Fusion of ETV6 to MDS1/EVI1 as a Result of t(3;12)(q26;p13) in Myeloproliferative Disorders

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Abstract

We identified a fusion between ETV6 on 12p13 and MDS1/EVI1 on 3q26 in a t(3;12)(q26;p13) found in two cases of myeloproliferative disorder. The resulting chimeric transcript consists of the first two exons of ETV6 fused to MDS1 sequences, which in turn is fused to the second exon of the EV11 gene. It has recently been reported that MDS1 can be expressed in normal tissues both as a single gene and fused to EV11. ETV6 does not contribute any known functional domain to the predicted fusion protein. Association with blast crisis and myelodysplastic syndrome-derived leukemia, bad prognosis, and relative complex karyotype are in agreement with observations made in other cases of t(3;12)(q26;p13). Furthermore, a comparison can be made with the formation of an AML1/MDS1/EVI1 fusion gene in translocations (3;21)(q26;q22).

Introduction

Involvement of both ETV6 and EV11 in pathogenesis of myeloproliferative and myelodysplastic disorders has been shown over the past few years. The ETV6 gene, a novel member of the ETS gene family of transcription factors, is a target gene of chromosomal abnormalities affecting 12p13 occurring in different hematopoietic malignancies of both myeloid and lymphoid origin. Golub et al. (1) identified ETV6 as a fusion partner of the platelet-derived growth factor receptor β gene (PDGFRB) in a t(5;12)(q33;p13) associated with chronic myelomonocytic leukemia. This translocation results in the expression of a chimeric protein consisting of the helix-loop-helix domain of ETV6 fused to the transmembrane and tyrosine kinase domains of platelet-derived growth factor receptor β.

Since then, other translocations involving ETV6 have been characterized. In myeloid disease, a t(12;22)(p13;q11) affecting ETV6 and MNI was described (2). In lymphoid disease (childhood ALL), a t(9;12)(q34;p13) leading to an ETV6-ABL fusion protein was reported (3), whereas t(12;21)(p13;q22) resulting in an ETV6/AML1 fusion and associated with a deletion of the second ETV6 allele was described as a frequent anomaly in childhood ALL (4, 5).

The Evi-1 gene was originally identified in mice by virtue of its retroviral activation in murine myeloid leukemia (6). Investigation of the expression pattern of the human EV11 gene in patients with AML showed aberrant expression due to chromosomal abnormalities of 3q26 in 7% of the examined cases (7). The involvement of EV11 in a t(3;21)(q26;q22) occurring in myeloid malignancies was demonstrated by Mitani et al. (8) and Nuclifora et al. (9). Moreover, the latter group provided evidence that in different patients, different breakpoints occur in a region of approximately 400–750 kb telomeric of the EV11 gene (9). These translocations caused intergenic splicing between AML1 on 21q22 and three genes on 3q26, namely EAP, MDS1, and EV11. Recently it has been shown that intergenic splicing between MDS1 and EV11 also occurs in normal kidney and pancreas (10).

FISH studies by Raynaud et al. (11) showed a similar heterogeneity of 3q26 breakpoints in five patients with a t(3;12)(q26;p13). Furthermore, it was shown by FISH that in three of these cases, the ETV6 gene was rearranged, but no molecular analysis of the breakpoints was performed.

Here we report two new cases with t(3;12)(q26;p13) in patients with myeloproliferative disorder. In both cases, this rearrangement results in the formation of a chimeric transcript consisting of the first two exons of the ETV6 gene fused to MDS1/EVI1.

