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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

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Non-Hodgkin's lymphoma represents 6–10% of pediatric malignancies, and diffuse large B-cell lymphoma (DLBCL) is one of the three major subtypes. The 2008 WHO classification included a new entity, Epstein–Barr virus (EBV)-positive DLBCL of the elderly, affecting patients >50 years. It has been demonstrated that EBV may play a role in tumor microenvironment composition, disturbing antitumor immune response and disease progression. As most studies were performed in adults, our aim was to assess EBV presence and latency pattern, as well as T-cell microenvironment in a pediatric DLBCL series of Argentina. The study was conducted on formalin-fixed paraffin-embedded biopsies from 25 DLBCL patients. EBV-encoded small nuclear early regions (EBERs) expression was performed by *in situ* hybridization, whereas EBV gene expression was analyzed using real-time PCR. Epstein–Barr virus latent membrane proteins (LMP)1, LMP2A, CD3, CD4, CD8 and Foxp3 expression were assessed by immunohistochemistry (IHC). Forty percent of cases showed EBV expression, with a significantly higher incidence among patients <10 years ($p = 0.018$), and with immunosuppressed ($p = 0.023$). T-cell subsets were not altered by EBV presence. Full EBV latency antigen expression (latency type III) was the most frequently pattern observed, together with BZLF1 lytic gene expression. One patient showed II-*like* pattern (LMP1 without LMP2A expression). Based exclusively on IHC, some patients showed latency II/III (EBERs and LMP1 expression) or I (EBERs only). These findings suggest that EBV association in our series was higher than the previously demonstrated for elderly DLBCL and that EBV latency pattern could be more complex from those previously observed. Therefore, EBV could be an important cofactor in pediatric DLBCL lymphomagenesis.

Key words: Epstein–Barr virus, diffuse large B-cell lymphoma, childhood, Epstein–Barr virus latency pattern, T-cell markers

Abbreviations: BL: Burkitt lymphoma; Ct: cycle threshold; CTL: cytotoxic T-lymphocytes; DLBCL: diffuse large B-cell lymphoma; EBERs: EBV-encoded small nuclear early regions; EBNA: Epstein–Barr virus nuclear antigen; EBV: Epstein–Barr virus; EFS: event-free survival; FFPE: formalin-fixed paraffin embedded; FITC: fluorescein isothiocyanate; GC: germinal center; HL: Hodgkin lymphoma; HPRT: hypoxanthine phosphoribosyltransferase; HRS: Hodgkin Reed–Sternberg cell; 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.

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Malignant lymphomas are the third most common malignancies in childhood, with non-Hodgkin's lymphoma (NHL) representing 6–10% of them.[1] Pediatric NHLs comprise three major subtypes: Burkitt lymphoma (43%) (BL), B-lymphoblastic lymphoma (7%) and diffuse large B-cell lymphoma (13%) (DLBCL).[2] DLBCL is rarely diagnosed in children <4 years of age, but the proportion of patients with DLBCL increases throughout childhood toward the second decade of life.[3] In Argentina, pediatric lymphomas are registered in the *Registro Oncopediátrico Hospitalario Argentino* (ROHA), which reported 11,445 pediatric tumors between 2000 and 2008, with lymphomas standing for 12.7% (57.5% NHL, 42.5% Hodgkin lymphoma [HL]) of them.[4]

Epstein–Barr virus (EBV) is one of the most common viruses in humans that infects >90% of the world population, establishing persistent latent infection in the host. Most people become infected with EBV during childhood or adolescence and gain adaptive immunity against the virus. Although EBV usually behaves as a harmless passenger, in rare cases, the virus transforming capacity might promote the development of B-cell lymphomas. The EBV-associated lymphomas and lymphoproliferative disorders include HL, several NHLs (*e.g.*, BL, acquired immunodeficiency syndrome-associated lymphoma, post-transplantation lymphoma

What's new?

Epstein Barr virus (EBV) is known to be associated with some cases of diffuse large B-cell lymphoma (DLBCL) in elderly patients. In this study, the authors found an even greater association between EBV and DLBCL in pediatric patients. They also found that latency patterns of EBV-antigen expression are more complex in pediatric DLBCL than in older patients, and that the virus is associated with immunosuppression in children. Thus, EBV could be an important cofactor in pediatric DLBCL lymphomagenesis.

