Human Cancer Biology

Clinical Cancer Research

Expression of Epstein-Barr Virus–Encoded Proteins in Extranodal NK/T-cell Lymphoma, Nasal Type (ENKL): Differences in Biologic and Clinical Behaviors of LMP1-Positive and -Negative ENKL

Naoko Kanemitsu[1], Yasushi Isobe[1], Azuchi Masuda[1], Shuji Momose[2], Morihiro Higashi[3], Jun-ichi Tamaru[3], Koichi Sugimoto[1], and Norio Komatsu[1]

Abstract

Purpose: Extranodal NK/T-cell lymphoma, nasal type (ENKL) is closely associated with Epstein-Barr virus (EBV). To elucidate its pathogenetic role, we examined the expression profiles of EBV-encoded proteins, especially focusing on latent membrane protein 1 (LMP1).

Experimental Design: Immunohistochemistry was carried out using clinical samples from ENKL cases, which were diagnosed between 1996 and 2010 at our institution. We statistically assessed the correlation between LMP1 positivity and the clinicopathologic data and further examined phosphorylation status of NF-κB RelA and Akt in ENKL cell lines.

Results: Most of the 30 examined cases showed pleomorphic morphology, natural killer cell immunophenotype, and a localized disease. Immunohistochemistry detected EBERs, but not EBNA2, in all cases. LMP1 and LMP2A were positive in 22 (73.3%) and 12 cases (40.0%), respectively. LMP1-positive cases tended to show a localized disease ($P = 0.060$, the Fisher exact test). Nuclear localization of phosphorylated RelA and detection of phosphorylated Akt were predominantly observed in LMP1-positive cases ($P = 0.002$ and $P < 0.001$, respectively, the Fisher exact test). RNA silencing experiments of LMP1 in Hank1 cells suggested a positive correlation between LMP1 expression and phosphorylation of RelA and Akt. With a median follow-up period of 26.7 months (range, 0.2–142.3 months), the 2.5-year overall survival rates for LMP1-positive and -negative cases were estimated at 78.3% and 12.5%, respectively ($P = 0.001$, log-rank test).

Conclusions: LMP1 expression shows correlations with phosphorylation of RelA and Akt and possibly has a favorable impact on clinical outcome in ENKL. *Clin Cancer Res; 18(8); 2164–72. ©2012 AACR.*

Introduction

Extranodal NK/T-cell lymphoma, nasal type (ENKL) is a rare disease that more frequently develops in East Asia and Central America than in the West (1, 2). More than 80% of the cases involve the nasal cavity and nasopharynx as initial sites, and approximately 70% of the cases show a localized disease (stage IE and IIE). These cases have recently been controlled by concurrent chemoradiotherapy (3–6). Nevertheless, the remaining advanced cases usually present with systemic coagulopathy and multiorgan failure and have poor prognosis (2–4, 7, 8). In more than 95% of the cases, neoplastic cells possess the Epstein-Barr virus (EBV) genome (1, 4, 8). In an immunocompetent setting, ENKL is most closely associated with EBV infection among EBV-related lymphomas, including Hodgkin lymphoma and Burkitt lymphoma (1, 9).

EBV is a lymphotropic herpes virus, which is carried in more than 90% of worldwide adult populations (9). EBV entry into the host cell results in lytic or latent infection. In lytic infection, EBV initially expresses the transactivator protein ZEBRA, which induces the lytic gene expression and leads to virion production and host cell lysis (10). The latent infection is characterized by expression of the latent gene products including EBV nuclear antigen (EBNA) 1, 2, and 3, latent membrane protein (LMP) 1 and 2, EBV-encoded small RNA (EBER) 1 and 2, and *Bam*H1 rightward transcripts (10, 11). After establishment of *in vitro* infection of B cells, EBV fully expresses these products, and their latency program is referred to as "type III latency" (10). These latent gene products can alter the infected cell

Authors' Affiliations: Departments of [1]Hematology and [2]Pathology and Oncology, Juntendo University School of Medicine, Tokyo; and [3]Department of Pathology, Saitama Medical Center, Saitama Medical University, Kawagoe, Saitama Prefecture, Japan

Note: N. Kanemitsu and Y. Isobe contributed equally to this work.

Corresponding Author: Yasushi Isobe, Department of Hematology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Phone: 81-3-3813-3111, ext. 3386; Fax: 81-3-3813-0841; E-mail: yisobe@juntendo.ac.jp

doi: 10.1158/1078-0432.CCR-11-2395

Translational Relevance

Latent membrane protein (LMP1) is a major oncoprotein of Epstein-Barr virus (EBV). We showed a correlation of LMP1 expression with biologic and clinical characteristics in extranodal NK/T-cell lymphoma, nasal type (ENKL), which is closely associated with EBV in immunocompetent patients. Although the expression showed a nonuniform distribution, immunohistochemistry detected LMP1 in more than 70% of the cases. LMP1 positivity had a strong correlation with activation of NF-κB RelA and Akt in ENKL. These events may lead to a survival advantage in the neoplastic cells. Nevertheless, LMP1-positive ENKL cases tended to present with a localized disease and clearly showed a favorable outcome. Our observations suggest that LMP1 expression affects the nature of ENKL. EBV may biologically and clinically continue to function in ENKL.

phenotype, resulting in oncogenic transformation of EBV-carrying cells (10, 11). Of these products, LMP1 is recognized as an oncoprotein, and it elicits the TNF receptor signaling pathway by recruiting TNF receptor–associated factors and other adaptor proteins in the infected cells (10, 11). LMP1 activates the NF-κB and phosphoinositide 3-kinase/Akt signaling through this pathway (10, 11). These events play important roles in B-cell immortalization and the transformation of rodent fibroblasts (10, 11).

Expression of the latent genes in EBV-infected T and natural killer (NK) cells is restricted in EBERs, EBNA1, LMP1, and LMP2 (12, 13). This program is called "type II latency" and helps EBV-infected T and NK cells to escape from host immune surveillance because EBV-encoded proteins except for LMPs show relatively low immunogenicity (14). Indeed, representative EBV-associated malignancies such as Hodgkin lymphoma and nasopharyngeal carcinoma (NPC) show "type II latency" (10). Although ENKL is considered to show type II latency, little is known about its detailed profile and contribution to the clinical and biologic aspects of ENKL (15, 16).

