Integrative Genomic Analysis of Pediatric Myeloid-Related Acute Leukemias Identifies Novel Subtypes and Prognostic Indicators

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ABSTRACT
Genomic characterization of pediatric patients with acute myeloid leukemia (AML) has led to the discovery of somatic mutations with prognostic implications. Although gene-expression profiling can differentiate subsets of pediatric AML, its clinical utility in risk stratification remains limited. Here, we evaluate gene expression, pathogenic somatic mutations, and outcome in a cohort of 435 pediatric patients with a spectrum of pediatric myeloid-related acute leukemias for biological subtype discovery. This analysis revealed 63 patients with varying immunophenotypes that span a T-lineage and myeloid continuum designated as acute myeloid/T-lymphoblastic leukemia (AMTL). Within AMTL, two patient subgroups distinguished by FL3-ITD and PRC2 mutations have different outcomes, demonstrating the impact of mutational composition on survival. Across the cohort, variability in outcomes of patients within isomutational subsets is influenced by transcriptional identity and the presence of a stem cell–like gene-expression signature. Integration of gene expression and somatic mutations leads to improved risk stratification.

SIGNIFICANCE: Immunophenotype and somatic mutations play a significant role in treatment approach and risk stratification of acute leukemia. We conducted an integrated genomic analysis of pediatric myeloid malignancies and found that a combination of genetic and transcriptional readouts was superior to immunophenotype and genomic mutations in identifying biological subtypes and predicting outcomes.

INTRODUCTION
Acute myeloid leukemia (AML) comprises a heterogeneous group of malignancies that are linked by the presence of blasts displaying morphologic and immunophenotypic features of myeloid cell differentiation. These characteristics served as the initial approach to subdivide AML into distinct clinical entities (1). Morphology and immunophenotype, however, are limited in biological, prognostic, and therapeutic significance. The identification of cytogenetic alterations and molecular lesions has allowed newer classification schemes to be developed with the most recent widely used approach being the World Health Organization classification of AML (2). Although the latter classification scheme divides AML into many distinct clinical, morphologic, and/or molecular subtypes, from a clinical perspective most current therapeutic pediatric protocols stratify patients into favorable, intermediate, and poor prognostic groups (3). Therapy in these groups is based on the relative risk of relapse, with poor prognostic groups proceeding to allogeneic hematopoietic stem cell (HSC) transplantation in first remission when a suitable donor is available.

With the development of genome-wide gene-expression profiling, array-based comparative genomic hybridization methodologies, and next-generation sequencing technologies, the field has gained a greater understanding of the molecular features involved in the occurrence of pediatric myeloid malignancies. Several pathologic lesions have been found to have prognostic implications contributing to a continuous refinement of risk stratification over time in the context of modern therapy. We previously applied an integrated analysis to a large cohort of pediatric acute megakaryoblastic leukemia

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(AMKL) that underwent next-generation sequencing with the goal of identifying biologically and clinically relevant subtypes so that we could gain a greater understanding of the biology of the disease as well as inform clinical decision-making (4). In that study, using gene-expression profiling coupled with somatic variants and outcome data, we were able to identify distinct molecular subtypes with varying outcomes. These results led to a recommendation to limit high-risk designation to a subset of patients, which has already been instituted in the ongoing multi-institutional AML16 trial for newly diagnosed pediatric patients with AML (NCT03164057) and several other collaborative group protocols. Here we apply a similar approach to a cohort of 435 pediatric patients with a spectrum of myeloid-related malignancies to provide a comprehensive view of this clinical entity and propose a refined classification scheme with clinical utility. Using this approach, we identify a previously undescribed subtype that spans a T-lineage and myeloid continuum, as well as new prognostic mutational events within previously described subtypes. Further, we demonstrate that mutational events, transcriptional profile, and evidence of a primitive hematopoietic progenitor gene-expression signature all associate independently with outcome. The most significant association occurs when all three of these factors are combined, arguing in favor of subgroup classification by comprehensive molecular profiling to optimize risk stratification in pediatric AML.

