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Clinicopathologic and prognostic features of TdT-negative pediatric B-lymphoblastic leukemia

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Little is known about B-lymphoblastic leukemia (B-ALL) that lacks expression of terminal deoxynucleotidyl transferase (TdT). To address this, we performed the largest study to date of TdT-negative B-ALL using data from St. Jude Total XV and XVI clinical trials. Compared to TdT-positive B-ALL ($n = 896$), TdT-negative B-ALL ($n = 21$) was associated with younger age (median, 1.4 versus 6.8 years, $P < 0.001$), higher white blood cell count (median, 52.8 versus 9.9×10^9/L, $P < 0.001$), absence of hyperdiploidy (0 versus 27.8%, $P = 0.002$), *KMT2A* rearrangement (100 versus 1.9%, $P < 0.001$), and inferior 5-year event-free survival (EFS) (76.2 versus 90.3%, $P = 0.047$). In the context of *KMT2A*-rearranged B-ALL ($n = 38$), TdT-negativity was significantly associated with the *MLLT1* rearrangement partner ($P = 0.026$) but was not independently predictive of survival, suggesting that the high-risk features of TdT-negative B-ALL are secondary to underlying *KMT2A* rearrangements. Finally, we compared the sensitivity of TdT-negativity to neuron-glial antigen 2 (NG.2) expression for the detection of *KMT2A* rearrangements and found that 63% of *KMT2A*-rearranged B-ALL cases not identified by NG.2 were TdT-negative. The results of this study expand the spectrum of immunophenotypic features that are specific for high-risk *KMT2A* rearrangements in pediatric B-ALL and can be readily implemented using existing standard acute leukemia flow cytometry panels.

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BACKGROUND

Terminal deoxynucleotidyl transferase (TdT) expression is an immunophenotypic marker of immaturity characteristic of lymphoblasts. With rare exceptions, the expression of TdT by an aberrant leukemic clonal B-cell population supports the diagnosis of B-lymphoblastic leukemia (B-ALL) while effectively ruling-out a mature B-cell neoplasm. However, ~2% of B-ALL cases lack TdT expression [1] and little is known about the clinicopathologic and genetic features of this unusual and potentially diagnostically challenging immunophenotypic subtype. To address this, we performed the largest study to date of TdT-negative B-ALL.

METHODS

With approval from the institutional review board of St. Jude Children's Research Hospital, all cases of B-ALL treated on the St. Jude Total Therapy XV and XVI clinical trials were identified [2, 3]. Cases of B-ALL that were reported as TdT-negative were selected for further review. Review of corresponding bone marrow aspirate or peripheral blood smears was performed for all cases to confirm that the leukemic population was morphologically consistent with lymphoblasts. Original flow cytometry scatter plots were reviewed for each case to evaluate TdT expression using residual T-lymphocytes as a negative internal control with a 10% cutoff used to define TdT negativity [4]. Cases in which the intensity of lymphoblast TdT expression matched that of the isotype control yet exceeded that of residual lymphocytes in >10% of events were regarded as TdT subset positive (Fig. 1). Corresponding clinicopathologic and survival data for cases of TdT-negative B-ALL were subsequently compared to those of TdT-positive B-ALL.

Flow cytometry

Four or eight color flow cytometry was performed on diagnostic peripheral blood and bone marrow aspirate samples using a FACSCalibur or FACSCanto II flow cytometer and analyzed with FACSDiva software (Becton Dickinson, San Jose, CA) as previously described [5, 6]. Anti-TdT antibody (clone HT-6; Agilent Technologies, Santa Clara, CA) was used to evaluate cytoplasmic TdT expression. A minimum of 20,000 cells per tube were counted and the overall sensitivity of the assay for the detection of a leukemic lymphoblast population was 0.05%.

Leukemic B-lymphoblasts were identified by a combination of CD45 versus side-scatter properties, co-expression of B-lineage markers (CD19, CD22, and cytoplasmic CD79a), and immunophenotypic markers of immaturity (CD34, CD133, and NG.2), absence of cytoplasmic CD3 and cytoplasmic MPO expression, and aberrant immunophenotypic features which distinguished the neoplastic population from hematogones. In cases which lacked expression of all immaturity markers (TdT, CD34, CD133, and NG.2), the diagnosis of B-ALL was supported by the presence of extensive leukemic disease, lymphoblast morphology, absence of surface light chain expression by flow cytometry, and cytogenetic features.

