Denaturing-HPLC-Based Assay for Detection of ABL Mutations in Chronic Myeloid Leukemia Patients Resistant to Imatinib

Simona Soverini,1,2 Giovanni Martinelli,1* Marilina Amabile,1 Angela Poerio,1 Michele Bianchini,1 Gianantonio Rosti,1 Fabrizio Pane,3 Giuseppe Saglio,4 and Michele Baccarani1 on behalf of the Italian Cooperative Study Group on Chronic Myeloid Leukemia (ICSG-CML) and the European LeukemiaNet—6th Framework Program of the European Community

Background: Despite strong efficacy of the BCR-ABL tyrosine kinase inhibitor Imatinib mesylate for the treatment of chronic myeloid leukemia (CML), resistance has been observed in a proportion of cases, especially those with advanced stages of the disease. Point mutations within the ABL kinase domain are emerging as the most frequent mechanism for reactivation of kinase activity within the leukemic clone. Regular testing for emerging mutations should be performed in clinical practice to assure appropriate therapeutic management of CML patients.

Methods: We developed a sensitive, high-throughput denaturing-HPLC (D-HPLC)-based assay for screening of ABL point mutations. For each sample, two partially overlapping fragments of 393 and 482 bp corresponding to the kinase domain were amplified by nested reverse transcription-PCR and analyzed under selected temperature and acetonitrile gradient conditions. Fifty-one bone marrow and/or peripheral blood specimens from 27 CML patients who showed cytogenetic resistance to Imatinib were screened in parallel by D-HPLC and by direct sequencing.

Results: In 12 of 27 (44%) patients, D-HPLC showed an abnormal elution profile suggesting the presence of a nucleotide change. Direct sequencing confirmed the presence of a point mutation in all cases. Conversely, all samples scored as wild-type by D-HPLC showed no evidence of mutations by direct sequencing. In two cases, novel amino acid substitutions at codons already known for being hot-spots of mutation were identified (F311I and E355D).

Conclusions: The proposed D-HPLC-based assay is highly specific and at least as sensitive as sequencing; with respect to the latter, it provides a much faster and less expensive semiautomated system for mutational screening. It may therefore potentially be a valuable tool for regular, large-scale testing of patients undergoing Imatinib treatment.

© 2004 American Association for Clinical Chemistry

The encouraging results from trials of Imatinib mesylate for the treatment of patients with chronic myeloid leukemia (CML)5 have established it as the new standard of care for chronic phase (CP) and accelerated phase (AP) of the disease (1–3). Imatinib is a potent and selective inhibitor of BCR-ABL tyrosine kinase, which is known to be deregulated in as many as 95% of CML patients. Despite high rates of hematologic and cytogenetic responses, primary refractoriness and acquired resistance have been observed in both late-CP and AP patients. Point mutations within the ABL kinase domain of the BCR-ABL

1 Institute of Hematology and Medical Oncology “Seràgnoli”, University of Bologna, Bologna.
2 Center for Applied Biomedical Research (CRBA), St. Orsola-Malpighi Hospital, Bologna.
3 CEINGE Advanced Biotechnologies and Department of Biochemistry and Medical Biotechnology, University of Naples “Federico II”, Naples, Italy.
4 Division of Hematology and Internal Medicine, Department of Clinical and Biological Sciences, University of Turin, Turin, Italy.
*Address correspondence to this author at: Institute of Hematology and Medical Oncology “Seràgnoli”, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy. Fax 39-051-6364037; e-mail gmartino@kaiser.alma.unibo.it.
Received January 5, 2004; accepted April 1, 2004.
Previously published online at DOI: 10.1373/clinchem.2004.031112

