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# EARLY T-CELL PRECURSOR LEUKEMIA: A SUBTYPE OF VERY HIGH-RISK ACUTE LYMPHOBLASTIC LEUKEMIA IDENTIFIED IN TWO INDEPENDENT COHORTS

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## Abstract

**Background—**Approximately one-fifth of children with acute T-lymphoblastic leukemia (T-ALL) succumb to the disease, suggesting unrecognized biologic heterogeneity that may contribute to drug resistance. We hypothesized that T-ALL originating from early T-cell precursors (ETPs), a recently defined subset of thymocytes that retain stem cell-like features, would respond poorly to lymphoid-cell directed therapy. We studied leukemic cells, collected at diagnosis, to identify cases with ETP features and determine their clinical outcome.

**Methods—**Leukemic cells from 239 patients with T-ALL enrolled at St. Jude and in the Italian national study AIEOP ALL-2000 were examined by gene expression profiling, flow cytometry and single nucleotide polymorphism array analysis. Probabilities of survival and treatment failure were calculated for subgroups considered to have ETP-ALL or typical T-ALL.

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**CONTRIBUTORS**
ECS initiated the study, collected data on immunophenotype and MRD in the St. Jude cohort and analyzed all data. CGM, XS and JRD collected and analyzed gene expression and SNP array data in the St. Jude cohort. MO and FGB were responsible for the initial laboratory diagnosis, and SCR for the cytogenetic analyses in the St. Jude cohort. DP and CC undertook the statistical analysis; JER took care of St. Jude patients and contributed to data collection; GB and AB were responsible for the data of the AIEOP cohort; CHP took care of St. Jude patients and led the St Jude clinical trials; DC designed the study, analyzed data and wrote the paper with the input of all other authors.

**CONFLICT OF INTEREST STATEMENT**
There are no potential conflicts of interest relevant to this article to report.

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**Findings—**Thirty patients (12.6%) had leukemic lymphoblasts with an ETP-related gene expression signature or its associated distinctive immunophenotype ($CD1a^-$, $CD8^-$, $CD5^{weak}$ with stem-cell/myeloid markers). Cases of ETP-ALL showed increased genomic instability. Patients with this form of leukemia had a very high proportion of remission failure or hematologic relapse: 72% (95% confidence interval, 40% to 100%) at 10 years versus 10% (4% to 16%) for typical TALL patients treated at St. Jude; and 57% (25% to 89%) at 2 years versus 14% (6% to 22%) for patients treated in the AIEOP trial.

**Interpretation—**ETP-ALL is a distinct, previously unrecognized, pathobiologic entity that confers a dire prognosis with use of standard intensive chemotherapy. Its early recognition, using the criteria outlined here, is essential for the development of an effective clinical management strategy.

## INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant clonal expansion of immature T cells that accounts for 10% to 15% of childhood and 25% of adult ALL cases. With wider use of intensive chemotherapy, the prognosis for childhood T-ALL has improved remarkably: nearly 80% of patients can be currently cured.[1;2] Further gains in treatment outcome will likely require methods to identify patients who continue to fail on contemporary protocols, so that alternative therapy can be introduced as early as possible. Clinical presenting features, such as older age and a high leukocyte count at diagnosis, are now regarded as unreliable predictors of outcome in T-ALL patients treated with intensive chemotherapy;[2;3] cell marker profiling has led to conflicting conclusions about its prognostic significance.[3–6] More recent studies have provided insights into the genetic abnormalities underlying T-ALL development, some of which appear to correlate with prognosis.[7–10] However, the prognostic associations of molecular abnormalities in T-ALL are not sufficiently compelling to justify their use in treatment planning.

So-called ETPs (early T-cell precursors) are a subset of thymocytes representing recent immigrants from the bone marrow to the thymus; they retain multilineage differentiation potential, suggesting their direct derivation from hematopoietic stem cells.[11–13] We hypothesized that a proportion of T-ALL cases originate from oncogenically transformed ETPs and might therefore respond poorly to lymphoid cell-directed chemotherapy. To test these predictions, we used a set of genes that are differentially expressed in ETPs[11;14–16] to identify cases of ETP leukemia and then undertook a comprehensive study to establish the biologic and clinical features of these leukemias. Our findings support the classification of ETP-ALL as a new pathobiologic entity associated with a dire outcome.

## METHODS

### Study population and treatment protocols

One hundred thirty-nine consecutive patients with T-ALL, ages 0.5–18.9 years (median, 8.8), were enrolled in Total Therapy Studies XIII, XIV and XV at St. Jude Children's Research Hospital in Memphis, Tennessee from January 1992 to December 2006. The diagnosis of T-ALL was made by at least two expert hematopathologists. In all 139 cases, leukemic lymphoblasts had ALL L1 or L2 morphology, with <3% blasts expressing cytochemical myeloperoxidase; none of the cases showed Auer rods. Leukemic cells consistently expressed CD7, cytoplasmic or surface CD3 (one case was not studied for this marker), and other T-cell markers; myeloperoxidase expression was absent (<3% positive cells) in all 94 cases studied by flow cytometry. An independent-validation population consisted of 100 consecutive patients with T-ALL, enrolled from December 2001 to August 2006 in the ALL-2000 protocol of the Associazione Italiana Ematologia Oncologia

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Pediatrica (AIEOP). Diagnostic bone marrow samples of AIEOP patients were immunophenotyped at a reference laboratory at the University of Padua (Italy); the only inclusion criterion was the availability of a full immunophenotypic profile. Patients received remission-induction and consolidation therapy, followed by risk-directed continuation treatment.[2;17–19] Patients who had 5% or more leukemic lymphoblasts after 5 to 6 weeks of remission induction therapy were considered to be induction failures. The studies were approved by the St. Jude Institutional Review Board or by the AIEOP Ethical Committee, with written informed consent from the parents or guardians, and assent from the patients.

**Molecular genetic analysis, flow cytometry, karyotyping, and minimal residual disease studies—**Gene expression profiling was done in all cases with available stored material (55 St Jude and 34 AIEOP) using U133A and U133 Plus 2.0 GeneChips (Affymetrix). Data were deposited in NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/, GSE8879). Immunophenotyping at diagnosis was performed by standard methods; cell staining was analyzed by FACScan, FACSCalibur or LSRII flow cytometers (all from Becton Dickinson) or an FC500 flow cytometer (Beckman Coulter). Leukemic blasts were identified by their light-scattering properties as well as expression of CD45, CD7 and/or cytoplasmic CD3. T-cell receptor (TCR) gene rearrangements were determined by the polymerase chain reaction in 58 St Jude cases.

