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Notch1 in primary effusion lymphoma: a clinicopathological study

Huan-You Wang, Franklin S Fuda, Weina Chen and Nitin J Karandikar

Department of Pathology, Dallas, TX, USA

Primary effusion lymphoma is a human herpes virus 8 (HHV-8)-associated large cell lymphoma of body cavities. Detailed large-scale clinicopathological studies are rarely reported, and the underlying mechanism of lymphomagenesis remains elusive. In the present report, we studied the clinicodemographic, immunophenotypic, and cytomorphological features on a cohort of 12 cases of primary effusion lymphoma. In contrast to HHV-8, which was positive in all nine cases tested (100%), HIV was found in 75% (9/12) of cases, whereas the three HIV-negative cases were either in elderly patients (one with hepatitis C virus infection and one with asbestoses exposure) or in a heart transplantation recipient. By flow cytometry, the antigens expressed in descending order were CD38, CD71, HLA-DR, CD30, and CD45RO. B-cell markers were largely negative. Cytomorphologically, all cases showed atypical to anaplastic morphology. Notch1, a member of transmembrane signal transduction family, was found in six of seven HHV-8-positive cases (86%). In agreement with *in vitro* studies using human primary effusion lymphoma cell lines, we have found that Notch1 was expressed in the majority of HHV-8-positive primary effusion lymphoma cases, corroborating the notion that Notch1 may have an important role in HHV-8-mediated lymphomagenesis of primary effusion lymphoma.
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Primary effusion lymphoma is a human herpes virus 8 (HHV-8) (also known as Kaposi's sarcoma-associated herpesvirus)-associated large B-cell neoplasm of the body cavity.[1] Primary effusion lymphoma usually develops in HIV-positive patients with HHV-8 and Epstein-Barr virus (EBV) as its etiological pathogens,[1,2] although not all primary effusion lymphoma cases are positive for both, indicating HHV-8 and EBV, either individually or in combination, may exert causative roles in the lymphomagenesis of primary effusion lymphoma.[1–3] However, the mechanism in which HHV-8 induces the development of primary effusion lymphoma is still elusive.

Human herpes virus is a member of gamma-2 herpes viruses, related genetically to simian herpesvirus saimiri, the prototype virus of this subgroup of the gammaherpesvirus subfamily.[4] HHV-8 has two replication phases in its lifecycle, namely latent and lytic, each of which expresses numerous proteins that promote cell proliferation, survival, and angiogenesis.[4] Although latency-associated nuclear antigen 1 is one of the most important proteins expressed by HHV-8 in its latency, viral interleukin-6 and viral G protein-coupled receptor are significant proteins during lytic infection.[4] Studies have shown that both viral interleukin-6 and viral G protein-coupled receptor contain intracellular Notch1-responsive promoters.[5] Using primary effusion lymphoma cell lines, intracellular Notch1 was shown to be capable of reactivating HHV-8 from its latency by means of activating the replication and transcription activator of HHV-8 in a dose-dependent manner.[6] Furthermore, it has been shown that intracellular Notch1 was aberrantly accumulated in HHV-8 latently infected primary effusion lymphoma cell lines, and this accumulation of intracellular Notch1 resulted in increased proliferation.[7]

All reports thus far have used *in vitro* studies on primary effusion lymphoma cell lines, which may or may not faithfully represent the actual primary effusion lymphoma patients. To this end, we studied Notch1 expression in HHV-8-positive primary effusion lymphoma specimens and found that the vast majority of these cases strongly express activated

Correspondence: Dr H-Y Wang, MD, PhD, Department of Pathology, UCSD School of Medicine, 3855 Health Sciences Drive, La Jolla, CA 92093-0987, USA.
E-mail: hywang2@ucsd.edu
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Notch1, indicating that Notch 1 has an important role in the HHV-8-mediated pathogenesis of primary effusion lymphoma. In addition, we present the detailed clinical, laboratory, and immunophenotypic features of these 12 primary effusion lymphoma cases, representing the second largest series in the studies of primary effusion lymphoma.