5 Nonstandard abbreviations: CML, chronic myeloid leukemia; CP, chronic phase; AP, accelerated phase; MCgR, major cytogenetic response; ASO, allele-specific oligonucleotide; RFLP, restriction fragment length polymorphism; PNA, peptide nucleic acid; D-HPLC, denaturing HPLC; BM, bone marrow; PB, peripheral blood; and ALL, acute lymphoblastic leukemia.
they induce resistance. Several mutations have been reported in association with tinib. In a recent report, an amino-acid change should be investigated in patients on escalation. Thus, to optimize therapeutic response, not all mutations within the ATP-binding site can prevent Imatinib from binding, either interrupting critical contact points between Imatinib and the protein or inducing a conformation to which Imatinib is unable to bind. Additional mutations, which confer a true resistant phenotype, and most of them are well characterized in terms of their ability and degree to which they induce resistance. Although some mutations (Y253F/H, E255K/V, and T315I) confer a true resistant phenotype and suggest withdrawal of Imatinib in favor of alternative therapeutic strategies, others (M244V, F311L, and F359V) may be overcome by dose escalation. Thus, to optimize therapeutic response, not only the presence of a mutation but also the actual amino-acid change should be investigated in patients displaying hematologic or cytogenetic resistance to Imatinib. A recent report (21) it was also suggested that in late-CP and AP patients treated with Imatinib, mutations can be detected by direct sequencing before clinical evidence of resistance, thus predicting the subsequent course of the disease; mutations in the nucleotide-binding loop of the kinase region (P-loop) are associated with a particularly poor prognosis; and (c) that patients with longer duration of CML before initiation of Imatinib therapy and patients who fail to achieve a major cytogenetic response (MCgR) in the first 6 months of therapy are at high risk of developing mutations and resistance and should be regularly monitored.

For these reasons, routine testing for emerging mutations should be performed to assure rational therapeutic management of CML patients. Sequencing has been widely used for this purpose (4–11, 21), but it is expensive and time-consuming, especially when subcloning of PCR products is performed. Several alternative methods have been reported, such as allele-specific oligonucleotide (ASO)-PCR (8), restriction fragment length polymorphism (RFLP)-based assays (11, 22), and peptide nucleic acid (PNA)-based clamping techniques (23). They are more sensitive but seem not to be suitable for large-scale screening of the entire spectrum of mutations reported to date. Here we present a novel, rapid, and straightforward method for monitoring and detecting leukemic cells containing Imatinib resistance-associated mutations based on PCR amplification and subsequent screening of PCR products by denaturing HPLC (D-HPLC) (24).

Materials and Methods
PAINTS AND SAMPLES
The study was performed retrospectively on a total of 51 bone marrow (BM) and/or peripheral blood (PB) samples obtained from 27 patients enrolled in two multicenter clinical trials of the Italian Cooperative Study Group on CML. For three patients, only BM or PB samples were available or evaluable. Seven patients were enrolled in the CML011/STI571 trial (newly diagnosed, early-CP CML patients treated with a combination of 400 mg/day Imatinib and PEGylated interferon; 78 patients enrolled between July and December 2001) (25), whereas the remaining 20 were enrolled in the CML002/STI571 trial (late-CP CML patients resistant or refractory to interferon, treated with 400 mg/day Imatinib; 200 patients enrolled between June and December 2000) (26). The trials were approved by the ethics committees of all participating centers and were run in accordance with the principles of the Helsinki Declaration. The patients who entered this molecular study were part of a larger series of patients (n = 56) who showed cytogenetic resistance to Imatinib therapy. Cytogenetic resistance was defined according to the following criteria: (a) patients failed to obtain at least a MCgR within the first 12 months of therapy, or (b) patients transiently obtained a MCgR or even a complete cytogenetic response within the first 12 months but subsequently lost it.

After receiving written informed consent, we collected BM and/or PB samples from all patients enrolled in the trials by the Bologna, Naples, and Turin centers before treatment and at regular time points up to the 12th month of therapy. Mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation and then stored at −80°C in guanidinium thiocyanate until use. ABL ABL mutations were used as negative controls. Although some ABL mutations (kindly provided by M.A. Santucci, University of Bologna) were used as positive controls. G250E- and H396R-mutated amplicons from two patients were subcloned into a pCR2.1-TA vector (Invitrogen) and used as positive controls. G250E- and H396R-mutated amplicons from two patients were subcloned into a pCR2.1-TA vector (TOPO TA Cloning Kit, Invitrogen) and used as positive controls. One PB sample from a CML patient known to be wild type for ABL mutations was used as negative control.

