RAPID COMMUNICATION

The t(1;19)(q23;p13) Results in Consistent Fusion of E2A and PBX1 Coding Sequences in Acute Lymphoblastic Leukemias

By Stephen P. Hunger, Naomi Gallili, Andrew J. Carroll, William M. Crist, Michael P. Link, and Michael L. Cleary

The t(1;19)(q23;p13) chromosomal translocation is observed cytogenetically in 25% of children with pre-B-cell acute lymphoblastic leukemia (ALL) and is associated with an adverse treatment outcome. The t(1;19) juxtaposes the E2A gene from chromosome 19 with the PBX1 gene on chromosome 1, leading to the production of fusion transcripts and resultant chimeric proteins that contain the transcriptional-activating motif of E2A and the DNA-binding homeodomain of PBX1. To investigate the molecular nature of E2A/PBX1 fusion in patients with t(1;19) ALL we used an RNA-based polymerase chain reaction (PCR) procedure to amplify a portion of the chimeric transcript. We detected E2A/PBX1 fusion transcripts in cells from 97% (37 of 38) of cases in which the t(1;19) had been observed cytogenetically. Molecular evidence of E2A/PBX1 fusion transcripts was also observed in a patient in whom a t(1;19) was not detected cytogenetically and in one patient with subclinical levels of minimal residual disease before overt clinical relapse. In all PCR-positive cases the junction of E2A and PBX1 coding sequences occurred at precisely the same location as demonstrated by hybridization of PCR products with a fusion site-specific detection oligonucleotide. These findings demonstrate the consistent fusion of E2A and PBX1 coding sequences resulting from t(1;19) and suggest that site-specific fusion of E2A and PBX1 is an important pathogenic event in t(1;19) ALL.

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ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) is frequently associated with clonal chromosomal rearrangements, and a number of consistently recurring translocations associated with specific subtypes of ALL have now been identified.3 In childhood ALL, translocations are detected in approximately one-half of all patients, and the presence of a translocation has been associated with an adverse treatment outcome.3 It is now evident that some nonrandom translocations lead to the activation of, or alteration in, genes (“proto-oncogenes”) involved in the control of cellular proliferation and/or differentiation, and that these alterations play a central role in the process of malignant transformation. Advances in molecular biology also enable these translocation products to be used as markers for the detection and monitoring of leukemia.

The t(1;19)(q23;p13) is the most common recurring translocation in childhood ALL.4 It was first described by Carroll et al5 in 1983 as a nonrandom translocation associated with pre-B-cell ALL (lymphoblasts which express cytoplasmic, but not surface, Ig). Subsequently, studies of the Pediatric Oncology Group (POG) showed that children with pre-B ALL had a significantly poorer response to treatment than other children with non-B, non–T-cell ALL,5,6 and that the subgroup with the t(1;19) was responsible for the adverse prognosis associated with pre-B ALL.7 Mellingen et al8 demonstrated that the E2A gene, which codes for the helix-loop-helix Ig enhancer binding factors E12 and E47,9 is consistently located at the breakpoint of the t(1;19). Subsequently, it has been shown that the t(1;19) leads to juxtaposition of the E2A gene from chromosome 19 with PBX1 (formerly designated prl), a novel homeobox gene on chromosome 1.10,11 E2A/PBX1 fusion mRNAs are formed and translated into apparent chimeric transcription factors which consist of the transcriptional-activating motif of E2A and the DNA-binding homeodomain of PBX1.10,11

We report here that chimeric transcripts can be detected by a standardized RNA-based polymerase chain reaction (PCR) procedure in the vast majority of patients with cytogenetically identified t(1;19)(q23;p13) and in one patient in whom a t(1;19) was not identified. We also demonstrate that the junction between E2A and PBX1 coding sequences occurs in the same location in all patients with detectable E2A/PBX1 fusion, suggesting that the breakpoints occur within one specific intron of each gene. Further, we illustrate the potential utility of PCR detection of minimal residual disease in a patient with t(1;19) ALL.