RESULTS
Genomic Landscape of Normal and Complex Karyotype Pediatric AML

The Children’s Oncology Group (COG)–NCI TARGET AML initiative molecularly characterized 993 pediatric AML cases, including 197 specimens that underwent comprehensive whole-genome sequencing (WGS; ref. 5). Of these, 94 carried one of three oncogenic fusions known to be strong drivers of leukemogenesis: RUNXI–RUNXIT1, CBFB–MYH11, and KMT2A rearrangements (KMT2A). Among all other somatic alterations detected, only 10 occurred in more than 5% of subjects, all of which had been described previously. This suggested that low-frequency molecular subsets may exist that require larger cohorts to fully elucidate. To address this limitation, we selected 122 pediatric AML normal, noncomplex, and complex karyotype specimens from five cooperative study groups (SJRH, DCOG, NOPHO, AIEOP, and BFM) that lacked RUNXI–RUNXIT1, CBFB–MYH11, and KMT2A by clinical testing for WGS and/or whole-exome sequencing (WES) and RNA sequencing (RNA-seq) to enrich for cases that carry low-frequency events (Supplementary Tables S1 and S2; Fig. 1A). Structural variations (SV), copy-number alterations (CNA), single-nucleotide variations (SNV), and indels were determined by our established pipelines, as well as an evaluation for regulatory rearrangements driving oncogene overexpression through enhancer hijacking (Supplementary Tables S3–S9 and Supplementary Figs. S1 and S2; ref. 6). When considering exonic SNV/indel, CNA, and SV calls, mutational burden ranged from 1 to 101 somatic events, including a case with TP53-associated chromothripsis that carried 89 lesions in total (Supplementary Table S9; Supplementary Figs. S1 and S3). In addition to known AML somatic mutations in genes such as CEBPA, GATA2, NPM1, WT1, FLT3, NRAS, Kras, ETV6, RAD21, SMC1A, STAG1, STAG2, STAG3, SMC3, and rearrangements in NUP98 and KAT6A, we identified rare events in known oncogenic drivers. These include internal tandem duplications (ITD) in GATA2, RUNX1, and CEBPA, as well as the repositioning of a distal ZEB2 enhancer, MYC enhancer, or ETV6 enhancer to ectopically activate BCL11B, MECOM, and MNX1 loci, respectively (Supplementary Table S6; Supplementary Figs. S4 and S5). Interestingly, 15 AML cases (12.3%) carrying loss-of-function mutations in polycomb repressive complex 2 (PRC2) genes were found to resemble an early T-cell precursor acute lymphoblastic leukemia (ETP-ALL) gene-expression profile (GEP) by gene set enrichment analysis (GSEA; Supplementary Fig. S6). ETP-ALL exhibits aberrant expression of stem cell and myeloid markers and has been shown to have a GEP consistent with transformation of a stem cell progenitor (7, 8). Further, mixed phenotype acute leukemias (MPAL) with T and myeloid lineage characteristics have previously been suggested to be in this spectrum of immature leukemias (9). We therefore hypothesized that these PRC2-mutated AML cases represented the myeloid end of this continuum. To provide a global transcriptional context to these ETP-like AMLs and evaluate a comprehensive cohort encompassing a range of pediatric myeloid malignancies, we integrated results from previously published AML (N = 169), MPAL (N = 80), AML (N = 45), and ETP-ALL (N = 19) data sets that had RNA-seq and either WES or WGS available for a total of 435 cases (Supplementary Table S10 and Fig. 1A; refs. 4, 5, 7–9).

Molecular Classifier of Pediatric Myeloid Malignancies Agnostic of Immunophenotype

T-distributed Stochastic Neighbor Embedding (t-SNE) visualization using a 381-gene list derived from the top 100 most variably expressed transcripts within each of the five sequencing data sets revealed a clear molecular classifier, identifying groups that had consistent mutational compositions but were agnostic of immunophenotype (Figs. 1B and C and 2; Supplementary Tables S10–S13; Supplementary Fig. S7). A bootstrap hierarchical clustering procedure defined subgroups with an overall reproducibility of 97.4% and highly concordant with the t-SNE transcriptional subgroups (adjusted Rand index = 0.72; Supplementary Table S14), indicating the subgroups identified by t-SNE are statistically meaningful. This classifier allowed the distinction of 63 cases with an ETP-ALL GEP comprising a mixture of AML (N = 12/63, 19%), acute undifferentiated leukemia (AUL; N = 1/63, 1.6%), MPAL (N = 31/63, 49.2%), and ETP-ALL (N = 19/63, 30.2%) leukemias (bootstrap reproducibility = 93.6%; Fig. 1B). All but one MPAL case within this subgroup coexpressed T-lineage antigens in addition to either myeloid and/or B-lineage antigens (Fig. 1B; Supplementary Table S10). Expression of MPO and CD3E confirmed that the reported immunophenotypes of these cases were correct (Supplementary Fig. S8). A separate validation cohort of 399 pediatric AML cases with microarray data confirmed the presence of this entity with 23 cases identified (Supplementary Fig. S9; Supplementary Tables S15 and S16). A five-gene classifier consisting of CD3G, COCH, SLC35D2, SPTLC3, and TBR4A was able to predict these cases in both the discovery and validation cohorts (AUC 0.977 and 0.88, respectively). A molecularly
Figure 1. Transcriptional identities correlate with key oncogenic driver events and are agnostic of immunophenotype. A, Study design. 122 normal, noncomplex, and complex karyotype pediatric specimens were selected. Exclusion criteria for sequencing include FAB M3 (acute promyelocytic leukemia, APML), FAB M7 (AMKL), core binding factor (CBF) leukemia [RUNX1–RUNX1T1, CBFβ–MYH11], and KMT2A cases. Cases underwent WGS, WES, and RNA-seq. Data were combined with four other pediatric data sets, including FAB M7, early T-cell precursor acute lymphoblastic leukemia, the TARGET data set, and pediatric AML cases were sequenced. NGS, next-generation sequencing. RNA-seq of 435 cases of pediatric AML, AMKL, MPAL, and ETP were combined and batch corrected. t-SNE visualization utilizing the top 100 differentially expressed genes within each data set. Immunophenotype of cases as determined by NGS, next-generation sequencing. Key oncogenic driver mutations as determined by next-generation sequencing. Ph-like, Philadelphia chromosome-like acute lymphoblastic leukemia; PTD, partial tandem duplication; Txn, transcription.

