

# Immunoglobulin V<sub>H</sub> Gene Mutational Analysis Suggests that Primary Effusion Lymphomas Derive from Different Stages of B Cell Maturation

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**Primary effusion lymphoma (PEL) is a recently described distinct subtype of non-Hodgkin's lymphoma associated with infection by the Kaposi's sarcoma-associated herpesvirus, also called human herpesvirus-8. Most cases of PEL are also associated with the Epstein-Barr virus (EBV). In order to better characterize the cellular origin of PEL, we investigated the immunoglobulin (Ig) heavy chain variable region (V<sub>H</sub>) genes expressed by tumor cells of the BC-1 and BC-3 cell lines derived from PELs and five original PEL specimens. In the six EBV-positive PELs examined, including the BC-1 cell line, the expressed V<sub>H</sub> gene sequences showed numerous point mutations relative to the putative germline V<sub>H</sub> gene sequences. In addition, the V<sub>H</sub> segment of one of these cases showed intraclonal sequence heterogeneity, indicating ongoing somatic mutation. In five cases, the distribution and type of mutations indicated that tumor cells had been selected by antigen. Because somatically mutated Ig genes are expressed by B cells that have reached a germinal center/post-germinal center stage of development, these findings suggest that the PEL cell of origin is a germinal center or post-germinal center B cell in most cases. In contrast, the V<sub>H</sub> gene segment expressed by tumor cells of the BC-3 cell line, which was originated from an EBV-negative PEL obtained from an HIV-negative patient, was unmutated, suggesting a pre-germinal center B cell origin for tumor cells of this particular PEL cell line. Taken together, these findings suggest that development of PELs may not be restricted to one stage of B cell differentiation and may represent transformation of B cells at different stages of ontogeny. (*Am J Pathol* 1998, 153:1609–1614)**

In 1989 Knowles et al,<sup>1</sup> and later other investigators, described an unusual type of aggressive non-Hodgkin's lymphoma that grows in the pleural, pericardial, and abdominal cavities as a lymphomatous effusion, usually in

the absence of a detectable tumor mass.<sup>2–4</sup> These so-called primary effusion lymphomas (PEL) have been recognized as a distinct clinicopathological and biological entity based on their unique constellation of clinical, morphological and immunophenotypic characteristics and consistent infection of the tumor cells by the Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus-8 (HHV-8).<sup>5–9</sup> PEL occurs predominantly but not exclusively among human immunodeficiency virus (HIV)-infected individuals.<sup>5,10–13</sup> These neoplasms exhibit cytomorphic features that bridge large cell immunoblastic and anaplastic large cell lymphoma, usually lack surface immunoglobulin (Ig) and B-cell-associated antigens but express CD45 and antigens associated with late stages of B cell differentiation such as CD30, CD38, CD71, CD138, and epithelial membrane antigen (EMA).<sup>5–7,13</sup> Genotypic analysis of the tumor cells has revealed clonal Ig gene rearrangement in all cases and Epstein-Barr virus (EBV) infection in most cases.<sup>6</sup>

Although the immunophenotypic and genotypic characteristics of PELs confirm their B cell lineage, the normal counterpart and stage of differentiation of the tumor cells remain controversial. The combination of the pleomorphic morphology, null cell immunophenotype, and CD30 antigen expression has led some authors to link PEL to anaplastic large cell lymphoma.<sup>10</sup> In contrast, other investigators have suggested that PEL cells represent a mature stage of B cell development closely associated with mature plasma cells based on the findings that the tumor cells coexpress CD30, CD38, CD138, and EMA.<sup>6,9</sup> Finally, some of the PELs display Ig heavy chain but not light chain gene rearrangement, causing some investigators to suggest that PELs may arise early in B cell development, following heavy chain gene but prior to light chain gene rearrangement.<sup>4</sup>

Analysis of nucleotide sequences of Ig heavy chain variable region (V<sub>H</sub>) genes can provide insight into the stage of B cell development at which clonal expansion occurs in B cell tumors. It has been shown that preger-

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minimal center B cells contain IgV<sub>H</sub> region genes with germ line sequence and postgerminal center B cells contain mutated Ig V<sub>H</sub> regions. Furthermore, antigen selected postgerminal center B cells display somatic mutations clustered in the complementary determining regions (CDR) sequences. Germinal center B cells are characterized by ongoing mutation, which is absent in postgerminal center B cells.<sup>14,15</sup>

In order to determine the stage of B cell development at which clonal expansion of the lymphoma cells occurs in PEL, we analyzed nucleotide sequences of IgV<sub>H</sub> regions expressed by seven PELs. Our results suggest that the development of PELs may not be restricted to one stage of B cell differentiation.

