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ORIGINAL ARTICLE

EBV detection in HIV-related oral plasmablastic lymphoma

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OBJECTIVES: Plasmablastic lymphoma (PBL) of the oral cavity is an aggressive neoplasm derived from B cell, considered to be the second more common among human immunodeficiency virus (HIV)-associated malignancies. As Epstein–Barr virus (EBV) infection has been associated with this neoplasm, the aim of the present study was to assess the presence of EBV in 11 cases of oral HIV-related PBL and investigate the controversial issue of the presence of Human herpesvirus-8 (HHV-8) in these tumors.

METHODS: DNA was extracted from nine cases of HIV-associated oral lymphomas, diagnosed as PBL, and genomic material was amplified by polymerase chain reaction to verify the presence of EBV. *In situ* hybridization (ISH) for EBV was performed in five cases. Immunohistochemical analysis was conducted to confirm previous diagnosis and verify HHV-8 infection.

RESULTS: The 11 cases had diagnosis confirmed by immunohistochemical analysis. Only nine cases presented an adequate amount of DNA for analysis, and EBV was detected in seven of them. The five cases tested for EBV viral infection by ISH showed positive signals. All 11 cases were negative for HHV-8.

CONCLUSION: The presence of EBV in all cases studied favors a direct role of this virus in the development of HIV-related PBL, and this finding could be considered when dealing with HIV patients.
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Keywords: plasmablastic lymphoma; EBV; HIV

Introduction

Among the secondary problems of patients with the human immunodeficiency virus (HIV) infection are lymphoproliferative diseases. Many studies of HIV-associated oral lesions have reported a 5–7.1% prevalence of non-Hodgkin's lymphoma (NHL) (Silverman *et al*, 1986; Feigal *et al*, 1991; Nittayananta and Chungpanich, 1997; Patton *et al*, 1998; Chidzonga, 2003). These tumors are predominantly aggressive high-grade B-cell lymphomas, being Burkitt's lymphoma, diffuse large B-cell lymphoma, primary effusion lymphoma (PEL) and plasmablastic lymphoma (PBL) the most common types (Green and Eversole, 1989; Vázquez-Piñeiro *et al*, 1997; Jaffe *et al*, 2001).

Plasmablastic lymphoma is a distinctive type of diffuse large B-cell lymphoma associated with acquired immunodeficiency syndrome (AIDS) and has unique immunophenotype in which plasmablastic lymphocytes are found in a high proportion (Delecluse *et al*, 1997). Predominantly it involves the oral cavity, in particular the gingival and palatal mucosa with a tendency to infiltrate adjacent bone (Flaitz *et al*, 2002; Gaidano *et al*, 2002; Sousa *et al*, 2002).

Histologically, PBL is composed of large neoplastic cells presenting phenotypes consistent with late B-cell maturation, probably arising from a postgerminal center B-cell, and is related to EBV (Delecluse *et al*, 1997; Flaitz *et al*, 2002).

Epstein–Barr virus (EBV) is a member of the human herpes virus family and has been associated with the development of lymphoproliferative diseases including malignant lymphoma, infectious mononucleosis, hairy leukoplakia, salivary glands tumors, and some gastrointestinal tract tumors (Purtilo *et al*, 1992; Ioachim *et al*, 1997; Okano and Gross, 2000). *In situ* hybridization (ISH) has been used to verify the presence of EBV in AIDS-associated lymphomas (Green and Eversole, 1989; Boiocchi *et al*, 1990; Chappuis *et al*, 1990), and results have varied from 15% to 80% of positivity.

Human herpesvirus-8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus, is also frequently involved in lymphoproliferative disorders occurring in association with HIV infection: PEL, Castleman's disease and multicentric Castleman's disease-associated PBL (Cesarman *et al*, 1995; Soulier *et al*, 1995; Oksenhendler *et al*, 2002). Most studies have found HHV-8 to be negative in oral PBL (Delecluse *et al*, 1997; Flaitz *et al*, 2002; Gaidano *et al*, 2002; Folk *et al*, 2006) but there is one report indicating the presence of HHV-8 in these tumors (Cioc *et al*, 2004).

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In the current study we present 11 new cases of PBLs of the oral cavity, their morphologic and immunohistochemical features and the presence of EBV DNA amplified using polymerase chain reaction (PCR) and ISH. The controversial presence of HHV-8 in oral PBL was investigated by immunohistochemistry.

