



Case study of primary imatinib resistance and correlation of BCR-ABL multiple mutations in chronic myeloid leukemia

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Background: The kinase inhibitor, imatinib (Gleevec™, Novartis) has proven to be an effective treatment for chronic myeloid leukemia, providing proof-of-principle for a molecularly targeted approach in oncology. Despite its success in BCR-ABL-positive chronic myeloid leukemia patients, primary imatinib resistance has emerged, resulting in disease relapse. Little is known about the underlying genetic causes of resistance, however, with emerging pharmacogenomic approaches, a more comprehensive picture is developing. In imatinib-resistant patients, point mutations have been detected in an ATP-binding domain of the *ABL* gene, which disturbs the binding of imatinib to this target leading to resistance. However, it remains to be determined whether these mutations confer primary imatinib resistance. **Methods:** Primary imatinib resistance was observed in a chronic myeloid leukemia patient with no hematological, cytogenetic and molecular response to 9 months treatment with 400 mg imatinib/day. A highly sensitive allele-specific oligonucleotide polymerase chain reaction was established to detect point mutations in BCR-ABL adenosine triphosphate-binding domain. **Results:** C944T and T1052C mutations were detected which cause complete and partial imatinib resistance, respectively. **Conclusions:** This is a case study of multiple point mutations conferring primary imatinib resistance in one patient at the same time. Elucidating the biological mechanisms of primary imatinib resistance will improve the ability to predict patient responsiveness and provide effective clinical management. Furthermore, the allele-specific oligonucleotide-polymerase chain reaction assay is very effective in detecting mutations related to imatinib resistance.

Chronic myeloid leukemia (CML) is a stem cell, myeloproliferative disorder manifested clinically as a marked increase in granulocytes, bone marrow hyperplasia and splenomegaly [1,2]. The initial symptoms that appear are nonspecific including fever, anemia, fatigue, weight loss and weakness [3]. CML primarily affects adults between the ages of 25 to 60 years and accounts for 15 to 20% of all leukemia cases [4].

CML is associated with the 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 to 8 years after clinical diagnosis of the disease and there is a very narrow window of opportunity for the treatment of this disease [7]. Hydroxyurea and interferon are first-line therapies for CML

patients, but patients commonly develop resistant [8]. STI-571, commonly known as imatinib (Gleevec™, Novartis), is currently the most efficacious, target-specific drug for the treatment of CML [9]. Imatinib binds to the adenosine triphosphate (ATP)-binding site of the tyrosine kinase domain of the BCR-ABL protein, a protein that triggers the carcinogenic pathway leading to the disease [10]. Thus, by occupying and blocking the ATP-binding site, imatinib prevents the signal transduction leading to the onset of CML [10]. However, a considerable number of incidences have been reported in patients who develop resistance to imatinib, leading to disease relapse [11]. This resistance has been attributed to mutations within the ATP-binding site of the tyrosine kinase domain of *BCR-ABL* gene, resulting in conformational changes in the BCR-ABL protein, hindering imatinib occupancy and binding [11–14].

Many *BCR-ABL* single base-pair mutations have been found in imatinib-resistant CML patients [15]. It has been reported that different mutations in the tyrosine kinase domain of the *BCR-ABL* transcript lead to different degrees of the drug resistance, depending upon the nature

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and location of the mutations [16]. Some of the mutations lead to moderate resistance and dose escalation can effectively overcome this problem [17]. However, some mutations can result in complete drug resistance [16–18]. Under such circumstances, combination therapies with imatinib or a form of substitution therapy have been reported to manage this resistance [19,20].