Conventional cytogenetic analysis and, in selected cases, fluorescence in situ hybridization were performed in all St. Jude cases, except six that lacked sufficient material. SNP analysis was performed in 54 of the St Jude cases (see Supplementary Methods).[20]

Minimal residual disease (MRD) was measured on days 15–19 and 43 of treatment in the St Jude cohort, and on days 33 and 78 in the AIEOP cohort with previously described flow cytometric or molecular methods.[21–23]

**Statistical analysis—**We initially identified ETP-ALL cases by unsupervised clustering analysis of the gene expression profiles of diagnostic samples of 55 St. Jude T-ALL patients, using a set of genes previously reported to be differentially expressed in ETP (Supplementary Table 1) [11;14–16] with corresponding probes available in the Affymetrix 133A GeneChip. The Linear Models for Microarray Analysis (Limma) and empirical Bayes t-test implemented in Bioconductor (bioconductor.org) were used to identify differentially expressed probe sets at a Benjamini-Hochberg false discovery rate (FDR) of <0.10.[24] Prediction analysis for microarrays (PAM) was used to build a predictive model to identify ETP cases with a training set of gene expression data from 55 St Jude patients; this model was then applied to identify analogous cases in an independent test set of gene expression data from 34 AIEOP patients.25 Enrichment of molecular signatures was assessed by gene set enrichment analysis (GSEA, see Supplementary Methods).26 We analyzed associations between the ETP immunophenotype and DNA copy abnormalities with the Mann-Whitney test, and associations with other surface markers or MRD with Fisher's exact test. Event-free survival (EFS) and (OS) estimates with 95% confidence intervals (CI) were made by the Kaplan-Meier method and compared with the Mantel-Haenszel (log-rank) test. The cumulative incidence of remission failure or relapse was estimated according to Kalbfleisch and Prentice, and compared with Gray's test,27 adjusting for competing risks (i.e, death during remission induction therapy or during remission, and second malignancies). The Cox proportional-hazards model was used to identify prognostic factors independently affecting EFS and OS. Associations between the cumulative incidence of relapse and known prognostic factors were analyzed with Fine and Gray's method,28 adjusting for the above competing risks. Analyses were implemented with S-Plus (Insightful), Statview and SAS (SAS Institute). All analyses of treatment response began after identification of the ETP-ALL cases in both the St. Jude and AIEOP cohorts; no changes in the classification (ETP-

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ALL versus typical T-ALL) were made thereafter. In some cases, there was insufficient material to perform all planned tests; each analysis was based on the entire set of available data for a given parameter (Fig. 1).

**Role of the funding source—**The funding sources of this study had no role in study design; collection, analysis, or interpretation of the data; or in the writing of the report. All the authors had access to the raw data. DC had full access to all of the data and the final responsibility to submit for publication

## RESULTS

Using a set of genes shown to be differentially expressed in ETPs compared with more mature thymic subpopulations (Supplementary Table 1),[11;14–16] we searched for leukemias with an ETP-related gene profile among 55 newly diagnosed cases of T-ALL. By unsupervised clustering analysis, we identified a cluster of 13 cases with gene expression profiles that strongly resembled those described for ETPs (Fig. 2a). Overexpressed genes in this group included *CD44*, *CD34*, *KIT*, *GATA2*, *CEPBA*, *SPI1*, *ID2* and *MYB*, while *CD1*, *CD3*, *CD4*, *CD8*, *RAG1*, *NOTCH3*, *PTCRA*, *LEF1*, *TCF12*, *LAT*, *LCK*, *TCF7*, *ZAP70* were underexpressed. We then examined the cell marker profile of the 13 clustered cases and found 9 with a strikingly distinct immature immunophenotype characterized by lack of CD1a and CD8 expression, weak CD5 expression with less than 75% positive blasts, and expression of one or more of the following myeloid or stem cell markers on at least 25% of lymphoblasts: CD117, CD34, HLA-DR, CD13, CD33, CD11b, and/or CD65. This cell marker profile clearly differed from that of most normal thymocytes (Fig. 2b). The four other cases that clustered with the ETP cases in the unsupervised analysis of ETP gene expression had somewhat different immunophenotypic features: 3 had expression of CD5 (with absence of myeloid or stem cell markers in one case); the fourth case had very high expression of surface CD3 and TCR gamma/delta.

Among 139 patients with T-ALL enrolled in three consecutive trials at St Jude Children's Research Hospital (including the 55 studied with gene expression arrays), 17 (12.2%) had leukemic lymphoblasts with the ETP immunophenotype defined above (Fig. 2c; Supplementary Table 2a). CD1a and CD8 were absent in all 17 patients; this occurred in only 15 of the remaining 122 patients ($p < 0.0001$). Second, expression of CD5 was low (10- to >200-fold lower than that of normal peripheral blood T-lymphocytes; Fig. 2b) resulting in a percentage of positive leukemic cells consistently below 75% (median, 45%); median CD5 expression among the 122 remaining cases was >99%, with more than 75% positive blasts in all cases, except for the one case with high TCR gamma/delta expression (Supplementary Table 2a). Third, all 17 cases expressed CD117, CD34, HLA-DR, CD13, CD33, CD11b, and/or CD65 on at least 25% of lymphoblasts, a feature found in 60 of the remaining 122 cases ($p < 0.0001$). TCR gene rearrangement analysis was performed in 58 of the 139 cases. It identified at least one rearranged TCR gene in 8 of the 9 ETP-ALL cases studied (Table 1), further corroborating the overall diagnosis of T-ALL. The same analysis detected at least one rearranged *TCR* gene in 47 of the 49 other T-ALL cases studied (36 *TCRB*, 27 *TCRG* and 20 *TCRD*).

We re-examined our database of gene expression profiles from 55 cases of T-ALL, using a supervised analysis, and identified 1082 probe sets that were differentially expressed in the 9 ETP-ALL cases (FDR of 0.10; top 150 probe sets shown in Fig. 2d and Supplementary Table 3).

To determine whether the gene profile associated with ETP-ALL in the St. Jude cohort could be validated in an independent cohort, we studied the gene expression data for 34 T-

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ALL patients enrolled in the AIEOP ALL-2000 study, including three whose lymphoblast immunophenotypic features resembled those of the St Jude ETP cases. Class prediction analysis by PAM, with the St. Jude cohort used as a training set and the AIEOP cohort as a test set, identified all three ETP-ALL-like cases in the AIEOP cohort (Supplementary Fig. 1). The predictive model also recognized two additional cases as ETP-ALL: both lacked CD1a and CD8, and expressed CD34 and CD117, but their expression of CD5 was not low enough to meet our stringent phenotypic criteria for ETP-ALL.

