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Jerzy Lasota · Markku Miettinen

Absence of Kaposi's sarcoma-associated virus (human herpesvirus-8) sequences in angiosarcoma

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Abstract Kaposi's sarcoma-associated virus (human herpesvirus-8 [HHV8]) sequences have been identified in both AIDS-associated and AIDS-non-associated Kaposi's sarcoma, but data relating to the detection of HHV8 sequences in angiosarcoma are variable. One study showed HHV8 sequences in 29% (7/24) of angiosarcomas, but others have not confirmed these results. We evaluated 33 angiosarcomas for HHV8 sequences to determine the frequency of this virus in angiosarcomas and its possible pathogenetic significance. Five cases of Kaposi's sarcoma from HIV-positive patients were used as positive controls. Five additional cases of Kaposi's sarcoma collected approximately 40–50 years ago were also analysed, and three were HHV8 positive. None of the angiosarcomas revealed HHV8 sequences after the standard 35 cycles of PCR. In 6 cases, nested PCR revealed the presence of HHV8 sequences. These results were not reproducible when outer primers (based on sequences outside of the earlier PCR products) were used for amplification. This suggests that the HHV8 sequences detected in 6 cases represent a low level of contamination. In contrast, HHV8 sequences were found in all Kaposi's sarcomas with well-preserved DNA after standard 35-cycle PCR amplification. These findings confirm a close association between Kaposi's sarcoma and HHV8 infection and suggest that HHV8 is not involved in the pathogenesis of angiosarcoma.

Key words Angiosarcoma · Human herpesvirus-8 · Kaposi's sarcoma · PCR

The opinions and assertions contained herein are the expressed views of the authors and are not to be construed as official or reflecting the views of the Departments of the Army or Defense

J. Lasota · M. Miettinen (✉)[1]
Armed Forces Institute of Pathology,
Department of Soft Tissue Pathology, Washington D.C., USA

Mailing address:
Armed Forces Institute of Pathology, Soft Tissue Department,
6825 16th Street N.W., Washington, DC 20306–6000, USA
Tel.: +1-202-782-2793, Fax: +1-202-782-9182

Introduction

Human herpesvirus-8 (HHV8) sequences, originally identified in AIDS-associated Kaposi's sarcoma by means of representative differential analysis by Chang et al. [9], have been found by PCR in both AIDS-associated and AIDS-non-associated Kaposi's sarcoma, and it has been suggested that they are important in its pathogenesis [1, 25]. The HHV8 sequences have also been found in AIDS-associated and AIDS-non-associated body cavity lymphomas that present with effusions [7, 8], in angiofollicular lymph node hyperplasia of Castleman [11, 31], and in angioimmunoblastic lymphadenopathy [21]. In primary effusion lymphoma experimentally transmitted in immunodeficient mice, the lymphoma cells have been found to contain HHV8 particles [29]. Very recently, HHV8 was identified in bone marrow dendritic cells from patients with multiple myeloma [30].

Because HHV8 has been thought to infect endothelial cells specifically [2], analysis of angiosarcomas is of particular interest from both pathogenetic and diagnostic points of view. Recently, the presence of HHV8 has been reported in angiosarcomas by some authors [15, 23] although not by others [10, 16, 18, 22].

To evaluate the possibility that HHV8 sequences might be present in angiosarcomas we studied 33 cases representing a broad range of different histological and clinicopathological types of angiosarcomas. Ten Kaposi's sarcomas, including 5 cases collected approximately 40–50 years ago, were also analysed. In 6 angiosarcomas, nested PCR amplification revealed HHV8 sequences. However, the presence of HHV8 sequences in these cases was not confirmed by amplification with outer primers. These findings suggest that the HHV8 sequences detected represented a low level of contamination. In contrast, HHV8 sequences were found in 5 Kaposi's sarcomas with well-preserved DNA after standard 35-cycle PCR amplification and in 3 of the 5 cases collected approximately 40–50 years ago. Lack of the HHV8 sequences in different types of angiosarcomas indicates that this virus is not involved in the pathogenesis of angiosarcomas.

