A Novel BCR-ABL Fusion Gene (e6a2) in a Patient With Philadelphia Chromosome-Negative Chronic Myelogenous Leukemia


A novel variant of the chimeric BCR-ABL mRNA transcript was detected in a patient with Philadelphia chromosome-negative (Ph−) chronic myelogenous leukemia (CML) by multiplex reverse-transcription polymerase chain reaction (RT-PCR). Sequence analysis of the fusion region of the amplified cDNA fragment showed an in-frame joining of exon e6 of the BCR gene and exon a2 of the ABL gene, giving rise to an e6a2 BCR-ABL transcript. This finding was confirmed by Southern blot analysis using a specific probe corresponding to intron 6 of the BCR gene, whereas conventional Southern blot for rearrangement of the major breakpoint cluster region (M-bcr) was negative. Western blot studies detected a BCR-ABL protein slightly larger than p185 BCR-ABL. Metaphase fluorescence in situ hybridization showed an insertion of ABL material into the BCR region without reciprocal BCR translocation. The findings in this case show that atypical BCR-ABL transcripts are detectable even in Ph− CML patients without M-bcr rearrangements. Multiplex PCR using primers that allow for amplification of all known BCR-ABL transcripts is an appropriate method to exclude these rare variants.

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MATERIALS AND METHODS

Patient history. A 41-year-old male patient was diagnosed as chronic-phase CML by trephine biopsy in March 1993. At that time, his peripheral blood showed mild leukocytosis with basophilic: white blood cell count (WBC) of $15.6 \times 10^9/L$, 13% basophils, 2% promyelocytes, 72% neutrophils, 13% lymphocytes, a platelet count of $216 \times 10^9/L$, a hemoglobin level of 15.0 g/dL, and an alkaline phosphatase score of 12 (normal value, 10 to 100). The activity of lactic dehydrogenase (LDH) was 196 U/L (normal range, 120 to 240 U/L). The spleen was not palpable. Trephine biopsy showed an increased cellularity of the bone marrow, marked eosinophilia, and decreased megakaryocytes, predominantly microforms. Cytogenetic analysis of 20 bone marrow metaphases by Giemsa banding showed a normal karyotype (46, XY). No specific therapy was commenced; the WBC count fluctuated between 10 and $55 \times 10^9/L$ during the following 20 months. In November 1994, the patient developed marked pain in the left shoulder; an x-ray showed osteolysis in the left proximal humerus with a soft tissue tumor. A biopsy of the tumor showed a myeloblastic infiltrate (CD33+ and CD34+). At that time, his peripheral blood showed a WBC count of $11.5 \times 10^9/L$, 8% basophils, 1% eosinophils, 1% myelocytes, 75% neutrophils, 11% lymphocytes, 4% monocytes, a platelet count of $480 \times 10^9/L$, a hemoglobin level of 15.4 g/dL, and increased LDH activity of 644 U/L. A bone marrow aspirate showed marked basophilia, eosinophilia, and 5% blasts, consistent with accelerated phase. After local irradiation and hydroxyurea therapy, the tumor recurred, accompanied by new foci. In January 1996, the patient underwent allogeneic bone marrow transplantation with marrow from a matched unrelated donor, but died of sepsis 16 days after transplantation.

RT-PCR. After red blood cell lysis, RNA was extracted from total WBCs by standard protocols. Conditions for reverse transcription and multiplex PCR for BCR-ABL using primers BCR-C, B2B, CA3+, and CS5+ have been described.15 Specific single-step PCR for BCR-ABL transcripts was performed using primers BCR-C (BCR exon e1) and CA3+ (ABL exon a3). Nested PCR for ABL-BCR transcripts was performed using primers NL1 and BE16 (first step) and GLy and B4c (second step) (Fig 1). Cloning and sequencing. Single-step BCR-ABL PCR products were digested with BamHI cloned in M13mp19, and sequenced using Sequenase version 2.0 T7 DNA polymerase.16 The sequencing products were run on an 8.0% polyacrylamide gel and exposed for 24 hours.

Southern blot analysis. Southern blot analysis for M-bcr rearrangements of the BCR gene was performed as reported.16 DNA was extracted from total WBC, digested with HindIII, EcoRI,

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Fig 1. Maps of BCR, ABL, and BCR-ABL mRNA transcripts. Primers BCR-C, B2B, CA3+, and C5e- were used for multiplex PCR for BCR and BCR-ABL; primers BCR-C and CA3- were used for single-step PCR for BCR-ABL; and primers Nbl', Gly', B4e-, and BE16- were used for nested PCR for ABL-BCR.

BamHI, and Bgl II; electrophoresed on a 0.7% agarose gel; blotted; and hybridized to 2-kb Bgl II/HindIII (5'M-bcr) and 1.2-kb HindIII/ Bgl II (3'M-bcr) probes. For analysis of rearrangements 3' of BCR exon 6, a specific 730-bp genomic DNA probe (BCR-6) was amplified by PCR using primers Int6' (S'-GAG TGC CTT ATG CTG GTT GTG) for BCR intron 6 and E7' (S'-CAG GTG TCC GAA CAT CTC GTC TC) for BCR exon 7.

