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Characterization of Childhood Precursor T-Lymphoblastic Lymphoma by Immunophenotyping and Fluorescent In-Situ Hybridization: A Report from the Children's Oncology Group

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Abstract

Background—T-lymphoblastic lymphoma (T-LBL) accounts for 25–30% of childhood non-Hodgkin's lymphoma and is closely related to T-lymphoblastic leukemia (T-ALL). Recently, we demonstrated distinct differences in gene expression between childhood T-LBL and T-ALL, but molecular pathogenesis and relevant protein expression patterns in T-LBL remain poorly understood.

Procedure—Children with T-LBL with disseminated disease were registered and treated on COG protocol 5971. Paraffin-embedded tumor tissue was obtained at diagnosis for immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) studies. We determined the pattern and intensity of staining for c-Myc, Skp2, Mib-1, p53, TCL-1, bcl-2, and bcl-6 proteins by IHC and c-Myc, p53, bcl-2, bcl-6, and TCR α/δ molecular alterations by FISH in 22 pediatric T-LBL cases.

Results—The majority of T-LBL samples expressed Mib-1 (59%) and c-Myc (77%) proteins in greater than 50% of the cells, but Skp2 (14%), p53 (14%), and bcl-2 (23%) expression was less common. FISH studies demonstrated 18% gains and 10% losses in c-Myc, 16% gains in p53, 12% gains and 6% losses in bcl-2, and 6% gains and 19% losses in bcl-6 with little direct correlation between the IHC and FISH studies.

Conclusions—Childhood T-LBL is a highly proliferative tumor associated with enhanced expression of c-Myc protein, but without detectable c-Myc molecular alterations. FISH studies did not identify consistent etiologies of molecular dysregulation, and future studies with other molecular approaches may be required to elucidate the molecular pathogenesis of childhood T-LBL.

Keywords

T-cell lymphoblastic lymphoma; immunohistochemistry; fluorescence in situ hybridization; immunophenotyping; molecular analysis

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INTRODUCTION

Precursor T-cell lymphoblastic lymphoma (T-LBL) accounts for approximately 33% of pediatric non-Hodgkin's lymphoma (NHL), most commonly involves the mediastinum or lymph nodes, [1,2], and is diagnosed by morphologic features, immunophenotype, and cytogenetics [3]. T-LBL is morphologically and immunophenotypically identical to T-cell acute lymphoblastic leukemia (T-ALL), although T-LBL has minimal marrow involvement (<25% bone marrow blasts) [1,3]. T-ALL and T-LBL have been thought to represent different presentations of a single entity [3]. Treatment of childhood T-LBL was based on NHL therapy (50–70% 3-year event free survival or EFS) [4], but recently use of T-ALL type therapy has improved 3-year EFS (75–80%) [5].

Genetic abnormalities in T-ALL commonly involve translocations between T-cell receptor (TCR) loci and genes coding for transcription factors, resulting in overexpression of the partner gene product, downstream events, and malignant transformation [1,6,7]. Processes affected include resistance or susceptibility to apoptosis, intracellular signaling, cell-cycle regulation, and proliferation [6,8]. Although genetic aberrations in T-ALL and other pediatric NHL have been extensively studied, the molecular genetics of T-LBL are not well-characterized. [7,9–11]. Gene expression profiling by microarray and immunohistochemical studies has shown differences in gene and protein expression profiles between T-ALL and T-LBL involving several functional groups such as adhesion molecules and transcription factors [12].

