Long-Template DNA Polymerase Chain Reaction for the Detection of the bcr/abl Translocation in Patients with Chronic Myelogenous Leukemia

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ABSTRACT

In most patients with chronic myelogenous leukaemia (CML) primitive hematopoietic progenitors carry the acquired reciprocal bcr/abl gene rearrangement t(9;22)(q34.1; q11.21). However, not all of the progenitor cells express the bcr/abl hybrid mRNA or the p210 fusion protein. These cells, therefore, might escape detection by techniques that are based on expression of the fusion gene. To circumvent this problem, we established a new detection method for the rearrangement at the DNA level. Because breakpoints might occur in a very large genomic region (> 200 kb), we developed a long-template DNA-PCR (LT-DNA-PCR). In 22 of 59 CML patients, fragments of up to 19 kb could be amplified. Furthermore, 6 of 7 leukapheresis products of three bcr/abl-positive patients which were collected after mobilization chemotherapy and had been shown to be negative for the bcr/abl rearrangement by FISH and by RT-PCR were clearly positive by LT-DNA-PCR. Using a specific pair of primers, it is possible to detect the presence of, and to characterize, the individual gene rearrangement. This approach could prove for diagnostic purposes as well as detection of minimal residual disease under cytotoxic therapy or after purging regimens, being independent of expression of the bcr/abl hybrid mRNA or the fusion protein.

INTRODUCTION

CML is a clonal hematological stem cell malignancy. Usually, a typical peripheral blood count leads to the diagnosis. In 95% of patients the Philadelphia chromosome (Ph) is present (1, 2). This is the result of an acquired reciprocal translocation t(9;22)(q34.1;q11.21) of the c-abl oncogene from chromosome 9 to the bcr on chromosome 22 (3). Due to this positioning effect a patient-specific chimeric fusion gene arises. Usually, fusion gene products of two slightly different sized mRNAs of approximately 8.5 kb are expressed, b2a2 and b3a2. Both of them encode for a M_w 210,000 fusion protein (p210 bcr/abl), a protein with increased tyrosine kinase activity (4, 5).

In the bcr, the translocation occurs somewhere in an intron within a 5.8-kb genomic region (6, 7). In abl, the chromosomal breakpoints are dispersed over an intron region as large as about 200 kb (8). Complete intron sequence is available only for the bcr introns, whereas in abl, approximately 95 kb of the 200 kb are yet unknown (Fig. 1A).

Two research groups independently reported that primitive CML progenitors carry the rearrangement but do not necessarily express the bcr/abl hybrid mRNA or the p210 fusion protein (9, 10). Because of this, these cells might escape detection by techniques that are based on expression of the fusion gene. Therefore, we intended to establish a method for detection of patient-specific rearrangements at the DNA level. Unfortunately, the breakpoints in CML are dispersed over such a large genomic region that at present, it is not possible to cover them by conventional PCR methods. In 1994, Cheng et al. (11) showed effective amplification of long targets from human genomic DNA using a recombinant Thermus thermophilus DNA polymerase. In accordance with their approach, we designed a set of LT-PCRs with one constant bcr primer and 10 abl primers at a distance of approximately 15 kb each. Depending on the translocation site the first abl primer 3’ of the translocation together with the constant 5’ bcr primer should be able to amplify the fusion region (Fig. 1B).

MATERIALS AND METHODS

Patient Samples. Peripheral blood from 59 patients with CML, 4 healthy volunteers, 3 control patients and three Ph^- cell-lines (BV 173, K-562, and LAMA 84) were analyzed (Table 1). Furthermore, samples from leukapheresis products of three patients with CP-CML collected after mobilization chemotherapy consisting of idarubicin, cytosine arabinoside, and etoposide were analyzed (Table 3). Samples were drawn after informed consent was obtained according to institutional guidelines.

RNA Isolation. Total cellular RNA from 1 × 10^5 to 1 × 10^6 cells from peripheral blood or thawed leukapheresis samples was extracted using a RNeasy Total Kit (Quiagen, Hilden, Germany) according to the manufacturer’s instructions.

