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# Fusion of the Homeobox Gene *HLXB9* and the *ETV6* Gene in Infant Acute Myeloid Leukemias with the t(7;12)(q36;p13)[1]

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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 *c7orf3,* a gene with unknown function. Reverse transcription-PCR of two cases with t(7;12), using primers for *c7orf3* 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[4] 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[5] (1). To date, >10 different fusion partners to *ETV6* have been identified in acute lymphoblastic leukemia, AML, and myelodysplastic syndromes.[5] 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 (2). *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 *MN1* in

t(12;22)(p13;q11) and *BTL* in t(4;12)(q11;p13) (4, 5). 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 (12)(p13p13),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 965c12 (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 RNeasy 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 $\mu$g 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 $\mu$g 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 HLX9-545F 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

Table 1 *Primers used for PCR and sequencing*

| Designation | Sequence (5′ → 3′) | Direction | Position | Gene (accession no.) |
|---|---|---|---|---|
| TELR1 | CCTGCTCCAGTAAATTGGCTGCAAGCG | Reverse | 187–213 | *ETV6* (NM_001987) |
| TELR2 | GGCAGGCGGATCGAGTCTTCCTCC | Reverse | 153–176 | *ETV6* (NM_001987) |
| TELF1 | GATCTCTCTCGCTGTGAGACATG | Forward | 5–27 | *ETV6* (NM_001987) |
| TEL301R | CTCTTTGGTCAGCAGCAGGAGAGC | Reverse | 301–324 | *ETV6* (NM_001987) |
| TEL135R | GTGGAAGAATGGTGAAAGAATCCGAGG | Reverse | 400–429 | *ETV6* (NM_001987) |
| TEL143R | CTGTGAGTGTATAGAGTTTCCAGGGTG | Reverse | 427–453 | *ETV6* (NM_001987) |
| TEL163R | CCTGGGCCTCCTCTGGACACAGTTATC | Reverse | 487–513 | *ETV6* (NM_001987) |
| TEL172R | GTTATGGTGCACATTATCCACGGATGG | Reverse | 514–540 | *ETV6* (NM_001987) |
| ETV6F1a | TGAGACATGTCTGAGACTCCTGCT | Forward | 19–42 | *ETV6* (NM_001987) |
| ETV6F1b | ACTCCTGCTCAGTGTAGCATTAAG | Forward | 34–57 | *ETV6* (NM_001987) |
| HLXB9-545F | CGCTCTCCTACTCGTACCCGCAG | Forward | 545–567 | *HLXB9* (NM_005515) |
| HLXB9-604F | ATCAAGCTGGGCGCCGGCACCT | Forward | 604–625 | *HLXB9* (NM_005515) |
| HLXB9-624F | CTTCCAGCTGGACCAGTGGCTG | Forward | 624–645 | *HLXB9* (NM_005515) |
| HLXB9-989R | GGCCCCAGCAGCTCCTCGGCTC | Reverse | 989–1010 | *HLXB9* (NM_005515) |
| HLXB9-1092R | TGCTGTAGGGGAAATGGTCCTCG | Reverse | 1092–1114 | *HLXB9* (NM_005515) |
| C7orf3-608F | TCAGTGGGCAGCTGGAGGAACTG | Forward | 609–631 | *C7orf3* (AF107455) |
| C7orf3-712F | GCCCAGCAGACTGATGATGGAGC | Forward | 712–734 | *C7orf3* (AF107455) |

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(7) and the der(12) (7). Although this YAC is reported to have a size of 160 kb, it most probably has a large deletion because it contains the markers *D7S550,* CHL.ATC3E04, *D7S2307,* and *D7S2380,*[6] the first three of which are all localized to NT_007905 (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 *HLXB9.*

In another of Tosi's patients, also with a t(7;12)(q36;p13), a breakpoint distal to both the PAC and one of the cosmids was observed (6), suggesting breakpoint heterogeneity.

