Kaposi’s sarcoma-associated herpesvirus infection and Kaposi’s sarcoma in Brazil

S. Ramos-da-Silva¹, D. Elgui-de-Oliveira¹,², L. Borges¹ and C.E. Bacchi²

¹Departamento de Patologia, Faculdade de Medicina de Botucatu, Universidade Estatal Paulista, Botucatu, SP, Brasil
²Consultoria em Patologia, Botucatu, SP, Brasil

Abstract

Kaposi’s sarcoma (KS) became a critical health issue with the emergence of acquired immunodeficiency syndrome (AIDS) in the 1980s. Four clinical-epidemiological forms of KS have been described: classical KS, endemic KS, iatrogenic KS, and AIDS-associated KS. In 1994, Kaposi’s sarcoma-associated herpesvirus (KSHV) or human herpesvirus type 8 was identified by Chang and colleagues, and has been detected worldwide at frequencies ranging from 80 to 100%. The aim of the present study was to evaluate the frequency of KSHV infection in KS lesions from HIV-positive and HIV-negative patients in Brazil, as well as to review the current knowledge about KS transmission and detection. For these purposes, DNA from 51 cases of KS was assessed by PCR: 20 (39.2%) cases of classical KS, 29 (56.9%) of AIDS-associated KS and 2 (3.9%) of iatrogenic KS. Most patients were males (7.5:1, M/F), and mean age was 47.9 years (SD = ± 18.7 years). As expected, HIV-positive KS patients were younger than patients with classical KS. On the other hand, patients with AIDS-associated KS have early lesions (patch and plaque) compared to classical KS patients (predominantly nodular lesions). This is assumed to be the result of the early diagnose of KS in the HIV-positive setting. KSHV infection was detected by PCR in almost all cases (48/51; 94.1%), irrespectively of the clinical-epidemiological form of KS. These results show that KSHV is associated with all forms of KS in Brazilian patients, a fact that supports the role of this virus in KS pathogenesis.

Key words
- Kaposi’s sarcoma
- Human herpesvirus type 8
- Kaposi’s sarcoma-associated herpesvirus
- Polymerase chain reaction
- Viral carcinogenesis
- Brazil

Introduction

In 1872, Kaposi’s sarcoma (KS) was described by Moritz Kaposi (1) as a rare and indolent angioproliferative neoplasm, mainly present as a skin lesion (2). Since the 1980’s, the disease has become a critical health issue with the emergence of the AIDS epidemic (2). The classical form of KS is characterized by solitary plaques or nodules in the lower extremities of elderly men from Eastern Europe and the Mediterranean region (3). Three other clinical-epidemiological forms are known: endemic KS, affecting children and young men in Central Africa (4); iatrogenic KS, observed in some patients under immu-
nosuppressive therapy (5), and AIDS-associated KS, an aggressive form of KS that occurs mainly in homosexual and bisexual men (6).

In 1994, Chang and co-workers (7) identified unique herpesvirus DNA sequences in KS lesions from HIV-positive patients. The new virus, Kaposi’s sarcoma-associated herpesvirus (KSHV) or human herpesvirus type 8 (HHV-8), is also demonstrable in tumors other than KS (8-11). The viral genome encodes several products that share structural and functional homology with human proteins that have pivotal role in cell proliferation, which may explain the viral contribution to a malignant transformation (12-18).

The worldwide frequency of KSHV detected in KS ranges from 80 to 100%. Frequently used methods to assess viral infection are in situ hybridization, serologic analysis and polymerase chain reaction (PCR) (19-21), the latter being the most sensitive. Using PCR, Moore and Chang (22) detected KSHV in 6/6 (100%) classical KS cases, in 4/4 (100%) HIV-negative homosexual patients, and 10/11 (91%) AIDS-associated KS cases. In a similar study, Dictor et al. (23) found the virus in all 14 KS cases from HIV-positive patients, as well as in 35/40 (88%) classic KS cases.

Data about KSHV infection in KS occurring in Brazilian patients are scarce (24-27). The objective of the present study was to identify the viral genome in KS lesions from HIV-positive and HIV-negative patients in Brazil.

Material and Methods

The study was approved by the Research Ethics Committee of the Botucatu Medical School, São Paulo State University (UNESP). Tissue biopsies from 51 KS lesions were obtained from the files of the Department of Pathology, Botucatu Medical School, and from private Surgical Pathology laboratories in São Paulo State, Brazil. Based on clinical and histopathological data, the cases were divided as follows: 20 (39.2%) cases of classical KS, 29 (56.9%) of AIDS-associated KS and 2 (3.9%) of iatrogenic KS. Skin biopsies with normal histology (N = 8) were used as negative controls for KSHV infection.