MATERIALS AND METHODS

Patients. Samples of leukemic cells, cryopreserved in liquid nitrogen at the time of initial diagnosis, were obtained from the POG cell bank from 34 patients with ALL and a cytogenetically identified t(1;19)(q23;p13). Samples of bone marrow and peripheral blood were obtained from one additional patient with t(1;19) ALL (who was not enrolled in a POG study) treated at the Children’s Hospital at Stanford. To search for patients with molecular evidence of E2A/PBX1 fusion in whom a t(1;19) was not detected cytogenetically, samples of leukemic cells from 14 pa-
tients with pre-B ALL who failed therapy because of relapse were obtained from the POG cell bank.

Approval for the ALL treatment and biologic studies was obtained from the Institutional Review Boards at the individual POG member institutions. All blood and bone marrow specimens were obtained with informed consent of the patients and/or their parents who were aware that specimens would be used for research purposes and that the subjects' privacy would be fully protected.

**Cytogenetics.** Cytogenetic analyses were performed on leukemic cells obtained at the time of diagnosis and mailed by overnight carrier to the POG reference laboratory in Birmingham, AL, using standard techniques as previously described. Analysis of leukemic cells from one patient was performed at the clinical cytogenetics laboratory of the Stanford University Hospital.

**RNA isolation.** Specimens from the POG cell bank were shipped on dry ice to Stanford University Medical Center. Samples were rapidly thawed, centrifuged briefly to pellet mononuclear cells, and then washed with phosphate-buffered saline (PBS). Total RNA was isolated by the guanidinium-acid phenol chloroform method essentially as described.

**PCR.** PCR specific for the t(1;19) was performed in parallel with amplification of a portion of the c-abl messenger RNA to assess the quality of RNA extraction. One microgram of total cellular RNA was reverse transcribed to cDNA by incubation for 45 minutes at 42°C in a total volume of 20 µL that contained: 1XPCR reaction buffer (10 mmol/L Tris/HCl pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% [wt/vol] gelatin), 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (BRL, Gaithersburg, MD), 20 U RNAsin (Promega, Madison, WI), 1 mmol/L of each dNTP (Pharmacia, Piscataway, NJ) and 50 pmol of the appropriate downstream primer. The reaction volume was then increased to 100 µL with additional PCR buffer, 2.5 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) and 50 pmol of the upstream primer. Samples were overlaid with 80 µL of light mineral oil (Sigma, St Louis, MO) and amplification was performed in a Perkin Elmer programmable thermocycler. Reaction times consisted of an initial denaturation at 94°C for 3 minutes, annealing at 55°C for 1 minute, and elongation at 72°C for 1.5 minutes, followed by 35 additional cycles (1 minute at 94°C, 1 minute at 55°C, 1.5 minutes at 72°C). During the final cycle, elongation took place at 72°C for 8.5 minutes. One-tenth (10 µL) of the PCR product was size-fractionated by electrophoresis in a 1.3% agarose gel and transferred onto an activated nylon membrane (Genatran-45; Plasco, Woburn, MA) by the method of Southern. Membranes were hybridized with an internal detection oligonucleotide probe end-labeled with 32P using polynucleotide kinase, and washed in 6X SSC for 15 minutes at room temperature followed by 15 minutes at 58°C (48°C for the SH2 detection oligonucleotide). Autoradiography was performed with Kodak (Rochester, NY) XAR-5 film for 15 to 60 minutes. Oligonucleotides were commercially synthesized and high performance liquid chromatography (HPLC) purified (Operon Technologies, Inc, Alameda, CA). The locations of oligonucleotides for t(1;19) amplification are schematically depicted in Fig 1, and their approximate locations. (V) indicates the sites where fusion of E2A and PBX1 coding sequences occurs.

**RESULTS**

PCR analyses were performed on RNA extracted from leukemic cells from 35 children with ALL carrying a cytogenetically identified t(1;19)(q23;p13). In 34 patients, amplification of an identically sized E2A/PBX1 chimeric sequence was detected. In one patient, PCR for t(1;19) was negative while the abl positive control sequence was ampli-

