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Epstein Barr virus in relation to apoptosis markers and patients' outcome in pediatric B-cell Non-Hodgkin lymphoma

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ABSTRACT

In this study, we investigated Epstein Barr virus (EBV) presence, associated to proliferation and apoptosis proteins in pediatric B-cell Non-Hodgkin lymphoma (B-NHL). EBERs, Ki67, active caspase 3, Bax and Bcl2 were analyzed on B-NHL tissue from 40 patients. Forty percent showed EBV expression, significantly higher among patients $\leqslant$10 years ($P = 0.027$), and associated with immunosuppression ($P = 0.020$), but not associated apotosis markers. However, EBV was associated with a worse event-free survival ($P = 0.016$), particularly under immunosuppression. Even though EBV did not seem to alter apoptotic pathways, it exhibited survival disadvantage and could be an important cofactor in B-cell lymphomagenesis in younger children.

1. Introduction

In Western countries malignant Non-Hodgkin lymphomas (NHLs) represent the fourth most common childhood cancer, accounting for about 6% of pediatric malignancies [1]. The majority of pediatric NHLs derives from B cells (B-NHL) and represents primary high-grade lymphomas. Among NHLs, the three most prevalent entities are Burkitt lymphoma (43%), diffuse large B-cell lymphoma (13%) and B-lymphoblastic lymphoma (7%) [2]. In Argentina, all cases of pediatric NHL diagnosed in public hospitals are registered in the *Registro Oncopediátrico Hospitalario Argentino Fundación Kaleidos* (ROHA). 11,445 pediatric tumors were reported during the period 2000–2008. Of these, 12.7% were lymphomas including 42.5% diagnosed as Hodgkin lymphoma (HL) and the remaining 57.5% as NHL [3].

More than 90% of adults worldwide are infected with Epstein Barr virus (EBV) [4]. EBV preferentially infects B-lymphocytes through the binding of the major viral envelope glycoprotein gp350 to the CD21 receptor on the surface of B cells [5], where it establishes a lifelong persistent infection. In developing countries, primary infection with EBV usually occurs during the first few years of life and is often asymptomatic. Meanwhile, in developed populations primary infection is frequently delayed until adolescence or young adulthood, and produces, in many cases, the characteristic clinical features of infectious mononucleosis [6].

Whereas establishment of viral persistence after primary infection with EBV normally results in an asymptomatic carrier state, EBV might also promote the development of B-cell lymphomas. The EBV-associated B-cell lymphomas include Burkitt lymphoma, Hodgkin lymphoma, diffuse large B-cell lymphoma of the elderly and post-transplant lymphomas [7].

Human tumor viruses display different direct or indirect mechanisms of cell transformation. In EBV mediated oncogenesis, several experiments reveal that viral proteins

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directly contribute to growth transformation. It has been demonstrated that Latent membrane protein 1 (LMP1) stimulates or inhibits various signaling pathways, resulting in transformation and survival of LMP1-expressing cells, especially in B cells [8]. Moreover, it has been demonstrated that LMP1 has an effect on different Bcl2 family members which in turn play a central role in the regulation of apoptosis. The Bcl2 family is composed of both pro-apoptotic and anti-apoptotic proteins [9]. LMP1 leads to overexpression of anti-apoptotic molecules such as Bcl2 [10] and to the inhibition of pro-apoptotic factors such as Bax [11]. It has been proposed that other EBV proteins (particularly EBNA1, and the EBV-encoded RNAs, EBERs), may possess also anti-apoptotic properties and contribute to increased tumorigenicity. Furthermore, EBV proteins EBNA3A and EBNA3C functionally interact with the pro-apoptotic protein Bim and consequently inhibit its expression [12].

