TCL1 Expression and Epstein-Barr Virus Status in Pediatric Burkitt Lymphoma

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Abstract

Elevated T-cell leukemia-1 (TCL1) oncoprotein expression might promote human Burkitt lymphoma (BL) because increased TCL1 causes Burkitt-like lymphomas in TCL1 transgenic mice. Epstein-Barr virus (EBV) infection has been implicated as a cause of increased TCL1 expression in multiple BL cell lines, suggesting a critical connection between EBV and TCL1-induced BL. The TCL1 expression and EBV status of 14 sporadic pediatric BL cases was determined by immunohistochemical staining for TCL1 and in situ hybridization for EBV-encoded RNA (EBER). Our results showed TCL1 protein in 11 cases, predominantly in the nucleus with strong-intensity staining. EBER was positive in 4 cases, with 3 of these cases also TCL1+. In the 10 cases that were EBER−, TCL1 was strongly positive in 8. These data indicate that the TCL1 oncoprotein is expressed strongly in most pediatric BL cases. However, persistent EBV is not essential for increased TCL1 expression, although elevated TCL1 and c-MYC coexpression might cooperate in the development of most pediatric and adult BL cases.

Aberrant T-cell leukemia-1 (TCL1) proto-oncogene expression is implicated in childhood and adult peripheral T-cell leukemia/lymphoma and might have a role in adult B cell malignant neoplasms of germinal center (GC) origin. In T-cell cancers, dysregulation is due to recurrent chromosomal rearrangements in the thymus involving the TCL1 locus at 14q32.1 and T cell receptor (TCR) gene regulatory elements at 14q11.2 (TCRα/δ locus) or 7q35 (TCRβ locus).1,2 TCL1 protein binds the serine/threonine kinase Akt and stimulates its hyperactivation and transport from the cytoplasm to the nucleus. This follows activation of Akt-stimulating signaling pathways, such as antigen receptor engagement in lymphocytes.3-7 Inappropriately augmented activation of Akt has been proposed as a mechanism by which dysregulated TCL1 expression promotes malignant transformation through increased survival and cell proliferation.3,7,8 Enforced TCL1 expression in transgenic mice also results in mature B- and T-cell tumors, providing support for the oncogenic potential of dysregulated TCL1 expression in human lymphoid cancers.7,9

During B-cell development, TCL1 protein expression remains high from early precursors in the bone marrow through preselected mantle cell stages of peripheral maturation.10,11 Subsequently, TCL1 expression is reduced in antigen-stimulated GC B cells, followed by gene silencing and loss of protein expression in memory B and plasma cells. This physiologic down-regulation of TCL1 in GC follicles parallels that of the antiapoptotic oncoprotein bcl-2 and might facilitate removal of B cells with inappropriate immunoglobulin-antigen affinities by apoptosis.9 However, a large number of GC- and post–GC-derived B-cell tumors, including diffuse large B cell lymphoma (DLBCL) and Burkitt lymphoma (BL), exhibit higher levels of TCL1 oncoprotein expression than that seen...
in follicle center B cells. In contrast with chronic T-cell malignant neoplasms, gene rearrangements do not seem to dysregulate TCL1 expression in mature B-cell tumors. \[12,13\]

Several studies have suggested a role for epigenetic modifications, such as DNA methylation, in controlling TCL1 expression during terminal B-cell differentiation. \[12,13\] With this in mind, a possible mechanism for up-regulated or continued high-level TCL1 expression in mature B-cell tumors is defective epigenetic regulation that fails to silence TCL1 during GC B-cell maturation. An additional possibility is that Epstein-Barr virus (EBV) infection, seen in more than 90% of endemic African BL cases and 15% to 20% of sporadic BL cases, positively regulates TCL1 expression and overrides TCL1 silencing in terminal B-cell development. \[14\] In this regard, the objectives of the present study were to evaluate TCL1 expression and EBV status in primary pediatric BL, evaluate a possible role for TCL1 in the pathogenesis of BL in the pediatric age group, and help determine a role for EBV in regulating TCL1 expression levels.