## Materials and Methods

### Pathological Samples

A group of seven non-Hodgkin's lymphomas containing KSHV sequences meeting the clinical, morphological, immunophenotypic, and molecular genetic criteria for PEL were included in this study.<sup>5,6,10</sup> Case 1 represents the KSHV-positive, EBV-negative BC-3 cell line established from a PEL occurring in an HIV-negative patient.<sup>16</sup> Case 2 represents the KSHV- and EBV-positive BC-1 cell line established from a PEL occurring in a patient with AIDS.<sup>17</sup> Cases 3–7 represent KSHV- and EBV-positive PELs collected from adult HIV-positive patients. Heparin-treated samples of the lymphomatous effusions were collected under sterile conditions during standard diagnostic procedures. Mononuclear cells were isolated and cryopreserved as described.<sup>5</sup>

### Immunophenotypic Analyses

The immunophenotype of the PEL tumor cells and cell lines was determined by direct and indirect immunofluorescent flow cytometry using the FACScan fluorescent activated cell sorter (Becton-Dickinson, Mountain View, CA) as previously described.<sup>18</sup> Monoclonal antibodies used include leukocyte common antigen (LCA, CD45), BerH2, Ki-1 (CD30), epithelial membrane antigen (EMA, Dako Corp, Santa Barbara, CA), B4 (CD19), B1 (CD20), B2 (CD21, Coulter Immunology, Hialeah, FL), Leu1 (CD5), Leu14 (CD22), Leu20 (CD23, Becton-Dickinson Immunocytometry Systems, Mountain View, CA), and T3 (CD3, United Biomedical, Hauppauge, NY). Antisera to total Ig, kappa ( $\kappa$ ) and lambda ( $\lambda$ ) Ig light chains were obtained from Dako.

### Isolation of RNA and First-Strand cDNA Synthesis

Total RNA was extracted from cryopreserved mononuclear cell suspensions using guanidine isothiocyanate techniques.<sup>19</sup> Five mg of RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Super-script RNase H Reverse Transcriptase, GIBCO-BRL,

Grand Island, NY), in conjunction with a poly(dT)<sub>12–13</sub> primer according to the manufacturer's instructions.

### PCR Amplification, Cloning, and Sequencing of the Expressed Ig V<sub>H</sub>-D-J<sub>H</sub> Genes

cDNAs were amplified by PCR using sense Ig V<sub>H</sub> gene family-specific (V<sub>H</sub>1, V<sub>H</sub>2, V<sub>H</sub>3, V<sub>H</sub>4, V<sub>H</sub>5, and V<sub>H</sub>6) leader primers in conjunction with an antisense consensus J<sub>H</sub> primer in independent reactions, as previously described.<sup>20</sup> After amplification, PCR products were electrophoresed in 2% agarose and visualized by ethidium bromide staining. Appropriately sized PCR products were isolated. PCR products were cloned in the pCR II vector using the TA cloning system (Invitrogen, San Diego, CA), following the manufacturer's instructions. Plasmid DNA was isolated from overnight cultures of randomly selected white colonies using Wizard Mini-preps (Promega, Madison, WI). DNA sequencing was performed using the Sequenase version 2.0 (United States Biochemical, Cleveland, OH) system according to the manufacturer's instructions. DNA sequences were analyzed using the MacVector version 4.5 (Eastman Kodak, New Haven, CT) software and the EMBL/GenBank data base.