[PTLD] and DLBCL), T or natural killer (NK) cell lymphoma, as well as epithelial carcinomas (*e.g.*, nasopharyngeal carcinoma [NPC], lymphoepithelioma-like carcinoma, and gastric adenocarcinoma), leiomyosarcoma and leiomyoma associated with immunosuppression.[5,6]

Based on several studies performed in Asian population, a provisional subtype of DLBCL was defined in 2008 classification by the World Health Organization (WHO). This new EBV-associated lymphoma was termed "Epstein–Barr virus-positive DLBCL of the elderly,"[7] which was defined as an EBV-positive clonal B-cell lymphoid proliferation, occurring in patients older than 50 years, without any known underlying immunosuppression or prior lymphoma. It was proposed that it is related to the senescence of the immune system inherent to the aging process, that causes a decrease in T-cell response which in turn involves defective immunosurveillance that results in an uncontrolled EBV-infected B-cell proliferation.[8] The prognosis of this entity is worse than the age-matched DLBCL without EBV infection. Interestingly, the incidence of EBV+ DLBCL of the elderly among DLBCL in Asian or Latin American countries ranges from 9 to 15%,[9–11] whereas it is only <5% in Western populations.[12,13] These preliminary data suggested that as with other EBV-associated disorders, there might be a geographical variation and/or ethnical predisposition for the development of EBV+ DLBCL of the elderly.

On the basis of the different EBV latency gene expression patterns related to EBV-associated tumors, three main types of virus latency have been identified. Latency I is the more restricted form of viral gene expression, characterizes BL and expresses only the EBV nuclear antigen (EBNA) 1 together with the EBV-encoded small nuclear early region (EBER). In contrast, latency III involves the unrestricted expression of all the six EBNAs together with the latent membrane proteins (LMP) 1 and LMP2. This type of latency mainly occurs in the setting of severe immune suppression and characterizes post-transplant and HIV-associated lymphoproliferative disorders, as well as it is usually observed in EBV-immortalized lymphoblastoid cell lines *in vitro*. Latency II is an intermediate form of virus latency in which, besides EBNA1 and EBERs, only LMP-1 and −2 are expressed. This pattern of EBV gene expression is observed in HL, T/NK cell lymphoma and NPC.[6] Only few studies focused on EBV latency pattern in EBV+ DLBCL of the elderly and reported viral latencies including patterns II and III.[10,11,13,14] EBV association with DLBCL in adult patients younger than 30 years has been described[15]; currently, data in regard to EBV association and viral gene expression patterns in pediatric DLBCL patients are hardly found.

On the other hand, tumor microenvironment is a complex network of non-neoplastic immune and stromal cells embedded in extracellular components, giving rise to a multifactorial crosstalk with neoplastic cells toward the induction of a supportive milieu.[16] Nowadays, there is growing interest in the analysis on how tumor microenvironment contributes to the pathogenesis of lymphoid malignancies. In DLBCL, it could have an important role in the development of the neoplastic cells and therapy failure[17] and is characterized by different types of T cells and stromal cells. On this regard, Lenz et al. and Linderoth et al. used microarrays to show that the microenvironment composition in DLBCL could be associated to treatment failure.[18,19] Given this fact, the precise characterization of the microenvironment composition, especially concerning T-cell population, is a matter of clinical importance. The role of the tumor microenvironment has been also demonstrated in another EBV-related lymphoma, namely HL, in which an increase in the proportion of cytotoxic T-lymphocytes (CTLs) is associated with an increased survival.[20] Moreover, others showed that patient's outcome depends not only on the proportion, but also on the functionality of CTLs.[17] Besides, the regulatory T-cell (Treg) population, which has the capacity to downregulate the activity of $CD8^+$ and $CD4^+$ T cells,[18] was implicated in worse prognosis of EBV-associated HL. Although the mechanism of this finding is poorly understood, the hypothesis is that Treg population causes a strong immunosuppressive environment around the Hodgkin–Reed Sternberg (HRS) cells, suppressing both the antitumor response and the EBV-specific immunity.[21,22]

To answer some of the questions arose from the role of EBV in the pediatric DLBCL pathogenesis, we assessed EBV association as well as viral gene expression in the DLBCL pediatric population from our hospital. We also characterized whether EBV infection alters T-cell tumor microenvironment composition, and finally correlated these findings with patient characteristics and outcome.

Material and Methods

Patients and samples

Formalin-fixed paraffin-embedded (FFPE) biopsy samples from 25 patients were collected retrospectively, on the basis of the availability of sufficient material, from the archives at Pathology Division, Ricardo Gutiérrez Children's Hospital in

Buenos Aires, Argentina, from 1987 to 2010. Institutional guidelines regarding human experimentation were followed, and they were in accordance to the Helsinki Declaration of 1975. The Ethical Committee of the institution has approved it, and that the subjects gave informed consent to the study. The medical records were reviewed with special attention to the previous medical history.