Patients and Methods

Case Reports. Case 1, a 26-year-old man, was admitted to the Department of Hematology of UZ St. Jan in Brugge in October 1995 because of bone pain, fatigue, and fever. Clinical examination showed a slight splenomegaly. Hematological data were as follows: hemoglobin, 8.7 g/dl; platelets, 37 × 10⁹; WBCs, 64 × 10⁹/liter with 27% neutrophils, 11% lymphocytes, and 11% lymphoblasts. Bone marrow was hypercellular with a proportion of 25% of blasts cells with a myeloid morphology, 19% promyelocytes, 14% myelocytes, and 14% eosinophils. The diagnosis of atypical chronic myelogenous leukemia in transformation was established. Induction chemotherapy following a standard protocol for AML (idarubicin, etoposide, and cytosine-arabinoside) was started. The patient was resistant to the therapy; however, after three courses of therapy, a complete remission was achieved. Two months later (February 1996) the patient received allogenic peripheral stem cell transplantation with bone marrow from a HLA-compatible brother. Remission duration was short (2 months), and the patient died 1 month later in early relapse. Cytogenetic analysis performed at the time of diagnosis revealed clonal chromosomal abnormalities in 100% (6 of 6) of analyzed bone marrow cells described as 46,XY, t(3;12)(q26;p13), t(9;15)(p21;q15;p13). A representative R-banded karyotype is shown in Fig. 1.

Case 2, a 33-year-old woman, was referred to the Department of Hematology of the Dr. Daniel den Hoed Cancer Center in Rotterdam. She was diagnosed with a refractory anemia with excess of blasts in transformation. Laboratory data showed hemoglobin of 11.1 g/dl; a platelet count of 311 × 10⁹/liter, and WBCs of 21.8 × 10⁹/liter with 8% of blasts. Bone marrow was hypercellular with 16.2% blasts, dysmyelopoiesis, and hyperactive megalakaryopoiesis with dysplastic micro-single lobed megakaryocytes. She was treated aggressively according to an AML protocol (Hovon-4). Only partial remission was obtained, and she died 6 months later while being prepared for bone marrow transplantation. Two cytogenetic analyses were performed in April and July 1988. In both instances, all bone marrow metaphases showed the same abnormal karyotype: 46, XX, t(3;12)(q26;p13), del(7)(q22) in 31 and 20 cells, respectively.

FISH. FISH was performed as described previously (12). The ETV6 locus at 12p13 was investigated with the following biotinylated LL12NCO1 cosmid

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2 The abbreviations used are: ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; FISH, fluorescence in situ hybridization; BAC, bacterial artificial chromosome; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA ends.
Fig. 1. R-banded karyotype of case I. Arrows, chromosomes involved in the rearrangements described in "Results."

Fig. 2. A, genomic structure of ETV6 and localization of the probes used for FISH. B, schematic representation of the fusion ETV6/MDS1/EVI1. Primers used for RT-PCR are indicated with open arrows. Black arrows, the breakpoints at the cDNA level. Oligo MDSR was used for detection of the ETV6/MDS1/EVI1 fusion.
probes (Lawrence Livermore National Laboratories, Livermore, CA) ordered as follows: 5' end, 179A6, 50F4, 212G9, 148B6, 3' end (13). The different ETV6 exons present in the cosmids are shown in Fig. 2A. FISH analysis on chromosome 3 was performed using two BACs, 264D15 and 328P24, isolated by screening high-density filters from a BAC library (Research Genetics, Huntsville AL) with a single-copy probe for MDS1. This probe was generated by PCR amplification using the primers MDSF (5'-GGCAACTACCCT-GAAATACC-3') and MDSR (5'-TGAATGCTTCACTGGATGTGGC-3'). Chromosome 12 was identified by cohybridization with the Texas Red-5-dUTP-labeled chromosome 12 alphoid probe pBR-12 (14) and simultaneous banding analysis using 4', 6-diamidino-2-phenylindole counterstaining. Chromosome painting using biotinylated library 3 and 15 (Cambio, Cambridge, United Kingdom), and Spectrum Orange-dUTP-labeled library 3 and 9 (Vysis, Stuttgart, Germany) was performed according to manufacturers' instructions. Between 6 and 10 abnormal metaphases were studied for each experiment. The FISH data were collected on a Leitz DMRB fluorescence microscope equipped with a cooled black and white charge coupled device camera (Photometries) run by SmartCapture software (Vysis).

**RT-PCR and Cloning.** Total RNA was isolated from bone marrow cells using the Trizol reagent (Life Technologies, Inc.). First-strand cDNA was
reverse-transcribed from 1 μg of total RNA with Murine Moloney Leukemia Virus reverse transcriptase (Life Technologies, Inc.) according to standard procedures using the primer R.Na (5'-CCAGTGACGACGGTGACGAAAGCTCAGGCTCAAA-ACCA-GC(N)₃'). A nested PCR was performed using primers specific for exon 2 of ETV6, namely ETV6F (5'-CCCTCCACACGACGCATACTCCC-3') and ETV6F2 (5'-CTCAGATGAGGGAAGACTCAGR-3') in combination with primers R.NR1 (5'-CCATGACAGGACGAGCGAGCTCAAA-ACCA-GC(N)₃') and R.NR2 (5'-GAGCTACGACTTAGCAGCAAG-3'). PCR products were cloned following standard procedures (15).