The authors examined mutations in the *ABL* gene ATP-binding domain of a CML patient receiving 400 mg/day of imatinib orally for 9 months. The patient had no hematological, cytogenetic or molecular response to imatinib. A highly sensitive allele-specific oligonucleotide (ASO)-polymerase chain reaction (PCR) method was used to detect three mutations: *C944T*, *T932C* and *T1052C* [21]. Interestingly, two mutations were discovered in this patient: the *C944T* mutation causing threonine-to-isoleucine substitution at amino acid 311 and the *T1052C* mutation leading to amino acid substitution from methionine-to-leucine at position 351. This is the first report of multiple point mutations in the *ABL* gene ATP-binding domain of a CML patient demonstrating primary imatinib resistance. This finding and its biological and clinical implications are discussed.

Methods

Patient's inclusion criteria

A CML patient receiving an oral dose of 400 mg/day imatinib for 9 months who has no hematological, cytogenetic and molecular response to the drug was investigated for *ABL* gene ATP-binding domain mutations responsible for resistance. Blood sample was taken from patient in ethylenediaminetetraacetic acid (EDTA). Consent from the patient was obtained for this study.

DNA extraction

Genomic DNA was extracted from patient's blood using the method described by Sambrook and colleagues [22] with a little modifications and optimization:

- 1 ml of blood was taken and mixed with 9 ml of buffer A (sucrose 10.95 g, 1 M Tris HCl 1 ml, 1 M MgCl₂ 500 µl and 1 ml of Triton per 100 ml of buffer), incubated on ice for 2 mins and spun at 1500 rpm for 15 mins at 4°C
- 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). 30 µl of 10% SDS and 3.5 µl of proteinase-K (10 mg/ml) were added. The mixture was subjected to shaking incubation at 37°C overnight

- An equal volume of buffer-equilibrated phenol was added and mixed on ice for 10 to 15 mins. It was centrifuged at 2500 rpm for 15 mins at 4°C
- Supernatant was taken, an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently at room temperature for 15–30 mins. Phases were separated by centrifugation at 2500 rpm for 15 mins at 4°C
- Supernatant was taken, 1/10 volume of 3 M sodium acetate and 1.5 volume of 100% isopropanol was added. DNA was precipitated
- Pellet was obtained by centrifugation at 14,000 rpm for 20–30 mins at room temperature. It was washed with 70% ethanol and air-dried
- Pellet was dissolved in Tris EDTA buffer, DNA quantity estimated spectrophotometrically and stored at -20°C

Allele-specific oligonucleotide PCR

Allele-specific oligonucleotides & normal primers ASO-PCR assay described by Roche-Lestienne and colleagues [21] was used for detection of three mutations in the *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.

PCR mix preparation

A 50 µl PCR reaction was performed containing 3 µl 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 healthy person with no Ph-positive leukemia were also included in the study.

PCR thermal profile

Thermocycling conditions for ASO-PCR were 12 minutes at 94°C followed by 35 cycles of denaturing at 95°C for 1 min, 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 mins.

Gel electrophoresis

Amplified products from ASO-PCR were electrophorized at 1.5% gel and visualized and analyzed after staining with 3% ethidium bromide.

Table 1. Identities and sequences of primers used in ASO-PCR.

Sr. #	Mutation	Primer identity	Primer type	Polarity	Primer sequence (5'-3')
1	<i>C944T</i>	A1	NP	Forward	GCC CCC GTT CTA TAT CAT CAC
		A2	ASO	Forward	GCC CCC GTT CTA TAT CAT CAT
		R1	Common	Reverse	GGA TGA AGT TTT TCT TCT CCA
2	<i>T932C</i>	B1	NP	Forward	CAC CCG GGA GCC CCC GT
		B2	ASO	Forward	CAC CCG GGA GCC CCC GC
		R4	Common	Reverse	CCC CTA CCT GTG GAT GAA GT
3	<i>T1052C</i>	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

The differences between normal and allele (mutation)-specific oligonucleotides indicated in bold.

ASO-PCR: Allele-specific oligonucleotide polymerase chain reaction; NP: Normal primer.

