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ETV6 fusions from insertions of exons 3-5 in pediatric hematologic malignancies

Sarah B. Mueller1, Yana Pikman2,3, Sarah K. Tasian4,5, Lewis B. Silverman2,3, Marian H. Harris1, Harrison K. Tsai1,6

1Department of Pathology, Boston Children’s Hospital, Harvard Medical School, Boston, MA, USA
2Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA
3Division of Hematology/Oncology, Boston Children’s Hospital, Harvard Medical School, Boston, MA, USA
4Division of Oncology and Center for Childhood Cancer Research, Children’s Hospital of Philadelphia, Philadelphia, PA, USA
5Department of Pediatrics and Abramson Cancer Center, University of Pennsylvania Perelman School of Medicine; Philadelphia, PA, USA
6Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA, USA

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Correspondence: Harrison.Tsai@childrens.harvard.edu

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To the Editor:

Current risk classification and treatment of patients with B-cell lymphoblastic leukemia (B-ALL) relies on rapid genomic testing for the identification of subtype-defining, prognostically significant, or potentially targetable alterations, which often occur as fusion events.1,2 Karyotype and fluorescence in situ hybridization (FISH) are the traditional methodologies used for detecting fusions; however, there is increasing recognition of false positive and false negative results with these tests depending on the structure of the rearrangement. Although FISH has greater resolution than karyotype, it is still limited to targeted aberrations of approximately 100 kb or larger, thus clinically important fusions with false negative FISH results due to relatively small insertions have been observed. ETV6::RUNX1 fusions define one of the most common pediatric B-ALL subtypes comprising 20-25% of cases.3 Although not universally favorable, its status is used in some treatment protocols to classify patients as provisionally low risk.4,5 These fusions typically result from chromosomal translocations t(12;21)(p13;q22) that are cryptic to karyotyping due to banding pattern similarities of the 12p and 21q chromosomal arms.5 As a result, FISH or RT-PCR is usually performed for diagnosis. Here we report two pediatric B-ALL cases that were negative for ETV6::RUNX1 rearrangements by FISH, but were deduced through targeted RNA next generation sequencing (NGS) to harbor ETV6::RUNX1 fusions characterized by focal insertions of ETV6 exons 3-5 into RUNX1 with concomitant intragenic deletions of the same exons from ETV6. We further report one pediatric AML case with an ETV6::EP300 rearrangement similarly involving insertion and deletion of ETV6 exons 3-5. Finally, by searching public whole-transcriptome sequencing data, we identify another ETV6::RUNX1-positive B-ALL with the same pattern and characterize the underlying insertion and deletion breakpoints on the DNA level.

Cases 1 and 2 were identified within two weeks of each other during routine review at Boston Children’s Hospital of a clinically validated targeted RNA NGS assay for detecting fusions based on ArcherDx FusionPlex Heme v2 (FPHeme; IDT, Coralville, IA, USA). Cytogenetics and FISH were performed at Integrated Oncology or partner institutions. Targeted DNA NGS was performed at Brigham and Women’s Hospital. Isoform analysis of RNA sequencing data was performed using the software isoformSR (https://github.com/ht50/isoformSR), as previously described.6 Clinical features and molecular findings are summarized in Table 1.

Case 1 was from a 4 year-old male with newly-diagnosed B-ALL with unrevealing genetic analysis, including normal karyotype and negative FISH for ETV6::RUNX1 and other subtype-defining rearrangements. FPHeme subsequently revealed an ETV6::RUNX1 fusion with standard breakpoints connecting ETV6 exon 5 (NM_001987.5) to RUNX1 exon 3 (NM_001754.5), together with an atypical reciprocal RUNX1::ETV6 fusion with rare breakpoints connecting RUNX1 exon 2 to ETV6 exon 3 and abnormally high expression (quantified by split-reads sequencing the junction) of an in-frame ETV6 exon-skipping isoform junction connecting exon 2 to exon 6 (i.e. skipping exons 3-5), satisfying previously established criteria for “outlier expression” relative to other FPHeme cases, (Figure 1A). We have previously shown outlier expression to be a sensitive and specific marker for underlying intragenic deletions in other genes, thus this case was most consistent with DNA deletion of ETV6 exons 3-5.6 Despite its unusual aspects, the targeted gene expression profile clustered with B-ALL cases harboring typical FISH-positive ETV6::RUNX1 fusions (Figure 1B). Theoretically, the observed fusion breakpoints could represent either a reciprocal translocation or a focal insertion; however, FISH analysis showed loss of one ETV6 signal in 68.5% of cells, similar to the 66% blast estimate by flow cytometry and confirmed to represent a single-copy, whole-gene ETV6 deletion by targeted DNA sequencing, making it impossible for the single remaining ETV6 allele to harbor both deletion of ETV6 exons 3-5 and a reciprocal translocation to RUNX1. The overall findings
therefore implied an intragenic deletion of $ETV6$ exons 3-5 with associated insertion between $RUNX1$ exons 2 and 3 (Figure 2).

