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RESEARCH ARTICLE

ANALYSIS OF C-KIT GENE MUTATION AS A MARKER FOR DIAGNOSIS OF PRIMARY OSTEOSARCOMA IN NORTH INDIAN POPULATION

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ABSTRACT

Background: Osteosarcoma, a frequent tumour of childhood and young adults, represents the most common primary malignant bone tumour. c-KIT gene is expressed in mast cell growth factor, cellular migration, proliferation, melanoblasts, haematopoietic progenitors and germ cells. We have designed our study with aim to explore the significance of c-KIT gene mutation in osteosarcoma patients.

Materials and methods: To determine the kind of mutation analysis in exon 9, 11, 13 and 17 of c-KIT gene in 60 osteosarcoma patients. We have done polymerase chain reaction (PCR) -single-strand conformational polymorphism (SSCP) followed by DNA sequencing.

Results: In osteosarcoma cancer the c-KIT gene mutation frequency was 3.33% (02/60) in exon 9, 8.33% (5/60) in exon 11, 15.0% (9/60) in exon 13 and 5.0% (3/60) in exon 17, respectively. We have detected two silent mutations Val497Val and Ile798Ile in exon 9, 17 in five cases and three missense mutations that is Phe584Ser, Lys642Glu and Val654Ala in exon 11, 13 in fourteen cases. The overall c-KIT gene mutation frequency in exons 9, 11, 13 and 17 was determined to be 31.6% (19/60).

Conclusions: The c-KIT gene may be used as a molecular prognostic/diagnostic marker of osteosarcoma patients.

INTRODUCTION

Osteosarcoma is a high-grade malignant tumor of children and young adults, accounts for the most common primary malignant bone tumor of long bones [1, 2]. Huvos *et al.* classified it into two categories as per response to preoperative neo-adjuvant chemotherapy as good responders and poor responders [3]. After multimodal treatment with chemotherapy and surgery, the approximate five years survival rate was observed upto 70%. With the advancement in molecular biology, role of various oncogenes and growth factors has been elucidated. Role of tyrosine kinase receptors in the pathogenesis of pediatric osteosarcomas has been studied and it was found that the tyrosine kinase receptors and their ligands, as c-met or platelet-derived growth factor receptor, influence the growth of these tumors. These growth factors act either in autocrine or paracrine fashion on tyrosine kinase receptor and regulate oncogenes responsible for development of osteosarcoma [4-9].

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Receptor tyrosine kinase (RTK) has molecular weight of 145 kd, encoded by c-KIT which is a cellular counterpart of v-KIT derived from the Hardy-Zuckerman 4-feline sarcoma virus and is located on chromosome 4q1- q12 [10]. The c-KIT gene receptor has 21 exons, ranging from 100- 300 bp [11]. The oncogene c-KIT acts through tyrosine kinase receptor and it plays on important role in regulating normal cell differentiation, maturation, and proliferation [12, 13]. After activation of tyrosine kinase receptor, it binds with its ligand and results in the formation of stem cell factor which acts at nuclear level resulting in uncontrolled growth and multiplication of cells [14]. The abnormal activation of tyrosine kinase receptor due to various mutation results in abnormal cell growth and tumour growth.

Mutation and deletion of exons 8, 9, 11, 13, 15, 17 in c-KIT gene have been reported in gastrointestinal stromal tumours, human solid tumours, mastocytosis, germ cell tumours, breast tumours and leukaemia cases [12, 15-19]. Imatinib mesylate

which act through inhibition of c-KIT gene pathway has been shown good results in the treatment of gastrointestinal stromal tumours [20, 21]. Another oral agent STI571 which acts through inhibition of c-KIT protein also has promising results in the treatment of these tumours [4]. Osteosarcoma is a highly malignant osteoid-forming spindle cell tumour of the bone, caused by various mutations in oncogenes. Presently, we lack a specific molecular marker related to biological activity of tumour which can be used as diagnostic tool. We want to study whether mutation in exon 9, 11, 13 and 17 of c-KIT gene is related with osteosarcoma or not. We also want to explore prognostic implications of these mutations in osteosarcoma.