Diagnosis was made from biopsies taken from the primary tumor and cases were classified by two pathologists (E.D.M and M.N.) as DLBCL according to the WHO scheme for B-cell NHL.[7] Cases were then subcategorized, when possible, as germinal center (GC) or non-GC according to the algorithm presented by Hans et al.[23]

EBERs in situ hybridization

EBERs *in situ* hybridization (ISH) was performed on FFPE tissue sections using fluorescein isothiocyanate (FITC)-conjugated EBERs oligonucleotides as probes (Dako, Carpinteria, CA). A monoclonal antibody anti-FITC labeled with alkaline phosphatase was used for the detection of hybridized sites (Dako). A case was defined as EBV positive when at least 20% of positive nuclear staining restricted to tumor cells was observed. We used a well-known mixed cellularity HL with specific staining in HRS cells as a positive control.

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 (Dako), CD20 (Dako), CD10 (Dako), bcl-6 (Dako) and MUM1 (Dako), CD4 (Leica, Newcastle, United Kingdom), CD8 (Dako), Foxp3 (AbCam, Cambridge, United Kingdom). Pretreatment of the sections with 10 mM sodium citrate buffer (pH 6) in microwave oven was performed. IHC detection of monoclonal and polyclonal antibodies was carried out using a universal streptavidin–biotin complex-peroxidase detection system (UltraTek HRP Anti-Polyvalent Lab Pack, ScyTek, UT) according to the manufacturer's instructions. Visualization of positive cells was performed using diaminobenzidine as chromogen. Appropriate positive controls were immunostained for each antibody, and negative controls were performed with the same method without the primary antibody. IHC was selective for each marker, in that reactive products were not observed in the absence of the respective primary antibody. The counting of CD3-, CD4-, CD8- and Foxp3-positive cells was performed as follows: a score system was adopted by using the 100× objective lens and counting ten fields selected on the basis of the best-preserved tissue areas that contained immunopositive cells. The number of immunopositive cells per field was divided by the total number of counted cells per field and the expression was defined as the percentage of positive cells in the total number of counted cells. Cells partly included in the fields were not counted.

Immunostaining was used to localize LMP1 and LMP2A expression in tumor cells on EBV+ (by EBERs ISH) tissue samples, using monoclonal antibodies CS1-4 (Dako) and clone 15F9 (AbCam), respectively. Both antibodies were incubated overnight at 4°C. Antigen unmasking with sodium citrate buffer (pH 6) in microwave oven for 10 min was performed. IHC detection of monoclonal and polyclonal antibodies was carried out using a universal streptavidin–biotin complex-peroxidase detection system (UltraTek HRP Anti-Polyvalent Lab Pack, ScyTek) according to the manufacturer's instructions. As a positive control, we used a well-known mixed cellularity HL with specific staining in HRS cells and negative control were performed without the primary antibody.

RNA extraction, reverse transcription and real-time PCR quantification

Approximately 2 × 10 μm sections from each of the 25 FFPE biopsy samples were used for nucleic acid extraction and purification. Total RNA was purified using the Recover-All Total Nucleic Acid Isolation Kit (Ambion, TX) following the specific protocol for RNA extraction which includes a DNase digestion step to ensure no DNA contaminations. RNA quantification was performed spectrophotometrically and a sample was considered adequate if the A_{260}/A_{280} ratio was 1.8–2.0. Good-quality RNA samples were chosen and 2 μg was used for cDNA synthesis using Superscript II RT kit (Invitrogen, CA) according to the manufacturer's instructions.

Expression of viral mRNAs was quantified by using the SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA) with a StepOne real-time detection system (Applied Biosystems, Foster City, CA). Expression levels of viral mRNA were normalized to the stably expressed housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) as an endogenous control and reference gene. All primers (Invitrogen, CA) were designed using the Primer Express package (Applied Biosystems, Foster City, CA) and were directed against highly conserved regions. Each set of primers was also tested for Raji and P3HR1 (cell line infected with type 1 and 2 EBV, respectively) cDNA to ensure that they worked across EBV sequence variations. PCR reactions were prepared in a final volume of 25 μL containing 1× SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA), 500 nM primers and 5 μL cDNA (equivalent to 100 ng RNA input). Universal thermal-cycling conditions were used. For calibration and generation of standard curves, each run also contained serial dilutions of Raji or P3HR1 cells cDNA (corresponding to 10 pg, 100 pg, 1 ng, 10 ng and 100ng input RNA) obtained as mentioned above. A melting curve analysis was performed to verify the specificity of the products and the cycle threshold (Ct) values were obtained for each gene at every point of the curve. All test samples were run in duplicate and expressed as an average. Template-

negative reaction served as control. Raji or P3HR1 cell lines were used as calibrators to normalize the EBV+ samples.

