TJC Turkish Journal of Cancer
Volume 39, No. 3, 2009

C-kit proto-oncogene deletion and point mutation at exon 8, 17 in human acute myeloid leukemia

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ABSTRACT

Human genomic DNA from 90 cases of acute myeloid leukemia (AML) were screened for mutations in the c-kit gene. C-kit is a receptor tyrosine kinase class III that is expressed by early hematopoietic progenitor cells and plays crucial role in hematopoietic stem cell proliferation, differentiation and survival. Exon 8 and 17 are the frequent sites of mutations in the leukemia cases. To determine the spectrum of mutations at exon 8 and 17 of c-kit gene in 90 acute myeloid leukemia AML cases, we have done Polymerase Chain Reaction (PCR) Single-strand conformational polymorphism (SSCP) followed by DNA sequencing. The c-kit mutation frequencies in exon 8 were 25.55% (23/90) and in exon 17 were 34.44% (31/90) in AML cases. We have detected two contrary deletions Tyr 418del and Asp 419del in exon 8 in 23 cases and three point mutations that is Asp816Val, Asp820Gly and Asn822Lys in exon 17, in 31 cases; respectively. Each and every mutations detected in our study are located in region of the receptor's extracellular domain and intracellular domain of second catalytic region. It gives the impression that incidence of mutation at exon 8 and 17 is elevated and possibly involved in clinical pathogenesis of AML and utilizable tool as the molecular prognostic marker. [Turk J Cancer 2009;39(3):95-103]

KEY WORDS: Mutation, proto-oncogene, SSCP, exons, AML, c-kit receptor

INTRODUCTION

Human c-kit, the cellular counterpart of v-kit derived from the Hardy-Zuckerman 4-feline sarcoma virus, is located on chromosome 4 and encodes a 145 kD receptor tyrosine kinase (RTK). Acute Myeloid Leukemia (AML) is one of the prevalent cancers of blood-forming cells in the bone marrow and heterogeneous disease in which hematopoietic progenitor cells attain genetic lesions that lead to a block in differentiation, increased self-renewal, and unregulated proliferation (1). Apparently, it is known that c-kit is a proto-oncogene and activating c-kit mutations are likely to contribute in the development of leukemia among humans (2,3). The c-kit is a cellular gene which codes for a transmembrane receptor kinase (Kit receptor, Kit-ligand or Steel factor) that is normally expressed in hematopoietic stem cells, mast cells, neural crest-derived melanocytes and germ cells. In context to humans, the c-kit gene has been mapped to chromosome 4q12 adjacent to the highly homologous platelet derived growth factor receptor (PDGFR) (4 - 6). Binding of stem cell factor initiates a sort of phosphorylation cascade that consequently leads to activation of various transcription factors that regulate apoptosis, cell differentiation and proliferation. A number of observations have suggested the role of c-kit, in connection to the development of a range of cells including hematopoietic cells in leukaemogenesis (7). The

augmented activation of c-kit gene with stem cell factor facilitate cell proliferation whereby in AML, an abnormal increase of proliferation occurs in two ways; first in case of AML, mutations of Asp816 in exon 17 and second in case of exon 8 c-kit gene lead to autonomous activation of c-kit gene, and also through over expression of c-kit gene in AML when c-kit gene is expressed in 80-90% of blast cells and point mutation of c-kit have been identified in 33-45% of AML cases (8-11). AML accounts for about 90% of all acute leukemias in adults, but is rare in children (12). The c-kit gene mutations in exon 17 is reported in gastrointestinal stromal tumors, Human solid tumors and Human germ cell tumors and exon 8 is reported in leukemia cases (13-18). C-kit gene exon 8 point mutations at Thr417 and deletions of codons 418, 419 and 420 have been reported in cases of AML (16,19,20). The report detailing exon 17 is Asp816Tyr substitution in the phosphotransferase domain of c-kit provides the first ever direct evidence for mutations in this protein leading to the development of human acute leukemia (21). It is notable here that, no any study has been performed or reported the frequency and the prevalence of mutations in exon 8 and exon 17 of c-kit gene in AML cases in Indian population; to date. We have screened the c-kit gene responsible for the mutations by Single Stranded Conformation Polymorphisms (SSCP) followed by direct sequencing in a total of 90 cases of AML. Furthermore, our study has also explored the extrapolative implications of these mutations and pointed out how these mutations are interconnected with progression and clinical-pathogenesis in myeloid malignancy.

