RAPID COMMUNICATION

Detection of ALL-1/AF4 Fusion Transcript by Reverse Transcription-Polymerase Chain Reaction for Diagnosis and Monitoring of Acute Leukemias With the t(4;11) Translocation

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The chromosomal breakpoints of t(4;11) translocation of acute lymphoblastic leukemia (ALL) have been recently identified at molecular level and shown to involve the AF4 (FEL) gene on chromosome 4 and the ALL-1 (MLL, Hrx) gene on chromosome 11. The ALL-1/AF4 fusion gene is transcribed into a chimeric mRNA. Using primer sets derived from ALL-1 and AF4 cDNAs by reverse transcription-polymerase chain reaction (RT-PCR), we were able to amplify the breakpoint sites of the fusion transcript of all 15 ALL cases with karyotypic or molecular evidence of the t(4;11). DNA fragments of different size were obtained as the consequence of different breakpoints on chromosome 11 and the presence of alternative splicing of ALL-1 exon 8. The feasibility of monitoring the residual cells carrying the t(4;11) in 2 ALL patients with different clinical outcome was evaluated. Overall, the presented results provide evidence that RT-PCR can be used as a rapid method for detecting this chromosomal abnormality and following the patient’s response to therapy.

MATERIALS AND METHODS

Patient samples. Bone marrow (BM) or peripheral blood (PB) samples were obtained from 15 ALL patients after informed consent. The patients ranged in age from 5 days to 57 years. Diagnosis was established according to standard morphologic and cytochemical criteria. Mononuclear cells obtained from BM or PB samples at diagnosis were isolated by Ficoll-Hypaque centrifugation. At this stage, the preparations, containing greater than 90% of leukemia blast cells, were used for immunophenotypic, DNA, and RT-PCR analyses. At the same time, chromosome preparations were obtained from direct BM cell cultures and from BM cell cultures not stimulated for 24 hours. The preparations were stained with quinacrine mustard (QFOQ banding technique) and 40 metaphases were routinely analyzed. Chromosome preparations were adequate in 13 of the 15 cases. Nine patients showed the specific t(4;11)(q21;q23) translocation. Four patients displayed an apparently normal karyotype.

DNA analysis. High molecular weight DNA was obtaining from leukemic samples following standard procedures. After digestion with HindIII, Bgl II, BamHI, EcoRI, and Xba I, DNAs were electrophoresed on 0.8% agarose gels, denatured, and blotted onto nitrocellulose papers. Filters were hybridized overnight with the 32p random priming-labeled probes, washed, and exposed for 48 to 72 hours for autoradiography using an intensifying screen. Hybridization and washing conditions have been described elsewhere.

Probes. The FA4 is a 480-bp genomic Alu-free fragment originating from the ALL-1 locus and derived from the previously reported Dde I fragment. The FA4 DNA insert spans some of the exon 8 sequences. The Y54.5 probe is a 0.5-kb Xba I-Eco RI fragment representative of the AF4 breakpoint cluster region on chromosome 4, as previously reported.

RT-PCR amplification of ALL-1/AF4 junctions. In vitro reverse transcription to cDNA of approximately 1 μg of total RNA extract from leukemic cells was performed with 2.5 U of cloned Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase in a volume of 20 μL containing 50 pmol of random examer primers, 1 mmol/L dNTP, and 20 U of RNase Inhibitor using a commercial kit (GeneAmp RNA PCR Kit; Perkin Elmer-Cetus, Norwalk, CT) for 15 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 5°C.

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Submitted May 19, 1993; accepted August 30, 1993.

Supported in part by Fondazione Tettamanti, by “Associazione P. Belli,” by grants and fellowship (to C.C.) of the Associazione Italiana per la Ricerca sul Cancro (A.B., A.R., and G.C.), and by the Consiglio Nazionale delle Ricerche (PF ACRO, Grants No. 92.02140.PF.39 to A.B. and No 92.02392.PF.39 to A.R.).

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Blood, Vol 82, No 10 (November 15), 1993: pp 2943-2947

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For amplification of the cDNA products, 80 μL of PCR mixture containing 1 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 200 μmol/L dNTP, 2.5 U of Taq DNA Polymerase (Perkin Elmer-Cetus), and 15 pmol of primers Ex5 and AF4.1 was added and the samples overlaid with mineral oil (Sigma, St Louis, MO). After an initial denaturation at 94°C for 1 minute, 30 cycles of amplification were performed on an DNA Thermal Cycler (Perkin Elmer-Cetus). One cycle of denaturation, annealing, and extension step consists of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, respectively. At the end, 5 μL of the first PCR product was used for a second round of amplification for a further 30 cycles using a nested primer (AF4.2). Finally, 1/10 of PCR products were run on a 2.5% Nusieve agarose gel stained with ethidium bromide and visualized under a UV lamp. The sequences of the primers used were Ex5, 5'-GAGGATCCTGGCCCCAAAAGAAAAG3'; AF4.1, 5'-TGAGCTGACCCGCTGCTGACG-3'; and AF4.2, 5'-AGGAAGCTTGGATGCTGCTGACG-3'.

