**MLL Rearrangements Impact Outcome in HOXA-deregulated T-lineage Acute Lymphoblastic Leukemia: A Children’s Oncology Group Study**

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Approximately 15% of newly-diagnosed patients present with T-lineage acute lymphoblastic leukemia (T-ALL). When matched for National Cancer Institute (NCI) risk criteria, patients with T-ALL are at greater risk of relapse than those with B-cell precursor ALL (BCP-ALL), warranting specialized therapies. Unlike BCP-ALL, where molecular abnormalities are commonly utilized for risk-adapted treatment, the recurring molecular lesions found in T-ALL are not. While most patients with T-ALL can be cured, survival is poor for those with refractory disease or relapse. With the advent of targeted therapies, efforts are underway to identify lesion-specific treatments for high-risk T-ALL.

In contrast to BCP-ALL, the molecular lesions that deregulate Homeobox A (HOXA) genes appear to be widely prevalent in T-ALL and acute myeloid leukemia (AML). Deregulated HOXA expression commonly occurs in immature, T-cell precursors (ETPs), or can be acquired by leukemic cells harboring lesions involving mixed lineage leukemia gene rearrangements (MLL-R) at 11q23, PICALM-AF10 at t(10;11)(p13;q14), SET-NUP214 and inv(7)(p15q34). Because there is a paucity of experience regarding the prognostic impact of HOXA-deregulating lesions in T-ALL, we utilized a retrospective cohort of 100 T-ALL patients enrolled on COG AALL0434 (NCT00408005) to analyze the cytogenetic and genomic features associated with treatment-related clinical outcomes. We enriched our cohort with 17 cases (17%) for whom induction failed (IF; M3 marrow at Day 29), allowing us to better evaluate the molecular lesions within this subset. The remaining cases were from patients who relapsed (REL; n = 8), or remained in complete continued remission (CCR; n =
Deregulated HOXA expression is a hallmark of MLL-R and AF10-R leukemias. To identify the prevalence of these and other rearrangements in our 100-member cohort, we performed an iterative evaluation of cytogenetics, FISH, and RNA sequence analyses. In 12 cases, we found MLL-R, including MLL-AF6 (KMT2A-MLLT4; n = 4), del3’MLL (n = 3), MLL-ENL (KMT2A-MLLT1; n = 3), MLL-PICALM (KMT2A-PICALM; n = 1) and MLL-AF17 (KMT2A-MLLT6; n = 1). Eight cases harbored re-arrangements of AF10, including PICALM-AF10 (n = 6), and two with DDX3X-AF10 lesions, one with a novel CASK gene fragment in a complex CASK-DDX3X-AF10 translocation (Table 1, Figure S1). Five cases harbored other previously described lesions, two with inv(7)(p15q34) (#18, #91), two with NUP98-R fusions, and one with HOXA10-TRBC. We identified four novel lesions: the first involving LINC01250-CCDC91, where a trans-Golgi transport regulator was fused to an intergenic region located 0.1 Mb upstream of ETV6 (#27); a second involving a fusion between RPP30-TLX1NB (#28), which also had a TRCB-MYB rearrangement; a third involving NUP98 rearranged to an intergenic region at 2q32 (#52); and the fourth involved a STAG2-LMO2 fusion (#96) (Table 1, S3 and S4). Although these newly-identified lesions showed HOXA deregulation, they represent distinct genetic subtypes of T-ALL where HOXA overexpression reflects stage of maturation arrest (data not shown).

To investigate upregulation of HOXA genes in T-ALL cases regardless genetic subtype of T-ALL we performed unsupervised hierarchical clustering using 25 probe sets for genes within the HOXA gene family. We identified a cluster of 20 cases that were characterized by increased expression of HOXA genes (FDR ≤0.05) (Figure S2, Table S5). Within this cluster, HOXA9/10 had > 60-fold increased expression over baseline (HOXA5 > 28 fold; HOXA3, HOXA7 and HOXA10 > 3 fold) (Table S5). We validated our profiling approach in an independent 90-member patient series, reported by Soulier et al., confirming that HOXA-deregulated T-ALL is enriched for MLL-R and AF10-R (Figure S2, Table S6 and S7).

