New Type of Bcr/Ab1 Junction in Philadelphia Chromosome-Positive Chronic Myelogenous Leukemia

By Giuseppe Saglio, Angelo Guerrasio, Claudia Rosso, Alfonso Zaccaria, Angela Tassinari, Anna Serra, Giovanna Rege-Cambrin, Umberto Mazza, and Felice Gavosto

A new and rare type of Bcr/Ab1 junction between exon C3 of the 3’ portion of the Bcr gene and Ab1 exon 2 has been identified in the leukemic cells of two Ph1-positive chronic myelogenous leukemia patients in chronic phase. This is the fourth type of Bcr/Ab1 junction so far identified in Ph1-positive hematologic malignancies and is a consequence of an unusual breakpoint position on chromosome 22 that falls approximately 20 kb downstream of the major breakpoint cluster region (bcr) of the Bcr gene. The new hybrid mRNA is 540 base pairs (bp) longer than that expressed by the K562 cell line and could codify for a Bcr/Ab1 protein carrying 180 additional aminocids with respect to the larger P210 protein so far identified. The hematologic phenotype expressed by the two patients carrying this unusual type of Bcr/Ab1 rearrangement does not significantly differ from that commonly seen in chronic myelogenous leukemia.

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THE PRESENCE of a Philadelphia chromosome (Ph1) in human hematologic malignancies parallels the presence of molecular defects involving the Ab1 proto-oncogene.1 As a consequence of the t(9;22) translocation giving origin to the Ph’ chromosome, the main body of the Ab1 proto-oncogene moves to chromosome 22 and rearranges with the Bcr gene.2 This event determines the formation of Bcr/Ab1 hybrids,3,4 which codify for abnormal proteins bearing a tyrosine-kinase activity contributed by the oncogene moves to chromosome 22 and rearranges with the Bcr gene.

In Ph1-positive chronic myelogenous leukemia (CML), the breakpoints on chromosome 22 occur almost invariably in a restricted region of the Bcr gene called bcr (breakpoint cluster region)3 or mbr 1 (major breakpoint cluster region). This area contains 4 exons that are numbered from 1 to 4, and a fifth exon (exon 5) lies immediately 3’ to its (Fig 1). Because this breakpoint cluster region may fall either in the intron separating exon 2 from exon 3 or in the intron separating exons 3 from exon 4, two different types of Bcr/Ab1 junction have been found in CML.9 In the first, bcr exon 2 is joined to Ab1 exon 2, whereas in the second bcr exon 3 is spliced to Ab1 exon 2. Therefore, the two chimeric messenger RNAs (mRNAs) differ for the presence of the bcr exon 3 sequences (75 bp) and the corresponding proteins for 25 amino acids.7 A higher degree of heterogeneity and a third type of Bcr/Ab1 junction has been identified in Ph1-positive acute leukemias.8-11 In fact, whereas approximately 50% of the cases show the same molecular rearrangements found in CML, in the remaining half, as a consequence of a breakpoint falling within the large intron 1 of the Bcr gene, only the first Bcr exon is joined to Ab1 exon 2.16 This results in the production of a Bcr/Ab1 protein of 190 Kd in molecular weight (P190).17-19

Here we describe the existence of a fourth type of Bcr/Ab1 junction present in two CML patients in chronic phase characterized by an unusual breakpoint position on chromosome 22 mapping in the 3’ portion of the Bcr gene.

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1819
various temperatures and times calculated on the probe oligonucleotide complementary Scakem agarose gel. Transferred on a Gene Screen membrane every extracted following standard procedures. Digested with different Southern blotting procedure. 6.7 chloroform method. Ten micrograms were run on CML patients previously described. (Promega, Madison, manufacturer’s instructions. Ten microliters were then diluted with bovine serum albumin, and previously labeled with RYA was obtained by the guanidinium thiocyanate and phenol-chloroform method. Ten microliters were then diluted with 10 mmol/l, EDTA), 5X SSPE, 0.1% SDS at room temperature for 10 minutes, and 5X SSPE and 0.1% SDS at various temperatures and times calculated on the probe oligonucleotide sequence. The dried filters were then exposed to x-ray films for 2 to 4 hours.

Oligonucleotides. The amplimers and oligonucleotide probes used for this study were synthesized with an Applied Biosystems 381A apparatus (Foster City, CA) and were (1) 5’ GCA GAG TGG AGG GAG AAC ATC CGG 3’, sense sequence between positions 519 and 542 of the bcr region exon 1 (primer a in Fig 1B); (2) 5’ ATA CTG TGC TAT GAA AAG TG 3’, sequence 876 to 895 of the bcr region exon 5 (primer b in Fig 1B); (3) 5’ AGC GAC ATC CAG GCA CTG AAG 3’, sequence 1242 to 1262 of the Bcr gene and corresponding to Bcr exon C3 according to Lifshitz et al. (primer c in Fig 1B); (4) 5’-TCA CTG GGT CCA GCG AGA