The specific primers used in real-time quantitative PCR (qPCR) for the amplification of latent genes (EBNA1, EBNA2, EBNA3C, LMP1 and LMP2A), lytic gene (BZLF1) and endogenous (HPRT) gene are listed in Supporting Information Table 1.

Real-time PCR data analysis

Relative quantification of EBV genes expression was performed as recommended by the manufacturers (ABI Prism 7700 Sequence Detection System User Bulletin #2; http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). Briefly, amplification of target genes cDNA and the reference gene HPRT cDNA were monitored continuously by changes in fluorescent intensity, using the StepOne software. The corresponding amplification plots were then used to determine the Ct value, defined as the number of cycles required by fluorescent signals to cross the threshold.

In most situations, the amplification efficiency of the gene of interest and reference (housekeeping) gene is different, and hence values for the relative quantification of viral genes were calculated by the Pfaffl Method.[24] From the standard curves of each gene analyzed, efficiencies ranged from 88 to 98%. The mean value of the Ct of an equivalent quantity of RNA input from Raji or P3HR1 cell lines was used as calibrator. When no signal was detected in a given sample, we assigned the arbitrary Ct value of the maximal cycle number ($Ct_{max} = 40$).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software, CA). Categorical variables were analyzed using Fisher's exact test. Mann–Whitney test was used to compare the means between T-cell population markers in relation with EBV presence. Correlations between data were determined employing Pearson's rank correlation index. Event-free survival (EFS) was defined as the time from initiation of treatment to the event, defined as nonresponse, death from any cause, tumor progression or second malignancy, or until the date of last follow-up. Survival distributions were estimated according to the Kaplan–Meier method, with differences compared by the log-rank test. All tests were two-tailed, and a p-value of <0.05 was considered statistically significant.

Results

Patient characteristics

Twenty-five DLBCL pediatric cases were included in this analysis. Median age at diagnosis was 8 years (range, 2–16 years). There was no gender predominance, with 12 males and 13 females. Advanced stages (III and IV) were observed in 9/17 (53%) of cases. Nineteen (76%) patients had extranodal presentation. Concerning clinical characteristics of the DLBCL patients, six were immucompromised, four had a primary immunodeficiency (two common variable immunodeficiency, one Burkley's syndrome and one ataxia-telangiectasia), one was PTLD and one was HIV+ (human immunodeficiency virus). On the basis of Hans' IHC classification,[23] 18 cases, with enough biopsy material, were classified being 7 (39%) of them GC subtype and 11 (61%) non-GC subtype.

Concerning therapy, all patients were treated with the consensus GATLA (*Grupo Argentino de Tratamiento de Leucemia Aguda*) treatment protocols (9-LNHP-94-GATLA and 1-LNHP-2000 GATLA). Patients' follow-up period ranged from 1 to 157 months (median, 36 months). Four of them were lost at follow-up after diagnosis. Among the 21 patients who accomplished follow-up 9/21 died, 1/21 relapsed after chemotherapy, and the remaining 12 patients remain alive.

Table 1. Demographic and histological characteristics of pediatric DLBCL series in relationship with EBV status (positive *vs.* negative)

Patients' characteristics	EBV status[1] Positive (%)	EBV status[1] Negative (%)	p-Value[2]
Age (years)			
≤10	9/15 (60)	6/15 (40)	0.018[3]
>10	1/10 (10)	9/10 (90)	
Gender			
Male	5/12 (42)	7/12 (52)	1.000
Female	5/13 (38)	8/13 (62)	
Histological subtype			
GC	3/7 (43)	4/7 (57)	1.000
Non-GC	6/11 (55)	5/11 (45)	
ND	1/7 (14)	6/7 (86)	
Clinical stage			
I/II	2/8 (25)	6/8 (75)	1.000
III/IV	3/9 (33)	6/9 (67)	
ND	5/8 (63)	3/8 (37)	
Primary site			
Nodal	4/6 (67)	2/6 (33)	0.175
Extranodal	6/19 (32)	13/19 (68)	
Immunological status			
Immunocompromised	5/6 (83)	1/6 (17)	0.023[3]
Immunocompetent	5/19 (26)	14/19 (74)	

[1]EBV status determined by EBERs ISH. [2]p-Value as determined by Fisher's exact test. [3]Statistically significant.
Abbreviations: GC: germinal center; ND: not determined (data not available).