RT-PCR. RT-PCR amplification using nested primers was performed according to standard protocols as described previously (12).
**DNA Isolation.** DNA was isolated using 1–10 ml of EDTA-blood or samples from leukapheresis products with a Qiagen Blood and Cell Culture DNA Midi Kit (Qiagen) according to the manufacturer’s instructions. Three \( \times 10^7 \) cells yielded a median of 97.6 \( \mu \)g genomic DNA, which was dissolved in Tris-EDTA buffer (pH 8.0) and stored at \(-20^\circ\)C.

**LT-DNA-PCR Primer Design.** A constant \( bcr \)–primer and 10 \( abl \)-primers at a distance of approximately 15 kb each were designed (Fig. 1B). All of the 22-mer primers were chosen with an annealing temperature of 68.0–68.7°C and a GC content of 50–55%. Primers were adjusted to 20 pmol per reaction. \( abl \) genomic localization (Fig. 1B; Table 2A) refers to GenBank sequences \( hsablgr1 \) (access code: UO7561), \( hsablgr2 \) (access code: UO7562), \( hsablgr3 \) (access code: UO7563). The sequence of used primers is shown in Table 2A.

**LT-PCR.** Initially, a set of ten PCR reactions per patient was performed. After identification of a patient characteristic amplification product the specific primer combination was used for subsequent analysis. A Perkin-Elmer GeneAmp XL PCR Kit (Perkin-Elmer, Foster City, CA) was used. Briefly, the reaction mix (100 \( \mu \)l) is divided into a lower reagent mix, the upper reagent mix and the sample. The lower reagent mix (40 \( \mu \)l) was composed of 12 \( \mu \)l of 3.3 \( XL \) Buffer II; 10 mM dNTPs, 20 pmol of each \( abl \) primer and the \( bcr \) B2 primer and 25 mM Mg(OAc)\(_2\). The volume was adjusted to 40 \( \mu \)l with \( dH_2O \). To this mix, an AmpliWax PCR Gem 100 (Perkin-Elmer) was added and melted at 75°C for 5 min. Thereafter, as soon as the wax hardened, the upper reagent mix was placed above the solid wax layer. The upper reagent mix (20 \( \mu \)l) consisted of 18.5 \( \mu \)l of 3.3 \( XL \) Buffer II and 3 units of \( rTth \) (recombinant \( T. thermophilus \)) polymerase. The sample was added to the upper reagent mix. It was composed of 0.5 \( \mu \)g of DNA (in 0.5 \( \mu \)l) and 39.5 \( \mu \)l of \( dH_2O \). Division of the lower and upper reagent mix allowed a hot-start technique and ensured that all of the tubes had a synchronized start time. Cycle conditions were as follows: denaturation time for 10 s at 93°C; annealing and extension time, starting at 10 min and increased by 10 s per cycle both at 68°C. Thirty-six cycles were performed with a final hold at 72°C for 10 min. All of the steps were performed using a Perkin-Elmer DNA Thermal Cycler.

**Restriction Enzyme Digestion.** Two \( \mu \)l of 10X restriction endonuclease buffer, 7 \( \mu \)l of \( dH_2O \), and 1 \( \mu \)l of restriction endonuclease (4–5 units) were added to 10 \( \mu \)l of PCR products and then incubated at 37°C for 60 min. The reaction was stopped by adding 0.5 \( \mu \)l of 5 mM EDTA buffer (pH 8.0).

**Agarose Gel Electrophoresis.** For the analysis of amplified products, agarose gel electrophoresis was performed to visualize PCR products. Fifteen \( \mu \)l of the amplification product were loaded per lane and run at a constant voltage of 80 V. A 0.8% SeaKemGold Agarose gel (FMC BioProducts, Rockland, ME) was used and stained with ethidium bromide.

**Hybridization with Specific \( bcr \) Probes and DNA Sequencing.** Aliquots of amplified products were dot-blotted and hybridized with eight digoxigenin probes about 400 bp apart spanning the \( bcr \) region between exons 13 and 15 (Fig. 1C). The DIG easy Hyb system (Boehringer, Mannheim, Germany) to detect complementary sequences was used according to the

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**Table 1 Patient and LT-DNA-PCR data**

<table>
<thead>
<tr>
<th>Samples</th>
<th>( n )</th>
<th>LT-DNA-PCR-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML, 1st CP</td>
<td>45</td>
<td>21</td>
</tr>
<tr>
<td>CML, 2nd CP</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CML, AP(^{**})</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>CML, BC</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CML cell lines</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Healthy volunteers</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>AML</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Ph(^+) ALL</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{**}\) AP, accelerated phase; BC, blast crisis; AML, acute myeloid leukemia; Ph\(^+\) ALL, Philadelphia chromosome-positive acute lymphocytic leukemia.
manufacturer’s instructions. The most 3’-located probe that gave a positive signal was subsequently used as sequencing primer for sequence analysis of the breakpoints at the 3’ end of the \textit{bcr} gene and the 5’ end of the \textit{abl} gene. Sequence analysis was performed by PCR cycle sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA) with an automated sequencer (DNA Sequencer, model 373A, PE Applied Biosystems).