RNA EXTRACTION AND REVERSE TRANSCRIPTION-PCR
Total cellular RNA was obtained by phenol–chloroform extraction, isopropanol precipitation, and washing with
700 mL/L ethanol, as described previously (27). RNA was quantified spectrophotometrically at 260 nm, and its integrity was assessed by electrophoresis on 2% agarose gels. Total cellular RNA (1 µg) was reverse-transcribed to cDNA by use of 5 µmol/L random hexamer primers and 200 U of M-MLV reverse transcriptase (GeneAmp RNA PCR Kit; Applied Biosystems).

**EXPERIMENTAL DESIGN OF D-HPLC ANALYSIS**

All samples were analyzed by D-HPLC on a Wave DNA Fragment Analysis System (Transgenomic Ltd.). To increase the sensitivity and specificity of ABL kinase domain amplification, we used a nested-PCR approach. The first round of amplification was done with 2 µL of cDNA and the following primers: F-BCR-A (positioned between exons 12 and 13 on BCR mRNA) and R-ABL-A (positioned on exon 8 on ABL mRNA; primer positions and sequences are detailed in Table 1). This procedure ensured that the wild-type, nonrearranged ABL transcript was not analyzed. An initial denaturation step of 5 min at 95 °C was followed by amplification for 30 cycles (denaturation for 40 s at 95 °C, annealing for 1 min at 60 °C, extension for 1 min at 72 °C) and final extension for 7 min at 72 °C. Reamplification of a 1-µL aliquot from a 1:50 dilution of the first PCR product was then performed with two internal primer pairs: F-ABL-B/R-ABL-B and F-ABL-C/R-ABL-C (Table 1 and Fig. 1). In this way, the ABL kinase domain was divided into two partially overlapping mRNA fragments, i.e., ABL-B (393 bp, spanning codons 206–335) and ABL-C (482 bp, spanning codons 262–421). This strategy was meant to obtain PCR products of optimal length for D-HPLC analysis (recommended length, 200–500 bp). For the second round of amplification, the following PCR conditions were used: initial denaturation step of 5 min at 95 °C, amplification for 35 cycles (denaturation for 30 s at 95 °C, annealing for 40 s at 55 °C, extension for 40 s at 72 °C), and final extension for 7 min at 72 °C. All PCR experiments were performed in a 50-µL final volume containing 1 U of Taq Gold DNA Polymerase (Applied Biosystems), 10× PCR buffer, 100 mM each deoxynucleotide triphosphate, 2.5 mM MgCl₂, and 1 µM each primer.

Using Wavemaker software, Ver. 4.1.40 (Transgenomic), we calculated the melting curves as described (28) to select optimal temperatures for the two ABL mRNA fragments. Aliquots (8 µL) of crude PCR samples, preheated for 10 min at 96 °C and then gradually reannealed for 10 min at room temperature, were loaded on a preheated C18 reversed-phase column at the following selected temperatures: ABL-B at 61.3, 62.3, and 62.8 °C; ABL-C at 60.2, 61.1, and 61.9 °C. DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mmol/L triethylamine acetate buffer (Transgenomic) at a constant flow rate (0.9 mL/min). The gradient was formed by mixing buffer A (0.1 mmol/L triethylamine acetate) and buffer B (0.1 mmol/L triethylamine acetate containing 250 mL/L acetonitrile). Eluted DNA was detected by the absorbance at 260 nm; each sample run took ~8 min. A wild-type sample was used as a negative control. The chromatogram from each tested patient was overlaid with the wild-type profile, and samples with an extra peak were scored as positive. To ensure that homozygous mutations did not escape D-HPLC detection, for all samples studied we also analyzed a 1:1 mixture of a wild-type negative control and PCR products from patient samples.