MATERIALS AND METHODS

Study samples collection

The study group included 90 cases of AML. Ethical approval was obtained from the institutional ethical committee of Era's Lucknow Medical College and Hospital, Lucknow, Uttar Pradesh, India. The peripheral blood or bone marrow samples were stained by Leishman stain method and the cases were classified, according to the FAB criteria (22). 90 AML patients were classified as M0 (n = 15), M1 (n = 15), M2 (n = 15), M3 (n = 15), M4 (n = 10), M5 (n = 10), M6 (n = 5) and M7 (n = 5).

Genomic DNA extraction

Samples were collected from 90 routinely-processed unstained bone marrow slides and peripheral blood diagnosed as AML, from Department of Haematology at Era's Lucknow Medical College and Hospital. Genomic DNA was extracted by Medox (DNA extraction kit, India) and DNA were stored in -20 degree Celsius temperature.

Polymerase chain reaction and single-strand conformational polymorphism

Polymerase chain reaction (PCR) was performed with 50 µl PCR reaction mixture containing 200-300 ng of template DNA, 10 pmol of each primer, 5 mmol/L of each mix dNTPs, 1X reaction buffer and 1 units of Taq polymerase enzyme (Fermentas, Germany) in an MJ Mini Thermocycler (Bio-Rad, UK). The cycling conditions denaturation at 94 °C for 30 seconds, followed by annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 sec-

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
08 samples	K	P	E	I	L	T	–	–	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
15 samples	K	P	E	I	L	T	Y	–	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

Fig 1. Amino acid sequences of the exon 8 of c-kit gene. The sequence starts at codon 412 and terminate at 448. The wild-type sequence is shown above. Deletion mutations are shown in shades ■ and detected in case numbers 02, 03, 04, 08, 10, 13, 24, 31, 33, 35, 36, 41, 47, 49, 52, 54, 67, 73, 77, 78, 85, 88 and 90

Codon	788	89	90	91	92	93	94	95	96	97	98	99	800	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Wild Type	C	I	H	R	D	L	A	A	R	N	I	L	L	T	H	G	R	I	T	K	I	C	D	F	G	L	A	R	D	I	K	N	D	S	N	Y	V	V	K	G	N
13 samples	C	I	H	R	D	L	A	A	R	N	I	L	L	T	H	G	R	I	T	K	I	C	D	F	G	L	A	R	D	I	K	N	D	S	K	Y	V	V	K	G	N
08 samples	C	I	H	R	D	L	A	A	R	N	I	L	L	T	H	G	R	I	T	K	I	C	D	F	G	L	A	R	V	I	K	N	D	S	N	Y	V	V	K	G	N
10 samples	C	I	H	R	D	L	A	A	R	N	I	L	L	T	H	G	R	I	T	K	I	C	D	F	G	L	A	R	D	I	K	N	G	S	N	Y	V	V	K	G	N

Fig 2. Amino acid sequences of the exon 17 of c-kit gene. The sequence begins at codon 788 and ends at 828. The wild-type sequence is shown above. Point mutations are shown in ■ and detected in case numbers 02, 03, 04, 13, 21, 22, 24, 25, 26, 29, 31, 33, 40, 41, 43, 49, 50, 52, 57, 58, 59, 63, 71, 75, 77, 78, 85, 86, 88, 90

