Detection of Two Alternative \textit{bcr/abl} mRNA Junctions and Minimal Residual Disease in Philadelphia Chromosome Positive Chronic Myelogenous Leukemia by Polymerase Chain Reaction

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The Philadelphia (Ph') chromosome in chronic myelogenous leukemia (CML) results in fusion of the \textit{bcr} gene and \textit{c-abl} oncogene, which transcribes into two types of chimeric \textit{bcr/abl} mRNAs: the L-6 junction and the K-28 junction. By means of a highly sensitive assay, combination of reverse transcription and polymerase chain reaction (RT/PCR), we analyzed 38 blood samples obtained from 31 patients with Ph'-positive CML and two patients with Ph'-negative \textit{bcr} rearranged CML. Among the 21 samples obtained in chronic phase, eight patients had the L-6 mRNA, 11 had the K-28 mRNA, and two had both the L-6 and K-28 mRNAs. Among the nine samples obtained in blast crisis, four contained the L-6 mRNA, two contained the K-28 mRNA, and three contained both the K-28 and L-6 mRNAs. This finding supports the concept of alternative splicing of \textit{bcr/abl} mRNAs transcribed in Ph'-positive CML. However, it appears to be a rare event. Of the eight samples obtained from eight patients who had achieved complete cytogenetic remission and negativity for \textit{bcr} region rearrangement for 6 months to 3 years after recombinant alpha interferon (ra-IFN) therapy, all of them showed evidence of minimal residual Ph'-positive clones as detected by the RT/PCR assay. This finding suggests that interferon therapy suppresses the proliferation of the Ph'-positive clones, but it does not completely eradicate the Ph'-positive stem cells.

\textbf{MATERIALS AND METHODS}

\textit{Patients' characterization and sample procurement.} Thirty-three patients diagnosed with CML entered this study. Thirty-one of them carried the Ph' chromosome by cytogenetic analysis prior to treatment. The remaining two patients had no Ph' chromosome, but the pretreatment bone marrow samples had clonal \textit{bcr} region rearrangement detected by Southern blot analysis. Thirty-eight blood samples were obtained in various clinical stages: 21 in chronic phase, nine in blast crisis, and eight in complete remission as determined by routine blood counts and morphologic examination, karyotyping, and Southern blot analysis of the bone marrow. These eight remission samples were obtained from six patients with Ph'-positive CML and two patients with Ph'-negative \textit{bcr} rearranged CML. All of these eight patients were treated with recombinant alpha interferon (ra-IFN) therapy. The duration of remission ranged from 6 months to 3 years.

\textit{Southern blot analysis and localization of the chromosomal breakpoints on the \textit{bcr} region.} High-molecular weight (mol wt) DNA was available in 33 samples (16 in chronic phase, nine in blast crisis, and eight in complete remission). DNA was digested with

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restriction endonuclease BamHI and BglII, size fractionated in a 0.8% agarose gel, and transferred onto a nylon membrane. The membrane was then hybridized with a 32P-labeled probe (Oncogene Science, Mineola, NY, Fig 1). After stringent washes with 0.1 x SSC/0.1% SDS, autoradiography was performed against a single intensifying screen at -70°C for 48 to 96 hours.

According to the rearrangement patterns detected by Southern blot analysis, the chromosomal breakpoints on the bcr region could be identified and sublocalized into four different zones (Fig 1). In cases that rearrangement was detected by BglII digestion but not by BamHI digestion, the breakpoint was identified as falling within zone 1. In cases that only one rearranged band was detected by both BglII and BamHI digests, the breakpoint was identified as falling within zone 2. In cases that two rearranged bands were detected by both BglII and BamHI digests, the breakpoint was identified as falling within zone 3. In cases that rearrangement was detected by BamHI but not by BglII, the breakpoint was identified as falling within zone 4.

