

# The Frequency of Immunoglobulin Heavy Chain Gene and T-Cell Receptor $\gamma$ -Chain Gene Rearrangements and Epstein-Barr Virus in ALK<sup>+</sup> and ALK<sup>-</sup> Anaplastic Large Cell Lymphoma and Other Peripheral T-Cell Lymphomas

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**We previously identified a relatively high frequency of B-cell proliferations along with simultaneous T-cell receptor  $\gamma$ -chain gene (*TRG*) and immunoglobulin heavy chain gene (*IGH*) rearrangements in a series of angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma, unspecified. Here, we report on a series of 74 peripheral T-cell lymphoma (PTCL) cases composed entirely of specific PTCL subtypes, including 28 cases of ALK<sup>+</sup> anaplastic large-cell lymphoma (ALCL), 35 cases of ALK<sup>-</sup> ALCL, and 11 cases that represent other specific PTCL subtypes. We performed *IGH* and *TRG* gene rearrangement studies and *in situ* hybridization for Epstein-Barr virus (EBV) to determine the frequency of *IGH* clonality and to investigate the relationship between EBV, clonality, and associated B-cell proliferations. Using BIOMED-2 PCR assays, we detected *TRG* clones in 64 of 74 (86%) cases and *IGH* clones in 6 of 74 (8%) cases, with all *IGH*-positive cases exhibiting a concurrent *TRG* clone. Despite the detection of occasional *IGH* clones, there was no correlation between *IGH* clonality and EBV, and B-cell proliferations were not identified in any of the cases. These findings suggest that other factors contribute to *IGH* clonality and demonstrate that, in the absence of an associated B-cell proliferation, *IGH* clonality occurs infrequently (8%) in specific PTCL subtypes. (J Mol Diagn 2008, 10:502–512; DOI: 10.2353/jmoldx.2008.080054)**

Peripheral T-cell lymphoma (PTCL) is an uncommon malignancy that accounts for less than 10% of non-Hodgkin lymphomas worldwide. By current World Health Organization criteria, the diagnosis and classification of PTCL is based on the combination of clinical, histologic, immunophenotypic, and genetic findings.<sup>1</sup> However, the diagnosis of PTCL is difficult even for experienced pathologists,

given the infrequency of cases, the often unusual histologic features, and perhaps most importantly, the lack of good immunophenotypic markers to assess for clonality in T-lineage neoplasms.

In cases of suspected PTCL, pathologists often evaluate for clonality and assess for lineage by performing PCR-based assays for clonal rearrangement in both the T-cell receptor  $\gamma$ -chain gene (*TRG*) and the immunoglobulin heavy chain gene (*IGH*). Although PCR-based assays for *TRG* typically detect clones in 80 to 90% of PTCL cases, several studies have shown that *IGH* clones are also detected relatively frequently (9 to 16%).<sup>2–6</sup> The detection of an *IGH* clone, with or without a concurrent *TRG* clone, complicates an already challenging diagnosis.

Recently, we investigated the etiology of *IGH* clones in PTCL by studying a large series of two of the most common subtypes of PTCL, angioimmunoblastic T-cell lymphoma (AITL) and PTCL-unspecified (PTCL-U).<sup>7</sup> A subset of cases were complicated by associated B-cell proliferations, a finding that we and others have described as an atypical infiltrate of B cells that is often associated with Epstein-Barr virus (EBV).<sup>8–11</sup> Using multiplex PCRs developed in a European collaborative study (BIOMED-2), we detected *TRG* clones in approximately 80% of cases and *IGH* clones in approximately 35% of cases, with the majority of *IGH* clones detected along with simultaneous *TRG* clones.

Interestingly, a positive *IGH* clone correlated, at least in part, with the presence of a B-cell proliferation, suggesting that B-cell proliferations contribute to *IGH* clonality. However, alternate explanations for the detection of simultaneous *TRG* and *IGH* clones include those that are technical in nature and so-called lineage infidelity. In this context, lineage infidelity refers to recombination of both *TRG* and *IGH* in the same clone. However, this phenomenon is more common in immature hematolymphoid neoplasms and occurs only rarely (<5%) in mature B- and T-cell non-Hodgkin lymphomas.<sup>12–15</sup>

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Regardless of the etiology, our findings raised several important questions. First, it remains unclear whether *IGH* clonality can occur in all PTCL subtypes or whether it is limited to AILT and PTCL-U, which represent the only two subtypes in which B-cell proliferations have been reported. If *IGH* clonality were limited to AILT and PTCL-U, this would suggest that B-cell proliferations are responsible for *IGH* clonality. Alternatively, although B-cell proliferations have not been described in other subtypes, this possibility has not been thoroughly investigated using the combination of immunohistochemical stains and assays for clonality and EBV. If *IGH* clones occur in other PTCL subtypes, they may be the manifestation of subtle or incipient B-cell proliferations that have been overlooked thus far.

To address these issues, we collected a series of 74 PTCL cases composed of subtypes other than AILT and PTCL-U, which corresponds to specific subtypes in which B-cell proliferations have not been reported. This series includes 28 cases of ALK<sup>+</sup> anaplastic large-cell lymphoma (ALCL), 35 cases of ALK<sup>-</sup> ALCL, and 11 additional cases representing other specific PTCL subtypes. We assayed all cases for *IGH* and *TRG* clonality, and we performed the experiments under "blinded" conditions to minimize experimental and interpretive bias. In addition, we evaluated cases for the presence of B-cell proliferations using immunohistochemical stains, and we performed *in situ* hybridization for EBV to investigate the relationship between EBV, *IGH* clonality, and possible B-cell proliferations.

## Materials and Methods

### Cases

Twenty-eight cases of ALK<sup>+</sup> ALCL, 35 cases of ALK<sup>-</sup> ALCL, 3 cases of subcutaneous panniculitis-like T-cell lymphoma, 3 cases of enteropathy-type T-cell lymphoma, 1 case of hepatosplenic T-cell lymphoma, 4 cases of T-cell large granular lymphocytic leukemia (T-LGL), and 24 cases of precursor B-cell acute lymphoblastic leukemia/lymphoma (pre-B-ALL) were selected from the Laboratory of Hematopathology, Stanford University Medical Center (Stanford, CA). Cases were received between January 1, 1995 and December 31, 2006, and diagnostic classification for all cases in this study was based on current World Health Organization criteria.<sup>1</sup> Cases of CD30<sup>+</sup>, ALK<sup>-</sup> PTCL that lacked the morphologic features of ALCL as defined by the World Health Organization criteria were classified as PTCL-undefined and excluded from the study. Cases were selected based on the availability of archived paraffin-embedded tissue or frozen bone marrow cells (decalcified specimens were excluded). The selected materials represented cases in which no previous DNA-based clonality study had been performed, and these cases have not been included in any previously published report, including our recent study on AILT and PTCL-U.<sup>7</sup> All cases showed greater than 10% involvement, and the majority showed >50% involvement. Use of tissue for this study was approved by

the Stanford University Panel on Medical Human Subjects [Protocol ID 79034; Institutional Review Board number 348 (Panel 1)].