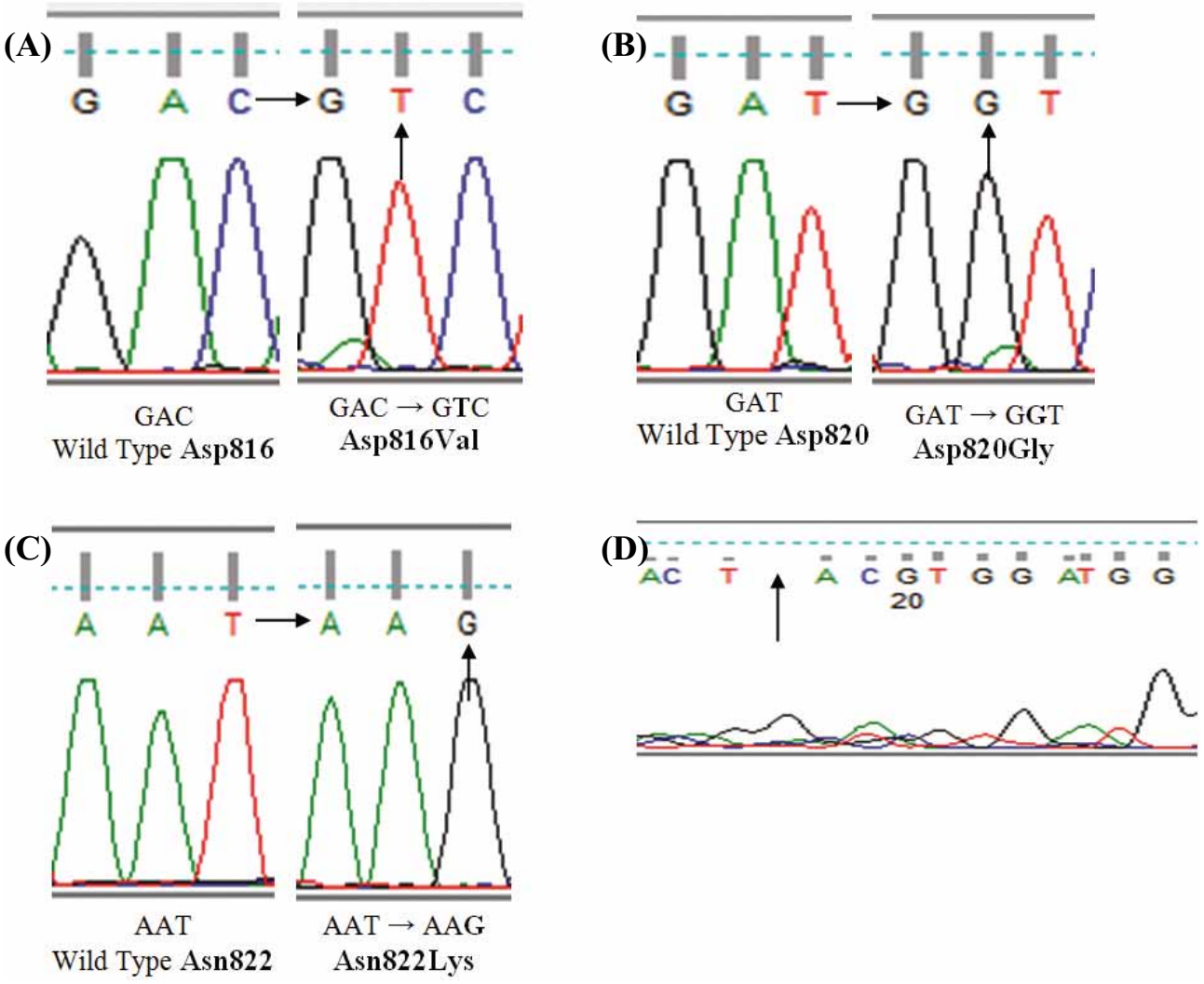

Fig 3(A&B&C&D). C-kit gene exon 17 point mutations (A): A→T, (B): A→G, (C): T→G (resulting in the amino-acid substitution (A): Asp816Val, (B): Asp820Gly and (C) Asn822Lys) and (D): exon 8 deletion mutations of 6 bp, TACGAC was found at codon 418 and 419

