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ARTICLE

Uncovering the Genomic Landscape in Newly Diagnosed and Relapsed Pediatric Cytogenetically Normal *FLT3*-ITD AML

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Fms-like tyrosine kinase 3 (*FLT3*) internal tandem duplication (ITD) mutations, common in pediatric acute myeloid leukemia (AML), associate with early relapse and poor prognosis. Past studies have suggested additional cooperative mutations are required for leukemogenesis in *FLT3*-ITD+ AML. Using RNA sequencing and a next-generation targeted gene panel, we broadly characterize the co-occurring genomic alterations in pediatric cytogenetically normal (CN) *FLT3*-ITD+ AML to gain a deeper understanding of the clonal patterns and heterogeneity at diagnosis and relapse. We show that chimeric transcripts were present in 21 of 34 (62%) of *de novo* samples, 2 (6%) of these samples included a rare reoccurring fusion partner *BCL11B*. At diagnosis, the median number of mutations other than FLT3 per patient was 1 (range 0–3), which involved 8 gene pathways; *WT1* and *NPM1* mutations were frequently observed (35% and 24%, respectively). Fusion transcripts and high variant allele frequency (VAF) mutants, which included *WT1*, *NPM1*, *SMARCA2*, *RAD21*, and *TYK2*, were retained from diagnosis to relapse. We did observe reduction in VAF of simple or single mutation clones, but VAFs were preserved or expanded in more complex clones with multiple mutations. Our data provide the first insight into the genomic complexity of pediatric CN *FLT3*-ITD+ AML and could help stratify future targeted treatment strategies.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Pediatric acute myeloid leukemia (AML) is a heterogeneous disease with a relatively low number of somatic mutations. Other pediatric cancers are known for the presence of chimeric transcripts. Few studies have looked at disease progression within pediatric AML.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This study addresses the question about the clonal heterogeneity between diagnosis and relapse in a cohort of cytogenetically normal Fms-like tyrosine kinase 3 (*FLT3*)-internal tandem duplication (ITD)-positive pediatric AML, as well as chimeric transcripts present in this population with hopes of gaining insight into therapy shortcomings.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ There is a knowledge gap about childhood AML disease progression between diagnosis and relapse. This study specifically enhances our knowledge about the stability of pediatric *FLT3*-ITD disease progression from diagnosis to relapse and lack of clonal resolution.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ This study highlights the therapy shortcomings in treatment of *FLT3*-ITD, an aggressive form of AML. It promotes further investigation into novel therapies strategies that may be more active against this aggressive form of AML.

Pediatric acute myeloid leukemia (AML) is a rare heterogeneous disease that accounts for 30% of all childhood leukemia.[1,2] It is distinct from the adult counterpart in both their genomic alterations and therapeutic response.[3] However, in both settings, pediatric and adult AML have relatively low somatic mutation burden compared with other tumor types.[4,5] Fms-like tyrosine kinase 3 (*FLT3*) internal tandem duplication (ITD) mutations are among the most common

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mutations in AML occurring in ~10–15% of pediatric and 30% of adult AML.[2] Within cytogenetically normal (CN) AML, *FLT3*-ITD is present in 18% of the pediatric and ~50% of the adult cases.[2] *FLT3*-ITD–positive AML is considered to be an aggressive disease with high risk of early relapse and decreased survival for both the pediatric and adult populations.[6–9] *FLT3*-ITD in cellular models leads to a proliferation and survival advantage.[10] Given the poor prognostic impact and growth advantage of *FLT3*-ITD, it makes it an interesting target therapeutically. A number of clinical trials have been published and others are underway investigating the prognostic outcomes of targeting FLT3 in AML.[11] However, the introduction of *FLT3*-ITD alone into murine models is insufficient to recapitulate the AML phenotype,[12,13] showing additional cooperative events are needed. Previously, Zwaan *et al*.[14] revealed that in newly diagnosed pediatric AML, *FLT3*-ITD mutations may co-occur with *MLL*-PTD, *NUP98-NSD1* fusions, and *WT1*, or *NPM1* mutations. In a study of 20 children with varying subtypes of AML, of note including only one sample that was CN and *FLT3*-ITD+, it was observed that the dominant variants/clones within the heterogeneous population persist from diagnosis to relapse, whereas subclonal variants, even established drivers, can be lost at relapse.[15] Relapse is thought to be driven by the selection or emergence of resistant subclones.[16,17] Despite this progress, studies evaluating the underlying biology and identification of candidate genes contributing to *de novo* and relapsed pediatric CN *FLT3*-ITD–positive AML have been largely under-reported in the literature.