The lesions were further classified as patch, plaque or nodules according to the stage of the disease. Early stages of KS are characterized by purple patches on the skin, which are histologically characterized by neoplastic cells scattered in the dermis. In the plaque stage, confluent groups of neoplastic cells are observed, as well as increased neovascularization and hemosiderin deposition. The nodular stage consists of tumors composed of high density of spindle-shaped cells and other components, such as endothelial cells, inflammatory infiltrate and erythrocytes.

At least two 25-µm sections were obtained from formalin-fixed paraffin-embedded tissues for DNA extraction. Briefly, sections were deparaffinized with xylene (10 min at 64ºC, 10 min at room temperature with shaking, and again 10 min at 64ºC). Next, the samples were centrifuged for 5 min at 15,800 g and xylene was removed and this sequence was repeated twice; the tissues were then dehydrated with ethanol and centrifuged for 10 min at 15,800 g. Ethanol was removed and these steps were repeated once. Subsequently, tissue sections were incubated overnight with proteinase K (400 ng/µL in 10 mM Tris, 1 mM EDTA, 0.5% Tween 20 buffer) at 56ºC.

After enzyme inactivation by heat (94ºC for 10 min), DNA purification was performed by adding 100 µL 5 mM NaCl plus 100 µL of the CTAB/NaCl solution (0.87 M NaCl, 0.34 M CTAB) to the samples. After 10 min of incubation at 65ºC, the samples were treated with 1:24 chloroform/isooamlic alcohol and centrifuged for 5 min at 15,800 g. The upper phase was transferred to a new tube with
ethanol, and kept at -20ºC for 10 min. After another centrifugation at 15,800 g, the precipitated DNA was treated with 70% ethanol at room temperature, and the pellet was resuspended in 50 µL TE solution (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). At this point, the quantity and quality of DNA were determined by UV-spectrophotometry and agarose-gel electrophoresis, respectively. Samples in which DNA concentration was higher than 0.4 µg/µL were diluted to 0.1 µg/µL to prevent inhibition of the PCR.

KSHV detection by PCR was initially carried out with KS330 primers (7). The samples without amplification in this first PCR assay were used in ORF26-directed nested-PCR (28). Doubtful cases were analyzed for amplification of viral ORF72 segment, which encodes a viral capsid protein (28). Samples negative for all PCR assays were further evaluated for amplification of the β-chain human globin gene with PCO26+/PCO26+ primers (29). PCR components were: buffer (28 mM Tris-Cl, pH 8.4, 70 mM KCl, 3.0 mM MgCl2), 0.2 mM each dNTP, 1.25 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), and 0.5 µM of upstream and downstream primers. The PCR cycling profile was 94ºC for 5 min (1X); 94ºC for 30 s, AT for 30 s, 72ºC for 1 min (35X); 72ºC for 7 min (1X), where AT is the annealing temperature for each primer pair: 57ºC for KS330 primers and 55ºC for both ORF26in and ORF72 primers. The PCR product was analyzed in 2% agarose gel stained with ethidium bromide. The expected amplicon sizes were 233 bp for KS330 primers, and 138 bp for both ORF26in and ORF72 primers.

Results

Using KS330 primers KSHV infection was detected in 31/51 (60.8%) KS cases. Using ORF26-directed nested-PCR (ORF26in primers), 14/20 (70%) of the originally negative cases proved to be KSHV-positive, while 3 of the 6 (50%) remaining cases had demonstrable viral ORF72 amplification. Thus, 94.1% of all KS cases were KSHV-positive. The KSHV genome was detected in all KS cases regardless of KS type. Normal skin biopsies, used as negative controls, were consistently negative for KSHV infection and suitable for amplification of a 123-bp β-globin gene segment. The specificity of KS330, ORF26in, and ORF72 PCR assays for KSHV detection was confirmed by automated sequencing of randomly chosen PCR products of KSHV-positive KS samples, as shown in Figure 1.

Patients with AIDS-associated KS were younger than those with classical KS. Regarding the stage of the disease, patients with classical KS predominantly presented nodular lesions, while HIV-positive patients had the early stages of KS (patch and plaque). These data are probably due to early identification of KS in HIV-positive patients, who often exhibited multiple lesions, facilitating clinical detection. Table 1 summarizes the available data on the KS cases evaluated, including clinical information and the KSHV infection status.

