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Fusion of *ETV6* with an intronic sequence of the *BAZ2A* gene in a paediatric pre-B acute lymphoblastic leukaemia with a cryptic chromosome 12 rearrangement

Ioannis Panagopoulos,[1] Bodil Strömbeck,[1] Margareth Isaksson,[1] Jesper Heldrup,[2] Tor Olofsson[3] and Bertil Johansson[1]

[1]Department of Clinical Genetics, Lund University, University Hospital, [2]Department of Paediatrics, Lund University, University Hospital, and [3]Department of Haematology, Lund University, University Hospital, Sweden

Running title: *ETV6/BAZ2A* fusion in ALL

Correspondence: Ioannis Panagopoulos, Department of Clinical Genetics, Lund University, University Hospital, SE-221 85 Lund, Sweden.
Tel: +46 46 172889; Fax: +46 46 131061
E-mail: ioannis.panagopoulos@med.lu.se

Summary

ETV6 at 12p13 is rearranged in a variety of haematological malignancies and solid tumours, with more than 20 different partners having been reported. These fusions result in either chimeric proteins or activation of the partner gene. However, there are a few examples of abnormalities resulting in truncated, and most likely, unproductive ETV6 proteins, suggesting that haploinsufficiency of *ETV6* and/or the partner is leukemogenic. We present a novel *ETV6* rearrangement, identified in a paediatric pre-B acute lymphoblastic leukaemia. FISH and molecular genetic analyses revealed a fusion of *ETV6* and *BAZ2A* (at 12q13), generated through a cryptic rearrangement between 12p13 and 12q13, consisting of exons 1 and 2 of *ETV6* and a sequence from intron 1 of *BAZ2A*. This transcript is not expected to produce any chimeric protein, but may encode a truncated form of ETV6, containing the first 54 amino acids (aa), followed by 16 aa from the 3´fusion sequence, reminiscent of *ETV6* fusions with *MDS2*, *LOC115548*, *PER1*, and *STL*. The rearrangement might also modify the regulation of *BAZ2A* by either activating a cryptic promoter or by coming under the control of the *ETV6* promoter. The present case emphasizes that “unproductive” *ETV6* rearrangements may play an important pathogenetic role in leukaemia.

Keywords: gene fusion, *ETV6*, *BAZ2A*, acute leukemia

Introduction

Cytogenetic analyses are today an integral part of the clinical management of patients with haematological malignancies, yielding diagnostic as well as prognostic information (Johansson *et al*, 2004; Mrózek *et al*, 2004). Furthermore, the detection of acquired chromosomal abnormalities, in particular translocations and inversions, has paved the way for identifying genes, located at the breakpoints, that if rearranged or otherwise deregulated are intimately associated with the leukemogenic process (Rowley, 2001). In fact, more than 200 such rearranged genes have been identified in haematological malignancies (Mitelman *et al*, 2005). Whereas most of these genes only have been reported to be involved in one, or a few, chromosomal abnormalities, some have been found to be quite promiscuous. For example, *ETV6* (previously *TEL*), a member of the ets family of transcription factors located at 12p13, has been shown to have several different partners in acute lymphoblastic leukaemias (ALLs), acute myeloid leukaemias (AMLs), chronic myeloproliferative disorders, and myelodysplastic syndromes (MDS). In fact, *ETV6* has to date been shown to be rearranged with more than 20 different genes, often encoding tyrosine kinases or transcription factors, in malignant haematological disorders as well as in solid tumours (Bohlander, 2005; Mitelman *et al*, 2005).

The molecular characterisation of the various chimaeras has shown that different regions of *ETV6* may be involved in the translocations and that the functional outcomes hence are quite variable. Fusion of the 5' region of *ETV6* to the 3' region of the partner gene can influence the functional activity of the latter gene, as seen in t(5;12)(q32;p13), complex rearrangements between 9q34 and 12p13, t(9;12)(p24;p13), and t(12;21)(p13;q22), involving *PDGFRB*, *ABL1*, *JAK2*, and *RUNX1*, respectively. The 5' part of *ETV6* can also drive the transcription of a structurally intact partner gene as in t(3;12)(q26;p13) with *MDS1/EVI1* and in t(12;13)(p13;q12) with the homeobox gene *CDX2*. In contrast, the 3' part of *ETV6* has been shown to be fused to the 5' part of the partner gene only in a few rearrangements, i.e., the

t(4;12)(q12;p13), t(7;12)(q36;p13), t(9;12)(p13;p13), t(12;13)(p13;q14), and t(12;22)(p13;q12), involving the *CHIC2*, *HLXB9*, *PAX5*, *TTL*, and *MN1* genes, respectively (Bohlander, 2005). These latter fusions most likely have an impact on the function of the HLH and ETS DNA binding domains of ETV6, which are still present in the hybrids.