Subjects and methods

Eleven cases of HIV-associated lymphomas were retrieved from the files of the Oral Pathology Service of the University of São Paulo. Clinical data including sex, age, and lesion sites are summarized on Table 1.

Immunohistochemical studies
The cases were routinely processed and visualized by a light microscope. Immunohistochemical study was performed on formalin-fixed paraffin-embedded tissue with a panel of monoclonal antibodies CD20 (1:500), CD45 (1:500), CD79a (1:50), VS38c (1:100) from Dako Corporation (Carpinteria, CA, USA), and evidence for monoclonality of B cells was achieved by analysis of immunoglobulin (Ig) light chains kappa (κ) and lambda (λ), both in a dilution of 1:100, from BioGenex (San Ramon, CA, USA). Dewaxed sections were incubated with primary serum and immunostained by the streptavidin–biotin–peroxidase method, followed by diaminobenzidine chromogen solution. Finally, sections were counterstained with Mayer's hematoxylin and examined by a light microscope. Appropriate positive and negative controls were included.

The presence of HHV-8 on paraffin-embedded tissue was investigated by immunohistochemistry using the mouse monoclonal antibody against HHV-8 LNA-1 (clone 13B10, dilution 1:50; Novocastra Laboratories, Newcastle upon Tyne, UK) by the labeled streptavidin–biotin peroxidase method (LSAB kit; Dako Corporation) and using the heat-mediated antigen retrieval in water bath, citrate buffer 0.01 M, pH 6.0, 30 min, 95°C, following the manufacturer's instructions. Sections of a Kaposis's sarcoma previously shown to have HHV-8 were used as positive control.

Analysis of EBV infection
Determination of tumor infection by EBV was performed by PCR and/or by Epstein–Barr early RNA (EBER) ISH.

DNA extraction method
DNA was extracted from nine formalin-fixed and paraffin-embedded cases diagnosed as PBLs (samples 1–9). The remaining two cases did not have enough material for DNA extraction.

Ten to twenty 10 μm thick sections of each block were collected in a 1.5-ml micro tube. One milliliter preheated xylene was added to each micro tube and kept at 56°C for 10 min. The tubes were then centrifuged at 9300 *g* for 5 min. The supernatant was discarded, followed by new changes of preheated xylene, until the complete removal of paraffin. The pellet was washed in a descending series of ethanol (absolute ethanol, ethanol 95%, 70%, and 50%). Each change was preceded by homogenization and centrifugation at 9300 *g* for 5 min. The samples were then placed in 200–400 μl of 500 μg/μl proteinase K digestion buffer (1 M NaCl; 1 M Tris-HCl, pH 8; 0.5 M EDTA, pH 8; 10% sodium dodecyl sulfate). Digestion was carried out for 3–5 days at 55–60°C, with daily replacement of proteinase K (250 μg/μl working concentration). When the protein digestion was deemed adequate, proteinase K was inactivated by heat at 95°C for 10 min. The material was then submitted to DNA extraction.

The method used for DNA extraction was the ammonium acetate method, as described by Rivero *et al* (2006). After proteinase K inactivation, the micro tubes containing genomic material received 200 μl of 4 M ammonium acetate. The micro tubes were vortexed for 20 s in high speed, incubated on ice for 5 min, and centrifuged at 13 000 *g* for 3 min. The supernatant was transferred to another tube; 600 μl of isopropanol was added, and centrifuged at 16 000 *g* for 5 min. The DNA pellet was washed in 70% ethanol and centrifuged at 16 000 *g* for 1 min. The supernatant was discarded. DNA was dissolved in 20 μl of TE solution (Tris-HCl 10 mM, pH 7.4, EDTA 1 mM, pH 8) and kept at 4°C until analysis with a spectrophotometer. Electrophoresis using 1% agarose gel was performed to verify DNA integrity.