Apoptotic cell death can be initiated by two alternative convergent pathways: the extrinsic pathway, which is mediated by cell surface death receptors, and the intrinsic pathway, which is mediated by the mitochondria. In both of them, cysteine aspartyl-specific proteases (caspases) that cleave cellular substrates are activated and particularly, the activation of the effector caspase 3 is crucial for the execution of apoptotic cell death. Defects in the apoptosis-inducing pathways can eventually lead to expansion of a population of neoplastic cells. Moreover, since chemotherapy and irradiation act primarily by inducing apoptosis, defects in the apoptotic pathway can make cancer cells resistant to therapy [13]. It is probable that those patients with a fatal outcome carry tumor cells intrinsically resistant to chemotherapy-induced cell death because in most of them, treatment fails to induce complete tumor remission [14–18].

Constitutively activated proteins of the apoptotic pathways may contribute to the clinical characteristics of EBV-positive tumors, since there are several *in vitro* studies that evidence the interaction between EBV antigens and key apoptotic proteins. In consequence, it may be interesting to investigate these links to further clarify their pathogenic role in B-NHL. Besides, both apoptotic proteins and EBV have been correlated with patient's outcome in B and T-cell NHLs [19–24]. Therefore, our aim was to investigate the relationship between EBV presence associated to cell proliferation as well as apoptosis-related proteins in pediatric B-NHL and to correlate these findings with patient's outcome. This analysis might shed light over the still obscure field of EBV-mediated pediatric B-NHL lymphomagenesis.

2. Materials and methods

2.1. Patients and tissue preparation

Formalin-fixed paraffin-embedded B-NHL tissue samples from 40 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 1990 to 2008. Institutional guidelines regarding human experimentation were followed, and they were in accordance to the Helsinki Declaration of 1975.

Diagnosis was made from biopsies taken from the primary tumor. Histological review was done by one of the investigators and cases were classified according to the World Health Organization (WHO) scheme for B-cell NHL [25].

2.2. Immunohistochemistry

Immunohistochemical staining for B-cell lymphoma differential diagnosis as well as proliferation and apoptosis analysis, was performed on formalin-fixed paraffin-embedded tissue sections with a panel of antibodies: CD3 (clone F7.2.38, 1:50), CD10 (clone 56C6, 1:50), CD20 (clone L26, ready to use), CD30 (Clone Ber-H2, ready to use), CD45 (Clone 2B11, ready to use), Bcl6 (Clone PG-B6p, 1:10), ALK (Clone ALK1, ready to use), Ki67 (Clone MIB-1, ready to use) (Dako, Carpinteria, USA) Bcl2 (clone E17, 1:100, Biogenex, San Ramon, CA, USA), Bax (polyclonal, 1:20, Biogenex), active caspase 3 (aCasp3) (polyclonal, 1:1000, R&D systems, Minneapolis, USA). Pretreatment of the sections with 10 mM sodium citrate buffer (pH 6) in microwave oven was performed. Immunohistochemical detection of monoclonal and polyclonal antibodies was carried out using a streptavidin–biotin complex-peroxidase detection system (Labeled Streptavidin Biotin, LSAB, Dako) according to the manufacturer's instructions. Visualizations were performed using diaminobenzidine (DAB) as chromogen. Appropriate positive controls were immunostained for each antibody, and negative controls were performed with the same method without the primary antibody.

The counting of Bax, Bcl2, aCasp3 and Ki67 was performed as follows: a score system was adopted by using the 100× objective lens and counting at least 10 fields selected at random on the basis that they contained immunopositive cells. The number of immunopositive cells was divided by the total number of counted cells 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.

2.3. EBERs in situ hybridization

EBERs *in situ* hybridization (ISH) was performed on formalin-fixed paraffin-embedded tissue sections using fluorescein isothiocyanate (FITC)-conjugated EBERs oligonucleotides as probes (Dako). A monoclonal antibody anti-FITC labeled with alkaline phosphatase was used for detection of hybridized sites (Dako). A case was considered EBV positive when positive nuclear staining specific restricted to tumor cells in at least one malignant cell was observed.