Materials and methods

Case Selection

A total of 12 cases of primary effusion lymphoma from 1996 to 2008 were retrieved from the Flow Cytometry database of the University of Texas (UT) Southwestern Medical Center at Dallas. In total, 11 cases were patients from UT Southwestern Medical Center at Dallas and 1 case was received as a consultation. Among 11 cases from UT Southwestern Medical Center, 2 cases with emphasis on cytogenetics were previously reported.[8] The diagnoses of primary effusion lymphoma for all cases were confirmed by clinical, laboratory, and morphological criteria, as outlined by the current WHO.[9]

Clinical, Laboratory, and Demographic Data

Detailed clinical, laboratory, and demographic data were obtained by thorough review of patient's medical record. Unpaired student's *t*-test was employed and *P*-values of less than or equal to 0.05 and 0.01 was considered significant and very significant, respectively.

Multiparameter Flow Cytometry

Flow cytometric analysis was performed on body fluid specimens using four-color FACSCalibur flow cytometer (Beckman Dickson, San Jose, CA, USA) as previously described.[10] The events were acquired using CELLQUEST software and analyzed by cluster analysis using Paint-A-Gate software (Beckman Dickinson). The following surface and cytoplasmic antigens were examined: CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD22, CD23, CD30, CD38, CD45, CD45RO, CD71, FMC-7, HLA-DR, surface kappa, surface lambda, cytoplasmic kappa, and cytoplasmic lambda.

Immunohistochemistry

The expression of HHV-8 and Notch1 was examined by immunohistochemistry using a BioTek Solutions Techmate 1000 automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA) using the Dako Envision + horseradish peroxidase/diaminobenzifine monoclonal detection system (Dako, Carpinteria, CA, USA). Both HHV-8 and Notch1 staining was carried out on seven cases of primary effusion lymphoma with available cellblocks. The presence of HHV-8 was assessed using latency-associated nuclear antigen-1 protein, a mouse monoclonal antibody to HHV-8 latent nuclear antigen-1 (NCL-HHV-8-LNA, clone name 13B10) purchased from Novocastra Laboratories (Newcastle upon Tyne, UK) with a dilution of 1:80. For Notch1, a mouse Notch1 monoclonal antibody (clone name mN1A) was purchased from Chemicon International (Temecula, CA, USA) with a dilution of 1:50. This Notch1 antibody has a high affinity for activated intracellular Notch1 molecules with no cross-reactivity with other members of Notch family (Notch2, Notch3, and Notch4).

In Situ Hybridization for EBV

Epstein-Barr virus status was assessed by detection of the EBV-encoded RNA (EBER) when cellblocks are available using EBER *in situ* hybridization kit purchased from Dako.

PCR for EBV, HHV-8, T-Cell Receptor, and Immunoglobulin Heavy Chain (IgH)

PCR detection of EBV, HHV-8, T-cell receptor (TCR), and IgH was performed on selected cases on the basis of the clinical circumstances and whether adequate materials were available at the time of diagnosis. PCR for EBV and HHV-8 was performed on periphery blood, and PCR for TCR and IgH was performed on body fluid samples. Briefly stated, DNA for EBV and HHV-8 was extracted from the peripheral blood using the QIAamp blood kit (Qiagen, Valencia, CA, USA). PCR for EBV and HHV-8 was performed using primer sets for EBV and HHV-8, respectively. The PCR products for EBV and HHV-8 were visualized by using agarose gel stained with ethidium bromide.

DNA from body fluid samples was extracted according to manufacturer's instruction (Qiagen, Germantown, MD). PCR for TCR and IgH was performed using the Biomed Primer sets for TCR and IgH (Invivoscribe, LLC, San Diego, CA, USA). The PCR products were fractioned using the pop 6 polymer in a 50-cm capillary on the ABI 3100 (Applied Biosystems, Foster City, CA, USA). A 'positive' IgH and TCR is defined as a monoclonal peak with an amplitude equal to or greater than three times the background bands; a 'weak positive' is defined as a monoclonal band with an amplitude two to three times the background bands.