Distinct subtype of acute leukemia termed acute myeloid/T-lymphoblastic leukemia (AMTL), with shared myeloid and T-lineage features, has previously been proposed by Gutierrez and Kentiss (10). In support of this entity, they noted shared gene mutations in prior sequencing reports of T-lineage and AML studies, including WT1, PHF6, RUNX1, and BCL11B. Consistent with this, transcriptionally defined AMTL cases in our discovery cohort carried mutations in these genes and were found to fall into one of two subgroups: a group characterized by FLT3-ITD (N = 26/63, 41.3%) and a second group enriched for loss-of-function alterations in one of three core PRC2 complex genes, including EZH2, SUZ12, and EED, or a splicing factor mutation that leads to inclusion of a cryptic exon resulting in truncated EZH2 transcripts predicted to undergo nonsense mediated decay (N = 37/63, 58.7%; Fig. 3A; Supplementary Fig. S10; ref. 11). Both subsets were found to carry cooperating events in transcription factors (WT1, NOTCH1, ETV6, PHF6, RUNX1, IKZF1, BCL11B TLX3); unique to PRC2 cases were activating events in RAS (NRAS, KRAS, NFI) and JAK/STAT (JAK1, JAK3, IL7R, SH2B3) signaling cascades, as well as loss-of-function mutations in genes that play a role in G1 checkpoint arrest (RB1, CCDN3, CDKN1B, and CDKN2A/B; Fig. 3A). In particular, network analyses identified a strong association between transcription factors associated with T-lineage differentiation (NOTCH1, PHF6, BCL11B, TLX3, TAL1, and IKZF2), PRC2 loss-of-function mutations, and JAK/STAT pathway alterations, whereas FLT3-ITD cases were enriched for RUNX1 and WT1 transcription factors (Supplementary Fig. S11; ref. 12). A comparison of overall survival clearly demonstrated that outcomes of the isotranscriptional AMTL subset are influenced by the mutational spectrum. Irrespective of whether the patient received AML, ALL, or a hybrid treatment approach, FLT3-ITD–positive AMTL cases were associated with a favorable outcome, whereas those with PRC2 mutations had a poor prognosis (P = 8 × 10−4; Fig. 3B; Supplementary Table S10). Consistent with this, AMTL cases in our AML validation cohort for which mutational data were available (N = 16/23, 69.6%) were similarly composed of FLT3-ITD–positive (N = 8/16, 50%) and FLT3-ITD–negative cases (N = 8/16, 50%); a subset of the negative cases (N = 3/8) had copy-number data available that confirmed deletional events in PRC2 genes in all three cases and an association with poor outcomes (P = 0.01; Supplementary Fig. S12; Supplementary Table S16). PRC2 loss-of-function mutations were
also present in a subset of core binding factor cases (N = 8/61, 13.1%; Supplementary Table S12). To determine if the presence of PRC2 mutations confers a poor prognosis in these patients as well, we evaluated outcomes in pediatric core binding factor AML cases from two previously published cohorts and found an inferior event-free survival in patients carrying two KIT activating mutations and PRC2 loss-of-function mutations (N = 5/142, 3.5%; P = 0.026; Supplementary Table S12 for genomic details and Supplementary Table S13 for genes and fusion events included in each of the groupings on the y-axis. AMP, amplification; AUL, acute undifferentiated leukemia. DEL, deletion; IP, immunophenotype; LOH, loss of heterozygosity; Ph-like, Philadelphia chromosome-like acute lymphoblastic leukemia; PTD, partial tandem duplication. Txn, transcription.