In this study, we examined the expression profiles of EBV-encoded gene products and evaluated its clinicopathologic features in ENKL. EBV seems to be associated with not only tumorigenesis but also the nature of ENKL.

Patients and Methods

Patients and diagnosis

We retrospectively analyzed data from patients with ENKL, who were diagnosed at Juntendo University Hospital (Tokyo, Japan) between August 1996 and June 2010. Clinical presentations of 2 cases were previously reported because of their distinctive clinical and immunophenotypic features (17, 18). A biopsy and further evaluation were conducted after obtaining written informed consent from each patient. All cases were diagnosed according to the current World Health Organization classification (1). Although T-cell receptor (TCR) gene rearrangement was not necessarily analyzed in all cases, flow cytometry defined the cases showing surface $CD3^+/CD5^+$ and surface $CD3^-CD5^-CD56^+$ immunophenotypes as T-cell and NK-cell types, respectively. In addition, immunohistochemistry for TCRβ (anti-TCRβ, Santa Cruz Biotechnology) was also used to confirm T-cell type ENKL. This study was conducted with the approval of the Institutional Review Board at Juntendo University.

In situ hybridization and immunohistochemistry

Latent EBV infection was determined by EBER *in situ* hybridization (ISH) using EBER PNA probes (Dako) according to the manufacturer's protocol. To detect EBV-encoded proteins, paraffin-embedded sections were treated with Tris-EDTA buffer (pH, 9.0) for 40 minutes at 98°C and incubated with primary mouse monoclonal antibodies as follows: CS1-4 (anti-LMP1; Dako), PE-2 (anti-EBNA2; Dako), and BZ-1 (anti-ZEBRA; Dako). As for LMP2A detection, we used 2 rat monoclonal antibodies, 15F9 and 4E11 (Santa Cruz Biotechnology) after treatment with 0.1 mol/L citrate buffer (pH, 6.0) for 7 minutes at 120°C. In addition, immunostaining for phosphorylated forms of RelA and Akt was carried out using rabbit monoclonal antibodies anti-phosphorylated RelA (Ser 536; Cell Signaling Technology) and anti-phosphorylated Akt (Thr 308; Santa Cruz Biotechnology), respectively. The antibody signals were enhanced by Envision (Dako) or a Catalyzed Signal Amplification System II (Dako).

Cell lines

To evaluate the association between LMP1 expression and phosphorylation of NF-κB RelA and Akt, 2 ENKL-derived cell lines, Hank1 and NK-YS [kindly provided by Dr. Masao Seto, Aichi Cancer Center (Nagoya, Japan) and Dr. Junjiro Tsuchiyama, Kawasaki Medical School (Kurashiki, Japan), respectively] were used (19, 20). Although Hank1 shows abundant expression of LMP1, the level in NK-YS is low. Another NK cell line, NKL [kindly provided by Dr Michael J Robertson, Indiana University (Bloomington, IN)] was used as an EBV-negative control (21). These cell lines were maintained in Iscove's Modified Dulbecco's medium (Invitrogen) containing 10% FBS (Invitrogen) and 100 U/mL human interleukin (IL)-2 (a gift from Shionogi, Osaka, Japan). In detection of phosphorylated Akt by Western blot analysis, these cell lines were starved of IL-2 for 48 hours and then treated with or without 500 U/mL IL-2 for 3 hours.

Western blot analysis

Cellular protein was electrophoresed on 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Primary antibodies were as follows: anti-LMP1 (3H2104, a, b, and c; Santa Cruz Biotechnology), anti-phosphorylated RelA (Ser 536; Cell Signaling Technology), anti-RelA (Cell Signaling Technology), anti-phosphorylated Akt (Thr 308; Santa Cruz Biotechnology), anti-Akt (Cell Signaling Technology), and anti-β-actin (Sigma-Aldrich). Antibody signals

were enhanced using ECL plus (GE Healthcare) according to the manufacturer's protocol. We quantified the expression levels of the 2 phosphorylated forms by comparison with their total protein levels with ImageJ version 1.44 (http://rsweb.nih.gov/ij/).

RNA interference for LMP1

siRNA duplexes against LMP1 were synthesized by Applied Biosystems (*Silencer* Select siRNA). The siRNA target sequence to LMP1 mRNA is aacugguggacucuauugguu. A control scrambled siRNA duplex was also purchased from Applied Biosystems. Transfection of siRNA was carried out using Nucleofector I (Lonza) and assayed for silencing 24 and 48 hours after transfection, respectively. Hank1 cells (1×10^6) were resuspended in 100 μL of Solution R with 200 nmol/L of each siRNA. Total RNA and whole-cell lysate were extracted from each 2×10^6 cells, respectively. Quantitative Real-time reverse transcriptase PCR analysis was conducted on a CFX96 real-time PCR detection system with SsoFast EvaGreen supermix (Bio-Rad Japan). The primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and LMP1 were designed as previously described (22, 23) and synthesized by Invitrogen. Relative mRNA levels were assessed by normalizing the obtained fluorescence data of LMP1 by those of GAPDH using the $\Delta\Delta C_t$ method.

Statistical analysis

The influence of LMP1 expression on clinicopathologic features of ENKL was evaluated by the Fisher exact test. Overall survival (OS) and progression-free survival (PFS) were measured and calculated by the Kaplan–Meier method, and statistical significance was assessed by the log-rank test. All *P* values were 2-sided, and values less than 0.05 were considered statistically significant. These analyses were conducted with PASW Statistics software version 18 (SPSS Japan).

Results

Morphologic and immunophenotypic features of ENKL

A total of 30 cases were diagnosed as ENKL. Tumor cells frequently showed an angiodestructive growth pattern, and this resulted in infiltrated tissue necrosis (Fig. 1A). Immunohistochemistry showed that all cases were positive for EBERs, TIA-1, and granzyme B (Table 1, Fig. 1B–D). The neoplastic cells consisted of a mixture of small- to medium-sized cells with a round-to-convoluted nuclear shape. This morphologic feature corresponds to the typical pleomorphic type (Fig. 1E and F). In contrast, 5 cases had a larger cell size and were described as "large cell" variant (Fig. 1G and H). Immunophenotypic features are listed in Table 1. Among the 30 cases, 27 (90.0%) were classified as NK-cell type ENKL, and 3 (10.0%) were T-cell type ENKL. All NK-cell type ENKL cases were positive for CD56. In contrast, one of 3 T-cell type ENKL cases was negative for this molecule. As shown previously (18), one NK-cell type ENKL aberrantly expressed CD20, which is frequently detected in B-cell lymphomas.