To further confirm the cell of origin of the ETP-ALL cases, we tested the gene expression signature for murine ETP, as described by Rothenberg et al.,[11] together with 1687 other gene sets applying GSEA to our entire set of microarray data. As shown in Supplementary Table 4, the set of genes upregulated in ETPs was highly enriched in ETP-ALL (p = 0.0021; FDR = 0.18), while the set of downregulated genes was highly enriched in typical T-ALL (p = 0.0062; FDR = 0.05); other highly enriched gene sets included genes also found in the ETP signature. The close correspondence between the gene expression signature of ETPs and the transformed lymphoblasts of ETP-ALL is illustrated in Supplementary Fig. 2, which shows the combined analysis of up- and downregulated genes in ETPs (p = 0.0059; FDR = 0.13). These findings support the ETP as the likely cellular target for transformation giving rise to the cases identified in this study.

Others have used levels of expression of certain oncogenic transcription factors to identify discrete subtypes of T-ALL.[7;29] Our cases of ETP-ALL had higher expression of *LMO1* (p = 0.0119), *LYL1* (p = 0.0002) and *ERG* (p = 0.0008) (Fig. 3a) but no clear distinction between ETP- and typical T-ALL cases could be made on the basis of the expression of these transcription factors. Expression of *TAL1*, *HOX11* and *LMO2* was not significantly different. Expression of Hairy-enhancer-of-split 1 (*HES1*), a target gene in the NOTCH signaling pathway[30], did not differ significantly between the ETP- and typical T-ALL cases, suggesting that the apparently aborted T-cell differentiation of these leukemias was not associated with their inability to transduce NOTCH signals. Likewise, expression of *FBXW7* or *PTEN*, genes often mutated or deleted in T-ALL,[31] did not differ between the two subgroups.

The 17 cases of ETP-ALL had highly variable karyotypes (Table 1). Notably, four cases had the 13q- abnormality compared with only four of the 116 typical cases with available chromosomal findings (p = 0.0095). No other significant differences were found. We used SNP arrays to screen for genetic lesions in 11 of the 17 ETP-ALL cases and in 43 of the 122 typical cases. Together, ETP-ALLs had significantly more DNA copy number abnormalities (mean 14.1 vs. 6.3 lesions, p = 0.0033), including both genomic gains (3.4 vs. 0.7 lesions, p = 0.0005) and losses (10.7 vs. 5.6 lesions, p = 0.0134; Fig. 3b, Supplementary Fig. 3 and Supplementary Table 5). The overall sizes of the genomic regions spanned by these gains and deletions were significantly larger in the ETP-ALL subgroup (mean size of gains per case, 97.5 Mb vs. 28.6 Mb, p = 0.0027; losses per case, 119.0 vs. 42.4 Mb, p = 0.0068; Fig. 3b, Supplementary Fig. 3 and Supplementary Table 5).

We found no significant associations between a diagnosis of ETP-ALL and clinical presenting features, except that 13 of the 17 patients were 10 years or older, compared to 49 of the 122 patients with typical T-ALL (p = 0.0187) (Supplementary Table 6).

The clearance of leukemic cells after the first phase of remission induction therapy was markedly inferior in patients with ETP-ALL: all 13 patients studied after 15 to 19 days of treatment had detectable MRD (≥0.01% leukemic cells in bone marrow), compared with 55 of the 91 having typical T-ALL (p = 0.0037; Fig. 4a). Levels of MRD were also significantly higher in patients with ETP-ALL: 10 of 13 had MRD ≥5% versus 12 of 91

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with typical T-ALL ($p < 0.0001$). Results of MRD measurements at the end of induction therapy (day 43) were consistent with these findings: of 14 patients with ETP-ALL who were studied at this interval, 10 had MRD compared with 28 of 116 with typical T-ALL ($p = 0.0007$; Fig. 4a). Six of the 14 patients had MRD ≥1%, indicating an extremely poor prognosis,[22] in contrast to only 6 of 116 in the comparison group ($p = 0.0003$).

The diagnosis of ETP-ALL was associated with a significantly worse outcome ($p < 0.0001$ by log rank tests for OS and EFS; Figs. 5a, b), with 10-year OS for ETP-ALL patients of 19% (95% CI, 0% to 92%) versus 84% (95% CI, 72% to 96%) for all remaining patients; 10-year EFS, 22% (95% CI, 5% to 49%) versus 69% (95% CI, 53% to 84%). All nine remission failures or relapses recorded in the ETP-ALL subgroup occurred in bone marrow, whereas 11 of the 22 relapses in the typical subgroup were confined to extramedullary sites. The cumulative incidence of remission failure or hematologic relapse was significantly higher in patients with ETP-ALL ($p < 0.0001$ by Gray's test; Fig. 5c). The 10-year cumulative incidence of remission failure or hematologic relapse was 72% (95% CI, 40% to 100%) for patients with ETP-ALL versus 10% (95% CI, 4% to 16%) for those with typical T-ALL. For patients who relapsed, the median time to relapse was 1.22 years for ETP-ALL and 1.74 years for typical T-ALL ($p = 0.14$). In univariate and multivariate analyses (Table 2), the diagnosis of ETP-ALL was by far the cofactor exerting the strongest negative impact on EFS (hazard ratio, 12.0; 95% CI, 4.6 to 31.3; $p < 0.0001$). Five of the 17 patients received allogeneic hematopoietic stem cell transplant because of >1% MRD on day 43 ($n = 4$) or persistent MRD during continuation therapy: 2 patients are in complete remission 3 and 6 years post-transplant, 1 died in remission 7 months post-transplant, and 2 relapsed 0.5 and 1.5 years post-transplant.