The ETV6-MDS1/EVI1 fusion was confirmed by hemi-nested RT-PCR on patient RNA using primers ETV6F1 and ETV6F2 in combination with EVIR (5'-CTATGCAAGCAACGCAAACTCC-3') specific for exon 2 of the EVII gene. In an attempt to clone the reciprocal fusion product, a 5' RACE experiment was performed using the nested primers ETV6R1 (5'-TTCAGCTCAAGCTTAGTCTTC-3') specific for exon 2 of the EVII gene. The PCR products from A were transferred to a nylon membrane. The membrane was hybridized with the 32P-labeled oligo MDSR, which only hybridizes to the 482-bp EVIl. The ETV6/MDS1/EVI1 cDNA appears as a product of 482 bp. Whereas the ETV6/EVI1 cDNA appears as a product of 144 bp. The primer R2N6 (5'-CCAGTGAGGACGAAAGCTCAGGCTCAAA-ACCA-GC(N)₃') in combination with primers R1 (5'-CTCAGATGAGGGAAGACTCAGR-3') and R2 (5'-GAGCTACGACTTAGCAGCAAG-3') were cloned following standard procedures (15).

The cytogenetic analysis of the second case revealed a t(3; 12) (FUSION OF ETV6 TO MDS1/EVI1) translocation. The potential involvement of the ETV6 gene located at 12p13 was then investigated by FISH with a pair of cosmids containing exon 1 or 8, respectively, of ETV6, namely 179A6 and 50F4. Hybridization signals from the first cosmid were found on the der(3) and der(9) chromosomes (Fig. 3A), whereas 148B6 hybridized with the der(12)t(3;12) and the der(12)t(9;15;12) (Fig. 3B), indicating a breakpoint between exon 2 and 3, as for the previous case. Next, the breakpoint of the t(3;12) was further investigated with two other cosmids mapped along ETV6 (50F4 and 212G9). As shown in Table 1, a hybridization signal from 50F4 was found on the der(3) but not on the der(12)t(3;12), whereas cosmid 212G9 hybridized to the der(3) and the der(12)t(3;12), indicating a breakpoint between exon 2 and exon 3 of ETV6 (Fig. 3C). The second signal from all these cosmids was found on a der(9)t(9;15;12). FISH with BAC probes 264D15 and 328P24 showed that both probes hybridized to the der(3)t(3;12), indicating a breakpoint telomeric to both EVII and MDS1 located at 3q26 (9).

The cytogenetic analysis of the second case revealed a t(3;12) del(7)(q22). FISH analysis showed split signals for 212G9 (Fig. 3D), thus suggesting a breakpoint between exon 2 and 3, as for the previous case.

The positioning of the breakpoint on chromosome 12 between exons 2 and 3 of the ETV6 gene allowed the identification of the fusion partner of ETV6 on chromosome 3. After cDNA synthesis from RNA of case 1 using the random hexanucleotide primer described above, a 3' RACE experiment was performed using two nested primers derived from exon 2 of ETV6. The amplification products were cloned, and 10 clones with an average insert length of 300 bp were analyzed by sequencing. Six clones contained uniquely ETV6 sequences. The remaining four clones showed a fusion of a novel sequence to exon 2 of the ETV6 gene, conserving the open reading frame of ETV6. A search of the sequence databases showed a perfect match with a sequence called CH3 (8). This sequence, first detected in a fusion of AML1 to EVII, was first thought to represent a 5' exon of EVII. It was recently shown, however, that the sequence is derived from MDS1 (10), which is also involved in chimeric transcripts as the result of t(3;21)(q26;q22). In view of the variant intergenic splicing described for EAP, MDS1, and EVII in t(3;21) cases, we investigated the eventual presence of ETV6-MDS1-EVI1 fusion transcripts in this patient. To this end, a primer was designed in the second exon of EVII (EVII1R, Ref. 8), and RT-PCR on the cDNA of patient 1 was performed as described above. Two bands of 482 and 144 bp (Fig. 4), respectively, were detected. Sequence analysis of the largest amplification product confirmed intergenic splicing between the first two exons of ETV6, MDS1, and the second exon of EVII, resulting in a chimeric mRNA with an open reading frame of 3816 nucleotides. Upon hybridization of the PCR products with an oligonucleotide with a MDS1 sequence, only the larger product showed a signal. Sequencing of the 144-bp product showed that this is the result of a splicing event between the second exon of ETV6 and the second exon of EVII without the intervening MDS1 sequence (Fig. 2B). Based upon the published sequences (8), the resulting chimeric ETV6/EVII transcript would contain two open reading frames. A small open reading frame starts at the first ATG in the ETV6 sequence and encodes a putative protein containing 80 amino acids, whereas a large open reading frame starts at an ATG sequence downstream of the first ATG in ETV6 (position 125, numbering according to Golub et al. (11)).

