

Germline deletion of *ETV6* in familial acute lymphoblastic leukemia

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Key Points

- *ETV6* germline deletions predispose to familial ALL.
- Germline deletions may be detected by analysis of whole genome and exome data that retain soft-clipped (partially mapped) reads.

Recent studies have identified germline mutations in *TP53*, *PAX5*, *ETV6*, and *IKZF1* in kindreds with familial acute lymphoblastic leukemia (ALL), but the genetic basis of ALL in many kindreds is unknown despite mutational analysis of the exome. Here, we report a germline deletion of *ETV6* identified by linkage and structural variant analysis of whole-genome sequencing data segregating in a kindred with thrombocytopenia, B-progenitor acute lymphoblastic leukemia, and diffuse large B-cell lymphoma. The 75-nt deletion removed the *ETV6* exon 7 splice acceptor, resulting in exon skipping and protein truncation. The *ETV6* deletion was also identified by optimal structural variant analysis of exome sequencing data. These findings identify a new mechanism of germline predisposition in ALL and implicate *ETV6* germline variation in predisposition to lymphoma. Importantly, these data highlight the importance of germline structural variant analysis in the search for germline variants predisposing to familial leukemia.

Introduction

Inherited and acquired alterations of genes encoding hematopoietic transcription factors are central oncogenic events in the pathogenesis of acute lymphoblastic leukemia (ALL).^{1,2} An inherited predisposition for ALL has also been suggested by studies showing that children with affected siblings have a 2 to 4 times increased risk of developing ALL.³ Recently, germline nonsilent mutations in genes, including *TP53*,⁴ *PAX5*,⁵ *IKZF1*,⁶ and *ETV6*,^{7,8} have been identified in both familial and sporadic ALL. However, sequencing of only the coding genome may fail to identify structural germline alterations and noncoding variants that affect gene regulation and predispose to ALL. Here, we present findings from analysis of a large kindred with 7 affected individuals presenting with pre-B-cell ALL, diffuse large B-cell lymphoma (DLBCL), thrombocytopenia, and aplastic anemia.

Methods

Kindred acquisition

Approval for the study was obtained from the South Eastern Sydney Illawarra Area Health Service–Northern Hospital Network Human Research Ethics Committee and the Sydney Children's Hospitals Network Human Research Ethics Committee. The study was approved by the Institutional Review Board of St. Jude Children's Research Hospital and was conducted in accordance with the tenets of the Declaration of Helsinki. Eligible relatives were identified through a detailed family history collected from the proband (V.1; Figure 1) and his