We hypothesized that subset analyses might identify lesions that were associated with refractory or relapsed disease. We found that MLL-R, but not AF10-R correlated with IF in T-ALL (P = 0.005) (Table S8). We found that cases with MLL-R had an inferior EFS compared to those that did not (P = 0.0035) (Figure 1A). Univariate and multivariate regression analyses indicated that MLL-R were significantly associated with IF (P = 0.003, 0.003) and EFS (P = 0.009, 0.008) after adjusting for the effects of age and WBC (Table S9 and S10). Patients bearing the ETP-ALL phenotype have been characterized as having poorly differentiated, stem-cell like immunophenotype. Because MLL- and AF10-R leukemias also demonstrate features of undifferentiated leukemias, and the COG immunophenotypic flow analyses for ETP-ALL status were not completely assessed for all patients, we utilized expression profiling to distinguish cases represented by immature, early T-cell precursors (ETPs) using the gene signature developed by Coustan-Smith et al. (Table 1, Figure S3). We found an association between ETPs and early treatment response (P = 0.01) (Table S8) and an inferior EFS (P = 0.029) (Figure S4), thus we assessed whether ETPs cases are enriched with translocations harboring MLL or AF10 genes. We found a

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marginally significant association between the presence of MLL-R and the ETPs status in our cohort (P = 0.07, Table S11). To investigate the effects of ETPs/MLL-R on T-ALL patient outcome, we next tested the relationship of MLL-R with IF, adjusting for ETPs phenotype, and found that the signature of ETPs/MLL-R was associated with IF in T-ALL (P = 0.01) (Table S12 and S13). We extended these observations to assess the impact of ETPs/MLL-R on EFS, and found a significant association with refractory disease and relapse (P = 0.005) (Figure 1B). Because MRD has emerged as a prognostic indicator of high-risk disease in T-ALL, we investigated the MRD status in MLL-R cases depending on the fusion partner or 3’-deletion. Disease progression was significantly associated with MLL-AF6, FISH-identified del3’MLL; exceptions occurring only if Day 29 MRD was < 0.1 (Table 1, Figure 1C). Interestingly, no patient with MLL-ENL failed therapy, despite Day 29 MRD levels ranging from < 0.01 to 2.8%, supporting reports that they do well with modern therapies. While AF10-R have been reported to confer an adverse risk in adult T-ALL\(^\text{10}\) in association with ETP features, we observed that only those with Day 29 Induction ≥10% failed treatment, in contrast to patients with MRD < 10%, who maintained a durable first remission when treated on AALL0434 and its single delayed intensification phase.

Because the recurring cytogenetic abnormalities that deregulate HOXA in T-ALL have not been systematically evaluated, the potential impact of such lesions on outcome has been unclear. Here, we profiled 100 patients for HOXA-deregulated T-ALL to determine whether the related molecular lesions might correspond with treatment outcome. The prognostic impact of MLL-R and AF10-R in T-ALL has been less clear, in part due to their rarity and difficulties in detection, and because of their variable T-cell stage of arrest. In our 100-case series, the molecular repertoire of T-ALL fusions, deletions and inversions was highly heterogeneous, with many lesions occurring with a frequency of 5% or less, and, in ~30% cases cytogenetic analyses were never performed (data not shown). It is therefore not surprising that the prognostic impact of molecular lesions remains an unanswered question in T-ALL.