Case 2 was from a 6 year-old female with newly-diagnosed B-ALL and similar findings of a normal karyotype and negative FISH for $ETV6::RUNX1$ and other fusions. FPHeme again revealed $ETV6::RUNX1$ and $RUNX1::ETV6$ fusions with the same exon structure as case 1, outlier expression of the same $ETV6$ exon-skipping isoform, and a gene expression profile clustering with typical cases of $ETV6::RUNX1$ B-ALL.

Given this recurrent pattern of insertions and deletions of intragenic exons, we retrospectively searched historical FPHeme cases (n=474) for outlier expression of any $ETV6$ exon-skipping isoform as a potential marker for cryptic insertions. We identified one additional case with outlier expression involving skipping of $ETV6$ exons 3-5 (case 3), but did not identify outlier expression involving skipping of other exons.

Case 3 was from a 5 year-old female with relapsed AML with detected in-frame reciprocal fusions $ETV6::EP300$ connecting exon 5 of $ETV6$ to exon 2 of $EP300$ (NM_001429.4) and $EP300::ETV6$ connecting exon 1 of $EP300$ to exon 3 of $ETV6$ (Figure 1A). The predicted chimeric protein contained the helix-loop-helix (HLH) domain of $ETV6$ inserted between the nuclear localization sequence and transactivation domains of $EP300$ and retained the $EP300$ chromatin modification region (Figure 2). Outlier expression of the $ETV6$ isoform skipping exons 3-5 was again observed, consistent with single-copy intragenic deletion (Figure 1A). Per report, interphase FISH analysis showed two strong $ETV6$ signals and one weak $ETV6$ signal. Metaphase FISH analysis showed that the weak $ETV6$ signal was located on a small G-size chromosome. $EP300$ FISH was not performed. Based on these data, we hypothesize that the weak $ETV6$ signal represented partial binding of the $ETV6$ probe to $ETV6$ exons 3-5 inserted into the $EP300$ gene on chromosome 22. Another case of $ETV6::EP300$, with unspecified structure, has been reported in the literature.7

Finally, we analyzed $ETV6$ exon-skipping isoforms in public total RNA sequencing data from a pediatric ALL cohort, revealing one $ETV6::RUNX1$ case (Sample ID: 36) with outlier expression of the isoform skipping exons 3-5, comprising 3.4% (1/29) of $ETV6::RUNX1$-positive B-ALL in the cohort, where it again co-occurred with the only instance of a reciprocal fusion connecting $RUNX1$ exon 2 to $ETV6$ exon 3.$^5,8$ Targeted DNA NGS showed single copy loss of $ETV6$, thus the exon-skipping isoform was expressed at a high variant allele fraction (95.3%; not shown), similar to case 1 (Figure 1A). Intron split-read analysis identified three sets of expressed DNA breakpoints derived presumably from pre-mRNA, compatible with deletion of 80.7 kb between $ETV6$ introns 2 and 5 (chr12:11,952,220 to chr12:12,032,917) and insertion of a near-exact 81.0 kb segment (chr12:11,951,870 to chr12:12,032,916) into $RUNX1$ intron 2 at breakpoints separated by 62 bp (chr21:36,296,008 and chr21:36,296,069) (Figure 1C). Per report, FISH analysis described a non-classical $ETV6::RUNX1$ rearrangement, where the translocated 5’ part of $ETV6$ to the derived 21 demonstrated a smaller green signal than usual, together with an absence of the small extra red signal that ordinarily represents the 5’ part of $RUNX1$ translocated to the derived 12. The FISH positivity of this case, in contrast to cases 1-2, might be related to differences in FISH probes or $ETV6$ insertion sizes, which theoretically could be as small as ~30 kb versus ~81 kb in this case.