### Histology and Immunohistochemistry

Histologic sections were prepared from formalin-fixed, paraffin-embedded tissue by cutting 3- to 4- $\mu$ m-thick sections and staining with H&E. Sections were stained for immunohistochemistry on either a BenchMark instrument (Ventana Medical Systems, Tucson, AZ) or Autostainer (Dako, Carpinteria, CA) using the biotin-avidin technique with diaminobenzidine as the chromogen.<sup>16</sup> All cases were stained with at least one B- and one T-cell marker. The following mouse monoclonal antibodies were used: AB75 (anti-CD2; Vision BioSystem, Norwell, MA), IF6 (anti-CD4; Vision BioSystem), 4C7 (anti-CD5; Vision BioSystem), CD7-272 (anti-CD7; Vision BioSystem), C8/144B (anti-CD8; Dako), 56C6 (anti-CD10; Vision BioSystem), MMA (anti-CD15; Ventana), L26 (anti-CD20; Dako), Ber-H2 (anti-CD30; Dako), MY10 (anti-CD34; Becton Dickinson, San Jose, CA), L60 (anti-CD43; Becton Dickinson), ROA6 (anti-CD45RO; Zymed, South San Francisco, CA), JCV117 (anti-CD79a; Dako), ALK1 (anti-ALK; Dako), E029 (anti-EMA; Dako), 24 (anti-PAX5; BD Transduction, Lexington, KY), GrB-7 (anti-granzyme; Dako), and 2G9 (anti-T-cell intracellular antigen-1; Immunotech, Quebec, Canada). The following rabbit polyclonal antibodies were used: anti-CD3 (Cell Marque, Hot Springs, AR) and anti-TdT (Supertechs, Bethesda, MD). Antigen retrieval was performed by using automated heat pretreatment (Ventana) for AB75, IF6, 4C7, CD7-272, C8/144B, 56C6, L26, Ber-H2, MY10, L60, JCV117, ALK1, and anti-CD3 or by using manual heat-induced epitope retrieval (Dako) with an ethylene diamine tetraacetic acid buffer for E029 or citrate buffer for GrB-7, 2G9, 24, and anti-TdT. For MMA and ROA6, staining was performed without antigen retrieval using the BenchMark instrument (Ventana).

### Flow Cytometry

Bone marrow aspirate specimens were diluted with 1 volume of Dulbecco's PBS (Sigma, St. Louis, MO), vortexed for 15 seconds, and incubated at 37°C for 20 minutes. After incubation, the mixture was centrifuged at 500  $\times$  *g* for 5 minutes, and the supernatant was discarded. The cells were resuspended in PBS with 20% fetal calf serum (HyClone, Logan, UT), washed three times in PBS, and diluted in PBS to 10<sup>4</sup> cells/ $\mu$ L for staining. An aliquot of cells was resuspended in PBS with 20% fetal calf serum and 20% dimethyl sulfoxide (Sigma) and archived at -70°C for subsequent clonality assays.

For detection of surface antigens, the following four-color antibody combinations were used (fluorescein isothiocyanate/phycoerythrin/peridinin-chlorophyll-protein/allophycocyanin): CD5/CD38/CD45/CD19, lambda/kappa/CD45/CD19, CD20/CD10/CD45/CD19, CD8/CD3/CD45/CD4, CD16/CD3/CD45/CD56, CD57/CD8/CD45/CD3, CD7/CD3/

CD45/CD2, TCR $_{\alpha\beta}$ /TCR $_{\gamma\delta}$ /CD45/CD3, and CD38/CD56/CD45/CD34. Antibodies to CD2 (S5.2), CD3 (SK7), CD4 (SK3), CD5 (L17F12), CD7 (M-T701), CD8 (SK1), CD10 (HI10A), CD34 (8G12), CD38 (HB7), CD45 (2D1), CD56 (NCAM16.2), CD57 (HNK-1), TCR $_{\alpha\beta}$  (WT31), TCR $_{\gamma\delta}$  (11F2), kappa (TB28-2), and lambda (1-155-2) were obtained from Becton Dickinson. For staining, 100  $\mu$ l of cells was mixed with antibodies at the manufacturer's recommended concentrations and incubated at 4°C for 20 minutes in the dark. After staining, the cells were treated with PharmLyse (Pharmingen, San Diego, CA) according to the manufacturer's protocol and resuspended in PBS for flow cytometry.

For detection of intracellular antigens, the following three-color antibody combinations were used (fluorescein isothiocyanate/phycoerythrin/peridinin-chlorophyll-protein): MPO/CD79A/CD45 and TdT/CD3/CD45. Antibodies to TdT (HT1 + HT4 + HT8 + HT9) and CD79A (HM47) were obtained from Beckman Coulter (Fullerton, CA), antibody to MPO (H-43-5) was obtained from Dako, and antibody to CD3 (SK7) was obtained from Becton Dickinson. For membrane permeabilization, 50  $\mu$ l of cells was treated with Fix & Perm (Caltag, Burlingame, CA) according to the manufacturer's protocol. After permeabilization, cells were mixed with antibodies at the manufacturer's recommended concentrations and incubated at room temperature for 20 minutes in the dark. Stained cells were resuspended in PBS for flow cytometry.

Flow cytometry was performed on a four-color FACS-Calibur instrument (Becton Dickinson), and data were acquired and analyzed using CellQuest software (Becton Dickinson). Specific cell populations were identified and gated based on forward versus orthogonal light scatter, CD45 expression versus orthogonal light scatter, and a variety of antigen expression patterns. Populations were evaluated for antigen expression by comparing the fluorescence intensity versus that of the same population stained with an isotype control.

### Isolation of DNA

DNA was obtained from either formalin-fixed, paraffin-embedded (FFPE) tissue blocks or archived bone marrow aspirates that were each assigned a unique three-digit number. The corresponding case identities were maintained anonymous from the experimenters and authors until final clonality results were assigned for every case. For FFPE tissue, DNA was obtained by cutting four to eight 20- $\mu$ m-thick sections and deparaffinizing by extracting three times in 1.0 ml of xylene (Fisher Scientific, Pittsburgh, PA). The extracted tissue was washed two times in 1.0 ml of 100% ethanol (Gold Shield Chemical Co., Hayward, CA) and then dried at 65°C. The tissue was resuspended in 2 volumes (50 to 200  $\mu$ l) of a mixture of 4 $\times$  PCR buffer II (Applied Biosystems, Foster City, CA), 0.1% sodium dodecyl sulfate, and 0.6 mg/ml proteinase K (Stratagene, La Jolla, CA) and incubated at 65°C overnight. Samples were further purified using a DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocols and eluting the DNA in 100 to 200  $\mu$ l of DNase/RNase-free water (Invitrogen, Carlsbad, CA).

For bone marrow aspirates, DNA from 1 to 2  $\times$  10<sup>6</sup> cells was isolated using a QIAamp DNA Blood mini kit (Qiagen) according to the manufacturer's protocols and eluting the DNA in 50 to 100  $\mu$ l of DNase/RNase-free water (Invitrogen).

### TRG and IGH Clonality Studies

Cases were evaluated for TRG and IGH clonality as described previously<sup>7</sup> using commercially available PCR-based kits (InVivoScribe Technologies, San Diego, CA) based on a European collaborative study (BIOMED-2 Concerted Action).<sup>17</sup> The PCRs were performed according to the manufacturer's protocols. Briefly, a 5- $\mu$ l aliquot of DNA sample was added to 45  $\mu$ l of each reaction mixture and 1.25 units of AmpliTaq Gold (Applied Biosystems). Amplification was performed using a PTC-200 thermocycler (M&J Research, Waltham, MA) by initially heating at 95°C for 7 minutes, followed by 35 cycles of 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 90 seconds. The final step was incubation at 72°C for 10 minutes.