EBV analysis

EBERs expression related to demographic and histological characteristics is summarized in Table 1. Latent EBV infection restricted to the nucleus of tumor cells, as determined by EBERs ISH, was detected in 10/25 (40%) DLBCL cases

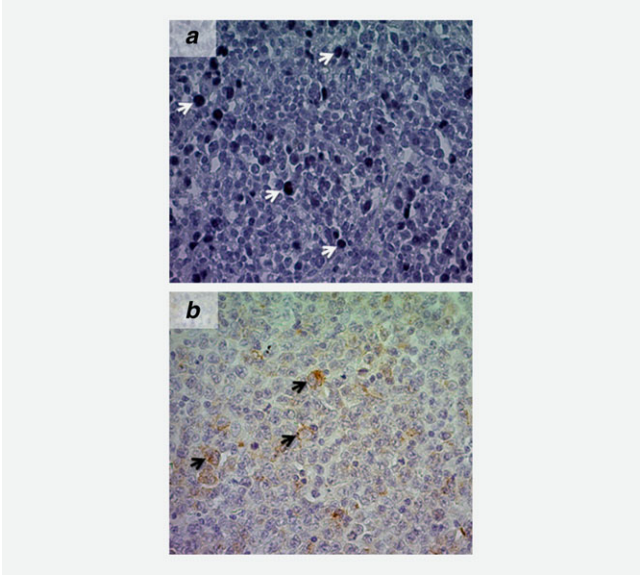

Figure 1. Representative expression of EBERs and LMP1 in pediatric DLBCL (patient 2). (*a*) Nuclear localization (arrows) of the EBERs in neoplastic cells by ISH. (*b*) Membranous and cytoplasmic localization (arrows) of LMP1 in neoplastic cells by IHC. Original magnification: 400×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 1*a*). As primary EBV infection occurs mainly in early childhood in Argentina, concerning EBV presence in DLBCL patients was sorted in two age groups: younger than 10 years ($\leq$10 years) and 11–16 years (>10 years). The number of EBV+ cases was significantly higher among patients $\leq$10 years than among patients >10 years ($p = 0.018$, Fisher's exact test). Five out of six (83%) immunocompromised patients and 5/19 (26%) immunocompetent patients showed EBERs+ ISH. EBV presence associated with immunosuppression status was statistically significant ($p = 0.023$, Fisher's exact test).

EBV positivity was more frequently observed in male sex cases (42 *vs.* 38%), advanced stages (33 *vs.* 25%), nodal involvement (67 *vs.* 32%) and non-GC subtype (55 *vs.* 43%); however, these differences did not reach statistical significance ($p > 0.05$, Fisher's exact test).

In all EBERs+ cases, we performed IHC for the detection of LMP1 and LMP2A viral proteins. LMP1 expression localized at the cytoplasm and surface membrane of neoplastic cells was positive in 6/10 (60%) EBV+ samples (Fig. 1*b*). By contrast, none of the samples was positive for LMP2A staining of neoplastic cells (data not shown).

Owing to biopsy preservation conditions, good-quality RNA for qPCR could be obtained from 12/25 samples ($n =$ 6 EBV+ and $n = 6$ EBV−). RNA from EBV+ samples was named as patient (pt.) 1, 2, 3, 7, 8 and 10. Among these samples, four out of six were immunocompetent and two out of six were immunosuppressed patients. Based on qPCR results, several distinct patterns of gene expression were identified and summarized in Table 2. Concerning EBV transcript expression, LMP1s were detected in all samples, with variable expression level among cases, whereas EBNA1, EBNA2, EBNA3C and BZLF1 were expressed in five cases. Finally, only one sample expressed LMP2A transcripts, but in a very low levels (data not shown). All six EBV− samples showed no expression of any EBV gene. Relative expression levels of EBV transcripts are shown in Figure 2.

Table 2 summarizes the results in the ten pediatric EBV+ DLBCL cases, where the latency program III was the most frequently observed, together with lytic gene expression (pt. 1, 2, 3, 7 and 10). Type II-*like* latency pattern with no presence of LMP2A and lytic transcripts was described in one patient (pt. 8). Finally, based exclusively on IHC results, one case presented latency I (pt. 9), and the remaining three EBERs+ samples displayed a latency type II/III (pt. 4, 5 and 6).

With regards to patient's outcome, in Kaplan–Meier survival analysis, the estimated 5-years EF was 57%. The 5-year EFS for EBV+ cases was 40%, compared to 73% observed in EBV non-associated cases although this difference was not statistically significant ($p > 0.05$, log rank test).