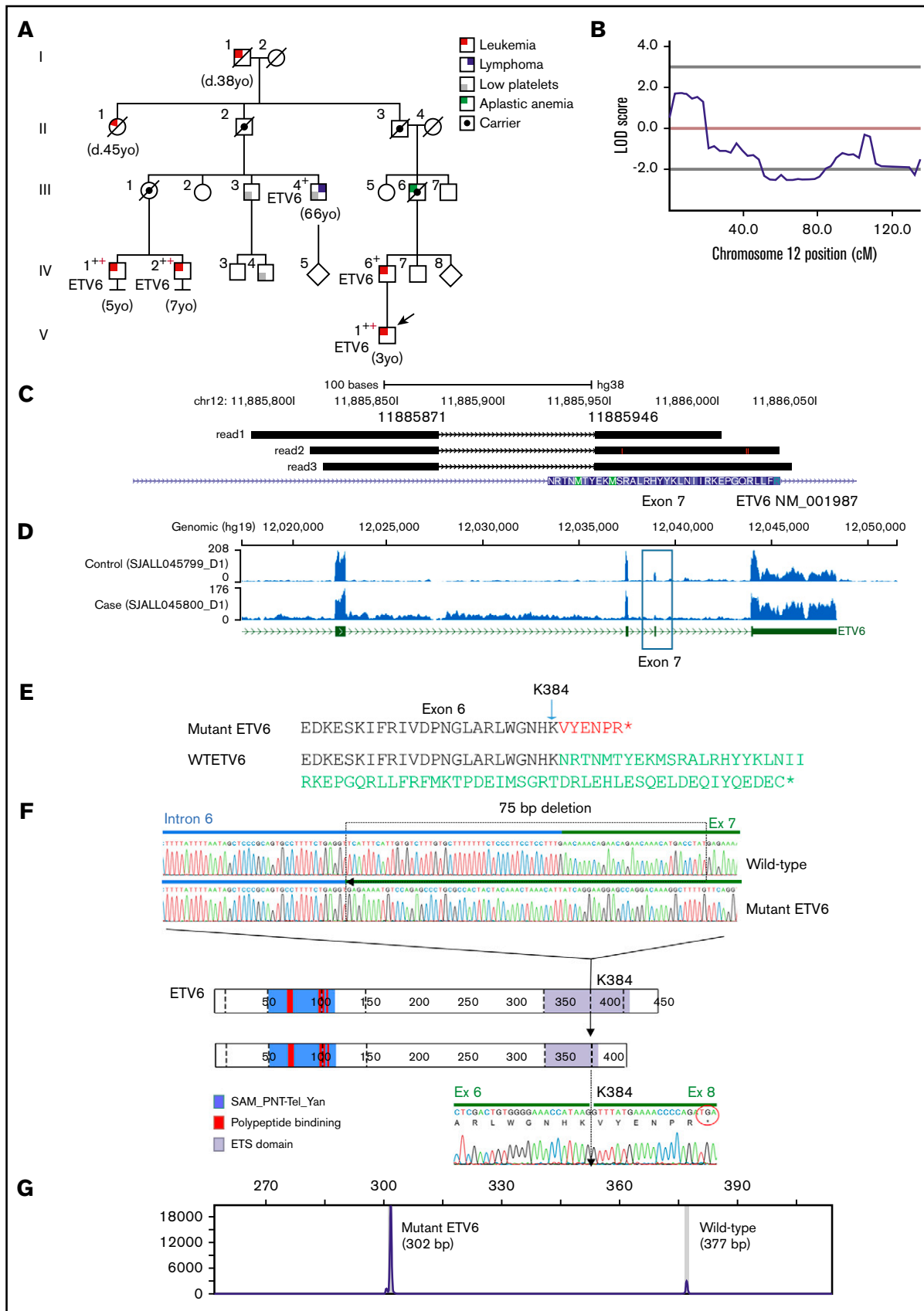


Figure 1.

first-degree relatives. Family members were contacted by phone and/or seen in person, invited to participate, and informed of the study outline, including benefits and risks of participation. Participants who provided verbal consent were sent formal correspondence, and informed written consent was obtained. Participants were asked for a blood sample for a full blood count and germline *ETV6* testing. All genetic testing and full blood count results were communicated back to the recruited individuals.

Nucleic acid preparation and sequencing

DNA used for whole-exome sequencing (WES) or whole-genome sequencing (WGS) were isolated from blood and lymphoma samples using organic methods or Machery-Nagel Nucleobond kits, fluorimetrically quantitated using the Qubit dsDNA BR Assay Kit and the Synergy plate reader (Biotek), and integrity assessed by 0.8% agarose gel electrophoresis. WGS library preparation and sequencing (III.4, IV.1, IV.2, IV.6, and V.1) was performed by HudsonAlpha Genomic Services Laboratory (Huntsville, AL). Uniquely barcoded samples underwent WGS on the HiSeq X10, per standard protocols. Approximately 360 million paired-end reads, each 150 bp in length, were generated for each sample. A mean coverage of 30× was achieved for each sample, resulting in >80% of the genome covered at >20×. For samples tested for the *ETV6* deletion, DNA was isolated using Nucleobond kits (Machery-Nagel).

For transcriptome sequencing, RNA was extracted from cryopreserved leukemic blasts and lymphoma tissue by TRIzol (Life Technologies). RNA was quantitated using the Qubit RNA BR Assay Kit and quality assessed using a RNA Screentape Assay on a 2200 TapeStation (Agilent). Libraries were prepared from total RNA with the TruSeq Stranded Total RNA Library Prep Kit according to the manufacturer's instructions (Illumina). Libraries were analyzed for insert size distribution on a 2100 BioAnalyzer High Sensitivity kit (Agilent Technologies) or Caliper LabChip GX DNA High Sensitivity Reagent Kit (PerkinElmer). Libraries were quantified using the Quant-iT PicoGreen ds DNA assay (Life Technologies) or low-pass sequencing with a MiSeq nano kit (Illumina) and sequenced with paired-end 100-bp setting on HiSeq 4000 (Illumina). The 30× coverage of exon bases was 35% for the ALL sample and 43% for the DLBCL sample.

