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

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 reverse transcription polymerase chain reaction (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

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

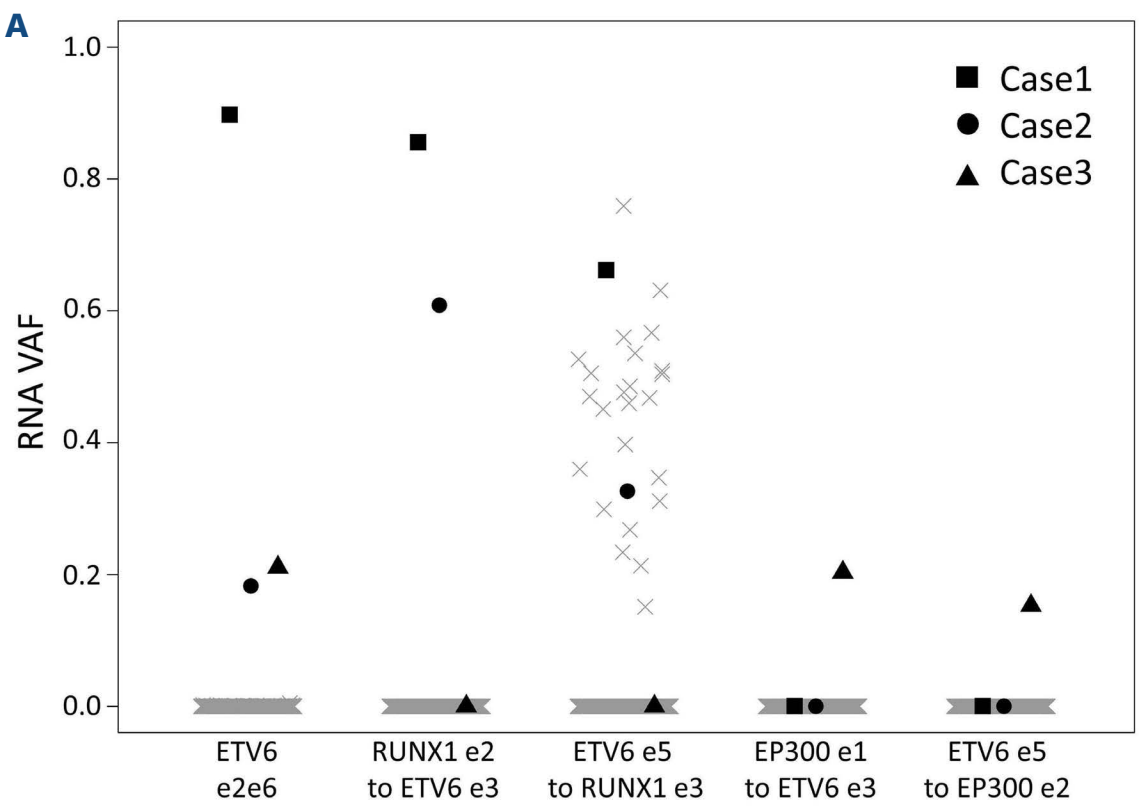
| | Case 1 | Case 2 | Case 3 |
|---|---|---|---|
| Age in years | 4 | 6 | 5 |
| Sex | M | F | F |
| Diagnosis | B-ALL | B-ALL | AML |
| BMA blast % | 66 | 95 | 52 |
| CNS involvement | No | No | No |
| Karyotype | 46,XY[20] | Failed | 47,XX,del(2)(p16p22), t(3;19)(p21;p13.3),del(9)(q21q31), del(12)(q24),+21[19] / 46,XX[1] |
| Clinical FISH for *ETV6::RUNX1* | Negative<br>1 copy *ETV6* in 68.5% | Negative | Negative |
| Fusions (RNA) | *RUNX1::ETV6* (e2 to e3)<br>*ETV6::RUNX1* (e5 to e3) | *RUNX1::ETV6* (e2 to e3)<br>*ETV6::RUNX1* (e5 to e3) | *EP300::ETV6* (e1 to e3)<br>*ETV6::EP300* (e5 to e2) |
| Breakpoints (RNA) | 1. (chr21:36421139-, chr12:11992074+)<br>2. (chr12:12022903+, chr21:36265260-) | 1. (chr21:36421139-, chr12:11992074+)<br>2. (chr12:12022903+, chr21:36265260-) | 1. (chr22:41489102+, chr12:11992074+)<br>2. (chr12:12022903+, chr22:41513191+) |
| Isoforms (RNA) | *ETV6* (e2 to e6)<br>*IKZF1* (e1 to e8) | *ETV6* (e2 to e6) | *ETV6* (e2 to e6) |
| *ETV6* del (DNA) | 1 copy del, whole gene | No | No |
| *IKZF1* del (DNA) | 1 copy del, exons 2-7 | No | No |
| Other molecular findings (DNA) | *KRAS* p.F156L<br>*KRAS* p.L19F<br>*NRAS* p.G12D<br>*SF3B1* p.E595K | *NFE2* p.Y172H (VUS) | 1 copy del *SETD2* (3p)<br>1 copy del *CUX1* (7q)<br>Gain *RUNX1, ERG, U2AF1* (21q) |