Table 1 Clinical data

Case	*Age (years)*	*Gender*	*Location of lesion*	*Duration of symptoms*	*HIV*[a]
1	32	Male	Hard palate[b]	8 months	Positive
2	29	Male	Hard palate	6 days	Positive
3	49	Male	Mandibular gingiva	15 days	Positive
4	49	Male	Mandibular gingiva	30 days	Not available
5	23	Female	Maxillary gingiva	2 months	Not available
6	41	Male	Gingiva	10 days	Not available
7	40	Male	Maxillary gingiva	Not available	Not available
8	41	Male	Gingiva	Not available	Not available
9	42	Male	Maxillary gingiva	4 months	Positive
10	29	Male	Mandibular gingiva	Not available	Positive
11	30	Female	Mandibular gingiva	Not available	Positive

[a]Some of the cases were known to be positive at the time of biopsy; the others were tested only after the diagnosis of plasmablastic lymphoma and HIV test was positive in all of them.
[b]In this case there was palate perforation with infiltration in the adjacent bone.

Polymerase chain reaction

DNA was amplified by PCR targeting the EBV sequence (269 bp) using the following primers: EBV sense (5′-GTC ATC ATC ATC CGG GTC TC -3′) and EBV antisense (5′-TTC GGG TTG GAA CCT CCT TG -3′) (Telenti *et al*, 1990; Baldanti *et al*, 2000). The reaction mixture consisted of 250 pM of each primer, formamide 1%, PCR buffer 1X (Tris-HCL 200 mM, pH 8.4; KCl 500 mM), 2 mM of $MgCl_2$, dNTP 0.28 mM (dATP, dTTP, dCTP, dGTP – Invitrogen, Carlsbad, CA, USA), Taq DNA polymerase 2 U (Invitrogen), 6 μl of extracted DNA 200 ηM and water (q.s.p. 25 μl). Thermal cycling was performed in a thermocycler (PTC-100; MJ Research, Inc, Waltham, MA, USA) with a hot start protocol (95°C, 3 min) followed by 40 cycles as follows: 95°C for 1 min (denaturation), 56°C for 1 min (annealing), and 72°C for 1 min (extension). A final elongation step at 72°C for 7 min was performed after later cycling. As positive control, DNA extracted from an EBV-positive case (hairy leukoplakia) was used. After PCR amplification, products were analyzed by electrophoresis in a 2% agarose gel and EBV sequence confirmed by DNA sequencing.

In situ *hybridization*

In situ hybridization was performed on formalin-fixed, paraffin-embedded tissue sections using fluorescein-conjugated EBV (EBER) RNA probe (Y5200; Dako Cytomation Inc.). Standard precautions to avoid RNase contamination were followed. Briefly, 3-μm sections of all eleven cases were deparaffinized, rehydrated, and digested with proteolytic enzyme (proteinase K at 37°C for 25 min). Thereafter, the probe was added and incubated at 55°C for 1.5 h; the sections were washed with a stringent solution (Stringent Wash, S3500; Dako), labeled with anti-FITC/AP (alkaline phosphatase-conjugated antibody to fluorescein), and rinsed with Tris-buffered saline (pH 7.5). A chromogen, BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium), was then added and counterstained with Mayer's hematoxylin. The presence of reactivity on sections was analyzed using a light microscope. The positive control used was a case of hairy leukoplakia, which had been previously tested. As negative control, the specific probe was omitted.

Results

The patients were all positive for HIV and the tumors were localized in the mucosa of the oral cavity with nine involving the gingiva. In two patients the tumor was localized in the palatal mucosa and in one of these cases there was palatal perforation. Nine patients were male and two were female with an average age of 36 years. Five patients were not aware of the HIV positivity at the time of diagnosis, which was confirmed latter.

All cases showed similar histologic findings characterized by diffuse lymphoid infiltrate composed of large cells with abundant and basophilic cytoplasm. The nuclei were large, presenting dispersed chromatin with a single central prominent nucleolus or two or more peripherically sited nucleolus reminding immunoblasts (Figure 1a).

Immunohistochemical studies showed that in all cases the tumor cells were positive for VS38c (plasma cell), and either kappa or lambda (Figure 1b–d). Some cases were weakly positive for the B-cell marker CD79a. All specimens were negative or showed a minimal expression of the B-cell antigen CD20.

Epstein–Barr virus was amplified in the tumor tissue of seven out of nine cases (77.8%) by PCR (cases 3–9). All the 11 cases tested for EBV infection by ISH clearly displayed positive signals in the nuclei of the tumor cells. The PCR and ISH results are shown in Figure 2.

