Fusion of the Homeobox Gene HLXB9 and the ETV6 Gene in Infant Acute Myeloid Leukemias with the t(7;12)(q36;p13)

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Abstract
Recently, we and others reported a recurrent t(7;12)(q36;p13) found in myeloid malignancies in children ≤18 months of age and associated with a poor prognosis. Fluorescence in situ hybridization studies mapped the 12p13 breakpoint to the first intron of ETV6 and narrowed down the region of 7q36 involved. By using the sequences made public recently by the Human Genome Project, two candidate genes in 7q36 were identified: the homeobox gene HLXB9 and E7orf3, a gene with unknown function. Reverse transcription-PCR of two cases with t(7;12), using primers for E7orf3 and ETV6, was negative. However, reverse transcription-PCR for HLXB9-ETV6 demonstrated alternative splicing; the two major bands corresponded to fusion of exon 1 of HLXB9 to exons 2 and 3, respectively, of ETV6. The reciprocal ETV6-HLXB9 transcript was not detected. It remains to be elucidated if the leukemic phenotype is attributable to the formation of the HLXB9-ETV6 fusion protein, which includes the helix-loop-helix and E26 transformation-specific DNA binding domains of ETV6 or to the disruption of the normal ETV6 protein.

Introduction
ETV6 (TEL), a member of the ETS family of transcription factors, located on 12p13, is known to be rearranged in a spectrum of hematological malignancies. These rearrangements often take the form of translocations, some of which can only be identified using molecular (cytogenetic) techniques. To date, >10 different fusion partners to ETV6 have been identified in acute lymphoblastic leukemia, AML, and myelodysplastic syndromes. The characterization of these chimeras has shown that different regions of ETV6 are involved in these translocations. Fusion of the HLH-domain of ETV6 to the 3′ region of the partner can influence and stimulate the activity of the partner gene, as seen in t(5;12)(q33;p13), t(9;12)(q34;p13), t(9;12)(p24;p13), and t(12;21)(p13;q22), involving PDGFRB, ABL1, JAK2, and AML1, respectively. ETV6 can drive the transcription of the partner gene as occurs in t(3;12)(q26;p13) with MDS1/EVI1 or in t(12;13)(p13;q12) with the homeobox gene CDX2 (3). A third type of fusion is where the partner gene forms a chimera with the 3′ part of ETV6, retaining the ETV6 HLH and ETS DNA binding domains, as shown for MNL1 in t(12;22)(p13;q11) and BTL in t(4;12)(q11;p13) (4). We, and others, recently described a novel recurrent translocation, (7;12)(q36;p13), occurring in children ≤18 months of age with mainly myeloid disorders (6, 7). This translocation was found in ~20% of Dutch patients with AML in this age group, making it the most common aberration next to translocations involving MLL (7). Survival data suggest that the presence of the t(7;12)(q36;p13), similar to MLL rearrangements, confers a poor prognosis (7). FISH studies revealed the involvement of ETV6 and helped to narrow down the relevant region of 7q36. Using material available from two of our patients, we have been able to show that HLXB9 is the partner gene of ETV6 in this translocation.

Materials and Methods
Case Reports, Cytogenetics, and FISH. The clinical, cytogenetic, and FISH features of both patients have been reported previously (7). In short, case 1 (patient 2 in Ref. 7) was a 4-month-old boy with AML M1, who died 3 months after diagnosis. The karyotype, refined by FISH, was 47,XY,der(7)t(7;12)(q36;p13),del(12p13),der(12)t(7;12),+19. Case 2 (patient 3 in Ref. 7) was an 18-month-old boy with AML M3 variant, without PML/RARA fusion, who died 39 months after diagnosis. The karyotype, refined by FISH, was 47,XY,t(7;12)(q36;p13),+19. No MLL rearrangements were observed in either case. The FISH studies were carried out using a range of cosmid probes for ETV6 (1). In both patients, the translocation was shown to have a breakpoint in intron 1 of ETV6, and in case 1, this was accompanied by a deletion of ETV6 distal to this. Significantly, in case 2, we found that the CEPH YAC 965cl2 (locus D7S550; size 160 kb), localized to 7q36, did span the breakpoint.

Nucleic Acid Isolation. Total RNA from both cases was extracted from cryopreserved bone marrow cells, stored in liquid nitrogen at the time of diagnosis, using the RNaseasy kit (Qiagen, Hilden, Germany). Total RNA extracted from the NB4 cell line known to have t(15;17)(q22;q12), using the Trizol reagent (Life Technologies, Inc., Stockholm, Sweden), was used as negative control RNA.

5′ RACE. The 5′ RACE was performed on 1 µg of total RNA from both cases using the SMART RACE cDNA amplification kit (CLONTECH, Palo Alto, CA) with oligo TELR1 (Table 1). Obtained clones were screened by Southern blotting with end-labeled oligo TELR2, located in exon 2 to ensure the inclusion of upstream ETV6 sequences, and with TELF1 located in exon 1 to rule out ETV6-containing gene products.