### Table 1. Oligonucleotide Sequences

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>t(1;19)</td>
<td>5'CACCGCTTCCGCCTAACAGC 3'</td>
</tr>
<tr>
<td>JN23 (downstream)</td>
<td>5'GCAACCCAGCCGGCCGGCC 3'</td>
</tr>
<tr>
<td>JN4 (upstream)</td>
<td>5'GGAGGAGAACCACACG 3'</td>
</tr>
<tr>
<td>SH2 (fusion site)</td>
<td>5'CCTACAGTGTCTTGGAG 3'</td>
</tr>
<tr>
<td>c-abl</td>
<td>5'TTTCACAGCTTTGACTCGG 3'</td>
</tr>
<tr>
<td>ABLX3 (upstream)</td>
<td>5'CCTTACACCGCGCGTACTCAT 3'</td>
</tr>
<tr>
<td>CML-ABL (detection)</td>
<td>5'TATCGTTAGAGTGTATCTCCACT 3'</td>
</tr>
<tr>
<td>PBX1</td>
<td>5'NG28 (upstream)</td>
</tr>
</tbody>
</table>
PCR ANALYSIS OF t(1;19) ALL

Fig 2. PCR analysis of patient samples. (a) Ethidium bromide stained gel displaying representative results of PCR reactions. Lane M shows position and sizes (base pairs) of molecular size markers (HindIII digest of φX174 DNA). Lanes numbered 1 contain products of t(1;19) PCR and lanes numbered 2 display products of the c-abl positive control amplification. RCH-ACV is a t(1;19)-containing cell line. A through D are patients with t(1;19) ALL: A is the one patient who was PCR negative (see text), B through D are examples of PCR-positive cases. Lane N contains RNA isolated from normal leukocytes. Lane BI contains the negative control with no input RNA. (b) The PCR products shown in (a) were transferred and analyzed for the presence of E2A/PBX1 fusion products by hybridization with the internal detection oligonucleotide JN26. (c) The PCR products shown in (a) were transferred and analyzed for the presence of c-abl amplification products by hybridization with the internal detection oligonucleotide CML-abl.

The 34 PCR-positive patients and three PCR-positive cell lines reported here all yielded a 161-bp fusion product as predicted from the nucleotide sequence of fusion cDNAs isolated from t(1;19)-carrying cell lines, and these products hybridized with an internal oligonucleotide (JN26) from chromosome 1 in all cases (Fig 2). To further investigate whether the junction of coding sequences was the same in each case, a “fusion-site oligonucleotide” (SH2) was synthesized that consisted of eight nucleotides from E2A (chromosome 19) and eight from PBX1 (chromosome 1) based on the previously published fusion cDNA sequence. This fusion-site oligonucleotide hybridized with all (37 of 37) PCR products obtained from t(1;19) ALL, but not with the native E2A or PBX1 cDNAs (see Fig 3). Thus, in all patients with E2A/PBX1 fusion products detected by PCR, the junction of E2A and PBX1 coding sequences occurred at precisely the same location. Our laboratory has previously reported that the breakpoints on chromosome 19 are clustered within 2 kb of one another in a single intron of the E2A gene. The previous observations in conjunction with the PCR results reported here suggest that the breakpoints on chromosome 1 must also occur within a single intron of the PBX1 gene, as the same exonic sequences are fused to E2A sequences in the fusion transcripts. At the genomic DNA level, it appears that the PBX1 gene is quite large and contains an intron of at least 50 to 100 kb within which the translocation breakpoints may be dispersed.

We hypothesized that PCR analysis should be able to detect molecular evidence of E2A/PBX1 fusion in some patients in whom a t(1;19) was not detected cytogenetically. There are several potential causes for such “false negative” karyotypes. For example, cytogenetic analysis may be unsuccessful when no analyzable metaphases are present after short-term culture; or spuriously normal karyotypes may occur when residual normal marrow elements preferentially undergo mitosis whereas the leukemic cells either die or do not enter mitosis. Microscopic interstitial translocation that are not visible at the cytogenetic level may also occur, as has been demonstrated in cases of “Ph-positive” chronic myelogenous leukemia (CML) with molecular evidence of bcr rearrangement by Southern blotting or bcr/abl fusion by PCR. To address this question, we obtained leukemic cells (cryopreserved at the time of initial diagnosis) from 14 children with cytogenetically t(1;19)-negative pre-B ALL who had relapsed on POG 8602 (the ALL treatment study). We postulated that this group of patients would be “enriched” for undetected cases of t(1;19) based...
on the substantially higher risk of relapse in pre-B cases with the t(1;19) compared with similar cases without the t(1;19).7 Of these 14 patients, five had a clonal abnormality other than t(1;19) and nine had either a normal karyotype (6) or unsuccessful cytogenetics (3). One of these 14 patients had clear molecular evidence of $EM/\text{PBX1}$ fusion by PCR, whereas the other 13 were negative (data not shown). The positive case was molecularly indistinguishable from the 34 patients with t(1;19) described above who were PCR positive, displaying a 161-bp fusion product that hybridized with both the chromosome 1 (JN26) and fusion-site (SH2) detection oligonucleotides. This patient had a 46 XX karyotype; interestingly, this sample took 4 days to reach the POG reference cytogenetics laboratory, suggesting that following transit the leukemic cells were unsuitable for obtaining a representative karyotype, although we cannot exclude the possibility of submicroscopic $EM/\text{PBX1}$ fusion.