Results

Analysis of C944T mutation

Cytosine-to-thymine mutation at position 944 of the *ABL* gene was determined by ASO-PCR. Patient and control DNA were amplified with ASO as well as normal primer to provide a PCR product of 158 bp. Although both patient and control DNA yielded specific bands with normal forward primer, PCR product was observed only for the CML patient when amplified using mutation-specific primer (Figure 1). The *C944T* mutation was detected in this patient.

Analysis of T932C mutation

Thymine-to-cytosine substitution at position 932 of the *ABL* gene in the imatinib-resistant CML patient was examined using ASO-PCR.

Patient and control DNA were amplified with ASO as well as normal primer to give a PCR product of 174 bp (Figure 2). Using normal primer, specific band was detected on the gel for the CML patient as well as healthy control but no PCR product was observed for patient DNA when amplified with mutation-specific primer (ASO).

Analysis of T1052C mutation

The *T1052C* mutation also confers imatinib resistance in CML patients. To detect this thymine-to-cytosine substitution at position 1052 of *ABL* gene in the imatinib-resistant CML patient, ASO-PCR was performed using mutation-specific primer as well as normal primer to give a PCR product of 112 bp. Although gel electrophoresis showed specific amplification both for the patient and healthy control, 112 bp ASO-PCR product was observed only for the CML patient (Figure 3). It is inferred that mutation *C1052T* mutation is also involved in conferring imatinib resistance in this patient.

Two mutations are responsible for primary imatinib resistance in the CML patient under study. Effectiveness and sensitivity of this ASO-PCR assay to detect point mutations associated with imatinib resistance in CML patients has been demonstrated.

Discussion

CML is a highly fatal disease which is very difficult to treat [23]. Patients often demonstrate resistance to the conventional chemotherapy such as hydroxyurea and interferon (IFN)- α [24]. In recent years, imatinib has emerged as a highly effective treatment for BCR-ABL-positive CML [25]. Imatinib halts the oncogenic activity of the BCR-ABL oncoprotein by binding to its ATP-binding site within the kinase domain (by

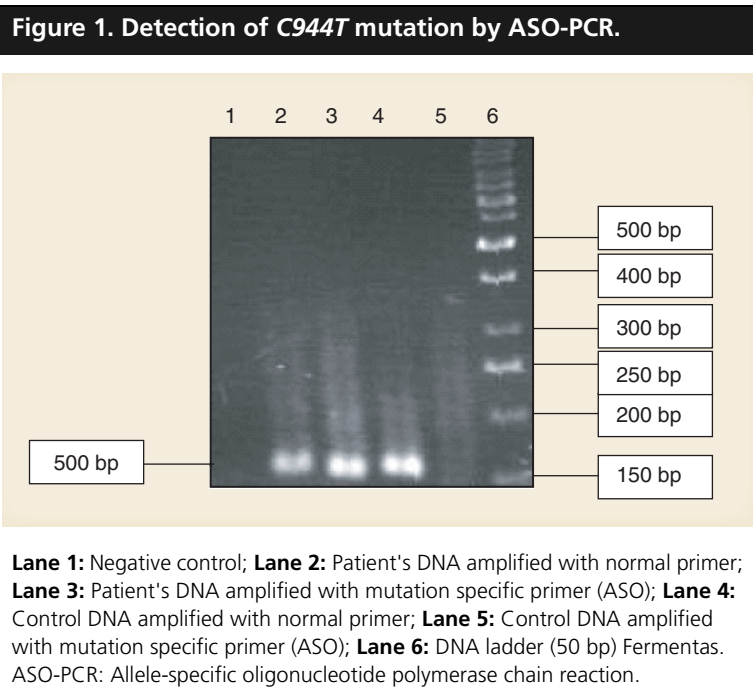
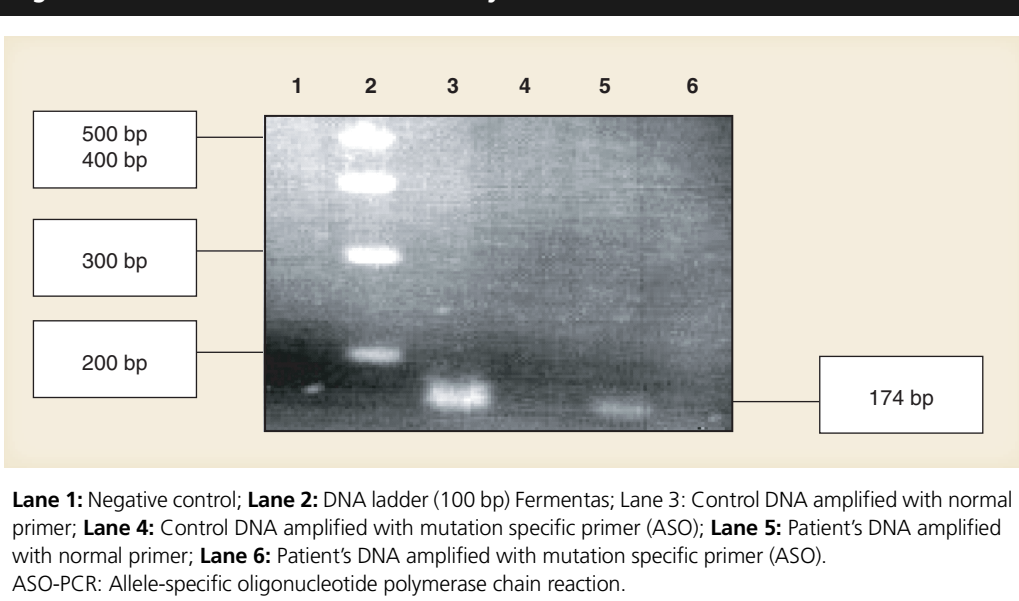