To validate our results, we studied response to therapy in a cohort of 100 T-ALL patients (96 white, 4 of other race; 79 male, 21 female; 62 aged 1–9 years, 38 ≥10 years), enrolled in the AIEOP ALL-2000 study (including the 34 with gene expression array data). Thirteen patients (13%) had immunophenotypic features characteristic of ETP-ALL (Supplementary Table 2b). Early development of drug resistance, a key feature of ETP-ALL, was also apparent among the AIEOP patients. Response to 1 week of prednisone alone (a strong prognostic factor in AIEOP studies[32] but not evaluated in St Jude trials) was markedly inferior in patients with ETP-ALL (Supplementary Fig. 4). The prevalence and levels of MRD in the AIEOP cohort were remarkably similar to findings in the St. Jude patients (compare Figs. 4a and 4b). After 78 days, 9 of the 10 patients with ETP-ALL and MRD measurements had detectable MRD, compared with only 34 of the 78 with typical T-ALL ($p = 0.0067$) (Fig. 4b). As in the St. Jude cohort, the diagnosis of ETP-ALL conferred a dismal clinical outcome (Figs. 5d, e; $p < 0.0001$ by log rank tests for OS and EFS). The 2-year OS of patients with ETP ALL was 45% (95% CI, 0% to 90%) versus 90% (82% to 99%) for those with typical T-ALL; 2-year EFS was 22% (0% to 59%) versus 71% (59% to 84%). The cumulative incidence of remission failure or hematologic relapse was much higher in patients with ETP ALL ($p < 0.0001$ by Gray's test; Fig. 5f). At 2 years, it was 57% (25% to 89%) for patients with ETP ALL versus 14% (6% to 22%) for those with typical TALL. For patients who relapsed, the median time to relapse was 1.21 years for ETP-ALL and 1.37 years for typical T-ALL ($p = 0.25$).

The ETP phenotype also appeared to have a prognostic impact in analyses limited to MRD-positive patients: 5-year cumulative incidence of relapse for the 10 patients with ETP ALL and MRD ≥ 0.01% on day 43 in the St Jude cohort was 50% ± 17% versus 25% ± 9% for the 28 MRD-positive patients with typical T-ALL ($p = 0.0173$). The 2-year estimates for the AIEOP patients who had MRD ≥ 0.01% on day 78 were 69% ± 21% for the 8 with ETP ALL cases versus 32% ± 10% for the 27 with typical T-ALL ($p = 0.0257$).

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## DISCUSSION

We have identified a unique biologic subtype of childhood leukemia, ETP-ALL, that is associated with a very high risk of remission induction failure or relapse in patients treated on contemporary protocols of intensive chemotherapy for ALL. We used the gene expression profile of normal ETP to identify their leukemic counterparts and define their immunophenotype. ETP-ALL cases have characteristic gene expression profiles, increased genomic instability and strikingly distinct cell surface features that readily enable the correct diagnosis: lack of CD1a and CD8 expression, weak CD5 expression, and expression of one or more myeloid- or stem cell-associated markers. The long-term response to therapy is one of the worst among recognized high-risk forms of childhood ALL, equal or inferior to that of *BCR-ABL*$^{+}$ ALL or infant ALL with *MLL* gene rearrangement.[2] Our findings suggest that the target cell for clonal expansion in these cases is a very early immigrant from the bone marrow to the thymus, a cell expressing abundant T-lineage, stem-cell and myeloid-associated transcripts, and possessing both lymphoid and myeloid developmental potential.[11–15]

Although other groups have linked T-ALL phenotypes to clinical outcome,[3;4;33] none of the previously described markers would have captured our group of 30 ETP-ALL cases. In particular, none of these cases could have been defined as "mixed-lineage leukemia" by the criteria used at St Jude (i.e., coexpression of CD3 and myeloperoxidase)[34] and only five would have met the EGIL/WHO criteria for "biphenotypic leukemia".35 Low expression of CD5 would identify most patients with ETP-ALL but nine cases had CD5 expression below the 75% threshold or borderline levels (76%–80%; Supplementary Tables 2a and 2b). Because cells in these cases expressed CD1a and/or CD8 and/or lacked stem cell/myeloid markers, they were excluded from the ETP ALL group. We tested whether expression of any myeloid-associated antigen alone could confer a poor outcome, but this feature by itself lacked prognostic weight (Supplementary Table 7). Only when found together with a lack of CD1a and CD8 expression and low CD5 expression did expression of myeloid-associated antigens correspond to the ETP genotype and predict an adverse outcome. The only classification dilemma was posed by a case (No. 110 in Supplementary Table 2a) whose cells had the ETP immunophenotype and clustered with ETP ALL by gene expression analysis but expressed high levels of surface CD3 with TCR gamma/delta, suggesting the diagnosis of hepatosplenic T-cell lymphoma rather than T-ALL. This exceptional case was not included in the ETP ALL group.

ETP-ALL cases showed a marked degree of genetic instability, among the highest yet recorded for any type of ALL.[20] Nonetheless, we could not identify a single genetic lesion previously associated with leukemic transformation that was common to all of our cases. Oncogenic transcription factors previously associated with an immature thymocyte phenotype, such as *LYL1* and *ERG*,[7;29] were expressed at higher levels in ETP versus typical T-ALL cases but the level of expression of these genes could not segregate these two leukemia subtypes. To determine the relative prognostic strength of the T-ALL classification based on ETP features to classifications based on *LMO1*, *ERG* or *LYL1* expression, we analyzed our series of 55 patients with available information on transcription factor levels. As shown in Supplementary Fig. 5, *ERG* and *LYL1* expression had prognostic significance but their predictive accuracy was lower than the classification based on ETP features.

Reliable prognostic factors for children with T-ALL have been lacking, with only marginal differences in outcome for subgroups defined by cell marker expression of leukemic lymphoblasts in early studies.[36,37] Hence, these patients have been treated uniformly in all major study protocols. Prednisone response[38] and, more recently, MRD are used as prognostic parameters but our analysis shows that, for patients with T-ALL, the ETP-ALL