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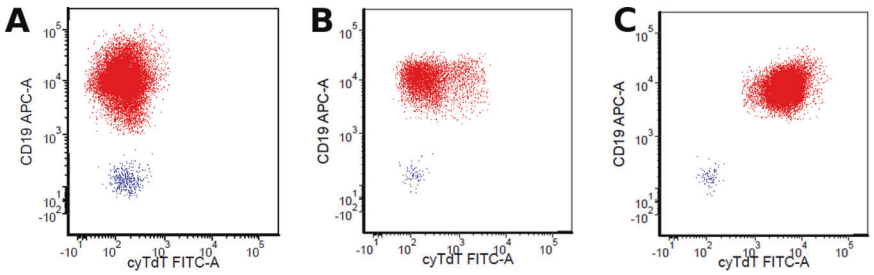

Fig. 1 Examples of variable TdT expression in B-ALL. Flow cytometry scatter plots depict three separate cases of B-ALL with different levels of TdT expression. Expression of TdT was measured using T-lymphocytes as a negative internal control and cases were classified as TdT-positive if >10% of B-lymphoblasts expressed TdT at an intensity greater than that of the T-lymphocytes. Blue events represent T-lymphocytes and red events represent leukemic B-lymphoblasts. **A** Example of B-ALL classified as TdT-negative based on <10% of B-lymphoblasts expressing TdT at an intensity greater than that of the T-lymphocytes. **B** Example of a case classified as TdT subset positive due to dim TdT expression in >10% of B-lymphoblasts. **C** Example of TdT-positive B-ALL with uniformly bright TdT expression.

Cytogenetics

Giemsa-banded conventional cytogenetic studies were performed on unstimulated fresh bone marrow aspirate or peripheral blood samples with a minimum of 20 metaphases counted. *KMT2A* rearrangements were also identified by fluorescence in situ hybridization using a *KMT2A* (11q23) dual color probe (Abbott Molecular Inc., Des Plaines, IL) with 200 interphase nuclei evaluated for each case and by reverse-transcriptase polymerase chain reaction (RT-PCR).

Evaluation of CNS disease

Assessment of central nervous system (CNS) involvement was performed by cytomorphologic evaluation of Wright-stained cerebrospinal fluid cytospin preparations and classified as follows: CNS 1, no detectable blasts; CNS 2, <5 WBC/μL with blasts; CNS 3, ≥5 WBC/μL with blasts; and traumatic lumbar puncture with blasts, ≥10 RBCs/μL with blasts.

Statistical analysis

Categorical variables were compared using Fisher's exact test while continuous variables were compared using the Wilcoxon rank sum test. Event-free survival (EFS) and overall survival (OS) probabilities were estimated using the Kaplan–Meier method and compared using the log-rank test. All reported *P* values are two-sided and not adjusted for multiple comparisons. Statistical analyses were performed with SAS version 9.4.

RESULTS

Immunophenotypic features of TdT-negative B-ALL

Twenty-one cases of B-ALL originally reported as TdT-negative were identified among the 917 B-ALL cases treated on Total XV and XVI (2.3%), all of which met the morphologic and immunophenotypic inclusion criteria. By flow cytometry, all cases were uniformly positive for CD19, CD22, and cytoplasmic CD79a while negative for cytoplasmic CD3, cytoplasmic MPO, and surface kappa/lambda light chains. The frequency of at least dim or partial expression of other markers was as follows: CD20 (47.6%), CD38 (100%), CD34 (42.9%), CD117 (0%), CD133 (61.9%), CD123 (71.4%), HLA-DR (100%), CD10 (23.8%), CD15 (47.6%), NG.2 (68.8%), CD13 (0%), CD14 (0%), CD33 (33.3%), CD2 (14.2%), CD5 (9.5%), and CD7 (52.3%). Three cases (14.3%) were negative for all immunophenotypic markers of immaturity (TdT, CD34, CD133, and NG.2); in these cases, the diagnosis of B-ALL was supported by the presence of extensive leukemic disease, morphologic features consistent with lymphoblasts, absence of surface light chain expression by flow cytometry, and the presence of *KMT2A* rearrangements.