The genomic and transcriptional region around *HLXB9* has been well characterized by Heus *et al.* (9) in their search for the gene defect causing preaxial polydactyly, mapped to a 1.9-cM region on 7q36 between *D7S550* and *D7S2423.* In this candidate region, centromeric to *HLXB9,* they identified three transcripts called *C7orf3, C7orf2,* and *C7orf4,* with *C7orf3* immediately adjacent to *HLXB9.* This, together with the results obtained by Tosi *et al.* (6), made *C7orf3* a possible candidate for fusion to *ETV6.* Alignment of the *HLXB9* and *c7orf3* cDNAs to the PAC revealed that the genes have opposite orientations: *c7orf3* is transcribed from centromere to telomere, whereas *HLXB9,* 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 *HLXB9* 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 *HLXB9* forward and various *ETV6* reverse primers on cDNA from both cases did amplify cDNA fragments, suggesting the presence of an *HLXB-9-ETV6* chimeric gene (Fig. 1, *Lanes 1* and *2*). The HLXB9-604F 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 HLXB9-604F and TEL135R primer combination amplified two cDNA fragments of 329 and 459 bp, whereas the primers HLXB9-604F 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 *HLXB9* gene did not amplify any cDNA product, suggesting that the reciprocal *ETV6-HLXB9* was not expressed (data not shown).

To verify the presence of *HLXB9-ETV6* chimeric transcripts, the 356- and 486-bp cDNA fragments were sequenced. This analysis revealed two types of chimeric *HLXB9-ETV6* transcripts. In the 356-bp transcript, nt 694 of *HLXB9* (NM_005515), corresponding to the end of exon 1, was fused to nt 188 of *ETV6* (NM_001987), corresponding to exon 3, resulting in an in-frame junction with a serine-to-cysteine (TCC to TGC) transition (Fig. 2). In the 486-bp
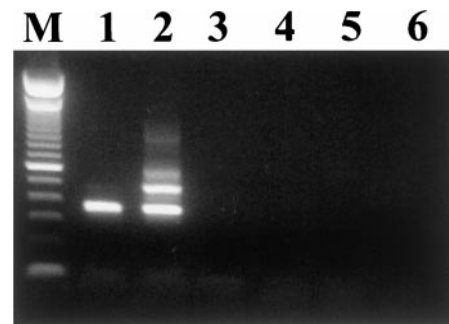


Fig. 1. RT-PCR for the detection of *HLXB9-ETV6* and *c7orf3-ETV6* transcripts in cases 1 and 2 and the NB4 control cell line. Nested PCR results are shown in *Lanes 1–3* using primers HLXB9-604F and ETV6143R: *Lane 1,* case 1; *Lane 2,* case 2; *Lane 3,* NB4. Nested PCR results using primers C7orf3-712F 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.

transcript, nt 694 of *HLXB9* was fused out of frame to nt 58, corresponding to exon 2, of *ETV6* (Fig. 2), confirming that the observed cDNA fragments are indeed alternatively spliced products.

*HLXB9,* also known as *HB9,* is a homeobox gene of, presumably, three exons resulting in an open reading frame of 1,206-bp coding for a 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 in complete remission (13). In addition, a mutation in *HLXB9* is the cause of dominantly inherited sacral agenesis (14), which together with anorectal malformation, a presacral mass, and urogenital malformation is referred to as the Currarino syndrome.