The development of the PCR9 has prompted a number of investigators to apply this technology to the detection of subclinical levels of minimal residual disease by amplification of specific sequences resulting from translocations or from Ig or T-cell receptor gene rearrangements.22,23 Yamada et al have recently reported the persistence of low levels of leukemic cells during the first 18 months of therapy in a small cohort of children with ALL and also described one patient in whom a large increase in leukemic cells, as detected by PCR, heralded relapse.23 We used PCR to monitor one patient with t(1;19) ALL during anti-leukemic therapy. As shown in Fig 4, there was clear evidence of low levels of $E2A/\text{PBX1}$ transcript detectable in a histologically normal marrow specimen obtained while the patient was in apparent complete clinical remission; approximately 10 weeks later he developed relapse in the bone marrow with high levels of $E2A/\text{PBX1}$ expression (data not shown).

**DISCUSSION**

In this report, we demonstrate that the t(1;19) chromosomal translocation in ALL results in a consistent molecular abnormality involving specific mRNA fusion of $E2A$ and $\text{PBX1}$ coding sequences. Using a standardized RNA-based PCR procedure to elucidate the molecular organization of the chimeric transcripts, it was observed that the junction of coding sequences occurs at precisely the same location in all cases with molecularly detectable $E2A/\text{PBX1}$ fusion. These findings provide compelling evidence that the site-specific fusion of E2A and PBX1 components of the chimeric protein is essential for the pathogenesis of t(1;19) ALL. These observations also have important implications for the molecular detection of t(1;19) in patients with ALL. $E2A/\text{PBX1}$ fusion transcripts were detected in over 95% of cases of t(1;19) ALL and in one patient who lacked cytogenetic...
evidence of t(1;19). Therefore, PCR analysis accurately identifies the t(1;19) in ALL and should be useful for identification of this translocation in cases that are cytogenetically unsuccessful and for detection of minimal residual disease.

Our laboratory has previously described the clustering of t(1;19) breakpoints within one intron of the E2A gene, and the data presented here strongly suggest that t(1;19) breakpoints must also lie within one specific intron of PBX1. This restricted localization of breakpoints within specific introns appears to be a consistent feature of leukemic translocations that result in the production of fusion proteins. The t(9;22)(q34;q11) is the prototype for this class of translocations. The breakpoints on chromosome 9 (ab1) are spread over a very large (~200kb) region at the 5' end of e-ab1, but exons 2 through 11 are always included in the final bcr/abl fusion message (exons 1a and 1b are variably included in the precursor RNA, but not in the mature bcr/abl fusion mRNA). The breakpoints in the bcr gene on chromosome 22 can occur either in the major breakpoint cluster region (generally between exons 2 and 3, or 3 and 4 of the bcr region, which are also known as exons 10-12 of the bcr gene) as is typically seen in CML, or within the first intron of the bcr gene (minor bcr) as is typically seen in the acute leukemias. The breakpoints on chromosome 9 (ab1) are spread over a very large (~200kb) region at the 5' end of e-ab1, but exons 2 through 11 are always included in the final bcr/abl fusion message (exons 1a and 1b are variably included in the precursor RNA, but not in the mature bcr/abl fusion mRNA). The breakpoints in the bcr gene on chromosome 22 can occur either in the major breakpoint cluster region (generally between exons 2 and 3, or 3 and 4 of the bcr region, which are also known as exons 10-12 of the bcr gene) as is typically seen in CML, or within the first intron of the bcr gene (minor bcr) as is typically seen in the acute leukemias. The breakpoints on chromosome 9 (ab1) are spread over a very large (~200kb) region at the 5' end of e-ab1, but exons 2 through 11 are always included in the final bcr/abl fusion message (exons 1a and 1b are variably included in the precursor RNA, but not in the mature bcr/abl fusion mRNA). The breakpoints in the bcr gene on chromosome 22 can occur either in the major breakpoint cluster region (generally between exons 2 and 3, or 3 and 4 of the bcr region, which are also known as exons 10-12 of the bcr gene) as is typically seen in CML, or within the first intron of the bcr gene (minor bcr) as is typically seen in the acute leukemias. The breakpoints on chromosome 9 (ab1) are spread over a very large (~200kb) region at the 5' end of e-ab1, but exons 2 through 11 are always included in the final bcr/abl fusion message (exons 1a and 1b are variably included in the precursor RNA, but not in the mature bcr/abl fusion mRNA). The breakpoints in the bcr gene on chromosome 22 can occur either in the major breakpoint cluster region (generally between exons 2 and 3, or 3 and 4 of the bcr region, which are also known as exons 10-12 of the bcr gene) as is typically seen in CML, or within the first intron of the bcr gene (minor bcr) as is typically seen in the acute leukemias.

