ABL gene kinase domain mutation scanning by denaturing high performance liquid chromatography sequencing method

Denatüre edici yüksek performanslı likit kromotografi yöntemi ile ABL geni kinaz bölgesi mutasyonlarının taranması

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Abstract

Objective: Despite the efficacy of the BCR-ABL tyrosine kinase inhibitor imatinib, the development of resistance against imatinib has been observed. The most important mechanisms known to cause resistance are point mutations in the ABL tyrosine kinase and the ATP domain. This study describes the use of denaturing high performance liquid chromatography (dHPLC) as a method to screen for mutations of the ABL gene.
Material and Methods: We used the dHPLC based assay for the screening of ABL point mutations. Forty chronic myeloid leukemia (CML) patients who showed resistance to imatinib were screened in parallel by dHPLC and direct sequencing.
Results: Nine of the 40 patients (23%) had mutations.
Conclusion: dHPLC can be a useful method for pre-screening. Analyzing the mutations and monitoring (high-risk) patients can improve their prognosis and survival rate. dHPLC can potentially become a valuable tool for regular testing of patients in the future. *(Turk J Hematol 2011; 28: 97-102)*
Key words: Chronic myeloid leukemia, imatinib resistance, mutation, dHPLC

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Özet

Amaç: Bir BCR-ABL tirozin kinaz inhibitörü olan imatinib'in etkinliğine karşın bazı hastalarda ilaca karşı direnç gelişimi gözlenmektedir.. Direnç gelişimine neden olan en önemli mekanizma, ABL geninin tirozin kinaz ve ATP bölgelerindeki nokta mutasyonlarıdır. Bu çalışma ABL gen mutasyonlarının taranmasında denatüre edici HPLC (dHPLC) yönteminin yerini ve önemini açıklama amacıyla yapılmıştır.
Yöntemler ve Gereçler: ABL nokta mutasyonları, dHPLC kullanılarak taranmıştır. İmatinib direnci gösteren 40 KML hastası DHPLC ve paralelinde doğrudan sekanslama ile incelenmiştir.
Bulgular: Kırk hastanın dokuzunda (%23) mutasyon saptandı.

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Sonuç: dHPLC yararlı bir ön tarama yöntemi olabilir. Mutasyon analizleri ile yüksek riskli hastaların monitörizasyonu prognozu iyileştirebilir ve sağ kalım oranını arttırabilir. Gelecekte dHPLC bu hastaların düzenli aralıklarla izleminde değerli bir araç olarak kullanılabilir.
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Introduction

Chronic myeloid leukemia (CML) is a proliferative stem cell disorder that affects patients in all age groups [1,2]. The clinical course of CML is characteristically triphasic, starting with a chronic phase (CP) of variable duration, followed by progression to an accelerated phase (AP) and finally resulting in blast crises (BC) [3-5]. CML is the first discovered human cancer associated with a consistent chromosomal abnormality-the chimeric *BCR/ABL* gene, known as Philadelphia (Ph) chromosome [6,7]. This translocation causes an unregulated tyrosine kinase activity and has a pivotal role in CML pathogenesis. Approximately 95% of CML patients have *BCR/ABL* rearrangement, indicating it is a suitable drug target for anticancer therapy [8,9].

Imatinib mesylate (IM) is a potent and selective inhibitor of BCR-ABL tyrosine kinase, the first rationally designed, molecularly targeted therapy for a human malignancy [10-13]. Despite the high rates of hematologic and cytogenetic responses, some patients show IM resistance in both phases. Resistance to IM can be defined as the lack of complete hematological response in patients with CP disease or as lack of return to CP for patients in an AP or BC [14,15].

Several underlying mechanisms for IM resistance have been studied in CML patients and cell lines. Drug resistance has mostly arisen as a result of point mutations in the *BCR-ABL* gene that reduce drug binding within the kinase domain or due to overexpression of BCR-ABL protein [15-18]. In clonal selection, *BCR-ABL* mutated cells have a higher survival rate due to the selective pressure of imatinib therapy. A number of mutations have been well characterized in terms of their ability and the degree to which they induce resistance [17,19-22]. Today, second-generation tyrosine kinase inhibitors (TKIs) have become available (Dasatinib, Sprycel, Bristol-Myers Squibb; Nilotinib, Tasigna, Novartis Pharma) in the market [23-25].

In clinical practice, cytogenetic monitoring (as fluorescence in situ hybridization-FISH) and reverse transcription quantitative polymerase chain reaction (RQ-PCR) have become gold standard monitoring assays, and they are becoming increasingly standardized between laboratories [26-28]. Furthermore, CML treatment should be monitored correctly and cABL kinase domain mutations should be investigated. However, there are no widely accepted guidelines at present for the screening of Ph+CML mutations [18,26,28]. Therefore, we report here the use of denaturing high performance liquid chromatography (dHPLC) and sequencing methods to screen for mutations in the nucleotide binding loop (P-loop), the catalytic domain and the activation loop of the *ABL* gene.