After amplification, 1  $\mu$ l of PCR product was added to 10  $\mu$ l of Hi-Di Formamide (Applied Biosystems) and 1  $\mu$ l of ROX-500 internal size standard (Applied Biosystems). The mixture was then denatured at 95°C for 5 minutes, chilled on ice for 5 minutes, and resolved by capillary electrophoresis on an ABI 3100 instrument using performance optimized polymer-4 (Applied Biosystems). The data were stored electronically and analyzed using GeneScan software (Applied Biosystems). Printed electropherograms were reviewed independently by two separate pathologists (B.T.T. and D.A.A.) who were blinded to the identity of specimens. Criteria for assigning a positive or negative result were similar to those used for clinical specimens at the Stanford Molecular Pathology laboratory. Briefly, the products of a primer set were considered clonal if one or two distinct peaks were present within the expected size range. In some cases, a polyclonal background was present in addition to the distinct peak(s). To be considered clonal, the height of at least one distinct peak had to exceed that of the polyclonal background by at least twofold. When a single primer set yielded three or more distinct peaks within the expected size range, the results for that primer set were classified as oligoclonal, regardless of whether a polyclonal background was present or absent. Cases in which one or more primer sets yielded clonal products were scored as positive. Cases in which all of the primer sets yielded polyclonal, oligoclonal, and/or undetectable products were scored as negative. Assays yielding a positive IGH study were scored as positive only if a repeat assay yielded identically sized products to the original study.

Control specimens included clonal DNA isolated from cell lines (InVivoScribe), polyclonal DNA isolated from human tonsil specimens (InVivoScribe), a sensitivity control composed of clonal DNA diluted in polyclonal DNA, and a blank composed of DNase/RNase-free water (Invitrogen). Multiple polyclonal DNA specimens, each isolated from different patients (InVivoScribe), consist-

**Table 1.** Patient Demographics of Collected Cases

Diagnosis	Cases (n)	Men [n (%)]	Women [n (%)]	Mean age (years)	Site (n of cases)
ALK <sup>+</sup> ALCL	28	16 (57)	12 (43)	27	Lymph node (23), soft tissue (2), leg (1), psoas (1), L1 vertebrae (1)
ALK <sup>-</sup> ALCL	35	21 (60)	14 (40)	54	Lymph node (25), abdomen (1), eye (1), stomach (1), mesentery (2), soft tissue (3), retroperitoneum (2)
SPTCL	3	1 (33)	2 (67)	50	Abdomen (1), palm (1), leg (1)
HSTCL	1	1 (100)	0 (0)	65	Liver (1)
T-LGL	4	2 (50)	2 (50)	56	Bone marrow (2), spleen (1), tonsil (1)
ETCL	3	0 (0)	3 (100)	59	Small bowel (2), stomach (1)
Total PTCL	74	41 (55)	33 (45)	44	Lymph node (48), bone marrow (2), spleen (1), leg (2), tonsil (1), stomach (2), small bowel (2), liver (1), abdomen (2), retroperitoneum (2), eye (1), psoas (1), mesentery (2), soft tissue (5), L1 vertebrae (1), palm (1)
Pre-B-ALL	24	10 (42)	14 (58)	16	Lymph node (13), bone marrow (3), skin (2), testis (1), ovary (1), mediastinum (1), nasal (1), periorbital (1), thigh (1)

ETCL, Enteropathy-type T-cell lymphoma; HSTCL, hepatosplenic T-cell lymphoma; SPTCL, subcutaneous panniculitis-like T-cell lymphoma.

tently yielded polyclonal signals and no clonal signals. For a run of clonality studies to be accepted into the data set, a positive clone had to be detectable from the sensitivity control, which was prepared at 2 or 3% (expressed as micrograms clonal DNA per microgram polyclonal DNA) for *TRG* or *IGH* assays, respectively. Individual DNA specimens had to yield detectable products of at least 300 nucleotides from the control amplification tube, which contains primers to amplify the human leukocyte antigen-DQ gene. In our validation of the InVivoScribe *TRG* and *IGH* kits, we were able to detect clones in 80 to 90% of known B- and T-cell neoplasms, and amplification of the provided polyclonal control DNA (InVivoScribe) or DNA isolated from paraffin-embedded reactive tonsil specimens in our laboratory yielded only polyclonal results.

### In Situ Hybridization for EBV

EBV-encoded small RNA was detected from 3- to 4- $\mu$ m-thick, formalin-fixed, paraffin-embedded tissue sections by *in situ* hybridization using a Ventana BenchMark instrument running a standardized program incorporating deparaffinization, hybridization to the Inform EBV-encoded small RNA probe cocktail, and staining with ISH iVIEW nitro blue tetrazolium (Ventana). Cases were evaluated for EBV-labeled cells under visible light microscopy, and the number of cells with blue-colored nuclei was visually estimated at 0, <1, 1 to 10, >10 to 100, or >100 per medium power field using a  $\times 15$  ocular and  $\times 20$  objective lens on a BX50 microscope (Olympus, Tokyo, Japan). The minimum number of labeled nuclei required for a nonzero score was three per section, which is more than that typically seen in a lymph node or tonsil removed from a healthy individual without evidence of immunodeficiency (B.T.T. and R.A.W., unpublished observations). For 30 of the 49 cases studied for EBV, a control hybridization was performed with an oligo-deoxythymidine

probe and successfully detected polyadenylated-mRNA in all 30 cases. For 19 of the 49 cases, sufficient material was not available for a control, but the proportion of EBV-positive cases was similar for the 30 cases with controls (30%) versus the 19 cases without controls (32%). In addition, an external positive control consisting of an EBV-positive malignancy (NK/T-cell lymphoma or nasopharyngeal carcinoma) was run with each set of hybridizations.

