Viral Studies in Burkitt Lymphoma

Association With Epstein-Barr Virus but Not HHV-8

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Key Words: Burkitt lymphoma; Kaposi sarcoma–associated herpesvirus; Human herpesvirus 8; KSHV/HHV-8; LANA protein; HIV; Immunohistochemistry; Polymerase chain reaction; PCR

DOI: 10.1309/2CNAWY6GAR0VQAXX

Abstract

Burkitt lymphoma (BL) is a highly aggressive non-Hodgkin lymphoma, composed of a monomorphic population of medium-sized B cells with a high proliferation rate and a consistent MYC translocation. Epstein-Barr virus (EBV) has been associated with BL with different frequencies depending on the clinical variant. Kaposi sarcoma–associated herpesvirus, or human herpesvirus 8 (HHV-8), infects a wide range of normal cells, having a well-established role in the pathogenesis of various neoplasms, including Kaposi sarcoma, primary effusion lymphoma, multicentric Castleman disease (MCD) and MCD-associated plasmablastic lymphoma. In secondary immunodeficiencies, such as HIV-1 infection and organ transplantation, HHV-8 is considered an opportunistic pathogen linked to the development of lymphomas in patients with AIDS and HIV+ patients. We studied the association of EBV and HHV-8 by immunohistochemical analysis, in situ hybridization, and polymerase chain reaction in a large number of well-characterized BLs. EBV was present in 45.0% of all BL cases with higher incidence in the pediatric group; most cases were EBV type A. We found no association of BL with HHV-8 in EBV+ BL or in EBV− cases, including the HIV+ BL group.

Burkitt lymphoma (BL) is a highly aggressive non-Hodgkin lymphoma with endemic, sporadic, and immunodeficiency-associated clinical variants composed of a monomorphic population of medium-sized B cells with a high proliferation rate and having a consistent MYC translocation.1,2 Viral infections, in particular Epstein-Barr virus (EBV), have been associated with BL; it is well established that this association occurs with different frequencies depending on the clinical variant.3 EBV is present in the majority of endemic cases of BL and nearly 30% of cases of sporadic BL.1 In Brazil, a high association of EBV with BL has been demonstrated in tropical areas, especially in the northeast region.4,5

Kaposi sarcoma–associated herpesvirus, or human herpesvirus (HHV)-8, is a virus able to infect mammalian cells, including lymphoid cells, dendritic cells, and fibroblasts. Several neoplasms have been associated with HHV-8, including Kaposi sarcoma, primary effusion lymphoma, multicentric Castleman disease (MCD), and MCD-associated plasmablastic lymphoma.6-8

In the context of secondary immunodeficiencies, such as HIV-1 infection and organ transplantation, HHV-8 is considered an opportunistic pathogen that has been linked to the development of lymphoproliferative diseases, including lymphomas and related diseases.9 HHV-8 has also been reported in association with lymphomas in patients with AIDS and HIV+ patients.10 In common variable immunodeficiency, a primary immunodeficiency disorder of unknown etiology, patients have a high risk of B-cell lymphomas; HHV-8 has been identified in at least some of the associated lymphomas and is considered an important factor in their pathogenesis.9 Du et al11 demonstrated monotypic HHV-8+ plasmablasts in MCD and MCD-associated plasmablastic lymphomas.
HHV-8–associated lymphomas have included cases of naive cell origin, germinal center (GC) and post–GC cells, unlike EBV-associated lymphomas, which are generally more restricted to GC or post-GC origin.\textsuperscript{12,13} HHV-8 and EBV coinfection has been documented in primary effusion lymphoma and in the setting of Castleman disease, typically associated with an immunosuppressed state.\textsuperscript{14}

In the literature, there is scarce information on the association of HHV-8 with BL in HIV+ and HIV– patients, and it is generally limited to data concerning African populations. Lazzi et al,\textsuperscript{15} in a study of East African patients, evaluated 16 BL cases and detected HHV-8 in nonneoplastic lymphoid cells in 1 case of an HIV– patient with a lymph node partially involved by BL. The molecular analysis in microdissected HHV-8+ cells in this case showed absence of clonality. It is worth mentioning that in this BL case, there was no association with Kaposi sarcoma. In a previous study on HIV-associated malignant lymphomas, also in African patients, Lazzi et al\textsuperscript{16} analyzed 29 cases of BL, with none showing HHV-8 by polymerase chain reaction (PCR). In 10 cases of African BL, Tao and Ambinder\textsuperscript{17} did not find HHV-8 or HHV-7 but found HHV-6 in 3 cases studied.