**Materials and Methods**

**Patient Selection**

Cases of BL were chosen from those meeting eligibility criteria for Children’s Cancer Group (CCG) treatment protocol CCG-5961 (French-American-British LMB 96 Treatment of Mature B-Cell Lymphoma/Leukemia: an SFOP LMB 96/CCG-5961/UKCCSG NHL 9600 cooperative study). \[15-18\] Before patients were enrolled, the participating CCG institutions obtained appropriate institutional review board approval of the protocol and informed consent for treatment for all patients.

**Pretreatment Evaluation**

All patients underwent complete history and physical examination with laboratory studies, including CBC count and lactate dehydrogenase measurement. Pathologic staging included bilateral bone marrow aspirates (biopsies optional), cerebrospinal fluid examination, and appropriate radiologic studies. Central nervous system involvement was defined as the presence of any L3 blasts or lymphoma cells in the cerebrospinal fluid, cranial nerve palsy, clinical signs of spinal cord compression, isolated intracerebral mass, or paraparesis or meningeal extension. Bone marrow was classified as involved if any L3 blasts or lymphoma cells were present. Radiologic investigations included at least a chest radiograph (posterior-anterior and lateral), abdominal ultrasound, and abdominal computed axial tomography scan or magnetic resonance imaging for stage of localized disease. Gallium scan was optional. The clinical stage of disease was determined using a modified Murphy classification. \[19\]

**Pathologic Evaluation**

For the cases in this study, biopsy tissue samples were preserved in formalin fixative and routinely processed and evaluated at the primary institution. Paraffin blocks or unstained sections from the biopsies were submitted to the CCG Pathology Center, Children’s Hospital, Columbus, OH, for central pathology review, including morphologic evaluation and immunophenotyping studies. Biopsy specimens from 14 pediatric patients with BL, sporadic type, at primary diagnosis were evaluated. The protocol cases were classified as BL according to the characteristic morphologic, immunophenotypic, and cytogenetic features as described in the revised European-American Lymphoma Classification \[20\] or the World Health Organization Classification. \[21\] The standard immunophenotyping panel for these cases included antibodies to CD20, CD79a, CD3, CD45RO, and terminal deoxynucleotidyl transferase as described previously. \[22\]

**TCL1 Immunohistochemical Analysis and Scoring**

Detection of TCL1 was performed on paraffin sections with a rabbit polyclonal antiserum to TCL1 using an immunoperoxidase method as previously described. \[8,11\] A positive result was interpreted as brown staining localized within the cytoplasm or nucleus of neoplastic cells. Scoring was recorded for the percentage of neoplastic cells staining positively along with the intensity of staining (scored visually 1+ [weak], 2+ [moderate], or 3+ [strong]). Negative control samples were prepared by substituting the primary rabbit antiserum to TCL1 with preimmune rabbit serum. Positive control tissue samples were paraffin sections of hyperplastic tonsil.

**In Situ EBV-Encoded RNA Hybridization**

Detection of EBV was performed with in situ hybridization on paraffin sections using a fluorescein isothiocyanate–conjugated oligonucleotide probe to EBV-encoded RNA (EBER; BioGenex Laboratories, San Ramon, CA) with an immunoperoxidase method as previously described. \[23\] A positive result was interpreted as brown staining localized within the nucleus of neoplastic cells. Positive control tissue samples were paraffin sections of EBV+ Hodgkin lymphoma. In addition, a fluorescein-labeled oligo (dT) probe (BioGenex Laboratories) was used as a positive control for detecting messenger RNA.

**Cytogenetic Evaluation**

Cytogenetic analysis was performed on biopsy specimens at the primary institution with G-banded karyotypes. The karyotypes were submitted for central cytogenetics review as follows: (1) 2 original karyotypes of each abnormal clone, and (2) 2 karyotypes of normal metaphase cells for each normal case. The central cytogenetics review was performed by a member of the CCG Cytogenetics Committee (W.G.S.). The karyotype results were reported using the 1995 International...
System for Human Cytogenetic Nomenclature. Karyotypes were classified according to the most abnormal clone.

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) procedures were performed on 4 to 5 µm unstained paraffin tissue sections using the LSI MYC Dual-Color Break-apart Probe (Vysis, Downers Grove, IL) and the LSI IGH/MYC/CEP8 Dual-Fusion Translocation Probe (Vysis). Before hybridization, the slides were pretreated using the VP2000 (Vysis) automated slide processor using the program specified for paraffin slides.