**FISH.** Cells from leukapheresis products were analyzed by interphase FISH for quantitation of Ph⁺ cells as described previously (13). The threshold of detection was determined at >6% to classify any specimen as Ph-positive. FISH was performed using a DNA probe mixture for the M\textit{bcr}/\textit{abl} translocation (Oncor, Gaithersburg, MD) according to the manufacturer’s instructions.

**RESULTS**

In 22 (37.3%) of 59 CML patients, fragments of up to 19 kb could be amplified by LT-PCR showing an individual patient characteristic PCR product (Table 1). The breakpoints were dispersed throughout intron 1b without any obvious clustering. In one patient, the breakpoint was in intron 1a. With the exception of primer 3–3, 3–7, and 3–8, every \textit{abl} primer generated fragments in selected patients. To prove the identity of the amplified fragments, restriction endonuclease digestion was used in four patients. Fragments matching exactly the calculated size according to the available sequence information could be generated (Fig. 2).

In addition, nine of the amplified products were further characterized by sequence analysis. In eight samples, the breakpoints on the \textit{abl} gene could be located in published sequences, whereas in one sample a localization was impossible (Fig. 3).

In DNA dilution experiments, the sensitivity of our LT-PCR method was determined to be 25 pg, the DNA content of approximately four primary CML cells (Fig. 4A) or the equivalent of four \textit{BV173} cells (data not shown; a fragment of <1 kb was amplified). RT-PCR was able to detect the RNA equivalent of about one cell (Fig. 4B).

Six of seven leukapheresis products from three patients that repeatedly had been found to be negative for the \textit{bcr}/\textit{abl} rearrangement by RT-PCR and interphase FISH were clearly positive by LT-DNA-PCR (Fig. 5). Only one of seven apheresis products was constantly negative by all three of the methods (Table 3). All three of the patients were positive by RT-PCR for \textit{bcr}/\textit{abl} before mobilization chemotherapy.

**DISCUSSION**

A point of controversy is the question whether all of the CML cells actively express the hybrid fusion gene or not (14). Primitive CML progenitor cells were shown to be RT-PCR-negative despite carrying the \textit{bcr}/\textit{abl} translocation. In these cells, \textit{bcr}/\textit{abl} mRNA might be either absent or minimally expressed. Antisense oligonucleotides targeted against the hybrid mRNA might, therefore, not be effective as eradication techniques (9). A second research group reported that 23% of Ph⁺ myeloid colonies were found to be \textit{bcr}/\textit{abl} mRNA-negative (10). Both of the studies have been criticized because of their inadequate controls for cDNA synthesis (15, 16).

Now that we are able to use PCR at the DNA level, the presence of gene expression as mRNA or protein is no prereq-
uisite for positive testing. There have been earlier attempts to establish detection methods at the DNA level. A bubble PCR technique was described to facilitate cloning of \textit{bcr/abl} break-points (14). DNA from patients with CP-CML was diluted into normal DNA and subjected to two-step PCR. It is necessary to clone and sequence the breakpoint from each patient and to design patient specific oligonucleotide primers. Therefore, this technique involves an appreciable amount of time and effort and, in addition, has a lower sensitivity than RT-PCR (17). On the other hand, the new LT-PCR is an easy way to perform a one-step PCR, once a patient-specific primer pair has been identified.

In comparison with RT-PCR, the DNA LT-PCR offers certain advantages. Because of the independence of gene expression, genomic DNA could be more informative for the detection of residual disease or for very immature selected progenitors (14). A technical advantage of LT-PCR is the handling of stable DNA instead of RNase sensitive RNA and using patient-specific primers would help to minimize the problem of PCR contamination. In this first study, we used a set of individual LT-PCR reactions to show the feasibility of the methodology. In future studies, however, multiplex LT-PCR with all of the different primer pairs in the same reaction tube might facilitate the practicability of this approach.