We used a well-known mixed cellularity HL with specific staining in Hodgkin Reed Sternberg (HRS) cells as a positive control.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, Cal-

ifornia, USA). Fisher's exact test or Chi square tests ($\chi 2$) were used for statistical analysis when appropriate. Mann–Whitney test was used to assess correlation between proliferation or apoptosis markers and EBV presence. Event-free survival (EFS) was defined as the time from initiation of treatment to the event. An event in EFS was failure to achieve complete remission (CR), relapse after a prior CR, or death from any cause. Survival distributions were estimated according to the Kaplan–Meier method. Differences in survival distributions were tested with the log rank test. All tests were two-tailed, and a P value lower than 0.05 was considered statistically significant.

3. Results

Forty B-NHL pediatric patients were included: 23 Burkitt's lymphoma (BL) and 17 diffuse large B-cell lymphoma (DLBCL). Three DLBCL patients were immucompromised, 2 of them have primary immunodeficiency and 1 was HIV+ (human immunodeficiency virus). One BL patient was also HIV+ [25]. Patients' median age was 7 years (age range: 1–16 years), and male:female ratio was 5:3.

EBERs expression associated with demographic and histological characteristics is listed in table 1. Latent EBV infection of tumor cells, as determined by EBERs *in situ* hybridization, was detected in 16 out of 40 (40%) B-NHL (Fig. 1). EBV presence was more prevalent in DLBCLs (8/17, 47%) than in BLs (8/23, 35%), but this difference was not statistically significant ($P = 0.522$, Fisher's exact test). EBV expression was not statistically associated with early (I/II) or advanced (III/IV) stage ($P = 0.673$, $\chi 2$ test), and with site of involvement (nodal vs. extranodal) ($P = 0.804$, $\chi 2$ test). Moreover, in those patients in whom LDH record was available, EBV presence did not show statistically significant association with LDH values, ($P = 0.141$, Fisher exact test). As previously mentioned, four patients were immunosuppressed. All immunocompromised patients all showed EBERs positive *in situ* hybridization, and EBV association with immunosupression was statistically significant ($P = 0.020$, Fisher's exact test). Since primary EBV infection occurs mainly in early childhood in Argentina, EBV presence in B-NHL biopsies was analyzed in two pediatric age groups: younger than 10 years ($\leqslant 10$) and 11–18 years (>10). The number of EBV positive cases was significantly higher among patients $\leqslant 10$ years than among patients >10 years ($P = 0.027$, Fisher's exact test).

Table 2 shows mean rank for EBV positive vs. EBV negative cases for Ki67, Bax, Bcl2 and aCasp3. In the course of our analysis, we first performed Fisher exact test comparing EBV status vs. each marker positivity considering several cut offs (10%, 20% and 30%), but the differences were not significant for any tested marker's cut off ($P > 0.05$). Therefore, we decided to use the Mann–Whitey *U* test to analyze the EBV expression status (EBV positive vs. EBV negative cases) in relation to mean rank percentage of Bcl2, Bax, aCasp3 or Ki67 (Table 2, Fig. 2A–D). No significant correlation was found between EBV status and each cellular apoptosis or proliferation marker investigated ($P > 0.05$).

Table 1
Demographic and histological characteristics of pediatric B-cell NHL. Correlation with EBERs expression.

Patients' characteristics	*N*	EBERs			*P*
		pos	*neg*	*% pos*	
Age (yr)					
$\leqslant 10$ years	29	15	14	52	0.027*
>10 years	11	1	10	9	
Sex					
Male	25	9	16	36	0.528[a]
Female	15	7	8	47	
Immunological status					
Immunocompromised	4	4	0	100	0.020*
Immunocompetent	36	12	24	33	
Histological subtype					
Burkitt lymphoma	23	8	15	35	0.522[a]
Diffuse large B-cell lymphoma	17	8	9	47	
EBV association		**16**	**24**	**40**	

[a] *p* as determined by Fisher exact test.
* Indicates statistically significance.