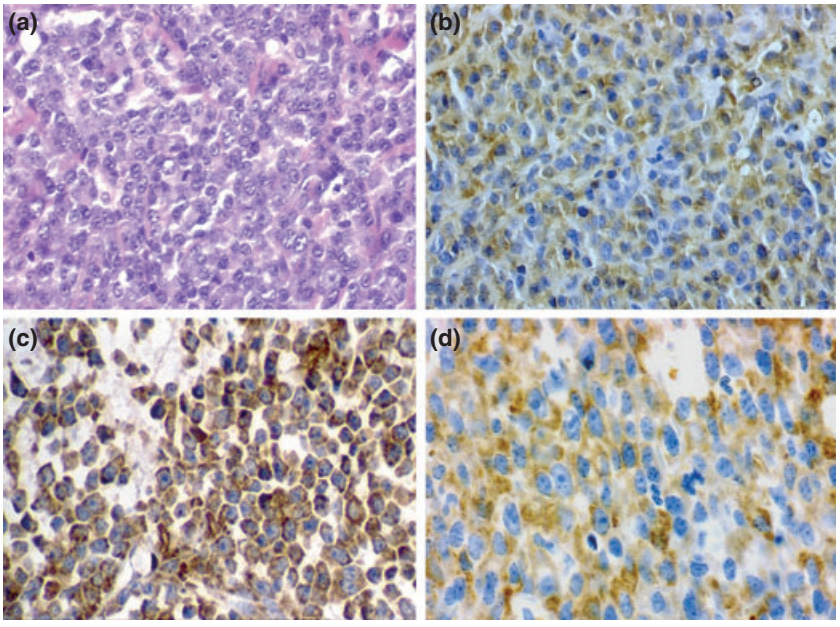

Figure 1 (**a**–**d**) Histologic and immunohistochemical aspects of oral plasmaflastic lymphoma (OPL). (**a**) Diffuse infiltrate of immunoblast-like cells with prominent nucleolus (hematoxylin–eosin stain, ×400). (**b**) Expression of lambda in most of the tumor cells (streptavidin–biotin, ×400). (**c**) Expression of plasma cell antibody (VS38) and (**d**) a detail of this expression (streptavidin–biotin, **c**, ×400; **d**, ×1000)

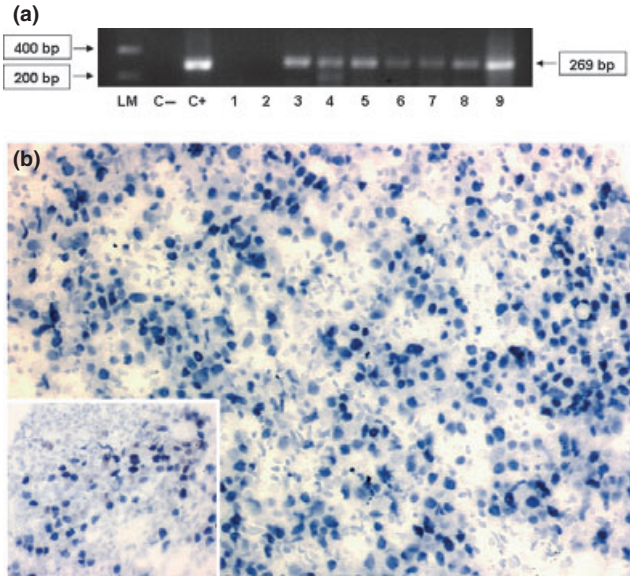

Figure 2 Detection of EBV infection in OPL. (**a**) Agarose gel (2%) showing EBV DNA amplification by PCR. No amplification: cases 1 and 2 (LM, low marker; bp, base pair; C−, negative control; C+, positive control; samples 1–9). (**b**) *In situ* hybridization with EBER probe showing positive nuclear signal (dark blue). Insert shows some cells with strong positive signal in a very small biopsy fragment of OPL