Tumor microenvironment analysis

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 percentage of CD3, CD4, CD8 and Foxp3 T-cell markers (Fig. 3*a*–3*d*) in EBV+ *versus* EBV− cases. Foxp3 expression on T-lymphocytes is an accurate marker of Tregs.[25] No significant correlation was found between EBV status and each cellular marker investigated ($p > 0.05$, Fisher's exact test). Within the tumor microenvironment, $CD3^+$ T cells as well as T-cell subsets showed no specific distribution, neither paracortical nor follicular. Percentages of different T-cell markers varied considerably between individual DLBCL cases (CD3: range 2–36%, median 17%; CD4: range 0.7–12%, median 4%; CD8: range 0.8–36%, median 11%; Foxp3: range 0–29%, median 0.6%). A direct correlation was observed between the percentages of CD3+ and CD4+, CD8+ or Foxp3+ cells and between CD8+ and Foxp3+ cells ($r = 0.561$, 0.800, 0.490 and 0.588; $p = 0.024$, 0.0002, 0.046 and 0.017, respectively). As regard to age groups ($\leq$10 or >10 years), we found no significant differences in the tumor microenvironment composition in any of the T-cell markers analyzed (data not shown).

Discussion

A number of studies have demonstrated the association between EBV infection and several subtypes of NHL.[5] Regarding patients with DLBCL, there are few studies looking for EBV involvement on its lymphomagenesis; furthermore, this association is still under confirmation. In our study, we assessed EBV association in a series of pediatric DLBCL (including immunosuppressed and immunocompetent patients) from our hospital. EBV association with DLBCL rose up to 40%, nevertheless, when excluding immunosuppressed patients, this association decreased to 26%. The overall percentage was very similar to those observed in the

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

		ISH		IHC		qPCR								
Patient No.	Immunological Status	EBERs	+ cells (%)[1]	LMP1	LMP2A	EBNA1	EBNA2	EBNA3C	LMP1	LMP2A	BZLF1	Latency Expression	Lytic Expression	Outcome
1	IC	+	40	−	−	+	−	+	+	−	+	III	+	Dead
2	IS	+	70	+	−	+	+	+	+	−	+	III	+	Dead
3	IC	+	30	+	−	+	+	+	+	−	+	III	+	Alive
4	IS	+	60	+	ND	ND	ND	ND	ND	ND	ND	II/III	ND	Dead
5	IS	+	80	+	ND	ND	ND	ND	ND	ND	ND	II/III	ND	Dead
6	IS	+	70	+	−	ND	ND	ND	ND	ND	ND	II/III	ND	Dead
7	IS	+	30	+	−	+	+	+	+	+	+	III	+	Dead
8	IC	+	20	−	−	−	−	−	+	−	−	II[2]	−	Alive
9	IC	+	90	−	−	ND	ND	ND	ND	ND	ND	I	ND	Alive
10	IC	+	50	−	−	+	+	+	+	−	+	III	+	LF

[1]≥20% EBERs+ tumor cells as a cut-off value. [2]Latency type II *like*.
Abbreviations: IS: immunosuppressed; IC: immunocompetent; EBNA: Epstein–Barr nuclear antigen; LMP: latent membrane protein; BZLF: intermediate-early EBV gene; qPCR: quantitative polymerase-chain reaction; IHC: immunohistochemistry; NA: no amplification; ND: not determined; LF: lost at follow-up.

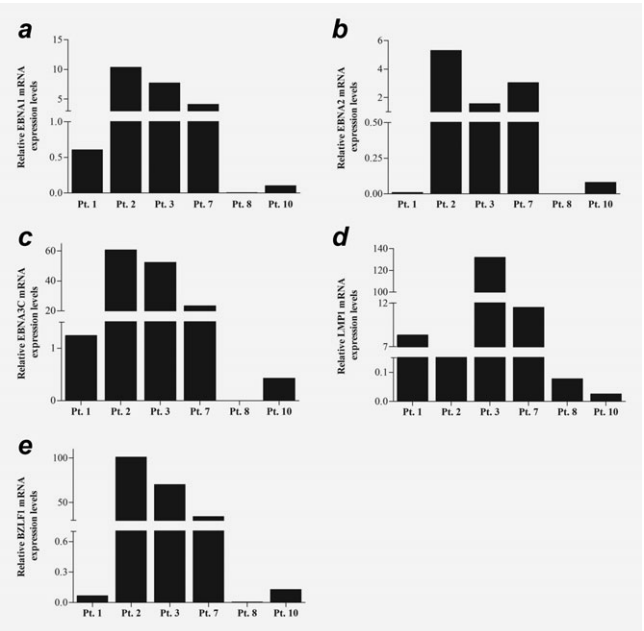

Figure 2. qPCR was performed to determinate the mRNA relative expression levels of (*a*) EBNA1, (*b*) EBNA2, (*c*) EBNA3C, (*d*) LMP1 and (*e*) BZLF1. HPRT was used as a reference gene. The bars represent expression levels in EBV+ DLBCL samples (patients 1, 2, 3, 7, 8 and 10), normalized to mRNA expression in EBV+ reference cell line as calibrator.