onds, repeated for 40 cycles followed by a final extension step at 72 °C for 8 minutes using the following primers exon 8 forward 5- GGCCATTTCTGTTTTCCTGT -3 and reverse 5- TCTGCTCAGTTCCTGGACAA -3 and exon 17 forward 5- TTCACTCTTTACAAGTTAAAATG -3 and reverse 5- GGACTGTCAAGCAGAGAATG -3. Single-strand conformational polymorphism analysis was performed according to Orita et al. (23) with few modifications. Samples were denatured at 94 °C for 5 minutes and immediately snap-cooled. 20 µl of amplified PCR product was loaded along with 20 µl denaturing dye on 12% 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 150 Volt. DNA on the gel was stained after electrophoresis with silver stain. Electrophoretic transpositions in single stranded DNA-PCR product among the samples were detected in contrast to the denatured-PCR products from the control carried out in contiguous pathway.

Sequencing

Amplicons were 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 BLAST (National Center for Biotechnology Information) and BioEdit software.

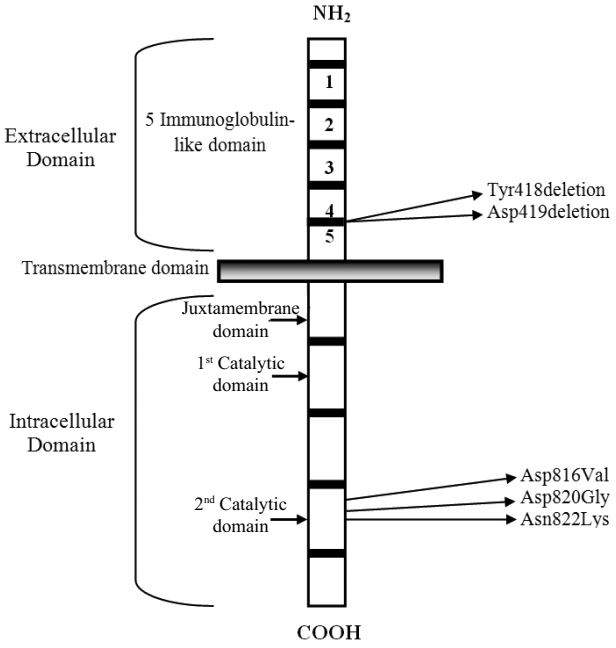

Fig 4. Schematic representation of c-kit gene and location of c-kit gene mutations portray our results of deletions and point mutations in exon 8 & 17 and the distinctive molecular domains encompass three functional structures, e.g. NH2-terminal extracellular domain, transmembrane domain and COOH-terminal intracellular domain, correspondingly

RESULTS

Out of 90 AML cases 50 were male and 40 were female with age ranging from 2 years to 65 years. The mean age of cases was 30.3±1.0 years (mean age of male cases were 23.0±4.2 years and mean age of female cases were 38.5±5.6 years). The median WBC count in cases was 40,000 cells/µl/cumm (ranges from 15,000 to 80,000 cells/µl/cumm) and the median count of blast cells was 70% (ranges from >40% to >80%). The cases were classified according to the French-American-British (FAB) criteria (22) as AML, M0 (n=15), M1 (n=15), M2 (n=15), M3 (n=15), M4 (n=10), M5 (n=10), M6 (n=5) and M7 (n=5). Thirty one deletion mutations were found in 23 AML cases in exon 8 and 31 point mutations were detected in 31 AML cases in exon 17. The c-kit mutation frequencies in exon 8 were 25.55% (23/90) and exon 17 were 34.44% (31/90) in AML cases. Details of clinical data of deletion and point mutation in exon 8 and 17 are shown in table 1.