For WES, genomic DNA was quantified using the Quant-iT PicoGreen assay (Life Technologies) and normalized to 50 ng per sample prior to library generation. Libraries were prepared using the Nextera Rapid Capture kit according to the manufacturer's instructions (Illumina). The resulting libraries were quantified using the Quant-iT PicoGreen assay, as well as analyzed for insert size distribution on a 2100 BioAnalyzer High Sensitivity kit (Agilent Technologies) or Caliper LabChip GX DNA

High Sensitivity Reagent Kit (PerkinElmer). The libraries were then combined in 12-plex maximum pools for sequencing by the Genome Sequencing Facility of the Hartwell Center for Biotechnology and Bioinformatics of St. Jude Children's Research Hospital. More than 90% of the exon bases have coverage of >30× for all samples.

Mapping, variant identification, and annotation

We used the Genome Analysis ToolKit for mapping and variant calling,⁹ based on the GRCh38 genome assembly. Resulting variant call format files were annotated using the Ensembl variant effect predictor.¹⁰ For germline copy-number variant and structural variant (SV) detection, we used CONCERTING¹¹ and CREST,¹² respectively.

Somatic genomic analysis

Single-nucleotide variant (SNV)/ insertion-deletion mutation (indel) calling and filtering was done using a previously reported pipeline.¹³ Briefly, the Genome Analysis ToolKit UnifiedGenotyper module was used to identify SNVs and indels from leukemia and germline samples, with common single-nucleotide polymorphisms/indels reported in dbSNP v142 and germline mutations detected from matched germline control samples removed. All the nonsilent SNVs/indels yield from the filtering pipeline were manually reviewed and only the highly reliable somatic ones were reported. Gene fusions was detected using FusionCatcher run on the raw FASTQ files.¹⁴

Linkage analysis

Prior to performing linkage analysis, we conducted Mendelian error checks using Pedstats.¹⁵ Variants with Mendelian errors were deleted from further analysis. The remaining markers were pruned with PLINK software¹⁶ using pairwise $r^2 < 0.1$ in sliding windows of 50 SNVs, moving in intervals of 5 SNVs. This resulted in a final exome-wide marker set of 147 558 SNVs, which we used for parametric linkage analysis using the Merlin software.¹⁷ We assumed an affected-only model with a disease allele frequency of 0.0001 and penetrance of 0.9. Individual III.4 was included in this analysis for phasing purposes alone, with no contribution from his disease status.

Variant filtering

All variants were filtered for minor allele frequency (MAF) against reference cohorts in the Exome Aggregation Consortium.¹⁸ Variants with MAF < 0.01 that were also classified by variant effect predictor as frameshift, nonsense, splice, or missense were then checked for evidence of sharing across 3 affected relatives (IV.1, IV.2, and V.1) with WES data and 4 affected relatives with ALL diagnoses and available WGS (IV.1, IV.2, IV.6 and V.1). Variants within *ETV6*, regardless of annotations, were checked for sharing across affected relatives.