Results

Clinical, Laboratory, and Demographic Features of PEL

The clinical, laboratory, and demographic features of all 12 primary effusion lymphoma cases were

Table 1 Clinical, demographic, and relevant laboratory features of patients with primary effusion lymphoma

Pa	*A/S*	*HIV*	*HHV-8*	*EBV*	*Hepatitis*	*Site of effusion*	*Symptoms of AIDS at the time of Dx*	*Time between Dx of HIV and PEL*	*WBC/Abs CD4*	*Follow-up*	*Others*
1	38/M	P	P	P	HBV and HCV	Pl	Present	Not known	NA	NA	
2	32/M	P	ND	ND	HCV	Pl	Present	Not known	8.7/ND	Expired, 2D	
3	66/M	N	P	P	HCV	A	N/A	N/A	3.9/252	Lost to follow-up after 6 months	
4	33/M	P	P	P	N	Peri-C	Present	17 years	6.8/63	Lost to follow-up after 113 months	
5	39/M	P	P	P	HBV	Pl	Present	3 months	3.7/55	Lost to follow-up after 48 months	
6	51/M	P	ND	ND	N	Pl	Present	11 months	4.7/7	Expired, 10D	Syphilis
7	48/M	P	P	ND	HBV	A	Present	17 years	6.9/ND	Lost to Follow-up	Syphilis, NSLC
8	52/M	N	P	P	N	Pl	N/A	N/A–3 years between heart Txp and PEL diagnosis	6.4/ND	Alive, 3 months	Heart Txp
9	39/M	P	P	N	N	Pl	Present	3 weeks	11.6/82	Expired, 1 month	KS and TB
10	40/M	P	P	N	HBV	Peri-C	Present	10 years	6.5/493	Alive, 10 months	KS
11	86/M	N	P	N	N	A and Pl	N/A	N/A	6.5/200	Expired, 1 month	Asbestos Exposure
12	51/M	P	P	N	N	A	Present	19 years	5.13/ND	Alive, 0.5 month	KS

A, ascites; Abs, absolute; AIDS, acquired immunodeficiency syndrome; A/S, age/sex; D, day; Dx, diagnosis; HBV, hepatitis B virus; HCV, hepatitis C virus; HgB, hemoglobin; HIV, human immunodeficiency virus; KS, Kaposi sarcoma; M, male; N, negative; N/A, not applicable; NA, not available; ND, not done; NSLC, non, small cell lung cancer; P, positive; Pa, patient; Peri-C, pericardia; Pl, pleural; Plt, platelet; TB, tuberculosis; Txp, transplant.

summarized in Table 1. All patients were male. Among the 12 primary effusion lymphoma patients, 75% (9/12) were positive for HIV, the remainder 25% (3 out of 12) were negative. The three HIV-negative patients included one elderly with hepatitis C virus (HCV) (case no. 3), one heart transplantation recipient (case no. 8), and one elderly with asbestos exposure (case no. 11). The median age at diagnosis of primary effusion lymphoma in HIV-positive patients was very significantly younger (41 years) than that of HIV-negative patients (68 years) ($P=0.002$). Four HIV-positive primary effusion lymphoma patients had concurrent hepatitis B virus (HBV) infection, two had concurrent HCV infection, and one (case no. 1) had concurrent HCV and HBV infections. Pleural effusion was the initial presentation in half of the patients (6/12), followed by ascites, pericardial effusion, both pleural and ascitic effusion in 3, 2, and 1 patient, respectively.

Among HIV-positive primary effusion lymphoma patients, full-blown symptoms of AIDS were present in all nine HIV-positive cases at the time when primary effusion lymphoma was diagnosed. The time lag between detection of HIV and diagnosis of primary effusion lymphoma in seven HIV-positive cases in which medical history was available for review was as short as 3 weeks (case no. 9) to as long as 19 years (case no. 12). In a heart transplant recipient (case no. 8), primary effusion lymphoma developed 3 years after heart transplantation.

