IJC
International Journal of Cancer

Epstein-Barr virus-positive diffuse large B-cell lymphoma association is not only restricted to elderly patients

M. Cohen[1], M. Narbaitz[2], F. Metrebian[2], E. De Matteo[3], M. V. Preciado[1] and P. A. Chabay[1]

[1] Molecular Biology Laboratory, Pathology Division, Ricardo Gutiérrez Children's Hospital, Buenos Aires, Argentina
[2] Histopathological Laboratory, National Academy of Medicine, Buenos Aires, Argentina
[3] Pathology Division, Ricardo Gutiérrez Children's Hospital, Buenos Aires, Argentina

Diffuse large B-cell lymphoma (DLBCL), the most common group of malignant lymphomas, account for 30% of adult non-Hodgkin lymphomas. The 2008 World Health Organization (WHO) classification included a new entity, Epstein-Barr virus (EBV)+ DLBCL of the elderly, affecting patients aged 50 years or older. However, some reports of younger EBV+ DLBCL cases, without evidence of underlying immunosuppression, can be found. The role of EBV in tumor microenvironment composition in DLBCL is still not well understood. Our aim was to assess EBV presence and latency pattern as well as tumor T-cell population in an adult DLBCL series of Argentina. The study was conducted on biopsies from 75 DLBCL patients. EBERs expression was performed by *in situ* hybridization, while EBV gene expression was analyzed using real-time polymerase chain reaction. LMP1, LMP2A, EBNA2, EBNA3A, CD4, CD8 and Foxp3 expression was assessed by immunohistochemistry. Nine percent of cases showed EBV expression, with similar frequency among patients younger than 50 years and 50 years or older (13% and 8%, respectively). T-cell subsets were not altered by EBV presence. Latency type II was the most frequently observed, together with lytic gene expression in EBV+ DLBCL, with ≥20% of EBERs+ cells. These findings suggest that EBV+ DLBCL in our series was similar to the previously described in Asia and Latin-America, displaying latency II or III expression profile and no age-specific characteristics. Finally, EBV+ DLBCL may be an entity that is not only restricted to patients who are older than 50 years of age, in consequence the age cutoff revision may be a current goal.

Key words: Epstein-Barr virus, diffuse large B-cell lymphoma, young adults, elderly, Epstein-Barr virus latency pattern, T-cell markers

Abbreviations: BL: Burkitt lymphoma; BZLF1: BamHI Z fragment leftward open reading frame 1; Ct: cycle threshold; CTL: cytotoxic T-lymphocytes; DLBCL: diffuse large B-cell lymphoma; EBV: Epstein-Barr virus; EBERs: EBV-encoded small nuclear early region; EBNA: Epstein-Barr virus nuclear antigen; FFPE: formalin-fixed paraffin-embedded; FITC: fluorescein isothiocyanate; GC: germinal centre; HL: Hodgkin lymphoma; HRS: Hodgkin Reed-Sternberg cell; HPRT: hypoxanthine phosphoribosyltransferase; IHC: immunohistochemistry; ISH: *in situ* hybridization; LMP: Epstein-Barr virus latent membrane protein; NHL: non-Hodgkin's lymphoma; qPCR: real-time quantitative PCR; Treg: regulatory T-cell

Additional Supporting Information may be found in the online version of this article.

Conflicts of interest: M.C. is a National Research Council (CONICET) doctoral fellow. P.A.C. and M.V.P. are members of the National Research Council (CONICET), Research Career Program.

Grant sponsor: National Agency for Science and Technology Promotion; **Grant number:** PICT 2011 no. 0478; **Grant sponsor:** National Research Council; **Grant number:** PIP 2012 res 4558-12
DOI: 10.1002/ijc.28942
History: Received 26 Nov 2013; Accepted 17 Apr 2014; Online 2 May 2014

Correspondence to: Melina Cohen, Laboratorio de Biología Molecular, División de Patología, Hospital de Niños R Gutiérrez, Gallo 1330, C1425EFD, Buenos Aires, Argentina, Tel.: 5411-49629138, Fax: 5411-49629138, E-mail: melucohen@yahoo.com.ar

Introduction

Epstein-Barr virus (EBV) is a human gamma-herpes virus that infects more than 90% of the adult population. The virus is able to infect and immortalize B lymphocytes both *in vitro* and *in vivo*. Even though its infection is mostly harmless, EBV is associated with a number of malignancies, such as Burkitt lymphoma (BL), classical Hodgkin lymphoma and diffuse large B-cell lymphoma (DLBCL).[1,2]

DLBCLs, the most common group of malignant lymphomas, account for 30% of adult non-Hodgkin lymphomas (NHLs). EBV+ DLBCL of the elderly, also known as age-related EBV+ B-cell lymphoproliferative disorder or senile EBV-associated B-cell lymphoproliferative disorder, was initially described by a Japanese group.[3] Later, it was included as a provisional entity in the 2008 World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues and is defined as a clonal EBV carrying B-cell proliferation arising in immunocompetent patients older than 50 years.[4] However, some reports of younger cases,[5,6] which included few patients who had no evidence of underlying immunosuppression and a diagnosis of EBV+ DLBCL, were described. The identification of these younger cases raise an interrogation if EBV+ DLBCL may be an entity that is not only restricted to patients who are older than 50 years of age.

The prognosis of EBV+ DLBCL of the elderly is worse than the age-matched DLBCL without EBV infection. The incidence of EBV among DLBCL from Asian or Latin

What's new?

Epstein-Barr virus+DLBCL was defined in the 2008 WHO classification of lymphoid neoplasm as occurring in patients over 50 with no immunodeficiency. This study aimed to shed light on the role of EBV in DLBCL pathogenesis and to assess whether viral presence and antigen expression are closely related to age differences. Similar EBV association was found between young and elderly DLBCL patients. EBV showed differential infectious characteristics that could reveal viral involvement in DLBCL pathogenesis. Age and EBV status did not alter T cell subsets in the tumor microenvironment. EBV+DLBCL may thus not be restricted to patients over 50.