**DIRECT SEQUENCING**

Direct sequencing was done in parallel on all samples by use of an ABI PRISM 377 DNA Analyzer (Applied Biosystems). A 20-µL aliquot of the same PCR product analyzed by D-HPLC was purified (QIAquick PCR Purification Kit; Qiagen) and sequenced by use of the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) according to manufacturer’s instructions. Sequences were

---

**Table 1. Primer pairs used for nested PCR.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence, 5’–3’</th>
<th>Positions*</th>
<th>Amplicon (length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-BCR-A</td>
<td>GAG CAG CAG AAG AAG TGT TTC AGA</td>
<td>BCR (nt 3075–3098)</td>
<td>A⁺ [1475 bp (b3a2) or 1401 bp (b2a2)]</td>
</tr>
<tr>
<td>R-ABL-A</td>
<td>CTC TAG CAG CTC ATA CAC CTG GG</td>
<td>ABL (nt 1484–1506)</td>
<td></td>
</tr>
<tr>
<td>F-ABL-B</td>
<td>CAT CAT TCA ACG TGT GCC GAC GG</td>
<td>ABL (nt 748–770)</td>
<td>B (393 bp)</td>
</tr>
<tr>
<td>R-ABL-B</td>
<td>GTT GCA CTC CCT CAG GTA GTC</td>
<td>ABL (nt 1120–1140)</td>
<td></td>
</tr>
<tr>
<td>F-ABL-C</td>
<td>GAA GAA ATA CAC CCT GAC GGT G</td>
<td>ABL (nt 930–951)</td>
<td></td>
</tr>
<tr>
<td>R-ABL-C</td>
<td>CGG ACT TGA TGG AGA A</td>
<td>ABL (nt 1393–1411)</td>
<td>C (482 bp)</td>
</tr>
</tbody>
</table>

* Primer positions are according to GenBank mRNA sequences X02596 (for BCR) and X16416 (for ABL).

⁺ The length of the amplicon obtained after the first PCR slightly varies according to whether the BCR-ABL junction is b2a2 or b3a2.
compared with the wild-type sequence by use of BLAST (ABL, accession no. X16416). Sequence analysis was performed on both strands for each fragment, and once a mutation was detected, its presence was confirmed at the DNA level.

**Results and Discussion**

In this report, we set up a straightforward, high-throughput D-HPLC-based assay to screen for the presence of mutations in the catalytic domain of ABL tyrosine kinase. The optimized conditions we present here make possible the use of D-HPLC for large-scale mutational screening of patients undergoing Imatinib treatment.

Taking advantage of the knowledge of ABL sequences and mutations reported to date, we defined the primers and the temperatures to be used for mutational analysis. A nested PCR protocol was set up to ensure high sensitivity and to avoid coamplification of the wild-type, nontranslocated ABL allele. In the second round of PCR, two sets of primers were designed to amplify two adjacent, partially overlapping fragments of optimal length for D-HPLC analysis (393 and 482 bp, respectively; recommended length, 200–500 bp; Fig. 1). Having established the DNA fragment sequences to be studied, we subsequently determined the melting temperatures to be used during D-HPLC screening. WAVEmaker software (Ver. 4.1.40) was able to visualize the position of melting subdomains with respect to the whole fragment sequence and to predict melting temperatures to maximize the resolution of heteroduplex and homoduplex peaks within each subdomain.

One wild-type sample was used as a negative control. Chromatograms from each tested patient were overlaid with the wild-type profile, and samples with extra peak(s) were scored as positive. To ensure that homozygous mutations would not escape D-HPLC detection, for all samples to be studied we also analyzed a mixture of a wild-type negative control and patients’ PCR products in a 1:1 ratio. Representative D-HPLC chromatograms for fragments B and C are illustrated in Fig. 2A. Wild-type PCR products showed a single peak corresponding to the wild-type sequence. By comparison, the mutant PCR products showed a double peak: the first one corresponding to the heteroduplex fraction, which has a shorter retention time, and the second one corresponding to the homoduplex product.