Briefly, the program included deparaffinization of the slides followed by dehydration, 0.2N hydrochloric acid treatment for 15 minutes, and incubation in sodium thiosulfate for 20 minutes at 80°C. The slides then were digested in protease for 15 minutes at 37°C, rinsed, and dehydrated. For FISH, 10 µL of the probe mixture was placed on the pretreated tissue section and coverslipped. The probe and target DNA were codenatured at 75°C for 5 minutes followed by an overnight hybridization using a HybRite (Vysis) automated FISH hybridization chamber. After hybridization, the slides were washed for 2 minutes in 2× standard saline citrate (SSC)/0.1% NP-40 (Vysis) at 72°C followed by 2 minutes in 2× SSC/0.1% NP-40 at room temperature. The slides were counterstained with 4',6-diamidino-2-phenylindole at a concentration of 125 ng/mL in antifade solution. Analysis was performed on an Olympus BX61 fluorescence microscope (Scientific Instrument, Culver City, CA) equipped with appropriate filters and Cytovision FISH system image capture software (Applied Imaging, Santa Clara, CA).

For the myc break-apart probe, 150 to 200 cells were scored for a “separation” of the green and red signal that was indicative of a rearrangement of the myc gene region at 8q24.1. The false-positive rate for this FISH test on paraffin tissue sections, as established before this study, is 0% to 15%.

For IGH/MYC/CEP8 probes, 150 to 200 cells were scored for the presence of the t(8;14)(q24.1;q32) as evidenced by juxtaposition of the red (myc) and green (IgH) signals forming a yellow “fusion” signal. The false-positive rate for this FISH system on paraffin tissue sections, as established before this study, is 0% to 10%.

Results

We evaluated 14 biopsy specimens diagnosed as BL from 14 pediatric patients. The clinical features of the 14 patients are summarized in Table 1. Children and adolescents in this study ranged in age from 2 to 16 years (median, 10 years). The male/female ratio was 6:1. The clinical stage of disease was determined according to the Murphy classification as follows: I, 2 cases; II, 2 cases; III, 9 cases; and IV, 1 case. The primary site of involvement included the following: intestine, 6 cases; abdomen, 3 cases; nasopharynx, 2 cases; and neck, orbit, and tonsil, 1 case each. These findings are similar to the reported male predominance and frequent sites of manifestation for sporadic BL in the pediatric age group.

In all 14 cases, the tumor biopsy tissue samples contained typical morphologic features of BL. Image 1, including monomorphic, medium-sized cells containing round nuclei with multiple nucleoli (2 to 5) and a moderate amount of cytoplasm. A starry-sky pattern was present owing to scattered apoptosis and associated macrophages. Immunohistochemical

Table 1  Clinical Features of Pediatric Burkitt Lymphoma in 14 Patients

<table>
<thead>
<tr>
<th>Case No./Sex/Age (y)</th>
<th>Murphy Stage</th>
<th>Primary Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/3</td>
<td>I</td>
<td>Tonsil</td>
</tr>
<tr>
<td>2/M/7</td>
<td>I</td>
<td>Neck</td>
</tr>
<tr>
<td>3/M/6</td>
<td>II</td>
<td>Nasopharynx</td>
</tr>
<tr>
<td>4/M/14</td>
<td>II</td>
<td>Nasopharynx</td>
</tr>
<tr>
<td>5/M/2</td>
<td>III</td>
<td>Intestine</td>
</tr>
<tr>
<td>6/M/2</td>
<td>III</td>
<td>Intestine</td>
</tr>
<tr>
<td>7/M/6</td>
<td>III</td>
<td>Abdomen</td>
</tr>
<tr>
<td>8/F/7</td>
<td>III</td>
<td>Intestine</td>
</tr>
<tr>
<td>9/M/10</td>
<td>III</td>
<td>Intestine</td>
</tr>
<tr>
<td>10/M/11</td>
<td>III</td>
<td>Abdomen</td>
</tr>
<tr>
<td>11/V/12</td>
<td>III</td>
<td>Intestine</td>
</tr>
<tr>
<td>12/M/13</td>
<td>III</td>
<td>Intestine</td>
</tr>
<tr>
<td>13/F/13</td>
<td>III</td>
<td>Abdomen</td>
</tr>
<tr>
<td>14/M/16</td>
<td>IV, + central nervous system</td>
<td></td>
</tr>
</tbody>
</table>