Discussion

Despite the increasing number of studies on KSHV and human neoplasia, to date the role of viral infection in the pathogenesis of KS has not been completely elucidated. It is well known that the KSHV genome encodes products that share homology with human cell proteins. For instance, viral bcl-2 (en-
Figure 1. Panel A. Kaposi's sarcoma-associated herpesvirus (KSHV) detection by PCR in Kaposi's sarcoma lesions (KS) and histologically normal skin samples (NS). BCBL-1 is the positive control (DNA extracted from a KSHV-positive primary effusion lymphoma cell line), whereas 1st No and 2nd No are PCR assays without sample DNA (“No DNA” negative controls). ORF26 nested-PCR (ORF26in) was performed with 0.5 µL of the respective PCR product from the previous KS330 PCR. Only KS28, KS36 and KS48 were positive for KSHV in KS330 PCR. However, all KS samples, but no NS sample, were found to be positive for KSHV after ORF26 in nested-PCR. In addition, samples KS20, KS24, KS28, KS36, KS48, and no NS sample were positive for KSHV by ORF72 PCR.

Panels B and C. PCR products of selected samples were submitted to automated nucleic acid sequencing, and the sequences were aligned with reference KSHV sequences from GenBank. In panel B, amplicons for KS330 (BCBL-1, KS36 and KS48) and ORF26in (KS20, KS24, KS36, KS48) KSHV-positive samples were aligned with a segment comprising nucleotides 987-1219 from KSHV sequence accession #U18551. In panel C, amplicons for ORF72 KSHV-positive samples (KS24, KS36 and KS48) were aligned with a segment comprising nucleotides 2225-2362 from the KSHV sequence accession #U40667. Differences in nucleotide sequences are indicated. Annealing regions for outer and inner PCR primers are indicated by shaded and white boxes, respectively.
coded by KSHV ORF16) and viral FLIP (FLICE inhibitory protein, ORF-K13) have anti-apoptotic properties, which may contribute to a poor prognosis in KS. The KSHV G-protein-coupled receptor can induce VEGF-mediated tumor growth, and viral cyclin-D (ORF72) may induce cell proliferation through pRB inactivation (30).

Using different techniques, KSHV infection has been detected in 80 to 100% of KS cases worldwide. Serologic studies detecting antibodies against KSHV latency-associated nuclear antigens have allowed viral detection in 80% of HIV-positive patients with KS, and in almost 2% of healthy blood donors from the United States (31). Interestingly, another antigen-based assay showed an even higher frequency of KSHV infection in the general population, i.e., 25% of randomly selected individuals were positive (20).

PCR-based procedures have proved to be reliable methods to evaluate KSHV infection in biological samples. Using this technique, Moore and Chang (22) detected the KSHV genome in frozen tissues from 11 cases of AIDS-associated KS (91%), and all cases of classical KS (N = 6), as well as HIV-negative homosexual patients with KS (N = 4). Similar results were obtained by Jin et al. (32), i.e., KSHV was detected in 100% of 17 KS biopsies, 12 from HIV-negative and 5 from HIV-positive patients. Combining PCR and in situ hybridization, Huang et al. (33) demonstrated KSHV infection in all KS biopsies from 12 HIV-positive and 2 HIV-negative patients, and the KSHV viral genome was also detected in 25% of semen and blood samples from HIV-positive individuals with KS.

These data indicate the high sensitivity of the PCR method for the detection of KSHV. However, in a study by Pan and coworkers (28), the authors concluded that KSHV detection by PCR may involve a high rate of false-negative results. For this reason, optimization of PCR procedures is mandatory, and nested-PCR approaches as well as amplification of different segments of the KSHV genome are recommended in order to obtain accurate results (28). Using a standard PCR procedure to detect the KSHV genome in DNA from formalin-fixed paraffin-embedded tissue from few cases, Caterino-de-Araujo et al. (25) initially found the virus in 3/7 (42.9%) KS cases; however, all of their samples were found to be positive after nested-PCR.

