Two different point mutations in ABL gene ATP–binding domain conferring Primary Imatinib resistance in a Chronic Myeloid Leukemia (CML) patient: A case report

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ABSTRACT

Imatinib (Gleevec) is the effective therapy for BCR-ABL positive CML patients. Point mutations have been detected in ATP-binding domain of ABL gene which disturbs the binding of Gleevec to this target leading to resistance. Detection of mutations is helpful in clinical management of imatinib resistance. We established a very sensitive (ASO) PCR to detect mutations in an imatinib-resistant CML patient. Mutations C944T and T1052C were detected which cause complete partial imatinib resistance, respectively. This is the first report of multiple point mutations conferring primary imatinib resistance in same patient at the same time. Understanding the biological reasons of primary imatinib resistance is one of the emerging issues of pharmacogenomics and will be helpful in understanding primary resistance of molecularly-targeted cancer therapies. It will also be of great utilization in clinical management of imatinib resistance. Moreover, this ASO-PCR assay is very effective in detecting mutations related to imatinib resistance.

INTRODUCTION

Chronic myeloid leukemia (CML) is a stem cell disorder (1). This is a myeloproliferative disease characterized by marked increase in granulocytes, marked bone marrow hyperplasia and spleenomegaly (2). The symptoms which appear in initial phase are non-specific including fever, anaemia, fatigue, weight loss, weakness, and others (3). CML primarily affects the adults between 25-60 years of age and accounts for 15-20% of all leukemia cases (4).

CML is associated with presence of Philadelphia (Ph) chromosome detectable microscopically which results from balanced chromosomal translocation t (9:22) (q34; q11), that is, translocation of proto-oncogene ABL from chromosome 9 to BCR gene at Chromosome 22 (5). BCR-ABL translocation is common in 95% of patients and Ph chromosome is found in all dividing multipotent stem cells (5). BCR-ABL fusion gene formed as a result of this translocation encodes a protein which has tyrosine kinase and oncogenic activity (6).

Median survival for CML patients is 3-8 years after clinical manifestation of the disease and physicians have very little time for treatment of this fatal disease (7). Hydroxyurea and interferon are first-line treatment for CML patients but usually patients show resistant to these therapies (8). STI 571 commonly called as Imatinib or Gleevec is currently the most specific drug for CML patients and is regarded as very effective therapy for CML

patients (9). This drug binds to ATP -binding site of tyrosine kinase domain in *bcr-abl* protein, a protein which triggers the carcinogenesis pathway leading to manifestation of the disease (10). Thus, by occupying and blocking the ATP binding site, it stops the signal transduction leading to onset of Leukemia (10). However, a considerable number of patients have been reported to show resistance to Gleevec, leading to relapses (11). Resistance against Gleevec has been attributed to mutations in the ATP–binding site of tyrosine-protein kinase domain of the BCR-ABL gene which lead to conformational changes in *bcr-abl* protein resulting in impairment of Gleevec binding (11-14). Many BCR-ABL single base pair mutations have been found in Gleevec resistant CML patients (15). It has been reported that different mutations in tyrosine-protein kinase domain of the BCR-ABL transcript lead to different degree of the drug resistance, depending upon the nature and location of the mutations (16). Some of the mutations lead to moderate resistance and dose escalation can efficiently overcome Gleevec resistance in these cases (17). On the other hand, some of the mutations lead to complete drug resistance (16-18). Under such circumstances, combination therapies with Gleevec or use of some substitution therapy have been reported to manage this resistance (19, 20).

We checked mutations in ABL gene ATP-binding domain of a CML patient who had been on oral dose of 400mg/day of Gleevec for nine months. Patient had no hematological, cytogenetic or molecular response to Gleevec. A very sensitive ASO-PCR method (21) was used to detect three mutations namely C944T, T932C and T1052C. Interestingly, we found two mutations in this patient: C944T mutation causing threonine to isoleucine substitution at amino acid 311 and T1052C mutation leading to amino acid substitution from methionine to leucine at position 351. This is the first report of double mutation in ABL gene ATP-binding domain of a Gleevec resistance CML patient. This new finding and its biological and clinical implications are discussed.

MATERIALS AND METHODS

Patient's inclusion criteria: A CML patient with oral dose of 400 mg/day for nine months of Gleevec who has no hematological, cytogenetic and molecular response to drug was investigated for ABL gene ATP-binding domain mutations responsible for Gleevec resistance. Blood sample was taken from patient in EDTA. Consent from patient was obtained for this study.