Materials and methods

Formaldehyde-fixed paraffin-embedded (FFPE) tissue from 33 angiosarcomas, 10 Kaposi's sarcomas and 36 fresh peripheral blood (PBL) samples from healthy blood donors was analysed. The FFPE tissue blocks were obtained from the files of Armed Forces Institute of Pathology, Washington D.C.

DNA for PCR amplification was obtained from peripheral blood mononuclear cells (PBMC) after density fractionation on Ficoll Histopaque 1077 (Sigma, St. Louis, Mo.) and from FFPE tissue using previously published procedures [17]. The quality of DNA templates was verified by PCR amplification of a 268-bp fragment of β-globin gene as described by Greer et al. [14] or by seminested PCR amplification of *bcl*-2 locus on chromosome 18. For the seminested PCR assay, a previously published MC8 forward primer [26] was used with reverse primers: MC10 (5'-GAG-CGCTAGGATTGTTACCT-3') and MC12 (5'AATACCTCTCAG-CAAAGCCATC-3'). The reverse primers were designed on the basis of previously published sequences [26]. The PCR products were 144 bp and 113 bp in the first and seminested reaction, respectively. The reaction mixture and PCR cycling conditions were similar to those used for the β-globin gene amplification [14].

The PCR assay types and lengths of the PCR products are shown in Table 1. Locations of the primers are shown in Fig. 1.

The presence of HHV8 sequences was evaluated by PCR as previously described [9]; approximately 0.5–1.0 μg of DNA templates were amplified for 35 cycles using primers designated in this study as KS1 and KS2. The amplification products were size fractionated in gels stained with ethidium bromide.

The negative cases were evaluated for HHV8 sequences using a nested PCR reaction. An aliquot (0.25 μl) of the PCR products was used as a template for the nested reaction and amplified for 30 cycles with the primers designed on the basis of previously published sequences [9] and designated in this study as KS3 (5'-CTC GAA TCC AAC GGA TTT GA-3') and KS4 (5'-AAT GAC ACA TTG GTG GTA TA-3'). The reaction mixture and PCR conditions were similar to those previously reported [9], except that the annealing temperature was 50°C. The specificity of the PCR and nested PCR amplification were confirmed by hybridization with HHV8-specific probe and by sequencing (following cloning) in selected cases.

Because of the severe degradation of DNA, a seminested PCR assay was designed to amplify a relatively small fragment of HHV8 sequence in archival material. The forward primers, designated as KS5 (5'-TCC CTC TGA CAA CCT TCA GA-3') and KS6 (5'-AGC AAC ACC CAG CTA GCA GT-3') were designed outside the sequences amplified by PCR assay and used with the reverse primer designated as KS7 (5'-TCA AAT CCG TTG GAT TCG AG-3'). The reverse primer corresponded to the sequence of the KS3 forward primer. The PCR products were 135 bp and 80 bp in the first and seminested reactions, respectively. The templates, reaction mixtures and cycling conditions were similar to those previously reported [9], except that the annealing temperature was 50°C. The amplification products were analysed on 10% polyacrylamide gels. The specificity of the seminested PCR reaction was confirmed by sequencing via cloning in plasmid (see below).

To confirm the results of the nested PCR, a new seminested PCR assay was developed. The forward primers KS5 and KS6 and a reverse primer KS8 (5'-AACAGCTGCTGCAGAATAGC-3') were used in the seminested PCR amplification. The products generated by the new seminested PCR assay were 194 bp and 139 bp in the first and seminested (second) reactions, respectively. The location of the primer sequences used in this study is shown in Fig. 1. The templates, PCR reaction mixtures and cycling conditions were similar to those used above for the simple and nested PCR, except that the annealing temperature was 55°C in the first reaction and in the seminested reaction. The specificity of the new seminested PCR amplification was confirmed by sequencing via cloning in plasmid (see below).

Efficiency of the nested and seminested PCR assays was investigated as previously published [33] by amplification of the serial dilutions of known amounts of purified PCR product from the standard PCR amplification of HHV8-positive Kaposi's sarcoma. Both nested and seminested PCR assays showed the same efficiency of detection, that is to say approximately 10 copies of virus.