Synthetic oligonucleotides used. Two oligonucleotides of 16 bases were used as primers. Primer bcr(+), 5′-CAGACTGCTTCA-CAGCA-3′, was derived from the sequence of the second exon of the bcr region. Primer abl(-), 5′-GACTGAAACTCGGAGT-S′, was complementary to the sequence of the second exon of the c-abl gene. In the presence of the L-6 junction mRNA, cDNA fragments of 80 base pairs (bp) were expected to be amplified. In the presence of the K-28 junction mRNA, cDNA fragments of 155 bp were expected to be amplified.

Two oligonucleotides of 20 bases were used as probes to confirm that the amplified cDNA fragments indeed contained sequences representative of the L-6 and K-28 junction mRNAs. Probe L-6 (5′-AATAAGGAAAGCCCTTCA-3′) and probe K-28 (5′-CAGAGTTCAGCCCTTCA-3′) were derived from the sequences at the bcr-abl junctional region of the L-6 and K-28 mRNAs, respectively.

Combined method of RT/PCR. Samples of total cellular RNA (1 to 10 μg) were dispensed in 20 μL of reverse transcription buffer containing 0.5 μg of primer abl(−), 1 μmol/L of dNTP’s (each of four), 20 u of AMV reverse transcriptase (United States Biochemicals, Cleveland), and 20 u of RNasin (Promega, Madison, WI) and incubated at 42°C for one hour. The sample was then suspended in 80 μL of PCR buffer containing 0.5 μg of primer bcr(+ ) and 2.5 u of Thermus Aquaticus DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). PCR (denaturation at 94°C for one minute, annealing at 42 to 55°C for one minute and extension at 72°C for two minutes) was then performed for 45 to 60 cycles as described. 14 Ten microliters of the PCR amplified product was loaded onto a 2.5% to 3% agarose gel or 4% NuSieve agarose gel and size fractionated at 60 V for five to six hours and then transferred to a nylon membrane. The membrane was then hybridized with a mixture of probes L-6 and K-28 that were 5′ end-labeled with 32P-α-ATP. Washing was performed with 2 x SSPE at room temperature for 30 minutes and at 60°C for 15 minutes. Autoradiography was performed against a single intensifying screen at -70°C for four to 16 hours.

**RESULTS**

**Feasibility and specificity of the RT/PCR assay.** We first studied the feasibility and the specificity of the RT/PCR assay to detect the chimeric bcr/abl mRNAs in Ph'-positive CML. We selected 24 Ph'-positive CML samples with clonal bcr region rearrangement shown by Southern blot analysis and six lymphoma samples without bcr rearrangement. By means of the RT/PCR technique, we were able to amplify cDNA fragments of 155 bp (representative of the K-28 junction mRNA) and/or 80 bp (representative of the L-6 junction mRNA) in 24 of 24 CML samples with clonal bcr rearrangement (Figs 2, 3; Table 1): nine had a band of 80 bp, 11 had a band of 155 bp, and four had two bands (155 bp and 80 bp). In contrast, none of the six lymphoma samples were amplifiable.

To further define the specificity of this assay, we studied whether a specific subtype of the bcr/abl mRNA junction was detected in samples with a specific chromosomal breakpoint on the bcr region. According to the patterns of bcr rearrangement by BglII and BamHI digests (Fig 3), we were able to identify the chromosomal breakpoint on the bcr region in these 24 Ph'-positive CML patients: eight within zone 1, 13 within zone 2, two within zone 3, and one within zone 4. The results were further correlated with the subtypes of the bcr/abl mRNA junctions detected by the RT/PCR assay (Table 1). We detected the L-6 junction mRNA in eight of eight patients with the breakpoint occurring within zone 1. Among the 13 patients with the breakpoint occurring within zone 2, we detected one with the L-6 junction, nine with the K-28 junction, and three with both L-6 and K-28 junctions. In the two patients with the breakpoint occurring within zone 3, we detected only the K-28 junction. In the patient with the breakpoint occurring within zone 4, both L-6 and K-28 junctions were detected. Thus, it appears that there is a strong correlation between the chromosomal breakpoint on the bcr region and the specific bcr/abl mRNA junction expressed. Samples with the breakpoint 5′ to the BamHI site
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of the second intron of the \textit{bcr} region (zone 1) contained only the L-6 junction mRNA. In cases that the breakpoint occurred at the more 3' region (zones 2 to 4), the majority of samples contained the K-28 junction and, intriguingly, a minority of them contained both L-6 and K-28 junctions even though only one clonal \textit{bcr} rearrangement was shown by Southern blot analysis.

