detected substitutions: Thr417Asp, Tyr418Phe, and Leu421His which are novel for the field. Moreover, point mutations in codons 423 and 448 of exon-8 have not been reported for any type of Leukemia. However, it was remarkable to find out that these point mutations produced the following new substitutions: Asn423Asp, Gln448Thr, and Gln448His (Table 4).

Table 4. Novel mutations detected during our study or mutations described already for c-kit gene at exon-8.

Mutations	Novel mutations	Mutation with different substitution		References
		Not reported substitution (Our result)	Reported substitution	
AAT → GAT	Asn423Asp			
CAG → ACA	Gln448Thr			
CAG → CAC	Gln448His			
ACT → GAT		Thr417Asp	Thr417Val	Kohl *et al.* (2005) Gari *et al.* (1999)
TAC → TTC		Tyr418Phe	Tyr418Arg	Kohl *et al.* (2005) Gari *et al.* (1999)
CTC → CAC		Leu421His	Leu421Gly	Kohl *et al.* (2005) Gari *et al.* (1999)

From all point mutations detected it seems that the residue that was more replaced was isoleucine following by tyrosine. However, because point mutations in the c-kit protein were located in extra- and cytoplasmic-domains, we thought that maybe these mutations were affecting hydrophobicity of these domains. Indeed, mutations Arg588Met (exon-11) and Tyr418Phe (exon-8) were substitutions where a hydrolytic residue was replaced by a

hydrophobic residue. However, most of the mutations involved a substitution in a hydrophobic residue for a hydrophobic or conversely. Physiologically, it seems that mutations in exon-11 are possibly more relevant regarding c-kit protein function. Interestingly 3 tyrosines, 1 threonine and 2 lysines residues were substituted it, recall that tyrosines and threonines are phosphorylation targets and lysine is an ubiquitination and sumoylation target.

Normally, these types of residues in membrane proteins are target for post-translational modifications and mutations in them may change the way of how proteins function (Miranda *et al.*, 2007; Vargas-Medrano *et al.*, 2011). In contrast, for exon-11, 4 serines, 2 threonines and 1 lysine were detected as the end residue product from a point mutation. Importantly, threonine and serine residues are phosphorylation targets and lysine residue is a ubiquitination and sumoylation target (Miranda *et al.*, 2007; Vargas-Medrano *et al.*, 2011).

These changes may affect the normal phosphorylation and ubiquitination maps for c-kit protein which can modify the way of how c-kit functions. However, experimental data for these hypotheses need to be first generated in order to determine if mutations described here have a significant effect on the c-kit protein activity.

Conclusions

In summary, this study is the first to report the presence of c-kit gene mutations in Leukemia cases in northern India. Mutations in exon-8 and -11 may be involved in c-kit over expression in Leukemia. Four novel mutations at codons Ile563Lys, Val569Leu, Tyr570Ser, and Pro577Ser in exon-11 and three novel mutations at codons Asn423Asp, Gln448Thr, and Gln448His in exon-8 c-kit gene might be useful as molecular genetic markers for Leukemia. Future studies in a larger group may be required to determine the prognostic implications and how these mutations are related with progression and pathogenesis of myeloid malignancy. Based on our *in silico* analysis, only mutations in exon-11 seem to play a crucial role in altering the biochemistry of c-kit protein, because point mutations at Tyr568Ser, Val569Leu, and Tyr570Ser are in places for autophosphorylation or magnesium binding which are crucial steps in signaling from ligand binding. However, this is something that needs to be elucidated by additional experiments. On the other hand, mutations in exon-8 may also be involved in ligand binding. From them, mutation Tyr568Ser is located in a magnesium binding site. In addition, the Tyr residue at this position is normally autophosphorylated by autocatalysis (Price *et al.*, 1997; Mol *et al.*, 2003; Sun *et al.*, 2009; Zadjali *et al.*, 2011). Moreover, mutations Val569Leu and Tyr570Ser are located in a domain that interacts with phosphotyrosine-binding proteins, and residue Tyr570 is normally autophosphorylated by autocatalysis.

The identification of novel mutations in c-kit in patients with AML not only provides new insight into the pathogenesis of this disease, but also may serve to provide a means of confirming a diagnosis and assessing prognosis for developing new intervention strategies. The incidence of mutations at exon-8 and -11 is high and might be involved in pathogenesis of AML. The mutations described here are recommended as prognostic markers in the northern Indian population. However, we do not discard the idea that these mutations could be found in other populations around the world.

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Supplementary table 1. Demographic profile of patients

Variables	AML (*n* = 60)	ALL (*n* = 10)	CML (*n* = 10)	CLL (*n* = 10)
M (%)/ F (%)	34 (56.7%) / 26 (43.3%)	06 (60.0%) / 04 (40.0%)	05 (50.0%) / 05 (50.0%)	06 (60.0%) / 04 (40.0%)
Age range	2-65	25-47	33-56	30-56
Mean (±SD)	36.43 (± 6.08)	35.70 (± 6.29)	43.70 (± 6.96)	46.30 (± 7.17)
Clinical features				
WBC count cells/ µl/ cumm	15000 - 60000	20000 - 40000	25000 - 450000	18000 - 35000
FAB	M_0 (n = 10), M_1 (n = 15), M_2 (n = 15), M_3 (n = 04), M_4 (n = 08) and M_5 (n = 08)	L1/ L2 (n = 10)	CML Chronic phase (n = 10)	CLL (n = 10)