For Southern blotting hybridization the gels were denatured, neutralized, and blotted on Zeta Probe GT Membrane (Bio Rad Lab, Hercules, Calif.), according to the procedure described by

Table 1 Different types of the PCR assays for detection of HHV8 used in this study

Type of PCR assay	First PCR reaction		Second PCR reaction	
	Primers	Products	Primers	Products
Standard	KS1/KS2	234 bp	–	–
Nested	KS1/KS2	234 bp	KS3/KS4	170 bp
Seminested[a]	KS5/KS8	194 bp	KS6/KS8	139 bp
Seminested[b]	KS5/KS7	135 bp	KS6/KS7	80 bp

[a] Used to evaluate reproducibility of the nested PCR results
[b] Used to evaluate archival samples of Kaposi's sarcoma

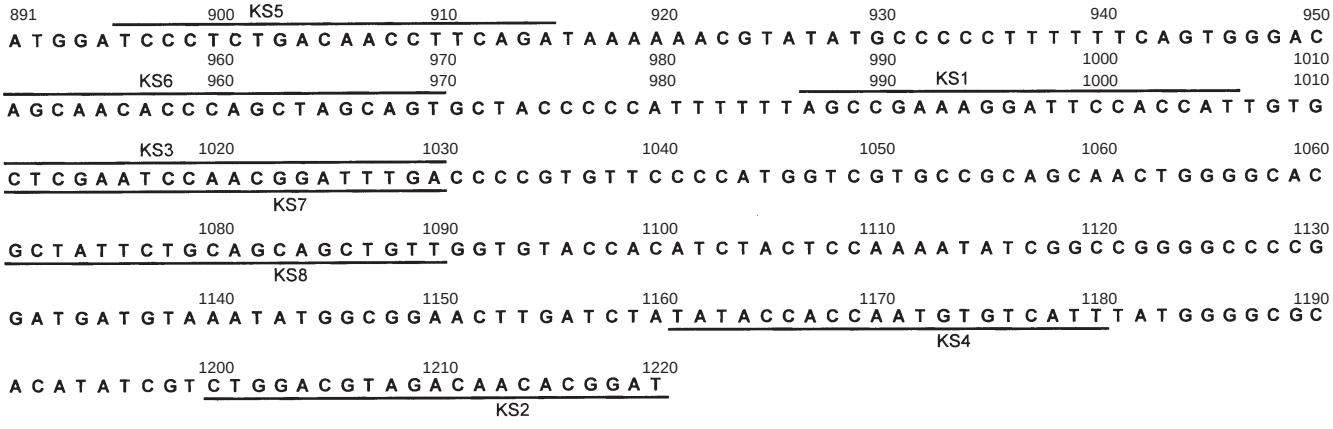

Fig. 1 Sequence of the human herpesvirus-8 amplified in this study based on the previously published 1853 bp-fragment of the HHV8 sequence by Chang et al. [9]. The *numbers* indicate base pairs as numbered by Chang et al. The primers used in this study are shown *above* (forward) and *below* (reverse) the sequence. The primers were used as follows: KS1/KS2 followed by KS3/KS4 primers for nested PCR; KS5 and KS6 forward primers and KS8 reverse primer for seminested PCR to confirm the results of the nested PCR; KS5 and KS6 primers and KS7 reverse primer for evaluation of the HHV8 in Kaposi's sarcomas diagnosed 40–50 years ago

Southern. The filters were hybridized with random prime ^{32}P-labelled internal, probe (HHV8 sequences from nucleotide 1011 to nucleotide 1180) for 16–24 h at 65°C, washed as recommended by the membrane manufacturer, and then exposed for 1–6 h using Kodak X-films (Rochester, N.Y.).

The PCR products were cut from the gels, purified, treated with Klenow polymerase (Boehringer Mannheim, Indianapolis, Ind.) in the presence of all four dNTPs, then blunt-end ligated into pBluescript-SK+ phagemid (Stratagene, La Jolla, Calif.) and transfected into DH5 α-competent cells (Gibco BRL, Gaithersburg, Md.). Several randomly selected recombinants were sequenced on a 373 DNA sequencer (Applied Biosystems, Foster City, Calif.). Computer analysis of the resulting DNA sequences was performed using the Lasergene software in connection with the data of the GeneBank 104/EMBL 51 database (January 1998 edition) and the published germline sequences [9, 24, 25].