Study samples collection

This study includes 60 biopsy proven cases of osteosarcoma in north Indian population and admitted in the department of Orthopaedic Surgery, King George's Medical University, Lucknow, India, a tertiary care centre. In these cases, fine needle aspiration cytology (FNAC) yielded positive results and on this basis, the tissue was diagnosed as osteosarcoma. Biopsy was done according to histological grading given by Enneking *et al* and Bickels *et al* [22, 23]. This study was carried out during May 2011 to April 2015. The study was approved by the institutional ethical committee. Written informed consent was obtained from every patient before recruitment in the study.

Isolation of genomic DNA

Samples were collected from 60 biopsy tissue diagnosed as osteosarcoma based on grading as per the internationally accepted standard [22, 23]. The genomic DNA was extracted by Fermentas (DNA extraction kit, Germany) and stored at -80ºC.

PCR Amplification of c-KIT gene

Primers for c-KIT gene were designed using GENE TOOL software. PCR was performed in a gradient thermocycler (ABI, USA) using thin walled 0.2ml PCR tubes. The final volume of the PCR reaction mixture was 25µl containing 10-40ng genomic DNA, 10picomoles of forward and reverse primers, 2X master mix (ABI, USA) at a concentration of 1X. Amplification was carried out using different primers for different exons (Table 1) and different PCR programs (Table 2) for different exonic regions of c-KIT gene. Further 5µl of amplified product was checked on 2% agarose gel with ethidium bromide staining [24].

Table 1 The primers sequence of c-KIT gene

Exon	Primers sequence	Product size (bp)	References
9	F 5'-GGCTTTTGTTTTCTTCCCTTT-3' R 5'-GAAGTCTTGCCCACATCGTT-3'	179	[19]
11	F 5'-ATTATTAAAAGGTGATCTATTTTTC-3' R 5'-ACTGTTATGTGTACCCAAAAAG-3'	257	[31]
13	F 5'-ATCAGTTTGCCAGTTGTGCT-3' R 5'-TTTATAATCTAGCATTGCC-3'	250	[16]
17	F 5'-TTCACTCTTTACAAGTTAAAATG-3' R 5'-GGACTGTCAAGCAGAGAATG-3'	220	[16]

Screening of c-KIT gene mutation by SSCP

Single-strand conformational polymorphism (SSCP) analysis was performed according to Orita *et al* and Hussain *et al*. [16, 25] with little modifications. Samples were denatured at 97˚C for 6 min with denaturing dye and immediately transferred to ice. 25 microliter of amplified PCR product was loaded along with 25µl of denaturing dye on 10% polyacrylamide gel. Gel was run in pre-cooled 1X TBE buffer. The gel tank was placed in a cold room at 4˚C and run for 10-12 hrs at 130 Volts. 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 (Figure 1).

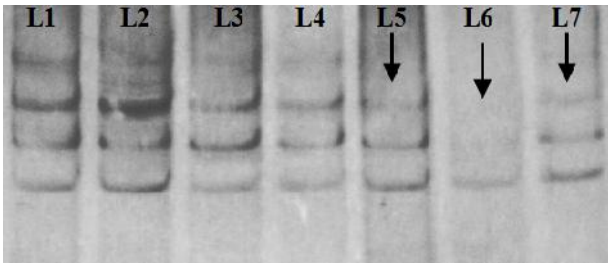

Figure 1 SSCP-PAGE showing electrophoresis mobility shift bands on native page. Control L1, No shift bands in L2- L4 and shift bands in L5-L7 (arrow).

Sequencing analysis of c-KIT gene

Amplified fragments of all samples were characterized by automated sequencing. The PCR product of each sample was first purified and then submitted in 25 µl quantity with 10 picomoles of appropriate primer. The sequencing was performed by automated direct DNA sequencing technique, which incorporates fluorescently labelled di-deoxy-nucleotides during cycle sequencing and separates the resulting products by capillary electrophoresis for detection on an ABI 3730XL DNA Analyser (Applied Biosystems, USA). Multiple alignment and sequence analysis were done using BLAST (Basic Local Alignment Search Tool), BioEdit, FinchTV and Auto Assembler Software (Applied Biosystems, USA). Mutations were reconfirmed by sequencing amplicons in both directions and in independent second samples.