Characteristics and outcomes of TdT-negative B-ALL from Total XV and XVI

The complete clinicopathologic features are summarized in Table 1. Compared to TdT-positive B-ALL, TdT-negative B-ALL was associated with younger age at diagnosis (median, 1.4 versus 6.8 years, $P < 0.001$), higher white blood cell count (median, 52.8 versus 9.9×10^9/L, $P < 0.001$), non-hyperdiploidy (DNA index < 1.16) (0 versus 27.8%, $P = 0.002$), and *KMT2A* rearrangement (100 versus 1.9%, $P < 0.001$). *KMT2A*-rearrangement represented the sole cytogenetic abnormality in 66.7% of TdT-negative cases; in the remaining cases, none of the additional cytogenetic abnormalities were recurrent. TdT-negative B-ALL had significantly lower 5-year EFS compared to TdT-positive B-ALL (76.2 versus 90.3%, $P = 0.047$; Fig. 2). The difference in 5-year OS between the two groups was not statistically significant (85.7 versus 95.5% for TdT-negative and positive B-ALL, respectively; $P = 0.091$). There was no significant difference in outcomes between cases which only harbored *KMT2A* rearrangements and those which had additional cytogenetic abnormalities.

TdT expression in the context of *KMT2A*-rearranged B-ALL

Among the 917 cases of B-ALL, *KMT2A* rearrangements were identified in 38 (4.1%), the clinicopathologic features of which are summarized in Table 1. Negative TdT expression was identified in 55.3% of *KMT2A*-rearranged B-ALL; among the TdT-positive cases, 47.1% were dim positive while 52.9% showed moderate to bright positivity (Fig. 1). Among *KMT2A*-rearranged B-ALL cases, negative TdT expression was significantly associated with the presence of the *MLLT1* rearrangement partner, which was present in 38.1% of TdT-negative cases versus 5.9% of TdT-positive cases ($P = 0.026$). However, there was no significant association between TdT expression and EFS (75.9% versus 58.8% for TdT-negative and positive *KMT2A*-rearranged B-ALL, respectively; $P = 0.3$).

NG.2 and negative TdT expression as immunophenotypic markers of *KMT2A* rearrangement

NG.2 expression was evaluated in 23 cases of *KMT2A*-rearranged B-ALL and was positive in 15 cases (65.2%). Among the eight cases of *KMT2A*-rearranged B-ALL that did not express NG.2, five were TdT negative (62.5%).

DISCUSSION

TdT is a DNA polymerase present in immature pre-B and pre-T cells that is responsible for the insertion of nucleotides to the V(D)J gene segment during immunoglobulin and T cell receptor gene rearrangement. Its expression is limited to the lymphoid compartment with regulation influenced by stage-specific transcription factors. Leukemic lymphoblasts, in general, express high levels of TdT; ALL cell lines were in fact one of the first sources of the purified enzyme [7]. However, ~2% of pediatric B-ALL cases lack TdT expression [1] and little is known about this unusual subtype. Here, in the largest series of TdT-negative B-ALL to date, we demonstrate that these potentially diagnostically-challenging cases represent an immunophenotypic variant of *KMT2A*-rearranged B-ALL.

Table 1. Characteristics of B-ALL patients from Total XV and XVI based on TdT expression.