Because a highly sensitive PCR procedure is prone to false-positive results caused by contamination of the sample by PCR products of previous analysis, strict measures against contamination were undertaken in all experiments. The following precautions were undertaken to prevent and monitor possible PCR contamination. Histological sectioning, DNA extraction, PCR amplification and electrophoresis of PCR products were processed in separate rooms. In each experiment, multiple negative control reactions were performed to detect potential random cross contamination; the positive control was processed at the same time using the same reagent mixture but at a different location. The work area and all laboratory equipment used to prepare nested PCR reactions were cleaned with DNA away (Fisher Scientific, Pittsburgh, PA) and UV-exposed for several hours after each experiment. Positive results were re-evaluated using a different PCR assay the second time.

Results

The 33 angiosarcomas and 10 Kaposi's sarcomas were classified according to Enzinger and Weiss [12]. Angiosarcomas represented different clinicopathological subtypes and locations: high-grade angiosarcomas of deep soft tissues (*n*=10), cutaneous angiosarcomas of scalp (*n*=5), cutaneous angiosarcomas of other sites (*n*=5), postmastectomy angiosarcomas of arm (*n*=3), intra-abdominal and gastrointestinal (*n*=3), splenic (*n*=2), and cardiac angiosarcomas (*n*=3), intracranial angiosarcoma of dura (*n*=1), and a lung metastasis of a soft tissue angiosarcoma (*n*=1). Three angiosarcomas were of the epithelioid type, and 1, from the skin of a finger, was a low-grade tumour corresponding to the so-called retiform haemangioendothelioma [5]. Two angiosarcomas of the deep soft tissues, a pericardial and abdominal wall angiosarcoma, and 1 cutaneous angiosarcoma from breast skin, arose in patients who had received irradiation for a malignant tumour in the area where the angiosarcoma subsequently developed. None of the cases was AIDS associated. In all cases, the diagnosis was based on the presence of various degrees of vasoformation by the tumour cells, supported by the demonstration of CD31 immunoreactivity.

Five Kaposi's sarcomas of the skin, collected from elderly patients approximately 40–50 years before, were also analysed. Histologically these tumours showed features typical of Kaposi's sarcoma (Fig. 2). AIDS-associated Kaposi's sarcomas were used as positive controls and represented variably haemorrhagic nodular spindle cell tumours in the skin (*n*=2) or internal organs (*n*=3) in HIV-positive patients.

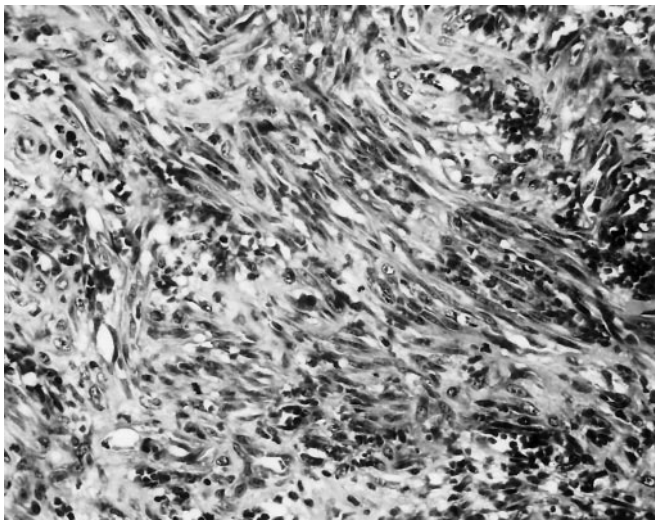

Fig. 2 Cutaneous Kaposi's sarcoma diagnosed 50 years ago shows atypical spindle cells in a haemorrhagic background. Haematoxylin and eosin ×300

Table 2 Summary of the results on PCR-assays for HHV8 on angiosarcoma, Kaposi's sarcoma and PBL samples obtained by different PCR assays

Lesions	No. of cases	No. of HHV8 positive cases in different PCR assays		
		Standard	Nested	Seminested[a]
Angiosarcoma	33	0	6	0
Kaposi's sarcoma	5	5	5	5
PBL samples	36	0	4	0

[a] Used to evaluate reproducibility of the nested PCR results

All the angiosarcomas and 5 AIDS-associated Kaposi's sarcoma cases showed amplification of 268 bp of β-globin gene. Five Kaposi's sarcoma cases diagnosed 40–50 years before did not amplify β-globin gene, but revealed amplification of 113 bp of genomic DNA after seminested PCR amplification.