Figure 1. Linkage mapping to chromosome 12 in a kindred with ALL and DLBCL. (A) A 5-generation kindred with 10 individuals with leukemia, DLBCL, aplastic anemia, and/or thrombocytopenia. Black crosses indicate samples subjected to WGS, and red crosses indicate samples subjected to WES. Squares and circles represent male and females, respectively. All family members with ALL or DLBCL sequenced harbored the *ETV6* deletion. Individuals III.3 and III.4 (DLBCL) both had thrombocytopenia and the deletion; IV.3 and III.2 had normal platelet counts and no *ETV6* deletion. (B) Multipoint linkage results highlighting region with a logarithm of the odds (LOD) score of 1.8. (C) Representative reads showing the exon 7 splice site deletion identified by WGS SV analysis (NM_001987.4:G.11885871_11885946del;NM_001987.4:c.1153-55_1173del). (D) RNA-sequencing coverage analysis showing reduced sequence depth at exon 7. (E) Protein truncation resulting from exon skipping. (F) Schematic representation of the *ETV6* protein, including the sterile α motif (SAM)/pointed domain of Tel/Yan protein, the polypeptide-binding domain, and the erythroblast transformation-specific domain (ETS). The top pair of electropherograms is from genomic PCR and Sanger sequencing and compares wild-type (WT) and deleted (75-bp) *ETV6*. Dotted line in the *ETV6* cartoon represents exon junctions. The lower electropherogram is from RT-PCR and Sanger sequencing and shows the skipping of exon 7 and splicing of exon 6 to exon 8 in tumor RNA. (G) Results from fragment size analysis showing amplification of both WT and deleted *ETV6* alleles.

Table 1. Platelet counts for family members

Patient	ETV6 status	Platelet count, × 10 ⁹ /L	Reference range
III-2	Negative	295	187-415
III-3	Positive	67	187-415
III-4	Positive	80	187-415
III-5	Negative	230	187-415
III-7	Negative	269	150-400
IV-3	Negative	245	187-415
IV-4	Positive	108	187-415
IV-6	Positive	183	187-415
IV-7	Negative	243	150-450
V-1	Positive	201	187-415

Three individuals (in bold) showed thrombocytopenia. Data are not available for IV-1 and IV-2

Genomic and RT-PCR of *ETV6*

Genomic polymerase chain reaction (PCR) was performed using Phusion High-Fidelity DNA Polymerase (M0530S NEB), 5× Phusion Buffer, 10 mM deoxyribonucleotide triphosphate, 10 μM forward primer (C5723: 5'-GGAGTAAACCTTGGTGACAGTGAAT-3'), 10 μM reverse primer (C5724: 5'-CTCCCCGTTATTTAAA-GAAAACAGC-3'), and template DNA. Reverse transcription (RT)-PCR was performed using Phusion High-Fidelity DNA Polymerase

(M0530S NEB), 5× Phusion Buffer, 10 mM deoxyribonucleotide triphosphate, 10 μM primers (C5732: 5'-CACTCCGTGGATTCAAACAGTCC-3' and C5733: 5'-TACTAACAACGGTGAAGGGTGAAG-3'), and complementary DNA template. For fragment size analysis, genomic DNA was amplified as described above with the forward primer being conjugated with a fluorescent dye (6-carboxyfluorescein) at its 5' end, and amplicons were analyzed by automated capillary gel electrophoresis. The results were plotted with AbiPrism GeneMapper v5 software (Applied Biosystems). The GeneMapper electropherograms displayed information about transcript length, peak height, and peak area.

Gene set enrichment and pathway analysis

Gene expression was quantified using RSEM¹⁹ on STAR²⁰-mapped BAM files. The gene ranks comparing the DLBCL sample and ALL sample were based on the log₂ ratio of gene fragments per kilobase of transcript per million mapped read values with addition of 1. The *GseaPreranked* module of gene set enrichment analysis²¹ was used with the rank file to explore the gene set collection of MSigDB and homemade leukemia gene sets.

Results

Description of kindred with ALL and DLBCL

The ages at diagnosis were 3 to 7 years for confirmed B-cell ALL, 38 to 45 years for leukemia not otherwise specified, and 66 years for DLBCL (Figure 1A). Three individuals exhibited chronic mild

Table 2. Rare coding variants identified by WES shared among 3 ALL-affected relatives