Outcomes of Isomutational Subsets Are Influenced by Transcriptional Identity

The favorable prognosis of AMTL cases carrying FLT3-ITD included those with cooperating WT1 mutations, several of which were classified as AML by immunophenotype (N = 10/26; 38.5% of FLT3-ITD AMTL cases carried WT1 mutations, two of which were AML). Historically, pediatric patients with AML with WT1 in AML MK-V -ITD/FLT3 cases that retain a stem cell progenitor–like state contributing to chemotherapy resistance. By guest on November 11, 2021. Copyright 2021 American Association for Cancer Research.
Integrated Molecular Diagnostics for Pediatric AML

Figure 3. Genomic and transcriptional features of AMTL. Sixty-three cases spanning AML, AUL, MPAL, and ETP immunophenotypes shared a common transcriptional identity (see Fig. 1B). A, Mutational spectrum of AMTL cases. Del, deletion; Ins, insertion; LOH, loss of heterozygosity. B, Outcomes of patients with AMTL according to FLT3-ITD/WT1 expression (log2) patients with AMTL according to FLT3-ITD/WT1 expression (log2). C, Expression of HOX locus genes in normal hematopoietic progenitor subsets and FLT3-ITD/WT1 double-mutant cases based on AMTL and MK-V transcriptional identity (see Fig. 1). D, Expression of HOX locus genes in normal hematopoietic progenitor subsets to define thresholds across Myeloid Leukemias. E, Enrichment of gene-expression signatures from HSC, CMP, and LP in FLT3-ITD/WT1 cases from AMTL and MK-V transcriptional clusters. n.s., not significant. F, pLSC6 score of FLT3-ITD/WT1 cases from AMTL and MK-V transcriptional clusters.

Ng and colleagues previously developed a 17-gene transcriptional score related to stemness, derived from functionally defined leukemia stem cells of adult patients with AML, which was predictive of prognosis (LSC17; ref. 18). More recently, a six-gene LSC score has been developed with significant prognostic value in pediatric AML (pLSC6; ref. 19). To determine if the more primitive nature of AML MK-V FLT3-ITD/WT1 cases was reflected in this score, we compared pLSC6 in AML MK-V and AMTL FLT3-ITD/WT1 patients (Fig. 3F). Consistent with enrichment of more primitive hematopoietic progenitor gene-expression signatures, AML MK-V FLT3-ITD/WT1 patients had a higher pLSC6 score ($P = 0.038$). To evaluate this more comprehensively across the cohort, we determined the pLSC6 score in normal hematopoietic progenitor subsets to define thresholds of low (lineage-committed cells), intermediate (multipotent progenitors), and high (pluripotent progenitors; Fig. 4A and B) values. Imposing these thresholds on our cohort, we identified a subset of patients with intermediate and high scores, which was significantly associated with an inferior overall survival ($N = 302/435, 69.4\%$ low pLSC6; $N = 119/435, 27.4\%$ high pLSC6).
intermediate pLSC6; \( N = 14/435, 3.2\% \) high pLSC6; \( P = 9.3 \times 10^{-7} \) discovery cohort; and \( N = 262/399, 65.7\% \) low pLSC6; \( N = 124/399, 31.1\% \) intermediate pLSC6; \( N = 13/399, 3.2\% \) high pLSC6; \( P = 2.1 \times 10^{-8} \) validation cohort; Fig. 4C; Supplementary Fig. S17). Although several subsets had uniform pLSC6 scores, such as \( CBFA2T3–GLIS2 \), \( RUNX1–RUNX1T1 \), \( CBFB–MYH11 \), and \( MNX1–rearranged \) cases, other subsets had variable scores demonstrating heterogeneity in leukemia “stemness” (e.g., \( KMT2A-r \) cases), highlighting pLSC6 as an independent variable in addition to mutational type and overall transcriptional signature (Fig. 4D; Supplementary Table S17; Supplementary Figs. S18–S20).

Transcriptional Identity, Mutations, and Stemness All Contribute to Outcome

To evaluate the relative contribution of each of the factors identified in our study to carry an association with survival, we utilized a Cox proportional hazards model to look at associations with overall survival. Transcriptional identity, oncogenic drivers, and leukemia stemness were all independently found to associate with outcome (Figs. 4C and 5A; Supplementary Tables S18 and S19; Supplementary Figs. S17, S19, and S20). The greatest association occurred when all three of these factors were combined \((P = 1.06 \times 10^{-12} \) discovery cohort and \( P = 1.19 \times 10^{-7} \) validation cohort). The impact of individual factors on outcome associations was variable in our discovery cohort, with \( CBFA2T3–GLIS2 \), \( ETS \) family rearrangements (\( FUS–ERG \), \( EWSR1–ERG \), \( FUS–FEV \), \( FUS–FLI1 \), \( MN1–FLI1 \), and \( EWSR1–FEV \)), and high pLSC6 score having the greatest negative association with outcome, whereas \( CEBPA \) mutations (mono- and biallelic) and low pLSC6 carried the greatest positive association with outcome (Supplementary Tables S20 and S21; Supplementary Fig. S21). Within biological subgroups identified in pediatric AML, certain factors carried greater weight than others (Table 1). Utilizing these rules for risk stratification, we compared outcomes in our discovery and validation cohorts for our proposed genomic classification (low, intermediate, and high risk) to those of the ongoing
Figure 5. Oncogenic driver events, transcriptional identity, and leukemia stemness all contribute to outcome in pediatric myeloid-related acute leukemias. **A**, Integrative Cox proportional hazards model to look at associations with overall survival in the discovery cohort [38]. Each bar represents the \( -\log_{10} P \) value of covariates and their association with survival. The covariates used in the model to calculate the \( P \) value are indicated below the graph with a check mark. Immunophenotype as a single covariate failed to reach statistical significance. **B**, Probability of EFS (pEFS) of an ongoing multi-institutional prospective pediatric AML trial (AML16) and the proposed classification scheme based on this article for the validation cohort. See Supplementary Figs. S22 and S23 for results of each independent cohort. **C**, Performance of the proposed genomic classification relative to that utilized in an ongoing prospective upfront pediatric AML study (NCT03164057) in terms of discrimination capability (left) and percentage of high-risk or low-risk classified patients (right) culminating in a risk classification utility score (top, right) for the validation cohort. See Supplementary Figs. S24 and S25 for results of each independent cohort. **D**, Working model. Mutational events in distinct hematopoietic progenitor subsets lead to transformation, and both components contribute to the transcriptional identity and leukemia stemness. Chemotherapy sensitivity and therefore outcomes are a composite of these factors.