Figure 2. Detection of T932C mutation by ASO-PCR.



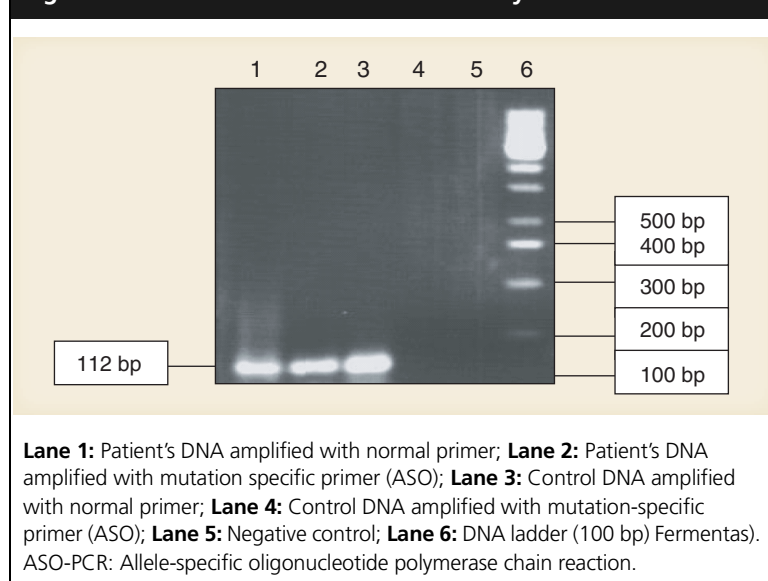
binding to a number of amino acids in the ATP-binding domain through the formation of hydrogen bonds [12]) and has fewer side effects than IFN, thus being well tolerated [26]. Recently, it was found that some mutations in *ABL* gene ATP-binding domain lead to conformational changes in the BCR-ABL oncoprotein, thus resulting in impairment of imatinib binding, leading to clinical resistance [27]. Some mutations cause complete imatinib resistance as they prevent binding to its target, while other mutations only affect this binding partially, leading to only moderate resistance to this drug

[8,14,18]. Thus, the detection of mutations associated with imatinib resistance is of a considerable clinical importance.