Another category of *ETV6* fusions seems to be "unproductive", i.e., includes chimaeras that do not result in functional products (Bohlander, 2005). We here present a further example of an unproductive *ETV6* fusion, consisting of exons 1 and 2 of *ETV6* and a sequence from intron 1 of the *BAZ2A* gene, in a paediatric pre-B ALL.

Patient, materials and methods

Case history and cytogenetic findings

A previously healthy 3-year-old girl was admitted to hospital in July 2004 because of fever, fatigue, and petechiae. The clinical examination revealed hepatosplenomegaly but no signs of central nervous system disease, mediastinal mass, or enlarged lymph nodes. The peripheral blood values were haemoglobin 64 g/l, white blood cell count 5.6 x 10^9/l, and thrombocytes 10 x 10^9/l. The bone marrow (BM) was hypercellular, containing blasts of L2 morphology. Flow cytometric two-colour analysis showed that 80% of the BM cells were positive for CD19, CD10, CD22, CD38, cytCD79a, and TdT but negative for CD34, CD20, cytIgM, and myeloid markers. A diagnosis of pre-B ALL was made. A monoclonal TCR-gamma gene rearrangement was identified and characterised, which – in parallel with flow cytometric analysis – was used for evaluating minimal residual disease by real time PCR. She started treatment according to the Nordic ALL-2000 protocol for standard intensive patients (drugs and dosages as in the Nordic ALL-1992 protocol; Gustafsson *et al*, 2000), and achieved complete remission (CR) on day 15. She remains in CR 18 months after the diagnosis.

The cytogenetic analysis of a diagnostic BM samples was unsuccessful because of lack of analysable metaphases. However, 3 cytogenetically abnormal metaphases of poor chromosome morphology could be analysed in a peripheral blood sample, yielding the karyotype 46,XX,del(1)(q25-32),t(?1;12)(q42;p13),?-21,?+mar[cp3]. Further genetic analyses revealed a DNA index of 1.00 and negativity for *MLL* rearrangement and for *BCR/ABL1* [t(9;22)(q34;q11)], *ETV6/RUNX1* [t(12;21)(p13;q22)], and *TCF3/PBX1* [t(1;19)(q23;p13)] fusions.

FISH analysis with the Vysis LSI TEL/AML1 ES Dual Color Translocation Probe (Abbott, Stockholm, Sweden), which TEL probe begins between exons 3 and 5, extending approximately 350 kb toward the 12p telomere, and contains exons 1A, 2, 1B, and 3 of *ETV6*, revealed a split signal for *ETV6* in 20% of the investigated nuclei. Based on the RACE results (see below), which indicated fusion of *ETV6* to a sequence mapping to the bacterial artifical chromosome (BAC) probe RP11-369C14, interphase FISH with this BAC, indirectly labelled with biotin-dUTP using Amersham's mega Prime kit and detected with streptavidin-diethylaminocoumarin (DEAC), and the TEL/AML1 probe was performed on BM cells.

RACE and RT-PCR analyses

Total RNA was extracted from the BM cells using the Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The 5´- and 3´-RACE were performed on 1 µg total RNA by the use of the Smart RACE cDNA amplification kit (BD Biosciences, Stockholm, Sweden) according to the manufacturer's protocol. For 5´-RACE, the Universal Primer A Mix (UPM) and the *ETV6* reverse primer TEL-34F were used in first round PCR (the *ETV6* and *BAZ2A* primers utilised for PCR amplification and sequence analyses are listed in Table I). The second round PCR was performed with the Nested Universal Primer A (NUP) and the *ETV6* reverse primer TEL-47F. For 3´-RACE, the UPM and the *ETV6* forward primer

ETV6-171F were used in first round PCR; the second round PCR was performed with the NUP and the forward primer ETV6-242F. Fifteen µL of the PCR products were analysed by electrophoresis through 1.3 % agarose gels, stained with ethidium bromide, and photographed.