multi-institutional AML16 prospective clinical trial for newly diagnosed pediatric patients with AML (NCT03164057; Fig. S8 and C validation cohort; Supplementary Figs. S22–S25 discovery and combined cohorts; Supplementary Tables S16, S22, and S23). For a given risk classification, we defined and computed the risk classification utility (RCU), which considers estimate outcomes for each risk group (outcome discrimination index) and the proportion of patients designated as high or low risk given that intermediate risk designates a patient lacking definitive high-risk or low-risk characteristics, and thus represents a patient whose status is unknown (Supplementary Table S24). A bootstrap procedure was then used to quantify the statistical variability and significance of comparisons of the RCU with the two classification schemes (Supplemental Table S25). In both the discovery and validation cohorts as well as in a combined analysis, our proposed classification was found to have a statistically significant greater RCU for EFS than AML16 (\( P = 0.036 \) discovery cohort, \( P = 0.018 \) validation cohort, and \( P = 0.036 \) combined cohorts; Fig. 5C; Supplementary Fig. S25). In particular, the proposed classification was superior at identifying high-risk patients within the intermediate- and low-risk groups, resulting in
Table 1. Biological subtypes identified in pediatric AML cases

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Immunophenotypes across the entire cohort (N)*</th>
<th>Proposed risk status based on overall survival (reference)**&lt;sup&gt;3,4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMTL</td>
<td>AML (12), MPAL (30), AUL (1), ETP (19)</td>
<td>FLT3-ITD mutation present: low PRC2 mutation present: high</td>
</tr>
<tr>
<td>CEBPA (mono- and bi-allelic)</td>
<td>AML (28), MPAL (3)</td>
<td>pLSC6 medium or high: high PRC2 low: low (41)</td>
</tr>
<tr>
<td>RUNX1-RUNX1T1</td>
<td>AML (27)</td>
<td>PRC2/KIT double-mutant present: high</td>
</tr>
<tr>
<td>CBFB-MYH11</td>
<td>AML (34)</td>
<td>PRC2/KIT double-mutant present: high</td>
</tr>
<tr>
<td>MNX1-r</td>
<td>AML (3), AMKL&lt;sup&gt;d&lt;/sup&gt;, AUL (1)</td>
<td>High (43)</td>
</tr>
<tr>
<td>ETS-r</td>
<td>AML (10), AMKL (1) MPAL (2)</td>
<td>High (44)</td>
</tr>
<tr>
<td>CBFA2T3-Glis2</td>
<td>AML (2), AMKL (11)</td>
<td>High (4, 45)</td>
</tr>
<tr>
<td>KMT2A-r</td>
<td>AML (56), AMKL (10), MPAL (9), AUL (2), ETP (1)</td>
<td>MK-V: high AMKL: high pLSC6 medium or high</td>
</tr>
<tr>
<td>GATA1</td>
<td>AML (3), AMKL (6), MPAL (1)</td>
<td>pLSC6 medium or high: high</td>
</tr>
<tr>
<td>HOX-r</td>
<td>AML (2), AMKL (13)</td>
<td>pLSC6 medium or high: high</td>
</tr>
<tr>
<td>NUP98-r</td>
<td>AML (17), AMKL (6), ETP (3)</td>
<td>High (4, 46–48)</td>
</tr>
<tr>
<td>NPM1</td>
<td>AML (25)</td>
<td>pLSC6 medium or high: high</td>
</tr>
<tr>
<td>DEK–NUP214</td>
<td>AML (5)</td>
<td>High (51)</td>
</tr>
<tr>
<td>FLT3-ITD&lt;sup&gt;e&lt;/sup&gt;</td>
<td>AML (28), MPAL (18), ETP (6)</td>
<td>WT1 and MK-V present: high pLSC6 medium or high</td>
</tr>
<tr>
<td>AML other</td>
<td>AML (39), AMKL (6), MPAL (24)</td>
<td>pLSC6 medium or high: high</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the number of cases across the discovery cohort with indicated immunophenotype. Genomic subtypes not identified in AML cases are not included in this table.