<table>
<thead>
<tr>
<th>KS forms</th>
<th>M/F ratio</th>
<th>Stage of disease</th>
<th>Age (years)</th>
<th>KSHV-positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>AIDS-associated</td>
<td>28:1</td>
<td>Patch</td>
<td>9/29 (31%)</td>
<td>36.9 ± 11.3</td>
</tr>
<tr>
<td>(N = 29)</td>
<td></td>
<td>Plaque</td>
<td>9/29 (31%)</td>
<td>36.3 ± 4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nodular</td>
<td>4/29 (13.8%)*</td>
<td>36.8 ± 14.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Visceral</td>
<td>6/29 (20.7%)</td>
<td>34.2 ± 13.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inconclusive</td>
<td>1/29 (3.5%)</td>
<td>53.0</td>
</tr>
<tr>
<td>Classical</td>
<td>4:1</td>
<td>Patch</td>
<td>2/20 (10%)</td>
<td>49.0 ± 28.3</td>
</tr>
<tr>
<td>(N = 20)</td>
<td></td>
<td>Plaque</td>
<td>3/20 (15%)</td>
<td>62.3 ± 16.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nodular</td>
<td>15/20 (75%)*</td>
<td>65.7 ± 14.7</td>
</tr>
<tr>
<td>Iatrogenic</td>
<td>1:1</td>
<td>Plaque</td>
<td>2/2 (100%)</td>
<td>41.5 ± 29.0</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD. P25, P75 = 25th and 75th percentiles, respectively. KSHV = Kaposi’s sarcoma-associated herpesvirus. *P < 0.05 between the indicated groups (Goodman’s proportion test).
In the present study, we detected 31/51 (60.8%) KSHV-positive cases in the first PCR round. Fourteen of 20 initially negative cases (70%) proved to be KSHV-positive with further analysis by nested-PCR. Additionally, 3 of the 6 (50%) remaining cases had demonstrable KSHV ORF72 amplification. Overall, KSHV infection was detected by PCR in 48/51 (94.1%) KS cases. These data emphasize the need for an optimized PCR procedure to minimize false-negative results, which may occur due to small quantity or high fragmentation of DNA extracted from formalin-fixed paraffin-embedded tissues, as well as a low viral burden in tissue biopsies and tumor scarcity in biopsy samples (e.g., early stage lesions of KS).

Some studies about KSHV transmission have demonstrated that the virus can be transmitted to transplanted patients by KSHV-infected donors (34). In this case, tumor development is associated with immunosuppressive therapy (35). Vertical KSHV transmission was demonstrated by Mantina et al. (36) who found viral DNA in two neonates; moreover, the authors emphasize that it may not be possible to determine if infection occurred in utero or intrapartum, but postnatal infection was ruled out due to the impossibility to detect DNA during the immediate postnatal period.

It was suggested that the sexual transmission of KSHV among homosexual men probably occurred because of anal receptive intercourse (37). A possible source of non-sexual horizontal transmission (e.g., through saliva) was suggested by a study that analyzed KSHV transmission in children before puberty (38). KSHV transmission by injection drug use was rare among drug users in Amsterdam (39), but another study demonstrated that KSHV seropositivity increased in association with injection drug use (40).

Few studies are available about KSHV frequency among KS patients in Brazil (24-27). Keller et al. (26) evaluated 66 blood samples from HIV-positive patients, and KSHV infection was detected by PCR in 29/39 (74.4%) of KS-positive and 1/27 (3.7%) of KS-negative cases. In serologic analysis, 38/39 (97.4%) of KS-positive individuals had antibodies to viral antigen and 7/27 (26%) of KS-negative individuals were positive to KSHV in at least one serologic analysis. They investigated immunodeficiency of HIV-positive patients through CD4 counts and observed a decrease in CD4 cells in HIV-positive patients with KS in the aggressive stage (26).

Analyzing 267 Brazilian patients, Zhang et al. (27) found anti-KSHV antibodies in 64-71% HIV-positive patients with KS. In a more recent study, Biggar et al. (24) detected a new KSHV subtype (namely subtype E) in different Brazilian Amerindians from the Northern region of Brazil using an immunofluorescence approach. The authors observed that KSHV infection was endemic in this particular group and were unable to identify KSHV-associated diseases, such as KS or HIV infection. They used 746 samples from 16 different tribes and detected a prevalence of KSHV infection ranging from 0 to 100% (overall, a 53% prevalence). KSHV immunofluorescence-positive cases were analyzed by quantitative PCR, and the viral genome was detected in 3/19 (16%) of samples from peripheral blood mononuclear cells and in 1/16 (6.3%) from saliva. It seems that oral transmission, rather than sexual transmission, was more important in this population since people with KSHV infection were 41% of the children (at least 10 years old) and 65% of the adults (30 years old) (24).

We could not find another study in the literature evaluating retrospectively KSHV infection in a larger number of KS biopsies than analyzed in the present study. Our results emphasize that KSHV is positively associated with all forms of KS and can be readily detected in tissue biopsies. Although PCR is extremely sensitive for detecting the KSHV genome in DNA samples from paraf-
fin-embedded tissues, the optimization of PCR-based procedures is necessary in order to minimize false-negative results.

Acknowledgments

The authors are indebted to Diagnostika Laboratory, Consultoria em Patologia Laboratory, and to Dr. Luiz Alberto Veronese for providing additional Kaposi’s sarcoma cases for this study. Additionally, we would like to thank Dr. Adalberto José Crocci for support with the statistical analysis, and Ms. Celene Gandin and Mr. Marcos Roberto Franchi for valuable technical support.

References