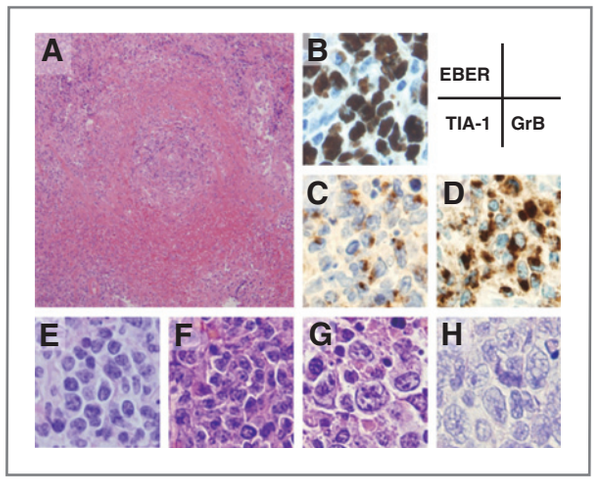

Figure 1. Morphologic features of ENKL. A, infiltrating neoplastic cells presented with an angiodestructive growth pattern and caused tissue necrosis. B–D, they were positive for EBERs, TIA-1, and granzyme B (GrB). E and F, most cases (25 of 30, 83.3%) showed typical pleomorphic cell morphology. G and H, a large cell variant was observed in 5 cases (16.7%).

Clinical characteristics of 30 cases with ENKL

Patient characteristics are summarized in Table 1. Among the 30 patients, no one had a past history suggestive of chronic active EBV-associated disease. They consisted of 19 males and 11 females with a median age of 62 years (range, 27–85 years). Reflecting initial manifestations such as nasal obstruction and bleeding, tumors developed from the nasal cavity or nasopharynx in most cases (27 cases, 90.0%). Four cases (13.3%) were initially involved the skin. According to the American Joint Committee on Cancer (AJCC) staging system, 23 cases (76.7%) presented with a localized disease (stages IE and IIE) and 7 (23.3%) had an advanced disease (stage IV). Among the 7 stage IV cases, 3 were accompanied by hemophagocytosis at diagnosis. The clinical characteristics of the present cases were concordant with those from recent reports (6).

Expression of EBV-associated gene products in ENKL

ISH clearly showed abundant expression of EBERs, indicating that most neoplastic cells are latently infected with EBV. In contrast, immunohistochemistry failed to detect EBNA2 in any cases (Table 1, Fig. 2A). Although ENKL is considered to show type II latency, LMP1 was detected only in 22 cases (73.3%). Another LMP, LMP2A, was detected in 12 cases (40.0%). This study therefore first confirmed the expression of LMP2A in a relatively large series of cases. Coexpression of them was observed only in 10 cases (33.3%). Although Hodgkin-Reed-Sternberg cells used as a positive control clearly expressed both proteins, the expression in ENKL showed a nonuniform distribution in the neoplastic cells (Fig. 2A). In addition, LMP1-positive cases not necessarily expressed LMP2A (Table 1). Morphologic variance was not correlated with expressions of either protein. These heterogeneous expression patterns were

Table 1. Patient characteristics and immunophenotypic findings of 30 cases with ENKL

				EBV-associated gene products					Immunophenotype							
Case	Age/sex	Initial involved sites	Cell type	EBER	EBNA2	LMP1	LMP2 (15F9/4E11)	ZEBRA	sCD3/ cyCD3	sCD5/ cyCD5	CD20	CD56	TIA-1/ GrB	TCR GR or TCRβ	Cell type	Treatment and outcome
1	27/M	Nasal cavity, testis, bone marrow	Typ	+	−	−	+/+	−	−/+	−/NA	−	+	+/+	−	NK	RT, CT, *DOD*
2	59/F	Nasal cavity	Typ	+	−	+	−/−	−	−/+	−/NA	−	+	+/+	−	NK	NA, *alive*
3	39/M	Nasal cavity	Typ	+	−	+	−/−	−	−/+	−/NA	−	+	+/+	−	NK	CT, Auto, *DOD*
4	59/F	Nasal cavity	Typ	+	−	+	−/−	−	−/+	−/NA	−	+	+/+	−	NK	RT, CT, Auto, *DWOD*
5	65/F	Skin	Typ	+	−	+	−/−	−	−/−	−/NA	−	+	+/+	−	NK	CT, RT, *AWOD*
6	62/M	Nasopharynx	Typ	+	−	+	−/−	+	−/−	−/−	−	+	+/+	−	NK	CT, *DOD*
7	60/M	Nasopharynx, cervical nodes	L	+	−	−	−/−	−	−/−	−/−	−	+	+/+	−	NK	CT, *DOD*
8	71/M	Skin, pleural effusion	Typ	+	−	−	−/−	−	−/−	−/−	+	+	+/+	−	NK	CT, *DOD*
9	62/F	Nasopharynx	L	+	−	−	−/−	−	−/−	−/NA	−	+	+/+	−	NK	CT+RT, *DOD*
10	71/F	Nasal cavity	Typ	+	−	+	+/+	+	NA/−	NA/−	−	+	+/+	−	NK	CT+RT, *AWOD*
11	65/M	Nasal cavity	L	+	−	+	−/−	−	−/−	−/−	−	+	+/+	−	NK	CT+RT, *AWOD*
12	73/M	Nasal cavity	Typ	+	−	+	−/−	−	−/−	−/−	−	+	+/+	−	NK	CT+RT, *AWOD*
13	68/M	Nasal cavity	Typ	+	−	+	−/−	−	−/−	−/−	−	+	+/+	−	NK	CT+RT, *AWOD*
14	32/F	Nasopharynx	Typ	+	−	+	−/−	−	−/+	−/−	−	+	+/+	−	NK	CT+RT, *AWOD*
15	45/M	Nasopharynx	Typ	+	−	+	+/+	−	−/+	−/−	−	+	+/+	−	NK	RT, CT, Allo, *AWOD*
16	32/M	Nasopharynx	Typ	+	−	+	+/+	+	−/+	−/−	−	+	+/+	−	NK	CT, Auto, *AWD*
17	68/F	Nasal cavity	Typ	+	−	−	−/−	−	−/+	NA/−	−	+	+/+	−	NK	RT, CT, *AWOD*
18	85/F	Nasal cavity	Typ	+	−	+	+/+	−	−/+	−/−	−	+	+/+	−	NK	CT, *DOD*
19	40/M	Nasopharynx, adrenal glands	Typ	+	−	+	+/+	−	−/+	−/−	−	+	+/+	−	NK	CT, RT, Allo, *DOD*
20	67/M	Nasal cavity	Typ	+	−	+	+/+	−	NA/+	NA/−	−	+	+/+	−	NK	CT+RT, *AWOD*
21	70/M	Nasal cavity, skin	Typ	+	−	+	−/−	−	−/+	−/−	−	+	+/+	−	NK	NA, *alive*
22	40/M	Nasal cavity, testis	Typ	+	−	−	−/−	+	−/+	−/−	−	+	+/+	−	NK	RT+CT, *DOD*
23	35/M	Nasal cavity	L	+	−	+	+/+	−	−/+	−/NA	−	+	+/+	−	NK	RT, *AWOD*
24	66/F	Nasal cavity	Typ	+	−	+	+/+	−	−/+	−/−	−	+	+/+	−	NK	RT, CT, *AWOD*
25	52/F	Nasal cavity	Typ	+	−	+	+/+	−	−/+	−/NA	−	+	+/+	−	NK	CT+RT, *AWOD*
26	52/M	Nasal cavity, cervical nodes	Typ	+	−	+	+/+	−	−/+	−/NA	−	+	+/+	−	NK	CT, RT, *AWOD*
27	75/F	Nasal cavity	Typ	+	−	+	−/−	−	NA/+	NA/−	−	+	+/+	−	NK	CT+RT, *AWOD*
28	75/M	Skin, nasopharynx	Typ	+	−	−	−/−	−	+/+	−/−	−	+	+/+	+	T	CT, *DOD*
29	33/M	Pharynx, small intestine	L	+	−	−	+/+	−	+/+	−/−	−	+	+/+	+	T	CT, Auto, *DOD*
30	28/M	Nasal cavity	Typ	+	−	+	−/−	−	−/+	+/NA	−	−	+/+	+	T	RT, *AWOD*