Materials and Methods

Patients

Peripheral blood (PB) samples were obtained from 40 imatinib-resistant or -intolerant CML patients, who were diagnosed between 1999 and 2007 in the Adult Hematology Division of Istanbul Medical Faculty, Istanbul. All patients showed resistance to 400 mg/day imatinib. Imatinib resistance was defined as inadequate initial response or loss of hematologic or cytogenetic response at any time during the treatment. All of the patient's materials were stored at -80°C after homogenization in RLT buffer (Qiagen, GmbH, Germany). One PB sample from a CML patient known to be wild-type for *ABL* mutations was used as a negative control. We obtained written informed consents from all patients

RNA isolation and cDNA synthesis

Total RNA was isolated by Qiagen RNeasy Protect kit (Qiagen, GnbH, Germany). RNA samples were treated with DNAse (1 U/μg) for the possible DNA contaminations during isolation. 1 μg of total RNA was used for cDNA synthesis by using random hex-

amers and MMLV reverse transcriptase according to the protocol of the manufacturer (Fermentase).

Nested PCR and experimental design of dHPLC analysis

Mutation detection in the nucleotide binding loop (P-loop), the catalytic domain and the activation loop of the *ABL* gene was performed via PCR-based dHPLC using a WAVE DNA fragment analysis system (Transgenomic). We used a nested-PCR approach for amplification of the ABL kinase domain with primers and PCR conditions described before [18]. First PCR was performed with 2 μl of cDNA and exons 12, 13 on BCR mRNA and exon 8 on ABL mRNA were amplified. The ABL kinase domain was divided into two partially overlapping fragments; codons 206- 335 (fragment B) and codons 262- 421 (fragment C) were amplified separately. This procedure ensures that the wild-type ABL transcript is not analyzed [18].

Using Wavemaker software (Transgenomic), we selected optimal temperatures for the two ABL mRNA fragments. Annealed PCR fragments (8-15 μl per sample) were injected into the DNASep HT cartridge for analysis at the following selected temperatures: Fragment B at 61.3, 62.3°C and fragment C at 60.2, 61.1°C.

Products were eluted at a constant flow rate of 1.5 ml/min with a linear acetonitrile gradient determined by Navigator software (Transgenomic) based on the size and GC-content of the amplicon. The gradient was formed by combining buffer A (0.1M TEAA) (Transgenomic) and Buffer B (0.1M TEAA with 25% acetonitrile) (Transgenomic). The elution profiles of DNA fragments, monitored by the system's UV detector, were used to produce chromatographs. A wild-type sample was used as a negative control. Less stable heteroduplexes denature earlier than homoduplexes and, thus, appear first in elution profiles. Patients' chromatograms were compared with the wild-type profile, and samples with different characters (eluted before normal homoduplexes) were scored as positive. In addition to positive scored samples, crude PCR products from patients scored as "negative" were also directly sequenced to exclude the presence of undetected genetic variations. Sequences were compared with the wild-type sequence and analyzed using CLC combined Workbench software (V.3.6.1, Denmark).

Results

In this paper, we set up a straightforward, high-throughput dHPLC-based assay to screen for the presence of mutations in the catalytic domain of ABL tyrosine kinase of 40 Turkish CML patients with imatinib resistance. Nine of 40 patients (23%) had point mutations, and mutation characteristics of the patients are shown in Table 1. Four mutations (M244V, G250E, Y253H, V304I) fell within the nucleotide-binding loop (P-loop) that was involved in ATP binding. We observed that two mutations (F359C, K357R) affected the catalytic domain and two mutations (T315I, F317L) were located in imatinib-binding residues. We identified two novel amino acid substitutions (V304I, K357R) affecting codons known as the hotspot region. Representative dHPLC chromatograms for fragments B and C are illustrated in Figure 1.

Discussion

The basis of current therapy for CML in the CP includes the three TKIs - imatinib, dasatinib and nilotinib - and allogeneic hematopoietic stem cell transplantation (allo-HSCT). The treatment algorithm appears simple and easy to follow. Imatinib is the recommended first-line treatment for newly diagnosed CP CML at an initial standard dose of 400 mg daily, dasatinib or nilotinib is second line, and alloHSCT is for instances of failure of drug therapy. A partial guide to the choice of second-generation TKIs may be provided by the detection of some mutations [2,5,15,17-19,21,26]. Therefore, it is recommended that molecular laboratories incorporate this analysis

Table 1. BCR-ABL mutation characteristics of patients

Patient	Base change	Amino acid substitution	Status
ABL11	ACT>ATT	T315I	R
ABL13	TTC>TTG	F317L	R
ABL15	GGG>GAG, TTC>TGC	G250E, F359C	R
ABL16	ATG>GTG	M244V	R
ABL17	TAC>CAC	Y253H	R
ABL20	ACT>ATT	T315I	R
ABL25	TTC>TTG	F317L	R
ABL29	GTC>ATC	V304I	N
ABL34	TTC>TTG, AAA>AGA	F317L and K357R	R, N

R: Previously reported; N: Previously undescribed

into their testing procedures [18,26]. In this paper, we used direct sequencing and dHPLC. Direct sequencing is the most widespread method applied in the routine monitoring of patients. However, its sensitivity is low (20%), and it may cause false-negative results. Therefore, we used dHPLC technique, which has a sensitivity of 1-5% in mutation detection, in addition to the sequencing [29-31].