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parameter is even stronger than MRD as a predictor of outcome. The very high risk of remission failure or subsequent relapse for patients with ETP ALL if treated with standard intensive chemotherapy indicates the need for alternative approaches to treatment. One option is myeloablative therapy followed by hematopoietic stem cell transplantation in first remission, a strategy that was shown to be superior to chemotherapy alone in children with T-ALL and poor early responses.[38] Hence, we have modified our approach to frontline treatment for patients with a diagnosis of ETP-ALL to include hematopoietic stem cell transplantation in first remission after consolidation and reintensification therapy. Alternatively, with continued study, it may be possible to repress genetic programs that prevent transformed ETPs from entering T-cell differentiation pathways so that they would become responsive to conventional lymphoid cell-directed therapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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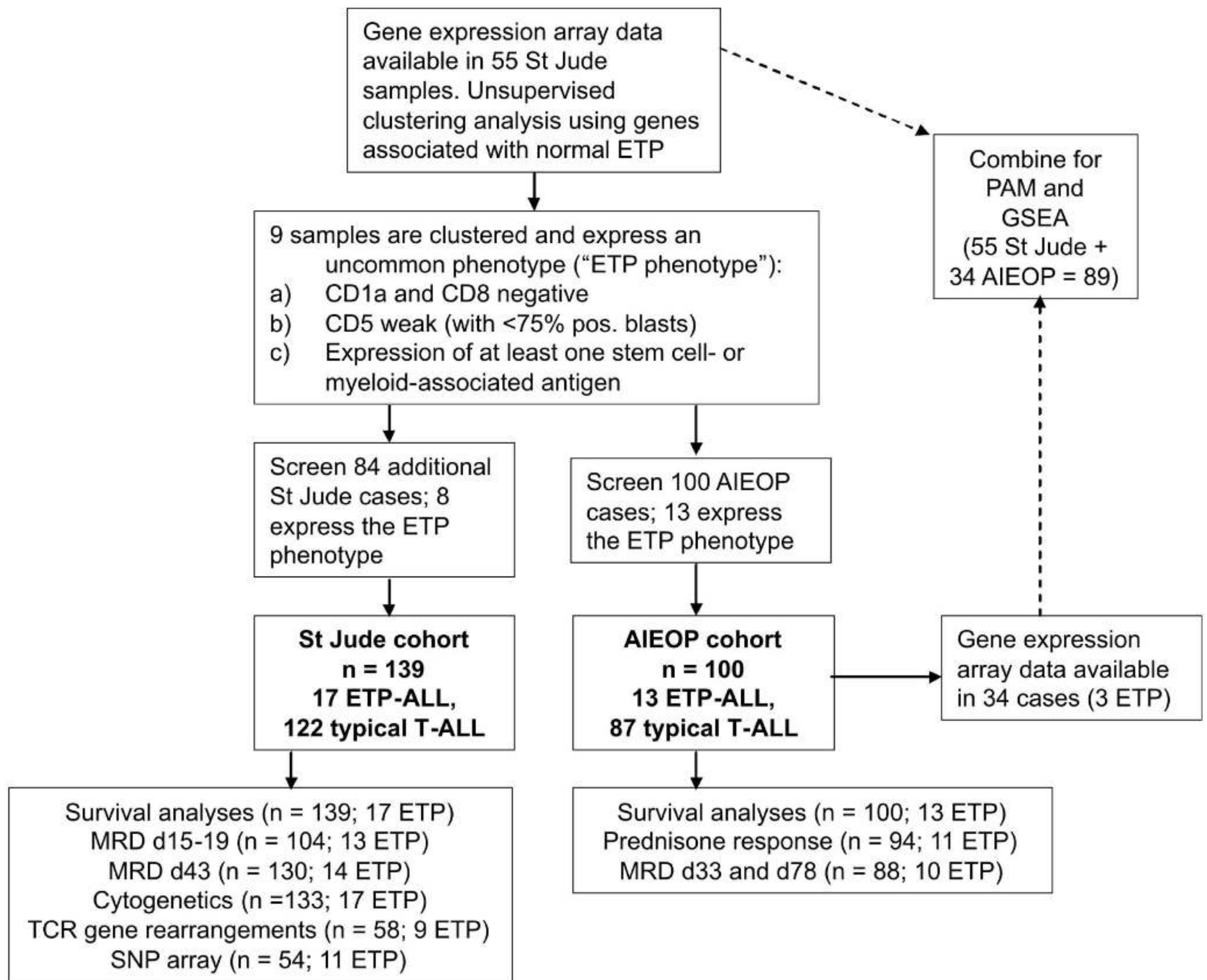


**Figure 1.**
Schematic representation of the studies performed.

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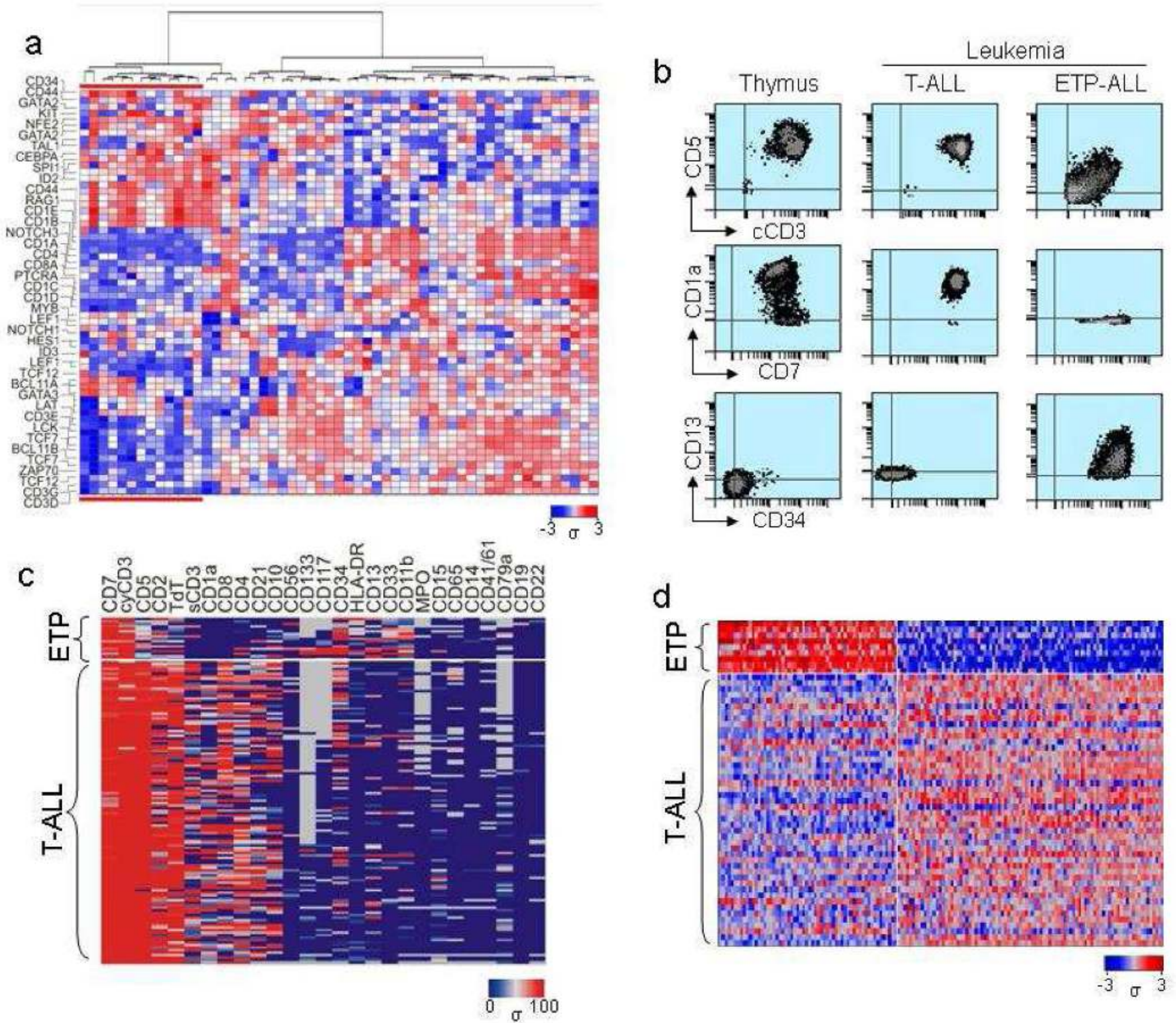