Supplementary table 2. Demographic profile of controls

Variables	Normal Healthy (*n* = 100)
M (%)/ F (%)	58 (56.7%) / 42 (43.3%)
Age range	2-65
Mean (±SD)	36.43 (± 6.08)
Clinical features	All morphological features normal and < 5% blast cells
WBC count cells/ µl/ cumm	4300- 10800

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Resumes of the author and co-authors

Syed Rizwan Hussain. He was born in Bokaro Steel City India on 11th August 1980. He did his Bachelor's degree in CBZ (Chemistry, Biology and Zoology) and Master's degree in Biomedical Sciences from India. He is pursuing his Ph.D. in Human Cancer Genetics from University of Baba Saheb Bhim Rao Ambedkar University India. He has a diverse area of research, such as Human Cancer Genetics, Molecular Diagnosis, Medical Clinical Biochemistry and Male Infertility. Currently, Syed Rizwan is a Research Assistant at Department of Biotechnology, Era's Lucknow Medical College and Hospital, India.

Amna Siddiqui. She was born in Jhansi India in October 9th 1979. She did Bachelor's degree in CBZ (Chemistry, Biology and Zoology) and Master's degree in Biotechnology from India. She completed her Ph.D. in Biotechnology from University of Bundelkhand India in 2010. She joined as a Sr. Manager in 2009 at Hindustan Bioenergy Ltd Lucknow India. She has diverse area of research such as Molecular biology, Microbiology, Plant biology and Human genetics. Currently, Dr. Amna is a Postdoctoral-Research Associate at Texas Tech University Health Science Center and Paul L. Foster School of Medicine. She got many scientific awards and fellowships in India.

Javier Vargas Medrano. He was born in Juarez City, Chih., México. From 1999-2005, Dr. Vargas attended college at the Autonomous University of Juarez City. During his Bachelor's degree, Dr. Vargas was involved in the field of Toxicology, studying the effect of pesticides on Ca^{2+}-ATPases, and he became author of many publications and conferences. Later, he attended graduate school at the University of Texas at El Paso, where he was involved in a research project studying the glycine transporter and its regulation, and its possible role as a pharmacological target in the treatment of schizophrenia. During his doctoral studies, he was awarded with two research assistantships funded by the National Institute of Health and Mental Health. Currently, Dr. Vargas is member of the American Chemical Society and he is a Postdoctoral-Research Associate at Texas Tech University Health Science Center and Paul L. Foster School of Medicine.

Hena Naqvi. She was born on 8th December 1982 at Gorakhpur India. She did her Bachelor's degree in CBZ (Chemistry, Biology and Zoology) and Master's degree in Biotechnology from India. She is pursuing her Ph.D. in Molecular Medical Genetics from C.S.M. Medical University, (Formerly-K.G. Medical University), Lucknow, India. She has worked as a junior lecturer in 2006-07 at Capital College, Bangalore, India. She joined as a Research Assistant in 2008 at Era's Lucknow Medical College and Hospital, Lucknow, India. Her area of research is Molecular biology, Human genetics and Male infertility. Currently, Ms. Hena Naqvi is working as Women Scientist A approved by Department of Science and Technology, Govt. of India at Era's Lucknow Medical College and Hospital, Lucknow, India.

Jonathon Mohl. He was born in St Louis, Missouri, United States (USA). He received a Bachelors of Science in Microbiology and Biochemistry at Colorado State University in Fort Collins, Colorado in 2002. While attaining this degree, he worked in a Mycrobacterial laboratory working on *M. avium* and *M. avium* ssp. *paratuberculosis*. He also worked as a teaching assistant in a molecular biology laboratory. After moving to El Paso, Texas, USA, he received a Professional Master of Science degree in Bioinformatics in 2009 at the University of Texas at El Paso. In the process of attaining his Master's degree, he worked at a bioinformatics programmer on the RNAVLab project and as a teaching assistant in the introductory bioinformatics courses. Currently, he is working in HIV research lab at the Texas Tech University Health Sciences Center in El Paso as a research aid.

Farzana Mahdi. He was born on 28th June 1966 at Lucknow, India. She did her Bachelor's degree in CBZ (Chemistry, Biology and Zoology) and a Master's degree in Organic Chemistry from India. She completed her Ph.D. in Life Sciences from Kanpur University 1992. She joined as an Assistant Professor in 2000 at Era's Lucknow Medical College and Hospital, Lucknow, India. She has diverse area of research such as Free Radical Biology, Toxicology, Clinical Biochemistry, Molecular aspects of metal toxicity, Male infertility, Human Cancer Genetics. Currently, Dr. Farzana is Life Member of Indian Society for Reproductive Biology and Comparative Endocrinology and Association of Clinical Biochemists of India. She is also a Founder Member of Society for Free Radical Research India. Currently, she is working as a Professor in Department of Biochemistry, Era's Lucknow Medical College and Hospital, Lucknow, India.

Fahim Ahmad. He was born in Daltonganj India in 12th October 1976. He did a Bachelor's and Master's degrees in Biotechnology from India. He completed his Ph.D. in Human Genetics from the University of Bundelkhand India in 2007. He joined as an Assistant Professor in 2009 at Isabella Thoburn College Lucknow, India. Dr. Ahmad has diverse area of research such as Human genetics, Molecular Virology and Cancer Genetics. Currently, Dr. Ahmad is member of Biotechnological Board in Jharkhand State and is a Postdoctoral-Research Associate at Texas Tech University Health Science Center and Paul L. Foster School of Medicine. He got many scientific awards and fellowships in India. His current project at Texas Tech University Health Sciences Center focuses on the mechanisms of genetic resistance to HIV-1 infection funded by the National Institute of Health.