Amplification of the β-actin mRNA was performed with 1 µg of the same RNA preparation used to identify ALL-I/AF4 junctions. Negative control were included in all PCR experiments.

**Sequencing.** The PCR products were purified to remove the excess primers and dNTPs by electrophoresis in 1% agarose and the DNA fragments recupered by the Geneclean kit. The purity of the DNA samples was checked by the dideoxynucleotide chain termination method with a modified T7 DNA Polymerase (Sequenase 2.0; US Biochemical Corp., San Francisco, CA). The products were run on a 20% polyacrylamide gel.

**Fig 1.** Partial cDNA sequence of the ALL-I/AF4 fusion gene. The positions of the primers Ex 5, AF4.1, and AF4.2 are indicated.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>WBC (× 10⁹/L)</th>
<th>Karyotype</th>
<th>Immunophenotype</th>
<th>RT-PCR Results</th>
<th>Clinical Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 mo/F</td>
<td>200</td>
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<td>+(702/588 bp)</td>
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<tr>
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<tr>
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<td>Pre-pre-B</td>
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<td>+(702 bp)</td>
<td>Relapse +30 mo; AlloBMT +34 mo</td>
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<td>53.7</td>
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<td>+(456 bp)</td>
<td>CCR +42 d</td>
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Abbreviations: WBC, white blood cell count; pre-pre-B, Tdt⁺, CD10⁻, CD19⁻; common, Tdt⁺, CD10⁺, CD19⁺; CCR, continuous complete remission; ND, not done; AlloBMT, allogeneic BM transplantation.
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Fig 2. Partial physical map of ALL-1 gene. Restriction sites are indicated by capital letters: B (BamHI), R (EcoRI), G (BglII), X (XbaI), and H (HindIII). Numbers refer to breakpoint position of different patients. FA4 indicates the genomic probe used to detect ALL-1 rearrangements by Southern blot analysis.

Fig 3. RT-PCR amplification of ALL-1/AF4 chimeric gene in the indicated patients representative of the breakpoint positions on ALL-1 gene. The two transcripts amplified in patient no. 1 are the consequence of alternative splicings on exon 8.

Fig 4. Sensitivity of PCR detection of the t(4;11) breakpoint junction. Total RNA isolated from ALL patient no. 12 was diluted by mixing with normal BM RNA. Dilutions include 0.9 ng of normal BM RNA mixed with 0.1 ng ALL RNA and 1 ng of normal BM RNA mixed with 0.01 ng, 0.1 ng, 0.01 ng, and 0.001 ng RNA, respectively. One microgram of ALL RNA was designated as the undiluted control. PCR was performed as described in Materials and Methods.

RESULTS

The main clinical, phenotypic, and karyotypic data of the 15 patients studied are shown in Table 1. Patients no. 2, 4, 8, 9, 12, 13, and 14 have been previously described. All cases presented an abnormal DNA configuration of ALL-1 gene. The 6 patients (nos. 9 through 14) in whom the cytogenetic analysis either failed or showed a normal karyotype were also studied to explore the AF4 DNA configuration. In all cases, new rearranged bands, together with germline fragments, were detected in at least two digestions with two different endonucleases (data not shown). According to the nucleotide sequence of ALL-1 and AF4 cDNAs, a set of amplimers was selected. Figure 1 shows the positions of Ex5, AF4.1, and AF4.2 relative to the sequence of ALL-1