For the RT-PCR, 4 µg of total RNA were reverse-transcribed in a 20 µL reaction volume containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM of each dNTP, 20 U RNAse inhibitor (RNA guard, Amersham Biosciences, Piscataway, NJ), 10 pmol random hexamers, and 400 units M-MLV Reverse Transcriptase (Invitrogen). The reaction was carried out at 37^{o}C for 60 min, heated for 10 min at 65^{o}C, and then kept at 4^{o}C. The 50 µL PCR volume contained 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM of each dNTP, 1 unit Platinum*Taq* DNA polymerase (Invitrogen), 0.5 µM of each of the forward and reverse primers (Table I), and 2 µL of the cDNA. Two µL of the first PCR product were then used as template in the second PCR with inner primer combinations (Table I). The quality of the cDNA synthesis was examined by amplification of a cDNA fragment of the *ABL1* gene (Panagopoulos *et al*, 2001). The PCR products were run on a PCT-200 DNA Engine (MJ Research, Waltham, MA), and all amplifications had an identical cycling profile: an initial denaturation at 94^{o}C for 5 min, followed by 30 cycles of 1 min at 94^{o}C, 1 min at 60^{o}C, and 1 min at 72^{o}C, and a final extension for 10 min at 72^{o}C. For sequence analyses, the RT-PCR-amplified fragments were purified using the Qiagen gel extraction kit (Qiagen, Hilden, Germany) and directly sequenced using the dideoxy procedure with an ABI Prism BigDye terminator v1.1 cycle sequencing kit (PE Applied Biosystems, Foster City, CA) on the Applied Biosystems Model 3100-Avant DNA sequencing system. The BLAST software was used for computer analysis of the sequence data (http://www.ncbi.nlm.nih.gov/BLAST/). The identified sequence (see below) has been submitted to the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk), and has been assigned the accession number AM181353.

Results

The interphase FISH analysis with the TEL/AML1 probe revealed a split signal of *ETV6* in 20% of the investigated nuclei, indicating that the translocation breakpoint at 12p13 was upstream of exon 4 of *ETV6* (Fig 1). Although the break in principle could be telomeric to *ETV6*, this gene was considered a likely target of the observed t(?1;12)(q42;p13). Hence, 5´- and 3´-RACE methodologies were utilised in order to find the putative partner gene to *ETV6*. Whereas 5´-RACE only amplified normal *ETV6* cDNA fragments, 3´-RACE showed a novel sequence fused to exon 2 of *ETV6* (Fig 2) in addition to normal *ETV6* transcripts, with the latter indicating that the wild type *ETV6* was not deleted. Database searches (http://www.ncbi.nlm.nih.gov/) revealed that this fragment corresponds to a sequence on BAC RP11-369C14 (accession number AC090681) which maps to 12q13. Co-hybridization of this BAC with the TEL/AML1 probe revealed a fusion signal of the TEL probe and the BAC RP11-369C14 (Fig 1). Subsequently, the BLAST analysis showed that the fusion point is in intron 1 of the *BAZ2A* gene, which is located in the BAC. Thus, the t(?1;12) was not a simple translocation; a cytogenetically cryptic rearrangement between 12p13 and 12q13 had also occurred.

The *ETV6/BAZ2A* cDNA fragment was confirmed by RT-PCR (Fig 3) using a forward primer (exon 1) specific for *ETV6* and a reverse primer (intron 1) specific for the intronic sequence of *BAZ2A* (ETV6-171F and BAZ2Aintr1-R1, respectively; Table I). Nested RT-PCR with the forward *ETV6* primers ETV6-171F and ETV6-242F (both in exon 1) and the reverse primers BAZ2A-551R and BAZ2A-498R, located in exon 3 of *BAZ2A* (NM_013449; http://www.ncbi.nlm.nih.gov/), i.e., distal to intron 1, did not amplify any product, nor did nested RT-PCR with forward primers in exon 1 of *BAZ2A* (BAZ2A-17F and BAZ2A-115F) and reverse primers in exon 4 of *ETV6* (TEL-143R and TEL-135R). These experiments

clearly show that there were no *ETV6/BAZ2A* or *BAZ2A*/*ETV6* fusions on the cDNA level involving exons from both genes; only a transcript consisting of exons 1 and 2 of *ETV6* fused to a sequence of intron 1 of *BAZ2A* was present.

Discussion

The *ETV6/BAZ2A* transcript identified in the present study is not expected to produce any chimaeric protein. However, it may code for a truncated form of ETV6, consisting of 54 aa from ETV6 followed by 16 aa from the intronic sequence of *BAZ2A*. This chimaera as such could be of pathogenetic importance. Alternatively – or additionally – the chromosomal rearrangement might deregulate *BAZ2A* by either activating a cryptic promoter located upstream of exon 2 of *BAZ2A* or through coming under the control of the *ETV6* promoter. The leukemogenic impact of a putative *BAZ2A* deregulation is currently unknown because the function of the *BAZ2A* gene, which is quite ubiquitously expressed and belongs to a family of bromodomain genes, is not well elucidated (Jones *et al*, 2000; Poot *et al*, 2000). However, it is known that the mouse homologue *Tip5* codes for a protein that is involved in chromatin remodelling (Strohner *et al*, 2001).