In the two patients described here, the t(7;12)(q36;p13) results in fusion of the NH$_2$ terminus of HLXB9 to the COOH terminus of ETV6 (Fig. 3). The fusion transcript creates a chimeric protein that contains the ETV6 HLH and the ETS domains, which are joined to the regulatory sequences and the first exon of HLXB9, but no longer contains the HLXB9 homeobox domain. The type of fusion protein formed is similar to the MN1-ETV6 and the BTL-ETV6 chimeras (4, 5). In the t(7;12)(q36;p13), the HLXB9-ETV6 fusion protein is under
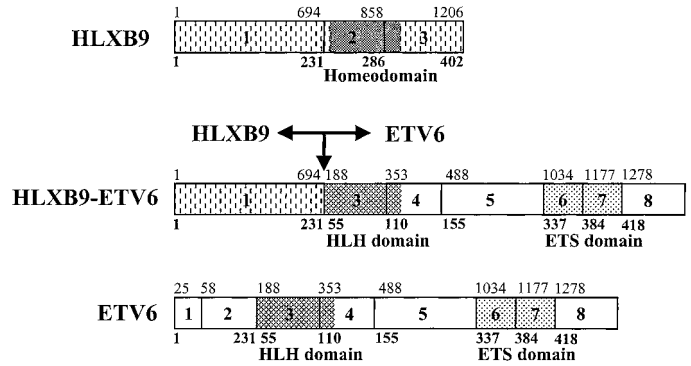


Fig. 3. Schematic representation of the HLBg and ETV6 proteins and the putative HLXB9-ETV6 chimeric protein resulting from the t(7;12)(q36;p13). *Arrow,* the observed breakpoints. nt numbers (cDNA level) are given above each protein, and amino acid numbers are given in bold type below each protein.

the influence of 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.

The fact that we could only detect the presence of the HLXB9-ETV6 transcript and not the reciprocal transcript suggests that the fusion protein driven from the HLXB9 promoter is the leukemogenic factor. However, additional t(7;12)(q36;p13) cases should be investigated to confirm that HLXB9 is involved in all these translocations. The young age of the patients identified with the t(7;12)(q36;p13) suggests that this 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.

## Acknowledgments

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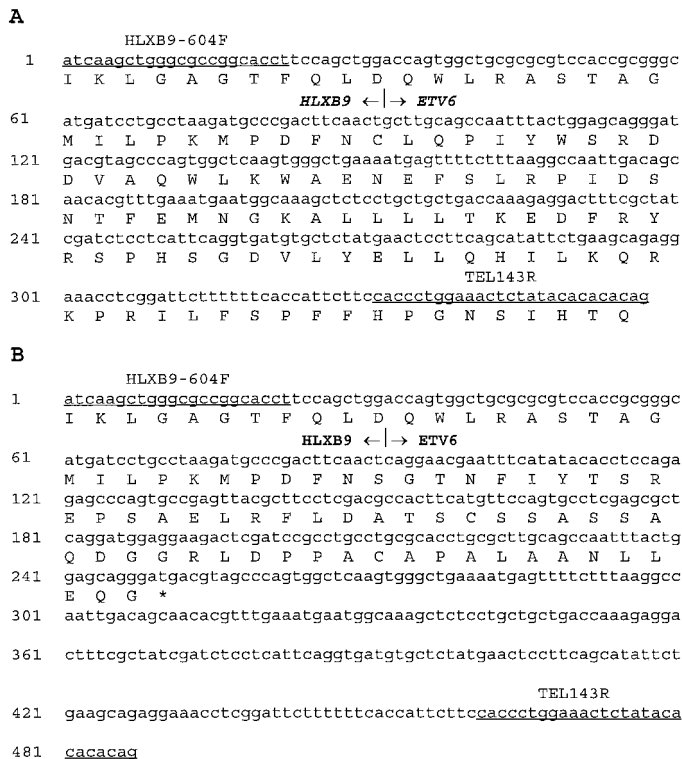


Fig. 2. The *HLXB9-ETV6* chimeric transcript. *A,* partial nt sequence of the 356-bp cDNA determined after direct sequencing. nt 694 of *HLXB9* is fused in frame to nt 188 of *ETV6. B,* partial nt sequence of the 486-bp cDNA determined after direct sequencing. nt 694 of *HLXB9* is fused out of frame to nt 58 of *ETV6. Arrow,* the junction of the *HLXB9* and *ETV6* genes.

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