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 × 10^9/L, 13% basophils, 2% promyelocytes, 72% neutrophils, 13% lymphocytes, a platelet count of 216 × 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 lactate 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. Cyto genetic 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 × 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 × 10^9/L, 8% basophils, 1% eosinophils, 1% myelocytes, 75% neutrophils, 11% lymphocytes, 4% monocytes, a platelet count of 480 × 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.3 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 NB+ and BE16+ (first step) and Gly+ and B4c- (second step) (Fig 1).

Cloning and sequencing. Single-step BCR-ABL PCR products were digested with BamH1, cloned in M13mp19, and sequenced using Sequenase version 2.0 T7 DNA polymerase.10 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.11 DNA was extracted from total WBC; digested with HindIII, EcoRI,
Fig 1. Maps of BCR, ABL, and BCR-ABL mRNA transcripts. Primers BCR-C, B2B, CA3-, and C5F 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.

*Bam*HII and *Bgl* II; electrophoresed on a 0.7% agarose gel; blotted; and hybridized to 2-kb *Bgl* II/*Hind*III (5'M-bcr) and 1.2-kb *Hind*III/ *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' (5'-GAG TGC CTT ATG CTG GTT GTG G) for BCR intron 6 and E7' (5'-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 × 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.
using a 215-kb fragment of the YAC clone D107F9 spanning the BCR gene and a 40-kb fragment of 3' coding and 3' flanking sequences of ABL of the clone cos-abl 8, as described. 13

Cell lines. As controls for PCR and Western blot experiments, the following cell lines were used: K562 (b3a2 BCR-ABL', blast crisis cell line), BV173 (b2a2 BCR-ABL', blast crisis cell line), B15 and SD1 (e1a2 BCR-ABL' ALL cell lines), and KG-1 (BCR-ABL' AML cell line).

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 Ni b+ and Be16- (first step) and Gyl- 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 2a 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).  

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

mosomes 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.
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.

Inspection of the intron/exon structure of the normal BCR and ABL genes shows a large number of potential in-frame BCR-ABL fusions. BCR exons e1, e6, e12 (b1), e13 (b2), e14 (b3), e19 (c3), and e20 (c4) are in frame with ABL exons a2 and a3, although joining of BCR exon e12 and ABL exon a2 would generate a stop codon at the point of fusion. Many of these combinations have been detected in patients with CML and ALL (Table 1). These BCR exons are also in frame with ABL exon a7, although e12a7 fusion would result in a stop codon. In addition, BCR exons e2, e3, e4, e5, e7, e8, e10, e11, e15, e16, e17, and e22 are in frame with ABL exons a4, a8, a9, a10, and a11; BCR exons e9 and e21 are in frame with ABL exons a5 and a6. However, analysis of BCR-ABL mutants has shown that the ABL SH2 domain, which is encoded by exons a3 and a4, is essential for transformation; therefore, it is extremely unlikely that any BCR-ABL fusion lacking either of these exons would cause CML.

Occasionally, e1a2 BCR-ABL transcripts and p185 BCR-ABL proteins resulting from m-bcr breakpoints have been described in chronic-phase CML. Most of these patients had an atypical clinical phenotype with monocytosis and dysplasia (reviewed in Melo et al). The hematologic phenotype of our patient was unusual in so far as the disease started with a phase of fluctuating leukocytes without necessity of treatment over 20 months, after which an extramedullary blastic infiltration occurred.

BCR-ABL proteins translated from unusual transcripts, despite lacking a variable number of amino acids coded for by the missing exons, are still oncogenic and can produce chronic-phase CML. 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.

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