An $ETV6::RUNX1$ fusion cryptic to FISH but detected by RT-PCR, which was designed to amplify any fusion connecting $ETV6$ exon 5 to $RUNX1$ exon 3 or exon 4 (including the insertion fusions described here), was previously reported in a pediatric B-ALL, although underlying genomic structure was not determined.$^5$ Importantly, in the absence of RNA sequencing or RT-
PCR, such FISH-negative cases would likely remain unclassified and, in some clinical protocols, may lead to unintended higher risk stratification and more intensive treatment regimens.\textsuperscript{3,4} Similarly, although uncommon in pediatric AML, \textit{ETV6} rearrangements are important to identify given their association with adverse risk regardless of fusion partner; chromosome 12p abnormalities/\textit{ETV6} rearrangements are accordingly an indication in pediatric AML for allogeneic hematopoietic stem cell transplantation in first remission in the current Children’s Oncology Group AAML1831 clinical trial (NCT04293562).\textsuperscript{7,10} The mechanistic consequences of insertion events will require elucidation. The gene expression profiles of cases 1-2 were similar to typical cases of \textit{ETV6::RUNX1} positive B-ALL in our historical cohort, suggesting functional similarity. An \textit{ETV6::RUNX1}-like gene expression profile, which may also confer a favorable prognosis, has been described as a provisional entity in otherwise unclassified B-ALL and has been associated with inactivating \textit{ETV6} variants and shown to be mediated by microsatellite enhancers ordinarily bound by \textit{ETV6}.\textsuperscript{2,11} Detection and screening for exon-skipping isoforms may be an effective way of recognizing fusions resulting from concurrent deletion and insertion of intragenic genomic material, which otherwise may be mistaken for typical balanced reciprocal translocations in standard short read sequencing data. Indeed, targeted RNA sequencing of case 3 at a partner institution reported the \textit{ETV6::EP300} fusion as connecting exons 1-5 of \textit{ETV6} to exons 2-31 of \textit{EP300}. The deletion-insertion pattern has also been described in the context of \textit{YAP1::KMT2A} rearranged sarcomas although screening our cohort did not reveal outlier expression of any \textit{KMT2A} exon-skipping isoforms in either \textit{KMT2A}-rearranged or non-rearranged hematologic cases.\textsuperscript{12}

In summary, clinically relevant \textit{ETV6} fusions from focal insertions of \textit{ETV6} may be more common in childhood leukemia than previously recognized, particularly in FISH-negative cases. Larger studies are necessary to determine their true frequency, assess clinical significance, and inform the use of NGS fusion testing in B-ALL.
References


5. Shurtleff SA, Buijs A, Behm FG, et al. TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. Leukemia. 1995;9(12):1985-1989.


### Tables

<table>
<thead>
<tr>
<th></th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
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<tbody>
<tr>
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<td>4</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Sex</td>
<td>M</td>
<td>F</td>
<td>F</td>
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<tr>
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<td>B-ALL</td>
<td>B-ALL</td>
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<td>66%</td>
<td>95%</td>
<td>52%</td>
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<td>no</td>
<td>no</td>
</tr>
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<td>Negative</td>
<td>Negative</td>
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<td>ETV6::RUNX1</td>
<td>1 copy ETV6 in 68.5%</td>
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<td>RUNX1::ETV6 (e2 to e3)</td>
<td>RUNX1::ETV6 (e2 to e3)</td>
<td>EP300::ETV6 (e1 to e3)</td>
</tr>
<tr>
<td></td>
<td>ETV6::RUNX1 (e5 to e3)</td>
<td>ETV6::RUNX1 (e5 to e3)</td>
<td>ETV6::EP300 (e5 to e2)</td>
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<td>Isoforms (RNA)</td>
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<td>ETV6 (e2 to e6)</td>
<td>ETV6 (e2 to e6)</td>
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<tr>
<td></td>
<td>IKZF1 (e1 to e8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETV6 del (DNA)</td>
<td>1 copy del, whole gene</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>IKZF1 del (DNA)</td>
<td>1 copy del, exons 2-7</td>
<td>no</td>
<td>no</td>
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<tr>
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<td>NFE2 p.Y172H (VUS)</td>
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<td>Gain RUNX1, ERG, U2AF1 (21q)</td>
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<td>SF3B1 p.E595K</td>
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AML: acute myeloid leukemia; B-ALL: B-lymphoblastic leukemia; BMA: bone marrow aspirate; CNS: central nervous system; e: exon; F: female; M: male; del: deletion; VUS: variant of unknown significance

**Table 1.** Patient characteristics and molecular findings.

Details of case 4 (Figure 1C) can be found in the supplementary table of the total RNA sequencing study (Sample ID: 36). The case was from a 4 year-old male with B-ALL with 89% blasts, a karyotype of 45,XY,-9,der(12)t(9;12)(q11.11;p11.22),der(18)t(17;18)(q21.31;q21.2)[8], positive FISH for *ETV6::RUNX1* with an unusual pattern of signals (described in the main text), RNA sequencing fusions of *RUNX1::ETV6* (e2 to e3) and *ETV6::RUNX1* (e5 to e3) (by re-analysis of the raw data), outlier expression of the isoform ETV6 e2 to e6 (by isoform analysis of the raw data), and 1 copy whole-gene deletions of *ETV6, KRAS, JAK2*, and *PAX5* as well as a VUS in *IKZF2* p.N35S (by review of the report from targeted DNA NGS testing, also performed at Brigham and Women’s Hospital).
Legends

Figure 1. RNA sequencing results.