RESULTS

Out of 60 biopsy proven cases with mean age 20.3 years SD ± 3.90, ranging from 10 years to 30 years, 14 cases have shown three missense mutations in exons 11, 13 and five cases have shown two silent mutations in exons 9, 17 (Figure 2, Figure 3 and Figure 4). One silent mutation detected in exon 9 (Val497Val), one missense mutation in exon 11 (Phe584Ser), two missense mutations detected in exon 13 (Lys642Glu and Val654Ala) and one silent mutation in exon 17 (Ile798Ile).We observed the c-KIT gene missense mutations rate in grade G2 (27.8% in a total of 5/18 cases) and grade G1/G2 (75.0% in a total of 9/12 cases). Found in c-KIT gene silent mutations rate in grade G1 (16.7% in a total of 5/30 cases). Details of the clinical data and missense and silent mutation in exons 9, 11, 13and 17 are shown in Table 3. Out of 60 osteosarcoma cases,

46 samples were found to have mutations by a shift in DNA position on SSCP-PAGE with respect to DNA from healthy donors (Figure 1). The results after comparing with previously reported findings are shown in Tables 4, c-KIT gene novel silent mutation in exon 17 for codon Ile**798**Ile is reported here for the first time.

The c-KIT gene mutation frequency was 3.33% (02/60) in exon 9, 8.33% (5/60) in exon 11, 15.0% (9/60) in exon 13and 5.0% (3/60) in exon 17, respectively. We have detected two silent mutations Val497Val and Ile798Ilein exon 9, 17 in five cases and three missense mutations that isPhe584Ser, Lys642Glu and Val654Ala in exon 11, 13 in fourteen cases. The overall c-KIT gene mutation frequency in exons 9, 11, 13 and 17 was determined to be 31.6% (19/60).

Analysis of c-KIT gene mutations in exon 9, 11, 13and 17

In 60 osteosarcoma cases, 12 samples have shown a shift in position in native SSCP-PAGE in exon 9. These were directly sequenced by an automated sequencer. One silent mutation was detected in two osteosarcoma patients (Figure 2, 3 and Table 3).

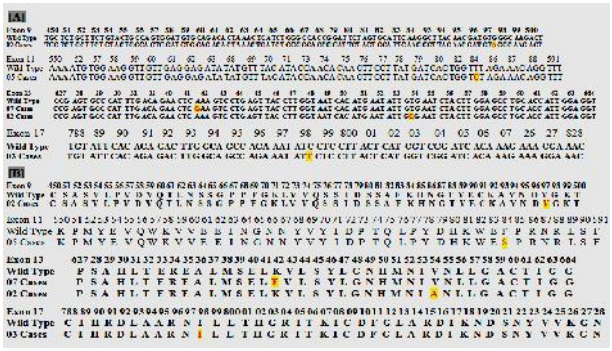

Figure 2 Exon 9, 11, 13 and 17 were mutational analysis of c-KIT gene in osteosarcoma cases. The wild-type sequence is shown above in the cases. Missense and silent mutations are shown in shade filled square:
[A] Nucleotides sequences
[B] Amino acids sequences.

Out of 60 osteosarcoma cases, 20 samples displayed a shift in position in native SSCP-PAGE in exon 11. These were directly sequenced by an automated sequencer. One missense mutation was detected in five osteosarcoma patients (Figure 2, 3 and Table 3).