There was no statistical significance with regard to hemoglobin level and platelet count (data not shown) between HIV-positive and HIV-negative primary effusion lymphoma patients. Unpaired student's *t*-test was not carried out because of small number of HIV-negative primary effusion lymphoma cases in which white blood cell count/absolute CD4 count were available.

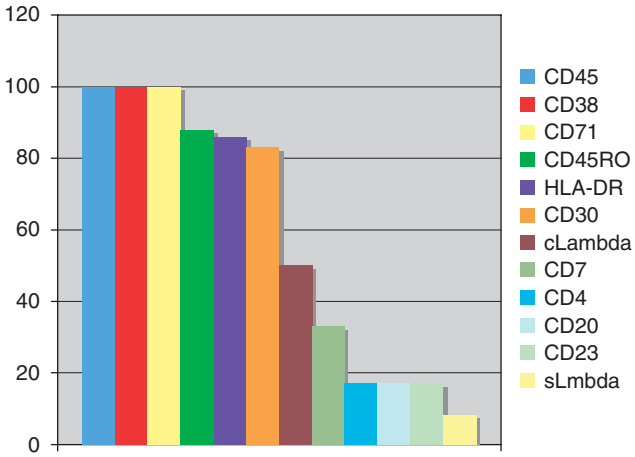


Figure 1 Frequency of surface and cytoplasmic antigen expression from primary effusion lymphoma patients in descending order.

Among 10 patients with complete follow-up, 4 died because of AIDS-related complications. The longest follow-up is 9 years and 4 months (case no. 4) before he was lost to follow-up.

Immunophenotypic Profiles of Primary Effusion Lymphoma

The summary of the frequency of common antigens is shown in Figure 1. All cases (100%, 12/12) were

positive for CD45, a leukocyte common antigen (Figure 2b). The next group of antigens with highest expression frequency was cell surface activation markers, including CD38 (bright, Figure 2b), CD71 (Figure 2c), HLA-DR (Figure 2d), and CD30 (Figure 2e) with 100% (12/12), 100% (12/12), 86%(6/8), and 83%(10/12) frequency, respectively. CD45RO (Figure 2h), a CD45 isoform expressed on memory T-cells, was positive in 88% of the cases (7/8). In contrast to rare expression of surface light chains (1/12, 8%), 50% of the cases (3/6) expressed cytoplasmic lambda immunoglobulin light chain