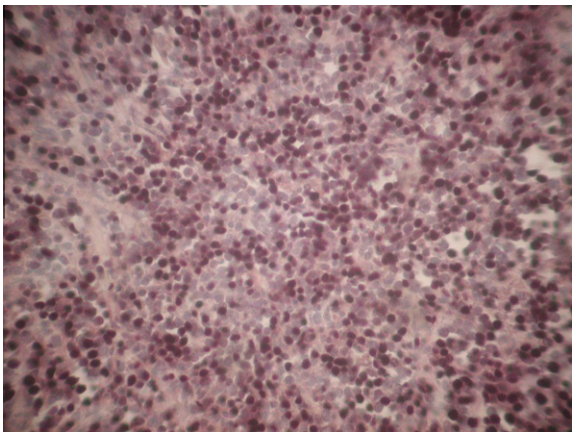

Fig. 1. EBERs *in situ* hybridization for EBV shows positive staining in the nucleus of neoplastic cells in a Burkitt lymphoma lymph node biopsy, original magnification 40×.

Table 2
EBV expression status (positive vs. negative) related to the percentage of expression of apoptotic and proliferation proteins in tumor cells (Mann–Whitney test).

	Viral status	N° cases (%)	Mean rank	*P*
Bcl2	EBV pos	15 (37.5)	17.85	0.241
	EBV neg	25 (62.5)	5.77	
Bax	EBV pos	16 (40)	27.77	0.348
	EBV neg	24 (60)	17.49	
aCasp3	EBV pos	16 (40)	2.36	0.571
	EBV neg	24 (60)	2.33	
Ki67	EBV pos	16 (40)	60.63	0.370
	EBV neg	24 (60)	51.11	

Survival analyses of well-defined risk factors were also evaluated, when available, in this series. The performance status was not available in the medical record of these patients. Concerning therapy, all patients were treated with the corresponding protocol according to each clinical characterization at diagnosis. However, since we have enrolled patients during a long period, the treatment protocols have slightly changed along the years, so we could not assume that all of them have received a homogeneous therapy. Prognostic factors (LDH, B-symptoms and stage) were analyzed in Kaplan Meier statistical survival analysis but they did not show statistical significance ($p > 0.05$, log rank test).

In Kaplan Meier survival analysis, patients follow up period ranged from 1 to 174 months (median 33.5 months), and the estimated 5 years EFS was 67.8%. The 5 year EFS for EBV-positive cases was 32.6%, compared with 75.6% observed in EBV-non-associated cases, this difference was indeed statistically significant ($P = 0.016$, log rank test) (Fig. 3). Since survival analysis exclusively in the immunocompetent subgroup of patients do not achieve statistical differences between EBV+ and EBV− patients ($P = 0.932$).

4. Discussion

BL and DLBCL constitute the majority of B-NHL in children and adolescents but still, only a few studies including