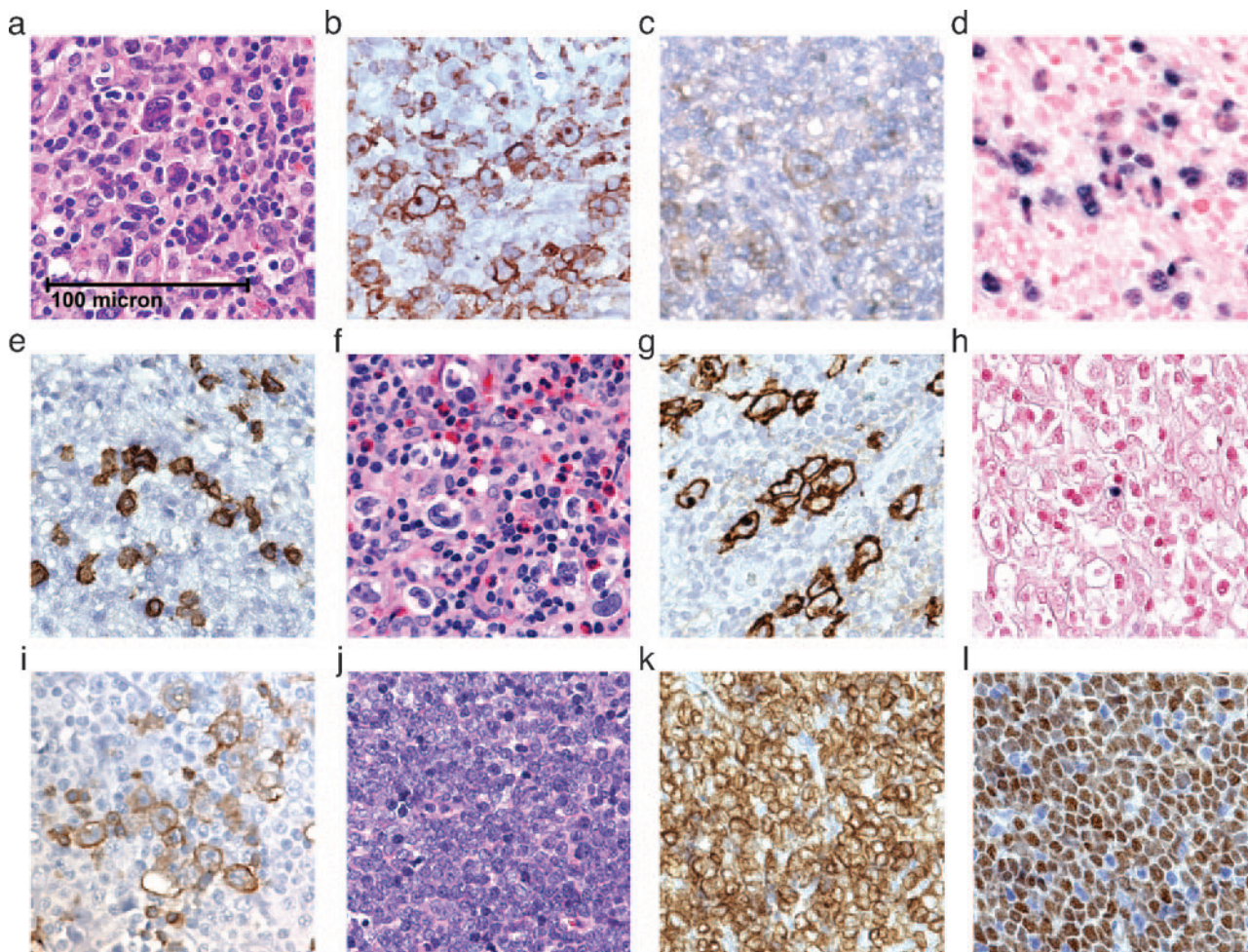
### Statistical Analysis

The data were analyzed using the software program JMP-IN version 5.1 (SAS Institute, Cary, NC). Age was entered as a continuous numerical parameter, and all other data were entered as categorical parameters. Differences in age between categorically defined groups were evaluated by *t*-test, and statistical significance was defined as  $P < 0.05$ . Correlations between categorical parameters were evaluated by Pearson  $\chi^2$  test and likelihood ratio test, and the lower  $P$  was recorded.

## Results

### Cases

We collected a series of 74 cases of PTCL from the Stanford hematopathology laboratory. This series is composed of 28 cases of ALK<sup>+</sup> ALCL, 35 cases of ALK<sup>-</sup> ALCL, 3 cases of subcutaneous panniculitis-like T-cell lymphoma, 1 case of hepatosplenic T-cell lymphoma, 4 cases of T-cell large granular lymphocytic leukemia (T-LGL), and 3 cases of enteropathy-type T-cell lymphoma. We also collected 24 cases of pre-B-ALL to serve as a positive control for *IGH* clonality. All cases were classified according to current World Health Organization criteria based



**Figure 1.** Histology of representative ALCL and pre-B-ALL cases. All panels shown at identical magnification. **a–e:** ALK<sup>+</sup> ALCL stained with H&E (**a**), immunohistochemistry with anti-CD2 (**b**), anti-CD30 (**c**), *in situ* hybridization for EBV (**d**), or anti-ALK (**e**). **f–i:** ALK<sup>-</sup> ALCL stained with H&E (**f**), anti-CD3 (**g**), *in situ* hybridization for EBV (**h**), or anti-CD30 (**i**). **j–l:** pre-B-ALL stained with H&E (**j**), anti-CD79a (**k**), or anti-TdT (**l**). Images were captured with a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) using a 60×/0.95 objective lens (Nikon) and a Spot CCD camera (Diagnostic Instruments, McHenry, IL) using Spot software version 4.6 for image acquisition.

on the combination of histologic, immunophenotypic, and clinical findings.<sup>1</sup>

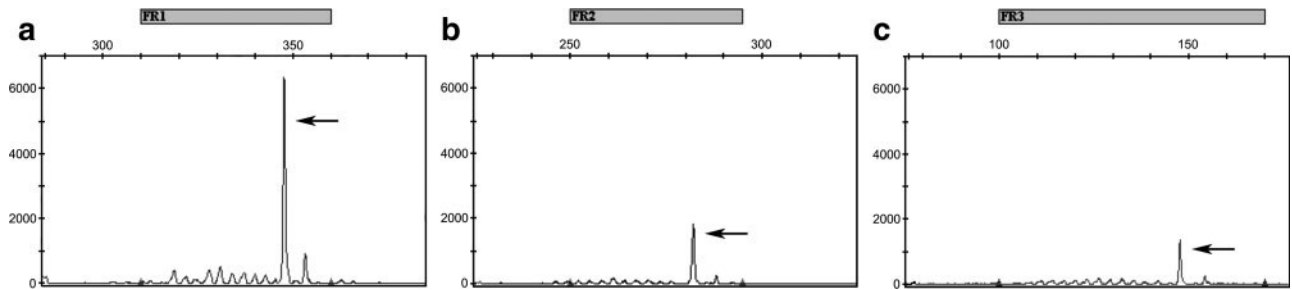
The patient demographics and anatomic sites of cases are summarized in Table 1. Each case was selected from a different patient, and the vast majority of cases represented the primary diagnosis. The majority of pre-B-ALL cases were from pediatric patients, and the mean age of pre-B-ALL patients was significantly lower than that of ALK<sup>+</sup> ( $P < 0.0368$ ) and ALK<sup>-</sup> ALCL patients ( $P < 0.0001$ ). For ALCL cases, the mean age of ALK<sup>+</sup> patients was significantly lower than that of ALK<sup>-</sup> patients ( $P < 0.0001$ ). Comparison between the diagnostic groups showed no significant difference in gender composition,

although males slightly outnumbered females among ALK<sup>+</sup> and ALK<sup>-</sup> ALCL patients, and females slightly outnumbered males among pre-B-ALL patients.

The ALK<sup>+</sup> and ALK<sup>-</sup> ALCL cases showed similar histologic features with large atypical cells with pleomorphic, often kidney-shaped nuclei and abundant cytoplasm with occasional eosinophilic inclusions. The immunophenotypic findings of the ALCL cases are summarized in Table 2, and representative histologic, immunohistochemical, and EBV *in situ* studies are shown for an ALK<sup>+</sup> and ALK<sup>-</sup> case in Figure 1, a–e and f–i, respectively. The majority of cases showed a predominance of large atypical CD30<sup>+</sup> cells, consistent with the common variant of ALCL. How-

**Table 2.** Immunophenotypic Findings of ALCL Cases

Diagnosis	Cases (n)	Positive cases/cases studied [n/n (%)]						
		CD2	CD3	CD15	CD43	CD30	ALK	EMA
ALK <sup>+</sup> ALCL	28	8/18 (44)	4/25 (16)	0/7 (0)	11/17 (65)	28/28 (100)	28/28 (100)	8/11 (73)
ALK <sup>-</sup> ALCL	35	9/14 (64)	16/32 (50)	1/12 (8)	16/23 (70)	35/35 (100)	0/35 (0)	4/13 (31)
Total	63	17/32 (53)	20/57 (35)	1/19 (5)	27/40 (68)	63/63 (100)	28/63 (44)	12/24 (50)



**Figure 2.** Electropherograms of PCRs for *IGH* rearrangements. The *x* axis indicates DNA length in nucleotides with expected product sizes above the axis. The *y* axis indicates relative fluorescence units. Shown are amplification products from a case of ALK<sup>-</sup> ALCL. **a**, **b**, and **c** represent different multiplex PCRs that use primers that bind to the FR1 (**a**), FR2 (**b**), or FR3 (**c**) region of *IGH*. This case was interpreted as positive with clonal peaks (arrows) detected by FR1, FR2, and FR3 primers.

ever, three ALK<sup>+</sup> cases showed mostly small- to medium-sized, CD30<sup>+</sup> and ALK<sup>+</sup> cells and were classified as the small cell variant.