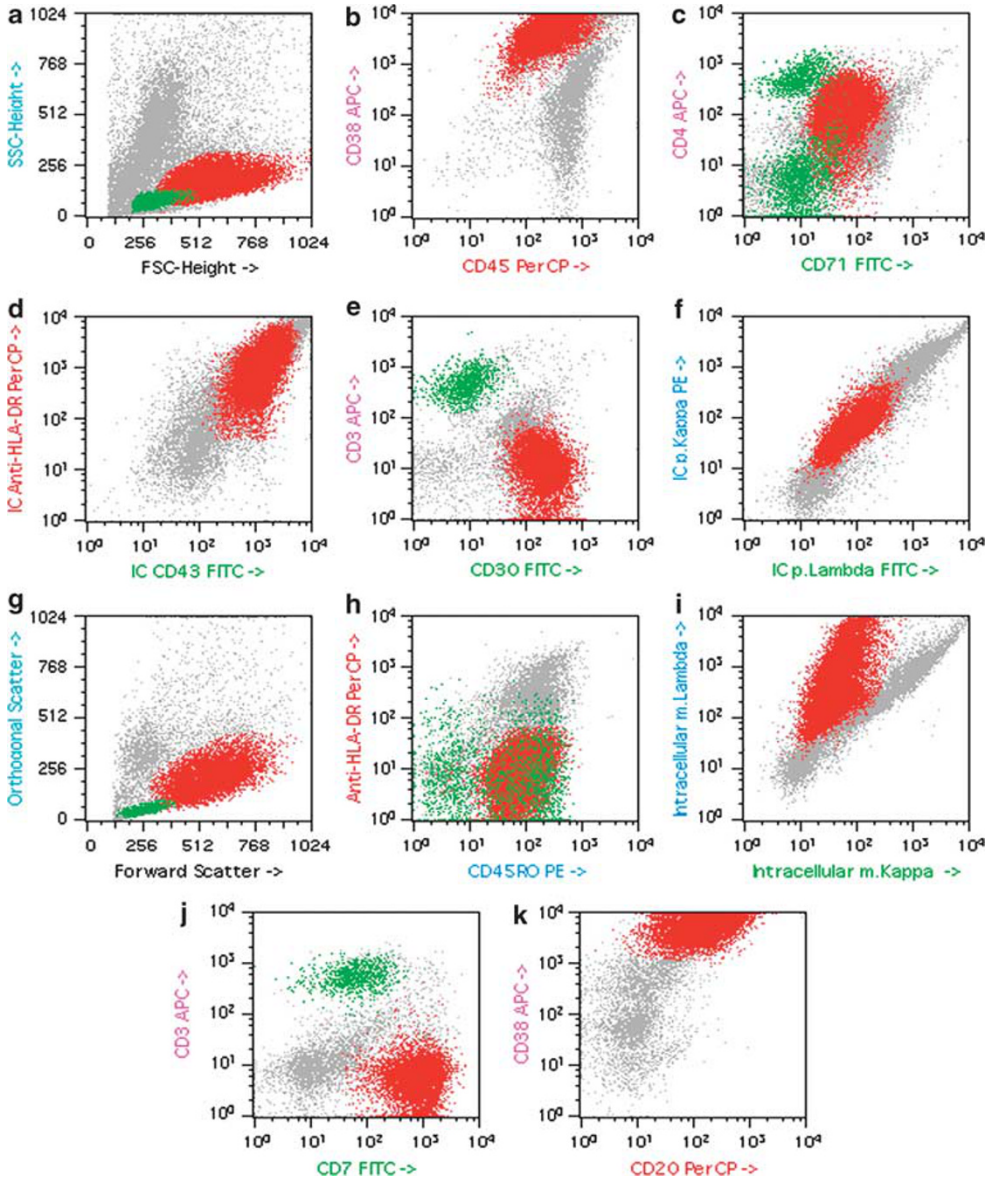
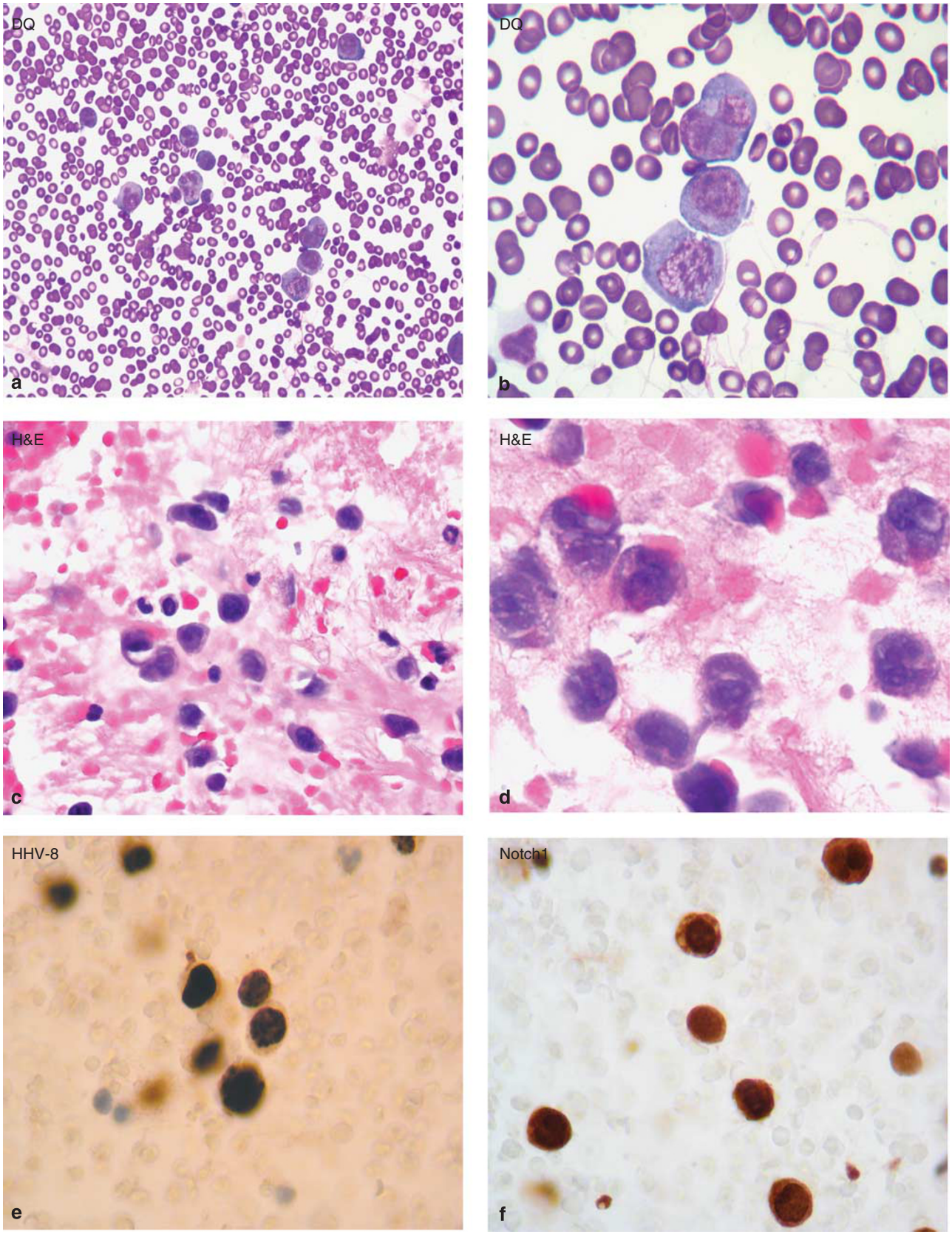