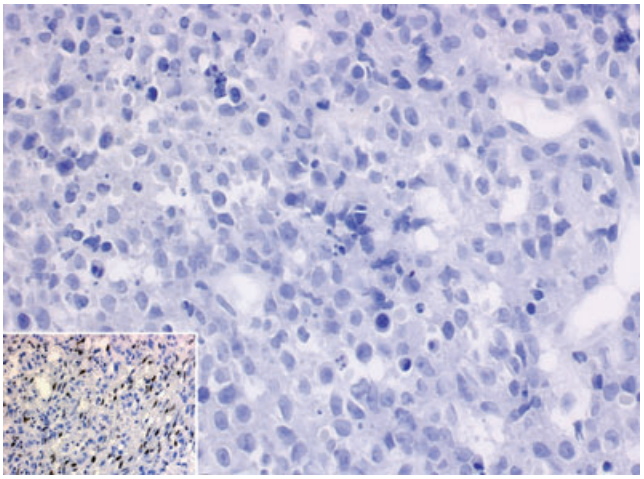

Figure 3 Immunohistochemistry showing negativity of tumor cells to HHV-8. Insert shows positive control (brown nuclei) (streptavidin–biotin, ×400; insert ×150)

All the 11 cases tested for HHV-8 infection by immunohistochemistry in oral PBL were negative, while the positive control, a sample of Kaposi's sarcoma, showed positivity (Figure 3).

Discussion

Diffuse large B-cell lymphomas (DLBCL) represent the largest group among NHL. PBL is a subtype of DLBCL first described by Delecluse *et al* (1997) as a highly malignant neoplasm arising in the oral cavity and jaws of HIV-infected patients, frequently related to EBV (Iamaroon *et al*, 2003). In the present report, the lymphomas were extranodal and, in all instances, the patients were positive for HIV, although the HIV status was not known in five patients prior to the diagnosis of PBL. This diagnosis leads to the investigation of HIV status and thus, in these five cases, PBL was the first manifestation of the HIV infection, as also reported by Colmenero *et al* (1991).

The tumors were localized in the mucosa of the oral cavity, nine (82%) occurred on the gingiva and two (18%) on the palate. The literature shows PBL occurring at a mean age of 39.6 years, with a range of 23–75 years; in this study the mean age was 36.8 years, with a range of 23–49 years. The gender distribution showed a male predilection of 4.5:1 similar to that shown in the literature of 3.91:1 (Scheper *et al*, 2005).

Diagnosis was established based on histologic findings and immunohistochemical profile as described by the WHO (Jaffe *et al*, 2001). PBLs are mainly characterized by a immunoprofile that includes positive expression for VS38c in the majority of the cells, variable positivity for CD79a, and negativity for CD20 (Carbone *et al*, 1997; Delecluse *et al*, 1997; Chetty *et al*, 2003; Colomo *et al*, 2004). This expressive positivity to VS38c and the monoclonality showed by an expression of κ or λ make this lymphoma unique (Lin *et al*, 2005). However, the immunophenotype is closely associated with that shown by plasmablastic plasma cell myeloma or multiple myelomas, but, clinical aspects, relation to HIV infection, and the presence of EBV allows a differentiation between these lesions (Vega *et al*, 2005).

In the present study, the presence of EBV was investigated in nine cases by PCR and detected in seven of them. In the two negative cases, ISH was performed and shown to be positive. ISH was also positive in the other two cases where PCR could not be performed. The failure in DNA amplification can be explained by the low amount of material for DNA extraction, as biopsy fragments were very small, despite the fact that PCR is a more sensitive technique than ISH.

The close association between PBL, HIV infection, and EBV is evident; however, the precise mechanism by which EBV is involved in tumor initiation and growth is not clearly understood. After an acute infection by EBV a small number of EBV-infected B cells remain latently infected and capable of reactivation at a later time. The gene products of latently infected B cells may contribute to B-cell immortalization increasing B-cell proliferation and protect them from apoptosis (Johannessen and Crawford, 1999).

The results presented here, together with other publications (Delecluse *et al*, 1997; Webster-Cyriaque *et al*, 1997; Brown *et al*, 2000; Carbone *et al*, 1997; Flaitz *et al*, 2002), indicate that infection by HHV-8 virus is not involved in the pathogenesis of oral PBLs contradicting the results of Cioc *et al* (2004).

On the basis of the known association between immunosuppression, EBV infection and lymphoproliferative disease, it is possible to hypothesize that latently infected B cells can be reactivated in the presence of immune dysregulation and so initiate lymphoproliferative diseases. Fan *et al* (2005) showed that high EBV

viral load is strongly correlated with the presence of lymphomas and undetectable plasmatic EBV does not exclude a diagnosis of lymphoma. Therapy directed against EBV may decrease levels of EBV and therefore, the detection of EBV might assist in diagnosis and management of PBLs.

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