We studied the association of EBV and HHV-8 in a large number of well-characterized BL cases in a Brazilian population.

**Materials and Methods**

**Case Material and Clinical Data**

We retrospectively obtained 311 cases of BL from the files of Consultoria em Patologia, a large reference consultation service in anatomic pathology located in Botucatu, São Paulo State, Brazil. The study group included all cases of BL with available representative formalin-fixed, paraffin-embedded (FFPE) blocks received in consultation between June 1997 and May 2007. Nodal and extranodal BL cases were included. Clinical data, including sex, age at diagnosis, and tumor location, were obtained from the referring pathologists and oncologists. We reviewed available H&E-stained slides of each case, and representative areas were selected for incorporation into tissue microarrays (TMAs). Morphologic subclassification of the cases was performed according to variants recognized by the World Health Organization classification.\textsuperscript{1}

**TMA Construction**

Six TMA blocks were constructed by using a tissue arrayer (Beecher Instruments, Sun Prairie, WI). Each individual case was represented by 3 tumor cores of 0.6 mm that were taken from the original paraffin blocks. Serial sections of 3 µm were cut from the tissue array blocks and used for immunohistochemical analysis. Proper positive and negative control cores for each marker were also included in the array block to provide adequacy of the antibodies used in the immunohistochemical studies as follows: tonsil (CD3, CD10, CD20, bcl-2, bcl-6, and Ki-67), lymphoblastic lymphoma (terminal deoxynucleotidyl transferase), EBV+ Hodgkin lymphoma (EBV latent membrane protein [LMP]-1), and Kaposi sarcoma and HHV-8–AIDS-related non-BL (Kaposi sarcoma–associated herpesvirus/HHV-8).

**Immunohistochemical Analysis and In Situ Hybridization**

An immunohistochemical study was performed for each TMA using Novolink polymer (Novocastra, Newcastle upon Tyne, England) as the detection system, and an epitope-retrieval method was applied as needed for each specific antibody; diaminobenzidine was the chromogen. Primary antibodies used were anti-CD20, anti-CD3, anti-CD10, anti-bcl-6, anti-Ki-67, anti-bcl-2, anti-EBV-LMP, and anti–HHV-8 latent nuclear antigen (LANA); for this last marker, only cells with a salt-and-pepper granular nuclear pattern of immunostaining were considered positive.\textsuperscript{1Table II.}

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen Retrieval</th>
<th>Source</th>
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<tbody>
<tr>
<td>CD20</td>
<td>L26</td>
<td>1:1,200</td>
<td>MW, CB</td>
<td>DAKO, Carpinteria, CA</td>
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<tr>
<td>CD3</td>
<td>SP7</td>
<td>1:200</td>
<td>S, CB</td>
<td>NeoMarkers/Lab Vision, Fremont, CA</td>
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<tr>
<td>CD10</td>
<td>56C6</td>
<td>1:100</td>
<td>S, CB</td>
<td>Novocastra, Newcastle upon Tyne, England</td>
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<tr>
<td>bcl-2</td>
<td>124</td>
<td>1:400</td>
<td>MV, CB</td>
<td>DAKO</td>
</tr>
<tr>
<td>bcl-6</td>
<td>PG-B6P</td>
<td>1:100</td>
<td>T + S, TRIS</td>
<td>DAKO</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MB-1</td>
<td>1:4,800</td>
<td>PC, CB</td>
<td>DAKO</td>
</tr>
<tr>
<td>LANA of human herpesvirus 8 (ORF-73)</td>
<td>LN-53</td>
<td>1:30,000</td>
<td>PC, CB</td>
<td>Advanced Biotechnologies, Columbia, MD</td>
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<td>TdT</td>
<td>Polyclonal</td>
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<td>DAKO</td>
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<tr>
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<td>CS1-4</td>
<td>1:500</td>
<td>S, CB</td>
<td>DAKO</td>
</tr>
</tbody>
</table>