The objective of the present study was to gain a deeper understanding of the clonal heterogeneity between diagnosis and relapse in larger cohort of pediatric patients with CN *FLT3*-ITD–positive AML, with hopes of gaining insight into therapy shortcomings. Given the low somatic mutational burden reported for AML, samples were sequenced by RNASeq[18] and a next-generation sequencing gene panel consisting of 80 genes[19] for chimeric transcripts and co-occurring mutations, respectively.

METHODS

Please refer to **Supplemental Materials and Methods** for more detailed protocols.

Patients and samples

Leukemic blasts from either diagnosis or relapse were collected from 37 individual patients (**Figure 1a**) with CN *FLT3*-ITD–positive AML that were treated on AML02 (ClinicalTrials.gov Identifier: NCT00136084; $N = 18$),[20] AML08 (NCT00703820; $N = 13$), RELHEM (NCT00908167; $N = 3$),[21] AML02/RELHEM ($N = 1$), or AML08/RELHEM ($N = 2$) clinical trials at St. Jude Children's Research Hospital. The median age was 12.4 years (range 2.7–19.2 years) with 11 adolescents (defined as 15–21 years), 25 children (3–14 years), and 1 toddler/infant (<3 years). At diagnosis, the median *FLT3*-ITD allelic ratio (AR) was 0.69 (range 0.04–18.15), median ITD length was 48 base pairs (range 21–183 bp), and median unique FLT3-ITD sequences was 1.5 (range 1–5). A summary of patient demographics at diagnosis are listed in **Table 1**, and characteristics of samples and analyses performed are listed in **Table S1**.

RNA sequencing and targeted gene panel

RNA library construction and analysis for RNA sequencing (RNASeq) has been described previously.[18] The RNASeq coverage average was 57X. Eighty protein-coding genes[19] were sequenced for mutation status using targeted amplicon sequencing with the MiSeq platform (Illumina). DNA libraries were prepared and analyzed as previously described by Eisfeld *et al*.[19] The average targeted gene panel coverage was 775X.

Confirmation of fusions and breakpoints

The RNASeq data were run through CREST[22] for identification of chimeric transcripts. The cDNA from samples identified to contain a chimeric transcript were analyzed by polymerase chain reaction to confirm the presence of fusions or breakpoints using the primers indicated in **Table S2**.

Statistical analysis

The Kaplan–Meier method was used to estimate the distribution of event-free survival (EFS), defined as the time elapsed from protocol enrollment until relapse, second malignancy, or death with times for subjects having none of these events censored at last follow-up. Overall survival (OS) was defined as the time elapsed from protocol enrollment to death with times for living subjects censored at last follow-up. The Cox proportional hazards regression model was used to evaluate the association of EFS with the *NUP98-NSD1* fusion, the *WT1* mutation, *NPM1* mutation, or *FLT3* AR (dichomotomized as <0.4 or ≥ 0.4). The *FLT3*-ITD AR has been previously published as a prognostic indicator, with AR $\geq$ 0.4 significantly affecting progression-free survival and relapse.[23]

RESULTS

Characterization of pediatric *de novo* CN *FLT3*-ITD–positive samples

Chimeric transcripts. Fusion genes represent a distinguishing feature of pediatric cancer, specifically leukemia.[3,18] Thus, we investigated the transcriptome from newly diagnosed CN *FLT3*-ITD–positive AML for potential fusion transcripts. Nineteen of 34 (56%) samples contained a previously described chimeric transcript (**Figure 1b, Table S3**) including: *MLL*-PTD ($n = 3$), *NUP98-NSD1* ($n = 14$), *CBFB-MYH11* ($n = 1$), and *DEK-NUP214* ($n = 1$), which were confirmed by real-time polymerase chain reaction and Sanger Sequencing. Two samples (6%) contained an in-frame fusion partner, *BCL11B* (B-cell chronic lymphatic leukemia/lymphoma B) with either of the transcriptional genes *RUNX1* or *ZEB2* (**Figure 1b, Table S3**). For both fusions, the breakpoint in BCL11B was at amino acid 21, thus allowing the fusions to maintain the six zinc finger domains found within BCL11B but losing the Friend of GATA (FOG) repression domain. The *ZEB2-BCL11B* fusion also contain the N-terminal ZEB2 nucleosome remodeling/deacetylase-interaction motif. RNA expression verified that *BCL11B* was expressed >40-fold when these fusion were present (**Figure 1c**). It was observed that when BCL11B was overexpressed in T-ALL cells, it triggers a chemo-resistance phenotype to etoposide.[24] Thus,