Abbreviations: Allo, allogeneic stem cell transplantation; Auto, autologous stem cell transplantation; AWD, alive with disease; AWOD, alive without disease; cyCD3, cytoplasmic CD3; cyCD5, cytoplasmic CD5; GrB, granzyme B; CT, chemotherapy; DOD, dead of disease; DWOD, dead without disease; L, large cell variant; NA, not available; RT, radiation therapy; sCD3, surface CD3; sCD5, surface CD5; TCR GR, TCR gene rearrangement; Typ, typical; ZEBRA, the EBV replication activator *Bam*HI-Z.

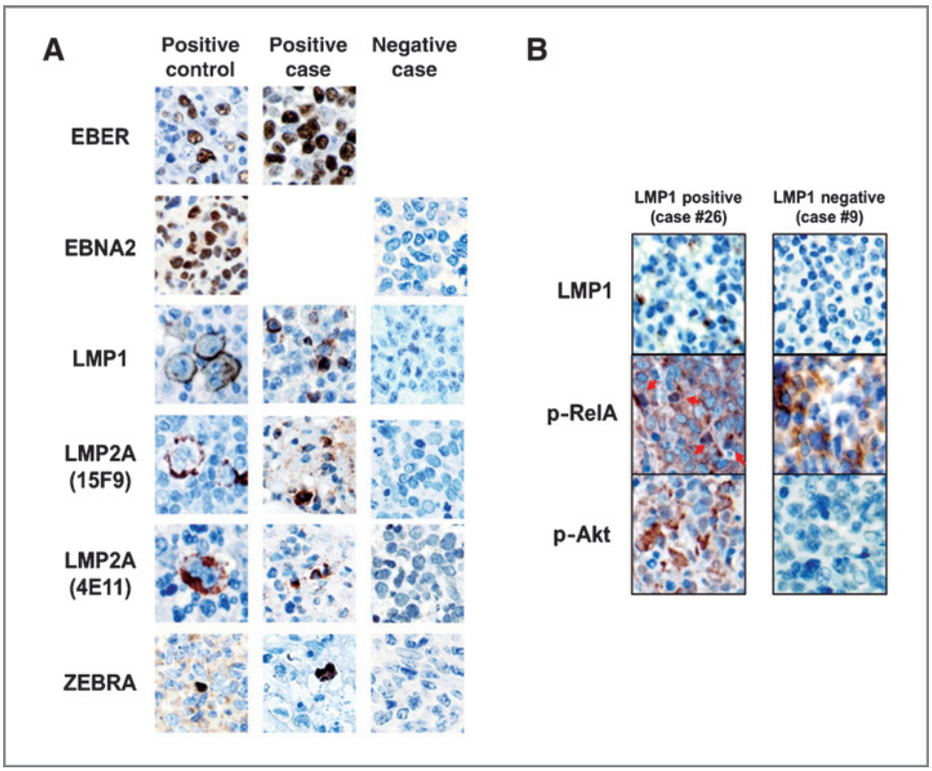

Figure 2. Immunohistochemistry for EBV-associated gene products and phosphorylated RelA and Akt in ENKL. A, ISH and immunohistochemistry confirmed that all examined cases were positive for EBERs but not EBNA2. LMP1 and LMP2A were positive in 22 (73.3%) and 12 cases (40.0%), respectively. In the positive cases, both proteins showed a patchy distribution in tumor lesions. Nuclear staining of ZEBRA was detected in a small proportion of the neoplastic cells in 4 cases. As positive controls, biopsy specimens from patients with immunodeficiency-associated lymphoproliferative disorders were used for staining of EBERs, EBNA2, and ZEBRA. In addition, those from patients with Hodgkin lymphoma were used for staining of LMP1 and LMP2A. B, nuclear localization of phosphorylated RelA was more frequently detected in LMP-positive cases (17 of 18 cases, 94.4%) than in LMP1-negative ones (1 of 8 cases, 12.5%). Phosphorylated Akt (p-Akt) was focally positive only in LMP1-positive cases (21 of 22 cases, 95.5%).

similar to those observed in NPC rather than Hodgkin lymphoma, which usually shows coexpression of LMP1 and LMP2A. We further evaluated the expression of ZEBRA. This immediately early protein was detected in a small proportion of neoplastic cells in 4 cases (Table 1, Fig. 2A). The result indicates that a small proportion of ENKL cells may enter the lytic cycle.

