

Analysis of the association between Epstein-Barr virus and classic Hodgkin's lymphoma in adult patients from Ceará (Brazil) by immunohistochemistry and *in situ* hybridization

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Análise da associação do vírus Epstein-Barr com a forma clássica do linfoma de Hodgkin em adultos do estado do Ceará: avaliação por imuno-histoquímica e hibridização in situ

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key words	resumo
Classical Hodgkin's lymphoma	The prevalence of Epstein-Barr virus (EBV) in patients with classical Hodgkin's lymphoma (CHL) is geographically variable. In the present study the prevalence of EBV in CHL was assessed in adult patients from Ceará, Brazil. Thirty-seven cases were immunohistochemically evaluated for EBV using latent membrane protein (LMP1) antibody and for EBV latency-associated RNA (EBER1) using <i>in situ</i> hybridization (ISH). Sex and age did not differ among patients as to the frequency of CHL. Nodular sclerosis was the predominant histological subtype. LMP1 was found in Reed-Sternberg cells in 67.5% of the cases whereas ISH detected EBER1 in 75.6%. Regarding histological subtypes EBV infection rates were not found statistically different in nodular sclerosis (NS) and mixed cellularity (MC) subtypes ($p = 0.66$).
Epstein-Barr virus	
LMP1	
EBER1	

abstract	unitermos
<i>A frequência do vírus Epstein-Barr (EBV) em pacientes com linfoma de Hodgkin Clássico (LHC) sofre variabilidade geográfica. No presente estudo investigamos a frequência do EBV em pacientes com LHC no estado do Ceará. Trinta e sete casos de linfoma de Hodgkin clássico foram avaliados por imuno-histoquímica para EBV usando o anticorpo monoclonal contra a proteína latente da membrana (LMP1) e pelo método de hibridização in situ para RNA associado ao EBV (EBER1). Não há diferença por sexo e idade dos pacientes no que concerne à frequência de LHC. O subtipo histológico esclerose nodular foi predominante. LMP1 esteve presente em células Reed-Sternberg em 67,5% e pela hibridização in situ, através da sonda EBER, foi evidente em 75,6% dos casos. Não observamos predominância significativa da associação de EBV com os subtipos histológicos esclerose nodular (EN) e celularidade mista (CM) ($p = 0,66$).</i>	Linfoma de Hodgkin clássico Vírus Epstein-Barr LMP1 EBER1

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Introduction

The incidence of classical Hodgkin's lymphoma (CHL) varies among population groups. Nodular sclerosis (NS) subtype is the most common one in developed countries, whereas the mixed cellularity (MC) subtype is more prevalent in developing countries^(5, 10). However, available data from Brazil seems to be divergent^(2, 20, 21).

Previous epidemiological findings suggest a relationship between classical Hodgkin's lymphoma and infection by Epstein-Barr virus (EBV)^(5, 7, 15, 17). The EBV-encoded oncogene latent membrane protein (LMP1) has been consistently shown to be expressed in Reed-Sternberg cells (RS), a fact with possible implication to the pathogenesis of the disease.

In developed countries, the incidence of EBV in CHL has been found to be approximately 50%⁽⁶⁾, whereas in Honduras, Peru, China and Mexico it is reported to be 100%, 94%, 61% and 67%, respectively^(3, 5, 23, 24).

The aim of the present study was to assess the prevalence of EBV-associated CHL of subtypes MC and NS in adult patients from the state of Ceará by immunohistochemistry (IHC) and by *in situ* hybridization (ISH).

Methods

Results of surgical biopsy specimens from 37 adult patients (18-64 years) with classical CHL were obtained from the files of medical institutions of Fortaleza (Ceará, Brazil) in the period 1997-2002. Thirty-seven cases with CHL were evaluated by IHC⁽¹³⁾ for EBV, LMP1 antibody (Catalog N° M897, DakoCytomation, USA) at the dilution of 1:50, amplified by streptavidin-biotin-peroxidase technique. RNA *in situ* hybridization was performed to detect EBV in H-RS cells with a biotinylated probe for Epstein-Barr encoded RNAs-1 (EBER-1, Y5200-Epstein-Barr virus RNA probe/FITC, ready-to-use, DakoCytomation, USA), a viral transcript present in high levels in infected cells (about 10⁷ copies per nucleus)⁽²²⁾. In all cases the diagnosis of CHL was confirmed by light microscopy and immunohistochemical phenotyping by two experienced hematopathologists (FVAF, FDRF). The cases were classified according to the World Health Organization Classification⁽¹⁴⁾. The panel of monoclonal antibodies included Ki-1 antigen/H-RS cells (CD30 [Catalog N° M751]), granulocyte-associated antigen/H-RS cells (CD15 [Catalog N° M733]), B cells (CD20 [Catalog N° MO 755]), T cells associated antigen (CD3 [Catalog N° M7254]), epithelial membrane antigen (EMA [Catalog. N°

MO 613]) and leucocyte common antigen (CD45 [Catalog N° MO701]), all from DakoCytomation, USA. As a negative control, normal mouse serum was used instead the primary antibody, followed by all other procedures.