Details of case 4 (Figure 1C) can be found in the *Online Supplementary Table S1* of the total RNA sequencing study (sample ID: 36).[8] 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)(q21.11;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 next generation sequencing testing, also performed at Brigham and Women's Hospital). 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; FISH: fluorescence in situ hybridization.

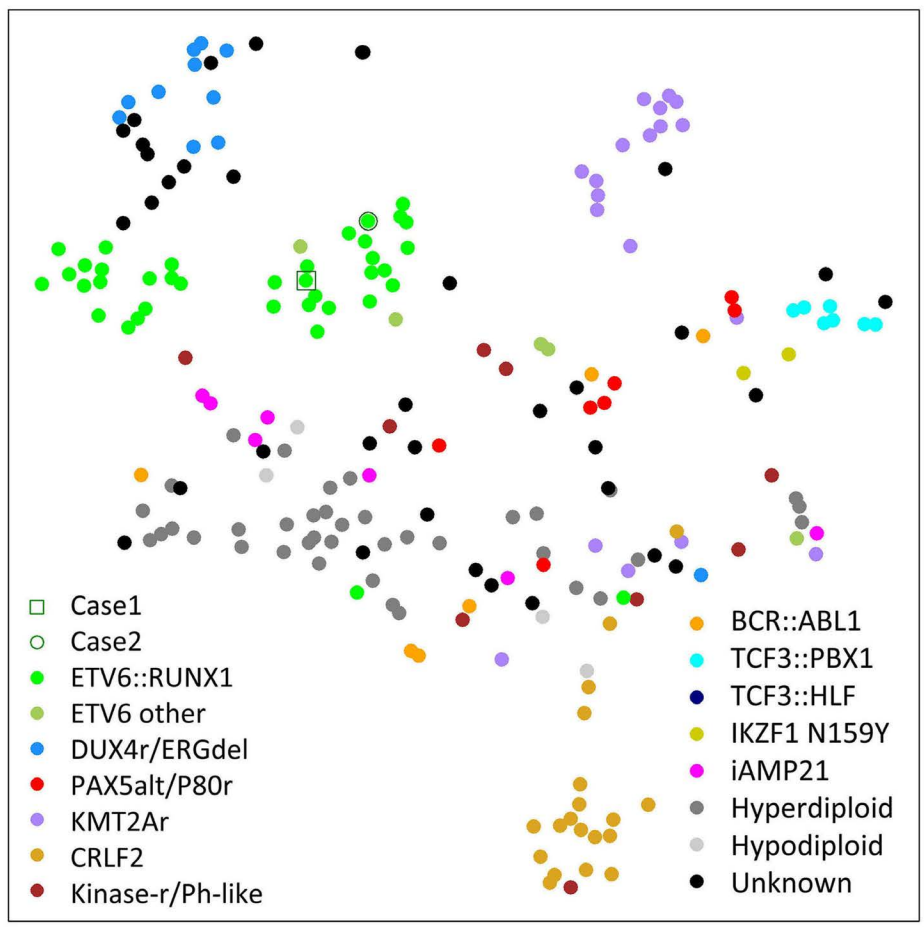
through targeted RNA next generation sequencing (NGS) to harbor *ETV6::RUNX1* fusions characterized by focal insertions of *ETV6* exons 3-5 into *RUNX1* with concomitant intragenic deletions of the same exons from *ETV6*. We further report one pediatric AML case with an *ETV6::EP300* rearrangement similarly 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 2 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.