LMP1 expression and phosphorylation of RelA and Akt in ENKL

In clinical samples, nuclear localization of phosphorylated RelA was more frequently detected in LMP1-positive cases (17 of 18 cases, 94.4%) than in LMP1-negative ones (2 of 7 cases, 28.6%; Table 2, Fig. 2B). In addition, phosphorylated Akt was focally positive only in LMP1-positive cases (21 of 22 cases, 95.5%; Table 2, Fig. 2B). Correlations between LMP1 positivity and these findings were statistically significant ($P = 0.002$ and $P < 0.001$, respectively, the Fisher exact test; Table 2).

We further evaluated the correlations in ENKL-derived cell lines. Western blot analysis clearly detected a retarded band of phosphorylated RelA in Hank1 compared with NK-YS and EBV-negative NKL (Fig. 3A). Phosphorylation of Akt at Thr 308 was more prominent in Hank1 and NKL compared with in NK-YS (Fig. 3B). This effect was increased in NK-YS and Hank1 cells cultured with abundantly IL-2–containing medium (500 U/mL; Fig. 3B). In Hank1, LMP1 expression was also upregulated after treatment of IL-2 (Fig. 3B). Although phosphorylation of Akt is not necessarily mediated only by LMP1, LMP1 expression may influence the effect in Hank1. To find direct evidence of LMP1 on phosphorylation of RelA and Akt, we carried out RNA interference (RNAi) for LMP1 in Hank1 (Fig. 3C). After 24 hours of knockdown, proportions of phosphorylated RelA and Akt were clearly decreased (Fig. 3D). Nevertheless, the potencies were weakened after 48 hours of knockdown (Fig. 3D). Hank1 had an ability to compensate for loss of LMP1. Thus, LMP1 partially engaged in phosphorylation of RelA and Akt in the ENKL-derived cells.

Prognostic factor, therapy, and impact of LMP1 expression on clinical outcome

According to the International Prognostic Index, approximately two thirds of the cases were scored in the low (12 cases, 40.0%) and low-intermediate (7 cases, 23.3%) risk categories. Although therapeutic options were considered in each case, 17 cases with a localized disease (56.7%) received radiation therapy. The applied chemotherapeutic regimens were not uniform but mainly contained methotrexate and L-asparaginase. Autologous and allogeneic stem cell transplantations were conducted in 4 and 2 cases, respectively. We evaluated correlations between LMP1 positivity and these clinical data. Although most parameters associated with prognosis were not statistically significant, LMP1-positive cases tended to show a localized disease ($P = 0.060$, the Fisher exact test; Table 2). With a median follow-up period of 26.7 months (range, 0.2–142.3 months), the 2.5-year OS rate

Table 2. Differences in clinical and biologic features between LMP1-positive and -negative cases

	LMP1 positive Cases (N = 22)	LMP1 negative Cases (N = 8)	*P*
Age, y			
≥65	10	3	1.000
<65	12	5	
Sex			
Male	13	6	0.672
Female	9	2	
Initial involved sites			
Nasal	20	7	1.000
Extranasal	2	1	
Clinical stage			
I/II	19	4	0.060
III/IV	3	4	
International Prognostic Index			
Low/low-intermediate	15	4	0.417
High-intermediate/high	7	4	
Radiation therapy			
Received	16	4	0.172
No	4	4	
NA	2	0	
MIB-1 index (%)			
≥80	3	2	0.589
<80	19	6	
Nuclear localization of pRelA (Ser536)			
Positive	17	2	0.002
Negative	1	5	
NA	4	1	
p-Akt (Thr308)			
Positive	21	0	<0.001
Negative	1	8	

Abbreviation: NA, not available.

for the present 30 cases was calculated at 58.0% [95% confidence interval (CI), 38.0%–78.0%; Fig. 4A]. In LMP1-positive and -negative cases, the 2.5-year OS rates were estimated at 78.3% (95% CI, 58.1%–98.5%) and 12.5% (95% CI, 0.0%–40.2%), respectively (Fig. 4B). Statistical difference was observed in survival between the 2 groups ($P = 0.001$, log-rank test). Among 20 cases responding to the therapy, 10 cases (7, LMP1 positive; 3 LMP1 negative) relapsed or showed disease progression. Between 2 LMP1-positive cases histologically reexamined, one (case #6) lost LMP1 expression in the recurrent lesion. In 28 cases evaluable for treatment response, PFS was also statistically significant between LMP1-positive and -negative cases (median, 75.9 vs. 15.5 months, $P = 0.013$, log-rank test; Fig. 4B). Thus, LMP1-positive ENKL cases showed a favorable clinical outcome compared with LMP1-negative ones.

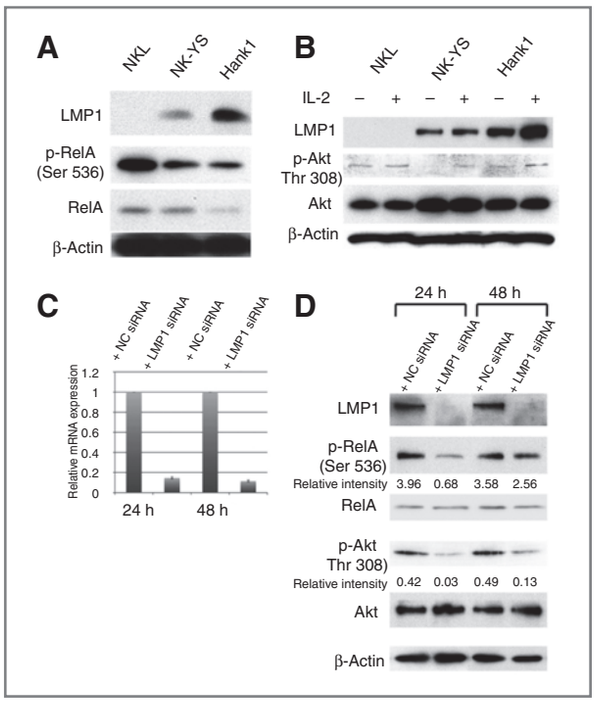

Figure 3. LMP1 expression and phosphorylated RelA and Akt in ENKL-derived cell lines. A and B, compared with poorly LMP1-expressing NK-YS, Western blot analysis clearly detected retarded bands of phosphorylated RelA (p-RelA) and Akt (p-Akt) in abundantly LMP1-expressing Hank1. In NK-YS and Hank1 cells, phosphorylation of Akt was increased by stimulation of 500 U/mL IL-2 for 3 hours. In Hank1 cells, LMP1 expression was also upregulated after IL-2 stimulation. C, we carried out RNAi for LMP1 in Hank1. D, after 24 hours of knockdown, proportions of p-RelA and p-Akt were clearly decreased. Nevertheless, the effects were weakened after 48 hours. Alterations in phosphorylated proportions in RelA and Akt were calculated with ImageJ software. NC, negative control.