CB, citrate buffer, pH 6; LANA, latent nuclear antigen; MW, microwave oven; ORF, open reading frame; PC, pressure cooker; S, steamer; T, trypsin; TdT, terminal deoxynucleotidyl transferase; TRIS, tris(hydroxymethyl)aminomethane. * Heat-induced epitope retrieval was used.
Sections from all TMAs were examined for the expression of Epstein-Barr viral early RNA (EBER) by in situ hybridization (ISH) using an EBER-1 probe, consistent with a 30-base oligonucleotide complementary to a portion of the EBER1 gene. Briefly, the slides were deparaffinized, dehydrated, predigested with Pronase (Sigma, St Louis, MO), and hybridized overnight at a concentration of 0.25 ng/µL of probe. After washing, detection was accomplished by using the avidin-biotin immunoperoxidase method with 3',3'-diaminobenzidine as the chromogen, without nickel chloride added for color enhancement. A brown or black color in the nucleus over background levels was considered a positive reaction. Appropriate positive (cases of EBV+ BL and EBV+ Hodgkin lymphoma) and negative control (EBV− tissue) cores were also included in the array.

Molecular Study

Molecular analysis for HHV-8 was performed in 32 cases, including 9 known HIV+ BLs and 23 HIV− BLs. The cases in the HIV− BL group were selected in a random manner. DNA isolation was performed as follows: FFPE histologic sections were submitted to deparaffinization by successive xylene baths and dehydration with 100% ethanol. After digestion of the material with Proteinase K at 50°C for 4 to 6 hours, 50 µL of a saturated sodium chloride solution (−6 mol/L) was added, and, after mixing for 30 seconds, the mixture was centrifuged at 4,000 rpm for 15 minutes. The supernatant was removed to a fresh tube, and one-tenth volume of 3 mol/L sodium acetate was added, followed by 2.5 volumes of 100% ethanol. Samples were placed at −20°C overnight and up to 6 hours, 50 µL of a saturated sodium chloride solution (~6 mol/L) was added, followed by 5 minutes in developer solution, 3% sodium hydroxide and 5 minutes in silver solution, 0.2% silver nitrate in fixative solution; and 5 minutes in silver nitrate in fixative solution; and 5 minutes in developer solution, 3% sodium hydroxide and 0.5% formaldehyde).

Results

Clinical Features

Of the 311 patients with BL, 221 (71.1%) were male and 90 (28.9%) were female. Of the patients, 149 (47.9%) were 16 years or younger, and 143 (46.0%) were older than 16 years; the age was unknown in 19 cases. The mean age was 23.1 years (range, 2-95 years). Extranodal BL constituted 201 (64.6%) of the cases, and primary lymph node involvement was observed in 99 (31.8%) of the cases; in 11 cases, it was not possible to determine nodal vs extranodal presentation. Extranodal primary disease was found in a higher proportion of pediatric patients (112/149 [75.2%]) than adults (80/143 [55.9%]); 11 cases in the extranodal group did not have age information available.