American patients ranges from 8% to 15%.[5,7,8] However, in western countries, this incidence is less than 5%.[9,10] These preliminary data suggested that, as seen in other EBV-associated disorders, there might be a geographical variation and/or ethnical predisposition for the development of EBV+ elderly DLBCL. It is noteworthy to mention that uniform criteria concerning the percentage of EBV+ cells that should be present to consider a DLBCL as EBV+ have not been established yet, which could imply a limitation when assessing disease prevalence. In fact, the number of EBV+ cells may range from 10% to almost all tumor cells present in the sample. The fact that differences in cutoffs for EBV-encoded RNAs (EBERs) positivity clearly impact on the frequency of EBV+ DLBCL was discussed by some authors.[5,11,12]

Three different latency patterns have been described in both EBV-related malignant disorders and EBV-derived cell lines. Several of these patterns were proposed to be associated with different degrees of immunosuppression. For example, Latency I, the most restricted form of viral gene expression, that characterizes BL, expresses the EBV nuclear antigen (EBNA) 1 together with the EBERs. Latency II is an intermediate form in which, besides EBNA1 and EBERs, latent membrane proteins (LMP) 1 and LMP2 are expressed. This pattern of EBV gene expression is observed in HL, T/NK cell lymphoma and primary effusion lymphoma, among others. Finally, latency III involves the unrestricted expression of all the six EBNAs together with LMP1 and 2. This type of latency mainly occurs in the setting of severe immunosuppression; in fact, post-transplant lymphoproliferative disorders (PTLDs) and AIDS-related lymphoproliferative disorders, as well as in EBV-immortalized lymphoblastoid cell lines *in vitro*, are characterized by this type of EBV latency pattern.[2,13] Few studies focused on the analysis of EBV latency patterns in EBV+ DLBCL of the elderly, which reported viral latencies including patterns II and III.[8,10,14,15]

It has been postulated that EBV+ DLBCL of the elderly might be caused by the senescence of the immune system as a part of the normal aging process, based largely on shared features with PTLDs, such as EBV infection and latency pattern, comparable morphology and presence of monoclonal T-cell populations.[8,16–18] Of note, immunosenescence refers to a continuous remodeling process, where the distribution of CD4+ and CD8+ T-cell populations is altered; however, the changes in CD4+ T-cell profile seem to be affected by advancing age at a higher degree than the one of CD8+ T-cell.[19] The presence of persistent infections, such as EBV or other persistent antigens, also induces a phenomenon called immune exhaustion, which contributes to the loss of immunosurveillance that could result eventually in an uncontrolled EBV-infected B-cell proliferation.[20,21]

It has recently been described that host immunity has a close relationship with tumor development. The expression of immune checkpoint molecules on T-cells represents an important mechanism that the immune system uses to regulate itself. Checkpoint molecules include cytotoxic T lymphocyte antigen-4, programmed death-1, lymphocyte activation gene-3, T-cell immunoglobulin and several others.[22] Recent data show that coexpression of checkpoint molecules occurs frequently on cancer-specific T-cells, suppressing host cytotoxic CD8+ T-cells (CTLs) and enhancing the activity of suppressive CD4+ regulatory T-cells (Tregs).[23] The recent advances in our understanding of lymphomas biology and immunology show that infiltrated immune cells and cytokines in the tumor microenvironment may play different functions that seem tightly related with the clinical outcome. In line with this, it has been proposed that in DLBCL they could have an important role, both in the development of the neoplastic cells and in therapy failure.[24]

On the other hand, EBV presence with a particular latent expression pattern would possibly render a CTL response specifically directed to viral antigens, which also appears to influence tumor infiltrating immune cells composition, and contributes to the impact on clinical presentation and outcome as well. Given this fact, the precise characterization of the microenvironment composition, especially concerning T-cell population, is a matter of clinical importance.

This study aims to investigate the frequency of EBV+ DLBCL together with viral gene expression in an Argentinean cohort of adults from a single center and to correlate EBV infection and age with certain T-cell markers, hoping to provide, in one hand, a better understanding of the role of EBV in the DLBCL pathogenesis, and on the other hand, an assessment of whether viral presence and antigen expression are closely related to age differences.

Material and Methods

Patients and samples

A total of 75 patients from National Academy of Medicine, Buenos Aires, Argentina, were enrolled in this study. They

had no prior lymphoma or any known underlying immunosuppression as well as were naive of treatment at diagnosis.

Formalin-fixed paraffin-embedded (FFPE) biopsy samples from the archives of the Histopathological Laboratory were collected retrospectively, on the basis of the availability of sufficient material from 2009 to 2012.

All tumors were diagnosed and classified by two pathologist (M.N. and F.M.), as DLBCL according to standard WHO criteria.[25] They were evaluated for histopathological features, such as necrosis and increase in apoptotic cells. Cases were then subcategorized, when possible, as germinal centre (GC) or non-GC according to the algorithm presented by Hans *et al.*[26]

This study has the approval of the Institutional Review Board and the Ethics Board of National Academy of Medicine and is also in accordance with the Helsinki Declaration of 1975, as revised in 1983. A written informed consent was obtained from all the included patients after the nature of the procedure had been fully explained. The medical records were reviewed with special attention to previous medical history.

Detection of EBERs by *in situ* hybridization

EBERs *in situ* hybridization (ISH) studies were performed using the peptide nucleic acid (PNA) *in situ* hybridization detection kit (Dako, Carpinteria, CA) according to the manufacturer's protocol as described previously.[27] A case was defined as EBV positive when at least 20% of positive nuclear staining restricted to tumor cells was observed.

Immunohistochemistry

Immunohistochemistry (IHC) staining for B-cell lymphoma differential diagnosis and tumor microenvironment immunophenotyping was performed on FFPE tissue sections with a panel of antibodies: CD3 (Cell Marque, Rocklin, CA), CD20 (Dako), CD10 (Cell Marque), bcl-2 (Dako), bcl-6 (Dako) and MUM1 (Dako), Ki67 (Dako), CD4 (Leica, Newcastle, UK), CD8 (Dako), Foxp3 (AbCam, Cambridge, UK) as described previously.[27] The counting of CD4, CD8 and Foxp3 positive cells was performed as follows: Numbers of total or immunopositive cells per high-power field were counted by two observers using ImageJ "cell counter" tool in 10 fields selected on the basis of the best-preserved tissue areas. The expression of the results was defined as number of immunopositive cells divided by the total number of counted cells. Cells partly included in the fields were not counted.

IHC was used to localize LMP1 and LMP2A viral antigen expression in tumor cells on EBERs+ tissue samples as described previously,[27] using monoclonal antibodies CS1-4 (Dako) and clone 15F9 (AbCam, Cambridge, UK) respectively. EBNA2 (clones 1E6 and R3, kindly provided by Dr. Kremmer, Forschungszentrum fur Umwelt und Gesundheit GmbH, Institut fur Immulogie, Munchen, Germany) and EBNA3A (AbCam) were also used to establish the latency type of EBV infection.