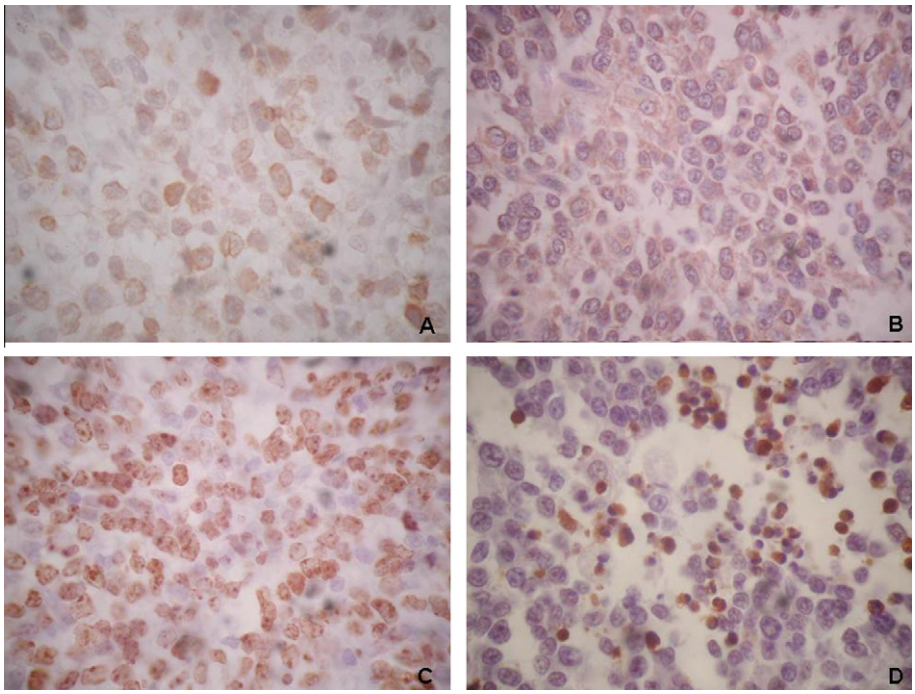

Fig. 2. (A) Bcl2 (100×), (B) Bax (100×), (C) Ki67 (100×), D) aCasp3 (100×) positive staining in neoplastic cells assessed by immunohistochemistry.

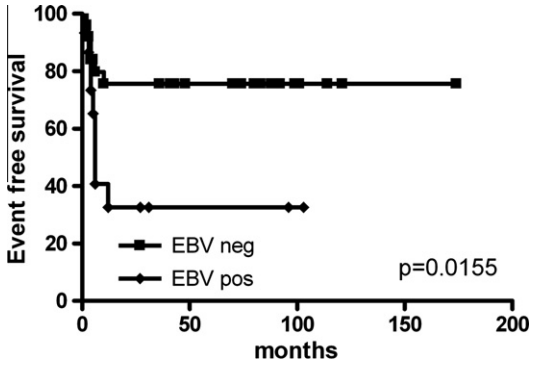

Fig. 3. Kaplan–Meier analysis. Patients' event-free survival curves correlated with EBV expression in 31 patients with survival available data (12 EBV+ patients and 19 EBV− patients).

pediatric patients were carried out [16,26]. In the present study, we analyzed EBV expression related to different components of the apoptotic pathway in pediatric BL and DLBCL cases from a single institution and found 40% of EBV expression among the studied cases. T-cell immunocompromised patients are at high risk of developing B-cell lymphomas, particularly in post-transplant patients [5], thus, EBV statistically association with immunosupressed condition observed in our series was not unexpected.

In Argentina, primary infection appears to occur in early childhood since by the age of 2, 75% of children have seroconverted, and 89% of patients are infected by EBV by the age of 3 [27]. Our group has previously observed a high rate of EBV association in patients younger than 10 years old in pediatric HL [28]. In our series, we confirmed that in pediatric B-NHL EBV expression is statistically associated with patients $\leqslant$10 years. In fact, a significant association between EBV infection and lower age was observed, since EBV+ cases showed a median age of 5 years, vs. a median of 10 years observed in EBV− cases (Mann–Whitney test; $P = 0.006$). This observation 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.

Usually, three epidemiologically distinct forms of BL with different rates of EBV association are recognized. First, the "endemic" form found in areas of equatorial Africa and Papua New Guinea, where malaria is holoendemic, and is 100% EBV genome-positive. Second, an adult form defined as "AIDS (Acquired immune deficiency syndrome)-BL" which proved to be very common among HIV-infected individuals, often appearing as one of the first symptoms of AIDS; having a rate of EBV association of 30–40%. Finally, the "sporadic" form, mainly found in children, which shows different degrees of EBV association depending on the geographic area. In Western countries it shows only 15–20% of EBV expression; but shows higher rates of association in other locations [29]. Interestingly, in the developing population from Brazil, variations in EBV association ranging from 29% in the South to 76% in the North have been described [30]. This fact probably suggests different epidemiological characteristics across Brazil which implies a distinct role of EBV in BL pathogenesis [31]. In our BL subgroup, EBV association matched that of the "sporadic" form, and raised up to 35% (8/23 EBV+

cases), which is lower than the 47% previously reported in adult Argentinean population [32].