For the other PTCL subtypes, the three subcutaneous panniculitis-like T-cell lymphoma cases showed a dense lymphoid infiltrate involving subcutaneous tissue with atypical CD3<sup>+</sup>, CD8<sup>+</sup> cells forming rings around adipocytes. The single hepatosplenic T-cell lymphoma case was a liver biopsy with numerous CD3<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>-</sup> lymphocytes in a characteristic sinusoidal pattern. The three enteropathy-type T-cell lymphoma cases were characterized by an infiltrate of large atypical CD3<sup>+</sup> lymphoid cells involving gastrointestinal mucosa, with the adjacent epithelium showing findings consistent with celiac spruce. For the four T-LGL cases, three were composed of CD3<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>+</sup> neoplastic cells that expressed one or more cytotoxic markers, including granzyme, T-cell intracellular antigen-1, and/or CD57. One T-LGL case was composed of CD3<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>-</sup> T-cells expressing the  $\gamma/\delta$  T-cell receptor and was classified as a  $\gamma/\delta$  T-LGL.

All of the pre-B-ALL cases were composed of numerous immature lymphoid cells (Figure 1, j-l). For 21 of 24 cases, specimens from extramedullary sites were chosen to obtain nondecalcified, formalin-fixed, paraffin-embedded tissue from anatomic sites that were similar to those of the PTCL cases (Table 1). Three pre-B-ALL cases, along with two of the T-LGL cases, were obtained from archived bone marrow aspirates. All of the pre-B-ALL cases exhibited a CD79a<sup>+</sup>, TdT<sup>+</sup>,

CD3<sup>-</sup> immunophenotype by immunohistochemical stains and/or flow cytometry.

### IGH Clonality Studies

We assayed cases for clonal rearrangements in the immunoglobulin heavy chain gene (*IGH*) using a multiplex PCR based on a European BIOMED-2 collaborative study.<sup>17</sup> The PTCL and pre-B-ALL cases were assayed concurrently, and the diagnoses of all cases were kept anonymous until clonality results were assigned for every case. The rationale for performing these studies in a “blinded” manner was to minimize any experimental or interpretive bias. In addition, the pre-B-ALL cases, which were expected to yield *IGH* clones, served as an internal positive control.

Representative *IGH* PCR data for an ALK<sup>-</sup> ALCL are shown in Figure 2, and the results for all cases are summarized in Table 3. Overall, *IGH* clones were detected in only 6 of 74 (8%) PTCL cases, including 3 of 28 (11%) ALK<sup>+</sup> ALCL cases, 3 of 35 (9%) ALK<sup>-</sup> ALCL cases, and 0 of 11 other PTCL cases. By contrast, *IGH* clones were detected in 23 of 24 (96%) pre-B-ALL cases, and the frequency of *IGH* clonality for pre-B-ALL cases was significantly higher than that of PTCL cases ( $P < 0.0001$ ).

In regard to the six ALCL cases with *IGH* clones, all six showed concurrent *TRG* clones, and five of six showed a CD3<sup>+</sup>, CD20<sup>-</sup> immunophenotype. The remaining case was negative for multiple B- and T-cell markers, including

**Table 3.** Clonality by PCR for *IGH* by Diagnosis

Diagnosis	Cases studied (n)	Positive cases [n (%)]	Positive cases detected by specific primer sets [n (% cases studied)]				
			FR1	FR2	FR3	D <sub>H</sub> 1-6	D <sub>H</sub> 7
ALK <sup>+</sup> ALCL	28	3 (11)	0 (0)	1 (4)	1 (4)	2 (7)	1 (4)
ALK <sup>-</sup> ALCL	35	3 (9)	1 (3)	1 (3)	2 (6)	1 (3)	0 (0)
SPTCL, HSTCL, T-LGL, and ETCL	11	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total PTCL	74	6 (8)	1 (1)	2 (3)	3 (4)	3 (4)	1 (1)
Pre-B-ALL	24	23 (96)	17 (71)	23 (96)	19 (79)	6 (25)	1 (4)

FR primer sets amplified complete rearrangements in *IGH* between variable segment (V<sub>H</sub>), diversity segment (D<sub>H</sub>), and joining segment (J<sub>H</sub>). D<sub>H</sub> primer sets amplified incomplete rearrangements between D<sub>H</sub> and J<sub>H</sub>. ETCL, Enteropathy-type T-cell lymphoma; HSTCL, hepatosplenic T-cell lymphoma; SPTCL, subcutaneous panniculitis-like T-cell lymphoma.

**Table 4.** Clonality by PCR for *IGH* by Specific Primer Set Combination

Primer sets yielding <i>IGH</i> clones	Cases [ <i>n</i> (% of cases studied)]		
	ALK <sup>+</sup> ALCL	ALK <sup>-</sup> ALCL	Pre-B-ALL
D <sub>H</sub> 1-6	1 (4)	1 (3)	0 (0)
D <sub>H</sub> 7	1 (4)	0 (0)	0 (0)
FR1, FR2, FR3	0 (0)	1 (3)	12 (48)
FR1, FR2, FR3, D <sub>H</sub> 1-6	0 (0)	0 (0)	4 (16)
FR1, FR2, FR3, D <sub>H</sub> 7	0 (0)	0 (0)	1 (4)
FR2	0 (0)	0 (0)	3 (12)
FR2, D <sub>H</sub> 1-6	0 (0)	0 (0)	1 (4)
FR2, FR3	0 (0)	0 (0)	1 (4)
FR2, FR3, D <sub>H</sub> 1-6	1 (4)	0 (0)	1 (4)
FR3	0 (0)	1 (3)	0 (4)
Negative	25 (89)	32 (91)	1 (4)
Total	28 (100)	35 (100)	24 (100)

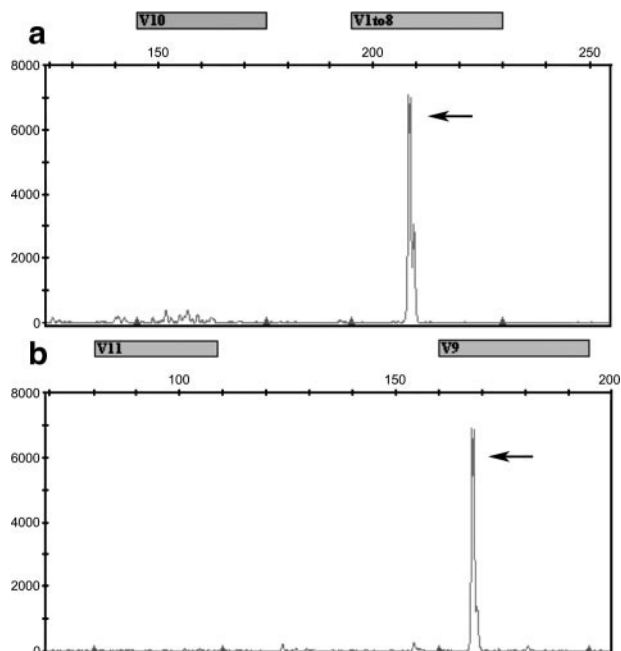
CD3 and CD20, and only CD30, CD43, and CD45 were expressed by the lymphoma cells. The combined morphologic and immunophenotypic findings were interpreted as supportive of the so-called null cell type of ALK<sup>-</sup> ALCL.