DISCUSSION

The c-kit gene is located in the human chromosome 4q11- q12. Exon 8 deletion mutations are localized between immunoglobulin-like loops 4 and 5 those are responsible for dimerization of the ligand-stimulating receptor at codon 418, 419 and exon 17 is located in intracellular region with juxtamembrane domain as separated in two domain regions first catalytic domain and second catalytic domain (24). In the Indian population, our study is the foremost, to report mutations in the exon 8 and exon 17 fragment of c-kit gene in cases of AML. Previously, a molecular study has elucidated a number of mutations in the exon 8 and exon 17 in dissimilar sorts of tumors. Moreover, mutation at the Codon 417, 418, 419, 420 and 421 correspondingly in Exon 8 has been put out by Kohl et al. (17). In particular terms, the deletion exists at the site of codon 419, in approximately 93% of all the exon 8 mutations and that was uncovered as a single abnormality in the AML patients (19). Likewise, the mutation enclosing the deletions of amino acid 417 up to 419 and an additional insertion of Isoleucine Thr417Ile has been mentioned by Gari et al. (16). The structural significance of the deleted region has been put forward by the involved amino acids, particularly Asp419, being highly conserved in the c-kit proteins of humans (6). In our study, we have shown two contrary deletions, detected at exon 8 in 23 cases (Figure 1 and Table 2) (Tyr 418del, Asp 419del). Specifically; deletion of tyrosine were found at codon 418 in eight cases, in addition, at codon 419, deletion of Aspartic acid were found in each of 23 cases. Deletion at Tyr418 and Asp419 were jointly found in eight AML cases with M0, M1 subtype, respectively, while deletion at Asp419 was found in 15 AML cases with M0, M1, M2, M3, M4, M5 and M7 subtypes. In all the 2 codons 418 & 419 deletion mutations were found in AML with frequent entries at codon 419. The c-kit gene mutations specifically in exon 17 have been detected at many codons 813, 816, 818, 820, 822, 823, 825 are reportedly involved in human germ cell tumors, gastro-intestinal stromal tumors, human solid tumors as well as in leukemia; recurrently (13-15). Exon 17 mutations at codon Asp816Val, Asp820Gly, Asn-822Lys have already been reported (15, 25-29). The prime effect of Asp816Val can be recognized through functional studies that established its pathogenetic role in mast cell transformation more precisely, mutations of c-kit in exon 17 most commonly established in more than 90% cases of Systemic Mastocytosis (SM) as well as AML, which are considered to be associated in pathogenesis of disease

Table 1
Outline of clinic based pathological data of c-kit gene deletion and point mutations in exon 8 and 17 in AML cases

Case No.	Age/ Sex	Histopathological findings			Codon Exon 8	Codon Exon 17
		AML	WBC	Blast Cells		
02	06/M	M1	50,000	>80%	Tyr**418**Del. Asp**419**Del.	Asp**820**Gly
03	60/F	M1	25,000	>60%	Asp**419**Del.	Asp**820**Gly
04	07/M	M5	60,000	>70%	Asp**419**Del.	Asn**822**Lys
08	50/M	M4	50,000	<80%	Asp**419**Del.	
10	65/F	M0	40,000	<70%	Asp**419**Del.	
13	18/M	M2	30,000	<60%	Asp**419**Del.	Asn**822**Lys
20	35/F	M1	15,000	>40%		Asp**820**Gly
21	18/M	M5	20,000	<50%		Asp**816**Val
22	45/M	M6	50,000	<80%		Asp**816**Val
24	50/M	M5	20,000	<50%	Asp**419**Del.	Asp**816**Val
25	30/M	M5	20,000	>50%		Asn**822**Lys
26	29/M	M6	25,000	>65%		Asn**822**Lys
29	55/M	M2	40,000	>60%		Asn**822**Lys
31	15/F	M1	30,000	>50%	Tyr**418**Del. Asp**419**Del.	Asp**816**Val
33	33/M	M1	60,000	>80%	Tyr**418**Del. Asp**419**Del.	Asn**822**Lys
35	36/F	M1	30,000	>70%	Tyr**418**Del. Asp**419**Del.	
36	40/M	M3	40,000	>50%	Asp**419**Del.	
40	40/F	M6	30,000	<60%		Asn**822**Lys
41	23/M	M7	20,000	>50%	Asp**419**Del.	Asp**820**Gly
43	45/F	M4	18,000	<50%		Asp**816**Val
47	15/M	M4	20,000	>50%	Asp**419**Del.	
49	40/F	M0	30,000	>50%	Tyr**418**Del. Asp**419**Del.	Asp**820**Gly
50	35/M	M0	55,000	>90%		Asp**820**Gly
52	29/M	M0	74,000	>60%	Tyr**418**Del. Asp**419**Del.	Asp**820**Gly
54	45/F	M3	80,000	>60%	Asp**419**Del.	
57	40/F	M7	30,000	<60%		Asn**822**Lys
58	23/M	M7	20,000	>50%		Asp**820**Gly
59	45/F	M4	18,000	<50%		Asn**822**Lys
63	15/M	M4	20,000	>50%		Asn**822**Lys
67	40/F	M0	30,000	>50%	Tyr**418**Del. Asp**419**Del.	
71	35/M	M0	55,000	>90%		Asp**816**Val
73	29/M	M0	74,000	>60%	Tyr**418**Del. Asp**419**Del.	
75	45/F	M3	80,000	>60%		Asp**820**Gly
77	40/F	M1	30,000	<60%	Asp**419**Del.	Asn**822**Lys
78	40/F	M4	30,000	<60%	Asp**419**Del.	Asn**822**Lys
85	23/M	M3	20,000	>50%	Asp**419**Del.	Asp**820**Gly
86	45/F	M4	18,000	<50%		Asn**822**Lys
88	15/M	M4	20,000	>50%	Asp**419**Del.	Asp**816**Val
90	40/F	M0	30,000	>50%	Asp**419**Del.	Asp**816**Val