**Outcomes approaching 80% overall survival or greater are designated as low risk and survival less than 40% are designated as high risk. Literature support of previously described subtypes and risk status is indicated in parentheses.

***Minimal residual disease is considered an independent risk factor, and residual levels of disease following induction chemotherapy warrant escalation of risk status.

**Reported in the literature (52).

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Integrated Molecular Diagnostics for Pediatric AML

The identification of AMTL cases that are devoid of T-lineage markers by flow cytometry and the distinction of the two subsets within AMTL that have differing outcomes. It has been shown through murine modeling that T-LP retain a broad lineage potential when transformed with oncogenes and specifically have the ability to differentiate into myeloid leukemia while retaining a lymphoid epigenetic memory, consistent with our findings (26). In this study, by Riemke and colleagues, a cohort of adult patients with AML was found to resemble the murine T-LP-derived myeloid leukemias by gene expression. This population, however, had a negative association with ETP-ALL by GSEA, and the mutation profile of these patients was predominated by mutations not found in pediatric AMTL, including NPM1, IDH2, and DNMT3A. This difference may be a result of distinct oncogenic events that are acquired by a T-LP as opposed to a difference in the cell of origin (Fig. 5D).

The existence of patients with FLT3-ITD/WT1 in AMTL that had superior outcomes, in contrast with previously published results, led us to compare outcomes of these patients across transcriptional subsets. The inferior overall survival of FLT3-ITD/WT1 double-mutant patients was restricted to those within the MK-V cluster. Of note, the vast majority of patients within this study were treated prior to the implementation of FLT3 inhibitors (2/291 patients with AML in the discovery cohort for whom treatment details were known received an FLT3 inhibitor at diagnosis, both of whom had events and are deceased; Supplementary Table S10). Although we cannot determine whether FLT3 inhibition would improve outcomes of MK-V FLT3-ITD/WT1 patients in our study, results from COG AAML1031 suggest that this targeted treatment approach can improve outcome in FLT3-ITD/WT1 patients with the caveat that the transcriptional identity in this study is unknown (4). The absence of FLT3 inhibition in our cohort allowed us to identify iso-mutational groups where disease outcome clearly associates with transcriptional identity and isozonotypic groups where outcome clearly associates with manipulational status. This finding has broad implications on variant interpretation in the era of precision medicine, as the impact on prognosis is not limited to the presence or absence of a given mutation. Furthermore, the incorporation of cell lineage–associated signatures also allowed us to distinguish patients who have the same genomic classification but differing outcomes (Table 1).

The highest power and outcome associations occur when all three of these factors are combined, arguing in favor of comprehensive diagnostics to optimize risk stratification in pediatric AML. A multivariate analysis to evaluate the prognostic informativeness of WT1 and FLT3-ITD mutational events after considering transcriptional identity, key driver mutation, and pLSC6 score supports this conclusion: Neither EFS nor overall survival was significantly associated with the presence of FLT3-ITD, a WT1 alteration, or the combination of these two after adjustment for pLSC6 score as a numeric predictor, transcriptional identity as a stratification factor, or driver mutation as a stratification factor (Supplementary Fig. S26; Supplementary Table S26). Further, neither EFS nor OS was significantly associated with FLT3-ITD, WT1, or the presence of both in models that considered only these variables as predictors (Supplementary Table S26).

The benefit of risk-adapted indications for HSC transplantation in pediatric AML has recently been shown by the BFM study group, with significantly higher EFS and higher rates of HSC transplants through improvements in genetic risk stratification (27). In a disease entity where the chemotherapy approach has remained largely unchanged over time with a limited number of novel therapeutic agents on the horizon, risk stratification, refined allograft indications, and supportive care continue to be major factors that have led to the improvement in outcome over time (28). It is, therefore, imperative that risk stratification be optimized to the maximum extent to cure more pediatric patients with AML. The vast majority of pathogenic calls and transcriptional information necessary to use our integrated approach can be obtained from paired WES and RNA-seq, which has been increasingly adopted in the clinical setting, arguing in favor of the feasibility of this approach (29–31). Targeted capture panels that detect SNV/indels and copy-number changes in combination with fusion detection assays are less comprehensive but also able to detect the vast majority of oncogenic lesions described in this study. In pediatric AML, all patients enrolled on the St. Jude AML16 study are already receiving Clinical Laboratory Improvement Amendments–certified WES, WES, and RNA-seq on diagnostic blasts. Although next-generation sequencing approaches are becoming increasingly standardized and prevalent in the field, bioinformatic analyses and interpretation of mutational impact within a case based on transcriptional identity and leukemia stemness will require additional expertise to implement. To enhance the clinical applicability of this study, we developed a panel of five genes whose expression can distinguish AMTL cases that can be combined with the previously developed sex–gene pLSC6 classifier–key determinants in our risk stratification model. In combination with key mutational events, this allows one to follow a hierarchical decision-making tree to stratify a patient (Fig. 6).