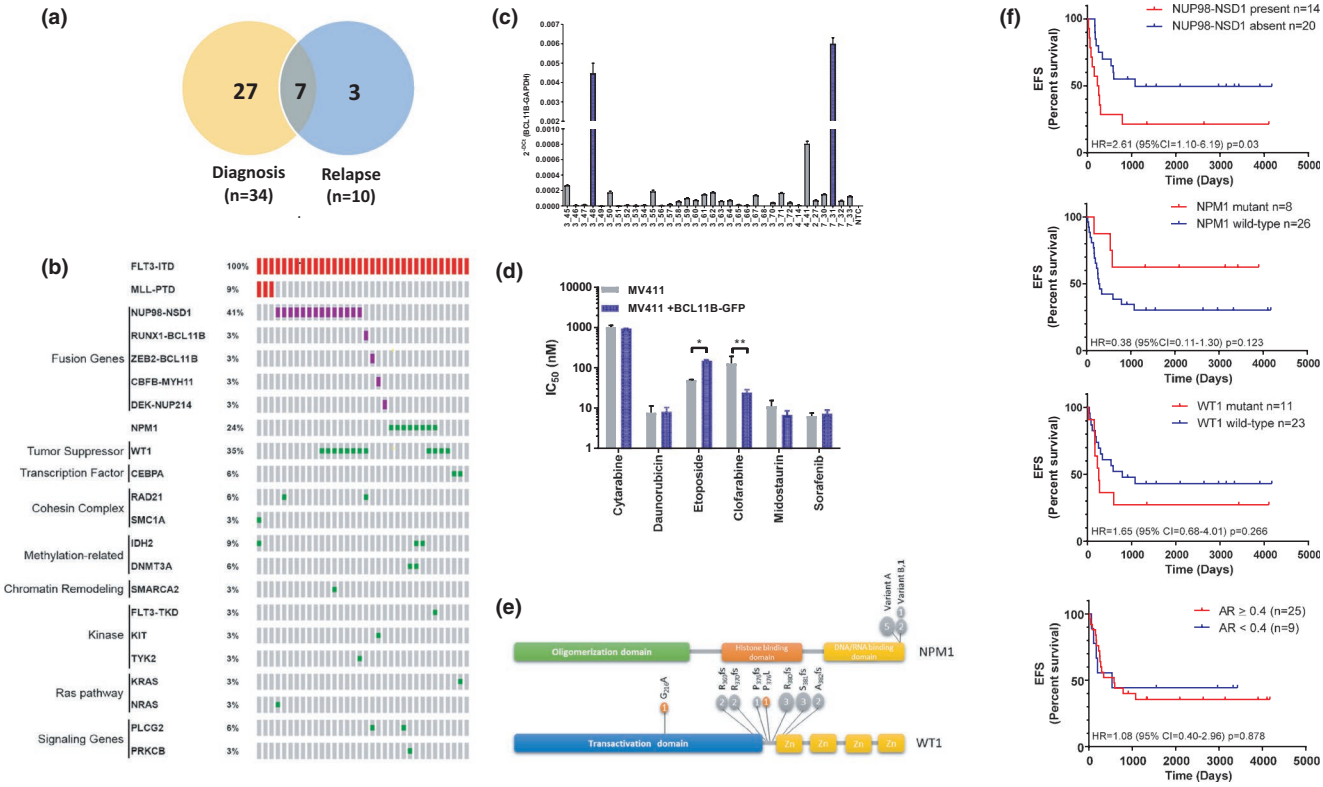

Figure 1 Characterization of *de novo* Fms-like tyrosine kinase 3 (*FLT3*) internal tandem duplication (ITD)-positive samples. (**a**) Venn diagram of patients' samples analyzed in this study. Matched samples are depicted by overlapping circles. (**b**) OncoPrint of duplications (red), fusion genes (purple), and individual mutations (indels and missense both in green) detected by RNASeq or targeted gene panel. (**c**) Real-time polymerase chain reaction quantifying *BCL11B* transcripts in *de novo* patient samples. Transcript levels are shown as $2^{-\Delta C_t}$ after standardization to *GAPDH*. (**d**) MV411 cells or MV411 cells expressing BCL11B-GFP were treated for 72 hours with dimethylsulfoxide or increasing concentrations of the indicated drug for 72 hours, and cell viability was measured by 3-(4 ,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Half-maximal inhibitory concentration (IC_{50}) measurements represent three experiments with six replicates each (n = 18). *P = 0.0096, **P < 0.0001. (**e**) Lollipop plots showing domain structure, and mutation locations within *WT1* and *NPM1*. Grey circles denote insertions or deletions, and orange circles denote missense mutations. Number of mutations at each location is indicated within the circle. (**f**) Kaplan–Meier plots show the rates of event-free survival (EFS) of 34 children and adolescents with *de novo FLT3*-ITD–positive acute myeloid leukemia (AML) with or without *NUP98-NSD1*, *NPM1*, or *WT1* and allelic ratio (AR) ≥ 0.4.

we sought to determine if overexpression of *BCL11B* in *FLT3*-ITD–positive cell lines would trigger a similar response. When *BCL11B* was overexpressed in a pediatric *FLT3*-ITD–positive cell line, MV4-11, there was no increase in growth rate (**Figure S1**). Cells were less sensitive to etoposide (half-maximal inhibitory concentration (IC_{50}) 149 nM vs. 50 nM, (P = 0.0096) but had an increase in sensitivity to clofarabine (IC_{50} 25 nM to 127 M, P < 0.0001). No altered sensitivity was seen for cytarabine, daunorubicin, or two FLT3 inhibitors midostaurin and sorafenib (**Figure 1d, Figure S1**).