Del.: deletion

Table 2
Comparison of reported mutations and the deletion mutations detected in our study of c-kit gene in exon 8 and exon 17

Case No	Substitution/ Mutations (Our Results)	Mutations (Our Results)	References
02	GAT→ G**G**T TAC→ Del. GAC→ Del.	Asp**820**Gly Tyr**418**Del. Asp**419**Del.	36 16, 17 16, 17, 18
03	GAT→ G**G**T GAC→ Del.	Asp**820**Gly Asp**419**Del.	36 16, 17, 18
04	AAT →AA**G** GAC→ Del.	Asn**822**Lys Asp**419**Del.	28 16, 17, 18
08	GAC→ Del.	Asp**419**Del.	16, 17, 18
10	GAC→ Del.	Asp**419**Del.	16, 17, 18
13	AAT →AA**G** GAC→ Del.	Asn**822**Lys Asp**419**Del.	28 16, 17, 18
20	GAT→ G**G**T	Asp**820**Gly	36
21	GAC→ G**T**C	Asp**816**Val	34
22	GAC→ G**T**C	Asp**816**Val	34
24	GAC→ G**T**C GAC→ Del.	Asp**816**Val Asp**419**Del.	34 16, 17, 18
25	AAT →AA**G**	Asn**822**Lys	28
26	AAT →AA**G**	Asn**822**Lys	28
29	AAT →AA**G**	Asn**822**Lys	28
31	GAC→ G**T**C TAC→ Del. GAC→ Del.	Asp**816**Val Tyr**418**Del. Asp**419**Del.	34 16, 17 16, 17, 18
33	AAT →AA**G** TAC→ Del. GAC→ Del.	Asn**822**Lys Tyr**418**Del. Asp**419**Del.	28 16, 17 16, 17, 18
35	TAC→ Del. GAC→ Del.	Tyr**418**Del. Asp**419**Del.	16, 17 16, 17, 18
36	GAC→ Del.	Asp**419**Del.	16, 17, 18
40	AAT →AA**G**	Asn**822**Lys	28
41	GAT→ G**G**T GAC→ Del.	Asp**820**Gly Asp**419**Del.	16, 17, 18 16, 17, 18
43	GAC→ G**T**C	Asp**816**Val	34
47	GAC→ Del.	Asp**419**Del.	16, 17, 18
49	GAT→ G**G**T TAC→ Del. GAC→ Del.	Asp**820**Gly Tyr**418**Del. Asp**419**Del.	36 16, 17 16, 17, 18
50	GAT→ G**G**T	Asp**820**Gly	36
52	GAT→ G**G**T TAC→ Del. GAC→ Del.	Asp**820**Gly Tyr**418**Del. Asp**419**Del.	36 16, 17 16, 17, 18
54	GAC→ Del.	Asp**419**Del.	16, 17, 18
57	AAT →AA**G**	Asn**822**Lys	28
58	GAT→ G**G**T	Asp**820**Gly	36
59	AAT →AA**G**	Asn**822**Lys	28