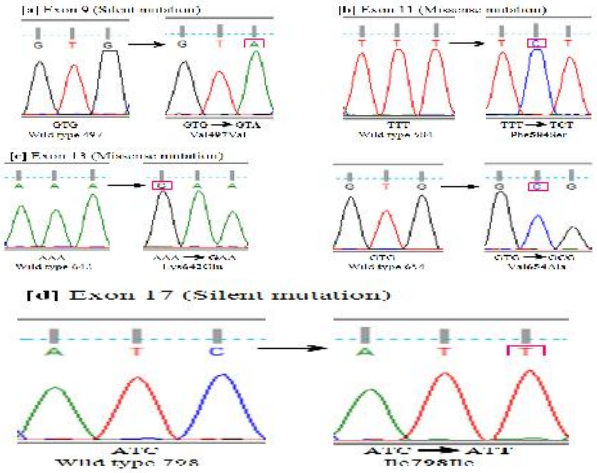

Figure 3 Nucleotides sequence chromatogram of c-KIT gene silent and missense mutations:
[a] Exon 9 nucleotides G→A and resulting in the amino-acid substitution Val**497**Val
[b] Exon 11 nucleotides T→C and resulting in the amino-acid substitution Phe**584**Ser
[c] Exon13 nucleotides A→G, T→C and resulting in the amino-acid substitution Lys**642**Glu and Val**654**Ala
[d] Exon 17 nucleotides C→T and resulting in the amino-acid substitution Ile**798**Ile.

In 60 osteosarcoma cases, 32 samples showed a shift in position in native SSCP-PAGE in exon 13. These were directly sequenced by an automated sequencer. Two missense mutations were detected in nine osteosarcoma patients (Figure 2, 3 and Table 3).

Out of 60 osteosarcoma cases, 10 samples have shown a shift in position in native SSCP-PAGE in exon 17. These were directly sequenced by an automated sequencer. One silent mutation was detected in three osteosarcoma patients (Figure 2, 3and Table 3). These findings, where we found mutations around the protein, it was important to address where in the protein these mutation were located. In order to determine the possible implications of these mutations in protein function, we analysed the protein sequence using the BioEdit and Pyre2 software. Val497Val silent mutation for exon 9 is located on extracellular domain (Immunoglobulin 5 region), Phe584Ser missense mutation for exon 11 is located on intracellular domain (Juxta membrane region), Lys642Glu and Val654Ala missense mutations for exon 13 are located on intracellular

Table 2 Details for c-KIT gene PCR conditions of exon 9, 11, 13 and 17.

Exon	Step 1 (Denaturation)	Step 2 (Annealing)			Cycles	Step 3 (Extension)
9	94˚C→ 5 mins	94˚C→ 1 min	55˚C→ 35 sec	72˚C→ 1 min	42	72˚C→ 6 mins
11	95˚C→ 5 mins	95˚C→ 1 min	56˚C→ 40 sec	72˚C→ 1 min	40	72˚C→ 8 mins
13	95˚C→ 5 mins	95˚C→ 1 min	55˚C→ 1 min	72˚C→ 1 min	40	72˚C→ 8 mins
17	94˚C→ 6 mins	94˚C→ 1 min	54˚C→ 55 sec	72˚C→ 1 min	41	72˚C→ 8 mins

Table 3 Detail of the clinical data and c-KIT gene mutation in osteosarcoma cases.

No of Cases	Stage	Grade	Exon 9	Exon 11	Exon 13	Exon 17
07	III	G1/G2			Lys**642**Glu	
05	IIA, IIB	G2		Phe**584**Ser		
03	IA, IB	G1				Ile**798**Ile
02	III	G1/G2			Val**654**Ala	
02	IA, IB	G1	Val**497**Val			

domain (First catalytic region), Ile798Ile silent mutation for exon 17 is located on intracellular domain (Second catalytic region) in osteosarcoma cases as shown in Figure 4.