Gene	Chromosome	Position (HG19)	AA change	REF/ALT	mRNA_accession	Class	MAF	In COSMIC	Gene list*
<i>NBPF1</i>	1	16902844	D679E	G/T	NM_017940	Missense			
<i>NOTCH2</i>	1	120611964	C19W	G/C	NM_024408	Missense		Yes	Yes
<i>PDE4DIP</i>	1	144915561	R622*	G/A	NM_014644	Nonsense			Yes
<i>TLR5</i>	1	223284214	S720R	A/T	NM_003268	Missense	0.005		
<i>MUC6</i>	11	1016662	P2047S	G/A	NM_005961	Missense			
<i>MUC5B</i>	11	1267049	T2980M	C/T	NM_002458	Missense	0.002		
<i>SORL1</i>	11	121384931	N371T	A/C	NM_003105	Missense	0.002		
<i>CD163L1</i>	12	7522073	T1307A	T/C	NM_174941	Missense	0.004		
<i>GDF3</i>	12	7842773	R266C	G/A	NM_020634	Missense	0.003		
<i>ZFYVE26</i>	14	68274585	N139S	T/C	NM_015346	Missense			
<i>CDC27</i>	17	45234303	A273G	G/C	NM_001114091	Missense		Yes	
<i>FKRP</i>	19	47259134	R143S	C/A	NM_001039885	Missense	0.007		
<i>ZNF814</i>	19	58385748	A337V	G/A	NM_001144989	Missense		Yes	
<i>APOB</i>	2	21245890	P877A	G/C	NM_000384	Missense			Yes
<i>XDH</i>	2	31562482	P1216H	G/T	NM_000379	Missense	0.002		
<i>MUC4</i>	3	195486102	R4960H	C/T	NM_018406	Missense	0.006		
<i>MUC7</i>	4	71347171	A237V	C/T	NM_001145006	Missense			
<i>FNDC1</i>	6	159687181	R1784W	C/T	NM_032532	Missense	0.008		
<i>HGC6.3</i>	6	168377029	P102S	G/A	NM_001129895	Missense			
<i>LOC100288524</i>	7	331427	N225K	C/A	NM_001195127	Missense			
<i>PRSS1</i>	7	142460335	K170E	A/G	NM_002769	Missense		Yes	
<i>SOX7</i>	8	10583367	A350S	C/A	NM_031439	Missense	0.001		

COSMIC, Catalogue Of Somatic Mutations In Cancer; mRNA_accession, RefSeq accession number for the mRNA transcript used for amino acid annotation; REF/ALT, reference allele/alternative allele.

*In-house curated list of cancer-predisposing genes.