The cell of origin of leukemia is defined as the normal hematopoietic cell from which the disease develops through the acquisition of mutations. A subset of cells termed “leukemia stem cells” are felt to propagate the disease over time, and studies have shown that similar to normal hematopoiesis, a hierarchical structure exists in leukemia, with the most primitive clone being identifiable through functional assays (32). Given the differentiation spectrum seen in leukemias, it can be a challenge to infer the cell of origin in bulk tumor populations. Despite this potential limitation, we found significant enrichment for more primitive progenitor cell signatures in patients with higher LSC6 scores. Our data are consistent with a model whereby a cell of origin acquires oncogenic driver mutations, and these two factors both contribute to the transcriptional identity of the leukemia and the stemness, all of which influence outcome (Fig. 5D).

In summary, comprehensive next-generation sequencing of pediatric AML can be utilized beyond pathogenic mutation calls to optimize risk stratification. Incorporation of transcriptional identity and leukemia stemness in clinical decision-making will further improve the identification of patients who may benefit from stem cell transplants in first remission and those who can be cured with chemotherapy alone.
**Figure 6.** Hierarchical decision-making tree for proposed risk stratification. *, T-MPAL, MPAL with T-lineage markers. MPAL cases coexpressing B-lineage markers contained ZNF384, Ph+, Ph-like, and KMT2Ar oncogenes and should be treated with ALL-directed therapy unless they prove nonresponsive to this approach. **, FLT3-ITD cases that are not AMTL and lack high-risk and low-risk features such as NUP98-r, monosomy 7, NPM1, and CEBPA. HR, high risk; IR, intermediate risk; LR, low risk.

**METHODS**

**Cohort**

Specimens sequenced in this study were provided from multiple institutions and collaborative groups. All samples were obtained with patient- or parent/guardian-provided written informed consent under protocols approved by the Institutional Review Board at each institution. Studies were conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects. Samples were de-identified prior to nucleic acid extraction and analysis. WGS, WES, RNA-seq and analysis for SVs, SNVs, indels, and CNA were performed as previously described (4, 7). TARGET AML, ETP, MPAL, and AMKL cohorts have been previously published and were obtained with permission from database of Genotypes and Phenotypes (dbGaP) and/or St. Jude Children’s Research Hospital (4, 5, 7–9). Transcript expression levels for gene-expression analyses were estimated from RNA-seq data as fragments per kilobase of transcript per million mapped fragments (FPKM) as previously described (4). Data for samples sequenced in this study have been deposited to the St. Jude Cloud (www.stjude.cloud; ref. 33) and European Genome-phenome Archive (study ID EGAS00001004701).

**RNA-seq Read Mapping, Gene-Expression Summary, and Batch Correction**

RNA reads were mapped using our StrongARM pipeline, described previously (13). Paired-end reads from RNA-seq were aligned to the following four database files using Burrows-Wheeler alignment: (i) the human GRCh37-lite reference sequence, (ii) RefSeq, (iii) a sequence file representing all possible combinations of nonsequential pairs in RefSeq exons, and (iv) the AceView database flat file downloaded from UCSC, representing transcripts constructed from human expression sequence tags. Additionally, they were mapped to the human GRCh37-lite reference sequence using STAR. The mapping results from the databases (ii–iv) were aligned to human reference genome coordinates. The final BAM file was constructed by selecting the best of the five alignments.

Reads from aligned BAM files were assigned to genes and counted using HTSeq with the GENCODE human release 15-gene annotation (34). The gene count matrix was used to generate an FPKM gene-expression data matrix using gene length information. A gene was called as “expressed” in a given sample if it had an FPKM value ≥0.01 based on the distribution of FPKM gene-expression values, and genes not expressed in any sample were excluded from downstream analysis. The gene-expression data were further quantile normalized using the normalizeBetweenArrays function available from the Limma package (35). The detected batch effect due to data source of St. Jude versus TARGET was corrected using the ComBat method (36).

**381-Gene Classifier**

For construction of the 381-gene classifier, the top 100 most variant genes from each of the five data sets (this article, ETP-ALL, MPAL, TARGET AML, and AMKL) were combined using log2-transformed FPKM values and median-adjusted deviation (4, 5, 7–9). This procedure effectively eliminated remaining batch effects (Supplementary Fig. S27). Visualization was performed using t-SNE using a perplexity value of 10 and 10,000 iterations (37). t-SNE coordinates from the run with the lowest final error (out of 10 runs) were selected for further analysis.