Also listed in Table 3 are the frequencies of clonal detection by specific primer sets in the PCR. The framework region (FR) primers, which amplify a complete V<sub>H</sub>-D<sub>H</sub>-J<sub>H</sub> rearrangement, detected more clones than the D<sub>H</sub> primers, which amplify an incomplete D<sub>H</sub>-J<sub>H</sub> rearrangement. For a given case, clonality was typically demonstrated by more than one primer set, which is summarized in Table 4. Of the 29 total *IGH* clones, 18 were detected simultaneously by the FR1, FR2, and FR3 primers, consistent with amplification of a complete rearrangement on one *IGH* allele by all three FR primers. In 5 of these 18 cases, the D<sub>H</sub>1-6 or D<sub>H</sub>7 primers also detected a clone, consistent with amplification of an incomplete rearrangement on the other *IGH* allele. Overall, 11 of the 29 total *IGH* clones were detected by the D<sub>H</sub>1-6 or D<sub>H</sub>7 primers, and in 8 of these 11 cases, clonality was also demonstrated by at least one FR primer.

Interestingly, the frequency of detection by single versus multiple primer sets differed between ALCL and pre-B-ALL cases (Table 4). Of the 6 ALCL cases with *IGH* clones, 4 were detected by only one primer set, and in 3 of these 4 cases, the clone was detected exclusively by the D<sub>H</sub>1-6 or D<sub>H</sub>7 primers. Only 2 ALCL cases were detected by multiple primer sets, with one ALK<sup>+</sup> case detected by the FR2, FR3, and D<sub>H</sub>1-6 primers and one ALK<sup>-</sup> case detected by the FR1, FR2, and FR3 primers (Figure 2, a, b, and c, respectively). By contrast, 20 of the 23 pre-B-ALL cases with *IGH* clones were detected by multiple primer sets, and only 3 were detected by a single primer set. None of the pre-B-ALL cases were detected solely by the D<sub>H</sub>1-6 or D<sub>H</sub>7 primers.

### *TRG* Clonality Studies

We assayed for clonal rearrangements in the T-cell receptor  $\gamma$ -chain gene (*TRG*) using a multiplex PCR based on the same European BIOMED-2 collaborative study as de-



**Figure 3.** Electropherograms of PCRs for *TRG* rearrangements. The *x* axis indicates DNA length in nucleotides with expected product sizes above the axis. The *y* axis indicates relative fluorescence units. **a:** A case of pre-B-ALL with a clonal peak (arrow) detected in the multiplex PCR that uses primers to detect rearrangements involving V<sub>γ</sub>10 or V<sub>γ</sub>1-8 of *TRG*. **b:** An ALK<sup>-</sup> ALCL case with a clonal peak (arrow) detected in the multiplex PCR that uses primers to detect rearrangements involving V<sub>γ</sub>11 or V<sub>γ</sub>9 of *TRG*.

scribed previously.<sup>17</sup> The *TRG* assays were also performed under blinded conditions, and as an added measure, the *TRG* and *IGH* studies were performed independently. For example, assays for *TRG* were performed and interpreted without any knowledge of the *IGH* results, and vice versa.

Representative *TRG* PCR data for a pre-B-ALL and ALK<sup>-</sup> ALCL case are shown in Figure 3, a and b, respectively, and the results for all cases are summarized in Table 5. Overall, *TRG* clones were detected in 64 of 74 (86%) PTCL cases, including 24 of 28 (86%) ALK<sup>+</sup> ALCL cases, 30 of 35 (86%) ALK<sup>-</sup> ALCL cases, and 10 of 11 (91%) other PTCL cases. For pre-B-ALL cases, *TRG* clones were detected in 16 of 24 (67%) cases, and the frequency of *TRG* clonality for pre-B-ALL cases was significantly less than that of PTCL cases ( $P < 0.03$ ). Further statistical analyses yielded no significant correlation between *TRG* clonality and *IGH* clonality or EBV. However, a positive *TRG* clone correlated with older age ( $P = 0.045$ ) and male sex ( $P = 0.009$ ).

Also listed in Table 5 are the number of clones detected by specific primer sets in the multiplex PCR, which target rearrangements between specific variable genes V<sub>γ</sub> and the joining segment J<sub>γ</sub> of *TRG*. For a given case, clonality was typically demonstrated by more than one primer set, which is summarized in Table 6 for all cases, and in Table 7, which provides details on the six PTCL cases with concurrent *TRG* and *IGH* clones. Overall, rearrangements in V<sub>γ</sub>1-8 were the most common, detected in 53% of cases, followed by rearrangements in V<sub>γ</sub>9, V<sub>γ</sub>10, and V<sub>γ</sub>11, detected in 37, 30, and 11% of cases, respectively.

**Table 5.** Clonality by PCR for TRG by Diagnosis

Diagnosis	Cases studied (n)	Positive cases [n (%)]	Positive cases detected by specific primer sets [n (% of cases studied)]			
			V <sub>γ</sub> 1-8	V <sub>γ</sub> 9	V <sub>γ</sub> 10	V <sub>γ</sub> 11
ALK <sup>+</sup> ALCL	28	24 (86)	14 (50)	10 (36)	10 (36)	2 (7)
ALK <sup>-</sup> ALCL	35	30 (86)	19 (54)	12 (34)	12 (34)	3 (9)
Other PTCL	11	10 (91)	8 (73)	8 (73)	5 (45)	4 (36)
SPTCL	3	3 (100)	3 (100)	3 (100)	2 (67)	1 (33)
HSTCL	1	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
T-LGL	4	3 (75)	2 (50)	3 (75)	1 (25)	1 (25)
ETCL	3	3 (100)	2 (67)	2 (67)	2 (67)	2 (67)
Total PTCL	74	64 (86)	41 (55)	30 (41)	27 (37)	9 (12)
Pre-B-ALL	24	16 (67)	12 (50)	6 (25)	2 (8)	2 (8)

ETCL, Enteropathy-type T-cell lymphoma; HSTCL, hepatosplenic T-cell lymphoma; SPTCL, subcutaneous panniculitis-like T-cell lymphoma.

In regard to the pattern of variable gene usage, there were few differences between the diagnostic groups (Table 5). For example, the V<sub>γ</sub>1-8 primers detected the most clones across all diagnostic groups, whereas the V<sub>γ</sub>11 primers detected the least. However, the frequency of V<sub>γ</sub>10 primer detection varied significantly by diagnosis, with V<sub>γ</sub>10 rearrangements detected in 27 of 74 (36%) PTCL cases but only in 2 of 24 (8%) pre-B-ALL cases ( $P = 0.004$ ). An additional finding that was unique to PTCL was the detection of TRG clonality by more than two primer sets (Table 6). Of the 64 PTCL cases with TRG clones, 8 cases were detected by three primer sets, and 2 cases were detected by all four primer sets. By contrast, none of the pre-B-ALL cases were detected by more than two primer sets.