The EBV-RNA *in situ* hybridization studies were performed using a 30-base oligonucleotide complementary to a segment of EBER1 gene.

Descriptive statistics and the Fisher test were used with a limit of $p < 0.05$.

Results

The demographic findings showed similar numbers of men and women with similar ages among the CHL patients (**Table 1**).

The histological analysis of the samples revealed a predominance of NS subtype (62.2%) (**Table 2**). The immunohistochemical and the *in situ* hybridization studies showed evidence of EBV respectively in 67.5% and 75.6% of the cases (**Table 3**). We did not find a statistically significant difference of EBV infection between NS and MC subtypes (LMP1: $p = 0.66$; ISH: $p = 0.1$).

Immunophenotypic findings showed that all RS cells immuno-expressed CD30 (a criteria of inclusion), and CD15 was present in 87.8% of the cases. EBV LMP1 was detected in 25 of the 37 cases (67.5%): 15/23 (65.2%) of NS, 8/11 (72.7%) of MC, 1/1 (100%) of lymphocyte depletion (LD) and 1/2 (50%) in unclassified cases (UC) (**Figure 1**). There was no immunostaining of either CD3, EMA, CD45 or CD20 in RS cells.

Table 1 Demography of the CHL patients (n = 37)

	Males	Females
Sex	18 (48.6%)	19 (51.3%)
Age average (years)	30	32

Table 2 Histological diagnosis of the CHL patients (n = 37)

Nodular sclerosis (NS)	23 (62.1%)
Mixed cellularity (MC)	11 (29.7%)
Lymphocyte depletion (LD)	1 (2.7%)
Unclassified (UC)	2 (5.4%)

Table 3 **Prevalence of EBV markers in CHL patients (n = 37)**

	LMP1		EBER1	
	25/37 (67.5%)	28/37 (75.6%)		
	(-/%)	(+/%)	(+/%)	(-/%)
NS (T = 23)	15 (65.2)	8 (34.8)	16 (69.5)	7 (30.4)
MC (T = 11)	8 (72.7)	3 (27.2)	10 (90.9)	1 (12.5)
LD (T = 1)	1 (100)	-	1 (100)	-
(UC) (T = 2)	1(50)	1(50)	1 (50)	1(50)

In situ hybridization studies evidenced that patients who scored as EBV-positive showed a strong hybridization signal in the nuclei of RS and Hodgkin's cells. Twenty-eight of 37 cases (75.6%) were positive for EBER1: 16/23 (69.5%) of NS, 10/11 (90.9%) of MC, 1/1 (100%) of LD and 1/2 (50%) of UC (**Figure 2**).

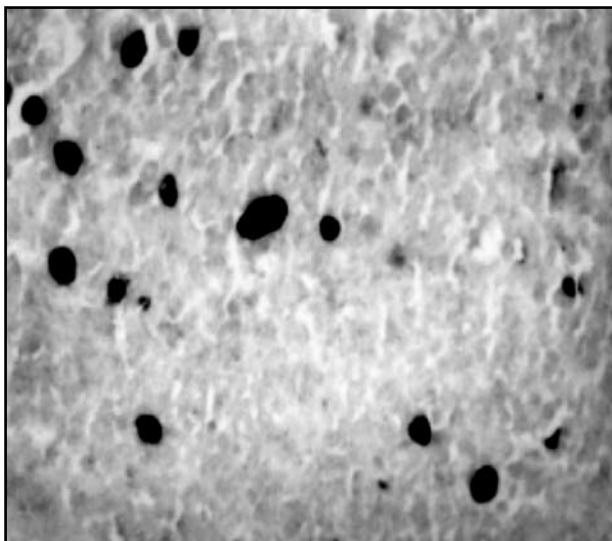


Figure 1 – Microscopic demonstration of EBV markers in classical Hodgkin's lymphoma NS-subtype by *in situ* hybridization (400x)

All cases with positive results by IHC were also positive by ISH. However, three negative cases for LMP1 were positive by ISH. All ISH and IHC results are summarized in Table 3.