RT-PCR Analysis. One, 2.5, and 5 µg of total RNA from cases 1, 2, and the cell line NB4, respectively, were reverse transcribed and PCR amplified as described previously (8). The primers used for PCR amplification are listed in Table 1. The HLXB9-ETV6 fusion transcript was detected using HLXB9-545F and TEL172R and reamplified with HLXB9-604F and TEL143R, HLXB9-604F, and TEL163R or HLXB9-604F and TEL135R. For amplification of a possible C7orf3-ETV6 fusion transcript, cDNA was amplified with C7orf3-608F and TEL172R and reamplified with C7orf3-712F and TEL143R. For the detection of the reciprocal ETV6-HLXB9 fusion transcript, ETV6F1a and HLXB9-1092R and ETV6F1b and HLXB9-989R were used as primer combinations.

Sequence Analysis. PCR-amplified fragments were directly sequenced using the dideoxy procedure with an ABI Prism BigDye terminator cycle
sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) with various primers (Table 1).

**Results and Discussion**

We have demonstrated previously that in the present cases with the t(7;12)(q36;p13), the breakpoint in ETV6 was located in intron 1. Furthermore, in case 1, a concomitant deletion of 5' sequences comprising at least part of intron 1, exon 1, and part of the upstream sequences of exon 1 was found (7). The latter finding strongly suggested that the fusion product encoded by the der(12)t(7;12), being 5' partner-3' ETV6, would be the pathogenically important chimera. Consequently, we initially used 5' RACE to attempt to identify the translocation partner, but only sequences corresponding to upstream ETV6 exons and introns were retrieved.

Our FISH experiments on case 2 using CEPH YAC 965c12 showed signals on both the der(12)t(7;12) (7). Although the breakpoint is at least 1 Mb more telomeric on 7q36, Tosi (6) described three YACs in the vicinity of the SHH gene, and the last is located on NT_007905 (National Center for Biotechnology Information, GenBank) in the chromosome7 (National Center for Biotechnology Information, GenBank) in the vicinity of the SHH gene, and the last is located on NT_007951, which is at least 1 Mb more telomeric on 7q36. Tosi et al. (6) described three cases with t(7;12)(q36;p13) that had the breakpoint on 7q36 within PAC_H_DJ1121A15. They also showed in these cases that two cosmids, derived from this PAC, were translocated to the der(12), indicating that the breakpoint should be proximal to the cosmids used. This PAC of 130 kb has been sequenced (GenBank accession no. AC006357) and is known to contain the homeobox gene ETV6, which is transcribed from centromere to telomere, whereas HLB9, as well as ETV6, are transcribed from telomere to centromere. Thus, the orientation of ETV6 is opposite to that of C7orf3, indicating that an in-frame fusion of these two genes cannot occur after a simple translocation but requires a more complex mechanism comparable with that which is presumed to occur in ETV6-ABL1 fusions (10). The orientation, however, of the HLB9 gene does match the criteria for an in-frame fusion with ETV6 by means of a simple translocation.

RT-PCR with two C7orf3 forward and two ETV6 reverse primer combinations did not amplify any cDNA product from the patients' samples (Fig. 1, Lanes 4 and 5), strongly suggesting that a C7orf3-ETV6 chimeric transcript was not present. However, RT-PCR using two HLB9 forward and various ETV6 reverse primers on cDNA from both cases did amplify cDNA fragments, suggesting the presence of an HLB9-9-ETV6 chimeric gene (Fig. 1, Lanes 1 and 2). The HLB9-604F and TEL143R primer combination amplified multiple cDNA fragments, with two major bands: one of 356 bp and the other of 486 bp (the 486-bp fragment was weakly amplified in case 1; Fig. 1, Lane 1). The HLB9-604F and TEL135R primer combination amplified two cDNA fragments of 329 and 459 bp, whereas the primers HLB9-604F and TEL163R amplified two fragments of 416 and 546 bp (data not shown). Nested PCR with two forward primers located in exon 1 of the ETV6 gene and two reverse primers located in exon 3 of the HLB9 gene did not amplify any cDNA product, suggesting that the reciprocal ETV6-HLB9 was not expressed (data not shown).

To verify the presence of HLB9-ETV6 chimeric transcripts, the 356- and 486-bp cDNA fragments were sequenced. This analysis revealed the presence of HLB9-ETV6 chimeric transcripts. The 356-bp transcript, nt 694 of ETV6, was located in intron 1. The 356-bp transcript, nt 694 of ETV6, was located in intron 1. The latter finding strongly suggested that the reciprocal ETV6-HLB9 was not expressed (data not shown).