To assess the specificity of our D-HPLC assay, we tested PCR samples from four cell lines known to be positive for some ABL mutations (Y253F, E255K, T315I, and M351T). In all cases, D-HPLC analysis yielded an abnormal elution profile compatible with the presence of a sequence variation, which was subsequently confirmed by direct sequencing.

To determine the detection limit, which has been reported to be variable in a range of 1–10% depending on the sequence and length of the fragments to be analyzed (29–31), we prepared limiting dilution experiments by mixing known quantities of amplified wild-type and mutant (G250E, Y253F, E255K, T315I, M351T, H396R) ABL PCR products. The percentages of mutant with respect to wild type were 0:100%, 50:50%, 30:70%, 20:80%, 10:90%, 5:95%, and 1:99%. The results indicated that D-HPLC has lower detection limits of 1–5% for the G250E, Y253F, E255K, T315I, and H396R mutations and 5–10% for the M351T ABL mutation (data not shown).

In 20 BM and/or PB samples from 12 of 27 (44%) patients, D-HPLC analysis showed an abnormal elution profile suggesting the presence of a sequence variation. Sequence analysis confirmed the presence of a point mutation in all cases. Conversely, none of the samples scored as wild type by D-HPLC showed evidence of mutations by direct sequencing. In most cases, additional peak(s) were detected at all three melting temperatures routinely used for D-HPLC screening. However, depending on the mutation and its position within the PCR fragment, one of the temperatures was usually most helpful in clearly resolving additional peak(s) from the homoduplex peak, the other two temperatures yielding only smaller modifications of the elution profile. In rare cases, the additional peak(s) were detected at one temperature only (F311I and F311L mutations in patients 9 and 3, respectively, were detected in fragment C only at 60.2 °C). These findings suggest that, at least in our experience, elution at all three temperatures established with the help of WAVEmaker software is helpful to establish or rule out the presence of a mutation.

The results of D-HPLC and sequence analyses for the whole panel of 51 BM and/or PB samples from the 27 patients analyzed are detailed in Table 2. Five mutations (M244V, G250E, Y253F, Y253H, and E255K) fell within the nucleotide-binding loop (P-loop), a region involved in ATP binding. One mutation (F359V) affected the catalytic domain. One mutation (H396R) fell within the activation loop, a region whose conformation regulates kinase activity. The F311L mutation maps close to residues directly involved in Imatinib binding (amino acids 315 and 317). Two nucleotide changes produced silent mutations. Moreover, we identified two novel amino acid substitutions affecting codons already known as hotspots of mutation (Fig. 2B): a GAG-to-GAT nucleotide change at codon 355, leading to a Glu-to-Asp amino acid substitution (E355D) and a TTC-to-ATC nucleotide change at codon 311, leading to a Phe-to-Ile substitution (F311I). Characterization of the effects of these two novel mutations on ABL protein structure and/or Imatinib binding is ongoing.

Imatinib mesylate has recently become the new standard of care for Philadelphia-positive (Ph+) CML and acute lymphoblastic leukemia (ALL) patients. Of concern, however, is the fact that significant proportions of ALL and AP-CML patients quickly relapse despite continued therapy. Because point mutations affecting critical sites within the ABL kinase domain seem to be one of the most
frequent mechanisms of resistance (with frequencies ranging from 26% to 90% depending on the phase of the disease) \((4–11)\), mutational screening of patients undergoing Imatinib treatment, especially those considered at higher risk of developing resistance—i.e., patients with longer duration of CML before initiation of Imatinib therapy and patients who fail to achieve a MCgR in the first 6 months of therapy \((21)\)—should routinely be performed to help decision-making on dose escalation or alternative treatment options.