Co-occurring mutations. Low somatic mutation burden compared with other tumor types is also a distinguishing feature of AML.[4,5] Thus, to categorize other genomic alterations that co-occur with *FLT3*-ITD, a targeted 80-gene panel[19] was utilized. At diagnosis, 15 genes classified as tumor suppressors, transcription factors, cohesin complex genes, methylation-related genes, chromatin remodeling genes, kinases, Ras pathway genes, and signaling genes, were observed to contain mutations with a variant allele frequency (VAF) of 0.10 or greater (**Figure 1b, Table S4**). Per patient, the median number of mutated genes in addition to *FLT3*-ITD was 1 (range 0–3). The most common co-occurring mutations within this pediatric cohort were in *WT1* and *NPM1*, making up 35% (n = 12) and 24% (n = 8) of the mutations observed, respectively (**Figure 1b,e, Table S5**). The median VAF for *WT1* was 0.40 (range 0.14–0.55), whereas it was 0.41 (range 0.29–0.51) for *NPM1* (**Table S4**). Two alterations within *WT1* were detected in three patients, which were resolved to different alleles. It is predicted that the *WT1* deletions and insertions would result in truncated WT1 protein around exon 7, with none keeping the integrity of the reading frame. Additional co-occurring mutations were seen in *RAD21*, *CEBPα*, *IDH2*, *DNMT3A*, and *PLCG2*, with VAF ranging from 0.43–0.55. In one *FLT3*-ITD diagnosis sample, no additional co-occurring mutations were observed using the targeted panel.

Genomic subtypes within FLT3-ITD–positive AML and clinical outcome. It is known that pediatric *FLT3*-ITD–positive AML is associated with worse prognosis,[7–9] especially those that contain *NUP98-NSD1*, and *WT1*.[3] However, in these studies, the cytogenetics of the *FLT3*-ITD

Table 1 Summary of patient demographics at diagnosis

Variables	*N*
Age (yrs)	
<10	10
≥10	24
Protocol	
AML02	18
AML08	16
Gender	
Female	13
Male	21
Race	
White	23
Black	7
Other	4
Karyotype	
CBF	0/34
M7	0/34
11q23	0/34
t(8;21)	0/34
inv(16)	0/34
MRD (induction I)	
<0.1%	8
≥0.1%	25
NA	1
(Min, Median, Max)	(0, 0.06, 0.74)
WBC (Min, median, Max; 10^9/L)	(2.5, 116, 412.2)
Platelet (Min, median, Max; 10^9/L)	(10.6, 59, 150)
Bone Marrow Blasts (%) (Min, Median, Max)	(2, 82, 97)