- 1 kb
- Cen
- Tel
- 12,13,15
- 1,2,3,4,5,6,7,8,9,10,11,14
- B R G X R
- 5 6 7 8 9 10 11 12 13
- 702 588 456
- O2
- Water
and AF4 previously reported. Primer Ex5, derived from ALL-1 exon 5, and primer AF4.1 were used as the first cycle of PCR; a second round of amplification was then performed using AF4.2 as a nested primer. As shown in Fig 2, our Southern analysis of ALL-1 gene displays two major breakpoint cluster regions. The first one is located between ALL-1 exons 6 and 7 (patients no. 12, 13, and 15), whereas the second one falls in the DNA region encompassing exon 7 to 9 (all the remaining patients). Among this second group, a breakpoint site between exon 8 and 9 has been precisely defined only for patients no. 5, 8, and 10, whereas in the other cases, our data do not allow us to define whether the breakpoints occur between exons 7 and 8 or between exons 8 and 9. Figure 3 shows the results of RT-PCR amplification of four different representative ALL cases. As illustrated, we observed a heterogeneous PCR pattern characterized by three different sized bands of 456, 588, and 702 bp, respectively. The 456-bp band was detected in patients no. 12, 13, and 15, whereas DNA analysis showed that, at the genomic level, the breakpoint occurred in the EcoRI-Bgl II DNA region between exon 6 and 7 (Fig 2). These findings have been confirmed by nucleotide sequence of PCR product from patient no. 12 that showed the fusion of ALL-1 exon 6 to AF4 sequences (data not shown). In the remaining 12 cases, in which breakpoints were telomeric to ALL-1 exon 7, we observed two bands of 588 and 702 bp, respectively. Nucleotide sequence showed that the longer PCR product is generated by the fusion of ALL-1 exon 8 and AF4 sequences (data not shown). The 588-bp PCR product is likely to result from the fusion of ALL-1 exon 7 to AF4 sequences, being equal in size to the difference between the 702-bp fragment less the 114-bp fragment of ALL-1 exon 8. Our findings are consistent with the presence of alternative splicing involving exon 8, which has been previously described. Having defined universal primers able to amplify the breakpoint sites in all t(4;11) ALLs, BM samples from 15 ALL patients were analyzed, achieving a positive amplification in all cases, as reported in Table 1. Moreover, to assess the specificity of the method and to rule out the carry-over contaminants, a variety of RNAs isolated from normal subjects (PB mononuclear cells) and from 5 acute myelogenous leukemia cell lines (GF-D8, KG-1, K562, U937, and HL-60) and four ALL cell lines (CEM, LL-13P, DAUDI, and PEER) were similarly analyzed. All these samples were consistently found to be negative for the presence of AF4/ALL-1 fusion transcripts (data not shown). To determine the sensitivity of PCR amplification in our study, RNA from patient no. 12 was serially diluted by mixing with normal BM RNA, reverse transcribed, and amplified as described in Materials and Methods. As shown in Fig 4 the PCR can detect the presence of ALL-1/AF4 fusion gene in the presence of less than 0.01 ng total RNA, which represents a final dilution between 10^{-3} and 10^{-6}. To evaluate the usefulness of this approach to the detection of minimal residual disease, we performed RT-PCR assays on serial samples of PB or BM mononuclear cells from patients no. 1 and 9. The 2 patients were selected because of the availability of frozen remission samples and because they were representative of different clinical outcomes. As shown in Fig 5, none of the BM samples tested in patient no. 1 after achieving complete remission resulted in detectable amplified products of the ALL-1/AF4 chimeric gene, suggesting the possible molecular eradication of the leukemic clone. In patient no. 9 (Fig 5B), the therapeutic protocol failed to induce a molecular clearing of the disease and the patient soon relapsed and died.
RT-PCR IN T(4;11) TRANSLOCATION

DISCUSSION

The identification and characterization of ALL-1 and AF4 genes, fused in ALL cases with the reciprocal t(4;11) translocation, has suggested the possibility of exponentially amplifying the result in ALL-1/AF4 chimeric transcript using the RT-PCR technique. This methodology is specific, very sensitive, and extensively used for molecular diagnosis of leukemia and monitoring of MRD in all cases with a specific and well-characterized chimeric transcript. Examples are the rearrangements of the bcr and c-abl genes in the t(9;22) of chronic myeloid leukemia and, more recently, the fusion of the PML and RAR-α genes in the t(15;17) of acute promyelocytic leukemia. The RT-PCR methodology applied in the present study allowed for the amplification of ALL-1/AF4 junctions in all 15 ALL patients with cytogenetic or molecular evidence of t(4;11) studied, suggesting that our amplimers encompass the different possible restriction fusion sites of ALL-1 and AF4. In fact, regarding ALL-1, as indicated also by our Southern and sequencing data, the two possible fusion sites between exons 6 and 7 and between exons 7 and 9 are downstream to our Ex5 oligoprimer. Similarly, with respect to AF4, the other restriction fusion site described 15 amino acids downstream to that reported by us in Fig 1, is upstream to our AF4.1 and AF4.2 primers. The resolution of the fragments obtained by the two rounds of amplification and the possibility of visualizing them by standard ethidium staining (as shown in Fig 3) made this approach the easiest and the most rapid method for identifying the t(4;11), even in the cases in which conventional cytogenetics failed. Furthermore, RT-PCR amplification, which allows for a more sensitive detection of MRD than do morphology and cytogenetic analysis, is important in monitoring the course of disease and in determining subsequent therapeutic approaches. Application of this approach to patient no. 9 showed that the ALL-1/AF4 fusion transcript persisted after intensive chemotherapy and between exons 7 and 9 are downstream to our Ex5 oligoprimer. Similarly, with respect to AF4, the other restriction fusion site described 15 amino acids downstream to that reported by us in Fig 1, is upstream to our AF4.1 and AF4.2 primers. The resolution of the fragments obtained by the two rounds of amplification and the possibility of visualizing them by standard ethidium staining (as shown in Fig 3) made this approach the easiest and the most rapid method for identifying the t(4;11), even in the cases in which conventional cytogenetics failed. Furthermore, RT-PCR amplification, which allows for a more sensitive detection of MRD than do morphology and cytogenetic analysis, is important in monitoring the course of disease and in determining subsequent therapeutic approaches. Application of this approach to patient no. 9 showed that the ALL-1/AF4 fusion transcript persisted after intensive chemotherapy treatments the patient subsequently relapsed.

In conclusion, we have described here a rapid RT-PCR method for amplifying the chimeric ALL-1/AF4 transcript. This latter is a new molecular marker with diagnostic and prognostic relevance particularly in ALL of early infancy in which the ALL-1 gene is altered in the 80% of cases.10

REFERENCES


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