Materials and Methods

Patients and cell samples. We have studied two Ph’-positive CML patients (G.S. and M.L.) lacking a rearrangement of the bcr region at Southern analysis. These patients were identified during a large molecular survey (more than 250 cases) on the Italian CML patients. At the time of the study both cases were already treated and maintained in hematological remission by therapy with a-interferon (patient G.S.) or busulfan (patient M.L.). Leukemic cells for this study were obtained from peripheral blood or bone marrow after centrifugation on a Ficoll-Hypaque gradient

Southern and Northern blots. DNA from the leukemic cells was extracted following standard procedures, digested with different restriction enzymes (including BglII, BclI, EcoRI, BamHI, KpnI, and HindIII), run on a 0.8% agarose gel, and subjected to the Southern blotting procedure as previously described.

RNA was obtained by the guanidinium thiocyanate and phenol-chloroform method. Ten micrograms were run on a denaturing gel containing 7% formaldehyde and 1% agarose, blotted to nitrocellulose filters, and hybridized as described.

The probes used for this study were (1) a 1.7-kb HindIII–BamHI fragment derived from the 3’ end of the Bcr cDNA; (2) a 1.5-kb BamHI cDNA fragment corresponding to the first and subsequent exons of the Bcr gene; and (3) a 2.2-kb EcoRI Abl cDNA fragment.

mRNA amplification and oligonucleotide hybridization. cDNA was synthesized from 5 μg of total RNA with 200 U of Moloney Murine Leukemia Virus (MoMLV) reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD). The reaction was performed in a 50-μL volume containing 100 pmol/L of a 3’ Abl primer (see below), 1 mmol/L dNTPs (each of four), and 20 U of RNasin (Promega, Madison, WI) at 37°C for 60 minutes according to the manufacturer’s instructions. Ten microliters were then diluted with 90 μL of a polymerase chain reaction (PCR) mixture containing 16.6 mmol/L ammonium sulphate, 67 mmol/L Tris-HCl pH 8.8, 6.7 mmol/L MgCl2, 10 mmol/L mercapto ethanol, 170 mg/mL bovine serum albumin, and 100 pmol of each of the two primers. After an initial denaturation at 97°C for 10 minutes, 2.5 U of Taq DNA polymerase was added and 40 cycles of denaturation, annealing, and extension were performed on an automated heat block (DNA thermal cycler, Perkin Elmer, Norwalk, CT) at 91°C for 1 minute, 48°C for 1 minute, and 55°C for 5 minutes, respectively, as previously described. 2.5 U of Taq DNA polymerase was added every 10 cycles.

At the end, 10 μL of the mixture was run on a 3% Nusieve, 1% Seakem agarose gel, transferred on a Gene Screen membrane (NEN, Dupont, MA), and hybridized to oligonucleotide probes complementary to specific Bcr/Abl junctions. The probes were previously labeled with γ 32P adenosine triphosphate (Amersham, Buckinghamshire, UK) by a T4 polynucleotide kinase reaction. Prehybridization and hybridization procedures were performed in 5X SSPE (1X SSPE = 0.18 mol/L NaCl, 10 mmol/L NaH2PO4, 1 mmol/L EDTA), 0.5% sodium dodecyl sulfate (SDS), and 5X Denhardt’s solution; washing conditions were 2X SSPE, 0.1% SDS at room temperature for 10 minutes, and 5X SSPE and 0.1% SDS at various temperatures and times calculated on the probe oligonucleotide.

Table 1. Clinical and Hematologic Findings of the CML Patients Bearing the Junction Between Bcr Exon C3 and Abl Exon 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Spleen (cm)</th>
<th>WBC Count (x 10^9/L)</th>
<th>Platelet Count (x 10^9/L)</th>
<th>Therapy</th>
<th>Survival (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.L.</td>
<td>F</td>
<td>76</td>
<td>4</td>
<td>28</td>
<td>1,020</td>
<td>Busulfan</td>
<td>56</td>
</tr>
<tr>
<td>G.S.</td>
<td>F</td>
<td>62</td>
<td>3</td>
<td>16</td>
<td>870</td>
<td>α-Interferon</td>
<td>30</td>
</tr>
</tbody>
</table>

Both patients are alive and in hematologic remission. The hematologic data were those at diagnosis.

Fig 2. Results of Southern blot analysis obtained on a control (lane 1) and on patient G.S. (lane 2) DNA by digestion with EcoRI and hybridization with a 1.7-kb HindIII–BamHI cDNA probe corresponding to the exons of the 3’ portion of the Bcr gene. Some of the visible bands are caused by cross-hybridization with other members of the Bcr gene family. The arrowhead points to an abnormal band visible on the DNA of the patient.
Fig 3. UV picture of the ethidium bromide-stained agarose gel showing the negative results obtained from RNA of the two patients (patient G.S. lane 3; patient M.L. lane 4) using the primers generally used to amplify the junctions normally present in CML and corresponding to the bcr region exon 1 (primer a in Fig 1B) and to Abl exon 2. Visible in lane 2 is a 319-bp band of amplification obtained from K562 and corresponding to a bcr exon 3/Abl exon 2 junction. Positive controls for the efficiency of the PCR reaction have also been included in lanes 5, 6, and 7 (K562, patients G.S. and M.L., respectively) and show the amplification of a 176-bp band corresponding to an Abl exon 2/Abl exon 3 RNA fragment.