samples were of mixed karyotypes. Thus, we evaluated our CN *FLT3*-ITD–positive samples to see if this worse prognosis association held true for EFS and OS. Additionally, we also looked at *NPM1*, which is known to be associated with a more favorable outcome[3] and *FLT3* AR (<0.4 compared with ≥0.4). A *FLT3* AR ≥ 0.4 has been previously reported to be associated with a worse prognosis in children.[23] Of these four features, only the presence of the *NUP98-NSD1* was significantly associated with a poorer EFS (P = 0.03; hazard ratio = 2.61; 95% confidence interval = 1.10–6.19; **Figure 1f, Figure S2**). *NPM1*, *WT1*, and AR ≥ 0.4 showed no significant association with EFS or OS in this cohort.

Characterization of pediatric relapse *FLT3*-ITD–positive samples

Chimeric transcripts. In matched diagnosis-relapse samples, fusion transcripts were maintained (**Figure 2a**) during disease progression. *NUP98-NSD1* and *DEK214-NSD1* fusions were also observed in two of the relapse samples without a matched diagnosis sample. A novel relapse-specific fusion between the 5′ untranslated region (UTR) of Leucine Zipper Protein 6 and Oxysterol Binding Protein Like 1A was observed at relapse that was not detected at diagnosis (**Figure 2a, Table S3**).

Co-occurring mutations. It was observed that there was no resolution of the ITD from diagnosis to relapse (**Figure 2b**). For other mutations, retention was observed regardless of the variant's dominance (**Figure 2a,b**) with either maintenance or increased VAF at relapse. No trend in VAF was observed in relation to transplant (**Figure 2b**). For patients 3, 5, 6, and 7 with germline DNA samples, no mutations were observed by targeted gene panel analysis (**Figure 2a**). A thought-provoking observation was that all of this cohort's relapse samples contained at least one *WT1* mutation. In most cases, the same *WT1* variant allele was maintained between diagnosis and relapse (**Figure 2b, Table S4**). However, in the case of patient 7, the *WT1* variant at diagnosis was no longer detected at relapse, but a new variant was acquired at relapse with a greater VAF than the original mutation. Additionally, the emergence of new *WT1* variants was observed in two more patients; patients 1 and 3 (**Figure 2b**). When more than one *WT1* variant was observed in a sample it was also resolved to different alleles. We found no evidence that the expression of WT1 was associated with mutation status, allelic status, or disease progression (**Figure S3**).

Clonal patterns of disease progression

It has been suggested that there are three major clonal patterns to relapse: (i) the regrowth of leukemic stem cells (LSCs) without additional mutations making the mutation profile (not accounting for VAF) at relapse indistinguishable from diagnosis, (ii) the outgrowth of diagnostic LSC clones with newly acquired mutations, or (iii) the outgrowth of preleukemic hematopoietic stem cells containing early acquired mutations and newly acquired mutations not present at diagnosis.[25,26] When analyzing diagnosis to relapse samples, although the mutation burden is low, a characteristic of AML, we observed all three clonal patterns (**Figure 2c, Figure S4**). Six of the seven samples had outgrowth of the LSC, with only one exhibiting outgrowth of hematopoietic stem cells, suggesting relapse in these patients was mainly due to the outgrowth of LSC. When looking at clonal patterns from diagnosis to relapse, it should be noted that there was a reduction in mutation VAF of simple clones, whereas in more complex clones the VAF seemed to either be preserved or expanded (**Figure S4**).

DISCUSSION

Pediatric *FLT3*-ITD–positive AML is an aggressive disease linked with early relapse and worse prognosis. Deciphering whether relapse arises from the acquisition of new mutations or is the outgrowth of diagnostic clones is important for understanding gaps in current treatment regimens. In the present genomic profiling study of CN pediatric *FLT3*-ITD AML, clonal stability of mutational patterns was observed from diagnosis to relapse. These observations are consistent with a report by Farrar *et al.*[15] involving children with AML of varying subtypes and cytogenetics, where mutations with a high VAF > 0.4 persisted from diagnosis to relapse. They did note fluidity in some cases between diagnosis and relapse with resolution of diagnostic variants, emergence of relapse variants, and marked changes in VAF from diagnosis to relapse. We did not observe this fluidity in our cohort of CN *FLT3*-ITD samples. In the study by Farrar *et al.*,[15] the one CN *FLT3*-ITD–positive sample did show