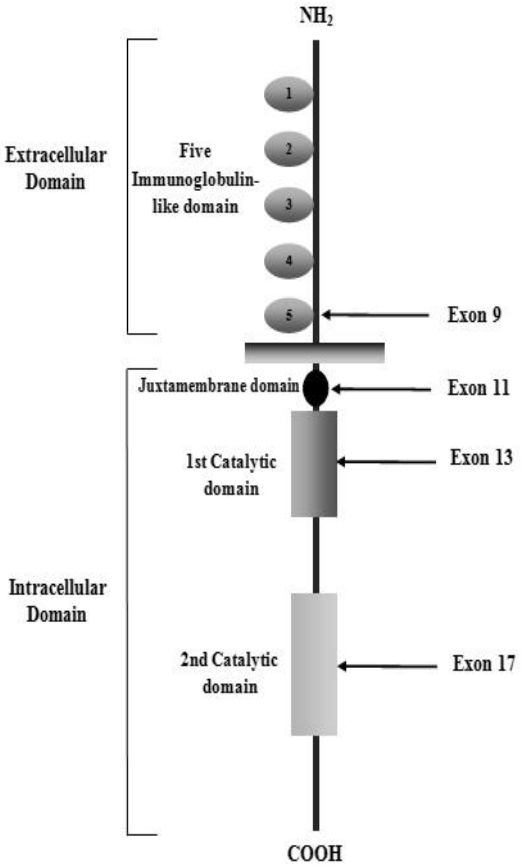

Figure 4 Diagram of c-KIT gene location of exon 9, 11, 13 and 17. The distinct molecular domains comprise two functional structures: extracellular and intracellular domains.

Table 4 Missense and silent mutations detected in our study of exons 9, 11, 13 and 17 in c-KIT gene and comparison between reported missense mutations.

No of Cases	Nucleotides Mutations (Our results)	Missense/Silent Mutations (Our results)	References
02	GTG→ G**T**A	Val**497**Val	[29]
05	TTT → **T**CT	Phe**584**Ser	[29]
07	AAA → **G**AA	Lys**642**Glu	[21]
02	GTG → G**C**G	Val**654**Ala	[30]
03	ATC → AT**T**	Ile**798**Ile	Novel

DISCUSSION

This present study is the first to report mutations in the KIT gene in osteosarcoma in north India patients. Several mutations in c-KIT gene have been documented to be associated with various types of cancer in previous molecular studies. Mutations in exon 11 of the KIT gene has been shown to more associated with neoplastic tumours in comparison to mutations in exons 9, 13 and 17 of the KIT gene [17, 26, 27]. About 65-92% of gastrointestinal stromal tumours are reported to have KIT gene activating mutations involving exon 11 located at the juxta membrane region [17]. Antonescu *et al* found that the majority of mutations at exon 11 are clustered within the classic hotspot region of the 50 end involving codons 550-560 while a second hotspot is located at 30 end involving codons 576-590 [28]. This study on the involvement of c-KIT gene in osteosarcoma oncogenesis have been reported to be performed with a smaller but more homogeneous cohort population of human osteosarcomas in children and young adults [12].

In the present data, samples were analysed on the basis of c-KIT gene mutations at exon 9, 11, 13 and 17 and we found that 19 osteosarcoma cases (31.6%) showed five point mutations whereas five cases (8.33%) showed two silent mutations and 14 cases (23.3%) showed three missense mutations. The silent mutation detected at exon 9 in two cases was Val497Val and another missense mutation at exon 11 was Phe584Ser detected. Both of these mutations were described previously by Sakurai *et al* in gastrointestinal stromal tumours [29] (Table 4). The missense mutation Lys642Glu andVal654Ala were detected in nine independent cases at exon 13, these mutations have also been reported previously in gastrointestinal stromal tumours [21, 30] while Ile798Ile novel silent mutation, found at exon 17 have not been reported previously and our study is first to report their occurrence in Indian population.

The c-KIT gene exon 9, 11, 13 and 17 mutations found in our study, were located between codons 450-500 in exon 9, 550-591 in exon 11, 627-664 in exon 13and 788-828 in exon 17, reported in previous studies and their associations with neoplasia have been summarized in Table 4. The majority of mutations detected in our study were found in patients with stage III and grade G1/G2 tumour as shown in Table 3 and may be associated with disease development and prognosis. Majority of these mutations are located in the intracellular domain region as with other known c-KIT gene mutations and they possibly code for activated kinase protein which downstream regulates various cell cycle pathway. Mutations of c-KIT gene at exon 8, 9, 13 and 17 observed in our study seems to play an important role in the pathogenesis of osteosarcoma, thus we can use c-KIT gene as a genetic marker in osteosarcoma patients. As we have observed majority of these mutations in late stage of disease, we can use it as a prognostic marker but still further studies are required with larger sample size to say it conclusively. After going through the literature, we found that our study is pioneer to report c-KIT gene mutations in osteosarcoma patients belonging to north India.

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