### EBV Studies

To investigate the role of EBV in IGH clonality, we performed *in situ* hybridization for EBV-encoded small RNA on 49 of the 74 (66%) PTCL cases, which were selected based on the availability of remaining tissue. Representative *in situ* hybridizations for an ALK<sup>+</sup> ALCL and ALK<sup>-</sup>

ALCL are shown in Figure 1, and the results for all cases are summarized in Table 8. A positive result, which was defined as more than three EBV-labeled cells per section, was observed in 15 of 49 (31%) cases, including 6 of 21 (29%) ALK<sup>+</sup> ALCL cases, 8 of 24 (33%) ALK<sup>-</sup> ALCL cases, and 1 of 4 (25%) other PTCL cases. For most EBV-positive cases, labeled nuclei were scattered and rare, which is unlikely to represent EBV within the lymphoma cells (Figure 1h). However, one ALK<sup>+</sup> ALCL case and two ALK<sup>-</sup> ALCL cases showed unanimous EBV labeling of large cells in a pattern consistent with an EBV-positive lymphoma (Figure 1d).

Despite the detection of EBV in 15 of 49 (31%) PTCL cases, the presence of the virus did not correlate with IGH clonality. Of the 6 PTCL cases with IGH clones, which included 3 ALK<sup>+</sup> ALCL cases and 3 ALK<sup>-</sup> ALCL cases, 5 cases were studied by *in situ* hybridization, and all were negative for EBV. Likewise, of the 15 PTCL cases that contained EBV-positive cells, all were negative for IGH clonality. Taken together, these findings suggest that EBV does not contribute to IGH clonality, and further analysis yielded no significant correlation between EBV and age, gender, ALK expression, or TRG clonality.

**Table 6.** Clonality by PCR for TRG by Specific Primer Set Combination

Rearrangement(s)	Cases [n (% of total PTCL or Pre-B-ALL cases studied)]							
	ALK <sup>+</sup> ALCL	ALK <sup>-</sup> ALCL	ETCL	HSTCL	SPTCL	T-LGL	Total PTCL	Pre-B-ALL
V <sub>γ</sub> 1-8	6	9	0	1	0	0	16 (22)	7 (29)
V <sub>γ</sub> 1-8, V <sub>γ</sub> 10	4	2	0	0	0	0	6 (8)	2 (8)
V <sub>γ</sub> 1-8, V <sub>γ</sub> 10, V <sub>γ</sub> 11	0	0	1*	0	0	0	1 (1)	0 (0)
V <sub>γ</sub> 1-8, V <sub>γ</sub> 11	0	0	0	0	0	0	0 (0)	1 (4)
V <sub>γ</sub> 1-8, V <sub>γ</sub> 9	3	4	1	0	1	1	10 (14)	2 (8)
V <sub>γ</sub> 1-8, V <sub>γ</sub> 9, V <sub>γ</sub> 10	1*	3*	0	0	1*	0	5 (7)	0 (0)
V <sub>γ</sub> 1-8, V <sub>γ</sub> 9, V <sub>γ</sub> 10, V <sub>γ</sub> 11	0	0	0	0	1*	1*	2 (3)	0 (0)
V <sub>γ</sub> 1-8, V <sub>γ</sub> 9, V <sub>γ</sub> 11	0	1*	0	0	0	0	1 (1)	0 (0)
V <sub>γ</sub> 10	2	5	0	0	0	0	7 (10)	0 (0)
V <sub>γ</sub> 10, V <sub>γ</sub> 11	1	0	0	0	0	0	1 (1)	0 (0)
V <sub>γ</sub> 11	1	2	0	0	0	0	3 (4)	0 (0)
V <sub>γ</sub> 9	4	2	0	0	0	1	7 (10)	3 (13)
V <sub>γ</sub> 9, V <sub>γ</sub> 10	2	2	0	0	0	0	4 (5)	0 (0)
V <sub>γ</sub> 9, V <sub>γ</sub> 10, V <sub>γ</sub> 11	0	0	1*	0	0	0	1 (1)	0 (0)
V <sub>γ</sub> 9, V <sub>γ</sub> 11	0	0	0	0	0	0	0 (0)	1 (4)
Negative	4	5	0	0	0	1	10 (14)	8 (33)

\*Cases in which clonality was demonstrated by three or more primer sets. ETCL, Enteropathy-type T-cell lymphoma; HSTCL, hepatosplenic T-cell lymphoma; SPTCL, subcutaneous panniculitis-like T-cell lymphoma.



**Table 7.** Results and Demographics for PTCL Cases with Simultaneous *IGH* and *TRG* Clones

Diagnosis	Age (years)	Sex	Site	<i>TRG</i> clonality results	<i>IGH</i> clonality results
ALK <sup>+</sup> ALCL	26	F	Posterior auricular lymph node	V <sub>γ</sub> 1-8	FR2, FR3, D <sub>H</sub> 1-6
ALK <sup>+</sup> ALCL	26	F	Axillary lymph node	V <sub>γ</sub> 1-8, V <sub>γ</sub> 10	D <sub>H</sub> 7
ALK <sup>+</sup> ALCL	22	F	Inguinal lymph node	V <sub>γ</sub> 1-8, V <sub>γ</sub> 9	D <sub>H</sub> 1-6
ALK <sup>-</sup> ALCL	54	M	Chest wall mass	V <sub>γ</sub> 11	D <sub>H</sub> 1-6
ALK <sup>-</sup> ALCL	69	F	Supraclavicular lymph node	V <sub>γ</sub> 1-8	FR3
ALK <sup>-</sup> ALCL	54	M	Axillary lymph node	V <sub>γ</sub> 10	FR1, FR2, FR3

### Discussion

Our main goal was to determine whether *IGH* clones occur in cases of PTCL that lack associated B-cell proliferations. To address this question, we performed clonality studies on a series of ALCL and other specific PTCL subtypes in which B-cell proliferations have not been reported. In addition, we concurrently assayed 24 cases of pre-B-ALL, which is a neoplasm known to commonly show both *IGH* and *TRG* rearrangements, and we performed these studies in a blinded manner. Our intent was to minimize any experimental or interpretive bias by eliminating a priori knowledge of the diagnosis. For example, if a case could be identified as a PTCL, interpretation of the data may favor the expected results, namely a positive *TRG* clone and a negative *IGH* clone.

Under these blinded conditions, we detected *IGH* clones in only 6 of 74 (8%) PTCL cases, consistent with previous reports on large series of PTCL (9 to 16%).<sup>2-6</sup> By contrast, we detected *IGH* clones in 23 of 24 (96%) pre-B-ALL cases, and similarly high rates of detection have been reported for pre-B-ALL.<sup>18-20</sup> In regard to PTCL, the frequency of *IGH* clonality differed significantly from our previous report, in which we detected *IGH* clones in 33% of AILT cases and 35% of PTCL-U cases ( $P < 0.004$ ).<sup>7</sup> We attribute this difference to the distinct composition of cases, with 20% of previous cases showing demonstrable B-cell proliferations. Collectively, our findings suggest that *IGH* clonality occurs infrequently (<10%) in most PTCL subtypes, with the exceptions of AILT and PTCL-U. This finding was also observed in a recent BIOMED-2 study, in which *IGH* clones were de-

tected in 10% of PTCL cases, but the majority of cases with *IGH* clones were classified as AILT or PTCL-U.<sup>21</sup>

Regarding the etiology of *IGH* clones, it remains our hypothesis that B-cell proliferations are largely responsible for this finding, at least in AILT and PTCL-U. However, *IGH* clonality can also occur in other PTCL subtypes, even in the absence of an associated B-cell proliferation or EBV. We investigated other possible etiologies, including pseudo-clonality, which refers to PCR amplification of a single cell that results in a nonreproducible product.<sup>22,23</sup> However, repeat testing of all positive *IGH* clones yielded reproducible products, making pseudo-clonality unlikely.