Because FISH analysis suggested a similar breakpoint on the der(12) for patient 2, RT-PCR was performed using nested ETV6 primers and the EVII primer described above. In this case, only the 482-bp product was detected, indicating the presence of a ETV6-

Table 1 Results of FISH analysis of patient 1

<table>
<thead>
<tr>
<th>Probe</th>
<th>Localization</th>
<th>3</th>
<th>9</th>
<th>der(12)t(3;12)</th>
<th>der(15)t(9;15;12)</th>
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<td>+</td>
<td>-</td>
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<td>179A6</td>
<td>12p13-ETV6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>12p13-ETV6</td>
<td>-</td>
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</tr>
<tr>
<td>212G9</td>
<td>12p13-ETV6</td>
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<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>148B6</td>
<td>12p13-ETV6</td>
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</tr>
<tr>
<td>328P24</td>
<td>3q26-MDS1</td>
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<td>+</td>
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* PRBl was used as an additional probe for 12p13 (21).
**MDS1-EVI1** fusion transcript but not the **ETV6-EVI1** splice variant. (Fig. 4).

To investigate the eventual presence of the reciprocal fusion RNA, a 5′ RACE experiment was performed with nested primers derived from exon 3 of **ETV6**. Sequencing of the PCR products revealed the absence of unknown sequences fused to exon 3 of **ETV6** (Fig. 5). Southern analysis with this sequence, however, showed that it was derived from exon 2 of the **ETV6** gene because it hybridized to the 3.1-kb EcoRI fragment at the T7 end of cosmid clone 212G9 (13). Sequencing of the genomic fragment showed that the cDNA sequence presented as an alternate first exon with a consensus 5′ splice donor site. This alternative transcript gives rise to a short open reading frame with a stop codon in exon 3 (Fig. 5).

**Discussion**

We show here that the reciprocal translocation t(3;12)(q26;p13) found in two patients with myeloproliferative disorder results in the intergenic splicing between exon 2 of **ETV6** on 12p13, **MDSI** sequences, and exon 2 of **EVI1** on 3q26. The resulting chimeric transcript shows one long open reading frame starting with the first ATG codon in **ETV6**. **ETV6** thus contributes no known functional domain to the predicted chimeric protein, and the oncogenic potential of the **ETV6/MDSI/EVI1** chimera might be the most relevant event.

Interestingly, in case 1, both alleles of **ETV6** are involved in chromosomal rearrangements. Deletion of the second **ETV6** allele is found in the majority of the childhood ALL cases with t(12;21)(p13; q22) (20) and was also described for t(9;15)(q34;p13) cases, suggesting that the wild-type **ETV6** could modulate the activity of the fusion products in those cases. Whether the t(9;15;12) results in the formation of yet another fusion gene or in the inactivation of the second **ETV6** allele remains under investigation. However, in the second case, the FISH analysis seems to indicate that the second **ETV6** gene is still present, suggesting that the inactivation of **ETV6** is not necessary for the expression of the oncogenic potential of the **ETV6/MDSI/EVI1** fusion reported here. However, the presence of a mutation in the second **ETV6** allele of case 2 was not excluded.

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**References**


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