To our knowledge, only about one-half of the sequence of the involved part of the \textit{abl} gene is known (Fig. 1). Nevertheless, it was possible to amplify the breakpoint region in more than one-third of the examined samples. In addition, further characterization of the amplified products by endonuclease restriction digest in four patients and sequence analysis at the breakpoint were successful in eight of nine analyzed samples. Once the complete intron sequence of \textit{abl} will be available, every breakpoint should be representable after appropriate primer design. With reference to the breakpoint site in the \textit{abl} gene, our results do not yet support data published by Jiang \textit{et al.} (18). In our findings, the breakpoints were equally dispersed over intron 1b and did not cluster, whereas Jiang \textit{et al.} suggested three cluster regions—30 ± 5.

Fig. 2 Agarose gel electrophoresis of one representative patient sample. A characteristic fragment of approximately 6000 bp could be amplified with the constant \textit{bcr} primer and the \textit{abl} primer 3–2 (Lane 2). Restriction enzyme digestion produced fragments of approximately 5400 bp and 600 bp, respectively. On the basis of sequence information, the calculated size of the smaller fragment is 614 bp. Lanes 1 and 4, \textit{\lambda}HindIII.

Fig. 3 Breakpoint localization in the \textit{abl} gene. Individual breakpoints were dispersed throughout the entire \textit{abl} intron sequence without clustering.

Fig. 4 A, sensitivity of LT-DNA-PCR. An approximately 4400-bp fragment could be generated with the constant \textit{bcr} primer and \textit{abl} primer 3–5 in one representative patient with 100% Ph\textsuperscript{+} hematopoiesis at the time of analysis. On the basis of sequence analysis, the calculated size is 4341 bp. For amplification, a dilution series of template DNA was used as follows: Lane 2: 0.25 \(\mu\)g; Lane 3: 25 ng; Lane 4: 2.5 ng; Lane 5: 250 pg; Lane 6: 25 pg; Lane 7: 2.5 pg; Lane 8: 250 fg; Lane 9: 25 fg; Lanes 10 and 11: not loaded, Lanes 1 and 12: \textit{\lambda}HindIII. B, sensitivity of RT-PCR. For amplification, a dilution series of BV173 mRNA was used as follows: Lane 2: 5 \(\mu\)g; Lane 3: 500 ng; Lane 4: 50 ng; Lane 5: 5 ng; Lane 6: 500 pg; Lane 7: 50 pg; Lane 8: 5 pg; Lane 9: 0.5 fg; Lanes 1 and 10: \textit{\phi}X174/HAEIII.
100 ± 13, and 135 ± 8 kb—downstream from exon 1b. In one case, the breakpoint was identified in intron 1a, a finding previously reported in three other patients (7, 17). Nevertheless, because of the relatively small number of cases examined, these findings should not be overrated. A potential disadvantage of LT-DNA-PCR could be single-copy target amplification, whereas, in RT-PCR, high expression per single cell might allow potentially more sensitive detection. However, our results comparing RT- versus LT-DNA-PCR showed comparable sensitivity no matter whether large or small fragments were amplified from DNA templates.

LT-PCR analysis of seven leukapheresis products of three CP-CML patients after mobilization chemotherapy showed reproducibly the presence of bcr/abl at the DNA level in six of them whereas analysis by RT-PCR and FISH failed to detect the fusion gene. The sensitivity of FISH does not allow detection of the rearranged bcr/abl gene unless >5% of rearranged cells are present within the analyzed cell population, a percentage also comparable to classical cytogenetic analysis. This fact is well known from patients receiving α-IFN therapy who become negative by cytogenetics or FISH, but are still positive by RT-PCR. Considering the sensitivity of RT-PCR and LT-DNA-PCR about equal, our results imply either that some rearranged cells do not express the bcr/abl fusion gene or that the expression is rather low, possibly just below the detection limit of RT-PCR.

The data that is shown support the usefulness of our method as a diagnostic tool and as a means to detect residual disease even in the absence of gene expression in CML patients. It will probably improve the evaluation of purging strategies in CML. In addition, a modification of the methodology could also be used to detect breakpoints associated with Ph-positive acute lymphocytic leukemia.

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