RNA extraction and real-time relative quantification PCR for EBV gene expression

Approximately 2 × 10 μm sections from each of the 75 FFPE biopsy samples were used for nucleic acid extraction and purification. Total RNA was purified using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, TX) and cDNA synthesis was performed using Superscript II RT kit (Invitrogen, CA) according to the manufacturer's instructions. To remove contaminated DNA in total RNA, a DNAse step was included for all RNA samples. RNA from cell lines used as calibrators was extracted by QIAamp DNA blood mini-kit (QIAGEN GmbH, Hilden, Germany) as manufacturer's instructions. For qualitative polymerase chain reaction (qPCR) assays of EBV gene transcription analysis, 2 μg of total RNA was reverse transcribed. The design and validation of specific primers for latent genes (EBNA1, EBNA2, EBNA3C, LMP1 and LMP2A), the immediate-early lytic gene BZLF1 and the endogenous HPRT gene were carried out as described.[27] qPCR was performed in a reaction volume of 25 μl with the SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA) with 5 μl diluted cDNA and the Universal thermal cycle, using a StepOne real-time detection system (Applied Biosystems). The normalized transcription values were calculated by the Pfaffl method.[28] The mean value of the Ct of an equivalent quantity of RNA input from Raji (EBV type I virus with a deletion in EBNA3C) or P3HR1 (EBV type II virus with a deletion in EBNA2) cell lines was used as calibrators when appropriate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 and InStat 3 software (GraphPad Software Inc., San Diego, CA). We compared demographics and histological characteristics between EBV+ and EBV− tumors. Categorical variables were analyzed using Fisher's exact test. Mann–Whitney test was used to compare the means between age groups or T-cell population markers in relation with EBV presence. Correlations between data were determined using Spearman's rank correlation index. All tests were two-tailed, and a $p < 0.05$ was considered statistically significant.

Results

Patient characteristics and histological features

Seventy-five DLBCL cases were included in this analysis. Median age at diagnosis was 63 years (range, 18–84 years). There was no gender predominance, with 37 males and 38 females (ratio 1:1 approximately).

The diagnosis was achieved in nodal location in 46 cases (61%), while extranodal onset was found in 29 patients (39%). Geographic necrosis was a prominent feature in 22 cases (29%). The proliferation rate was high, with Ki-67-positive cells representing more than 70% in most

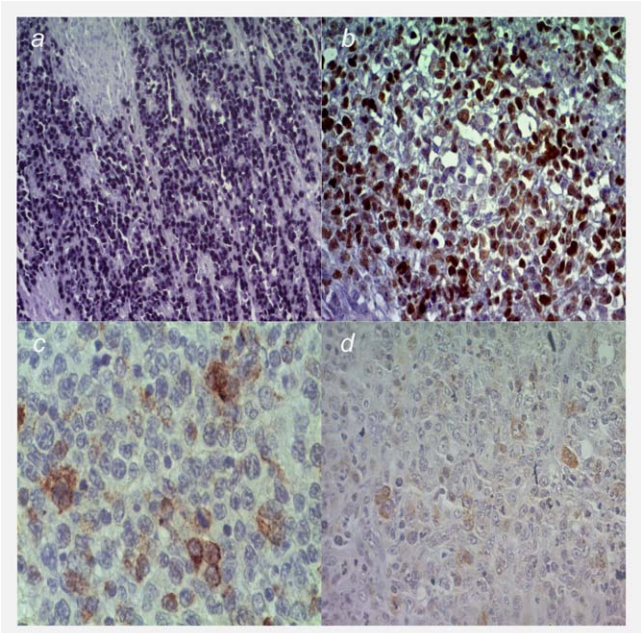

Figure 1. Representative expression of EBV-encoded small nuclear early region (EBERs), latent membrane protein 1 (LMP1), −2A (LMP2A) and EBV nuclear antigen 2 (EBNA2) in a DLBCL case (pt. 1). (*a*) Nuclear localization of the EBERs in neoplastic cells by ISH. (*b*) Nuclear localization of the EBNA2 in neoplastic cells by IHC. (*c* and *d*) Membranous and cytoplasmic localization of LMP1 and LMP2A in neoplastic cells by IHC. Original magnification: ×200 (*a*) and ×400 (*b–d*). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

cases (64%), reflecting the aggressiveness of this lymphoma. On the basis of Hans IHC classification, 53 cases with enough biopsy material available were classified as GC subtype ($n = 27$, 51%) and non-GC subtype ($n = 26$, 49%).

EBV analysis

In this study, patients with DLBCL were screened for the presence of EBV in the malignant cells by means of EBERs ISH. EBV was detected in 7 of 75 (9.3%) DLBCL cases (Fig. 1*a*), showing EBERs nuclear positive staining in ≥20% of the malignant cells. The median of the EBERs+ cells percentage was 80% in the studied group (range 20–90%). Interestingly, 8 of 75 (10.7%) cases showed EBERs expression restricted to 5–10% of tumor cells. However, given that EBV infection in those eight cases displayed certain particular characteristics, as mentioned following, we considered a 20% of EBERs positive tumor cells as cutoff to define EBV positivity.

EBV positivity was more frequently observed in male sex cases (14% vs. 5%), nodal involvement (13% vs. 3%) and non-GC subtype (15% vs. 7%); however, these differences did not reach statistical significance ($p > 0.05$, Fisher's exact test). EBERs expression related to demographic and histological characteristics is summarized in Table 1.

As EBV+ DLBCL of the elderly occurs in patients older than 50 years, our cohort of patients was sorted in two age groups: younger than 50 years ($n = 23$) and older than 50 years ($n = 52$). Unexpectedly, the frequency of EBV+ cases was slightly higher among patients younger than 50 years (3 of 23, 13%) than in patients aged 50 years and older (4 of 52, 8%), but this difference was not statistically significant ($p > 0.05$, Fisher's exact test). Furthermore, the median age of EBV+ cases was 35 years and 75 years for patients younger than 50 years and patients aged 50 years and older, respectively; while it was 35 years and 70 years for EBV− cases from both groups ($p > 0.05$, Mann–Whitney test).

In those biopsies displaying EBERs+ tumor cells, namely, cases (pt.) 1–15, IHC for the detection of LMP1, LMP2A, EBNA2 and EBNA3A viral proteins was performed to discriminate latencies II and III patterns. LMP1 and LMP2A expressions localized at the cytoplasm and surface membrane of neoplastic cells were detected in 7 of 15 (47%) and 3 of 15 (20%) EBV+ samples, respectively (Figs. 1*c* and 1*d*). All LMP2A+ cases were LMP1+ as well. EBNA2 staining in the nucleus of tumor cells was observed in 2 of 13 (15%) samples (Fig. 1*b*), whereas EBNA3A was absent in all samples studied. Concerning only cases considered as EBV+, the expression of LMP1 was found in 7 of 7 (100%), LMP2A in 3 of 7 (43%) and EBNA2 in 2 of 6 (33%), as showed in Table 2.