Since first described by Coustan-Smith et al., patients with the ETP-ALL phenotype have received much attention for their unique biological profile and increased risk for relapse.\(^\text{11}\) Recently, ETP-ALL patients have been reported to have similar outcomes as non-ETP patients on the UKALL 2003 and COG AALL0434 studies.\(^\text{12}\) When analyzed as a continuous variable on the AALL0434 study, Induction Day 29 MRD ≥10% was highly predictive of relapse, if not outright induction failure, but not ETP-ALL.\(^\text{13}\) Moreover, HOXA deregulation does not confer a worse prognosis in T-ALL,\(^\text{5}\) but we identified a subset of HOXA-deregulated cases having high end-induction MRD with MLL-R and AF10-R that failed therapy, suggesting that such patients might benefit from early identification, follow-up MRD monitoring, and/or alternate approaches to therapy. We have also shown that patients with MLL-driven immature cells, having ETPs features, were likely to fail therapy, especially when involving MLL-AF6 or del3’MLL rearrangements. While MLL-AF6 lesions have been reported to confer a worse prognosis in AML,\(^\text{14}\) we are the first to show their impact on outcome in T-ALL. In contrast, our results support the findings by Nigro et al.\(^\text{15}\) showing that in pediatric T-ALL, AF10-R tend to be more commonly arrested in more differentiated state, and without an adverse effect on outcome (Figure 1).
There is pressing need to re-evaluate the role of routine cytogenetics/FISH testing in T-ALL. Because IF is a relatively rare event in the current era of modern therapies, the identification of molecular biomarkers relevant to disease resistance and treatment failure has been challenging. Enrichment of the tested cohort in IF cases allowed us to show that MLL rearrangements are determinants of high-risk disease in T-ALL. In addition to testing all samples for MRD at the end-Induction and end-Consolidation, we propose that cytogenetic tests be performed on all T-ALL patients at diagnosis specifically including testing for MLL-R and AF10-R. In cases where Day 29 MRD is ≥0.1%, follow-up MRD testing might be used to intensify conventional therapy, pursue targeted therapies, or consider transplant in first remission. Further studies are warranted to validate our findings in larger retrospective cohorts or early clinical trials.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Event free survival in molecular interrogation cohort of 100 T-ALL (COG AALL0434). (A) EFS for MLL-R (n = 12; black line) vs those with MLL germline (MLL-WT, n = 88; blue dash line) (log rank Mantell-Cox, P = 0.0035). (B) EFS for MLL-R having the ETPs phenotype by GEP (MLL-R/ETPs, n = 6; black solid line) vs. MLL-R cases without the ETPs signature (MLL-R/non-ETPs, n = 6; blue dash line) vs. non-rearranged MLL with ETPs phenotype by GEP (MLL-WT/ETPs, n = 20; purple, dot line) vs non-rearranged MLL without the genomic ETPs signature (MLL-WT/non-ETPs, n = 68; yellow dash-dot line).
(log rank Mantell-Cox, P = 0.0057). \textit{MLL-R/ETPs vs. MLL-WT/non-ETPs, P = 0.0004; MLL-R/non-ETPs vs. MLL-WT/non-ETPs, P = 0.0752; MLL-R/ETPs vs. MLL-WT/ETPs, P = 0.1364; MLL-WT/ETPs vs. MLL-WT/non-ETPs, P = 0.1399}. (C) Post-Induction Day 29 MRD levels in T-ALL patients (COG AALL0434) with specific \textit{MLL-R} (n = 10) and \textit{AF10-R} (n = 8) (Table 1) measured by flow cytometry (circles – \textit{MLL-AF6}, squares – FISH-identified del3’\textit{MLL}, diamond - \textit{MLL-ENL}, triangle - \textit{AF10-R}; red - IF; green - REL; black - CCR).
Table 1

Karyotypic, FISH and molecular classification for 23 patients treated on AALL0434 with features of HOXA overexpression and/or MLL-R/AF10-R. All FISH analyses were done in CLIA-approved reference centers. No cases of AF10-R were initially found at diagnosis.