The chimaeric *ETV6/BAZ2A* is similar to the *ETV6/STL* and *ETV6/MDS2* fusions, which have been found in a cell line derived from a pre-B-ALL carrying a t(6;12)(q23;p13) (Sato *et al*, 1997) and in an MDS with a t(1;12)(p36;p13) (Odero *et al*, 2002), respectively, in the sense that they are all predicted to code for a short ETV6 protein, containing the first 54 aa, followed by only a few aa. In the present case, 16 aa from the *BAZ2A* gene are present, whereas 4 and 14 aa of *STL* and *MDS2*, respectively, follow ETV6 in the fusions involving the latter genes (Sato *et al*, 1997; Odero *et al*, 2002). There are also similarities to three other *ETV6* fusions. In the *ETV6/PER1* hybrid, generated through the t(12;17)(p13;p13) in AML (Penas *et al*, 2003), exon 1 of *ETV6* is fused to the anti-sense strand of *PER1*. Similarly, the

first two exons of *ETV6* have been shown to be rearranged with the anti-sense strand of *LOC115548* at 5q13 in a complex rearrangement in a case of AML (Belloni *et al*, 2004). Furthermore, out-of-frame *ETV6* chimaeric transcripts involving the *ACSL6* (*ASC2*) gene have been reported in AML and MDS with t(5;12)(q31;p13) (Yagasaki *et al*, 1999). None of these *ETV6* rearrangements are expected to code for any functional products. Thus, formation of a fusion protein does not seem to be the pathogenetically important outcome of these translocations. However, it should be stressed that no functional studies of these "abortive" *ETV6* rearrangements have, as yet, been performed.

Another possible leukemogenic mechanism in the present case could be deregulation of a gene in the vicinity of *BAZ2A*. In fact, it has been shown that the t(5;12), with *ACSL6* as the 3' partner, leads to overexpression of *IL3*, which is located near the breakpoint at 5q31, and that the t(4;12)(q11-12;p13), with *CHIC2* as the 5' partner, results in deregulation of the homeobox gene *GSH2* close to the 4q11-12 breakpoint (Cools *et al*, 2002). Alternatively, the pathogenetic impact may be mediated through haploinsufficiency of *ETV6* and/or *BAZ2A*, as has recently been suggested for the *ETV6/LOC115548* hybrid in AML (Belloni *et al*, 2004).

In conclusion, taking into account the various *ETV6* abnormalities, including the present one, resulting in non-functional hybrid proteins, it is tempting to postulate that rearrangements of *ETV6* as such may play an important pathogenetic role in leukaemia.

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Legends to the figures

Fig 1. Interphase FISH with the Vysis LSI TEL (green)/AML1 (red) ES Dual Color Translocation Probe and the BAC RP11-369C14 (purple). Two green, two red, two purple, and one purple/green fusion signal are seen, with the latter indicating fusion of *ETV6* (at 12p13) with BAC RP11-369C14 (at 12q13).

Fig 2. Nucleotide and predicted amino acid sequences of the chimaeric cDNA fragment of exons 1 and 2 of *ETV6* and a sequence from intron 1 of *BAZ2A*, which was amplified using 5´-RACE. The forward and reverse primers are indicated in bold. The junction between exon 2 of *ETV6* and the sequence corresponding to BAC RP11-369C14 is denoted by an arrow. The asterisk indicates a stop codon.

Fig 3. Detection of the hybrid cDNA fragment of exons 1 and 2 of *ETV6* and a sequence from intron 1 of *BAZ2A* (lane 3) using the primer set ETV6-171F and BAZ2Aintr1-R1 (Table I; Figure 2). No fragment was obtained when genomic DNA was used as a template (lane 4). Nested RT-PCR with forward *ETV6* primers (ETV6-171F and ETV6-242F located in exon 1) and reverse *BAZ2A* primers (BAZ2A-551R and BAZ2A-498R located in exon 3) did not amplify any product (lane 1), nor did nested RT-PCR with forward primers in exon 1 of *BAZ2A* (BAZ2A-17F and BAZ2A-115F) and reverse primers in exon 4 of *ETV6* (TEL-143R and TEL-135R) (lane 2). The quality of the cDNA synthesis was examined by amplification of a cDNA fragment of the *ABL1* gene (lane 7). Lane 5, blank (water) for nested PCR with forward *ETV6* primers and reverse *BAZ2A* primers. Lane 6, blank for nested PCR with forward *BAZ2A* primers and reverse *ETV6* primers. M, 1 kb DNA ladder.

Table I. Primers used for PCR and sequencing.