DNA Extraction: Genomic DNA was extracted from patient's blood using the method described by Sambrook *et al.* in 1989 (22) with little modifications and optimization. Briefly:

1. One ml of blood was taken. It was mixed with 9 ml of Buffer A (Sucrose 10.95g, 1M Tris HCl 1ml, 1M MgCl$_2$ 500 μl and 1 ml of Triton per 100 ml of buffer), incubated on ice for 2 minutes and spun at 1500 rpm for 15 minutes at 4°C.
2. Pink pellet of nuclei was resuspended in 320 μl of cold Buffer B (25 ml of EDTA pH 8, 20 ml of 5M NaCl per 100 ml of buffer). Thirty two μl of 10% SDS and 3.5 μl of Proteinase-K (10mg/ml) were added. The mixture was subjected to shaking incubation at 37°C overnight.
3. An equal volume of buffer-equilibrated phenol was added and mixed on ice for 10-15 minutes. It was centrifuged at 2500 rpm for 15 minutes at 4°C.
4. Supernatant was taken, an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently at room temperature for 15-30 minutes. Phases were separated by centrifugation at 2500 rpm for 15 minutes at 4°C.
5. Supernatant was taken, 1/10 volume of 3M sodium acetate and 1.5 volume of 100% isopropanol was added. DNA was precipitated.
6. Pellet was obtained by centrifugation at 14,000 rpm for 20-30 minutes at room temperature. It was washed with 70% ethanol and air-dried.
7. Pellet was dissolved in Tris EDTA buffer, DNA quantity estimated spectrophotometrically and stored at -20°C.

Allele-specific oligonucleotide (ASO) PCR: Allele Specific Oligonucleotides (ASO) and Normal Primers (NP): ASO-PCR assay described by Catherine Roche-Lestienne *et al.* (2002) (21) was used for detection of three mutations of ABL gene ATP-binding domain namely C944T, T932C and T1052C. DNA was amplified with normal primer (NP) or mutation specific forward primers (ASO) and a common reverse primer. The identities and sequences of the primers are given in Table 1.

Table 1: Identities and sequences of Primers used in ASO-PCR (Difference between normal and Allele (mutation) Specific Oligonucleotides indicated).

Sr. #	Mutation	Primer identity	Primer type	Polarity	Primer sequence (5/-3/)
1	C944T	A1	NP	Forward	GCC CCC GTT CTA TAT CAT CA**C**
		A2	ASO	Forward	GCC CCC GTT CTA TAT CAT CA**T**
		R1	Common	Reverse	GGA TGA AGT TTT TCT TCT CCA
2	T932C	B1	NP	Forward	CAC CCG GGA GCC CCC G**T**
		B2	ASO	Forward	CAC CCG GGA GCC CCC G**C**
		R4	Common	Reverse	CCC CTA CCT GTG GAT GAA GT
3	T1052C	C1	NP	Forward	CCA CTC AGA TCT CGT CAG CCA **T**
		C2	ASO	Forward	CCA CTC AGA TCT CGT CAG CCA **C**
		R5	Common	Reverse	GCC CTG AGA CCT CCT AGG CT

PCR Mix preparation: A 50 μl PCR reaction was performed containing 3μl of DNA, 10X PCR buffer (Fermentas, USA), 1.5 mM MgCl2, 300 M each of dATP, dGTP, dCTP and dTTP, 1.5 U Taq DNA polymerase (Fermentas, USA) and 25 pM each of forward and reverse primers. A negative control and a control from a clinically-declared healthy person with no Philadelphia positive leukemia were also included in the study.

PCR Thermal Profile: Thermo cycling conditions for this ASO-PCR conditions were 12 minutes at 94°C followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 64°C (for mutation 1 and 2) or 68°C (for mutation 3) for 1 minute and extension at 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes.

Gel Electrophoresis: Amplified products from ASO-PCR were electrophorised at 1.5% gel and visualized and analysed after

staining with 3% ethidium bromide.