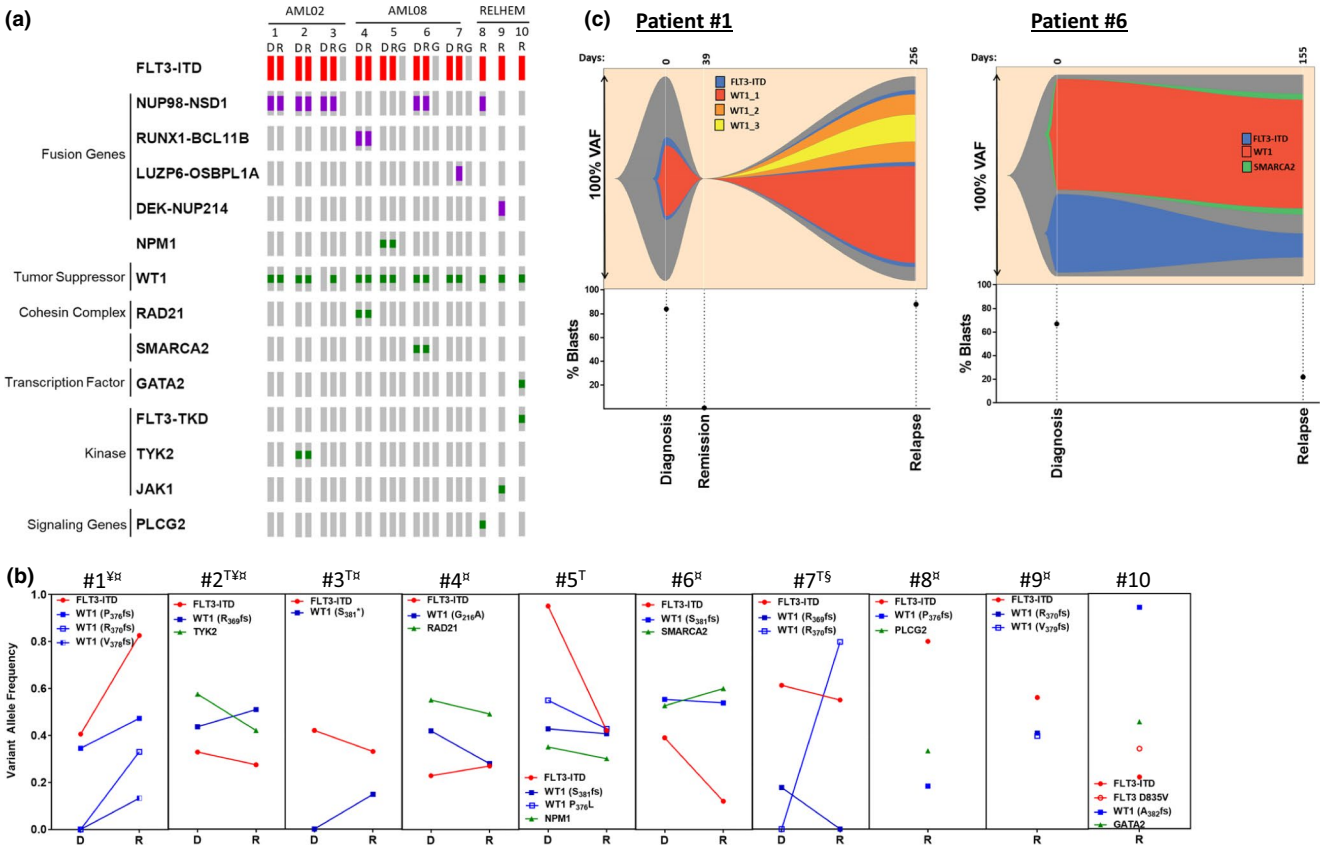

Figure 2 Characterization of relapse Fms-like tyrosine kinase 3 (*FLT3*) internal tandem duplication (ITD)-positive samples. (**a**) OncoPrint of duplications (red), fusion genes (purple), and individual mutations (indels and missense both in green). Sample identified as: D, diagnosis; R, relapse; and G, germline. (**b**) Mutation variant allele frequency (VAF) is shown from diagnosis to relapse. T, transplant; ¥, FLT3-ITD VAF was determined by RNASeq; ¤, contain fusion transcripts at diagnosis and relapse; and §, contains a relapse specific fusion transcript. (**c**) Fishplots depict two clonal patterns identified between diagnosis and relapse.

similar clonal stability. It could be conceivable that stability in some of the variants present may be due to germline mutations. However, in the majority of cases, germline samples were not available, and this could not be evaluated. When germline samples were available, no diagnosis or relapse variants were detected suggesting pathogenic potential of these mutations and stability from diagnosis to relapse.