To date, sequencing of PCR products (either subcloned or not) has been widely used to search for known and unknown ABL kinase domain variants in CML and Ph+ ALL patients \((4–11)\). A major limitation of sequencing, however, is the low sensitivity. Several authors have reported that direct sequencing of ABL PCR products generally has a detection limit of 20% \((5,11)\). Subcloning of PCR products followed by sequencing improves the detection, with the lower detection limit depending on the number of clones tested. By cloning and sequencing of 108 clones/sample (thus reaching a lower detection limit <1%), Hofmann et al. \((32)\) reported that rare cells bearing the E255K mutation could retrospectively be detected before Imatinib administration in two patients with advanced Ph+ ALL. The mutation was found in a single clone from each patient. Despite being able to detect the presence of a point mutation in pretreatment samples, such a method is expensive, cumbersome, and time-consuming, and its application for screening of large series of patients who are candidates for or undergoing Imatinib treatment is unlikely.

A screening method combining PCR with single-strand conformation polymorphism analysis was also described by the same Hofmann et al. \((32)\). The method was tested in a cohort of Ph+ ALL patients previously evaluated by direct sequencing \((10)\). Although PCR with single-strand conformation polymorphism analysis can generally find conformational changes of DNA with a minimum detection limit of \(\sim 2–5\%\), the authors reported that it was inferior to sequencing; there were two Imatinib-resistant

---

Fig. 2. D-HPLC profiles \((A)\) and direct DNA sequencing \((B)\) of mutant DNA. \((A)\), D-HPLC profiles of two representative mutants \((\text{mut})\) overlaid with the wild-type \((\text{wt})\) profiles. \((\text{Left panel})\), fragment B at 61.3 °C; \((\text{right panel})\), fragment C at 60.2 °C. See the text for details. \((B)\), direct DNA sequencing showing the previously unreported GAG-to-GAT \((\text{left panel})\) and TTC-to-ATC \((\text{right panel})\) nucleotide changes at codons 311 and 355, respectively.
Table 2. Results of D-HPLC and sequence analyses for fragments B (codons 206–335) and C (codons 262–421) in the panel of 51 BM and/or PB samples from the 27 CML patients analyzed.  

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time from start of therapy, months</th>
<th>Source</th>
<th>D-HPLC analysis</th>
<th>Sequence analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML 011 protocol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>BM</td>
<td>MUT</td>
<td>MUT</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>NA</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>BM</td>
<td>WT</td>
<td>MUT</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>BM</td>
<td>WT</td>
<td>MUT</td>
</tr>
<tr>
<td>CML 002 protocol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>BM</td>
<td>MUT</td>
<td>MUT</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>BM</td>
<td>MUT</td>
<td>WT</td>
</tr>
<tr>
<td>13</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>15</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>16</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>17</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>BM</td>
<td>MUT</td>
<td>WT</td>
</tr>
<tr>
<td>19</td>
<td>12</td>
<td>BM</td>
<td>MUT</td>
<td>WT</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>BM</td>
<td>WT</td>
<td>MUT</td>
</tr>
<tr>
<td>21</td>
<td>6</td>
<td>BM</td>
<td>WT</td>
<td>MUT</td>
</tr>
<tr>
<td>22</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>23</td>
<td>6</td>
<td>BM</td>
<td>MUT</td>
<td>WT</td>
</tr>
<tr>
<td>24</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>25</td>
<td>6</td>
<td>BM</td>
<td>WT</td>
<td>MUT</td>
</tr>
<tr>
<td>26</td>
<td>6</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>27</td>
<td>12</td>
<td>BM</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

* a In 20 samples from 12 patients, D-HPLC analysis showed an abnormal elution profile compatible with the presence of a sequence variation. Sequence analysis confirmed the presence of a point mutation in all cases. Conversely, all samples scored as wild type by D-HPLC showed no evidence of mutations by direct sequencing.

b Previously untreated, early-CP CML patients, treated with 400 mg/day imatinib + Pegylated α-interferon.