Several groups have also described the molecular organization of the t(15;17)(q22;q11-21) translocation in acute promyelocytic leukemia. The breakpoints on 17 are spread over a very large (~200kb) region at the 5' end of e-ab1, but exons 2 through 11 are always included in the final bcr/abl fusion message (exons 1a and 1b are variably included in the precursor RNA, but not in the mature bcr/abl fusion mRNA). The breakpoints in the bcr gene on chromosome 22 can occur either in the major breakpoint cluster region (generally between exons 2 and 3, or 3 and 4 of the bcr region, which are also known as exons 10-12 of the bcr gene) as is typically seen in CML, or within the first intron of the bcr gene (minor bcr) as is typically seen in the acute leukemias.

Another intriguing possibility is that a small subset of rearrangements may occur within different regions of E2A and/or PBX1, analogous to the minor cluster region in the t(14;18) and the minor breakpoint cluster region in the t(9;22). Identification and investigation of such variant t(1;19) cases could yield important insights into the function of subregions of the E2A/PBX1 fusion protein. Analysis of additional patients should help to clarify the significance of such PCR negative cases.

Suryanarayan et al have recently reported similar findings with PCR analysis of children with Ph1-positive ALL; in all cases studied, patients with cytogenetically detected
t(9;22)(q34;q11) had chimeric berabl sequences that could be amplified by PCR.\textsuperscript{12} Further, PCR was able to identify one patient with berabl fusion who did not have a cytogenetically detected Ph.\textsuperscript{1} The results of these two studies demonstrate that PCR analysis to detect translocations associated with an adverse prognosis can potentially be an important adjunct to cytogenetics. Current treatment for children with ALL often includes patient stratification and/or treatment assignment based on clinical and biologic features present at diagnosis. The t(1;19) and t(9;22) have been reported to confer a higher risk of treatment failure in several studies.\textsuperscript{2-4} Therefore, it is important to identify accurately all patients with these translocations so that appropriate therapy is given, and so that risk factors can be properly evaluated.

At the present time, risk-directed therapy involves primarily clinical features (age, leukocyte count, organomegaly) or biologic factors (immunophenotype, DNA content or ploidy) present at the time of initial diagnosis. Therapy is generally not modified unless overt relapse occurs. If patients at increased risk of treatment failure (based on the detection of minimal residual disease at specific timepoints during remission) could be reliably identified, innovative strategies (such as marrow transplantation) could be applied early, while the patients are in good overall medical condition and when the leukemic burden is at a minimum. Clearly, further studies are necessary to determine whether PCR detection of minimal residual disease can reliably identify such patients. Amplification of chimeric sequences resulting from high-risk translocations is an attractive strategy to address these questions. The demonstration that a standardized RNA-based PCR can accurately identify fusion sequences in all patients with t(9;22)\textsuperscript{12} and the overwhelming majority of patients with t(1;19) is an essential prerequisite for such investigations.

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