Morphologic Features

In the original material reviewed to select the areas for TMA, 309 cases of BL showed entirely diffuse architecture, and rare cases exhibited a focal nodular pattern (2 cases). Most of the cases showed cytologic features of the “classic” subtype (271/311 [87.1%]) Image 1A, 28 (9.0%) of the cases showed histologic features of atypical BL, and 12 (3.9%) cases, there was insufficient material in the paraffin block to proceed with PCR. Two primers encompassed this region (E2 up, 5′-AGGCTGCCCACCTGAGGAT-3′ and E2 low, 5′-GCCACCTGGCAGCCCTAAAG-3′) containing a 16-bp deletion in EBV type A, yielding an amplification product with 170- and 186-bp fragment lengths for types A and B, respectively. In some cases, a seminested reamplification was performed using the E2 up and E2R low (5′-GCTGCCACCT-GGCCGAAT-3′) primers, rendering amplification products of 111 and 127 bp for types A and B, respectively. In each 25-µL PCR reaction, 100 ng of DNA, 0.2 µmol/L of 5′ and 3′ oligonucleotide primers, 0.2 mmol/L of dNTP, 2.5 µL of 10x PCR buffer, 1.25 U of Platinum Tag DNA polymerase (Invitrogen), and 1.5 mmol/L of magnesium chloride were used. The mixture was subjected to 35 cycles of amplification (30 seconds at 96°C, 30 seconds at 60°C, and 1 minute at 72°C in a PTC 200 thermocycler [MJ Research, Watertown, MA]). Before cycling, the samples were denatured at 96°C for 2 minutes. After the last cycle, the polymerization step was extended by 10 minutes, according to Araujo et al.4

PCR-amplified products for both studies were analyzed in a 7% polyacrylamide gel with silver staining (5 minutes in a fixative solution, 5% acetic acid and 10% of ethanol; 5 minutes in silver solution, 0.2% silver nitrate in fixative solution; and 5 minutes in developer solution, 3% sodium hydroxide and 0.5% formaldehyde).
showed plasmacytoid differentiation.

Immunohistochemical Analysis and ISH

All cases had an immunophenotype consistent with BL, as described in the World Health Organization classification. CD20 and CD10 were positive in all cases; bcl-6 positivity was found in 250 cases (80.4%) and was more frequent in the pediatric than in the adult population. bcl-2 was negative in all cases.

ISH for EBV showed 134 positive cases (45.0%), the majority in the pediatric population (76 [56.7%]) and 52 (38.8%) in patients older than 16 years. In 6 EBV+ BL cases, the age was unknown. In the pediatric group, 39 (51%) were EBV+; in the adult group, 36.3% were EBV+. In 13 cases, ISH for EBV was inconclusive because there was insufficient neoplastic tissue available in the cores.

None of the cases showed nuclear granular positivity for LANA protein with HHV-8 antibody. LMP-1 expression was not observed in any of the cases. All positive control samples for LMP-1 and LANA protein showed the expected immunostaining reactivity.

Molecular Analysis

Molecular analysis for HHV-8 was performed in 32 cases, including 9 known HIV+ and 23 HIV–BLs; 19 were EBV−, and the other 13 cases presented EBV infection. No BL case had HHV-8 viral DNA, including cases of sporadic BL and HIV-associated BL.

EBV molecular subtyping analysis showed that 96 (73.3%) of 131 cases were EBV type A. The rest were type B (27 [20.6%]). It is worth noting that in 8 cases (6.1%), the DNA obtained from the FFPE blocks had less than 100 bp, giving inconclusive results for the EBV subtyping PCR analysis. No case showed both EBV subtypes in the same sample.
The spectrum of HHV-8–associated lymphoid tumors is different from that associated with EBV.\textsuperscript{21,22} HHV-8 is closely associated with Kaposi sarcoma but is associated with only a few categories of lymphoproliferative diseases, mostly occurring in HIV-infected patients, with primary effusion lymphoma (PEL), a nonsolid B-cell lymphoma, having the most significant association. Extranodal marginal zone lymphoma, multiple myeloma, Kikuchi disease, and hemophagocytic lymphohistiocytic syndrome are lymphoproliferative disorders with a variable and generally infrequent association with HHV-8.\textsuperscript{6,23-27} In contrast, the evidence strongly supports a role for EBV in the pathogenesis of a wide spectrum of human lymphoid malignancies, including B- and T-cell lymphomas, natural killer neoplasms, and Hodgkin lymphoma and nonhematologic tumors such as nasopharyngeal carcinoma and gastric tumors.\textsuperscript{1,12,13}