	TdT+ (*n* = 896)	TdT– (*n* = 21)	*P* value[a]*, (TdT+ vs TdT−)	TdT+ *KMT2A-r* (*n* = 17)	*P* value[a]** (TdT- *KMT2A-r* vs TdT+ *KMT2A-r*)
Age at diagnosis			0.0001[W]		0.20[W]
Median years (range)	5.1 (0.16–18.89)	1.4 (0.12–18.73)		5.03 (0.16, 18.45)	
<1 year, *n* (%)	5 (0.6)	6 (28.6)		3	
1–9 years, *n* (%)	681 (76.0)	10 (47.6)		9	
>10 years, *n* (%)	210 (23.4)	5 (23.8)		5	
Gender, *n* (%)			0.83		0.52
Male	492 (54.9)	11 (52.4)		11 (64.7)	
Female	404 (45.1)	10 (47.6)		6 (35.3)	
Race, *n* (%)			0.18		0.62
White	724 (80.8)	14 (66.7)		12 (70.6)	
Black	120 (13.4)	5 (23.8)		2 (11.8)	
Other	52 (5.8)	2 (9.5)		31 (17.7)	
WBC (x 10^9/L), *n* (%)			<0.0001[W]		0.83[W]
<50	747 (83.4)	9 (42.9)		8 (47.1)	
50–<100	87 (9.7)	4 (19.5)		2 (11.8)	
≥100	62 (6.9)	8 (38.1)		7 (41.2)	
CNS status, *n* (%)			0.063		0.90
CNS2/3 or	292 (32.6)	8 (38.1)		8 (47.1)	
Traumatic w/blasts		3 (14.3)		2 (11.8)	
CNS-1, traumatic w/o blast	604 (67.4)	10 (47.6)		7 (41.2)	
DNA index, *n* (%)					
≥1.16	249 (27.8)	0	0.002	0	1
<1.16	647 (72.2)	21 (100)		17 (100)	
MRD day 15, *n* (%)			1		1
<5%	796 (88.8)	18 (85.7)		16 (94.1)	
≥5%	87 (9.7)	2 (9.5)		1 (5.9)	
Not done	13 (1.5)	1(4.8)		0	
MRD day 42, *n* (%)			0.75		1
<0.01%	757 (84.5)	16 (76.2)		14 (82.4)	
≥0.01%	131 (14.6)	3 (14.3)		2 (11.8)	
Not done	8 (0.9)	2 (9.5)		1 (5.9)	
KMT2A-r partner, *n* (%)					
AFF1/AF4		5 (23.4)		9 (52.9)	0.094
MLLT1/ENL		8 (38.1)		1 (5.9)	0.026
MLLT3/AF9		6 (28.6)		1 (5.9)	0.10
MLLT10/AF10		2 (9.5)		1 (5.9)	1
Other (not recurrent)		0		5 (11.8)	0.012
Immunophenotype, *n* (%)					
CD34+		9 (42.9)		11 (64.7)	0.21
CD10+		5 (23.8)		2 (11.8)	0.43
CD15+		10 (47.6)		9 (52.9)	1
NG.2+		11/16 (68.8)		4/7 (57.1)	0.66
5-year EFS (95% CI)	90.3 (88.3–92.3)	75.9 (59.5–96.8)	0.047[L]	58.8 (39.5–87.6)	0.3[L]
5-year OS (95% CI)	95.5 (93.9–96.7)	85.7 (72.0–100)	0.091[L]	76.5 (58.7–99.5)	0.56[L]

W Wilcoxon rank sum test, *L* log-rank test.
**P* values for comparisons of all cases of TdT-positive B-ALL to TdT-negative B-ALL.
***P* values for comparisons of TdT-positive *KMT2A*-rearranged B-ALL to TdT-negative *KMT2A*-rearranged B-ALL.
[a]Fisher's exact test unless otherwise specified.

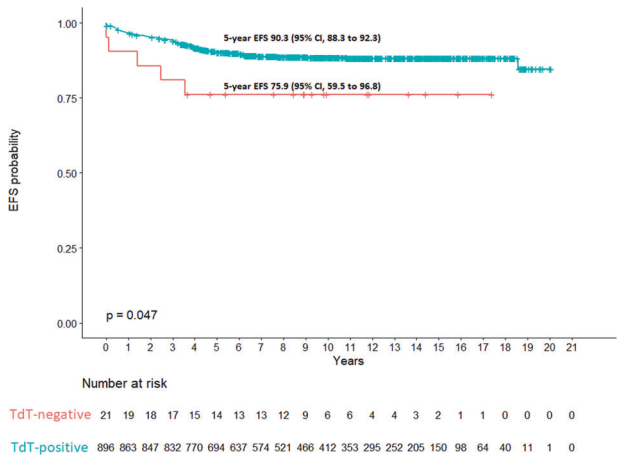

Fig. 2 Event-free survival of TdT-negative and TdT-positive B-ALL. Event-free survival (EFS) of TdT-positive and TdT-negative B-ALL patients from Total XV and XVI clinical trials was estimated using the Kaplan-Meier method and compared with the log-rank test. Tick marks indicate data censored at the time of last follow-up.

Compared to TdT-positive B-ALL, TdT-negative B-ALL was significantly associated with younger age at diagnosis (median, 1.4 versus 6.8 years), higher white blood cell count (median, 52.8 versus 9.9×10^9/L), uniform absence of hyperdiploidy (0 versus 27.8%), and inferior 5-year EFS (76.2 versus 90.3%). Notably, however, when TdT-negative *KMT2A*-r B-ALL was compared to a cohort of similarly treated TdT-positive *KMT2A*-r B-ALL cases, TdT expression was not associated with a significant difference in EFS, suggesting that the high-risk features of TdT-negative B-ALL are best explained, at least in part, by the presence of underlying *KMT2A* rearrangements.