### Analysis of Mutations

We calculated the number of expected replacement (R) mutations in complementary determining regions (CDR) and framework regions (FR) for the Ig V<sub>H</sub> genes using the formula  $R_{\text{CDR}}$  or  $R_{\text{FR}} = n \times \text{CDR } R_f$  or  $\text{FR } R_f \times \text{CDR}_f$  or  $\text{FR}_f$ , where  $n$  is the total number of observed mutations;  $R_f$  is the replacement frequency intrinsic to each Ig V<sub>H</sub> gene,<sup>21</sup> and  $\text{CDR}_f$  and  $\text{FR}_f$  are the relative sizes of the CDRs and FRs. A binomial probability model was used to evaluate whether the observed R mutations in CDRs and the scarcity of silent (S) mutations in the FRs were due to chance alone:  $p = n!/k!(n-k)!q^k(1-q)^{n-k}$ , where  $q$  = probability that an R mutation will localize to CDR or FR ( $q = \text{CDR}_{\text{rel}}$  or  $\text{FR}_{\text{rel}} \times \text{CDR } R_f$  or  $\text{FR } R_f$ ), and  $k$  = number of observed R mutations in the CDR or FR.<sup>21</sup>

## Results

### Immunophenotypic Analysis

The immunophenotypic features of the PELs included in this study are summarized in Table 1. All but one PEL (case 5) expressed CD45. However, only one case (case 3) expressed surface Ig. Also, all seven cases lacked B cell-associated antigens CD19 and CD20 and only case (case 6) expressed CD22. Five cases (cases 1–4 and 6) expressed CD30, three cases (cases 2, 6, 7) expressed EMA and two cases (cases 2 and 6) expressed HLA-DR. All seven cases lacked CD21 and CD23 and T cell-associated antigens.

**Table 1.** Results of Immunophenotypic Analysis of Seven Cases of PEL

Antigens	Case Number						
	1 (BC-3)	2 (BC-1)	3	4	5	6	7
slg	-	-	+	-	-	-	-
κ	-	-	-	-	-	-	-
λ	-	-	+	-	-	-	-
HLA-DR	-	+	-	-	-	+	-
CD3	-	-	-	-	-	-	-
CD5	-	-	-	-	-	-	-
CD19	-	-	-	-	-	-	-
CD20	-	-	-	-	-	-	-
CD21	-	-	-	-	-	-	-
CD22	-	-	-	-	-	+	-
CD23	-	-	-	-	-	-	-
CD30	+	+	+	+	-	+	-
CD45	+	+	+	+	-	+	+
EMA	-	+	-	-	-	+	+

+, positive; -, negative.

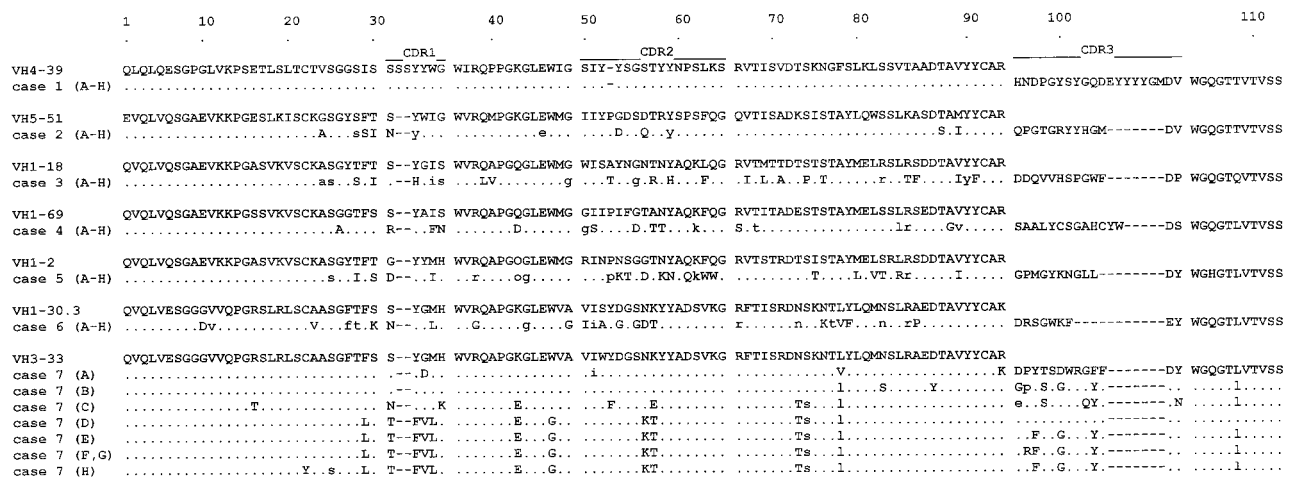
*Sequence Analysis of the Expressed Ig V<sub>H</sub>-D-J<sub>H</sub> Genes*