ASO-PCR is a very specific and sensitive technique for detection of known mutations [29]. This method is even more sensitive than mutation detection by sequencing of the *ABL* ATP-binding domain [30] as DNA sequencing can only be used for point mutation detection when the proportion of mutated cells is more than 30% [21]. In cases where the number of mutated cells is less than 30% of the total cells in a patient's sample, at least ten independent clones from the patient must be analyzed for mutation detection, which is quite costly and time consuming [21]. Conversely, ASO-PCR is comparatively more sensitive, specific, very economical and a quick method for the detection of mutations [31]. The ASO-PCR established in this study is capable of detecting one mutated, imatinib-resistant cell out of 10,000 normal cells [21].

In the present study, we found two mutations in a imatinib-resistant CML patient by ASO-PCR. It is the first report of multiple mutations in an imatinib-resistant CML patient. A thymine-to-cytosine mutation at position 1052 of the *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 imatinib to its target and thus leads to partial drug resistance [21]. This mutation has previously been reported [18].

Figure 3. Detection of mutation T1052C by ASO -PCR.



It has been authoritatively documented that partial imatinib resistance can be actively overcome by dose escalation from 600 to 1600 mg/day [32]. In addition, a cytosine-to-thymine mutation at position 944 on the *ABL* gene was detected, which is known to cause a threonine-to-isoleucine amino acid substitution at position 315 in ATP-binding domain of BCR-ABL oncoprotein. This mutation has already been reported by various authors [12–14,18,33] and on the basis of crystallization studies of the structure of ABL kinase domain, it has been determined that threonine 315 is among the amino acids that form hydrogen bonds with imatinib by providing an oxygen atom [12]. When isoleucine substitutes threonine as a result of the *C944T* mutation, it does not provide oxygen atom for binding [12]. Moreover, it contains an extra hydrocarbon group in its side chain which results in steric hindrance to imatinib [12]. Thus, binding of drug is completely impaired leading to complete drug resistance [18,33]. Complete imatinib resistance can be managed clinically by combination therapies [34]. Imatinib plus arsenic oxide, pegylated IFN- α and/or cytarabine have provided promising results for CML patients who show complete resistance [20,35,36]. Moreover, some new drugs such as farnesyl transferase inhibitors and Raf kinase inhibitors for example, which interrupt oncogenic signaling pathways involving the BCR-ABL oncoprotein are undergoing clinical trials with promising initial results [37]. Another drug, Genasense[®] (Genta Inc.), which degrades BCR-ABL mRNA has provided promising results in imatinib-resistant CML patients [38]. Thus it is

inferred in the present situation that mutation detection related to imatinib resistance has a great clinical importance. Imatinib resistance can be detected very early – 1 to 3 months after initiation of therapy [21] – and molecular typing of imatinib-resistant patients on the basis of nature and location of mutations in ATP-binding domain can help in the optimization of therapy [18,21,35,39].

It should be kept in mind that the impact of mutations on imatinib resistance reported to date is based on a single mutation for each patient. As this is the first report of multiple mutations conferring imatinib resistance in one patient and each mutation confers different levels of resistance, the biological significance of more than one ABL ATP-binding domain mutations conferring imatinib resistance in same patient with a difference in resistance mechanism is still to be determined. However, we theoretically assume that such multiple mutations will result in more complicated imatinib-resistance patterns, which might be much more difficult to manage clinically than CML patients with the single mutation. As little is known about the underlying genetic and molecular reasons for primary imatinib resistance, we hope that this new discovery will help not only unravel the pharmacogenomic basis of primary resistance to such molecularly targeted therapies but will also help in formulating new methods to manage such complicated cases of resistance.

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Highlights

- Although imatinib is currently the most effective monotherapy for BCR-ABL-positive leukemia, primary imatinib resistance (non-responsiveness) remains the main hurdle to be overcome and understood.
- This report describes a positive correlation between the BCR-ABL ATP domain mutations and primary imatinib resistance in chronic myeloid leukemia patients.

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