Investigations into the molecular pathogenesis of T-LBL derive from T-ALL, but little is known specific to T-LBL [13]. In the present study, we investigated possible molecular pathogenesis of T-LBL, with emphasis on whether protein overexpression by IHC correlates with underlying molecular abnormalities by FISH. Rather than focus exclusively on abnormalities previously reported in T-ALL, we took a broad approach including gene and protein abnormalities seen in both adult and pediatric lymphomas where commonly implicated genes include c-Myc [14–16], p53 [17–19], bcl-2 [18], bcl-6 [20], TCL-1 [21], and Skp2 [22]. These genes represent a variety of cellular functions and are reported abnormalities occurring in NHL or T-ALL. Bcl-2 abnormalities are seen in adult B-cell lymphomas (follicular lymphomas and 33% of diffuse large B-cell lymphomas), bcl-6 abnormalities are seen in adult and pediatric diffuse large B-cell lymphoma (~ 40%), and c-Myc abnormalities are more common in pediatric (Burkitt lymphoma) than adult lymphomas. Translocations involving TCR α/δ are common in mature adult T-cell disorders. p53 and Mib-1 overexpression are common among high-grade B-cell lymphomas, and TCL-1 and Skp2 abnormalities have also been reported in a variety of NHLs. Mib-1, c-Myc, Skp2, p53, bcl-2, TCL-1, and bcl-6 proteins were evaluated by IHC and c-Myc, p53, bcl-2, bcl-6, and TCR α/δ were evaluated by FISH. To focus on correlation between IHC and FISH results, we were limited by commercial availability of the necessary materials.

METHODS

Patients and Tumor Samples

Deidentified, formalin-fixed, paraffin-embedded T-LBL tumor samples were randomly obtained from 22 patients enrolled in Children's Oncology Group (COG) trial CCG 5971 for treatment of disseminated LBL using ALL type therapy (archival material). Studies were IRB approved.

Immunohistochemical Staining (IHC)

IHC for c-Myc, Skp2, Mib-1, p53, bcl-2, TCL1, and bcl-6, utilizing commercially available antibodies, was performed on all 22 cases following heat-induced epitope retrieval (HIER) (Table I) using a Ventana ES automated stainer (Ventana, Tucson, AZ) with appropriately staining positive and negative controls. Positive staining was nuclear in all cases, as expected. Staining was independently evaluated by two authors (SLP and KJS) without knowledge of the FISH results, and results were expressed as an average. Staining patterns were classified according to percentage of tumor cells with positive staining (negative, ≤50%, >50%), and staining intensity (strong 3+–4+; moderate 2+–3+; weak 1+–2+).

Fluorescence in Situ Hybridization (FISH)

FISH was performed for c-Myc, p53, bcl-2, bcl-6, and TCR α/δ. Due to limited availability of slides, not all cases were stained with each probe, and priority for testing was based upon the IHC data with c-Myc, p53, and bcl-2 given highest priority.

FISH was performed on tumor tissue sections using two-color break apart LSI MYC (8q24), LSI BCL2 (18q21), LSI BCL6 (3q27) and LSI TCR α/δ (14q11.2) probe sets and a single color LSI p53(17p13) probe obtained from Vysis/Abbott, Inc. (Downer's Grove, IL). Slides were pretreated using the VP2000 automated slide processor (Vysis/Abbott, Inc.). FISH was performed by co-denaturation on a HYBrite™ instrument (Vysis/Abbott, Inc.) at a denaturation temperature of 80 ° C for 5 minutes, followed by an overnight hybridization at 37 °C, post-wash in 2×SSC/0.3% NP-40 at 73 ° C for 2 minutes and counter-stain with DAPI II. Results were classified as normal, gain, loss, or positive for rearrangement for each genetic locus based upon scoring of 100 cells. Cutoffs for losses and gains on paraffin-embedded tissue were determined by counting 100 cells on 10 normal control tissues for each probe (1000 total cell count), and averaging values. Losses and gains were designated as values that deviate +/− 2SD from the average (see supplemental Table I). Cutoffs for rearrangement were also defined using paraffin-embedded institutional control materials.

RESULTS

IHC Studies

High expression of c-Myc and Mib-1 proteins were identified in the majority of cases (77% and 59%, respectively), with strong positive staining in >50% of tumor cells (Table II and Table IV, Figure 1A–B). In addition, strong positive staining in ≤50% of tumor cells was identified in an additional 18% of cases for c-Myc and 41% of cases for Mib-1. Only 1 case had negative staining for c-Myc, and no cases were negative for Mib-1.

Expression of Skp2, p53, and bcl-2 proteins was more variable with most cases containing 10–40% positively staining tumor cells, and a predominance of moderate intensity staining (Table II and Table IV, Figure 1C–E). Skp2 and p53 staining in >50% of tumor cells was seen in 14% of cases. Bcl-2 staining showed 23% of cases with strong positive staining (4+) in >50% of tumor cells, 50% of cases with moderate intensity (2+–3+) staining of ≤50% of the tumor cells, and 27% (6 cases) were negative.