**Figure 2.**

Gene expression and immunophenotypic features of ETP-ALL. **(a)** Unsupervised clustering analysis of diagnostic bone marrow samples from 55 pediatric patients with T-ALL, using a set of genes differentially expressed in ETP (Supplementary Table 1).[11;14–16] One cluster (indicated by red bars) had a gene expression profile resembling that of ETP cells. **(b)** Flow cytometric contour dot plots illustrating the immunophenotypes of normal thymocytes (discarded material from infants undergoing open heart surgery), and representative cases of ETP and typical T-ALL. Each sample was labeled simultaneously with the indicated antibodies (cCD3 = cytoplasmic CD3) and analyzed with an LSRII flow cytometer and DIVA software using a log density and biexponential setting. Vertical and horizontal lines in each plot correspond to zero immunofluorescence. **(c)** The heat map shows percentages of positive leukemic cells for each of the listed markers among the 139 St Jude T-ALL cases studied at diagnosis; gray indicates missing data. Each row represents one case and each

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column the percentage of positive leukemic cells with the indicated marker. **(d)** Heat map of the top 150 differentially expressed genes (see Supplementary Table 3), ranked by *P* value.

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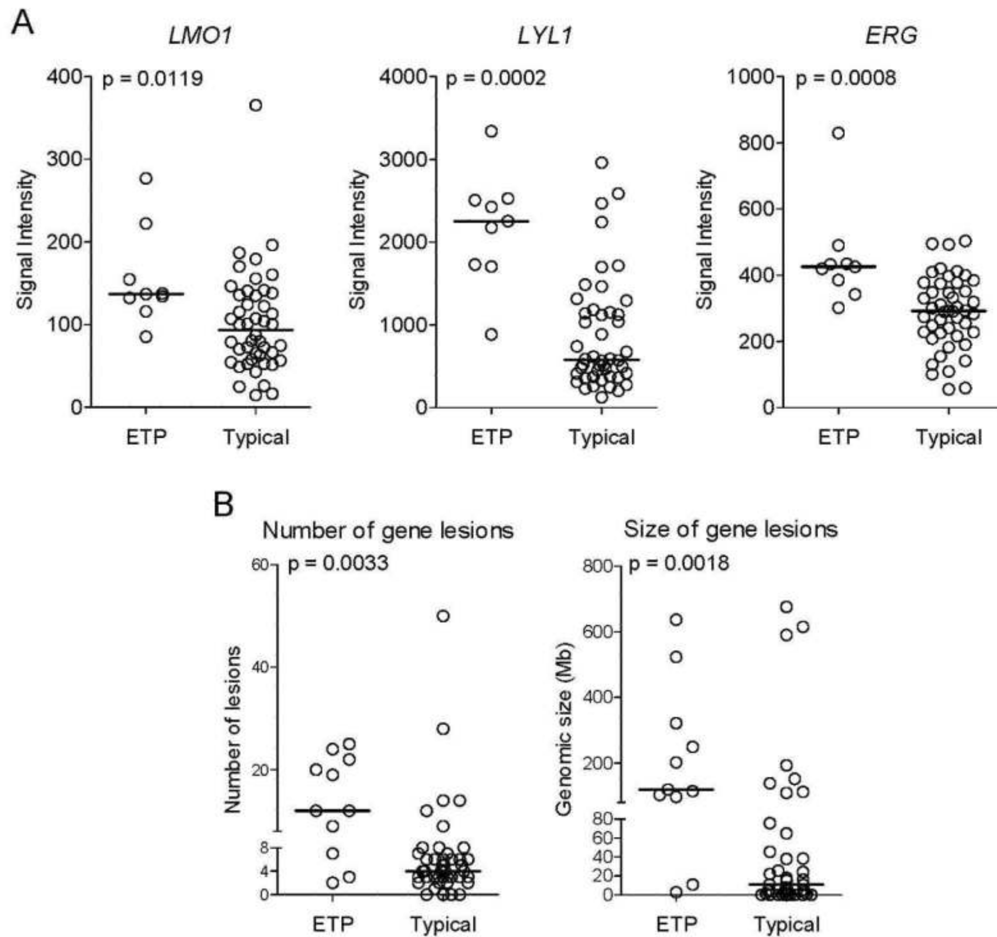


**Figure 3.**

Genetic abnormalities in ETP-ALL. **(a)** Expression of transcription factors previously implicated in the pathogenesis of T-ALL7;29 in cases with an ETP origin (n = 9) or typical T-ALL (n = 46) immunophenotypes, as measured by the Affymetrix 133A GeneChip. Bars indicate median values. **(b)** SNP array analysis of genetic lesions in 11 ETP-ALL and 43 typical T-ALL cases. Total numbers of genomic gains and losses and the sizes of genomic lesions are shown. Further details can be found in Supplementary Fig. 3 and Supplementary Table 5. Bars indicate median values in all panels.