Discussion

In the present study, most ENKL cases showed a uniform pattern of onset, typical pleomorphic cell morphology, and a similar NK cell immunophenotype. Although ENKL presented as a simple disease entity, immunohistochemistry confirmed heterogeneous expression patterns of LMP1 and LMP2A. The result indicates that expressions of EBV-associated proteins vary among cases. In addition, a small proportion of neoplastic cells seemed to enter the lytic cycle. Namely, EBV can disrupt its latency in ENKL cells. The detection frequency was lower for LMP2A than for LMP1. Fox and colleagues recently reported the existence of a novel LMP2A protein derived from alternatively spliced mRNA in ENKL cells (24). Besides sensitivity, the 2 antibodies used may fail to detect the protein due to different immunogenicity.

During *in vitro* EBV infection of NK or T cells, LMP1 is expressed without the presence of EBNA2, which is the main

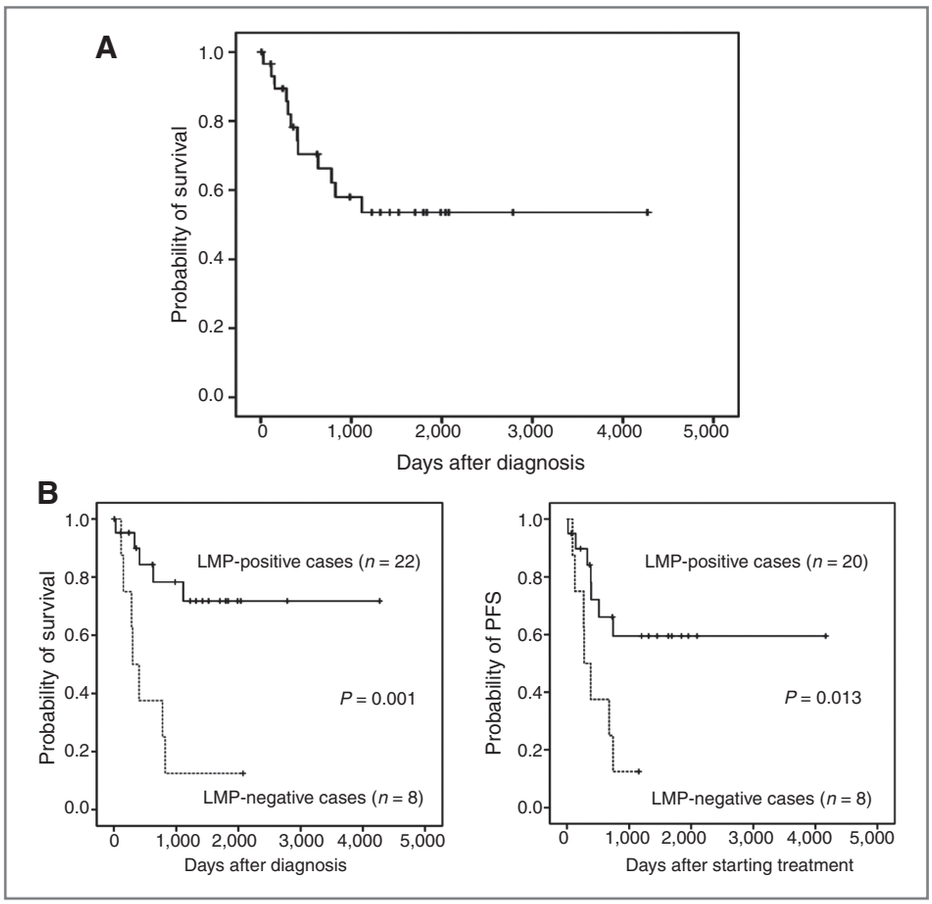

Figure 4. OS and PFS of the present cases. A, the 2.5-year OS rate for the present 30 cases was calculated as 58.0%. B, between LMP1-positive and -negative cases, the OS and PFS rates were statistically significant ($P = 0.001$ and 0.013, respectively, log-rank test).

inducer of LMP1 in EBV-infected B cells (10, 12, 13). We previously showed that LMP1 expression in EBV-infected NK cells was observed after establishment of latent infection (12). LMP1 expression in ENKL seems to be controlled by the surrounding environment such as cytokines (25). Although stimulation of IL-10 and IFN-γ likely influence LMP1 expression, ENKL cells themselves secrete these cytokines affecting the surrounding cells and may induce tissue necrosis and vascular damage (16, 26–28). We assume that the expression of LMP1 in ENKL may be controlled under the dynamic relationship between the neoplastic and surrounding cells.

Although the physiologic functions of LMP1 in Hodgkin lymphoma and NPC are well investigated (10, 11), the pathogenetic role in ENKL has been not fully evaluated. We confirmed that LMP1 expression in ENKL was correlated with both nuclear localization of RelA and detection of phosphorylated Akt. Indeed, the activation of NF-κB and phosphorylation of Akt in ENKL cells has mainly been discussed in relation to stimulation effects of cytokines such as IL-2 and IL-15 (29, 30). Although environmental factors may exert a supportive role in the activation of NF-κB and phosphorylation of Akt, RNAi for LMP1 in Hank1 cells confirmed a major effect of LMP1 on these events in ENKL cells. Similar to Hodgkin lymphoma and NPC, LMP1 potentially engages the activation of NF-κB and Akt even in ENKL.