AG-3', antisense sequence 306 to 327 of Abl exon 2\(^5\), (5) 5'-ATT GAT CCC GCT GCT CAG CAG ATA CTC-3', antisense sequence 559 to 586 of Abl exon 3\(^5\), (6) 5'-GCT GAA GGG CTT TGA CGT CGA AGG 3', antisense sequence spanning the junction between Bcr exon C3 and Abl exon 2.

Direct sequencing. DNA sequencing was performed directly on double-strand amplified DNA previously purified on acrylamide gel.

RESULTS

The main clinical data of the two patients have been summarized in Table 1. In both cases, parameters at diagno-

Fig 4. (A) UV picture of the ethidium bromide-stained agarose gel showing the PCR products obtained from RNA of the two patients (patient G.S. lanes 2, 5, and 8; patient M.L. lanes 3, 6, and 9) using the primers described in the text and mapped in Fig 1B. The 504-bp band visible in lane 2 was obtained using Bcr primer b and Abl/exon 2 primer. The 780-bp band in lane 3 was obtained using Bcr primer b together with Abl/exon 3 primer. The 138-bp band in lane 4 was obtained using Bcr primer c and Abl/exon 2 primer. Lanes 1, 4, and 7 show the negative results obtained from RNA of the K562 cell line, using the same primers. Lane 10 is a negative reaction in which RNA addition was omitted. (B) Autoradiography of the filter obtained by blotting the agarose gel shown in A after hybridization with an oligonucleotide probe (sequence f in Materials and Methods) spanning the junction between Bcr exon C3 and Abl exon 2. Only the bands in lanes 2 and 3, 5 and 6, and 8 and 9 are positive.
sis reflect a chronic form of CML. Both patients showed an initial hematological phase characterized by a marked thrombocytosis with only a moderate increase of the WBC count.

Both patients were positive for the presence of a Ph chromosome and negative for rearrangements of the bcr region. Patient M.L. was previously reported to present an unusual type of breakpoint in the initial hematological phase characterized by a marked thrombocytosis with only a moderate increase of the WBC count. 

With a cDNA probe corresponding to the 3' part of the Bcr gene, a similar region. Patient M.L. was previously reported to present an unusual type of breakpoint in the initial hematological phase characterized by a marked thrombocytosis with only a moderate increase of the WBC count. Both patients showed an abnormal band also hybridized with a cDNA probe corresponding to the Bcr gene. A 138-bp stretch crossing the connection that was sequenced. The vertical arrow between positions 404 and 405 marks the 117-bp stretch crossing the connection that was sequenced. The primers used for amplification and sequencing are underlined.

**DISCUSSION**

We have identified in the leukemic cells of two Ph1-positive CML patients the presence of a hybrid mRNA showing a new type of junction between the Bcr and the Abi genes. This is the fourth type of Bcr/Abi junction so far identified in Ph-positive hematologic malignancies and is rare; it has been found only in two of more than 250 cases examined. This junction is associated with the presence of an unusual breakpoint position on chromosome 22 falling in the 3' portion of the Bcr gene. The new hybrid mRNA is 540 bp longer than that expressed by the K562 cell line and may codify for a Bcr/Abi protein carrying 180 additional amino acids with respect to the larger P210 protein so far identified. Unfortunately, several attempts to verify the size of this protein by immunoprecipitation have been unsuccessful. This is common in CML patients in chronic phase and, as recently reported by Shibata et al., is probably due to a...
phosphotyrosine phosphatase activity present in the mature myeloid cells of CML. Nevertheless, our data show that in a small percentage of Ph'-positive CML patients in chronic phase, variant Bcr/Abl junctions may exist. The study and characterization of the molecular defects present in these patients may be important to evaluate the existence of a possible relationship between the presence of certain types of Bcr/Abl proteins and the hematologic phenotype.

In this regard, the presence of P190 is generally associated with an acute leukemia phenotype. Some investigators also have reported that CML patients retaining the bcr region exon 3 sequences in their Bcr/Abl rearrangement have a worse prognosis compared with those who lack these sequences. These observations suggest an influence of the Bcr/Abl junction on the biological activity of the hybrid protein and, as a consequence, on the clinical phenotype. By contrast, the patients described in this report carry an abnormal Bcr/Abl mRNA retaining most of the Bcr sequences, but they do not differ significantly in terms of hematologic phenotype from the average CML patients carrying the usual types of Bcr/Abl junction.

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