Staining for TCL1 and bcl-6 was negative in 91% and 86% of cases, respectively, with weak to moderate intensity staining in <25% of cells in remaining cases (Table II and Table IV, Figure 1F–G).

FISH Studies

FISH analysis was performed on available slides with 16 cases having 4 or 5 probes, and 6 cases with 3 or fewer probes evaluated. There was one hybridization failure for bcl-2 (case

4), and multiple cases had suboptimal hybridizations, seen more commonly for bcl-2 and TCR.

64% (14/22) of cases were normal for all of the gene targets evaluated including 7 cases in which all 5 gene targets were assessed. Abnormalities in at least 1 target were seen in 36% (8 cases) (Table III and Table IV). Abnormal cases tended to have multiple abnormalities, particularly with gains in c-Myc (4 cases with other abnormalities). Case 2 demonstrated gains in c-Myc, p53, bcl-2, and bcl-6 (TCR was not assessed in this case), while case 6 demonstrated gains in c-Myc, p53, bcl-2, and TCR and loss of bcl-6. Two cases (8 and 10) showed isolated losses of c-Myc. The only other isolated loss was of bcl-6 in case 16. Case 18 showed TCR rearrangement as a sole FISH abnormality without an identifiable c-Myc or bcl-2 abnormality. Representative images of FISH for c-Myc and bcl-6 (gains and losses) are shown in Figure 2.

Correlation of IHC and FISH results

In general, IHC and FISH results did not correlate well, although a few interesting patterns were noted (Table IV). There was high expression of c-Myc protein by IHC in the majority of cases; however 72% (16/22) were normal by FISH. Three of the 4 cases with c-Myc gains by FISH had 2+–3+ staining in >50% of tumor cells while the other had 2+ staining in less than half. Cases with c-Myc losses by FISH (2/2) had moderate intensity (2+–3+) staining in ≥50% of tumor cells. Thirteen of 16 (81%) cases normal for c-Myc by FISH had 3+ or 4+ staining by IHC in >50% of the tumor cells. One negative c-Myc staining case was normal by FISH.

p53 and bcl-2 demonstrated variable IHC with most cases having moderate intensity staining in <50% of tumor cells. FISH for p53 was predominantly normal with only 3/19 (16%) of cases showing gains. One case with p53 gain by FISH had strong positive IHC staining, and the other 2 had weak staining in <50% of tumor cells.

Bcl-2 FISH demonstrated 82% (14/17) cases with normal hybridization, 2/17 cases (12%) had gains and 1/17 (6%) demonstrated a loss. Interestingly, 1 of 2 cases with gains by FISH had negative staining by IHC, and 1 had staining in <50% of the tumor cells. The single case with a loss by FISH also showed moderate staining in less than half of the tumor cells. Cases that were normal by FISH demonstrated a spectrum of IHC results ranging from negative staining to 4+ staining in 100% of the tumor cells.

Staining for bcl-6 was negative in the majority of cases by IHC, and FISH for bcl-6 was normal in 12/16 cases (75%). Three cases demonstrated a loss of bcl-6 by FISH, and two of these were negative by IHC while one had 2+ staining in <50% of tumor cells. The single case with a gain by FISH had the most bcl-6 staining by IHC of any of the cases (3+ staining in half of the tumor cells).

FISH for TCR was performed in 14 cases; twelve (86%) did not have identifiable abnormalities, 1 case (7%) demonstrated a gain, and 1 case (7%) was positive for rearrangement with no other abnormalities observed.

All study tumor samples were morphologically composed of sheets of lymphoblasts with rare intervening non-neoplastic cells. Thus, the predominance of normal FISH results does not suggest sample contamination by non-neoplastic cells, but rather that FISH abnormalities involving these targets were not present or detectable. Performance of the FISH studies on paraffin-embedded tumor tissue is a limitation due to decreased sensitivity, as well as increased potential losses due to truncation of signal (incomplete cells) and increased gains (overlapping cells) when compared to traditional cultured specimens.