Table 2
Comparison of reported mutations and the deletion mutations detected in our study of c-kit gene in exon 8 and exon 17 (continued)

Case No	Substitution/ Mutations (Our Results)	Mutations (Our Results)	References
63	AAT →AA**G**	Asn**822**Lys	28
67	TAC→ Del. GAC→ Del.	Tyr**418**Del. Asp**419**Del.	16, 17 16, 17, 18
71	GAC→ G**T**C	Asp**816**Val	34
73	TAC→ Del. GAC→ Del.	Tyr**418**Del. Asp**419**Del.	16, 17 16, 17, 18
75	GAT→ G**G**T	Asp**820**Gly	36
77	AAT →AA**G** GAC→ Del.	Asn**822**Lys Asp**419**Del.	28 16, 17, 18
78	AAT →AA**G** GAC→ Del.	Asn**822**Lys Asp**419**Del.	28 16, 17, 18
85	GAT→ G**G**T GAC→ Del.	Asp**820**Gly Asp**419**Del.	36 16, 17, 18
86	AAT →AA**G**	Asn**822**Lys	28
88	GAC→ G**T**C GAC→ Del.	Asp**816**Val Asp**419**Del.	34 16, 17, 18
90	GAC→ G**T**C GAC→ Del.	Asp**816**Val Asp**419**Del.	34 16, 17, 18

Del.: deletion

(30-33). Resultantly, confirmation to this finding came across with the identification of Asp816Val in modified mast cells in the patient with aggressive mastocytosis and an associated hematological disorder (34,35). The earlier detection of a proximal mutation Asp820Gly in aggressive mast cell disease indicates to the fact that c-kit exon 17 is present in the second catalytic domain (36). In our studies, we have detected eleven point mutations, i.e. Asp816Val, Asp820Gly and Asn822Lys, at exon 17 in 31 cases, respectively (Figure 2, Table 2). In addition, mutation at the codon 822 was spotted in thirteen cases whereas; mutation at codon 820 was detected in ten cases as well as mutation at codon 816 was identified in 8 cases (Figure 3). Most of the c-kit activating mutations apart from mutation identified between codon 813 to 825, which is found to be entirely in agreement with the others (25-29,34,36) Basically, these mutations are huddled in the same region as other known c-kit 17 mutations and in all probability coding for a constitutively activated protein; also. Noteworthy to quote here, that we have identified deletion and point mutations both at exon 8 and 17 in eleven cases of AML; respectively. The c-kit gene exon 8 and 17 mutations detected during our study, positioned between codons (412- 448) in exon 8 and (788- 828) in exon 17 is obvious endorsement of the earlier reported mutations within the diverse population which has been summarized in figure 4 and table 2. Summarily, this study is the first to report the presence of c-kit gene mutations in AML cases in Indian population. These observations suggest that c-kit gene mutations in exon 8 and 17 represent gain-of-function mutations that are susceptible to c-kit selective protein tyrosine kinase inhibitors. These findings point toward an important functional part of c-kit gene in exon 8 and 17 mutations in the pathogenesis of AML and provide the source for the c-kit gene that might represent functional molecular genetic markers in AML. Future studies in a larger group might be required to establish the extrapolative implications and to explore these mutations and their linkage with progression and clinical-pathogenesis in myeloid malignancy.

ACKNOWLEDGEMENT

This complete study was supported by intramural grants on behalf of the Era's Lucknow Medical College and Hospital, Lucknow-226 003, Uttar Pradesh (India).

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