To deeply characterize EBV latency patterns in those EBERs+ cases, we assessed viral gene expression by means of qPCR. Concerning EBV transcripts expression, we observed variable levels of the different genes among cases. EBNA1 was detected in nearly all samples, whereas EBNA2 and BZLF1 were expressed in 11 cases, EBNA3C in 10 cases,

Table 1. Demographic and histological characteristics of adult DLBCL series related to EBV status (positive *vs*. negative)

Patients' characteristics	EBV status[1]		P^2
	Positive (%)	Negative (%)	
Age (years)			
Younger than 50	3 of 23 (13)	20 of 23 (87)	0.6687
50 years and older	4 of 52 (8)	48 of 52 (92)	
Gender			
Male	5 of 37 (14)	32 of 37 (86)	0.2553
Female	2 of 38 (5)	36 of 38 (95)	
Primary site			
Nodal	6 of 46 (13)	40 of 46 (87)	0.0502
Extranodal	1 of 29 (3)	28 of 29 (97)	
Histological subtype			
Non-GC	4 of 26 (15)	22 of 26 (85)	
GC	2 of 27 (7)	25 of 27 (93)	0.4203
ND	1 of 22 (5)	21 of 22 (95)	

[1]EBV status determined by EBERs ISH (≥20% EBERs+ cells as a cutoff value).
[2]*p* as determined by Fisher's exact test.
Abbreviations: GC: germinal-centre; ND: not determined (insufficient material).

Table 2. Types of latency pattern and lytic expression on EBV+ DLBCL cases related to clinical outcome

Pt. no.	Age (years)	ISH		IHC			Latency expression[1]	qPCR					Latency expression[2]	Lytic expression	Outcome
		EBERs	(%)	LMP1	LMP2A	EBNA2	EBNA3A		EBNA1	EBNA2	EBNA3C	LMP1	LMP2A	BZLF1			
1	18	+	90	+	+	+	−	III[3]	+	+	+	+	+	+	III	+	Dead
2	38	+	90	+	−	−	−	II	+	+	+	+	−	+	III[3]	+	LF
3	35	+	80	+	−	ND	ND	II/III	+	+	+	+	+	+	III	+	Dead
4	68	+	80	+	+	−	−	II	+	+	+	+	+	+	III	+	Dead
5	76	+	30	+	+	−	−	II	+	+	+	+	+	+	III	+	Dead
6	76	+	25	+	−	−	−	II	+	+	+	+	+	+	III	+	LF
7	73	+	20	+	−	+	−	III	+	+	+	+	−	+	III[3]	+	LF

[1]Latency pattern defined by IHC.
[2]Latency pattern defined by qPCR.
[3]Latency type, when some transcripts are not detected or detected in low levels.
Abbreviations: Pt.: patient; EBNA: Epstein-Barr nuclear antigen; LMP: latent membrane protein; BZLF: intermediate-early EBV gene; qPCR: quantitative polymerase-chain reaction; IHC: immunohistochemistry; ISH: *in situ* hybridization; ND: not determined; LF: lost at follow-up.

LMP1 was in 9 cases and LMP2A in 8 cases. In several cases, the levels of transcripts detected were very low, so we did not consider them for analysis and for latency pattern definition. Relative expression levels of EBV transcripts are shown in Figure 2. In the ≥20% EBERs+ cases, qPCR analysis revealed all latency transcripts expression with a lytic gene (pt. 1–7), including pt. 2 and 7, where LMP2A expression was not found. In the <20% EBERs+ cases, several scenarios were disclosed. High levels of all latent transcripts together with BZLF1 were observed in pt. 8. The remaining cases (pt. 9–15) exhibited variable transcripts presence, but all with modest levels of expression (Fig. 2).

A direct correlation among all EBV transcripts quantified and the median percentage of EBERs+ cells was observed (EBNA1 $r =$ 0.86, EBNA2 $r = 0.82$, EBNA3C $r = 0.75$, LMP1 $r = 0.74$, BZLF1 $r = 0.75$; $p < 0.001$, LMP2A $r = 0.59$; $p < 0.05$). There was also a direct correlation between all the EBV latent and BZLF1 genes analyzed ($r > 0.8$; $p < 0.001$), except for LMP2A, which correlate only with EBNA1 and LMP1 ($r = 0.70$ and $r = 0.53$; $p < 0.05$).

Although several works used only qPCR results to analyze latency pattern, here we decided to define it based on IHC results of various latency proteins. Several distinct patterns of expression were identified and summarized in Table 2. Four cases (pt. 2, 4–6) revealed latency II, sometimes without LMP2A expression (pt. 2, 6 and 7). Two cases (pt. 1 and 7) displayed latency III pattern based on EBNA2 immunopositivity. One case (pt. 3) was classified as latency II/III as a result of LMP1 protein detection only, due to lack of enough material to perform EBNA2 and −3A IHC. As regard to age groups, we could not find any significant association with a particular latency program. Finally, those cases (pt. 8–15) with so few EBERs+ cells were not considered for latency pattern definition. However, their viral characteristics and clinical outcome are described in Supporting Information Table S1.

Tumor microenvironment analysis

Since immunosenescence has been postulated to be implicated in the mechanism behind this entity, we investigated whether there was a difference between both age groups (younger than 50 years and 50 years and older) with reference to tumor microenvironment composition. We found no significant differences in the tumor microenvironment composition when assessing CD4, CD8 and Foxp3 T-cell markers (Fig. 3*a*–3*c*) between younger and older cases (Supporting Information Fig. S1*a*). The total cell numbers counted for each group was comparable (Supporting Information Fig. S1*b*). Regression analysis did not indicate a correlation between the proportions of T-cells and age ($p > 0.05$, Spearman's rank correlation index).