We found *BCR-ABL* mutations localized in the P-loop, in the catalytic domain. The P-loop normally acts as nucleotide-binding loop for the phosphate groups of ATP, and its mutations are the most serious and common mutations. The catalytic domain is involved in the catalytic process [14-16,32-35]. In our study group, we detected (M244V, G250E, Y253H, V304I) substitutions in P-loop domain, (K357R, F359C) mutations in catalytic domain and (T315I, F317L) mutations in the imatinib-binding site. M244V, G250E, F317L, and F359C mutations might be overcome by dose-escalation [5,14,18,26,36]. However, Y253H mutation confers a highly resistant phenotype, suggesting withdrawal of imatinib in favor of alternative therapeutic strategies [18,26]. Two patients showed T315I mutation, which interfered with a critical hydrogen bond that forms between the ABL kinase and imatinib. It is known to be resistant to imatinib as well as the second-generation TKIs. Other than mutations, clinical relevance of other differences between dasatinib and nilotinib is unknown [5,26,36,37].

dHPLC is a sensitive and simple way to detect low level of mutations, based on differentiation in elution profiles. The results of the dHPLC showed several heteroduplex peaks on the chromatograms of samples (Figure 1). All abnormal elution profiles were parallel to sequencing results. As a result, dHPLC seems to be a reliable method for use in the pre-screening process. Certainly, the sequencing must confirm a mutation and tell us the exact mutation type.

Mutations can occur among imatinib-resistant and -intolerant patients, and sensitive detection of the mutation status might be important for the patients who are resistant to imatinib. The European LeukemiaNET (ELN) recommends mutation analysis in occurrences of suboptimal response or failure of 400 mg daily imatinib treatment. The suboptimal response refers to no cytogenetic response after three months of therapy, less than partial cytogenetic response after six months of therapy, only achieving a partial cytogenetic response at 12 months of treatment, or less than major molecular response after 18 months of treatment. Furthermore, loss of the major molecular response at any time during the treatment or detection of a mutation is so-called suboptimal response. Treatment failure refers to not achieving a complete hematological response after three months of treatment, no cytogenetic response after six months of therapy, less than a partial cytogenetic response after 12 months of treatment, or incomplete cytogenetic response after 18 months of treatment. Losing the complete hematological response or complete cytogenetic response at any time during the treatment and detection of a mutation that is poorly sensitive to imatinib or the second- generation TKIs or clonal chromosome abnormalities are also considered failure. By determining the mutational status of a patient, the right therapy option (second-generation TKIs, alloSCT, experimental drugs, etc.) can be selected for each patient [38]. dHPLC system shows higher sensitivity (95-99%) for detecting minor clones in the mixed follow-up patient samples. Conventional direct sequencing has lower detection sensitivity to perform such analysis (about 80-90%) [29,31]. Therefore, we suggest using dHPLC screening as a routine approach to screen ABL kinase domain mutations, which can be confirmed by sequence analyses.

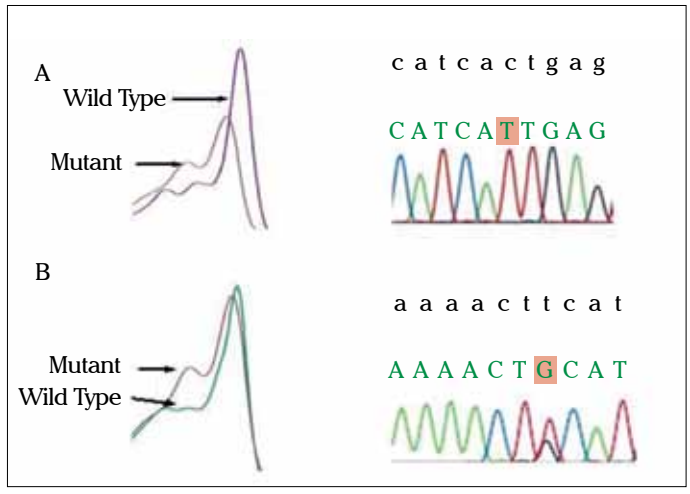

Figure 1. dHPLC chromatograms and direct sequencing of mutant DNA for fragments B and C. (A) dHPLC profiles of two representative mutants overlaid with the wild-type profiles (left panel), fragment B at 61.3°C; (right panel); ACT>ATT, T315I. (B) dHPLC profiles of two representative mutants overlaid with the wild-type profiles (left panel), fragment C at 60.2°C; (right panel); TTC>TGC, F359C

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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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