

The Frequency of Mutations in Exon 11 of the c-kit Gene in Patients With Leukemia

Lösemili Hastalarda c-kit Geni Ekson 11 Mutasyonlarının Sıklığı

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Abstract

Objective: To determine the frequency of mutations in exon 11 of the c-kit gene in patients with leukemia.

Material and Methods: The study included 50 leukemia patients (31 with acute myeloid leukemia, 5 with acute lymphoblastic leukemia, 9 with chronic myeloid leukemia, and 5 with chronic lymphocytic leukemia) that underwent PCR-SSCP, followed by direct DNA sequencing.

Results: In all, 28 of the leukemia patients were male and 22 were female, with a mean age of 31.88 years (range: 2-65 years). In total, 20 mutations in 19 patients were identified, including Lys550Asn, Tyr568Ser, Ile571Thr, Thr574Pro, Gln575His, Tyr578Pro, Asp579His, His580Gln, Arg586Thr, Asn587Asp, and Arg588Met, as well as novel point mutations at codons Ile563Lys, Val569Leu, Tyr570Ser, and Pro577Ser. Ile571Leu substitution was observed in 2 patients and Trp582Ser substitution was observed in 3 patients.

Conclusion: The results suggest that mutations in exon 11 of the c-kit gene might be useful as molecular genetic markers for leukemia

Key Words: C-kit, Leukemia, SSCP, Mutation

Özet

Amaç: Lösemili hastalarda c-kit geni ekson 11 mutasyonlarının sıklığını belirlemek.

Gereç ve Yöntemler: Bu çalışma PCR-SSCP ve sonrasında direkt DNA sekanslama yapılan 50 lösemi hastası (31 akut myeloid lösemi, 5 akut lenfoblastik lösemi, 9 kronik myeloid lösemi ve 5 kronik lenfositik lösemi) hastası ile yapıldı.

Bulgular: Genel olarak lösemi hastalarının 28'i erkek ve 22'si kadındı ve ortalama yaş 31,88 (aralık: 2-65) yılı. Toplam olarak 19 hastada Lys550Asn, Tyr568Ser, Ile571Thr, Thr574Pro, Gln575His, Tyr578Pro, Asp579His, His580Gln, Arg586Thr, Asn587Asp ve Arg588Met ve ayrıca Ile563Lys, Val569Leu, Tyr570Ser ve Pro577Ser kodonlarında yeni nokta mutasyonları olmak üzere 20 mutasyon saptandı. Ile571Leu yerine konması 2 hastada ve Trp582Ser yerine konması 3 hastada görüldü.

Sonuç: Sonuçlar c-kit geni ekson 11 mutasyonlarının lösemi için moleküler genetik işaretler olarak faydalı olabileceğini düşündürmektedir.

Anahtar Sözcükler: C-kit, Lösemi, SSCP, Mutasyon

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Received/Geliş tarihi : January 7, 2011

Accepted/Kabul tarihi : May 17, 2011

Introduction

Leukemia is a heterogeneous disease characterized by hematopoietic progenitor cells that acquire genetic lesions, leading to loss of differentiation, an increase in self-renewal, and unregulated proliferation. In 2000, approximately 256,000 children and adults worldwide developed some form of leukemia, and 209,000 died due to leukemia, which accounts for about 3% of the almost 7 million cancer-related deaths and about 0.35% of all deaths that year. A study that examined the incidence of cancer according to 16 sites of the body reported that leukemia was the 12th most common class of neoplastic disease and the 11th most common cause of cancer-related death [1].

Leukemia is classified based on the presence of specific cytogenetic abnormalities, as well as the French-American-British (FAB) classification of leukemic cells [2]. A number of studies suggest that c-kit—a member of the type III receptor tyrosine kinase (RTK) family—is important for the development of a range of cells including hematopoietic cells, and plays a role in leukemogenesis—[3]. High-level expression of c-kit has been reported in 60%-80% of acute myeloid leukemia (AML) patients [4,5] and point mutation of c-kit has been observed in 33.35%-45% of AML patients [6]. Nevertheless, many of these studies were screened for c-kit mutations, only in a limited portion of the c-kit coding sequence and others were limited by small study populations.

It is known that c-kit is a leukemia proto-oncogene and that activated c-kit mutations are likely to contribute to the development of leukemia in humans [7-10]. The activation sphere of the c-kit receptor has resulted in constitutive c-kit kinase activity and c-kit receptors that harbor such mutations when introduced into mammalian cells in the downstream signaling pathways lead to factor-independent growth in vitro and leukemogenesis in vivo [7,11]. The c-kit gene is a member of the type III TKR family, which includes platelet-derived growth factor receptors (PDGFRs) [12-14].