Sensitivity of the RT/PCR assay and detection of minimal residual disease. In order to determine the sensitivity of the RT/PCR assay, we performed a mixing experiment. Total cellular RNA samples prepared from two blood samples were used: one obtained from a patient in blastic phase of CML, and the other from a normal individual. The CML sample was known to contain the L-6 junction mRNA. This sample was serially diluted with the normal sample (the total amounts of total cellular RNA used were 10 \mu g) and then RT/PCR was performed. After 60 cycles of amplification, we were able to detect the chimeric \textit{bcr/abl} mRNA even when we loaded a sample equivalent to 0.1 ng of the original sample (Fig 4).

We also studied eight patients who had achieved complete remission for 6 months to 3 years after re-IFN therapy. Prior to treatment, six of these eight patients had the Ph' chromosome by karyotyping and \textit{bcr} rearrangement by Southern blot analysis; the remaining two had clonal \textit{bcr} rearrangement even though no Ph' chromosome could be detected by cytogenetic analysis. At the time of remission, all these eight samples appeared to be normal by means of routine blood counts, morphologic examination, karyotyping, and Southern blot analysis. However, minimal residual \textit{bcr/abl} mRNAs were detected in all these eight samples by the RT/PCR assay (Fig 5).
Table 1. Detection of Chimeric bcr/abl mRNA in Ph'-Positive CML by Polymerase Chain Reaction: Correlation of Subtypes of Chimeric bcr/abl mRNA With the Breakpoint on the bcr Region

<table>
<thead>
<tr>
<th>Breakpoint on bcr</th>
<th>Patients Analyzed</th>
<th>Patients With L-6 mRNA Only</th>
<th>Patients With K-28 mRNA Only</th>
<th>Patients With K-28 and L-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone 1</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zone 2</td>
<td>13</td>
<td>1</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Zone 3</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Zone 4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>9</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

Disease status and simultaneous expression of two alternative mRNA junctions. Since we had observed simultaneous expression of both K-28 and L-6 mRNAs in the same patient, we were interested in studying whether it occurred more frequently in any particular phase of the disease. We analyzed 38 blood samples obtained in different disease stages: 21 in chronic phase, nine in blast crisis, and eight in complete remission. Among the 21 samples obtained in chronic phase, eight had the L-6 junction, 11 had the K-28 junction, and two had both L-6 and K-28 junctions. Among the nine samples obtained in blast crisis, four contained the L-6 junction, two contained the K-28 junction, and three contained both L-6 and K-28 junctions. Among the nine samples obtained in complete remission, four contained the L-6 junction, two contained the K-28 junction, and three contained both L-6 and K-28 junctions. Among the eight samples obtained in complete remission, six expressed the L-6 junction and two expressed both L-6 and K-28 junctions (Table 2). Thus, simultaneous expression of two alternative mRNA junctions in the same sample is a rare event in our study. But it appears that it may occur more frequently in blast crisis (three of nine) than in chronic phase (two of 21).

There were five patients in whom matched samples in different phases of disease were available. In four, we analyzed samples both in chronic phase and in remission. Three of them had the L-6 junction detected in chronic phase as well as in remission. The remaining patient had the K-28 junction in chronic phase and, interestingly, both K-28 and L-6 junctions were detected in remission sample. There was one patient in whom we studied samples in late chronic phase (5 years after initial diagnosis) and in blast crisis. In both samples, both K-28 and L-6 junctions were detected.