Discussion

The prevalence of Hodgkin's lymphoma and the distribution of major subtypes have been described as variable in different populations. In developing countries, Chang *et al.*⁽⁵⁾ described a MC subtype as predominant. In the present study NS subtype was found more prevalent

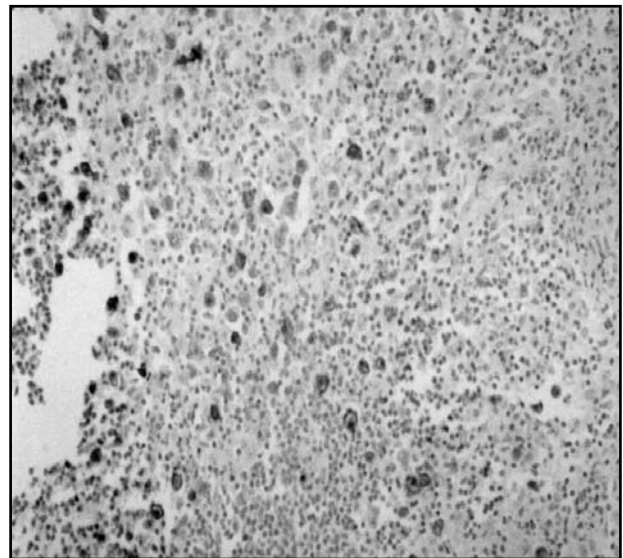


Figure 2 – Microscopic demonstration of EBV markers in classical Hodgkin's lymphoma NS-subtype by immunohistochemistry (200x)

in CHL adult patients from Ceará, contrasting to a previous study in children, in whom MC subtype predominated⁽¹⁾. However, more recent unpublished findings from our laboratory show a trend for an inversion in subtypes detected in children. Remarkably, the incidence of MC and NS subtypes of CHL in adult patients from Ceará reported herein differs from previous studies^(12, 19). The present study points to a gradual shift in prevalence, from MC to NS subtype in adult patients. This finding is in agreement with some of the recent Brazilian reports^(2, 20, 21). The factors responsible for this change are not well known. It is possible that changes in host response and/or living conditions resulting from investments in nutrition and health programs might have played a role on it.

In our series, the prevalence of CHL in males and females was not found different, as also shown in other previous studies⁽²³⁾.

Our results clearly agree with findings reported in literature regarding the detection of EBV in CHL^(4, 9-11). It is possible that the higher viral load associated with the poor socioeconomic status of the patients from Ceará might be a contributing factor in worsening prognosis in CHL. EBV was associated with CHL in patients from Ceará in 75.6% of cases, according to ISH results. The association between NS subtype and EBV was even higher (57.5%) than that found for unpublished data from adult patients in several regions of France (30.3% [data selected from the Pathology files of Laennec Hospital – Paris V University by Rocha Filho, in the period of 1997-1999]). These results support the hypothesis that EBV is more frequently implicated in

the pathogenesis of CHL in adult patients in Northeastern Brazil than in France. Previous studies involving patients from this Brazilian region found prevalence rates for EBV in children and adolescents as high as those of the adult patients sampled in this study⁽¹⁾.

The association between EBV and CHL subtypes was found to be significantly higher in patients from Ceará (approximately 1.4 times) than that reported for patients from São Paulo⁽¹⁸⁾. In a study from Rio de Janeiro, EBV was detected in 55% of cases, its presence being significantly correlated (88%) with the MC subtype⁽¹⁶⁾.

In agreement with previous reports⁽⁸⁾ the present findings suggest that LMP1 expression may be adequate for identifying EBV-associated CHL. Moreover, ISH is strongly suggested in cases with negative results, especially in MC subtype.

Conclusions

Results from the present study support the hypothesis that EBV is often implicated in the pathogenesis of CHL in adult patients from Ceará. We hypothesize that such high

rates are due to a possible high EBV viral load observed in our patients, along with the early onset of CHL between children and adolescents, which may have contributed to the comparatively stronger association between the virus and the disease we observed. Other factors such as ethnicity, nutrition and climate might also play a role demanding further studies.

The role of EBV in CHL remains to be clarified, and the evaluation of the clinical implications of the presence of EBV for evolution, prognosis and pathogenesis of the disease requires further studies.

The techniques of ISH (EBER₁ RNA) and IHC applied to tissues fixed in formaldehyde and embedded in paraffin, as employed in our studies, may turn out to be quite valuable for future epidemiological studies on the association of EBV virus with CHL in other regions of Brazil.

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