EBV presence in DLBCL has been described to a lesser extent by other research groups which have focused on adult and elderly patients. Actually, Park et al. [19] identified 9% of EBV-associated DLBCL in an adult series, with a higher association rate among the elder patients. Alternatively, Yamauchi et al. described an EBV association of only 5% in patients younger than 30 years old [33]. However, when comparing both previous results with our pediatric series, we found a notably higher EBV association with DLBCL (47%) than the others. This fact indicates that EBV might have an important role in pediatric DLBCL development and requires further investigation.

Some viruses are known to encode proteins which suppress or delay apoptosis. Such "death prevention" viral genes could contribute to cancer development if they allow cells that were destined to self-destruction to continue proliferating. Several tumor-virus-encoded proteins exhibit apoptosis inhibiting activity [34]. In fact, EBV-induced growth transformation is not simply a matter of triggering proliferation by activating cell cycle, but it also involves modulation of the apoptotic pathway to enhance cell survival [35]. In both extrinsic and intrinsic apoptotic pathways, activation of the effector caspase 3 is important for the execution of apoptotic cell death. Thus, we correlated EBV expression with caspase 3 activation, but we failed to observe correlation between EBV and aCasp3 quantitative expression.

In order to analyze EBV role as an apoptotic pathway inhibitor, intrinsic apoptotic pathway, particularly Bcl2 and Bax protein expression, were studied. First EBV gene that was identified as having anti-apoptotic properties was LMP1, which acts by inducing the anti-apoptotic proteins, such as Bcl2 [36]. Furthermore, Komano and Takada have reported the up-regulation of Bcl2 oncoprotein upon EBV infection in BL cell lines, without LMP1 expression [37]. When Bcl2 expression was correlated with EBV expression in our series, Bcl2 up-regulation was not observed in EBV positive cases, so it seems that the *in vitro* proposed mechanism did not mimic the *in vivo* scenario, as previously observed in EBV+ gastric carcinoma [38]. It has been also demonstrated that pro-apoptotic Bax protein may be an important target of the anti-apoptotic *in vitro* activity of LMP1 and additionally, decreased Bax levels have been observed in various EBV-associated tumors including HL [10]. As previously observed for Bcl2, EBV was not statistically associated with decreased Bax expression in our series, suggesting that functional inactivation and/or inhibition of Bax is not involved in our pediatric EBV-associated B-NHL.

Park et al. analyzed the largest adult series of DLBCL by far, and proved that EBV positive DLBCL showed substantially poorer response to chemotherapy compared with their EBV negative counterpart [19]. Chuang et al. described that presence vs. absence of EBV did not correlate with prognosis in pediatric and adult BL [39]. In line with this, EBV positivity was not related with prognosis in a pediatric B and T-cell NHL series described by Aktas et al. [20]. In our pediatric B-NHL series EBV showed statistically association with worse EFS, but it was mainly due to the combination of EBV presence in an immunodeficient background, since EBV+ immunocompetent patients do not achieve significant worse EFS. Nevertheless, it should be kept in mind that EBV expresses all latency proteins in immunosupressed associated malignancies, and some of these proteins have been proved to have proliferative effects, which combined expression might affect EFS. Unexpectedly, in our patients EBV association with worse EFS could not be related to alteration of apoptosis pathway, since EBV was not statistically associated either with apoptotic index changes, or deregulation of pro and anti-apototic proteins.

The EBV association of 40% in our pediatric B-NHL series supports the idea that EBV acts as a cofactor in tumor development in immunocompetent patients, and a key factor of B-cell lymphomagenesis under immunodeficiency status. Perhaps, on EBV positive patients, viral antigen expression could be used as a tool for novel tumor-targeted therapy in addition to conventional therapy [40].

Conflict of interest

None declared.

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