Type III TKRs share a sequence homology and a similar structure, with 5 immunoglobulin-like repeats in the extracellular domain, 1 transmembrane domain (TM), 1 juxtamembrane domain (JM), 2 intracellular tyrosine kinase domains (TK1 and TK2) divided by a kinase insert domain (KI), and 1 C-terminal domain [15]. The genomic locus that encodes the c-kit gene receptor has 21 exons, ranging from 100 bp to 300 bp [16]. Mutations in exon 11 of the c-kit gene have been reported in gastrointestinal stromal tumors, solid tumors, and germ cell tumors [17-

19]. To date, no study has reported the frequency or prevalence of mutations in exon 11 of the c-kit gene in leukemia patients in India. As such, the present study aimed to identify mutations in exon 11 of the c-kit gene in Indian patients with malignant leukemias (acute myeloid leukemia [AML], acute lymphoblastic leukemia [ALL], chronic myeloid leukemia [CML] and chronic lymphocytic leukemia [CLL]) and to determine if c-kit gene mutations could be used as molecular genetic markers for leukemia.

Material and Methods

Patients

The study included 50 leukemia patients and 50 healthy controls. Ethical approval of the study protocol was granted by the Era's Lucknow Medical College and Hospital Ethics Committee. Clinical data of patients as well as control samples were recorded. Blood or bone marrow samples were stained according to the Leishman stain method and the patients were classified according to FAB criteria [20] as follows: AML (n = 31); ALL (n = 5); CML (n = 9); CLL (n = 5).

DNA Extraction

Specimens were collected from 50 routinely processed unstained bone marrow slides and blood diagnosed as leukemic by the hospital's hematology department and were then stored at -20°C . Genomic DNA was extracted according to Moskaluk et al. 1997 [21] with some modification. Lysis buffer and proteinase K (10 mg mL^{-1}) were added to samples, followed by incubation at 55°C for 1-2 h. Then, $10\ \mu\text{L}$ of 10% SDS, $120\ \mu\text{L}$ of 5M NaCl, and $300\ \mu\text{L}$ of RNase-free water were added, followed by thorough mixing and shaking. Next, $400\ \mu\text{L}$ of phenol:chloroform (4:1) was added, followed by centrifugation at 15,000 rpm for 10 min at 4°C . The supernatant was collected into a new tube. For precipitation chilled absolute alcohol was added and centrifuged at 11,000 rpm for 5 min at 4°C . The precipitate was washed with 70% alcohol and dissolved in $100\ \mu\text{L}$ of HPLC water.

Polymerase Chain Reaction and Single-Strand Conformational Polymorphism

Polymerase chain reaction (PCR) was performed with $25\ \mu\text{L}$ of PCR reaction mixture containing 200 ng of template DNA, 10 pmol of each primer, $10\ \text{mmol L}^{-1}$ of each mix dNTP, 1X reaction buffer, and 0.3 U of Taq polymerase enzyme (Fermentas, Germany) in an MJ Mini thermocycler (Bio-Rad, UK). The cycling conditions were as follows: 35 cycles of denaturation at 94°C for 30 s, followed by annealing at 56°C for 30 s, and extension

at 72°C for 30 s, followed by a final extension step at 72 °C for 10 min using the following primers [19]: forward: 5-ATTATTTAAAAGGTGATCTATTTTTC-3; reverse: 5-ACTGTTATGTGTACCCAAAAAG-3. Single-strand conformational polymorphism analysis was performed according to Orita et al. [22] with some modifications. Samples were denatured at 94° C for 8 min, and then immediately transferred to ice. Then 15 µL of amplified PCR product was loaded along with 15 µL of denaturing dye on 8% polyacrylamide gel. The gel was run in pre-cooled 1Xx TBE buffer. The gel tank was placed in a cold room (4 °C) and run for 15 h at 140 V. The PCR product on the gel was silver stained after electrophoresis. Electrophoresis mobility shifts in the patients' single-stranded or double stranded PCR products were detected via comparison with the controls' PCR products that were run in adjacent lanes.

Sequencing Analysis

Amplicons were sequenced using an automated sequencer (ABI 3730XL DNA Analyzer, Applied Biosystems, Foster City, California, USA) and examined using FinchTV software. Amplicons with mutations were reconfirmed via sequencing in both directions and in independent second samples. Sequences were analyzed using BioEdit JustBio software.

Results

Among the 50 leukemia patients 28 were male and 22 were female, with a mean age of 31.88 years (range: 2-65 years). The patients were classified according to FAB criteria [20] as AML (n = 31), ALL (n = 05), CML (n = 09), and CLL (n = 05). A shift in position was noted in 39 of the 50 patient samples via native SSCP-PAGE. In 19 of the 31 AML cases 20 point mutations were observed, whereas none were detected in the ALL, CML, or CLL patients. Point mutation details are shown in Table 1.