Based on the above mentioned results, we analyzed DLBCL cases altogether and found that percentages of

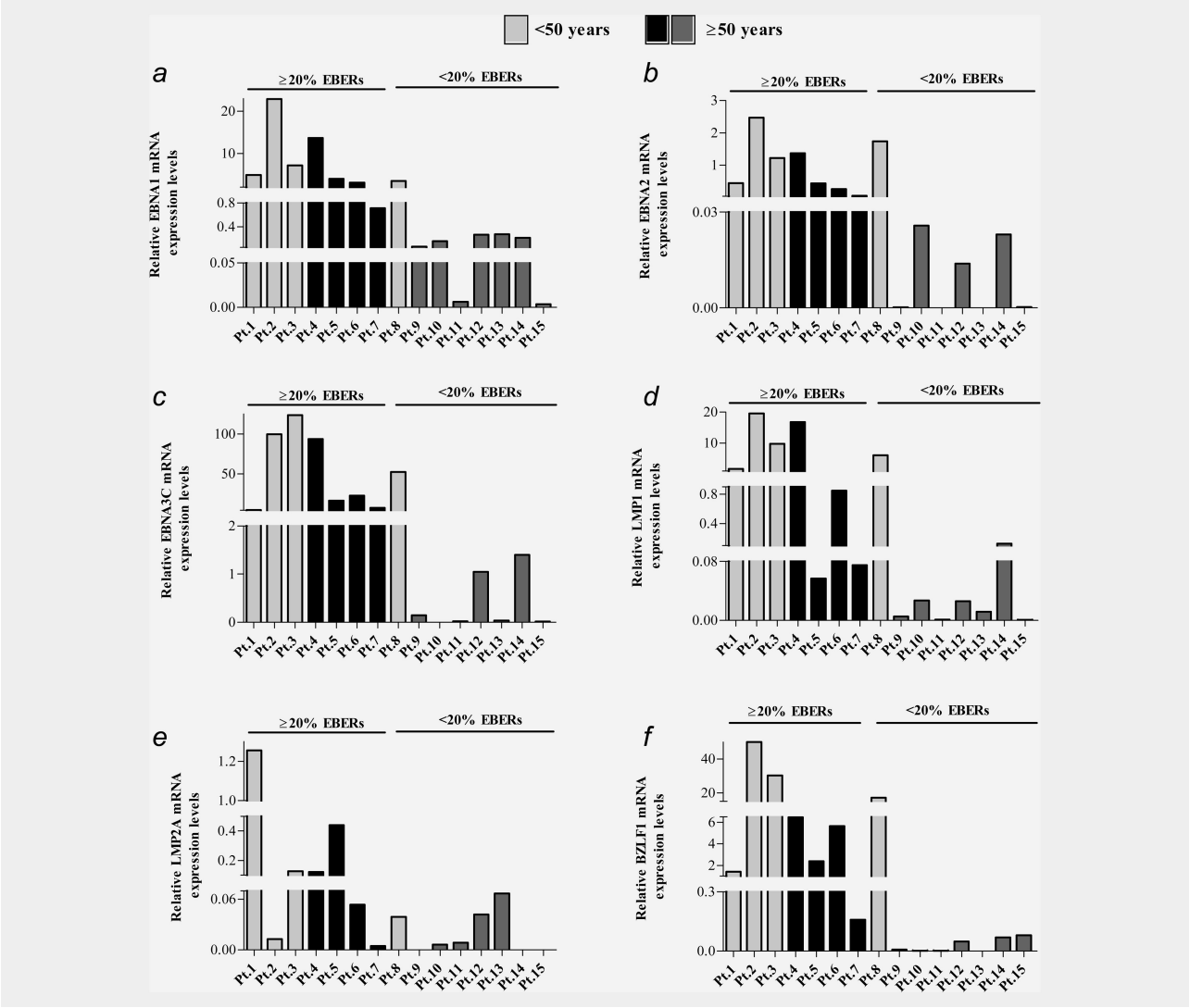

Figure 2. qPCR was performed to determinate the mRNA relative expression levels of (*a*) EBNA1, (*b*) EBNA2, (*c*) EBNA3C, (*d*) LMP1, (*e*) LMP2A and (*f*) BZLF1. HPRT was used as a reference gene. The bars represent expression levels in EBERs+ DLBCL samples (pt. 1–15), normalized to mRNA expression in EBV+ reference cell line as calibrator. Light-grey bars indicate patients younger than 50 years, black bars indicate patients aged 50 years and older and dark-grey bars indicate patients aged 50 years and older with <20% EBERs+ cells.

different T-cell markers varied considerably between individual DLBCL cases (CD4: range 0–29%, median 5.9%; CD8: range 0–51%, median 4.5%; Foxp3: range 0–23%, median 2.6%). Numbers of CD4+ and CD8+ T-cells were higher than Foxp3+ T-cells ($p < 0.05$, Mann–Whitney test). A direct correlation was observed between the percentages of CD4+ and Foxp3+ cells ($r = 0.5$, $p < 0.001$) and, to a lesser extent, between CD8+ and Foxp3+ cells ($r = 0.3$, $p < 0.05$ Spearman).

Given that EBV may be able to modulate the tumor microenvironment composition and local EBV-specific immunity, the impact of EBV status on T-cell population was investigated. Table 3 lists mean rank percentages of CD4, CD8 and Foxp3 T-cell markers in EBV+ vs. EBV− cases. No significant difference was found among each cellular marker investigated concerning EBV status ($p > 0.05$, Mann–Whitney test).

Discussion

The pathogenic role of the EBV in NHL is still under discussion; however, it is well known that EBV is able to drive cellular proliferation as a potential carcinogen.[29] Recently, EBV+ DLBCL was defined in the 2008 WHO classification of lymphoid neoplasm as an EBV+ clonal B-cell lymphoproliferation that occurs in patients older than 50 years without any immunodeficiency or prior lymphoma.[30] With the aim of disclosing EBV involvement on DLBCL lymphomagenesis, this work addressed the prevalence of EBV infection in a series of adult DLBCL from a single center of Argentina.

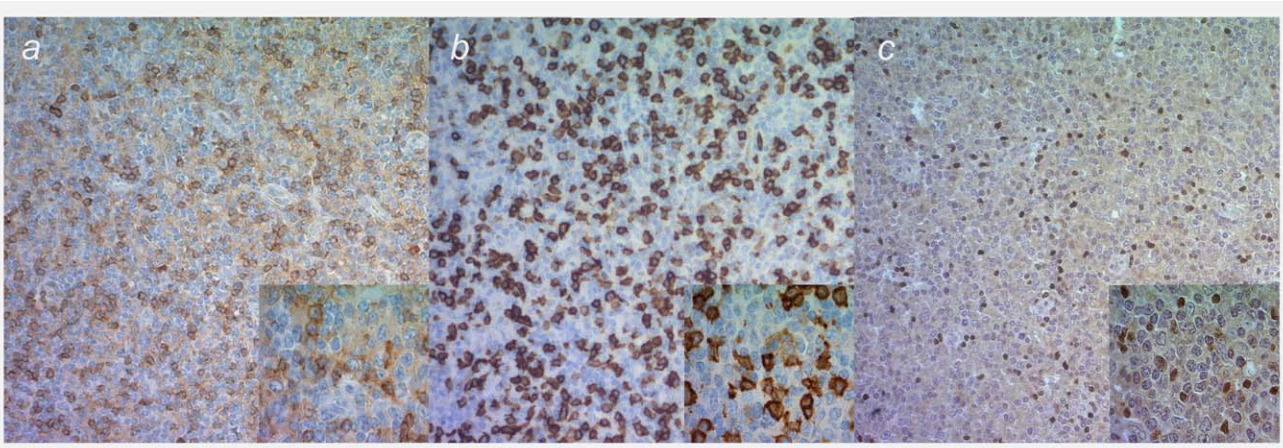

Figure 3. Representative IHC of (*a*) CD4, (*b*) CD8 and (*c*) Foxp3 shows positive staining of T-cells within tumor microenvironment in a EBV+ DLBCL case (patient 3). Original magnification: ×200. An enlarged view is shown in the inset (×1,000). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