DISCUSSION

In this report, we demonstrate that the RT/PCR assay facilitates the detection of chimeric bcr/abl mRNAs in Ph'-positive CML. It is highly specific. This assay permits the detection of extremely small quantities of bcr/abl mRNAs by using only a small amount of total cellular RNA sample. Using this highly sensitive and specific assay, we investigated the possibility of alternative splicing in the CML type of bcr/abl mRNAs and we also studied whether minimal residual Ph'-positive clones were present in patients in remission after IFN therapy.

Correlating the chromosomal breakpoint on the bcr region with the subtypes of bcr/abl mRNA junction, we tested the accuracy of the splicing mechanism of the bcr/abl transcripts.
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Table 2. Detection of Chimeric \textit{bcr/abl} mRNA in CML by Polymerase Chain Reaction: Correlating the Subtypes of Chimeric \textit{bcr/abl} mRNA With Disease Status

<table>
<thead>
<tr>
<th>Disease Status</th>
<th>Patients Analyzed</th>
<th>L-6 mRNA Only</th>
<th>K-28 mRNA Only</th>
<th>K-28 and L-6 MRNAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic phase</td>
<td>21</td>
<td>8</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Blast crisis</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Complete remission by karyo-</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>type and southern</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>18</td>
<td>13</td>
<td>7</td>
</tr>
</tbody>
</table>

in CML. In cases that the breakpoint occurred within the second intron of the \textit{bcr} region, the L-6 junction mRNA was expected. In fact, we observed that eight of eight with the breakpoint 5' to the \textit{Bam}HI site in the second intron of the \textit{bcr} region expressed the L-6 junction mRNA. In cases that the breakpoint occurred within the third intron of the \textit{bcr} region, the K-28 junction mRNA was expected. In fact, we observed that 12 of 16 samples with the breakpoint 3' to the \textit{Bam}HI site of the second intron of the \textit{bcr} region expressed the K-28 junction mRNA, one expressed the L-6 junction mRNA, and three expressed both L-6 and K-28 junctions. Such a strong correlation between the chromosomal breakpoint on the \textit{bcr} region and the specific mRNA junction transcribed indicates that the fused \textit{bcr/abl} gene is capable of transcribing and splicing accurately into the chimeric \textit{bcr/abl} mRNA over a long distance (up to more than 100 kb). However, in rare instances, simultaneous expression of both mRNA junctions occurred in the same patient even though only one Ph'-positive clone was detected by Southern blot analysis. There are two possible explanations for this observation. The first explanation is that there were two populations of Ph'-positive clones, one of which was too small to be detected by Southern blot analysis. The second possibility is that this phenomenon is the result of alternative splicing as proposed by Shitivelman et al.1,2 We favor the second explanation because in our study, simultaneous expression of both mRNA junctions occurred exclusively in cases with the breakpoint falling within the more 3' \textit{bcr} region.

We observed that simultaneous expression of two alternative mRNA junctions may occur more frequently in blast crisis than in remission and chronic phase. A prospective study in more patients with longitudinal follow-up will help us elucidate the significance of coexpression of two alternative \textit{bcr/abl} MRNAS.

The sensitivity and specificity of PCR amplification are strongly related to the annealing temperature of PCR. In Fig 2, PCR was performed at higher annealing temperature. More specific PCR amplification could be achieved, but the signals detected were relatively weaker even though 60 cycles of PCR were performed. In Figs 4 and 5, PCR was performed for 60 cycles at lower annealing temperature. The sensitivity to detect the \textit{bcr/abl} cDNA fragments appeared to be higher, and more nonspecific amplification was generated.

We studied eight patients who had achieved complete cytogenetic remission after α-IFN therapy. All of these patients showed evidence of minimal residual Ph'-positive clones by the RT/PCR assay. Furthermore, two of these eight patients had achieved sustained complete remission for 3 years. This observation suggests that α-IFN suppresses the proliferation of the Ph'-positive clones, but it does not eradicate the Ph'-positive stem cells completely. Long-term clinical follow-up is necessary to determine whether these patients will eventually relapse. Additional studies are needed to evaluate the proliferative capability of the residual Ph'-positive clones and whether the subtype of \textit{bcr/abl} mRNA relates to therapeutic response and long-term prognosis.

REFERENCES


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