(A) ETV6 exon skipping isoform and ETV6 fusion transcripts detected on targeted RNA sequencing (FPHeme) over historical cohort (n=474) consisting of cases 1-3 (black square, circle, and triangle) and others (gray x’s). Cases 1-3 had outlier expression of the ETV6 isoform junction e2e6 connecting exon 2 to exon 6, consistent with an underlying intragenic deletion of the skipped exons 3-5, as well as fusion breakpoints consistent with insertion of exons 3-5 between exons 2 and 3 of the partner gene RUNX1 (cases 1-2) or between exons 1 and 2 of the partner gene EP300 (case 3). Case 1 was also associated with single copy whole gene deletion of ETV6 and accordingly had the highest expressed variant allele frequency (VAF) of e2e6. (B) Clustering by tSNE of the targeted transcriptional profiles (100 genes) of cases 1 and 2 among all B-ALL cases (n=209) colored by subtype including ETV6::RUNX1 fusions (green dots). (C) Screening of public total RNA sequencing data identified one more pediatric ETV6::RUNX1-positive B-ALL case (Sample ID: 36; SRR15301257) with ETV6 e2e6 at high VAF (95.3%; not shown), fusion breakpoints consistent with insertion of ETV6 exons 3-5 between RUNX1 exons 2 and 3, and single copy whole gene deletion of ETV6 by targeted DNA NGS. Split-read analysis of intronic alignments identified 3 sets of expressed DNA breakpoints within split-reads: (i) chr12:11952219+ (ETV6 intron 2) to chr12:12032918+ (ETV6 intron5) illustrated in the example paired split-read SR1 (light blue), (ii) chr21:36296008-(RUNX1 intron 2) to chr12:11951870+ (ETV6 intron 2) illustrated in the example paired split-read SR2 (yellow), and (iii) chr12:12032916+ (ETV6 intron 5) to chr21:36296069- (RUNX1 intron 2) illustrated in the example paired split-read SR3 (gray). The breakpoints of SR1 implied a deletion of the intervening 80.7 kb segment between the ETV6 intron 2 and intron 5 breakpoints. The breakpoints of SR2 and SR3 implied a focal insertion of the near-exact 81 kb segment of ETV6 (chr12:11951870+ to chr12:12032916+) into RUNX1 intron 2 at breakpoints separated by 62 bp (chr21:36296008- and chr21:36296069-), thus also associated with a duplication of this short 62 bp segment. The inclusion of an additional ~350 bp from intron 2 in the inserted segment compared to the deleted segment suggested some amount of repair/replication within intron 2 during formation of the deletion. In theory, the breakpoints of SR2 and SR3 alternatively could represent a reciprocal translocation with duplication of ETV6 exons 3-5, however single copy loss of the other ETV6 allele made it impossible for the remaining allele to harbor both a reciprocal translocation and a deletion of the same region. The intronic split-reads harboring DNA deletion and rearrangement breakpoints were presumably derived from pre-mRNA, as described in other studies.6

Notation – VAF: expressed variant allele fraction. 5’ and 3’: refers to the 5’ and 3’ ends of the split-read alignments. +/- refers to the DNA strand of the alignment; note that ETV6 is transcribed from the plus strand (+) of chromosome 12 whereas RUNX1 is transcribed from the minus strand (-) of chromosome 21.

Figure 2. Schematics of ETV6 insertions.

Structure of the rearrangements detected in cases 1 and 2 (left side) and case 3 (right side) with the predicted chimeric protein products.
A

RNA VAF

Case1
Case2
Case3

ETV6 e2e6
RUNX1 e2 to ETV6 e3
ETV6 e5 to RUNX1 e3
ETV6 e3 to EP300 e1
ETV6 e5 to EP300 e2

B

Case1
Case2
ETV6::RUNX1
ETV6 other
DUX4r/ERGdel
PAX5alt/P80r
KMT2Ar
CRLF2
Kinase-r/Ph-like

BCR::ABL1
TCF3::PBX1
TCF3::HLF
IKZF1 N159Y
IAMP21
Hyperdiploid
Hypodiploid

C

Case4

chr12:11,951,784-11,952,371
chr12:12,032,833-12,033,001
chr21:36,295,821-36,296,243

ETV6 intron 2
ETV6 intron 5
RUNX1 intron 2
ETV6 NM_001987.4

RUNX1 NM_001754.4

QCFMR

GLQP...

RIT ECILG...

ASDGT GLQP...

RIAD DFGSLF...

HLH domain

Transactivation domain

Runt homology domain

NLS

HLH domain

Transactivation domain

Chromatin modification region (Bromo, Ring, and histone acetylation domains)