Image 1  Pediatric Burkitt lymphoma (H&E, original magnification x150).
staining demonstrated a B-cell phenotype, with all 14 cases positive for CD20 and CD79a, negative for T-cell markers CD3 and CD45RO, and negative for the precursor marker terminal deoxynucleotidyl transferase (data not shown). None of the cases had bone marrow involvement characteristic of precursor B-cell acute lymphoblastic leukemia.

Cytogenetic karyotype studies Table 2 in 10 cases were abnormal, with 9 cases (1, 4, 6, 7, and 9-13) showing the typical t(8;14)(q24.1;q32) causing a c-MYC/IgH translocation, and 1 case (14) showing a variant c-MYC translocation t(8:22)(q24.1;q11), characteristic of BL.28,29 The other 4 cases without cytogenetic abnormalities on karyotype studies were evaluated by FISH studies and demonstrated c-MYC translocations in all 4 cases, including 3 cases with c-MYC/IgH translocations.

TCL1 oncprotein expression was positive in 11 BL cases (79%) in immunohistochemical analysis Image 2. TCL1 staining of neoplastic cells was localized predominantly in the nucleus with strong intensity, along with weak staining in the cytoplasm in all positive cases Table 3. TCL1 was detected in the majority of neoplastic cells (range, 60%-100%) in 10 of 11 cases. The TCL1 staining intensity in BL was considerably stronger, particularly in the nucleus, than the staining intensity of TCL1 in reactive follicle center cells of hyperplastic lymphoid tissue, consistent with increased TCL1 oncprotein expression in BL Image 3.

In situ hybridization for EBER was positive in 4 BL cases (29%; Table 3) Image 4. EBER staining was restricted to the nucleus in all 4 cases. Among these EBER+ cases, 3 cases also were TCL1+. In the 10 EBER– cases, 8 were TCL1+ (Table 3).

**Discussion**

Aberrant TCL1 expression promotes mature lymphoid malignant neoplasms in humans and transgenic mice. An association between elevated TCL1 expression and BL has been shown in cell lines and in a small number of primary BL samples, presumably from adult patients.2,10-12,30 In the present
study, strong TCL1 oncoprotein expression was detected in most cases of sporadic pediatric BL patient samples, consistent with previous cell line and adult BL data and suggesting that elevated TCL1 oncoprotein expression is a common event in the pathogenesis of pediatric BL. Increased TCL1 expression was observed in specimens containing and lacking EBV. The possibility that EBV− cases evolved from EBV+ B cells using a “hit-and-run” mechanism cannot be excluded formally, although a recent study of Hodgkin lymphoma failed to detect evidence for this mechanism in EBV-negative Hodgkin lymphoma cases.31 Our data minimally indicate that increased TCL1 expression does not depend on persistent EBV infection in BL.

Two main mechanisms have been suggested for high-level TCL1 expression in BL, including TCL1 promoter hypomethylation and EBV infection, although possible post-transcriptional regulation has not been addressed.12,32 The DNA methylation status of TCL1 in primary follicle center B cells (from which BL originates) and post-GC memory and plasma B cells is not known. However, a limited analysis of the TCL1 core promoter in EBV− post-GC AF-10 myeloma and BC-1 primary effusion lymphoma cell lines shows that dense CpG (5′-CG-3′ palindrome; p, phosphate group) methylation is not correlated with the lack of TCL1 expression in these lines.13 Further studies are needed to determine the role of epigenetic regulation of TCL1 silencing during terminal B-cell differentiation and potential epigenetic alterations that cause continued high-level expression in BL.