In vitro studies suggest that LMP1-expressing ENKL cells may have a survival advantage compared with LMP1-negative ones. Contrary to this hypothesis, LMP1 expression clinically showed a favorable outcome in ENKL. Because LMP1-positive cases tended to show a localized disease, the clinical stage probably influences the prognosis. Among the LMP1-positive cases, one showed that cytotoxic T-cell infiltration into the lesion, which seemed to be associated with tumor regression (17). LMP1 has antigenicity and further upregulates expressions of adhesion molecules and surface antigens in the host cells (14, 31–34). Although LMP1 may render the cell malignant phenotype, these alterations should be involved in host immune response and may affect the manner of tumor progression. Previous *in vitro* studies show that cytotoxic T cells against LMP1 can directly kill ENKL-derived cell lines (35, 36). Therefore, LMP1 may have reciprocal effects on the expressing cells, that is, even though LMP1-expressing cells show a malignant phenotype, an extrinsic immune system can eradicate these cells *in vivo*. In Hodgkin lymphoma, the presence of LMP1 seems to influence its clinical behavior in concert with the patient's age and histology (37, 38). In addition, LMP1-

positive NPC is reported to be less prone to relapse, despite its aggressive clinical behavior (39, 40). Although the impact of LMP1 on clinical outcome remains controversial, we assume that LMP1-positive ENKL cells are prone to targets of cytotoxic T cells, despite their pernicious nature.

In conclusion, we observed nonuniform expression patterns of LMP1 and LMP2A in ENKL clinical samples. LMP1 expression is correlated with phosphorylation of RelA and Akt and potentially has a favorable impact on clinical outcome in ENKL. To address this issue, further prospective evaluation is needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Isobe, K. Sugimoto, N. Komatsu
Development of methodology: N. Kanemitsu, Y. Isobe, A. Masuda, M. Higashi, J. Tamaru
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Kanemitsu, Y. Isobe
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Kanemitsu, Y. Isobe, S. Momose, J. Tamaru
Writing, review, and/or revision of the manuscript: N. Kanemitsu, Y. Isobe, A. Masuda, K. Sugimoto, N. Komatsu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Isobe, A. Masuda, S. Momose, J. Tamaru
Study supervision: Y. Isobe, K. Sugimoto, N. Komatsu

Acknowledgments

The authors thank Dr. Kengo Takeuchi (The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan) and Dr. Masaru Hosone (Nihon Medical University School of Medicine, Tokyo, Japan) for reviewing histologic findings in the present cases, Dr. Masao Seto (Aichi Cancer Center, Nagoya, Japan), Dr. Junjiro Tsuchiyama (Kawasaki Medical School, Kurashiki, Japan), and Dr. Michael J. Robertson for provision of NK cell lines, and Dr. Kazuo Oshimi (Eisai Boston Institute, Boston, MA) and Asami Ohno for critical advice and technical assistance, respectively.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 19, 2011; revised February 7, 2012; accepted February 14, 2012; published OnlineFirst February 27, 2012.

References

1. Chan JKC, Quintanilla-Martinez L, Ferry JA, Peh SC. Extranodal NK/T-cell lymphoma, nasal type. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. World Health Organization classification of tumors of haematopoietic and lymphoid tissues. Lyon, France: IARC Press; 2008. p. 285–8.
2. International T-cell Lymphoma Project. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. J Clin Oncol 2008;26:4124–30.
3. Lee J, Suh C, Park YH, Ko YH, Bang SM, Lee JH, et al. Extranodal natural killer T-cell lymphoma, nasal-type: a prognostic model from a retrospective multicenter study. J Clin Oncol 2006;24:612–8.
4. Suzuki R, Suzumiya J, Yamaguchi M, Nakamura S, Kameoka J, Kojima H, et al. Prognostic factors for mature natural killer (NK) cell neoplasms: aggressive NK cell leukemia and extranodal NK cell lymphoma, nasal type. Ann Oncol 2010;21:1032–40.
5. Yamaguchi M, Tobinai K, Oguchi M, Ishizuka N, Kobayashi Y, Isobe Y, et al. Phase I/II study of concurrent chemoradiotherapy for localized nasal natural killer/T-cell lymphoma: Japan Clinical Oncology Group Study JCOG0211. J Clin Oncol 2009;27:5594–600.
6. Kim SJ, Kim K, Kim BS, Kim CY, Suh C, Huh J, et al. Phase II trial of concurrent radiation and weekly cisplatin followed by VIPD chemotherapy in newly diagnosed, stage IE to IIE, nasal, extranodal NK/T-cell lymphoma: Consortium for Improving Survival of Lymphoma Study. J Clin Oncol 2009;27:6027–32.
7. Oshimi K. Progress in understanding and managing natural killer-cell malignancies. Br J Haematol 2007;139:532–44.
8. Au WY, Weisenburger DD, Intragumtornchai T, Nakamura S, Kim WS, Sng I, et al. Clinical differences between nasal and extranasal natural killer/T-cell lymphoma: a study of 136 cases from the International Peripheral T-Cell Lymphoma Project. Blood 2009;113:3931–7.
9. Rickinson AB, Kieff E. Epstein-Barr virus. In: Knipe DM, Howley PM, et al., editors. Fields virology. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2007. p. 2655–700.
10. Kieff ED, Rickinson AB. Epstein-Barr virus and its replication. In: Knipe DM, Howley PM, et al., editors. Fields virology. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2007. p. 2603–54.
11. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004;4:757–68.
12. Isobe Y, Sugimoto K, Yang L, Tamayose K, Egashira M, Kaneko T, et al.: Epstein-Barr virus infection of human natural killer cell lines and peripheral blood natural killer cells. Cancer Res 2004;64:2167–74.
13. Yoshiyama H, Shimizu N, Takada K. Persistent Epstein-Barr virus infection in a human T-cell line: unique program of latent virus expression. EMBO J 1995;14:3706–11.
14. Hislop AD, Taylor GS, Sauce D, Rickinson AB. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. Annu Rev Immunol 2007;25:587–617.
15. Chiang AK, Tao Q, Srivastava G, Ho FC. Nasal NK- and T-cell lymphomas share the same type of Epstein-Barr virus latency as nasopharyngeal carcinoma and Hodgkin's disease. Int J Cancer 1996;68:285–90.
16. Xu ZG, Iwatsuki K, Oyama N, Ohtsuka M, Satoh M, Kikuchi S, et al. The latency pattern of Epstein-Barr virus infection and viral IL-10 expression in cutaneous natural killer/T-cell lymphomas. Br J Cancer 2001;84:920–5.
17. Isobe Y, Aritaka N, Sasaki M, Oshimi K, Sugimoto K. Spontaneous regression of natural killer cell lymphoma. J Clin Pathol 2009;62:647–50.
18. Ando J, Sugimoto K, Ando M, Isobe Y, Sasaki M, Oshimi K. CD20-positive extranodal NK-cell lymphoma, nasal type. Eur J Haematol 2008;80:549–50.
19. Tsuchiyama J, Yoshino T, Mori M, Kondoh E, Oka T, Akagi T, et al. Characterization of novel human natural killer-cell line (NK-YS) establishment from natural killer cell lymphoma/leukemia associated with Epstein-Barr virus infection. Blood 1998;92:1374–83.
20. Kagami Y, Nakamura S, Suzuki R, Iida S, Yatabe Y, Okada Y, et al. Establishment of an IL-2-dependent cell line derived from `nasal-type' NK/T-cell lymphoma of $CD2^+$, $sCD3^-$, $CD3\varepsilon^+$, $CD56^+$ phenotype and associated with the Epstein-Barr virus. Br J Haematol 1998;103:669–77.
21. Robertson MJ, Cochran KJ, Cameron C, Le JM, Tantravahi R, Ritz J. Characterization of a cell line, NKL, derived from aggressive human natural killer cell leukemia. Exp Hematol 1996;24:406–15.
22. Iwata S, Wada K, Tobita S, Gotoh K, Ito Y, Demachi-Okamura A, et al. Quantitative analysis of Epstein-Barr virus (EBV)-related gene expression in patients with chronic active EBV infection. J Gen Virol 2010;91:42–50.
23. Bell AI, Groves K, Kelly GL, Croom-Carter D, Hui E, Chan ATC, et al. Analysis of Epstein-Barr virus latent gene expression in endemic Burkitt's lymphoma and nasopharyngeal carcinoma tumor cells by using quantitative real-time PCR assays. J Gen Virol 2006;87:2885–90.