WT, wild type; MUT, sequence variant.

d NA, samples were not available: BM sample for patient 4 and PB samples for patients 6 and 7.

e Late-CP CML patients resistant or refractory to α-interferon and 400 mg/day imatinib.
samples known to have the E255K mutation that did not show any shifted band (32).

Alternative methods that allow the detection of specific ABL mutations have been developed. They include PCR-RFLP analysis or modifications (11, 22), ASO-PCR (8), and PNA-based PCR clamping techniques (23). With conventional RFLP analysis (11), a detection limit of 5% can be reached, but the assay is limited to a very small number of ABL mutations (Y253F/H, E255K/V, T315I, and M351T) for whom the nucleotide change determines the abolishment or creation of a restriction site. Recently, an enhanced PCR-RFLP method for T315I and Y253F mutations has also been described (22). It relies on the artificial introduction of a TaqI restriction site in the mutant, but not in the wild-type sequences, by use of mismatched, allele-specific primers. This procedure is meant to rescue only mutation-containing molecules, thus enhancing the sensitivity of the downstream detection methods. This approach was very sensitive, being able to reliably detect artificially generated mutated amplicons in the presence of 1000-fold excess of wild-type molecules. Another extremely sensitive technique for detection of specific point mutations is ASO-PCR. In their report, Roche-Lestienne et al. (8) used an ASO-PCR method with a detection limit of 1:10 000 (10^{-4}) to retrospectively identify the presence of F311L, T315I, and M351T mutations in 5 patients of 24 screened at the time of resistance by direct sequencing. In all of these patients, the mutations turned out to be detectable in the samples collected before Imatinib initiation. More recently, Kreuzer et al. (23) developed a PNA-based PCR clamping technique with a detection limit of 1:500 (0.2%) for Y253H, E255K, and T315I mutations. With this method, the E255K mutation was again retrospectively detected at diagnosis in a single AP-CML patient who never achieved either cytogenetic or hematologic remission.

Despite the fact that the methods cited above were highly sensitive when used for longitudinal screening of resistant patients known to have a specific point mutation, some drawbacks exist. ASO-PCR can easily produce false-positive results because a single, erroneous primer hybridization can lead to the generation of artificial templates. Moreover, it is not suitable for widespread testing because a panel of more than 20 specific primers should be used to screen for all of the mutations reported to date (4–11, 21, 23). The latter disadvantage also applies to PNA-based PCR clamping techniques, which rely on expensive sets of primers and fluorescent probes for detection of point mutations. More importantly, all of these techniques can not be used for further identification of new point mutations occurring in the kinase domain of ABL protein.

Here we describe a novel method that could potentially play an important role as a primary screening tool. To the best of our knowledge, this is the first application of D-HPLC for screening of ABL point mutations in CML or Ph+ ALL patients. D-HPLC is a reversed-phase ion-pairing HPLC method specifically developed for detection of DNA sequence variations such as point mutations, small insertions, and deletions (33). Under conditions of partial heat denaturation within a linear acetonitrile gradient, heteroduplexes that form in PCR samples with internal sequence variations display reduced column retention time with respect to their homoduplex counterparts. The elution profiles for such samples are distinct from those with a homozygous sequence, making the identification of samples harboring polymorphisms or mutations a straightforward procedure. After D-HPLC analysis, only those samples with an abnormal elution profile are subjected to sequencing to determine the precise sequence abnormality. Of note, D-HPLC instrument can be coupled to a fragment collector, which allows automated fragmentation and purification of the eluted PCR product corresponding to the mutated peak, thus increasing the sensitivity of the downstream steps required to determine the precise sequence abnormality (31, 34). Although our instrument is at present not equipped with a fragment collector, data from the literature suggest that, in cases of low-concentration mosaicisms, mutations that can not be characterized by direct sequencing of unfraccionated PCR products are successfully resolved after the selective collection of the low-concentration mutant heteroduplex peaks.