However, established institution-specific cutoffs specific to paraffin-embedded tissue were used when interpreting the results. Unfortunately, although some of the FISH results do imply numerical chromosomal changes, karyotypic data was available for only two patients in the study. Case 8 demonstrated a t(7;14), but was negative for TCR rearrangement by FISH, and case 16 demonstrated complex abnormalities that did not correlate with FISH findings.

DISCUSSION

The molecular abnormalities present in immature T-cell neoplasms and pediatric NHL are heterogeneous, involving different components of cellular regulation. The aim of this study was to investigate the molecular pathogenesis of T-LBL, with particular emphasis on whether detection of protein overexpression by IHC correlates with underlying molecular abnormalities by FISH. In T-ALL, conventional cytogenetic techniques have shown that translocations commonly involve regulatory elements of the TCR α/δ (14q11.2) or TCR β (7q34) loci and various cellular oncogenes such as TAL1, c-Myc, LMO2, NOTCH1, and unrecognized partner genes that may or may not be normally expressed during T-cell development [6–9,11,13]. Multiple secondary abnormalities usually occur, with early abnormalities providing a proliferation advantage and/or differentiation block that combines with later events to result in transformation [9]. Activation of cellular oncogenes, production of abnormal fusion genes, and deletions of tumor suppressor genes may cause malignancy [6]. Identification of abnormalities is often done by conventional cytogenetic analysis, but genetic abnormalities may be cryptic [6,8,9]. Use of FISH analysis or IHC staining to detect overexpression or aberrant expression of proteins have been used as a surrogate for cytogenetic analysis. Using this strategy, we evaluated expression of a broad panel of proteins associated with NHL, including c-Myc, p53, bcl-2, and bcl-6 by IHC and FISH in cases of T-LBL to determine whether possible molecular abnormalities can be reliably predicted by IHC.

Analysis of the data indicated that correlation between IHC and FISH results was weak, suggesting that IHC staining alone is not sufficient to identify specific molecular abnormalities, but may better reflect aberrant protein production that arises from a variety of mechanisms. For example, strong expression of c-Myc protein by IHC was present in the majority of our cases (77%) but often did not correlate with an identifiable c-Myc abnormality by FISH (72% normal by FISH); perhaps suggesting that upstream or downstream genetic abnormalities, such as NOTCH-1 mediated signaling, are contributing to dysregulation of c-Myc, as c-myc is an important downstream target of NOTCH-1 [6,14–16].

Similarly, the tumor suppressor p53 was positive in most cases but showed variable staining, with most cases having staining in <50% of tumor cells. However, only 16% of cases were abnormal by FISH (gains), supporting the concept that protein overexpression is not directly or exclusively due to FISH-detectable molecular abnormalities [17–19]. Reported expression of bcl-2 in T-LBL has been variable [18], and we observed variable bcl-2 expression in 73% of cases with strong staining in only 23%. However, 82% of FISH results were normal, and of the three abnormal cases by FISH, only 2 cases had any detectable IHC staining for bcl-2.

Other important regulators of lymphomas, such as bcl-6, are often overexpressed predominantly due to rearrangements placing the bcl-6 gene adjacent to an exogenous promoter. Interestingly, although it is most often associated with B-cell neoplasms, bcl-6 was recently shown to be overexpressed in a subset (8 of 16) of T-LBL cases, although, subsequent molecular analysis showed germline bcl-6 configuration [20]. Our bcl-6 results

confirmed bcl-6 overexpression in T-LBL (14% of cases) and predominantly normal FISH (75% of cases).