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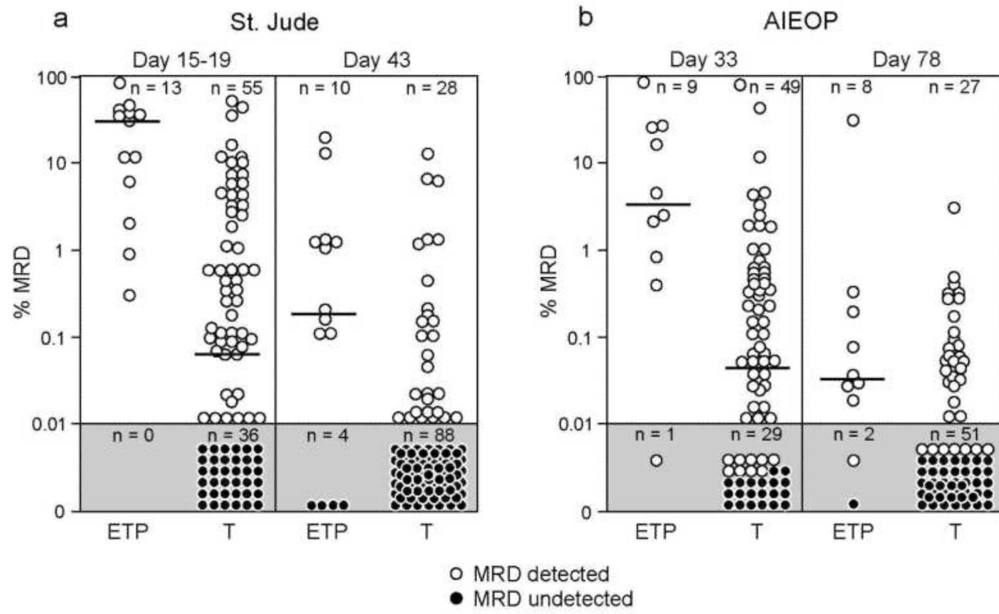


**Figure 4.**

Prevalence of MRD during the early phases of therapy for patients with ETP or typical T-ALL. MRD levels were measured by flow cytometry **(a)** or by polymerase chain reaction amplification of antigen-receptor genes **(b)**. Horizontal bars indicate median values, if above 0.01%.

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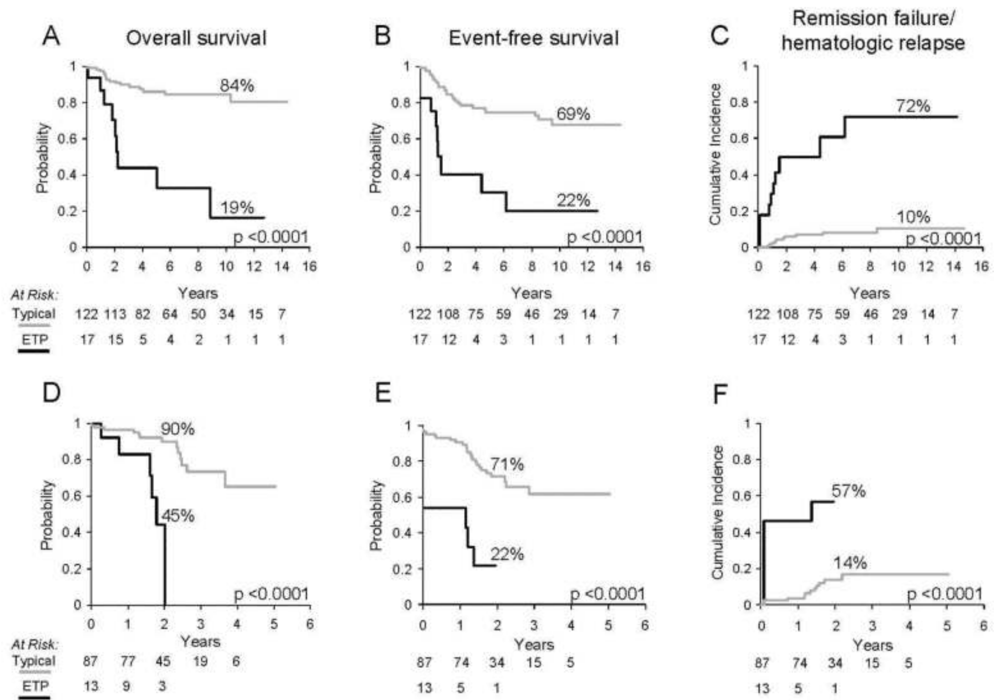


**Figure 5.**

Kaplan-Meier plots of **(a, d)** overall survival, **(b, e)** event-free survival, and **(c, f)** the cumulative incidence of remission failure or hematologic relapse in patients with typical T-ALL (gray line) versus ETP-ALL (black line) treated on either St. Jude (**a–c**) or AIEOP protocols **(d–f)**. The event-free survival curves start at the end of remission induction (day 43 for St. Jude and day 33 for AIEOP patients). Outcome estimates at 10 and 2 years of follow-up are shown; *P* values are from the log-rank test.

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## Table 1

Selected clinical and biologic presenting features of St. Jude patients with ETP-ALL