To analyze the Ig V<sub>H</sub>-D-J<sub>H</sub> genes expressed by the seven PELs, first-strand cDNAs were synthesized from total RNA of lymphoma cells and then PCR-amplified using the appropriate primers. The amplified DNAs were cloned and sequenced. Eight sequences from independent bacterial isolates were analyzed in each sample. The sequences obtained were compared with the corresponding germline sequences using EMBL/GenBank database to determine the respective homology of each gene with its closest germline counterpart. The closest germline V<sub>H</sub> genes and their degrees of similarity are shown in Table 1. The deduced amino acid sequences of the V<sub>H</sub>-D-J<sub>H</sub> segments of the seven PELs and predicted amino acid changes when compared with the reported closest germline V<sub>H</sub> gene sequences are depicted in Figure 1. All the V<sub>H</sub>-D-J<sub>H</sub> sequences were in-frame and no stop codons were found, suggesting that these cases expressed func-

tional Ig. In cases 1-6, only identical and unique sequences were detected from analysis of the multiple isolates. Cases 3-5 used germline genes from the V<sub>H</sub>1 family and showed 88.4%, 93.5%, and 90.1% homology to their closest germline genes, respectively. Cases 1, 2, and 6 utilized germline genes from V<sub>H</sub>4, V<sub>H</sub>5, and V<sub>H</sub>3 families and displayed 100%, 94.9%, and 90.1% homology to their corresponding germline genes, respectively. In case 7, V<sub>H</sub>-D-J<sub>H</sub> gene sequences derived from eight independent isolates revealed seven unique but collinear sequences. Lymphoma cells of this case rearranged a member of V<sub>H</sub>3 family and sequences displayed 95.2% to 99.0% homology with the germline precursors (Table 2). D segment sequences in cases 1 and 4 displayed the highest degree of identity to those of germline genes HD5-5 and HD2-15, respectively. The CDR3 sequences of the other PELs did not display any identity to those of known germline D genes. The HJ4 gene was used in five cases and the HJ6 gene in two cases.

*Mutation Analysis*

In six cases, the expressed V<sub>H</sub> genes showed numerous nucleotide differences from the closest germline gene sequences obtained from EMBL/GenBank database. Because germline V<sub>H</sub> genes were not identified in these patients, some of these nucleotide differences may represent individual polymorphism. However, sequence polymorphism among V genes is generally low<sup>23</sup> and in cases where we<sup>24</sup> and others<sup>25,26</sup> have analyzed germline genes of the patients, sequences corresponded exactly to those obtained from EMBL/GenBank database in most cases. Therefore, it has been concluded that polymorphism in V<sub>H</sub> genes is not sufficient to require analysis<sup>27-29</sup> and nucleotide differences in these cases have been classified as somatic mutation. Analysis of the distribution of somatic mutations in each sequence has been carried out by the method of Chang and Casali.<sup>21</sup> In this method, the binomial distribution model is used to



**Figure 1.** Deduced amino acid sequences of Ig V<sub>H</sub>(D)-J<sub>H</sub> regions of the tumor-derived clones (A-H) from seven patients with PEL. Comparisons are made with the closest germline V<sub>H</sub> genes. The proportion of the CDR3 corresponding to the D and J<sub>H</sub> segments are not shown. The individual FR and CDR regions are indicated according to Kabat et al (1991). Identity with the V<sub>H</sub> germline sequences is shown by dots, replacement mutations by uppercase letters, and silent mutations by lowercase letters.