RESULTS

Analysis of Mutation C944T

Cytosine to Thymine mutation at position 944 of ABL gene was checked by ASO-PCR. Patient DNA and DNA from a healthy control were amplified with Allele-specific oligonucleotide as well as normal primer to give a PCR product of 158 bp. Although both patient and healthy control DNA yielded specific bands with normal forward primer, PCR product was observed only for patient when amplified using mutation specific primer (Fig 1). So, mutation C944T was found in this patient.
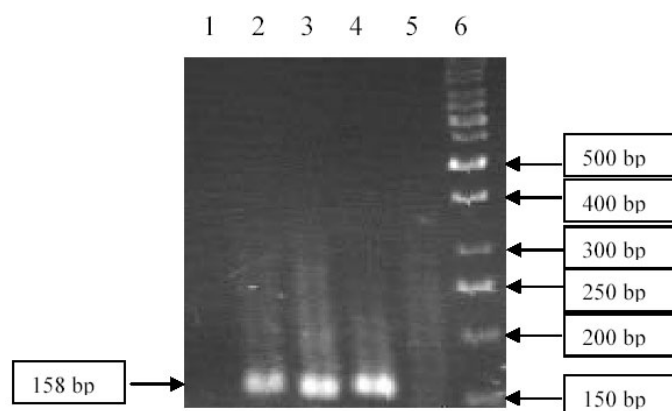
Fig. 1: Detection of Mutation C944T Mutation by ASO-PCR. Lane 1: Negative control, Lane 2: Patient's DNA amplified with normal primer, Lane 3: Patient's DNA amplified with mutation specific primer (ASO), Lane 4: Control DNA amplified with normal primer, Lane 5: Control DNA amplified with mutation specific primer (ASO), Lane 6: DNA ladder (50 bp) Fermentas.

Analysis of Mutation T932C

Thymine to cytosine substitution at position 932 of ABL gene of Gleevec-resistance CML patient was checked by ASO-PCR. Patient DNA and DNA from a healthy control were amplified with allele-specific oligonucleotide (mutation specific primer) as well as normal primer to give a PCR product of 174 bp (Fig. 2). Specific band using normal primer was detected on gel for patient as well as healthy control but no PCR product was observed for patient DNA when amplified with mutation specific primer (ASO).
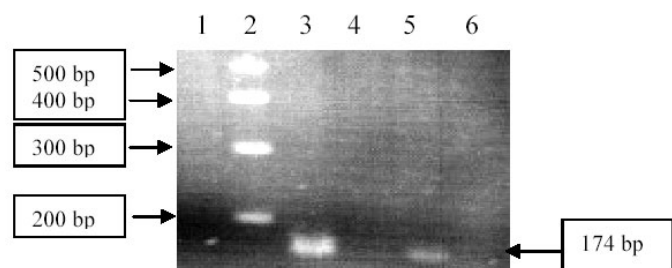
Fig. 2: Detection of Mutation T932C Mutation by ASO-PCR. Lane 1: Negative control, Lane 2: DNA ladder (100 bp) Fermentas, Lane 3: Control DNA amplified with normal primer, Lane 4: Control DNA amplified with mutation specific primer (ASO), Lane 5: Patient's DNA amplified with normal primer, Lane 6: Patient's DNA amplified with mutation specific primer (ASO).

Analysis of Mutation T1052C

This mutation also confers Gleevec resistance in CML patients. For checking this thymine to cytosine substitution at position 1052 of ABL gene in Gleevec-resistance CML patient, ASO-PCR was performed using mutation specific primer (Allele -specific oligonucleotide) as well as normal primer to give a PCR product of 112 bp. Although gel electrophoresis showed specific amplification both for patient and healthy control, 112 bp ASO -PCR product was observed only for patient (Fig. 3). It is inferred that mutation C1052T mutation is also involved in this patient in conferring Gleevec resistance.
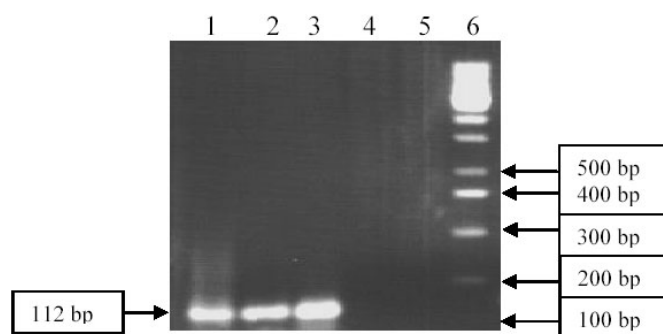
Fig. 3: Detection of Mutation T1052C by ASO-PCR. Lane 1: Patient's DNA amplified with normal primer, Lane 2: Patient's DNA amplified with mutation specific primer (ASO), Lane 3: Control DNA amplified with normal primer, Lane 4: Control DNA amplified with mutation specific primer (ASO), Lane 5: Negative control, Lane 6: DNA ladder (100 bp) Fermentas.

On the basis of our results, we conclude that two mutations are responsible for primary Gleevec resistance in the CML patient under study. We also conclude that this ASO-PCR is very effective and sensitive to detect point mutations conferring imatinib resistance in CML patients.