Figure 2 Immunophenotypic profiles of two representative cases of primary effusion lymphoma by four-color flow cytometry. Dot plots (**a–f**) represent case no. 3 and (**g–k**) represent case no. 8. PEL cells are painted in red and small mature normal T-lymphocytes are in green. **a** and **g** show forward and side light scatter properties. PEL cells are positive for CD45 (**b**), CD38 (bright) (**b**), CD4 (dim) (**c**), CD71 (**c**), CD43 (**d**), HLA-DR (**d**), CD30 (**e**), CD45RO (**h**), intracellular lambda immunoglobulin light chain (**i**) (case no. 8), CD7 (**j**) and CD20 (**k**), but negative for surface CD3 (**e**, **j**) and intracellular immunoglobulin light chains (**f**) (case no. 3).

Figure 3 Cytomorphology and representative expression of HHV-8 and Notch1. (**a** and **b**) A Diff-Quick stain shows that the primary effusion lymphoma cells are medium-sized to large cells with oval to irregular nuclear contours, slightly open chromatin, prominent nucleoli, and relative abundant pale basophilic cytoplasm (original magnification, **a**, ×200, **b**, ×1000). (**c** and **d**) A cellblock shows similar morphology (H&E, original magnification, **c**, ×400, **d**, ×1000). (**e**) The primary effusion lymphoma cells from a cellblock show strong nuclear immunoreactivity for HHV-8 (original magnification, ×1000). (**f**) Primary effusion lymphoma cells from a cellblock are strongly and diffusely positive for both nuclear and cytoplasmic staining for Notch1. The nuclear stain appears stronger than cytoplasm (original magnification, ×1000).