**Table 2.** Analysis of Ig V<sub>H</sub>-D-J<sub>H</sub> Genes Expressed by the Neoplastic Cells in Seven Cases of PEL

Case No.	Intraclonal Diversity	Clone	Closest Germline Gene	Nucleotide Identity %	Ig V <sub>H</sub> Gene								D Gene	J <sub>H</sub> Gene
					CDR1 and CDR2				FR1, FR2, and FR3					
					R	S	R:S	P	R	S	R:S	P		
1 (BC-3)	no	A-H	HV4-39	100	0 (0.00)	0	0.00	0.00	0 (0.00)	0	0.00	0.00	HD5-5	HJ6
2 (BC-1)	no	A-H	HV5-51	94.9	4 (9.03)	3	1.33	$7.08 \times 10^{-3*}$	5 (2.58)	3	1.67	$6.84 \times 10^{-2}$	ND	HJ6
3	no	A-H	HV1-18	88.4	7 (20.56)	4	1.75	$0.20 \times 10^{-5*}$	15 (5.70)	8	1.87	$1.34 \times 10^{-4*}$	ND	HJ4
4	no	A-H	HV1-69	93.5	8 (11.49)	2	4.00	$4.95 \times 10^{-2*}$	5 (3.19)	4	1.25	$1.18 \times 10^{-1}$	HD2-15	HJ4
5	no	A-H	HV1-2	90.1	12 (18.28)	2	6.00	$9.18 \times 10^{-3*}$	8 (4.88)	7	1.14	$5.45 \times 10^{-2}$	ND	HJ4
6	no	A-H	HV1-30.3	90.1	8 (17.89)	2	4.00	$1.64 \times 10^{-4*}$	10 (4.82)	9	1.11	$1.01 \times 10^{-2*}$	ND	HJ4
7	yes	A	HV3-33	98.6	1 (2.46)	1	1.00	$1.39 \times 10^{-1}$	2 (0.7)	0	2.00	$1.15 \times 10^{-1}$	ND	HJ4
		B	HV3-33	99.0	0 (1.85)	0	0.00	0.00	2 (0.50)	1	2.00	$6.91 \times 10^{-2}$	ND	HJ4
		C	HV3-33	96.3	5 (6.78)	1	5.00	$1.31 \times 10^{-1}$	3 (1.8)	2	1.50	$1.76 \times 10^{-1}$	ND	HJ4
		D	HV3-33	96.0	6 (7.39)	0	6.00	$1.62 \times 10^{-1}$	4 (2.0)	2	2.00	$8.83 \times 10^{-2}$	ND	HJ4
		E	HV3-33	96.0	6 (7.39)	0	6.00	$1.62 \times 10^{-1}$	4 (2.0)	2	2.00	$8.83 \times 10^{-2}$	ND	HJ4
		F-G	HV3-33	96.0	6 (7.39)	0	6.00	$1.62 \times 10^{-1}$	4 (2.0)	2	2.00	$8.83 \times 10^{-2}$	ND	HJ4
		H	HV3-33	95.2	6 (8.63)	0	6.00	$7.74 \times 10^{-2}$	5 (2.3)	3	1.67	$4.95 \times 10^{-2*}$	ND	HJ4

ND, not detected; R, number of detected and (expected) R mutations; S, number of detected S mutations; P, probability; \*, statistically significant ( $P < 0.05$ ).

calculate the probability that an excess in CDRs or scarcity in FRs of R mutations resulted from chance alone. The results of the analysis are summarized in Table 2. The distribution of R and S mutations indicates a high R:S ratio in the CDRs of cases 4–7; however, this ratio did not exceed 2.9 for cases 2 and 3. Applying the binomial distribution model revealed more R mutations in the CDRs than expected in five sequences, with significant ( $P < 0.05$ ) clustering. All mutated sequences had an R:S ratio equal to or less than 2 in their FR. Using the binomial distribution model, there were fewer R mutations in the FRs than expected due to chance in three sequences, with significant ( $P < 0.05$ ) conservation of sequences.

### Discussion

We have characterized the Ig V<sub>H</sub> gene configuration expressed by tumor cells of seven PELs in order to help determine their cellular origin. Both EBV-positive and EBV-negative cases were analyzed in order to cover the clinicopathological spectrum of the disease. The V<sub>H</sub> gene of the BC-3 cell line, which was the only EBV-negative PEL examined here and the only PEL obtained from an HIV-negative individual, showed complete nucleotide sequence identity with its putative germline precursor, suggesting a naive pregerminal center B cell origin of this PEL. In contrast, the six EBV-positive PELs, including the BC-1 cell line, displayed highly mutated V<sub>H</sub> genes. The average mutation frequency for the six cases analyzed was 7.7%, a value considerably higher than the 3–4% mutation frequency determined for nonmalignant germinal center and memory B cells,<sup>30</sup> suggesting that most PELs, and all of those containing EBV in this series, have reached a mature stage of B cell development.