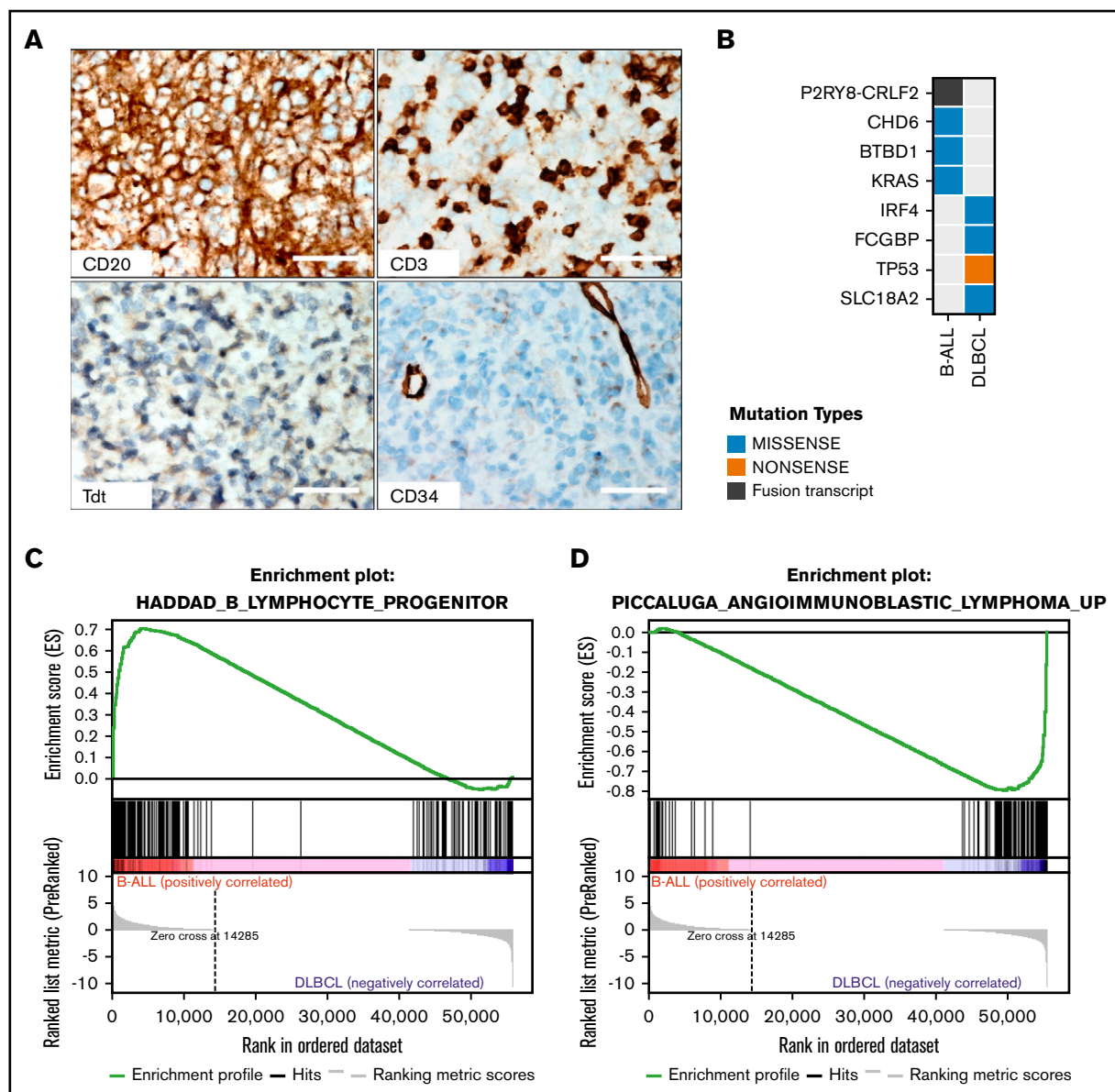


Figure 2. Characterization of tumors in the *ETV6*-mutated kindred. (A) Immunohistochemistry of the DLBCL tumor is consistent with mature B-cell lineage, with expression of CD20 and lack of expression of the immature markers CD34 and Tdt and the T-cell marker CD3, which highlights admixed small T cells (original magnification $\times 40$; scale bars, 50 μm). (B) Mutational analysis of WGS data showing distinct ALL and DLBCL mutational spectra of each sample. (C) Gene set enrichment analysis (GSEA) from RNA sequencing of tumor sample showing enrichment of B lymphoid progenitor genes in the B-cell ALL sample (D) and genes expressed in lymphoma in the DLBCL sample. Collectively, the pathologic and genomic features support a true DLBCL in case III.4 rather than a lymphomatous presentation of ALL. Tdt, terminal deoxynucleotidyltransferase.

thrombocytopenia (Table 1), and 1 had aplastic anemia. Samples were collected after ≥ 2 years of remission. Using WES and WGS, we performed genome-wide linkage analysis and targeted analysis of germline SNVs, indels, and SVs to identify the putative cause of ALL in this family.

Identification of a germline deletion of *ETV6* by integrated linkage and sequencing

We first screened for rare coding (frameshift, nonsense, and missense) and splice site mutations from WES data from germline samples in 3 members of the pedigree (indicated by red crosses

in Figure 1A). This analysis revealed 22 variants shared among the 3 affected members (Table 2), none of which were likely to have a role in leukemogenesis. Using a linkage disequilibrium-pruned subset of the variants from WES, we then performed linkage analysis to determine whether we could, in an unbiased manner, identify genomic regions shared by the affected family members. This analysis identified a 19-cM region on chromosome 12p with a peak multipoint linkage score of 1.8 (Figure 1B). Importantly, this region alone achieved a logarithm of the odds score of > 1 from the genome-wide scan. Examination of the linkage region revealed the presence of *ETV6*, a known ALL predisposition gene. Given that no