**A**




**B**

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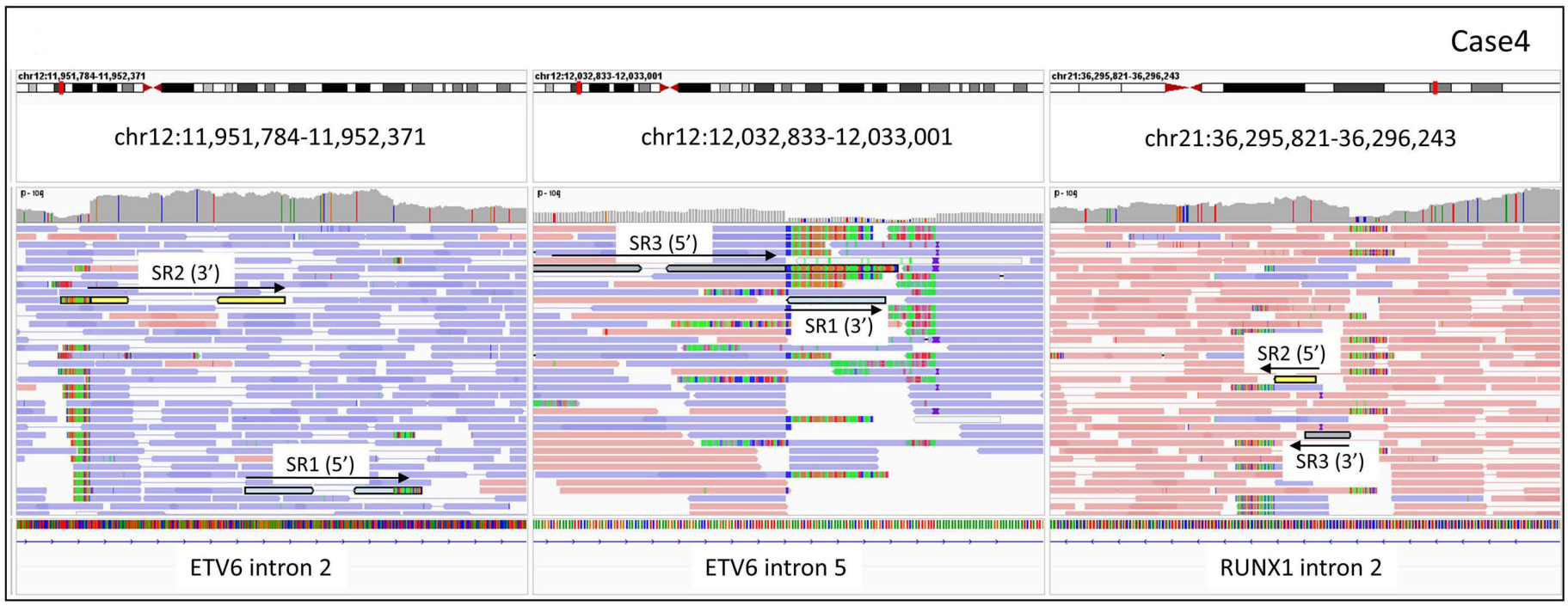


**Figure 1. RNA sequencing results.** (A) *ETV6* exon skipping isoform and *ETV6* fusion transcripts detected on targeted RNA sequencing (FPHeme) over historical cohort (N=474) consisting of cases 1-3 (black square, circle, and triangle) and others (gray x's). Cases 1-3 had outlier expression of the *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 and 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 t-distributed stochastic neighbor embedding (t-SNE) of the targeted transcriptional profiles (100 genes) of cases 1 and 2 among all B-cell lymphoblastic leukemia (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 next generation sequencing. 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* intron 5) 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.

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::ETV*6 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 acute myeloid leukemia (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.[6,8] Targeted DNA NGS showed single copy loss of *ETV6*, thus the exon-skipping isoform was expressed at a high variant allele fraction (95.3%; *data not shown*), similar to case 1 (Figure 1A). Intronic split-read analysis identified three sets of expressed DNA breakpoints derived presumably from