A role for EBV in regulating TCL1 expression is unresolved. TCL1 expression was reported in endemic but not in sporadic BL lines, suggesting that EBV regulates TCL1.2 EBV-immortalized fetal cord and B-lymphoblastoid cell lines also demonstrate TCL1 expression.2,11,33 Most recently, latency I EBV+ Akata BL cells that spontaneously lose EBV produced

### Table 3
TCL1 Expression and EBER Status in Pediatric Burkitt Lymphoma

<table>
<thead>
<tr>
<th>Case No.</th>
<th>TCL1+ Tumor Cells (%)</th>
<th>TCL1 Stain</th>
<th>EBER Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Distribution</td>
<td>Intensity</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>3</td>
<td>&gt;90</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>Nucleus</td>
<td>2+</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>9</td>
<td>&gt;90</td>
<td>Cytoplasm</td>
<td>3+</td>
</tr>
<tr>
<td>10</td>
<td>&gt;90</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>&gt;90</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>13</td>
<td>&gt;90</td>
<td>Nucleus</td>
<td>3+</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>Cytoplasm/Nucleus</td>
<td>1+</td>
</tr>
</tbody>
</table>

EBER, Epstein-Barr virus–encoded RNA; TCL1, T-cell leukemia-1; +, positive; −, negative; 1+, weak; 2+, moderate; 3+, strong.

* A hyperplastic tonsil from an unaffected patient.

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![Image 3](image3.jpg)

**Image 3** T-cell leukemia-1 (TCL1) expression in germinal center B cells of lymphoid follicular hyperplasia. Immunohistochemical stain for TCL1 oncoprotein exhibits low intensity immunoreactivity (brown stain) in the cytoplasm of follicle center centrocytes and centroblasts (diaminobenzidine chromogen, hematoxylin counterstain, original magnification ×150).

![Image 4](image4.jpg)

**Image 4** Epstein-Barr virus status in pediatric Burkitt lymphoma. In situ hybridization for Epstein-Barr virus–encoded RNA exhibits reactivity (brown stain) in the nucleus of Burkitt lymphoma cells (diaminobenzidine chromogen, hematoxylin counterstain, original magnification ×150).
subclones with reduced TCL1 expression. EBV reinfection into the EBV– BL subclones restored TCL1 expression to about 60% of its original level. In contrast with these observations, EBV– DLBCL cases often expressed abundant TCL1. Also, in a separate study, sporadic and endemic BL lines expressed TCL1, whereas matched EBV+ and EBV– lines showed similar levels of TCL1 expression. Results from the present study also showed that high-level TCL1 expression does not depend on concurrent EBV infection. Combined, the data indicate many possibilities, but to establish or negate a direct role for EBV requires linking viral components directly with TCL1 gene and/or posttranscriptional regulation.

TCL1 oncprotein levels decrease in follicular B cells, similar to the decreased expression of BCL-2 in GCs. Because B cells undergo selection pressure for appropriate antibody affinities at this stage of development, this finding supports the idea that TCL1 is cell-protective and is down-regulated to facilitate B-cell selection. Increased TCL1 expression in GC-derived pediatric BL suggests an aberrant cell-protective mechanism, like BCL-2 in follicular lymphoma. In mouse B cells, bcl-2 is poorly oncogenic, with slow development (>12 months) and low penetrance (10%-15%) of the malignant phenotype. Coexpression of c-myc with bcl-2 dramatically increases the rate and penetrance of B-lymphoid tumors in transgenic mice. There is an interesting parallel between this situation, in which prosurvival bcl-2 augments c-myc transformation, and the situation reported herein for TCL1, in which prosurvival TCL1 is overexpressed in BL cases that contain c-MYC/IgH translocations causing dysregulated expression of the c-MYC proto-oncogene. This parallel suggests a potential synergistic, cell-protective role for increased TCL1 oncprotein expression in the development of c-MYC–expressing pediatric BL cases. Transgenic mice with increased TCL1 in B-cell lymphocytes have a very high frequency of aggressive B-cell lymphomas that resemble BL and DLBCL. It will be interesting to determine the c-myc expression status in Burkitt-like tumor cells that arise in these transgenic mice. These mice might provide a useful model for EBV– BL to study the pathogenesis and potential novel therapeutic strategies, perhaps including molecules directed toward the interaction of TCL1 with Akt. Overall, the TCL1 oncprotein is expressed strongly in most pediatric BL cases. However, persistent EBV is not essential for increased TCL1 expression, which might be required along with dysregulated c-MYC for the development of most BL cases.

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