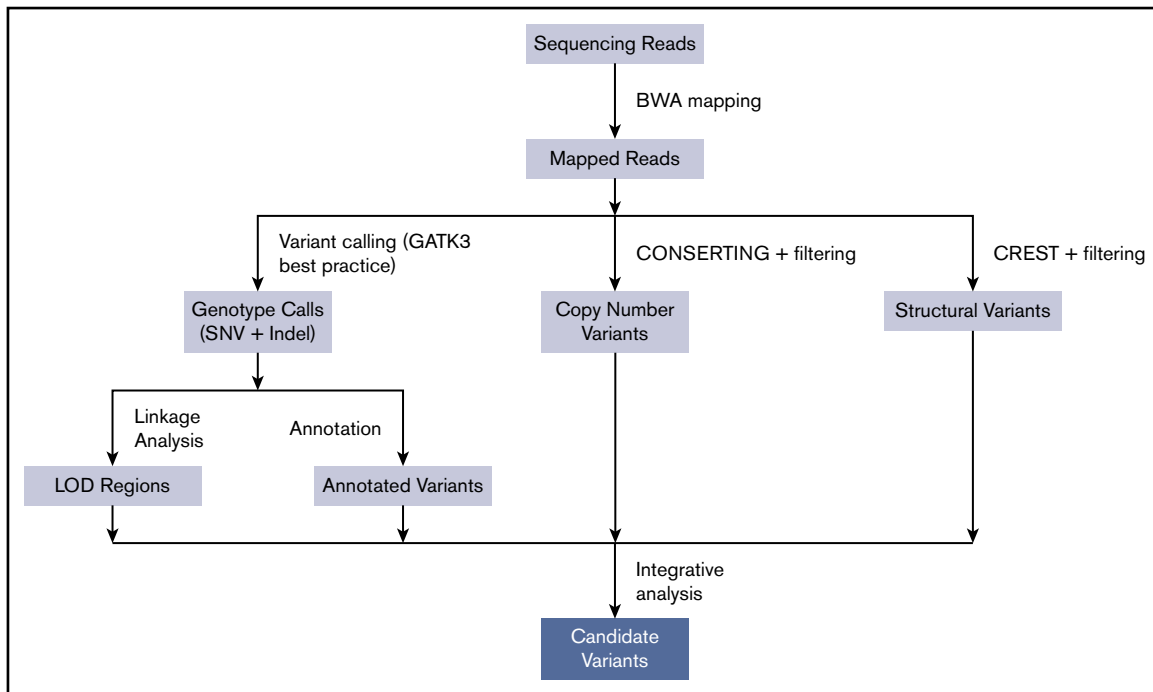


Figure 3. Workflow showing optimal practices for identification of germline variants predisposing to familial leukemia, incorporating analysis of soft-clipped reads. BWA, Burrows-Wheeler Aligner; LOD, logarithm of the odds.

coding or splice mutations in *ETV6* had been identified in our WES data, we postulated that a noncoding variant in *ETV6* might be the underlying driver of the linkage peak.

Recurrence testing and deletion verification

To identify noncoding as well as coding variations in the region of linkage, we performed WGS of nontumor DNA in 5 of the affected family members with leukemia or lymphoma and available material (indicated by black crosses in Figure 1A). No SNVs/indels within noncoding regions of *ETV6* were shared by all 5 affected relatives or by the 4 relatives with ALL. We next analyzed the region of linkage for SVs using the CREST algorithm¹² and identified a 75-nt germline deletion (NM_001987.4: G.11885871_11885946del) at the intron 6–exon 7 junction of *ETV6* shared by all affected individuals (Figure 1C). The presence of this deletion was supported by soft-clipped reads, in which only part of the read sequence mapped to a single genomic location (Figure 1C-E). Typically, a deletion of this size would not be detected when sequence data are hard-clipped, resulting in loss of noncontiguously mapped sequence, for quality control purposes.^{22,23} The evidence supporting an SV obtained from analysis of from soft-clipped reads by CREST or other alternative SV detection algorithms highlights the utility of this information that may be lost in alternate WGS/WES mapping and analysis approaches.