| ID  | Age | Gender | WBC | CNS | ETPs by GEP | Day 29 MRD (%) | Karyotype | FISH | RNA seq | Lesion |
|-----|-----|--------|-----|-----|-------------|---------------|-----------|-------|---------|--------|
| 7   | 9   | Male   | 6.8 | 1   | ETPs       | 25.8          | 46,XX,t(6;11)(q27q23)(10)/46,X Y[10] | MLL-AF6 | MLL-AF6 |
| 25  | 7   | Female | 196 | 2   | 84         | MLL-AF6       | MLL-AF6 |
| 43  | 7   | Female | 144.9 | 1   | ETPs       | 93            | MLL-AF6   | MLL-AF6 |
| 79  | 16  | Female | 58.1 | 1   | < 0.01     | 46,XX,t(7;12)(q34p13),del(11)(q22q23)8/46,XX[2] | MLL-AF6 | MLL-AF6 |
| 67# | 8   | Male   | 577.5 | 2   | 0.48       | -46,XY[4;7](q12q36)6/43-46,XY,add(20)(p12)[cp9] | del3'MLL | del3'MLL |
| 89  | 18  | Male   | 6.6 | 1   | ETPs       | 94.3          | 46,XX,del(1)(p10)add(16p34),del(11)(q23),−21,+mar[3]/46,XY[37] | del3'MLL | del3'MLL |
| 77# | 16  | Male   | 379.8 | 1   | 65.6       | -46,XY,der(3)(3;5)(q12q34);t(4;7)(q21q22),add(5)(q13),add(9)(p22,add(9)(q22),del(11)(q23),del(13)(q12q44),del(18)(q11);q21q23);j9/46,XY[1] | del3'MLL | del3'MLL |
| 23  | 12  | Male   | 48.3 | 1   | ETPs       | 0.11          | 46,XY,t[11;19]q23;11(3)3(20)/46,XY[3] | MLL-ENL | MLL-ENL |
| 31  | 9   | Male   | 139.1 | 2   | < 0.01     | 46,XY,t[11;19]q23;11(3)3(20)/46,XY[3] | MLL-ENL | MLL-ENL |
| 85 * | 2   | Male   | 440.1 | 1   | 2.8        | MLL-ENL       | MLL-ENL |
| 59  | 17  | Female | 260.6 | 1   | ETPs       | < 0.01        | 47,XX,del(1)(q32),del(5)(q22),der(11)(q11;17)(q23q21)+15,−17,mar[cp8]/46,XY[12] | MLL-AF17 | MLL-AF17 |
| 21  | 12  | Male   | 389  | 1   | ETPs       | 50.8          | 46,XY,der(11)(q11;14)(p13q11.2),t(11;15)(q22q22),der(14)(q11;14)(p13q11.2),der(15)(q11;15)(q21q22)[4]/46,XY[16] | MLL-PICALM | MLL-PICALM |
| 1   | 7   | Male   | 9.6  | 1   | < 0.01     | 46,XY[20]     | PICALM-AF10 | PICALM-AF10 |
| 94  | 5   | Female | 158.9 | 1   | < 0.01     | 46,XY[20]     | PICALM-AF10 | PICALM-AF10 |
| ID  | Age | Gender | WBC | CNS | ETPs | Day 29 MRD (%) | Karyotype                                                                 | FISH       | RNA seq   | Lesion       |
|-----|-----|--------|-----|-----|------|----------------|--------------------------------------------------------------------------|------------|-----------|--------------|
| 46# | 7   | Male   | 19.6| 1   | 1    | < 0.01        | 45,XY;dc(7;12)(p11.2;p11.2<del)(9)(p32)(10;11)(p12;q14),-21[3]/47,XY/17 | PICALM-AF10| PICALM-AF10|              |
| 68  | 7   | Female | 91.9| 2   | 4.2  |               | 46,X;add(X)(q26);del(5)(q31),t(10;12)(q21;q13)/46,X[7]                  | PICALM-AF10| PICALM-AF10| PICALM-AF10  |
| 72  | 27  | Male   | 19  | 1   | ETPs | 28.4          | 46,XY,t(10;11)(p13;q21)[8]/45,del(9)(p13q14),-9,-9,mar(4)[4]/46,XY[8]  | AF10-R     | PICALM-AF10|              |
| 11  | 14  | Male   | 243.4| 1 | 11   |               | 46,XY,add(10)(p13q11)(q21)x2,add(12)(p11.2)[5]/46,XY                  | PICALM-AF10| PICALM-AF10|              |
| 16  | 5   | Male   | 142.1| 1 |      | < 0.01        | 46,Yt(X;10)(p10;p10)[18]/46,XY[2]                                      | AF10-R     | CASK-DDX3X-AF10| CASK-DDX3X-AF10 |
| 34  | 14  | Male   | 72.5| 3   | 0.6  |               | 46,XY,der(9)(qter->q34;p24-<qter)/46,XY                                | AF10-R     | DDX3-AF10| DDX3-AF10    |
| 52  | 12  | Male   | 116.7| 1 | 48.3 |               | 45,XY,add(2)(q21)x2,add(11)(p11.2),del(12;17)(p10p10)[20]            | NUP98-R    | NUP98-IGR(2q32.3)|              |
| 18  | 11  | Male   | 123 | 2   |      | < 0.01        | 46,XY,inv(7)(p15q34),del(12)(p12)[17]/46,XY[10]                        | TCRB-HOA10 | TCRB-HOA10|              |
| 27  | 7   | Female | 351.4| 1 | ETPs | 88.3          | 46,XX,del(2)(q33),del(12)(p12)[20]                                    | LIN012      | IGR(12p13.2)-CCDC91|              |

*MLL-ENL* fusion that was missed at diagnosis.

*(Italic)* cases (#67, #77, #46) that did not fall within 20-member HOXA cluster.

**Bold:** Supplemental FISH screening at Mayo Clinic; LINC – long intergenic noncoding RNA; IGR - intergenic region.