In BL, EBV has been demonstrated in up to 100% of the endemic form and in only 15% to 30% of sporadic cases in the United States.\textsuperscript{1} In some parts of the world (North Africa and South America), the incidence is intermediate between true sporadic and endemic variants.\textsuperscript{1} In Brazil, there are limited data about the frequency of EBV in cases of BL. Previous studies showed that the frequency of EBV in cases of Brazilian BL varied from 58% to 87%.\textsuperscript{4,5,28-30} It is important to emphasize that in the 5 previous studies on BL in Brazil, all EBV+ cases were described in the pediatric population only. Our study on BL showed 134 EBV+ cases (45.0%), with 76 cases (56.7%) occurring in patients in the age group of 16 years or younger and 52 cases (38.8%) in patients older than 16 years, which represents 36.4% of the adult group. To the best of our knowledge, this is the first study in Brazil reporting the frequency of EBV in adult cases of BL. Our overall results place Brazil as a country with intermediate association of BL with EBV.\textsuperscript{1}

EBV strains can be categorized into 2 types (A and B), and different geographic prevalence of these strains has been observed.\textsuperscript{4} By studying the \textit{EBNA}-2 gene, we determined that 96 (73.3%) of 131 cases contained type A EBV and 27 (20.6%) contained type B EBV. The simultaneous presence of both types of EBV in the same tumor was not observed in any sample. This pattern, with a predominance of type A, is similar to that observed in sporadic cases and previous studies of Brazilian BL\textsuperscript{4,31} and different from the pattern described in BL occurring in Equatorial Africa.\textsuperscript{32} LMP-1 was negative according to the highly restricted pattern of EBV latent proteins observed in BL.\textsuperscript{33}

HHV-8–associated lymphomas include cases of naive cell origin, GC and post–GC cells, unlike EBV–associated lymphomas, which are of GC or post-GC origin.\textsuperscript{12,13} Moreover, HHV-8 and EBV coinfections have been documented in the setting of primary effusion lymphoma and Castleman disease, typically associated with an immunosuppressed state.\textsuperscript{14,34,35}

The biologic heterogeneity of AIDS–non-Hodgkin lymphoma is highlighted by their histogenetic differences. HHV-8–associated lymphomas, which often develop in persons with advanced AIDS, present predominantly as PEL. HHV-8 has also been recently detected in solid extracavitary-based lymphomas.\textsuperscript{36-38} The HHV-8–associated solid lymphomas are unusual lymphomas that occur more specifically in HIV+ patients, are extracavitary and arise in nodal and/or extranodal sites, and sometimes are associated with coinfection with EBV; histologically, they usually display PEL-like morphologic features and a plasma cell–related phenotype.

Lazzi et al,\textsuperscript{15,16} in different studies of African patients (East Africa), found no case with tumoral lymphoid cells infected by HHV-8 in 16 and 29 BL cases, respectively. Tao and Ambinder\textsuperscript{17} studied HHV-8, HHV-7, and HHV-6 DNA in 10 cases of BL in Africa and found only HHV-6 in 3 of their cases.

Molecular identification of HHV-8 is known to produce false-positive and false-negative results.\textsuperscript{34} The latter is thought, in part, to be related to HHV-8 sequence variation, which can range up to 35% in certain regions of the viral

Image 2 Human herpesvirus (HHV)-8 DNA detection by polymerase chain reaction (PCR). A, Size control PCR. B, HHV-8–specific PCR. C+, DNA extracted from peripheral blood; C+1, positive HHV-8 Kaposi sarcoma; C+2, positive HHV-8–AIDS-related non-Burkitt lymphoma; M, DNA molecular weight marker; No, DNA absence; 1-2, HIV+ Burkitt lymphoma cases; 3-4, HIV– Burkitt lymphoma.
genome. On the other hand, LANA immunohistochemical studies are thought to be a more reliable marker of HHV-8 infection. In the present study, we found no evidence for HHV-8 in BL, including cases with HIV+ status using immunohistochemical and molecular methods. We were, however, able to confirm the association of EBV with BL, found in 45.0% of the cases of BL in the present series. In no case was coinfection of EBV and HHV-8 found.

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Supported by grants 5R01CA082274 and 5R01CA112217 from the National Cancer Institute (Dr Harrington) and the AIDS Malignancy Consortium (National Cancer Institute), Bethesda, MD.

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