Other explanations include lineage infidelity and solitary germinal centers. Proof of lineage infidelity requires single cell analysis, which others have achieved by performing PCR on single cells isolated by microdissection.<sup>6,24</sup> However, the technical complexity of these experiments limits the number of cases that can be studied, and although evaluation for lineage infidelity is beyond the scope of this report, it remains a possible cause of *IGH* clonality. Several studies have shown that a single germinal center isolated by microdissection can yield an *IGH* clone by PCR.<sup>25,26</sup> Therefore, we re-examined all of our PTCL cases for germinal centers. However, none of the cases with *IGH* clones contained solitary or multiple germinal centers, and there was no correlation between germinal centers and *IGH* clonality.

It should be noted that three of the six *IGH* clones in PTCL were detected exclusively by the D<sub>H</sub>1-6 or D<sub>H</sub>7 primers. There are several explanations for this finding,

**Table 8.** EBV Studies by *in Situ* Hybridization for EBER

Diagnosis	Cases studied (n)	Positive cases [n (%)]	EBV-labeled cells per medium-power field (n)					
			0	Less than 1	1 to 10	Greater than 10 to 100	Greater than 100	
ALK <sup>+</sup> ALCL								
With <i>IGH</i> clone	3	0 (0)	3	0	0	0	0	
Without <i>IGH</i> clone	18	6 (33)	12	4	1	0	1*	
Total	21	6 (29)	15	4	1	0	1	
ALK <sup>-</sup> ALCL								
With <i>IGH</i> clone	2	0 (0)	2	0	0	0	0	
Without <i>IGH</i> clone	22	8 (36)	14	4	1	1	2*	
Total	24	8 (33)	16	4	1	1	2	
SPTCL, HSTCL, T-LGL, and ETCL	4	1 (25)	3	0	1	0	0	
Total PTCL	49	15 (31)	34	8	3	1	3	

One SPTCL, one HSTCL, one T-LGL, and one ETCL were tested for EBV, and only the T-LGL case, which did not have an *IGH* clone, showed EBV-labeled cells. EBER, EBV-encoded small RNA; ETCL, enteropathy-type T-cell lymphoma; HSTCL, hepatosplenic T-cell lymphoma; SPTCL, subcutaneous panniculitis-like T-cell lymphoma.

\*Cases in which EBV-labeled cells corresponded to the neoplastic cells.

which was also observed in a recent BIOMED-2 study on PTCL.<sup>21</sup> Because B-cell maturation requires complete  $V_H$ - $D_H$ - $J_H$  rearrangement of at least one *IGH* allele,<sup>27</sup> a clone detected solely by  $D_H$  primers must be the result of  $D_H$ -primed amplification of an incomplete  $D_H$ - $J_H$  rearrangement on one allele and the inability of the FR primers to amplify a complete  $V_H$ - $D_H$ - $J_H$  rearrangement on the other allele. Lack of amplification may be caused by translocation or mutation of the FR primer binding sites, and among mature B-cell lymphomas, isolated  $D_H$  primer detection is most common in those that have undergone somatic hypermutation.<sup>28</sup> If these events also occur in PTCL, this would suggest that *IGH* clonality is caused by germinal center or postgerminal center B cells. Another intriguing possibility is EBV-mediated survival and clonal expansion of a B cell that has acquired a large deletion or nonsense mutation in its functional *IGH* allele. This phenomenon, also known as EBV rescue of a "crippled" B cell, occurs in classical Hodgkin lymphoma<sup>29</sup> and post-transplant lymphoproliferative disorders.<sup>30-32</sup> However, in our study, all of the cases with *IGH* clones lacked EBV. An entirely alternate explanation for isolated  $D_H$  primer detection involves lineage infidelity, with a sole  $D_H$ - $J_H$  rearrangement on one *IGH* allele. In this context, a complete rearrangement on the other allele is not a developmental requirement because the events occur in a neoplastic T cell rather than a B cell.

The assays for *TRG* clonality also provided results that were consistent with the reported literature. For PTCL cases, *TRG* clones were detected in 64 of 74 (86%) cases, which included 24 of 28 (86%)  $ALK^+$  ALCL cases, 30 of 35 (86%)  $ALK^-$  ALCL cases, and 10 of 11 (91%) other PTCL cases (Table 4). These frequencies are similar to those of previous reports<sup>2,33,34</sup> and of recent studies using the BIOMED-2 *TRG* PCR.<sup>21,35,36</sup> Although few reports include large numbers of ALCL, Brüggemann et al<sup>21</sup> detected *TRG* clones in 24 of 34 (71%)  $ALK^+$  cases and 8 of 9 (89%)  $ALK^-$  cases in a BIOMED-2 study. For the pre-B-ALL cases, we detected *TRG* clones in 16 of 24 (67%) cases, and this frequency is similar to comparable studies on pre-B-ALL.<sup>18,20,37</sup>

We also evaluated *TRG* variable gene usage by analyzing which specific primer sets yielded a clonal product (Table 5), and the frequencies of specific variable gene rearrangements were similar to our previous study.<sup>7</sup> Rearrangements in  $V_\gamma$ 1-8 were most common, occurring alone or in combination with other variable genes in 53 of 80 (66%) *TRG* clones (Table 5). By corollary, 27 of 80 (34%) *TRG* clones would have been missed if the  $V_\gamma$ 1-8 primers were used alone. In general, the pattern of variable gene usage was similar across diagnostic groups, with the exception of  $V_\gamma$ 10 rearrangements, which were frequent in PTCL, but nearly absent in pre-B-ALL. We previously reported that  $V_\gamma$ 10 rearrangements are relatively common in AILT and PTCL-U,<sup>7</sup> and others have shown  $V_\gamma$ 10 rearrangements are virtually absent in pre-B-ALL.<sup>38</sup>

For most *TRG* clones, one or two rearrangements were detected, consistent with one or two rearranged *TRG* alleles, respectively (Table 5). However, of the 80 *TRG* clones, 8 showed three variable gene rearrangements,

and 2 showed four variable gene rearrangements (Table 6). Interestingly, all of the cases with more than two rearrangements were PTCL cases. Because diploid cells have two *TRG* alleles, explanations for this finding include a second minor clone or oligoclonal, genome instability, and aneuploidy. Regardless of the etiology, we and others have previously shown that more than two *TRG* rearrangements are detected by PCR in a minority (~10%) of histologically proven PTCL.<sup>7,21,39</sup> In this study, more than two rearrangements were detected in 10 of 74 (14%) PTCL cases, accounting for 10 of the 64 (16%) *TRG* clones in PTCL.

Taken together, the *TRG* and *IGH* assays provided correct lineage assignment for the majority of PTCL cases; however, simultaneous *TRG* and *IGH* clones were detected in 6 of 74 (8%) cases, resulting in ambiguous lineage. Although an *IGH* clone was not detected without a concurrent *TRG* clone, the potential for incorrect lineage assignment is readily apparent. Therefore, for cases of suspected PTCL, we recommend using DNA-based clonality studies primarily to provide support for a neoplastic process, and any unexpected results should be evaluated within the context of the histopathologic findings. Although an *IGH* clone may suggest a diagnosis of AILT or PTCL-U, *IGH* clonality can also occur in other PTCL subtypes, and this finding should not negate the diagnosis established by the combination of morphologic, immunophenotypic, and clinical findings.

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