The intraclonal heterogeneity of the expressed V<sub>H</sub>-D-J<sub>H</sub> gene sequences was variable among the six EBV-positive PELs. In five of these six cases, the expressed V<sub>H</sub>-D-J<sub>H</sub> sequences were extensively mutated but these sequences did not show intraclonal heterogeneity. These findings suggest that the tumor cells have traversed the germinal center but are no longer influenced by the somatic hypermutation mechanism. It could be concluded

from this that the neoplastic event has occurred at a postgerminal center stage of B-cell maturation. The similar pattern of somatic mutation and antigen expression, including the absence of surface Ig in multiple myeloma and most of these PEL cases, suggests a plasma cell origin for some PEL cases.<sup>31</sup>

In contrast to homogenous Ig gene sequences of PELs, considerable intraclonal heterogeneity, similar to the situation in follicle center cell lymphoma, was detected in one case.<sup>32,33</sup> The ongoing somatic mutation indicates that the tumor cells are still influenced by the mutation mechanism after malignant transformation. This finding is highly suggestive of a germinal center origin of this particular PEL. However, certain memory B cells may recirculate through the germinal center and undergo further rounds of somatic mutation and antigen selection that further improve and expand B-cell memory.<sup>34</sup>

The germinal center/postgerminal center cellular origin of EBV-positive PELs analyzed is also suggested by the distribution of mutations in the V<sub>H</sub> genes of the tumor cells. Replacement mutations that affect CDRs can be positively or negatively selected by antigen, as these regions form the antigen binding sites. If a DNA segment displays more R mutations than would be expected by chance, it is likely that positive pressure was exerted on the gene product to select for these mutations.<sup>21</sup> In all but one EBV-positive PEL, the significant clustering of R mutations in the CDRs indicates that tumor cells have been selected by antigen or, in other words, that the tumor cells have undergone affinity maturation.

EBV-infected tumor cells of different lymphoid neoplasms frequently express mutated Ig V<sub>H</sub> genes. For example, it has been shown that the tumor cells of endemic Burkitt's lymphomas,<sup>35</sup> posttransplantation lymphoproliferative disorders,<sup>36</sup> and Reed-Sternberg cells of Hodgkin's disease<sup>37</sup> express mutated Ig V<sub>H</sub> genes. Our findings strengthen this observation by providing evidence that EBV-positive tumor cells of PEL also express mutated V<sub>H</sub> genes. However, the exact role of EBV infection in Ig gene somatic mutation of neoplastic cells is controversial. It has been demonstrated that EBV-infected normal B lymphocytes display V<sub>H</sub> genes with so-



matic hypermutation and evidence intraclonal heterogeneity.<sup>38</sup> Therefore, it appears that EBV infection plays a role in preneoplastic events by driving B cell proliferation, activation, and mutation of the Ig gene.

Several lines of evidence suggest that KSHV infection plays a central role in the development of PELs. KSHV carries different genes that may behave as oncogenes, including a gene homologous to bcl-2 (ORF16), a gene homologous to the cellular D-type cyclins (ORF72/cyclin D), and a G-protein-coupled receptor displaying constitutive activation (ORF74/GPCR).<sup>39,40</sup> However, nonneoplastic lymphoid cells may be infected by KSHV. Ambroziak et al<sup>41</sup> have shown that the CD19-positive B cell population of HIV-infected and uninfected individuals may carry KSHV without clinical evidence of PEL or Kaposi's sarcoma, and Luppi et al<sup>42</sup> found KSHV sequences in benign lymph nodes with florid germinal center hyperplasia. This finding suggests that latent infection of KSHV precedes malignant transformation of the cells and additional adequate stimuli may be necessary to initiate malignant transformation of the KSHV-infected cells, not unlike the EBV scenario. A possible explanation for the heterogeneous mutation profile of V<sub>H</sub> genes found in PELs is that malignant transformation is not restricted to specific stages of B cell development. Based on this hypothesis, B cells may carry KSHV in all stages of B cell maturation and events leading to the neoplastic transformation may occur independently of the stage of B cell development.

B cell tumors generally are thought to represent normal B cells that froze at a point in B cell differentiation.<sup>43</sup> Analysis of Ig genes expressed by B cell tumors shows that most of the non-Hodgkin's lymphoma types represent a certain stage of B cell development at which clonal expansion occurred. Our results demonstrate that tumor cells of PEL may express unmutated, highly mutated, and intraclonal divergent Ig V<sub>H</sub> gene sequences suggesting that development of PELs is not restricted to one stage of B cell maturation. These results also suggest that different stages of B cell differentiation can be the targets of malignant transformation associated with KSHV infection.

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