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

T-cell marker	EBV status[1]	Mean rank[2]	*p*-Value[3]
CD3	Positive	14.7	0.432
	Negative	19.5	
CD4	Positive	3.6	0.315
	Negative	4.9	
CD8	Positive	13.0	0.659
	Negative	10.5	
Foxp3	Positive	4.8	0.812
	Negative	2.2	

[1]EBV status determined by EBERs ISH. [2]*p*-Value as determined by Fisher's exact test. [3]The number represents the mean of the (no.+ cells/field)/(no. total cells/field) × 100.

previous reports restricted to African children, which analyzed series of NHL that included a subset of DLBCL, and described an association of 43–44.4% between EBV and this lymphoma subtype[26,27] although the immunological status of these patients was not clearly specified. Although it should be kept in mind that the immunocompetent analyzed group is small, when only these patients were considered, EBV association still remained higher than the previously reported for elderly patients from Asia (8–10%), Latin American countries (9–15%)[9–11] and also from Western populations (<5%).[12,13] Further analysis in a larger series would reinforce these findings.

A higher prevalence of EBV in lymphomas diagnosed in children younger than 10 years old has been previously described by our group in pediatric HL[28] and B-NHL.[29] In our present series, we confirmed that in pediatric DLBCL, EBV expression is statistically associated with patients of ≤10 years. This was not unexpected, as in Argentina children are infected quite early in their life and nearly 70% of children are seropositive by the age of 2.[30] This observation together with the high association in childhood HL as well as B-NHL confirms that EBV could be an important cofactor in B-cell lymphomagenesis in younger children, may be arising as a late complication of EBV primary infection. In several EBV-associated malignancies, there is a clear male prevalence.[31]

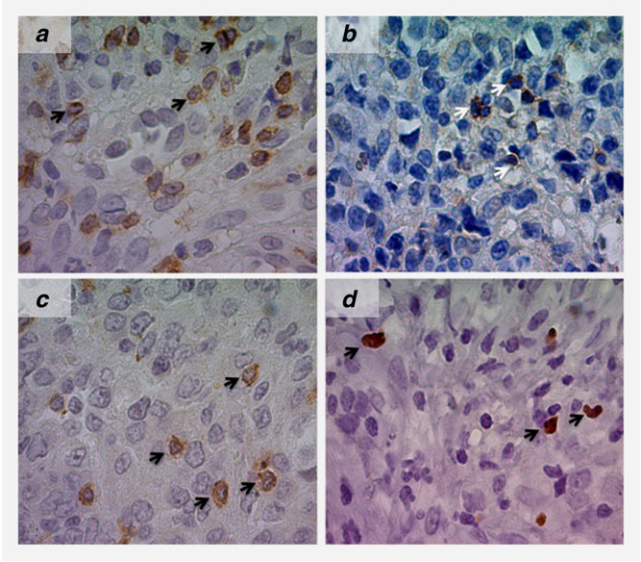

Figure 3. Representative IHC of (*a*) CD3, (*b*) CD4, (*c*) CD8 and (d) Foxp3 shows positive staining of T cells (arrows) within tumor microenvironment in a pediatric DLBCL (patient 1). Original magnification: 1,000×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

We previously showed that in pediatric EBV+ NHL, there was not gender predominance.[32] Despite an apparently male predominance in our series, there were no statistically significant differences as regards gender and EBV association.

NHLs have typically been the most frequent malignancy of immunosuppressed subjects, whether secondary to HIV, prior to organ transplantation or as a congenital condition.[33] It is likely that deficient T-cell function is responsible, at least in part, for this association. Such immunosuppression conditions tend to increase the likelihood of EBV malignancies, by deregulating T-cell immune surveillance that normally controls EBV latent infection in immunocompetent patients, and by reactivating this latent infection in some infected cells to a lytic one. A high percentage of EBV+ NHL, which included a few DLBCL cases, was reported in immunosuppressed infected children.[34,35] In agreement with these findings, we found statistically significant association between immunosuppressed patients and EBV.

Each EBV-encoded latent gene contributes in a different way to achieve transformation.[6] LMP1 is essential for EBV-induced B-cell transformation *in vitro*, induces many changes associated with B-cell activation stimulating growth and differentiation of B-cells and also upregulates expression of the antiapoptotic proteins. LMP2A prevents reactivation of EBV in latently infected cells by blocking tyrosine kinase phosphorylation. EBNA1 allows the EBV genome to be maintained in the B cell as an episome and also has antiapoptotic effects *in vitro*. EBNA2 is the main viral transcription factor that induces expression of LMP1 and LMP2, as well as cellular proteins that enhance the growth and transformation of B cells. A role for EBNA3A and EBNA3C together in apoptosis protection has also been reported, as they co-operate with, or mediate their effects through downregulation of proapoptotic Bim protein.[36] EBERs transcripts may also be important for oncogenesis and resistance to apoptosis.