24. Fox CP, Haigh TA, Taylor GS, Long HM, Lee SP, Shannon-Lowe C, et al. A novel latent membrane 2 transcript expressed in Epstein-Barr virus-positive NK- and T-cell lymphoproliferative disease encodes a target for cellular immunotherapy. Blood 2010;116:3695–704.
25. Kis LL, Takahara M, Nagy N, Klein G, Klein E. IL-10 can induce the expression of EBV-encoded latent membrane protein-1 (LMP-1) in the absence of EBNA-2 in B lymphocytes and Burkitt lymphoma- and NK lymphoma- derived cell lines. Blood 2006;107:2928–35.
26. Boulland M-L, Meignin V, Leroy-Viard K, Copie-Bergman C, Briere J, Touitou R, et al. Human interleukin-10 expression in T/natural killer-cell lymphomas. Am J Pathol 1998;153:1229–37.
27. Teruya-Feldstein J, Jaffe ES, Burd PR, Kanegane H, Kingma DW, Wilson WH, et al. The role of Mig, the monokine induced by interferon-γ, and IP-10, the interferon-γ-inducible protein-10, in tissue necrosis and vascular damage associated with Epstein-Barr virus-positive lymphoproliferative disease. Blood 1997;90:4099–105.
28. Shen L, Chiang AKS, Liu WP, Liang RHS, Srivastava G. Expression of HLA class I, β2-microglobulin, Tap1 and IL-10 in Epstein-Barr virus-associated nasal NK/T-cell lymphoma: implications for tumor immune escape mechanism. Int J Cancer 2001;92:692–6.
29. Chow C, Liu AY, Chan WS, Lei KIK, Chan WY, Lo AWI. Akt plays a role in the survival of the tumor cells of extranodal NK/T-cell lymphoma, nasal type. Haematologica 2005;90:274–5.
30. Chan K-K, Shen L, Au W-Y, Yuen H-F, Wong K-Y, Guo T, et al. Interleukin-2 induces NF-κB activation through BCL10 and affects its subcellular localization in natural killer lymphoma cells. J Pathol 2010;221:164–74.
31. Cherney BW, Sgadari C, Kanegane C, Wang F, Tosato G. Expression of the Epstein-Barr virus protein LMP1 mediates tumor regression *in vivo*. Blood 1998;91:2491–500.
32. Gottschalk S, Edwards OL, Sili U, Huls MH, Goltsova T, Davis AR, et al. Generating CTLs against the subdominant Epstein-Barr virus LMP1 antigen for the adoptive immunotherapy of EBV-associated malignancies. Blood 2003;101:1905–12.
33. Mehl AM, Floettmann JE, Jones M, Brennan P, Rowe M. Characterization of intercellular adhesion molecule-1 regulation by Epstein-Barr virus-encoded latent membrane protein-1 identifies pathways that cooperate with nuclear factor κB to activate transcription. J Biol Chem 2001;276:984–92.
34. Peng M, Lundgren E. Transient expression of the Epstein-Barr virus LMP1 gene in B-cell chronic lymphocytic leukemia cells, T cells, and hematopoietic cell lines: cell-type-independent-induction of CD23, CD21, and ICAM-1. Leukemia 1993;7:104–12.
35. Demachi-Okamura A, Ito Y, Akatsuka Y, Tsujimura K, Morishima Y, Takahashi T, et al. Epstein-Barr virus (EBV) latent membrane protein-1-specific cytotoxic T lymphocytes targeting EBV-carrying natural killer cell malignancies. Eur J Immunol 2006;36:593–602.
36. Kobayashi H, Nagato T, Takahara M, Sato K, Kimura S, Aoki N, et al. Induction of EBV-latent membrane protein 1-specific MHC class II-restricted T-cell responses against natural killer lymphoma cells. Cancer Res 2008;68:901–8.
37. Diepstra A, van Imhoff GW, Schaapveld M, Karim-Kos H, van den Berg A, Vellenga E, et al. Latent Epstein-Barr virus infection of tumor cells in classical Hodgkin's lymphoma predicts adverse outcome in older adult patients. J Clin Oncol 2009;27:3815–21.
38. Claviez A, Tiemann M, Lüders H, Krams M, Parwaresch R, Schellong G, et al. Impact of latent Epstein-Barr virus infection on outcome in children and adolescents with Hodgkin's lymphoma. J Clin Oncol 2005;23:4048–56.
39. Hu L-F, Chen F, Zhen Q-F, Zhang Y-W, Luo Y, Zheng X, et al. Differences in the growth pattern and clinical course of EBV-LMP1 expressing and non-expressing nasopharyngeal carcinomas. Eur J Cancer 1995;31:658–60.
40. Khabir A, Karray H, Rodriguez S, Rosé M, Daoud J, Frikha M, et al. EBV latent membrane protein I abundance correlates with patient age but not with metastatic behavior in north African nasopharyngeal carcinomas. Virol J 2005;2:39.