Western blot analysis. Western blotting was performed on lysates of 1 to 10 x 10⁶ cells from the patient's peripheral blood leukocytes, from leukocytes from a Ph⁺ ALL patient, and from K562 and B15 cell lines; run on a 6.5% polyacrylamide gel; blotted; incubated with 1:20,000 dilution of monoclonal anti-ABL antibody 8E9 for 2 hours; and developed by enhanced chemoluminescence (ECL) methods as described previously.¹¹

Fluorescence in situ hybridization (FISH). Interphase and metaphase dual-color FISH for BCR and ABL genes were performed.
Fig 2. (A) Multiplex PCR for BCR-ABL and BCR transcripts. Lane 1, e6a2 BCR-ABL CML patient (1,123 bp); lane 2, b3a2 BCR-ABL CML patient; lane 3, b2a2 BCR-ABL CML patient; lane 4, Ph−/BCR-ABL− CML patient; lane 5, normal control; lane 6, SD1 cell line (e1a2 BCR-ABL, 481 bp); lane 7, K562 cell line (b3a2 BCR-ABL, 385 bp); lane 8, BV173 cell line (b2a2 BCR-ABL, 310 bp); lane 9, blank; lane M, 123-bp marker. BCR bands (1808 bp) are positive for all cell lines and patients. (B) Single-step PCR for BCR-ABL transcripts using primers BCR-C (BCR exon e1) and CA3− (ABL exon a3). Lane 1, SD1 cell line (e1a2 BCR-ABL); lane 2, e6a2 BCR-ABL CML patient; lane 3, b2a2 BCR-ABL CML patient; lane 4, b3a2 BCR-ABL CML patient; lane 5, blank; lane M, 123-bp marker.

RESULTS

RT-PCR. In the initial screening by multiplex PCR, an atypical large amplification product was detected in addition to the normal BCR band (Fig 2A). Specific single-step RT-PCR using primers BCR-C (BCR exon e1) and CA3− (ABL exon a3) confirmed the amplification of an abnormal BCR-ABL transcript (Fig 2B). ABL-BCR transcripts were not detectable after either one- or two-step PCR using primers NB1+ and BE16− (first step) and Gy1+ and B4e− (second step).

Cloning and sequencing. BamHI-digested BCR-ABL cDNA PCR products were cloned and sequenced. In all of the five clones investigated, identical fusions between exon e6 of the BCR gene and exon a2 of the ABL gene, resulting in an e6a2 BCR-ABL transcript, were found.

Southern blot analysis. Results of the Southern blot for M-bcr rearrangements were negative. Hybridization with a specific probe (BCR-6) for rearrangements 3′ of BCR exon e6 resulted in a 4.1-kb BamHI fragment and a 3.8-kb HindIII fragment in addition to the expected 6.4-kb BamHI and 8.2-kb HindIII germline fragments. Twelve M-bcr CML patients showed germline configuration using probe BCR-6.

Western blot analysis. A strong BCR-ABL protein signal was detected on Western blots of the patient’s leukocytes. The ratio of BCR-ABL/ABL was 2.0, as measured by densitometry. The patient’s protein was slightly larger than the p185 BCR-ABL protein (Fig 3).

FISH. Single-color metaphase FISH using the BCR probe detected BCR material on the long arms of both chromosomes 22, but not on chromosome 9. Dual-color FISH using the ABL and the BCR probe showed a colocalization of BCR and ABL on the long arm of one chromosome 22, without BCR translocation. Sixty-two of 100 interphase nuclei of a peripheral blood smear showed a colocalization of BCR and ABL. At that time, 82% of peripheral blood leukocytes were myeloid and 18% were lymphoid.

DISCUSSION

Using multiplex PCR, we were able to detect atypical e6a2 BCR-ABL transcripts in a patient with hematologic features of CML, but no demonstrable Ph chromosome and no M-bcr rearrangement in Southern blot analysis. To our knowledge, this patient is the first Ph− CML case with a breakpoint outside the M-bcr and the first case to express an e6a2 BCR-ABL. This new in-frame hybrid mRNA is 861 bp shorter than that expressed by the K562 (b3a2) cell line K562 CML B15 ALL KG1 e6a2 ela2 ela2− p210 BCR-ABL− p185 BCR-ABL− p145 ABL−
and is predicted to encode a BCR-ABL protein 287 amino acids smaller than the b3a2 p210 protein. Our data show that breakpoints outside M-bcr have to be considered and that variant BCR-ABL fusion transcripts and proteins can be expressed not only in Ph+, but also in Ph− CML patients.

It was shown that BCR-ABL proteins translated from unusual transcripts, especially those resulting from m-bcr breakpoints, are still oncogenic and can produce BCR-ABL+ disease. Ph negativity and M-bcr negativity in patients with clinical signs of CML are not sufficient for diagnosis of Ph−/BCR-ABL− CML. To definitively exclude BCR-ABL+ disease, Ph− CML patients should be investigated by multiplex PCR, Western blotting, or FISH, even if the expected M-bcr rearrangement is not detectable by conventional Southern blot.

**ACKNOWLEDGMENT**

The M-bcr probes for Southern blot analysis were provided by Prof C.R. Bartram (Heidelberg, Germany), the B15 cell line was provided by Dr S. Smith (Department of Pediatrics, University of Chicago, Chicago, IL), the SD1 cell line was provided by Prof B.D. Young (St Bartholomews Hospital, London, UK), the YAC clone D107F9 was provided by Prof Th. Cremer (Heidelberg, Germany), and the cos-ABL 8 clone was provided by Dr N. Heisterkamp (Los Angeles, CA).

**REFERENCES**


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**Table 1. Described or Theoretically Possible In-Frame BCR-ABL mRNA Fusions**

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<tr>
<td>e1a2</td>
<td>Reviewed in Melo et al8</td>
<td>50% of adult Ph+ ALL and 75% of childhood Ph+ ALL</td>
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<td>e6a2</td>
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<td>b2a2 [e13a2] and b3a2 [e14a2]</td>
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A Hochhaus, A Reiter, H Skladny, JV Melo, C Sick, U Berger, JQ Guo, RB Arlinghaus, R Hehlmann, JM Goldman and NC Cross