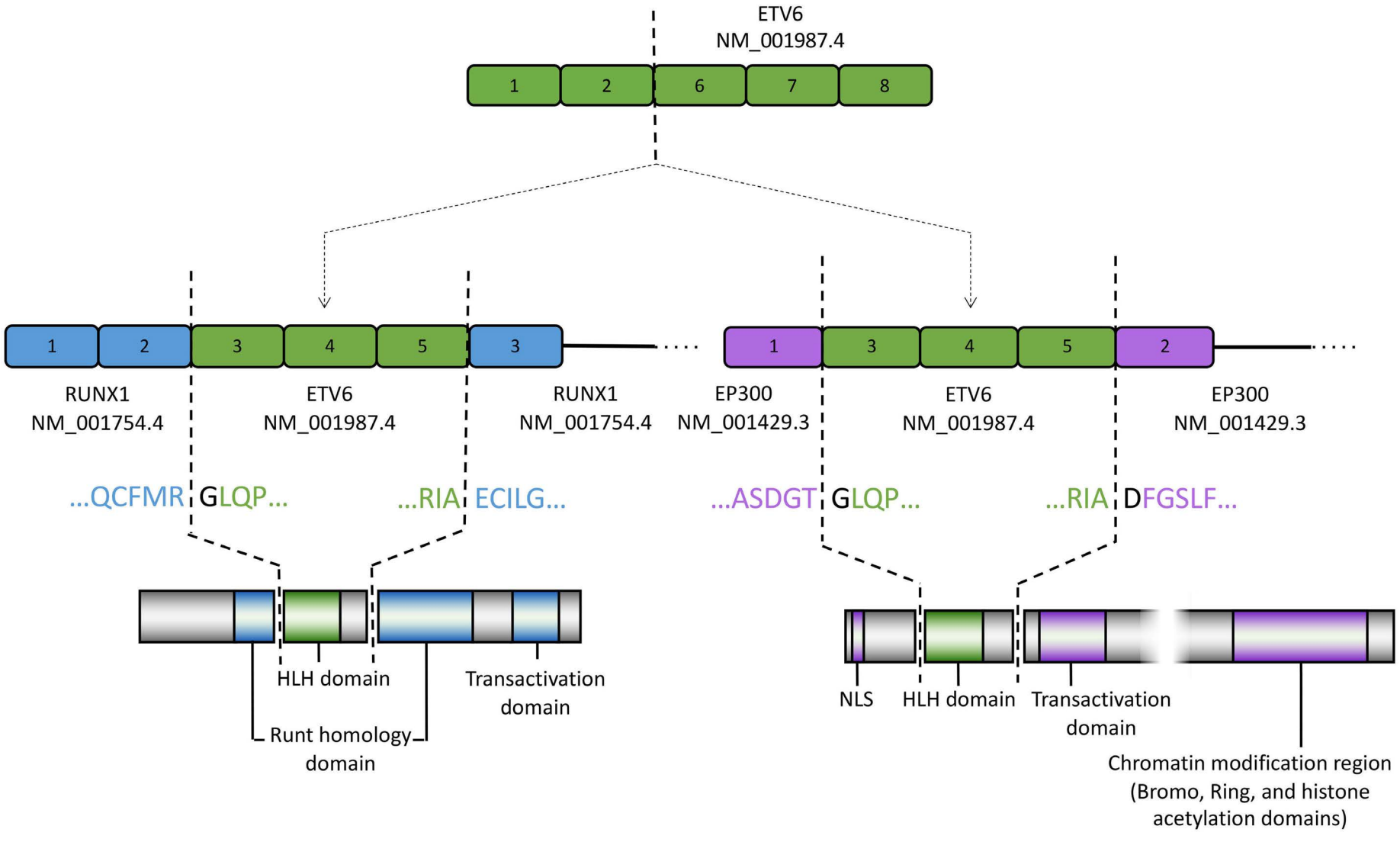


**Figure 2. Schematics of ETV6 insertions.** Structure of the rearrangements detected in cases 1 and 2 (left side) and case 3 (right side) with the predicted chimeric protein products.

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.[9] 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.[3,4] Similarly, although uncommon in pediatric AML, *ETV6* rearrangements are important to identify given their association with adverse risk regardless of fusion partner; chromosome 12p abnormalities/*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 (*clinicaltrials gov. Identifier: NCT04293562*).[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 *ETV6::RUNX1* positive B-ALL in our historical cohort, suggesting functional similarity. An *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 *ETV6* variants and shown to be mediated by microsatellite enhancers ordinarily bound by *ETV6*.[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 *ETV6::EP300* fusion as connecting exons 1-5 of *ETV6* to exons 2-31 of *EP300*. The deletion-insertion pattern has also been described in the context of *YAP1::KMT2A* rearranged sarcomas although screening our cohort did not reveal outlier expression of any *KMT2A* exon-skipping isoforms in either *KMT2A*-rearranged or non-rearranged hematologic cases.[12]

In summary, clinically relevant *ETV6* fusions from focal insertions of *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.

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**Contributions**

SM and HKT designed the study, performed data analysis, and wrote the manuscript. SKT, YP, LBS, and MHH contributed data. All authors edited the manuscript.

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**Data-sharing statement**

Select data is available upon request to the corresponding author.

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