DISCUSSION

CML is a very fatal disease which is very difficult to treat (23). Patients usually resist the chemotherapy like hydroxyurea and interferon-á (24). In recent years, Gleevec has fascinated the physicians, clinicians, patients and researchers by its high effectiveness against BCRABL positive CML patients (25). As it stops the oncogenic activity of bcr-abl onco-protein by binding to its ATP-binding site in kinase domain, it has fewer side effects than interferon and is thus well-tolerated (26). It binds to some amino acids in ATP -binding domain by making hydrogen bonds (12). Recently it was found that some mutations in ABL gene ATP–binding domain lead to conformational changes in bcr-abl oncoprotein, thus resulting in impairment of Gleevec binding and leading to clinical resistance (27). Some mutations cause complete Gleevec resistance as they completely stop the Gleevec binding to its target while other mutations only affect this binding partially, leading to only moderate resistance to this drug (14, 18, 28). Thus, detection of mutations associated with Gleevec resistance is of a considerable clinical impact.

Allele specific oligonucleotide (ASO) PCR is a very specific and

sensitive technique for detection of known mutations (29). This method is even sensitive than mutation detection by sequencing of ABL ATP-binding domain (30) because DNA sequencing can only useful for point mutation detection when proportion of mutated cells is more than 30% (21). In cases where number of mutated cells is less than 30% of the total cells in patient's sample, at least 10 independent clones from the patient must be analysed for mutation detection which is quite costly and time consuming (21). On the other hand, ASO-PCR is comparatively more sensitive, specific, very economical and quick method for detection of mutations (31). The ASO-PCR established by us is able to detect one mutated, Gleevec resistant cell out of 10,000 normal cells (21).

In present study, we found two mutations in a Gleevec-resistant CML patient by ASO-PCR method. It is the first report of multiple mutations in a Gleevec resistant CML patient. A Thymine to cytosine mutation at position 1052 of ABL gene was detected. This mutation leads to amino acid substitution from Methionine to Threonine at position 351 of ABL ATP binding domain (18). This mutation only partially impairs the binding of Gleevec to its target and thus leads to partial drug resistance (21). This mutation has previously been reported (18). It has been authoritatively documented that partial Gleevec resistance can be actively overcome by dose escalation of Gleevec from 600-1600mg/day (32). On the other hand, a cytosine to thymine mutation at ABL gene position 944 was detected which causes threonine to isoleucine amino acid substitution at amino acid position 315 in ATP-binding domain of bcr-abl oncoprotein. This mutation has already been reported by various authors like (12-14, 18, 33). It has been determined on the basis of crystal structure of abl kinase domain that Threonine 315 is among those amino acids which make hydrogen bonds with Gleevec by providing an oxygen atom (12). When isoleucine takes the place of threonine as a result of C944T mutation, it does not provide oxygen atom for binding (12). Moreover, it contains an extra hydrocarbon group in side chain which results in steric hindrance to Gleevec (12). Thus, binding of drug is completely impaired leading to complete drug resistance (18, 33). Complete Gleevec resistance can be managed clinical by combination therapies (34). Gleevec plus arsenic oxide, Gleevec plus Pegylated interferon-á and Gleevec plus Cytarabine have promising results for CML patients who show complete resistance to Gleevec (20, 35, 36). Moreover some new drugs like Farnesyl transferase inhibitors, Raf kinase inhibitors etc. which stop cancer pathway signaling by bcr-abl oncoprotein have been are under clinical trials with promising initial results (37). Another drug called Genasense which degrades BCR-ABL mRNA has promising results against Gleevec-resistant CML cases (38). Thus it is inferred in present situation that mutation detection related to Gleevec resistance has a great clinical importance. Gleevec resistance can be detected very early in one to three months after initiation of therapy (21) and molecular typing of Gleevec resistant patients on the basis of nature and location of mutations in ATP-binding domain can help in adjustment of therapy accordingly (18, 21, 35, 39).

It should be kept in mind that impact of mutations on Gleevec resistance reported so far is based on a single mutation for each patient. As this is first report of double mutation conferring Gleevec resistance in same patient and each mutation has different impact on drug resistance, biological significance of two or more ABL ATP-binding domain mutations conferring Gleevec resistance in same patient with a difference in resistance mechanism is still to be determined. It is, however, expected that such multiple mutations could result in much complicated Gleevec resistance patterns which might be found more difficult to manage clinically.

ACKNOWLEDGMENTS

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