The results of this study confirm that pediatric T-LBL, in general, has a high proliferative rate and increased c-Myc expression. Low expression of other important lymphoma – associated proteins such as p53, bcl-2, and skp2, and almost no expression of bcl-6 and TCL-1 were observed by immunohistochemistry. Furthermore, this initial study has demonstrated that immunohistochemical staining is an unreliable method for identifying protein overexpression arising secondary to underlying FISH molecular abnormalities. Although limited by a small number of cases, no correlation was observed between protein overexpression by IHC and FISH detection of molecular abnormalities, using appropriate controls and cutoffs. Although FISH studies may be less sensitive in paraffin-embedded tissue, our results were normal in the majority of cases and suggest chromosomal copy number changes in the abnormal cases, rather than translocations involving the target genes. Future studies could be designed to specifically relate T-ALL and T-LBL using FISH probes more specific to these tumors, and additional studies to further elucidate the molecular abnormalities in T-LBL, with particular focus on utilizing oligonucleotide microarray and/or array comparative genomic hybridizations, may correlate with aberrant protein expression and help better elucidate the molecular pathogenesis of childhood T-LBL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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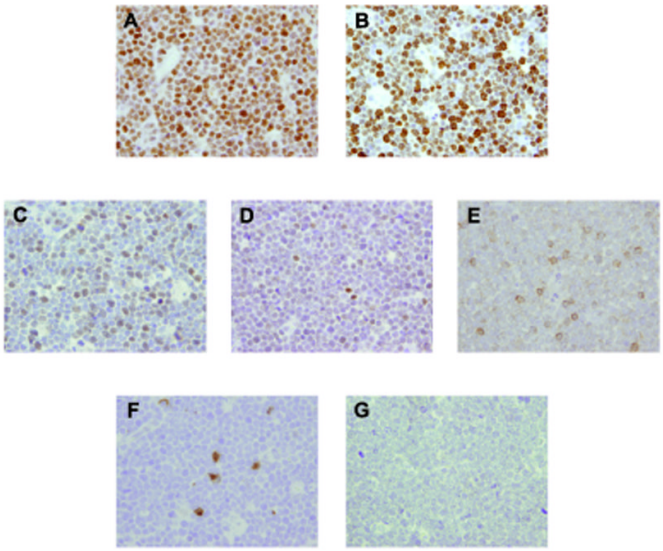

Figure 1.
Representative images of the immunohistochemical stains, all images 40× magnification: c-Myc (A), Mib-1p53 (D), bcl-2(E), TCL (F), bcl-6 (G).

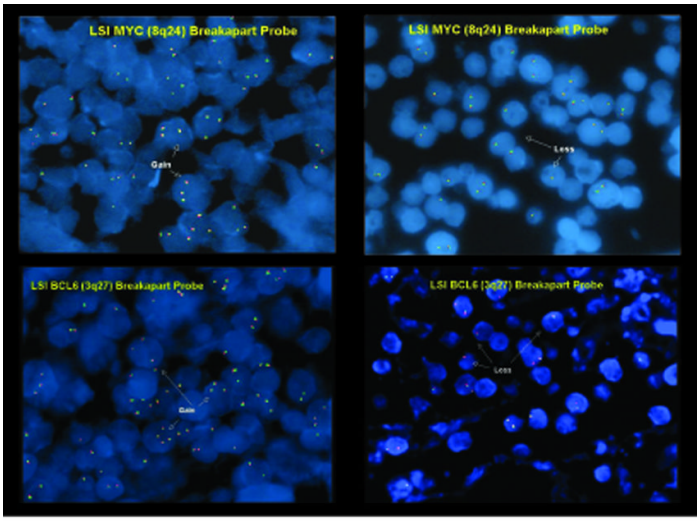

Figure 2.
Representative images for c-Myc and bcl-6 FISH.

Table I

Antibodies Used for Immunohistochemical Staining

Antibody	Source	Clone	Dilution	Retrieval	Staining
c-Myc	Santa Cruz Biotechnology	1–262	1:200	PC/CB	Nuclear
Ki-67	Dako	MIB-1	1:100	PC/CB	Nuclear
Skp2	Santa Cruz Biotechnology	H-435	1:50	PC/CB	Nuclear
p53	Dako	DO-7	1:80	PC/CB	Nuclear
bcl-2	Dako	124	1:80	PC/CB	Nuclear
TCL1	Upstate	N/A	1:1000	PC/CB	Nuclear
bcl-6	Biocare Medical	PF16	1:25	PC/Borg	Nuclear

Santa Cruz Biotechnology, Santa Cruz, CA; Dako, Glostrup, Denmark; Biocare Medical, Concord, CA; Upstate, Lake Placid, NY **PC/CB**, Antigen retrieval by pressure cooker with citrate buffer (pH 6.0); **PC/Borg**, Antigen retrieval by pressure cooker with Borg buffer (pH 9.5, Biocare Medical, Walnut Creek, CA).