| Pt | Age (yr) | Sex | WBC (× $10^3$/ $mm^3$) | Mediastinal mass | FAB type[a] | EGIL score[b] | | *TCR* gene rearrangement[c] | | | Karyotype |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | T-cell | Myeloid | TCRB | TCRG | TCRD | |
| 1 | 11.9 | M | 76.7 | No | L2 | 4 | 3 | - | - | - | 46,XY,del(11)(q23)[2]/46,XY[29].nuc ish[MLLx2] |
| 2 | 17.3 | M | 9.1 | No | L1 | 5 | 1 | - | - | - | 46,XY[11] |
| 3 | 15.4 | M | 3.8 | No | L2 | 4.5 | 1 | - | - | - | 54,XY,+Y,+4,add(5)(p15),+8,+9,+19,+21,+21, +22[14]/46,XY[14] |
| 4 | 16.0 | M | 41.9 | No | L1 | 4 | 1 | - | - | - | 47,XY,t(3;11)(q12;p15),+19[15]/46,XY[1] |
| 5 | 17.0 | M | 4.5 | No | L2 | 4 | 1 | - | - | - | 46,XY[25] |
| 6 | 13.8 | F | 2.8 | No | L1 | 5.5 | 1 | - | - | - | 47,XX,+4,t(10;12)(p11.2;q15)[3]/47,XX,idem, del(5)(q22q35)[3]/46,XX[14] |
| 7 | 10.4 | M | 460.5 | Yes | L2 | 4.5 | 1 | - | - | - | 46,XY,del(5)(q22q33),+10,del(13)(q12q14), add(15)(p11.1),del(17)(p11.2),−19[12]/46,XY[3] |
| 8 | 16.9 | M | 273 | Yes | L2 | 6 | 2 | - | - | - | 46,XY,del(5)(q13q22),inv(12)(p11.2q24.1), del(13)(q12q22)[8]/46,idem,del(1)(q21q42)[10]/46,XY[2] |
| 9 | 10.1 | M | 2.2 | No | L1 | 4.5 | 2 | Yes | Yes | Yes | 47,XY,der(1)add(1)(p36.3)add(1)(q32),t(4;7) (q21;p22),del(9)(p13),der(11)add(11)(p13)add(11)(q21),add(12)(p11.2),add(18)(q23),+mar[12]/47,idem,-der(1),-der(11), add(19)(p13.3)[7]/<br>46,XY[6] |
| 10 | 7.9 | M | 44.8 | No | L1 | 6 | 3 | Yes | No | No | 46,XY,del(1)(p22p32),inv(2)(p11.2q13)c,der(4)t(4;10)(q21;q26),der(5)t(5;10) (p13;p11.2),der(10) t(4;10;4;10;5)(4qter->4q?::10p11.2->10q11.2::4q?::10q::5p13->5pter)[18]/<br>46,XY,inv(2)(p11.2q13)c[2] |
| 11 | 3.4 | M | 31.8 | No | L2 | 3.5 | 1 | Yes | No | No | 47,XY,+9,der(11)del(11)(p11.2p15)inv(11)(p11.2q22)[19]/46,XY[1] |
| 12 | 12.8 | F | 16.6 | No | L1 | 6 | 1 | No | No | Yes | 46,XX,t(4;11)(q21;p15),del(5)(q22q35),del(12) (p12)[20] |
| 13 | 6.2 | F | 63.8 | No | L1 | 3.5 | 3 | Yes | No | No | 47,X,-X, ins(12;9)(p12;q13q34),+16,+19[9]/48,X,del(X) (q24),idem[9] |
| 14 | 10.6 | M | 53.8 | No | L1 | 4.5 | 2.5 | No | No | No | 46,XY[20] |
| 15 | 12.1 | M | 181.6 | No | L1 | 3.5 | 2 | Yes | Yes | Yes | 46,XY,add(7)(q35),del(11)(q21q23.1),del(13) (q12q22)[20].nuc ish [MLLx2] |
| 16 | 18.9 | M | 13.1 | No | L1 | 6 | 1 | Yes | No | No | 46,XY,+6,inv(10)(p13q22),add(12)(p13),der(12) t(12;14)(p13;q11.2),del(13)(q22q24),−14[17]/46,XY[3] |
| 17 | 9.9 | M | 3.9 | No | L1 | 4 | 3 | Yes | No | Yes | 48,XY,+19,+22[20] |

[a] Morphology according to the French-American-British (FAB) classification of ALL. Unusual morphologic features (not seen in typical ALL) included prominent surface blebs (n = 5), associated myeloid dysplasia with dysplastic eosinophils (n = 2), phagocytosis by blasts of platelets and nucleated elements (n = 2), and cytochemical myeloperoxidase expression in 1% to 2 % of putative leukemic blasts (n = 4).

[b] Scoring for T-lymphoid or myeloid cell differentiation follows criteria of the European Group for the Immunological Characterization of Leukemias (EGIL) and World Health Organization.[35] Findings indicative of T-ALL include CD3 expression, score of 2; CD2, CD5, CD8 or CD10 expression, score of 1, and TdT, CD7 or CD1a expression, score of 0.5. Those indicative of AML included myeloperoxidase expression, score of 2, CD117, CD13, CD33 or CD65 expression, score of 1, and CD14, CD15 or CD64 expression, score of 0.5. Leukemias with scores greater than 2 for myeloid and 1 for lymphoid are termed biphenotypic by EGIL criteria.

[c] Dashes indicate not done.

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## Table 2

Univariate and multivariate analysis of event-free survival according to the diagnosis of ETP-ALL and selected variables in St. Jude patients[a]

| | Univariate | | | | Multivariate | | | |
|---|---|---|---|---|---|---|---|---|
| | | 95 % CI | | | | 95 % CI | | |
| Variable | Hazard Ratio | Lower | Upper | p value | Hazard Ratio | Lower | Upper | p value |
| ETP-ALL | | | | | | | | |
| Yes vs No[b] | 4.58 | 2.25 | 9.33 | <0.0001 | 10.65 | 3.73 | 30.42 | <0.0001 |
| Sex | | | | | | | | |
| Male vs. Female | 1.09 | 0.52 | 2.30 | 0.82 | 1.24 | 0.57 | 2.69 | 0.59 |
| Race[c] | | | | | | | | |
| White vs. Other | 0.76 | 0.39 | 1.46 | 0.40 | 0.95 | 0.44 | 2.04 | 0.89 |
| Age (yr)[d] | | | | | | | | |
| <1 vs. 1–10 | 1.81 | 0.24 | 13.45 | 0.56 | 1.62 | 0.18 | 14.31 | 0.66 |
| >10 vs. 1–10 | 0.81 | 0.42 | 1.56 | 0.53 | 0.42 | 0.18 | 1.00 | 0.05 |
| WBC ($10^9$/L)[d] | | | | | | | | |
| ≥10–49 vs. <10 | 0.86 | 0.34 | 2.19 | 0.75 | 0.85 | 0.31 | 2.35 | 0.75 |
| 50–99 vs. ≤10 | 0.20 | 0.04 | 0.94 | 0.04 | 0.14 | 0.03 | 0.68 | 0.02 |
| ≥100 vs. ≤10 | 0.99 | 0.43 | 2.27 | 0.99 | 0.92 | 0.35 | 2.37 | 0.85 |
| CNS involvement | | | | | | | | |
| Yes vs. No | 1.84 | 0.97 | 3.51 | 0.06 | 2.38 | 1.06 | 5.33 | 0.04 |
| Mediastinal mass | | | | | | | | |
| Yes vs. No | 0.98 | 0.51 | 1.89 | 0.95 | 1.39 | 0.54 | 3.55 | 0.49 |
| Treatment protocol | | | | | | | | |
| XIII and XIV vs. XV[e] | 1.15 | 0.58 | 2.27 | 0.69 | 0.88 | 0.34 | 2.33 | 0.80 |
| MRD on day 43 | | | | | | | | |
| ≥0.01% vs. <0.01% | 2.30 | 1.12 | 4.72 | 0.02 | 1.30 | 0.56 | 3.03 | 0.54 |

[a]By the Cox proportional-hazards model.

[b]Based on the 17 patients with ETP-ALL versus the 114 with a typical immunophenotype (eight patients were excluded because of incomplete information on one or more variable)

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[c] As reported by parents (or patients, if older than 18 years of age)

[d] Age and WBC were also analyzed as continuous variables. They were not significant predictors and did not substantially alter the results of the multivariate analysis when included as such in the model (data not shown)

[e] Because of the small number of patients enrolled in Study XIV (see Supplementary Table 6), this cohort was combined with patients in Study XIII