Negative TdT expression is a frequent feature of *KMT2A*-rearranged B-ALL. Overall, 55.2% of pediatric *KMT2A*-rearranged B-ALL was found to be TdT-negative and, interestingly, this distinctive immunophenotypic feature was significantly associated with the *MLLT1* rearrangement partner. In total, eight out of nine cases (88.9%) of B-ALL cases with *KMT2A-MLLT1* were TdT-negative and this accounted for 38.1% of all TdT-negative *KMT2A*-rearranged B-ALL cases compared to 5.9% of TdT-positive cases ($P = 0.026$). The single case with *KMT2A-MLLT1* that was classified as TdT-positive showed only dim subset positivity, indicating that negative or occasionally dim TdT expression is a defining immunophenotypic feature of this particular *KMT2A*-rearranged B-ALL subtype. Consistent with the absence of TdT expression found in this study, a report on immunoglobulin gene rearrangements in infantile *KMT2A*-rearranged B-ALL found that rearrangements were less frequent in *KMT2A*-rearranged disease compared to childhood B-ALL [8]. Further, the maturity status of the rearrangement was mainly determined by the *KMT2A* partner with *KMT2A-MLLT1* positive patients having significantly less *IGK* and *IGL* gene rearrangements compared to *KMT2A-MLLT3* [8].

The observation that TdT-negative B-ALL is consistently *KMT2A*-rearranged aligns with what little data is available in the literature. In a study of 186 children with newly diagnosed B-ALL, only five cases were found to be TdT-negative among which three harbored *KMT2A* rearrangements [1]. Interestingly, Yasmeen et al described four cases of *KMT2A*-wildtype B-ALL which were reported as TdT-negative by flow cytometry but TdT-positive by immunohistochemistry [9]. Given that these cases were TdT-positive by immunohistochemistry, it is possible that the application of the flow cytometric criteria for TdT expression used in our study would have led to the classification of at least some of these cases as TdT dim-positive. Alternatively, this discrepancy may reflect subtle differences in gating strategies or in the overall sensitivity of the assays for the detection of low-level TdT expression. As bone marrow biopsies for B-ALL diagnosed by peripheral blood flow cytometry are not routinely performed at our institution, confirmatory TdT immunohistochemistry was not possible in this study.

The data presented in this study expand the spectrum of immunophenotypic features that are specific for *KMT2A* rearrangement in B-ALL and can be reliably used to predict the presence of these high-risk rearrangements at the time of initial flow cytometric diagnosis. Currently, expression of NG.2 represents the most well-established standalone immunophenotypic marker specific for *KMT2A*-rearrangement in B-ALL [6, 10]. However, anti-NG.2 antibody is not a standard component of most diagnostic flow cytometry panels currently utilized in the clinical setting. Further, even in this series where NG.2 expression was evaluated in a significant subset of cases, it yielded a false negative result in 34.8%. Notably, however, among the *KMT2A*-rearranged B-ALL cases that were NG.2-negative, 62.5% were TdT-negative, demonstrating that even in a setting where NG.2 expression is routinely evaluated, negative TdT expression represents a complementary immunophenotypic parameter for predicting *KMT2A* rearrangement.

In summary, the results of this study demonstrate that negative TdT expression in pediatric B-ALL is a specific immunophenotypic marker for *KMT2A* rearrangement and that this association likely underlies the poor clinical outcomes documented in these cases. As TdT expression is already routinely evaluated by most standard acute leukemia diagnostic flow cytometry panels, recognition of negative TdT expression at the time of initial flow cytometric diagnosis can be used to anticipate the likelihood of *KMT2A* rearrangement and ensure that appropriate confirmatory genetic testing is performed.

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AUTHOR CONTRIBUTIONS

Study conception: M.M.K. and J.K.C.; Study design: M.M.K., J.K.C., H.I., and C.-H.P.; provision of study material: C.-H.P., S.J., J.K.C., H.I., and T.A.G.; data collection and assembly: Y.Z., J.K.C., M.M.K., and Y.L.; data analysis and interpretation: MMK, JKC, YZ, CC, CHP, HI; manuscript writing: MMK; manuscript editing and final approval: all authors.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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