Using genomic PCR and Sanger sequencing, we experimentally verified the 75-bp deletion which comprised 54 nt in intron 6 and 21 nt in exon 7 of *ETV6* in the proband (Figure 1F). Reverse transcription of RNA followed by PCR demonstrated that the deletion resulted in skipping of exon 7, a frameshift and premature stop codon in exon 8, resulting in a truncated *ETV6* transcript (Figure 1G). We performed screening of the family using PCR

and identified 2 further members of the kindred who had chronic thrombocytopenia and carried the mutation (Figure 1A). Subsequently, we screened an additional 4500 ALL patients from a previous analysis of germline variants in ALL⁸ and 4200 pediatric cancer patients studied by the Pediatric Cancer Genome Project²⁴ and St. Jude Life study²⁵ using WES data and analysis of soft-clipped reads. No additional cases with *ETV6* germline deletions were identified, indicating that the deletion is rare and possibly private to this kindred. We next reanalyzed the WES data from this kindred using optimal SV analysis approaches and, remarkably, observed that remapping of the WES data using algorithms that preserve soft-clipped reads recapitulated identification of the deletion owing to its location at the intron 6–exon 7 boundary that is captured by the exome bait (data not shown).

Genomic profiling of ALL and DLBCL

Germline *ETV6* alterations are known to predispose to platelet defects and ALL,^{7,8,26,27} but have not been reported to predispose to more mature lymphoid neoplasms.²⁸ In light of the observation of DLBCL in addition to ALL in carriers of the germline *ETV6* deletion in this kindred, we next examined the pathologic characteristics and genomic alterations in tumor samples from individuals in the pedigree with ALL and DLBCL who had available material by immunohistochemistry, WGS, and transcriptome sequencing. The DLBCL (III.4) tumor cells were positive for CD20 and negative for CD3, CD34, and terminal deoxynucleotidyltransferase (Figure 2A), consistent with this sample being DLBCL rather than B lymphoblastic lymphoma. The B-cell ALL tumor from the proband (Figure 1A, V.1) exhibited high hyperdiploidy; *P2RY8-CRLF2* rearrangement; mutations of *CDH6*, *BTBD1*, and *KRAS*, but not

somatic alteration of *ETV6*; and enrichment for the gene expression signature of Ph-like ALL and B lymphoid progenitors (data not shown and Figure 2B-C). These data are consistent with prior observations that individuals with ALL harboring germline *ETV6* mutations are enriched for hyperdiploidy⁸ and that *P2RY8-CRLF2*, a rearrangement that deregulates JAK-STAT signaling common in Ph-like ALL, is also observed in B-cell ALL with high hyperdiploidy.²⁹ RNA sequencing and gene expression profiling of the DLBCL sample showed enrichment for genes upregulated in lymphoma (Figure 2D) also supporting the notion that this sample is representative of typical DLBCL rather than lymphoblastic pre-B-cell lymphoma and that the germline *ETV6* mutation may also predispose to the development of lymphoid malignancies more mature than ALL. Interestingly, a deletion that removed exon 2 of *ETV6* and was inherited from mother to child had been previously identified through single-nucleotide polymorphism array analysis in a nonsyndromic ALL patient.³⁰ This supports the need for SV detection algorithms or DNA microarray integration studies of similar patients.

Discussion

Through integrated linkage mapping and structural variation analysis in a large kindred of B-cell ALL and DLBCL, we identified a germline deletion that causes exon 7 skipping and protein truncation of *ETV6*. This is the first report of a potentially pathogenic germline SV in *ETV6* in familial ALL and potentially extends the known importance of *ETV6* germline variants in predisposition to lymphoid malignancies. The observation of DLBCL in a carrier of the variant suggests that *ETV6* alterations predispose to lymphoma, but this single case warrants further analysis of additional DLBCL cases and kindreds. Importantly, the use of a tool that utilizes soft-clipped (partially mapped) reads was able to identify the variant initially in WGS data but also upon reanalysis of WES data. This is now part of our routine analysis (Figure 3). These findings demonstrate the utility of WGS to identify SVs predisposing to cancer and the potential for optimal analysis of WES data to identify SVs, particularly when the at least 1 boundary of the SV falls in the region of the exome capture bait.

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Authorship

Contribution: D.G.H., K.O., C.Q., E.R., V.J., and G.W. analyzed data; R.S.M., K.A.S., R.S., K.T., G.C.-T., M.T., M.W., and D.S.Z. provided patient samples and clinical data; M.L.C., I.I., and D.P.-T. performed laboratory assays; V.L. performed pathologic analyses; C.G.M. and E.R. wrote the manuscript; and C.G.M. oversaw the study.

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