We observed the maintenance of *WT1* mutations with relapse. Other studies carried out in adult AML populations have shown associations among *WT1* mutations, relapse, and disease resistance.[27–29] Taken together, one could propose a potential role for *WT1* mutations in disease progression in conjunction with *FLT3*-ITD. The exact mechanism would need to be studied further to delineate. The main AML mutational hot spot for *WT1* centers around exon 7 for both adult and pediatric cases.[3,28] The location of the mutations in exon 7 are N-terminal to the zinc finger domains, suggesting that these *WT1* mutants would maintain the transactivation domain but be truncated or lose the integrity of the zinc finger domains. Loss of zinc finger domains in the Ikaros family of transcriptional regulators results in a classical dominant negative phenotype inhibiting DNA binding of their dimeric partners.[30] Thus, given that the RNA expression levels are not altered one could hypothesize that *WT1* mutants may be functioning in a dominant negative way preventing the wild-type WT1 from accessing the DNA or altering interactions with additional proteins, such as p53 or TET2. However, the penetrance of such a dominant negative phenotype would need to be further explored.

Fusion transcripts are a common feature in pediatric AML and are known to be AML drivers.[3,18,31,32] A rare recurring fusion partner, *BCL11B*, was identified in this study, including a novel fusion *RUNX1-BCL11B*. *ZEB2-BCL11B* has been identified in a young adult with *FLT3*-ITD AML[33] with the same breakpoints reported here. Furthermore, a single *ZEB2-BCL11B* fusion was also identified in the Children's Oncology Group cohort[3] of unknown *FLT3*-ITD status. BCL11B, both a repressor and transactivator, has been implicated in the pathogenesis of both adult hematological malignancies and lymphomas.[34,35] A *HELIOS-BCL11B* fusion was identified in adult T-cell leukemia/lymphoma by Fujimoto *et al.*,[34] that has the same breakpoint within *BCL11B* as reported here. They observed that the *HELIOS-BCL11B* fusion caused a decrease in transcriptional suppression, as well as altered subnuclear localization. Fuijimoto *et al.*,[34] hypothesized that this altered localization and loss of transcriptional suppression may lead to aberrant transcription leading to leukemogenesis in adult T-cell leukemia/lymphoma. Thus,

there is potential for *BCL11B* to have a role in the pathogenesis of pediatric *FLT3*-ITD AML.

In summary, our data represent a broad depiction of the genomic landscape of pediatric (CN) *FLT3*-ITD–positive AML from diagnosis to relapse. The categorization of co-occurring genomic lesions, as well as recurring chimeric transcripts, highlights the multifaceted biology behind *FLT3*-ITD–positive pediatric AML. Genomic analysis of relapse samples provides insight into the pathogenesis of *FLT3*-ITD–positive AML highlighting unseen vulnerabilities in current frontline treatment strategies given the absence of clonal resolution and dominance of *WT1* mutations. In the dawn of targeted therapies, comprehensive sequencing, including RNAseq for fusion transcripts should be considered at diagnosis and throughout treatment to recognize problematic and complex clones that may require combination therapies, including chemotherapy and/or targeted therapies to achieve deep and durable remission. Optimistically, having this better understanding of the genomic heterogeneity during progression of pediatric *FLT3*-ITD AML will allow for advancement in current and future treatment strategies, as well as guide us in the improved mutation testing.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

Supplementary Materials and Methods
Figure S1. Cell viability assessment for MV411 overexpressing BCL11B.
Figure S2. Association of selected molecular features on overall survival.
Figure S3. Expression of *WT1* transcripts.
Figure S4. Visualizing clonal patterns from diagnosis to relapse using fishplots.
Table S1. Characteristics of diagnosis samples, paired diagnosis/relapse samples, and relapse samples.
Table S2. Primers utilized in study.
Table S3. Related to **Figure 1**. Validated chimeric transcripts.
Table S4. NGS-targeted gene panel validation list.
Table S5. Related to **Figure 1**. NPM1 mutations.

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