Discussion

The present study is the first to report mutations in exon 11 of the c-kit gene in leukemia patients from Northern India. Previous molecular studies have reported several mutations in exon 11 in different types of tumors. Mutations in exons 9, 13, and 17 of the c-kit gene are less frequently detected than those in exon 11. These mutations are considered rare in gastrointestinal stromal tumors, with a reported frequency of <10%, but are not uncommon in hematopoietic malignancies and germ cell neoplasms [23-25]. It was reported that 65%-92% of gastrointestinal stromal tumors harbor kit-activating mutations, the majority of which are localized at the juxtamembrane region involving exon 11 [26,27].

Table 1: Point Mutations in Exon 11 of the c-kit gene

Patient no.	Leukemia Type	Nucleotide	Codon
09	AML	TAT→TCT	Tyr568Ser
11	AML	AGG→ATG	Arg588Met
12	AML	TGG→TCA	Trp582Ser
13	AML	ATA→CTA	Ile571Leu
17	AML	ATA→CTA	Ile571Leu
18	AML	AAA→AAC	Lys550Asn
20	AML	GTT→CTT	Val569Leu
21	AML	TAC→TCC	Tyr570Ser
22	AML	GAT→CAT	Asp579His
23	AML	CCT→TCC	Pro577Ser
24	AML	CAC→CAA	His580Gln
27	AML	TAT→CCT	Tyr578Pro
35	AML	TGG→TCA	Trp582Ser
39	AML	AAC→GAC	Asn587Asp
40	AML	ATA→AAA	Ile563Lys
43	AML	ATA→ACA	Ile571Thr
44	AML	AGA→ACA	Arg586Thr
45	AML	CAA→CAC	Gln575His
50	AML	ACA→CCA TGG→TCA	Thr574Pro Trp582Ser

The majority of exon 11 mutations are clustered within the classical 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. [28], which includes frame deletions of 1 to several codons (typically involving codons 557-560) and point mutations and internal tandem duplications (typically involving the 30 end). In the present study heterogeneous point mutations in AML patients were observed, some of which were and were not previously reported. In 19 of the 31 AML patients 20 point mutations were observed; a point mutation at Lys550Asn in 1 patient and at Ile571Leu in 2 other patients were previously reported [29-32]. Mutation at codon 582 (Trp582Tyr and Trp582His) was reported by Tae Won Kim et al. [30]

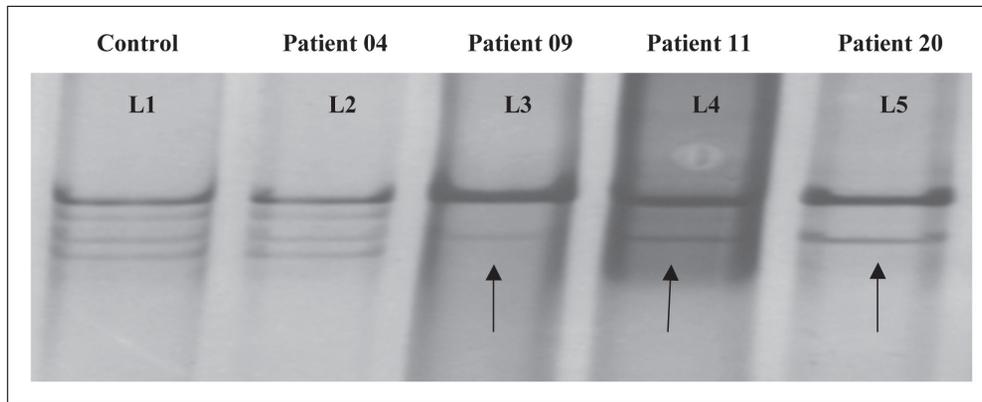


Figure 1: SSCP-PAGE analysis of exon 11 (257 bp) shows electrophoresis mobility shift on native page. Lane 1 is the control, and lanes 2-5 are patients (no shift in patient 04, and a shift in patients 09, 11, and 20).