**HSC Progenitor Gene-Expression Analysis**

Single-cell HSC progenitor (HSCP) counts, SPRING plot coordinates, and population assignments were taken from Pellin and colleagues (17). For comparing HSCP and leukemia gene expression, single-cell counts per gene were summed up for each of the 11 different HSCP populations, normalized to the number of cells in each population, and log-transformed. Resulting gene-expression values were scaled together with log2FPKM expression values of the 435 leukemias using the normalize between arrays function of Limma (method quantile), pLSC6 scores and Spearman correlation coefficients were calculated using these values. For some analyses, multilymphoid progenitors and pre-B/natural killer values were averaged to generate LP values.
plSC6-high, -medium, and -low cutoff values were based on HSCP population values, with the most primitive populations designated as high (populations 1, 2, 3, 7, 9, 10, and 11 from Pellin and colleagues), the more committed populations designated medium (populations 4, 5, 6, and 8), and values lower than these low. Exact cutoff values were calculated using linear extrapolation.

**Statistical Analysis**

All analyses were done in R. Survival and global test analyses were performed as previously described (4). Treatment details for patients are included in Supplementary Tables S1 and S10. The integrative statistical model was evaluated using the global test assuming interaction between the explanatory variables (38). Transcriptional identity and key oncogenic driver were defined as categorical and leukemia stemness (plSC6) as a continuous variable, and assuming interaction between these three exploratory variables. Individual associations are shown in Supplementary Fig. S18, and main contributing covariates clarified in Supplementary Table S15 by using plSC6 as a categorical variable (low vs. medium/high).

**Validation Cohort**

A pediatric AML microarray gene-expression cohort of 443 cases was constructed based on previously published data (19, 39, 40). AML M5 cases with t(15;17) were excluded from this cohort prior to assembly, because this subclass was absent from the discovery cohort and has excellent therapy options and disease outcome. Of these, 44 were also included in the discovery cohort and functioned as controls for the equivalence of the RNA-seq and microarray measured gene expression. Three hundred ninety-nine cases, which did not overlap, were used for gene-expression validation of results obtained in the discovery cohort. For 386 of these cases, disease outcome data were available (Supplementary Table S15) and were used for outcome validation analyses.

Key oncogenic driver determination was based on a combination of clinical testing and/or laboratory testing from the cohorts as previously published (see Supplementary Table S15, column K). Cases in which mutational status was unknown were removed from analyses as appropriate.

Transcriptional identity of the validation cohort cases was determined by co-clustering of microarray mRNA expression values of overlapping classifier genes (n = 249) of single cases with the complete RNA-seq cohort using Spearmann correlation distance-based t-SNE, exactly as done for the RNA-seq cohort clustering. For overlapping genes, probe sets with highest specificity and selectivity (https://genecards.weizmann.ac.il/geneannot/index.shtml) were used, omitting probe sets recognizing more than one gene. For robustness assessment of transcriptional identity calls, we made use of the stochastic initial seeding of the t-SNE algorithm by performing 10 clustering repeats. Cases with clustering inconsistency in more than 2 of the 10 runs (25/443 cases, 5.6%) were not assigned a transcriptional identity label. Transcriptional identity of 95% (41/43) of the microarray profiled cases also present in the discovery cohort. For 386 of these cases, disease outcome data were available (Supplementary Table S15) and were used for outcome validation analyses.

For a given risk classification, censored event-time endpoint (such as EFS or overall survival), and cohort outcome data set, we defined the risk of each risk group and Kaplan–Meier estimate of survival and hazard ratio for each risk group (Fig. 4B and C). We plotted the utility curve as Kaplan–Meier survival estimate of the low-risk group versus that of the high-risk group (Fig. 4C).
Fornerod et al.

An ideal utility curve is a flat line at y = 1; in this case, there is some time point at which the Kaplan–Meier estimate of high-risk patients is 0 and that of low-risk patients is 1. A utility curve along the line y = x could reasonably be obtained by completely random assignment of patients into low-risk or high-risk groups. The “outcome discrimination index” was defined and computed as twice the area above the line y = x and below the utility curve. The outcome discrimination index is 1 if the utility curve is ideal and 0 if the utility curve does not have any point above the line y = x that can be obtained by random risk classification assignments. We defined and computed the “meaningful classification proportion” as the proportion of patients designated as high or low risk because intermediate risk typically designates a patient lacking definitive high-risk or low-risk characteristics (Fig. 4C; Supplementary Fig. S27). Finally, the RCU was defined and computed as the product of the meaningful classification proportion and the outcome discrimination index (Fig. 4C; Supplementary Fig. S27). The RCU equals 1 if and only if all patients have a meaningful classification and the outcome discrimination is 1.

A bootstrap procedure was used to quantify the statistical variability and significance of comparisons of RCU of four risk classification schemes. The RCU of each risk classification scheme was computed for the discovery cohort, and 100,000 bootstraps of the discovery cohort, the validation cohort, and the combined cohort was determined (Supplementary Table S25).

**Authors’ Disclosures**

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**Authors’ Contributions**


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