Table 1 Primers used for PCR and sequencing

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence (5' → 3')</th>
<th>Direction</th>
<th>Position</th>
<th>Gene (accession no.)</th>
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<tr>
<td>TELR1</td>
<td>CTCGCCCTGAATATGTTGCTGGCAGCG</td>
<td>Reverse</td>
<td>187–213</td>
<td>ETV6 (NM_001987)</td>
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<td>TELR2</td>
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<td>Reverse</td>
<td>153–176</td>
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<td>TELF1</td>
<td>GATCTCTCTCTCCTGTTGAGACATG</td>
<td>Forward</td>
<td>5–27</td>
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</tr>
<tr>
<td>TEL130R</td>
<td>CTTTGGTGACAGCAAGCGAGAGAGC</td>
<td>Reverse</td>
<td>301–324</td>
<td>ETV6 (NM_001987)</td>
</tr>
<tr>
<td>TEL133R</td>
<td>GTCGACAGAATGGGAAAGAATCGGGG</td>
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<td>400–429</td>
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<tr>
<td>TEL143R</td>
<td>CTGTAAGTGTAGTATGGTATGCCCAGG</td>
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<td>427–453</td>
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<tr>
<td>TEL163R</td>
<td>CCTGGGCCCTTCTCTGAGACATGATTC</td>
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<td>487–513</td>
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<td>TEL172R</td>
<td>GTATGAGTGCACATTATCAACGGATTG</td>
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<td>514–540</td>
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<tr>
<td>ETVF81a</td>
<td>TGACAGAATGGGAAAGAATCGGGG</td>
<td>Forward</td>
<td>19–42</td>
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<td>ETVF81b</td>
<td>ACTCTGCTCTCCTGAGATTAGTAGTT</td>
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<td>34–57</td>
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<td>HLBX9-545F</td>
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<td>Forward</td>
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<td>C7orf3-712F</td>
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<td>Forward</td>
<td>712–734</td>
<td>C7orf3 (AF107455)</td>
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</table>

Fig. 1. RT-PCR for the detection of HLBX9-ETV6 and C7orf3-ETV6 transcripts in cases 1 and 2 and the NB4 control cell line. Nested PCR results are shown in Lanes 1–3 using primers HLBX9-604F and ETV6143R: Lane 1, case 1; Lane 2, case 2; Lane 3, NB4. Nested PCR results using primers C7orf3-712F and TEL143R are shown in Lanes 4–6: Lane 4, case 1; Lane 5, case 2; Lane 6, NB4.

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6 Internet address: http://www.genet.sickkids.on.ca/chromosome7.
In the t(7;12)(q36;p13), the HLXB9-ETV6 fusion protein is formed is similar to the MN1-ETV6 and the BTL-ETV6 chimeras (4), containing the HLXB9 homeobox domain. The type of fusion protein is driven from the HLXB9 promoter, which under normal conditions is down-regulated during differentiation. However, in the leukemia described here, the down-regulation is disturbed because the cells do not differentiate. It remains to be elucidated if this differentiation block might be caused by the formation of the HLXB9-ETV6 fusion protein or by the disruption of the normal ETV6 protein equilibrium.

In the two patients described here, the t(7;12)(q36;p13) results in fusion of the NH2 terminus of HLXB9 to the COOH terminus of ETV6. This results in a fusion protein of 403 amino acids (11). The gene has a high GC content (75%), which might explain the problems encountered in the attempted 5’RACE PCR. Transcripts of 2.2 kb are expressed in the colon, small intestine, and pancreas. In addition, HLXB9 is highly expressed in CD34+ bone marrow cells as transcripts of 5.3 and 3.9 kb; this expression is not observed in CD34- cells. In the CD34+ cells, HLXB9 expression increases upon exposure to interleukin 3 and granulocyte macrophage colony-stimulating factor, followed by down-regulation upon differentiation, showing it to be a marker of immature hematopoietic cells (12). The gene could therefore be involved in the regulation of growth and differentiation of progenitor cells. Elevated HLXB9 expression has also been observed in acute lymphoblastic leukemia or AML, but not in chronic lymphocytic leukemia or chronic myeloid leukemia patients, or in acute leukemias of the lymphoblastic leukemia or AML, but not in chronic lymphocytic leukemia or chronic myeloid leukemia patients, or in acute leukemias in complete remission (13). In addition, a mutation in the ETV6 gene on 12p13 is also known as the Currarino syndrome.

In the two patients described here, the t(7;12)(q36;p13) results in fusion of the NH2 terminus of HLXB9 to the COOH terminus of ETV6 (Fig. 3). The fusion transcript creates a chimeric protein that is fused in frame to nt 188 of ETV6 transcript and not the reciprocal transcript suggests that the leukemia may originate in utero as has been demonstrated for leukemias associated with MLL rearrangements (15) and the t(12;21)(p13;q22), also involving ETV6 (16). This could provide clues to which etiological factors may play a role in the development of this type of leukemia.

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cations involving ETV6 in children 18 months of age or younger with myeloid disorders. Leukemia, 15: g15–g20, 2001.