Designation	Sequence (5´→ 3´)	Direction	Position	Locus (accession no.version)[a]
ETV6-171F	CTTAAATGACCGCGTCTGGCTGG	Forward	171-193	*ETV6* (NM_001987.3)
ETV6-242F	CTGAGAACTTCCTGATCTCTCTCGC	Forward	242-266	*ETV6* (NM_001987.3)
TEL-34F	ACTCCTGCTCAGTGTAGCATTAAG	Forward	284-307	*ETV6* (NM_001987.3)
TEL-47F	CTCAGGATGGAGGAAGACTCG	Forward	395-415	*ETV6* (NM_001987.3)
TEL-135R	GTGGAAGAATGGTGAAAGAATCCGAGG	Reverse	679-650	*ETV6* (NM_001987.3)
TEL-143R	CTGTGAGTGTATAGAGTTTCCAGGGTG	Reverse	703-677	*ETV6* (NM_001987.3)
BAZ2A-17F	GCAGTTCAGGCTGTGTGGTTCGCA	Forward	17-40	*BAZ2A* (NM_013449.2)
BAZ2A-115F	CGTGAAGTGGAGGCCCAAGGACAG	Forward	115-138	*BAZ2A* (NM_013449.2)
BAZ2A-498R	GGGTCCTTGAGGTTGCTGCCAG	Reverse	519-498	*BAZ2A* (NM_013449.2)
BAZ2A-551R	CCAAGGATGCCGTTGAGTGGGTA	Reverse	573-551	*BAZ2A* (NM_013449.2)
BAZ2Aintr1-R1	CAAGTCCATCTGATGTCAGCATCC	Reverse	57361-57338	RP11-369C14 (AC090681.13)
BAZ2Aintr1-R2	GTCTGATGGGGTTGATTTGATGC	Reverse	57302-57280	RP11-369C14 (AC090681.13)

[a]http://www.ncbi.nlm.nih.gov/

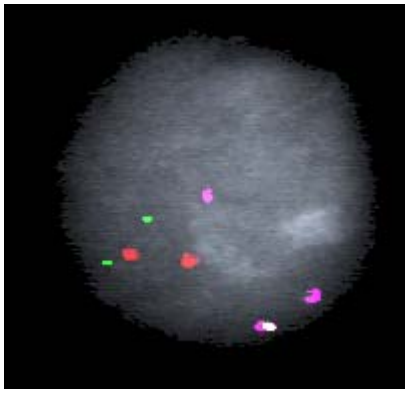

Figure 1

ETV6-171F

CTTAAATGAC CGCGTCTGGC TGGCCGTGGA GCCTTTCTGG GTTGGGGAGA

ETV6-242F

GGAAAGGAAA GTGGAAAAAA C**CTGAGAACT TCCTGATCTC TCTCGC**TGTG

AGACATGTCT GAGACTCCTG CTCAGTGTAG CATTAA***GC***AG GAACGAATTT

M S E T P A Q C S I K Q E R I S

CATATACACC TCCAGAGAGC CCAGTGCCGA GTTACGCTTC CTCGACGCCA

Y T P P E S P V P S Y A S S T P

CTTCATGTTC CAGTGCCTCG AGCGCTCAGG ATGGAGGAAG ACTCGATCCG

L H V P V P R A L R M E E D S I R

ETV6 exon 2 ↓ nt 57206 of BAC RP11-369C14 (AC090681)

CCTGCCTGCG CACCTGCgga aaatcaactt gctgttctgt caagtgccaa

L P A H L R K I N L L F C Q V P I

ttcaggtaag caaaggctga agaaagggga acttaaaatc a**gcatcaaat**

Q V S K G *

caaccccatc agacagcaac ttaaaggaac taatcagggg caagaaaaag

BAZ2intr-R2

gatgctgaca tcagatggac ttgtttttac tgtttccaga atatcaaaaa

BAZ2intr-R2

ccccaacagc cccagtgaga agagcaatga tgctgctatc ttactagtga

tgatgccact ttcacacaat cataagtgtc tatctgctcc catactggat

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Figure 2

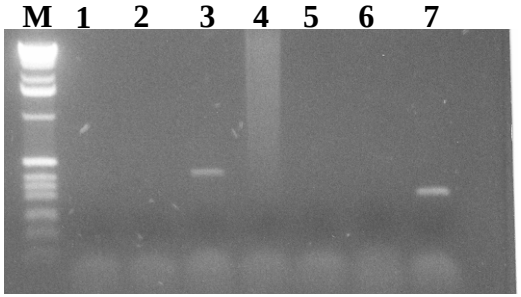

Figure 3