Previous studies suggested that EBV+ DLBCL in elderly patients generally have a viral latency type II or III pattern.[10,11,13,14] Besides, the same was reported in a study performed on pediatric B-NHL.[37] Based on our results (Table 2) in the pediatric EBV+ DLBCL series, the latency program III was the most frequently observed, together with lytic gene expression. BZLF1 lytic gene expression could mirror a percentage of cells undergoing lytic phase, as previously observed for BL.[38] LMP2A expression in the absence of LMP1 was observed in NPC,[39] but unexpectedly, LMP1 expression without LMP2A demonstrated in our study was not previously observed. Therefore, further studies, particularly *in vitro*, would be necessary to better elucidate this observation. The absence of LMP1 protein expression by IHC together with transcript detection by qPCR in two cases could be explained by the low levels of LMP1 transcript detected, which might not render enough protein quantity to be noticed by IHC. Hence, in our hands, latency patterns have proven to be more complex than indicated on the previously published reports.

To our knowledge, our study is the first attempt to compare EBV+ DLBCL latency profiles in immunosuppressed/immunocompetent pediatric patients. It was previously demonstrated that EBV+ lymphomas in immunocompromised patients were associated with a latency III pattern.[6] Our data, restricted to pediatric DLBCL, had indicated that the presence or absence of immunosuppression apparently did not influence the EBV viral latency pattern. This findings are in line with the analysis of Nguyen-Van et al.[40] who also observed in a small series like ours, similar results in 14 EBV+ DLBCL cases, in both immunocompetent and immunocompromised patients aged more than 50 years.

A direct 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.[41] On the other hand, despite extensive study, the control of LMPs gene expression in different latent states is not fully understood. In B cells displaying latency III type infection, LMP1 and LMP2A/B expression are dependent on expression of EBNA2.[42] However, in latency II pattern, it has been demonstrated that LMP1 expression can be absolutely independent of EBNA2 expression.[43] In our case series, the expression of LMP1 correlated with the other genes analyzed but did not reach statistically significance. In our series, neither EBV presence nor latencies patterns seemed to have a clear prognostic impact although most patients with a latency type II/III had an aggressive disease and died after diagnosis.

The role of EBV in the induction of T-cell subpopulation balance has attracted the interest of many researchers. Although EBV-specific CTLs can be detected in HL and NPC as well as

they have been shown to kill LMP1- and LMP2-expressing cells *in vitro*, they are unable to eliminate EBV-infected tumor cells *in vivo*.[44,45] This failure may be owing to the increased recruitment of Tregs that control the activation of autoaggressive $CD4^+$ and $CD8^+$ T cells, thereby preventing autoimmunity.[18] It was demonstrated *in vitro* that viral EBNA1 protein is able to attract Tregs.[21] However, Alvaro et al.[46] showed that Treg number in primary HL do not vary significantly with EBV status, and thus, alternative *in vivo* mechanisms must exist to recruit these cells in the majority of EBV-tumors. On the other hand, in B-cells, expression of the immunomodulator interleukin-10 can be induced by some EBV gene products, including those from immediate-early lytic gene, BZLF1, and two latent products, LMP1 and EBERs.[47–49] Therefore, these antigens may induce immunomodulation to bias microenvironment in favor of immune suppression, affecting T cells in their functionality without major changes in their numbers. We have not found a relationship between EBV presence and $CD4^+$ or $Foxp3^+$ cell number in pediatric DLBCL, confirming the lack of correlation between viral presence and different subtypes of T cell. On the other hand, although an increased number of CTLs has been linked to EBV expression in HL,[50] there was no relationship between $CD3^+$ and $CD8^+$ cells with EBV presence in our pediatric DLBCL series (Table 3).

Conclusions

In summary, our study adds to the growing body of literature of EBV-positive DLBCL, and is the first report demonstrating the association of EBV in pediatric DLBCL patients in a Latin American country. This finding, together with prior epidemiologic observations in adult population and the known oncogenic potential of EBV *in vitro*, suggests that EBV plays a role in the pathogenesis of DLBCL. We hypothesize that infection with EBV early in life increases the risk of developing this malignancy. Furthermore, these data emphasize the need for epidemiological studies involving different centers from our geographic region, to confirm this hypothesis.

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