DQ
a
DQ
b
H&E
c
H&E
d
HHV-8
e
Notch1
f

(Figure 2i), whereas the remaining three cases had no detectable intracellular kappa or lambda, although one of them (case no. 12) had negative to dim-positive surface lambda light chain expression. CD7, a pan-T-cell-associated antigen, was seen in one-third of the cases (33%, 4/12) (Figure 2j). CD4 (Figure 2c), CD20 (Figure 2k), and CD23 (data not shown) had similar expression frequency (17%, 2/12). It is noteworthy that none of the 12 cases expressed surface CD3, a T-cell-specific antigen, or CD19, a pan B-cell marker.

Cytomorphology

The cytomorphological features were similar in all 12 primary effusion lymphoma cases. The neoplastic lymphoid cells were medium-sized to large cells with atypical to anaplastic morphology, including irregular to indented nuclear contours, slightly condensed chromatin, multiple prominent nucleoli, and relatively abundant basophilic cytoplasm (Figure 3a through d).

Expression of EBV and HHV-8 in Primary Effusion Lymphoma Patients

As summarized in Table 1 and explained in detail in Table 2, the expression of EBV and HHV-8 in this cohort of primary effusion lymphoma cases was analyzed by multiple methods dictated by the clinical circumstances at the time of morphological diagnosis and during the course of this study. The PCR for EBV was positive in all three cases tested (100%, 3/3) (Table 2). *In situ* hybridization for EBV (EBER) was positive in one out of five cases tested (20%, 1/5) (Table 2), in which PCR was not performed. *In situ* hybridization was not performed for one case (no. 7) owing to exhaustion of cellblock. The PCR for HHV-8 was positive in all five cases tested (100%, 5/5) (Table 2).

To confirm the expression of HHV-8 in lymphoma cells, immunohistochemistry of HHV-8 using latency-associated nuclear antigen-1 antibody was performed on six primary effusion lymphoma cases, and results showed HHV-8 positivity in all the six cases (100%, 6/6) (Table 2 and Figure 3e).

Notch1 Immunoreactivity

Notch1 was positive in six out of seven primary effusion lymphoma cases (86%), from which cellblocks were available. The staining pattern was both nuclear and cytoplasmic, with the former stronger than the latter (Figure 3f).

IgH and TCR Gene Rearrangements in Primary Effusion Lymphoma

Among the five patients (patient nos. 3, 8, 10, 11, and 12) from whom samples were obtained for PCR detection of IgH and TCR gene rearrangements, two patients each were positive for IgH (nos. 11 and 12) (40%, 2/5) and TCR (nos. 10 and 12) (40%, 2/5) gene rearrangement, respectively (Table 2). The sample obtained from patient no. 12 was particularly interesting in that it was positive for IgH rearrangement, and also weakly positive for TCR rearrangement.

Discussion

For the first time, we have shown the protein expression of Notch1 in the majority of primary effusion lymphoma cases (86%, 6 out of 7), which not only confirms the *in vitro* studies using primary effusion lymphoma cell lines[6,7] but also strongly supports the notion that Notch1 may have a significant role in the majority of HHV-8-mediated lymphomagenesis of primary effusion lymphoma. The interactions between Notch1 and HHV-8 occur in both the lytic and latent phases of HHV-8 lifecycle. For example, intracellular Notch1 can activate the replication and transcription activator

Table 2 Summary of EBV, HHV8, Notch1, TCR, and IgH gene rearrangement of PEL

Patient	*EBV by PCR*	*EBV by ISH*	*HHV8 by PCR*	*HHV8 by IHC*	*Notch1*	*TCR*	*IgH*
1	ND	P	ND	P	N	ND	ND
2	ND	ND	ND	ND	No cellblock	ND	ND
3	P	ND	P	ND	No cellblock	N	N
4	ND	ND	P	ND	No cellblock	ND	ND
5	P	ND	P	ND	No cellblock	ND	ND
6	ND	ND	ND	ND	No cellblock	ND	ND
7	ND	ND	ND	P	Strong P	ND	ND
8	P	ND	P	ND	Strong P	N	N
9	ND	N	ND	P	Strong P	ND	ND
10	ND	N	ND	P	Strong P	P	N
11	ND	N	P	P	Moderate P	N	P
12	ND	N	ND	P	Strong P	WP	P