Table 3. EBV expression status (positive *vs*. negative) related to the expression of several T-cell markers in tumor microenvironment

T-cell marker	EBV status[1]	Mean rank[2]	*p*[3]
CD4	Positive	11.1	0.363
	Negative	7.6	
CD8	Positive	10.3	0.495
	Negative	6.9	
Foxp3	Positive	5.2	0.582
	Negative	4.8	

[1]EBV status determined by EBERs ISH (≥20% EBERs+ cells as a cutoff value).
[2]The number represents the mean of the no. + cells/no. total cells ×100.
[3]*p* as determined by Mann–Whitney test.

In this study, we analyzed a series of 75 adult DLBCL cases for the presence of EBV infection by ISH. Per definition, EBERs in DLBCL should be present in the neoplastic cells, although the expression of them can range from 10% to almost all of the tumor cells in the sample.[31] Current literature does not establish a definitive cutoff as was reviewed by Wada *et al.*[11] Hofscheier and coworkers. mentioned EBV positivity in <20% of the large cells in 5% of DLBCL cases in the Mexican population and suggested that in rare cases, secondary EBV infection of an established B-cell clone could be the explanation.[14,32] Hoeller *et al.* also reported that two of their EBV+ DLBCL cases showed <20% positive cells.[10] In addition, EBV gene expression exhibited particular characteristics, given that previous studies suggested that EBV+ DLBCL in elderly patients generally have viral latency type II or III pattern.[8,10,14,15] We observed differences in the viral gene levels and protein expression, in cases with more than 20% of positive cells and those with less than 20% of positive cells. This fact leads us to define EBV patterns in the former group, whereas, due to the small amount of EBERs+ cells, the EBV latency could not be defined in the latter, even though morphological and immunophenotypic properties were similar in both groups. As observed in Table 2, patients with ≥20% of positive cells displayed most frequently the latency program II together with BZLF1 lytic gene expression, in agreement with previous works. Particularly, EBNA2 expression was higher (33%) than observed in Japan and Mexico.[8,10] Based on the above mentioned reports and differential viral expression, we decided to apply ≥20% of positive cells as cutoff to define an EBV+ DLBCL case. EBV positivity (median 80% of positive cells) was observed in seven cases, representing an overall prevalence of 9.3%, while eight cases (11%) of DLBCL showed EBV positivity in 5–10% of the large cells. Furthermore, the LMP1 expression observed in EBV+ DLBCL cases, together with an immediate early gene expression, could reveal an active role of EBV in lymphoma development. Remarkably, BZLF1 lytic gene expression is suggestive of an abortive lytic cycle linked to tumor progression, as proposed by Strong *et al*, who recently reported high immediate early lytic expression in DLBCL cases without other lytic genes.[33]

A strong correlation among all EBNAs quantified was observed, which is predictable, as in latency III, these EBNA mRNAs are generated by alternative splicing of long primary transcripts initiated either from the tandemly repeated Wp promoter or the upstream Cp promoter.[34] Alternatively, in B cells displaying latency III type infection, LMP1 and LMP2A/B expressions are dependent on expression of EBNA2.[35] In our series, the expression of LMP1 significantly correlated with the other genes analyzed, while LMP2A correlation was only observed with EBNA1 and LMP1, in agreement with the low levels detected for this particular target. On the other hand, BZLF1, a immediate-early lytic gene, correlated with latency genes expression. Our observation suggested that EBV+ DLBCL cases are expressing a mixture of latency II or III and a lytic EBV gene. A striking finding was the marked

correlation among all EBV transcripts (latent and lytic) quantified and median percentage of EBERs+ cells. Recently, it was described by means of large-scale functional genomics analysis of EBV that viral lytic genes are coexpressed with cellular cancer-associated pathways, suggesting that the lytic cycle may play an unexpected role in virus-mediated oncogenesis.[36]

Based on WHO classification scheme and for comparison purposes, we divided the series into two groups, younger than and older than 50 years. We managed to recruit 23 young cases and 52 elderly cases in the period studied which revealed that the prevalence of EBV+ DLBCL in both groups was similar (13% vs. 8%, respectively). Nearly 4,800 lymphoma cases are diagnosed every year in Argentina and 50% of them are NHLs. About 90% of NHLs occur after 35 years of age.[37] Despite this data, there is no detailed registry of DLBCL incidence in our country. It is reported that half of patients with DLBCL are more than 65 years old and the probability of having a DLBCL grows with age, which could explain the difference in sample size between young and elderly groups from this single center. The prevalence of EBV+ DLBCL of the elderly in Argentinian patients (8%) was comparable with the data published in Latin-American and Asian literature (8–15%), and what is more, the median age at presentation in older patients (75 years) was similar to the one described for Japanese and Peruvian series (71 and 70 years, respectively).[8,38] These observations are not unexpected, since other EBV-associated neoplasms such as nasopharyngeal carcinoma and NK/T-cell nasal lymphomas have been shown to be more prevalent in Asian and Latin American populations.[39,40]

Beltran *et al.* recently described a group of patients with EBV+ DLBCL, who displayed the histological and immunophenotypical characteristics described for this entity, but the 50 years cutoff criteria was not fulfilled.[6] Moreover, Oyama *et al.* excluded seven patients younger than 40 years of age (7% of their EBV+ DLBCL patients),[8] whereas a report from Korea by Park *et al.* included patients with EBV+ DLBCL as young as 20 years.[5] In addition, Hoeller *et al.* excluded two European patients who were younger than 50 years, representing 20% of all their EBV+ DLBCL cases.[10] Therefore, it was not surprising that EBV in our DLBCL cases was also associated with a proportion of cases that were under 50 years old (13%), even though it was noteworthy that the difference between both groups was not significant ($p > 0.05$). Furthermore, we observed that latency III was not restricted only to patients older than 50 years, in which a less restrictive latency might be expected under an unfavorable immunological status triggered by aging process. This remarkable finding suggests that the entity "EBV+ DLBCL of the elderly" can occur in young immunocompetent patients and might not be an age related-EBV association. In fact, our group had previously found in an Argentinean cohort an association between EBV and pediatric DLBCL showing similar or even higher frequencies than the adult counterpart,[27] in agreement with few more studies in pediatric NHL from developing countries.[41–43] Although the number of patients is low for a comparison, the similarities between both groups strongly suggest that the age cutoff established should be revised.