Our results from a panel of 51 BM and/or PB samples panel from the 27 patients evaluated in parallel by D-HPLC and direct sequencing showed that D-HPLC is a reliable method for prescreening of PCR products. All samples showing an abnormal elution profile had a nucleotide change by automatic sequencing and vice versa. With respect to sequencing, the method described here is much faster: in contrast with the very cumbersome evaluation of sequence data, the evaluation of results by D-HPLC is quite simple because the investigator must differentiate only between single and multiple peaks in the elution profiles. Moreover, PCR-generated products do not need further manipulation before analysis; they are loaded directly on the D-HPLC column, and up to 196 samples can be screened in a single run. Use of a microtiter plate autosampler allows the mutational screening to be almost totally automated. Another major advantage is the fact that D-HPLC analysis is less expensive than sequencing (35).

Our preliminary experiments with serial dilutions of wild-type and mutant ABL PCR products at different ratios showed that D-HPLC has lower detection limits of 1–5% for G250E, E255K, Y253F, T315I, and H396R and 5–10% for M351T. These percentages are in agreement with those reported in the literature (29–31). Although we have not checked the ability of our method to detect the whole spectrum of mutations reported in the literature, the six mutations tested to date are not only the ones found most frequently in CML patients, but also those that show a dramatic increase in IC50 (drug concentration required to inhibit proliferation by 50%) to values...
that can not be reached simply by escalating the dose of Imatinib, thus indicating the need for an alternative treatment option. As mentioned previously, it has recently been reported that, at least in some CML cases, specific mutations (E255K, F311L, and T315I) could be found in a small proportion of leukemic cells before the initiation of Imatinib treatment \((8, 23)\). Whether this is a general phenomenon or is restricted to specific sub-group(s) of patients is still unclear. The five patients reported by Roche-Lestienne et al. \((8)\) were cyogenetically refractory to Imatinib. The single patient reported by Kreuzer et al. \((23)\) was refractory both hematologically and cyogenetically. The inability of other studies to detect mutations in pretreatment samples might be attributable to a lack of sensitivity, to differences in patient populations, or both. D-HPLC may play an important role as a primary screening tool, allowing the detection of ABL kinase domain mutations with detection limits ranging from 1% to 10%. Despite being theoretically more sensitive than direct sequencing and at least as sensitive as cloning techniques, it is currently unclear whether D-HPLC would allow early detection of mutations conferring resistance. In the present study, 6 of 10 patients who turned out to have a missense mutation were in hematologic response, which was maintained for several months after the mutation was detected. Moreover, in a prospective study on 144 late-CP and AP-CML patients treated with Imatinib, Branford et al. \((21)\) showed that mutations could be detected by direct sequencing before clinical evidence of resistance, thus predicting the subsequent course of the disease. Longitudinal studies on larger series of patients in different phases of the disease and with different kinetics of response/resistance to Imatinib (i.e., upfront or secondary resistance) and correlations to clinical outcome are required to determine whether D-HPLC screening may be predictive of subsequent development of Imatinib resistance. If this is not the case, a combined molecular diagnostic approach could alternatively be adopted: those patients scored negative by DHPLC pre-screening could be further investigated at least for those mutations that determine a true resistant phenotype (i.e., Y253F/H, E255K, and T315I) by more sensitive methods such as enhanced PCR-RFLP analysis \((22)\).

We thank Dr. M.A. Santucci for providing the ABL-mutated cell lines. We also thank Biagio Pellicani for contributions to the sequence analyses and Dr. Vilma Mantovani, Daniela Basta, and Marinella Cenci (Center for Applied Biomedical Research) for valuable contributions to the D-HPLC analyses. We also thank Fondazione Cassa di Risparmio in Bologna for supporting the Center for Applied Biomedical Research. This study was supported by Associazione Italiana per la Ricerca sul Cancro, Cofin 2003 (to M. Baccarani), AIR, Fondazione Del Monte di Bologna e Ravenna, FIRB, and Ateneo 60% grants.

References


16. Deininger MW, Goldman JM, Lydon N, Melo JV. The tyrosine kinase...