IgH, immunoglobulin heavy chain; IHC, immunohistochemistry; N, negative; ND, not done; P, positive; PCR, polymerase chain reaction; TCR, T, cell receptors; WP, weak positive.

promoter, leading to expression of replication and transcription activator,[6] which is a switch molecule from latency to lytic replication cycle.[11] In addition, intracellular Notch1 is elevated in latent phase of HHV-8 in primary effusion lymphoma cell lines,[5] and the increased intracellular Notch1 is potentially able to reactivate HHV-8 from its latency by binding to DNA-binding protein recombination signal sequence-binding protein-J kappa to convert this complex to a positive transcriptional complex from a corepressor complex.[12] In fact, the crystal structure of C promoter binding factor clearly shows that intracellular Notch1 binds the hydrophobic pocket on C promoter binding factor.[13]

The expression of Notch1 in primary effusion lymphoma patients may also shed light on the potential therapeutic use of Notch1 inhibitors on primary effusion lymphoma patients. It has been recently shown that γ-secretase inhibitor, which blocks the activation of Notch1, engages the retinoblastoma pathways and elicits cell cycle exit in T-cell acute lymphoblastic leukemia cell lines,[14] and inclusion of γ-secretase inhibitor in the therapeutic regimen in T-cell acute lymphoblastic leukemia provides a better treatment outcome.[15]

It is of equal interest to point out that one HHV-8-positive primary effusion lymphoma case (patient no. 1) was negative for Notch1 expression, which implies HHV-8-mediated mechanisms/pathways other than Nocth1 may also have a role in the lymphomagenesis of primary effusion lymphoma. For example, among many molecules and pathways, the transcriptional activities of *MYC* are increased[16] and the degradation of MYC protein is prolonged[17] by HHV-8 in primary effusion lymphoma cell lines; HHV-8 also regulates the cellular transcription factor nuclear factor-κ B activation pathway, which in turn protects HHV-8-infected cells against spontaneous apoptosis,[18] and maintains the latent viral life cycle.[19,20]

Epstein-Barr virus, assessed by *in situ* hybridization for the presence of EBER, is usually present in primary effusion lymphoma according to the current WHO definition.[9] However, among the 12 primary effusion lymphoma cases reported here, four patients (Case nos. 9–12) were negative for EBV. Literature review has clearly shown that primary effusion lymphomas without EBV coinfection have been well documented. For instance, EBV was not detected in 1 out of 12 and 3 out of 12 HHV-8-associated primary effusion lymphoma in HIV-positive patients.[21,22] Furthermore, absence of EBV coinfection tends to be more prevalent in primary effusion lymphoma in HIV-negative patients. For example, at least 10 cases of HIV-negative patients with primary effusion lymphoma were found to be without EBV coinfection.[3,21–24] Different from the reported CD20 expression among all seven EBV-negative primary effusion lymphoma cases from Japan,[24] CD20 was not expressed in any of our four EBV-negative primary effusion lymphoma cases.

Although HHV-8 is usually associated with primary effusion lymphoma,[25] however, HHV-8-negative primary effusion lymphoma is infrequently reported in the literature.[26–28] Two cases (Case nos. 2 and 6), in whom HHV-8 was not tested owing to the fact that either it was not tested at the time of initial diagnosis or there was no cellblock available at the time of this study, were included in this study, because these two cases had all the characteristic morphological and immunophenotypic features of HHV-8-positive primary effusion lymphoma.

In summary, we have shown expression of Notch1 in majority of the primary effusion lymphoma cases. This finding, together with the studies using primary effusion lymphoma cell lines, supports the notion that Notch1 is the downstream effector of HHV-8-mediated lymphomagenesis in primary effusion lymphoma. In addition, we also provide comprehensive studies with regard to clinical and demographic features of primary effusion lymphoma and detailed immunophenotypic analysis.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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