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
Patient 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	R	L	S	F
Patient 11	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	M	L	S	F
Patient 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	D	H	K	S	E	F	P	R	N	R	L	S	F
Patient 13	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
Patient 17	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
Patient 18	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
Patient 20	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
Patient 21	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	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F
Patient 22	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
Patient 23	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	S	Y	D	H	K	W	E	F	P	R	N	R	L	S	F
Patient 24	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	W	E	F	P	R	N	R	L	S	F
Patient 27	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
Patient 35	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	S	E	F	P	R	N	R	L	S	F
Patient 39	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	D	R	L	S	F
Patient 40	K	P	M	Y	E	V	Q	W	K	V	V	E	E	K	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
Patient 43	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	T	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F
Patient 44	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	T	N	R	L	S	F
Patient 45	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	H	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F
Patient 50	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	P	Q	L	P	Y	D	H	K	S	E	F	P	R	N	R	L	S	F

Figure 2: Amino acid sequences of exon 11 of the 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 ■.

and Ying-Yong Hou et al. [17] reported Trp582Try and Trp582Gln; however, in 3 of the present studied patients there was a different substitution in the same codon in which tryptophan was replaced by serine (Trp582Ser). Mutations at codons Tyr568Asp, Ile571Thr, Thr574Tyr, Gln575Ile, Tyr578Phe, Asp579Gln, Asp579Pro, His580Leu, His580Tyr, His580Pro, Arg586Trp, Arg586Ile, Arg586Phe, Arg586Asp, Asn587Glu, Asn587Pro, Asn587His and Arg588Phe, Arg588Tyr, and Arg588Lys have been reported [17,30,29,32]; however, in the present study we observed substitution muta-

tions at Tyr568Ser, Thr574Pro, Gln575His, Tyr578Pro, Asp579His, His580Gln, Arg586Thr, Asn587Asp and Arg588Met which have not been previously reported. We also detected 4 novel point mutations—Ile563Lys, Val569Leu, Tyr570Ser, and Pro577Ser—at codons 563, 569, 570 and 577 respectively in exon 11 of the c-kit gene which have not been previously reported in any neoplasia patients. The mutations in exon 11 of the c-kit gene observed in the present study between codons 550 and 591 are in agreement with previously reported mutations in different populations (Figure 2 and Table 2).

Table 2: Comparison of Mutations in Exon 11 of the c-kit gene Identified in the Present Study and Those Previously Reported

Mutations	Novel Mutations	Mutation with different substitution		Existing Reported Mutations	References
		Substitution not previously reported (present results)	Reported Substitution		
ATA→AAA	Ile563Lys				
GTT→CTT	Val569Leu				
TAC→TCC	Tyr570Ser				
CCT→TCC	Pro577Ser				
TAT→TCT		Tyr568Ser	Tyr568Asp		[31]
ATA→ACA		Ile571Thr	Ile571Leu		[32]
ACA→CCA		Thr574Pro	Thr574Tyr		[17]
CAA→CAC		Gln575His	Gln575Ile		[17]
TAT→CCT		Tyr578Pro	Tyr578Phe		[30]
GAT→CAT		Asp579His	Asp579Gln Asp579Pro		[30]
CAC→CAA		His580Gln	His580Leu His580Tyr His580Pro		[17,30]
TGG→TCA		Trp582Ser	Trp582Tyr Trp582His Trp582Gln		[17,30]
AGA→ACA		Arg586Thr	Arg586Trp Arg586Ile Arg586Phe Arg586Asp		[17,30]
AAC→GAC		Asn587Asp	Asn587Glu Asn587Pro Asn587His		[17,30]
AGG→ATG		Arg588Met	Arg588Phe Arg588Tyr Arg588Lys		[17,30]
AAA→AAC				Lys550Asn	[31]
ATA→CTA				Ile571Leu	[32]

K: Lysine (Lys); P: proline (Pro); M: methionine (Met); Y: tyrosine (Tyr); E: glutamic acid (Glu); V: valine (Val); Q: glutamine (Gln); W: tryptophan (Trp); I: isoleucine (Ile); N: asparagine (Asn); G: glycine (Gly); D: aspartic acid (Asp); T: threonine (Thr); H: histidine (His); R: arginine (Arg); L: leucine (Leu); S: serine (Ser); F: phenylalanine (Phe); AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; CML: chronic myeloid leukemia; CLL: chronic lymphocytic leukemia; PCR: polymerase chain reaction; SSCP: single-strand conformational polymorphism; PAGE: polyacrylamide gel electrophoresis; DNA: deoxyribonucleic acid; A: adenine; T: thymine; G: guanine; C: cytosine; n: number of samples.

In conclusion, the present study is the first to report the presence of c-kit gene mutations in Indian leukemia patients. The observed mutations in exon 11 of the c-kit gene may be involved in c-kit over expression in leukemia. These observations suggest that mutations in exon 11 of the c-kit gene might be useful molecular genetic markers for leukemia. Additional research with larger study groups may clarify the prognostic implications of these mutations, and their association with the pathogenesis and progression of myeloid malignancy.

Acknowledgments

This study was supported by an intramural grant from Era's Lucknow Medical College and Hospital, Lucknow, India.

Conflict of Interest Statement

The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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