Concerning other demographical aspects, several EBV associated malignancies displays a clear male prevalence.[44] However, despite an apparent male predominance in our series, there were no statistically significant differences with regard to gender and EBV association. In line with this, we had previously showed that in pediatric EBV+ DLBCL there was not gender predominance either.[27]

In contrast to the first publications,[3,45] most recent reported series—including ours—have found a predominant nodal presentation.[9,10,14,46] Morphologically, the distinction between the polymorphic and the monomorphic subtype is no longer regarded as clinically relevant.[8] Although several pathological characteristics have been described in EBV+ LDBCL of the elderly, we also corroborated that there is no morphologic or immunohistochemical pattern that is specific of this entity.[15,31]

The intratumoral immunological alterations induced by EBV+ have been extensively demonstrated in HL, in which viral presence was involved in the attraction of many of the microenvironmental cells into the lymphoma background. This scenario in DLBCL remains unclear. Moreover, in recent years, it has been proposed that defective immune surveillance for EBV may develop late in life and be associated with the development of EBV positive B-cell lymphoproliferative disorder.[47] This defective immune surveillance for EBV is associated with immunological deterioration as a result of aging process,[3,48–50] and explains the correlation between an increased incidence of this lymphoma type together with the increased age of the patients found in the Eastern series. Surprisingly, we did not observe major differences linked with age of the relative numbers of any T-cell population analyzed (Supporting Information Fig. S1*a*). This fact, together with EBV association with both age groups, might confirm previous statements that the definitional cutoff of 50 years proposed by the WHO could be arbitrary,[4,48] since cases below the age of 50 years can be found and suggest that, at least for T-cell populations analyzed, immunosenescence could be ruled out. Concerning EBV status, no significant difference was found among each cellular marker investigated, as we previously found in pediatric EBV+ DLBCL cases.[27]

To the best of our knowledge, this is the first report evaluating the prevalence of EBV in DLBCL adult cases in Argentina. In this study, no significant difference was found between EBV– and EBV+ DLBCL cases and viral latency profile with regard to age at presentation. In addition, lack of differences in T-cell population analyzed in DLBCL microenvironment in both age groups reinforces the idea that the age criterion and possibly the designation of this disease might be revised and perhaps reformulated in the next version of the WHO classification.

Acknowledgements

The authors thank Carlos Nastasi, Histopathological Laboratory, National Academy of Medicine, Argentina, for his helpful histotechnical work.