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Table II

Results of Immunohistochemical Stains

Antibody	Negative	≤50% of cells with positive staining	>50% of cells with positive staining
c-Myc	1 (5%)	4 (18%)	17 (77%)
MIB-1	0 (0%)	9 (41%)	13 (59%)
Skp2	4 (18%)	15 (68%)	3 (14%)
p53	2 (9%)	17 (77%)	3 (14%)
bcl-2	6 (27%)	11 (50%)	5 (23%)
TCL1	20 (91%)	2 (9%)	0 (0%)
bcl-6	19 (86%)	3 (14%)	0 (0%)

Table III

Fluorescence in Situ Hybridization

Target Gene	# of cases analyzed	Normal	Gains	Losses	Split
c-Myc	22	16(72%)	4(18%)	2(10%)	
p53	19	16(84%)	3(16%)	0	
bcl-2	17	14(82%)	2(12%)	1(6%)	
bcl-6	16	12(75%)	1(6%)	3(19%)	
TCR	14	12(86%)	1(7%)	0	1(7%)

Table IV

FISH and IHC Results

Case	# of targets evaluated	C-Myc FISH	C-Myc IHC	bcl-2 FISH	bcl2 IHC	bcl-6 FISH	bcl-6 IHC	p53 FISH	p53 IHC	TCR FISH
1	5	Normal	100% (3+)	Normal	100% (4+)	Normal	Neg	Normal	50% (2+)	Normal
2	4	Gain 29%	75% (3+)	Gain 24%	25% (3+)	Gain 39%	50% (3+)	Gain 29%	75% (4+)	Not done
3	1	Normal	75% (3+)	Not done	75% (2+)	Not done	Neg	Not done	25% (2+)	Not done
4	4	Normal	100% (3+)	Fail	50% (3+)	Normal	25% (2+)	Normal	25% (2+)	Normal
5	2	Gain 36%	75% (2+)	Not done	Neg	Not done	Neg	Gain 79%	25% (1+)	Not done
6	5	Gain 58%	100% (3+)	Gain 29%	Neg	Loss 32%	Neg	Gain 51%	25% (1+)	Gain 45%
7	5	Normal	100% (4+)	Normal	25% (3+)	Normal	Neg	Normal	25% (2+)	Normal
8	5	Loss 57%	75% (2+)	Normal	Neg	Normal	Neg	Normal	Neg	Normal
9	5	Normal	50% (3+)	Normal	50% (2+)	Normal	Neg	Normal	50% (3+)	Normal
10	5	Loss 53%	50% (3+)	Normal	25% (2+)	Normal	Neg	Normal	25% (2+)	Normal
11	5	Normal	Neg	Normal	Neg	Normal	Neg	Normal	25% (2+)	Normal
12	3	Normal	100% (4+)	Normal	50% (2+)	Not done	Neg	Normal	100% (3+)	Not done
13	2	Normal	100% (4+)	Normal	100% (4+)	Not done	Neg	Not done	75% (3+)	Not done
14	4	Normal	75% (3+)	Normal	Neg	Normal	Neg	Normal	Neg	Not done
15	5	Normal	100% (3+)	Normal	75% (2+)	Normal	Neg	Normal	50% (3+)	Normal
16	5	Normal	50% (2+)	Normal	25% (2+)	Loss 39%	Neg	Normal	25% (3+)	Normal
17	1	Normal	100% (3+)	Not done	100% (4+)	Not done	Neg	Not done	25% (3+)	Not done
18	5	Normal	100% (4+)	Normal	Neg	Normal	Neg	Normal	25% (3+)	Rearranged 70%
19	5	Normal	100% (4+)	Normal	25% (3+)	Normal	Neg	Normal	25% (2+)	Normal
20	5	Normal	75% (3+)	Normal	25% (2+)	Normal	Neg	Normal	25% (2+)	Normal
21	2	Normal	75% (3+)	Not done	25% (2+)	Not done	Neg	Normal	50% (2+)	Normal
22	5	Gain 26%	25% (2+)	Loss 31%	25% (2+)	Loss 45%	25% (2+)	Normal	25% (1+)	Not done