References

1. Rickinson AB, Kieff ED. Epstein Barr virus. In: Knipe DM, Howley PM, eds. Fields virology, 5th edn., vol. 2. Philadelphia: Walters Kluwers/Lippincott Williams and Wilkins, 2007. 2655–700.
2. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. *Nat Rev Cancer* 2004;4:757–68.
3. Oyama T, Ichimura K, Suzuki R, et al. Senile EBV+ B-cell lymphoproliferative disorders: a clinicopathologic study of 22 patients. *Am J Surg Pathol* 2003;27:16–26.
4. Nakamura S, Jaffe ES, Swerdlow SH. EBV positive diffuse large B-cell of the elderly, 4th edn., vol. 2. Lyon, France: IARC Press, 2008.
5. Park S, Lee J, Ko YH, et al. The impact of Epstein-Barr virus status on clinical outcome in diffuse large B-cell lymphoma. *Blood* 2007;110: 972–8.
6. Beltran BE, Morales D, Quinones P, et al. EBV-positive diffuse large b-cell lymphoma in young immunocompetent individuals. *Clin Lymph Myel Leuk* 2011;11:512–6.
7. Morales D, Beltran B, De Mendoza FH, et al. Epstein-Barr virus as a prognostic factor in de novo nodal diffuse large B-cell lymphoma. *Leuk Lymph* 2010;51:66–72.
8. Oyama T, Yamamoto K, Asano N, et al. Age-related EBV-associated B-cell lymphoproliferative disorders constitute a distinct clinicopathologic group: a study of 96 patients. *Clin Cancer Res* 2007;13:5124–32.
9. Gibson SE, Hsi ED. Epstein-Barr virus-positive B-cell lymphoma of the elderly at a United States tertiary medical center: an uncommon aggressive lymphoma with a nongerminal center B-cell phenotype. *Hum Pathol* 2009;40:653–61.
10. Hoeller S, Tzankov A, Pileri SA, et al. Epstein-Barr virus-positive diffuse large B-cell lymphoma in elderly patients is rare in Western populations. *Hum Pathol* 2010;41:352–7.
11. Wada N, Ikeda J, Hori Y, et al. Epstein-barr virus in diffuse large B-Cell lymphoma in immunocompetent patients in Japan is as low as in Western Countries. *J Med Virol* 2011;83:317–21.
12. Chuang SS, Ichinohasama R, Yang CC, et al. Multicentric primary intestinal EBV-positive diffuse large B cell lymphoma of the elderly presenting with perforation. *Int J Hematol* 2010;91:534–8.
13. Tan LH. A practical approach to the understanding and diagnosis of lymphoma: an assessment of the WHO classification based on immunoarchitecture and immuno-ontogenic principles. *Pathology* 2009;41:305–26.
14. Hofscheier A, Ponciano A, Bonzheim I, et al. Geographic variation in the prevalence of Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly: a comparative analysis of a Mexican and a German population. *Mod Pathol* 2011;24:1046–54.
15. Castillo JJ, Beltran BE, Miranda RN, et al. Epstein-barr virus-positive diffuse large B-cell lymphoma of the elderly: what we know so far. *Oncologist* 2011;16:87–96.
16. Montes-Moreno S, Odqvist L, et al. EBV-positive diffuse large B-cell lymphoma of the elderly is an aggressive post-germinal center B-cell neoplasm characterized by prominent nuclear factor-kB activation. *Mod Pathol* 2012;25:968–82.
17. Dojcinov SD, Venkataraman G, Pittaluga S, et al. Age-related EBV-associated lymphoproliferative disorders in the Western population: a spectrum of reactive lymphoid hyperplasia and lymphoma. *Blood* 2011;117:4726–35.
18. Nguyen-Van D, Keane C, et al. Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly expresses EBNA3A with conserved CD8+ T-cell epitopes. *Am J Blood Res* 2011;1:14.
19. Koch S, Larbi A, Derhovanessian E, et al. Multiparameter flow cytometric analysis of CD4 and CD8 T cell subsets in young and old people. *Immunity Ageing* 2008;5:6.
20. Hakim FT, Gress RE. Immunosenescence: deficits in adaptive immunity in the elderly. *Tissue Antigens* 2007;70:179–89.
21. Zinkernagel RM, Moskophidis D, Kundig T, et al. Effector T-cell induction and T-cell memory versus peripheral deletion of T cells. *Immunol Rev* 1993;133:199–223.
22. Nirschl CJ, Drake CG. Molecular pathways: coexpression of immune checkpoint molecules: signaling pathways and implications for cancer immunotherapy. *Clin Cancer Res* 2013;19:4917–24.
23. Rozali EN, Hato SV, Robinson BW, et al. Programmed death ligand 2 in cancer-induced immune suppression. *Clin Dev Immunol* 2012; 2012:656340.
24. de Jong D, Enblad G. Inflammatory cells and immune microenvironment in malignant lymphoma. *J Intern Med* 2008;264:528–36.
25. Swerdlow SH, Campo E, Harris NL, et al. WHO classification of tumours of haematopoietic and lymphoid tissues, vol. 2. Lyon, France: IARC Press, 2008.
26. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 2004; 103:275–82.
27. Cohen M, De Matteo E, Narbaitz M, et al. Epstein-Barr virus presence in pediatric diffuse large B-cell lymphoma reveals a particular association and latency patterns: analysis of viral role in tumor microenvironment. *Int J Cancer* 2013; 132:1572–80.
28. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl Acids Res* 2001;29:e45.
29. Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. *N Engl J Med* 2004;350:1328–37.
30. Sabattini E, Bacci F, Sagramoso C, et al. WHO classification of tumours of haematopoietic and lymphoid tissues in 2008: an overview. *Pathologica* 2010;102:83–7.
31. Castillo J. EBV-positive diffuse large b-cell lymphoma of the elderly. In: Tao J, Sotomayor E. Hematologic cancers: from molecular pathobiology to targeted therapeutics, vol. 14. Netherlands: Springer, 2012:83–98.
32. Quintanilla-Martinez L, Lome-Maldonado C, Ott G, et al. Primary non-Hodgkin's lymphoma of the intestine: high prevalence of Epstein-Barr virus in Mexican lymphomas as compared with European cases. *Blood* 1997;89:644–51.
33. Strong MJ, O'Grady T, Lin Z, et al. Epstein-Barr virus and human herpesvirus 6 detection in a non-Hodgkin's diffuse large B-cell lymphoma cohort using RNA-seq. *J Virol* 2013;87:13059–62.
34. Rowe M, Kelly GL, Bell AI, et al. Burkitt's lymphoma: the Rosetta Stone deciphering Epstein-Barr virus biology. *Semin Cancer Biol* 2009;19: 377–88.
35. Johannsen E, Koh E, Mosialos G, et al. Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by J kappa and PU.1. *J Virol* 1995;69:253–62.
36. Arvey A, Tempera I, Tsai K, et al. An atlas of the Epstein-Barr virus transcriptome and epigenome reveals host-virus regulatory interactions. *Cell Host Microbe* 2012;12:233–45.
37. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893–917.
38. Beltran BE, Castillo JJ, Morales D, et al. EBV-positive diffuse large B-cell lymphoma of the elderly: a case series from Peru. *Am J Hematol* 2011;86:663–7.
39. Suzuki R. NK/T-Cell Lymphomas: pathobiology, prognosis and treatment paradigm. *Curr Oncol Rep* 2012;14:395–402.
40. Quintanilla-Martinez L, Ridaura C, Nagl F, et al. Hydroa vacciniforme-like lymphoma: a chronic EBV+ lymphoproliferative disorder with risk to develop a systemic lymphoma. *Blood* 2013;122: 3101–10.
41. Tumwine LK, Orem J, Kerchan P, et al. EBV, HHV8 and HIV in B cell non Hodgkin lymphoma in Kampala, Uganda. *Infect Agent Cancer* 2010;5:12.
42. Cool CD, Bitter MA. The malignant lymphomas of Kenya: morphology, immunophenotype, and frequency of Epstein-Barr virus in 73 cases. *Hum Pathol* 1997;28:1026–33.
43. Kasprzak A, Spachacz R, Wachowiak J, et al. Epstein-Barr virus (EBV) infection in B-cell non-Hodgkin's lymphomas in children: virus latency and its correlation with CD21 and CD23 molecules. *Folia Histochem Cytobiol* 2007;45:169–79.
44. Hsu JL, Glaser SL. Epstein-barr virus-associated malignancies: epidemiologic patterns and etiologic implications. *Crit Rev Oncol Hematol* 2000;34:27–53.
45. Shimoyama Y, Asano N, Kojima M, et al. Age-related EBV-associated B-cell lymphoproliferative disorders: diagnostic approach to a newly recognized clinicopathological entity. *Pathol Int* 2009;59:835–43.
46. Quintanilla-Martinez L, de Jong D, et al. Gray zones around diffuse large B cell lymphoma. Conclusions based on the workshop of the XIV meeting of the European Association for Hematopathology and the Society of Hematopathology in Bordeaux, France. *J Hematopathol* 2009;2:211–36.
47. Cohen JI, Kimura H, Nakamura S, et al. Epstein-Barr virus-associated lymphoproliferative disease in non-immunocompromised hosts: a status report and summary of an international meeting, 8–9 September 2008. *Ann Oncol* 2009;20:1472–82.
48. Shimoyama Y, Yamamoto K, Asano N, Oyama T, et al. Age-related Epstein-Barr virus-associated B-cell lymphoproliferative disorders: special references to lymphomas surrounding this newly recognized clinicopathologic disease. *Cancer Sci* 2008;99:1085–91.
49. Shimoyama Y, Oyama T, Asano N, et al. Senile Epstein-Barr virus-associated B-cell lymphoproliferative disorders: a mini review. *J Clin Exp Hematopathol* 2006;46:1–4.
50. Lazuardi L, Jenewein B, Wolf AM, et al. Age-related loss of naive T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. *Immunology* 2005;114:37–43.