None of the 33 angiosarcomas and 36 PBL samples from healthy blood donors showed a visible band of PCR products upon agarose gel electrophoresis. In contrast, all 5 Kaposis's sarcoma samples used as a positive controls showed a visible band upon agarose gel electrophoresis. Table 2 summarised the results of HHV8 amplification by different PCR systems.

Five Kaposi's sarcoma cases diagnosed 40–50 years before were evaluated for the presence of HHV8 sequences by a seminested PCR assay. In 3 cases, a sharp single PCR band was seen upon gel electrophoresis. Multiple negative controls were included in each run to rule out random contamination (Fig. 3). Sequence analysis confirmed the specificity of the PCR amplification.

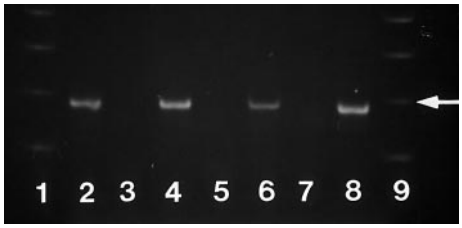

Fig. 3 Gel electrophoretic analysis of HHV8 PCR-products. DNA was obtained from archival Kaposi's sarcoma and amplified by seminested PCR assay; 10% polyacrylamide gel stained with ethidium bromide. *Lanes 1, 9* Molecular weight markers (φX174/HinfI), *lanes 2, 4 6* cases of Kaposis's sarcoma collected 40–50 years ago, *lanes 3, 5, 7* negative controls, *lane 8* positive control (AIDS-associated Kaposi's sarcoma, processed in a separate laboratory). The 82-base pair fragment of the molecular weight marker is shown by an *arrow*

Aliquots of the PCR products of 33 angiosarcomas and 36 PBLs were re-amplified using a nested PCR procedure. Six of 33 angiosarcomas (18%) and 4 of 36 PBLs (11%) that were negative after standard PCR amplification showed a visible band of nested PCR products upon agarose gel electrophoresis. The products were cloned and sequenced. Sequence analysis revealed identical sequences in all products and homology with HHV8 sequences.

To exclude or confirm the possible contamination, all samples that were positive according to nested PCR were re-evaluated for the presence of HHV8 sequences using a new seminested PCR assay. The forward primers used in the new seminested PCR assay were designed outside of the previously amplified sequences. None of the previously positive 6 angiosarcomas and 36 PBL samples revealed amplification of HHV8 sequences. However, this seminested PCR assay showed the same amplification efficiency of as the nested PCR assay.

Discussion

The presence of human herpesvirus-8 sequences in Kaposi's sarcoma in patients with or without HIV infection has been extensively documented [1, 9, 25]. Using PCR in situ hybridization, HHV8 sequences have been localized in Kaposi's sarcoma spindle cells and in endothelial cells lining the vascular spaces of Kaposi's sarcoma lesions [2]. These findings indicate possible tropism of the virus for endothelial cells and suggest that other endothelial cell malignancies could be associated with HHV8 infection.

We evaluated the possible presence of HHV8 sequences in different clinicopathological types of angiosarcomas, in an attempt to obtain a better understanding of the possible relationship between HHV8 infection and development of these tumours. Our nested PCR studies revealed HHV8 sequences in 6 of 33 angiosarcomas. Because we could not confirm the HHV8 positivity in any of these cases with an outer primer PCR system, we concluded that the initial positive observations represented minimal random contamination. In contrast, all 5 cases of AIDS-associated Kaposi's sarcoma showed consistent HHV8 positivity with all PCR systems.

Our results on the HHV negativity of angiosarcomas agree with several reports [10, 16, 18, 22]. However, McDonaugh et al. [23] found that 7 of their 28 (28%) angiosarcomas were HHV8 positive. Possible explanations of the discrepancy between our results and those of McDonaugh et al. include the possibility that different types of angiosarcomas were analysed, since the nature of the angiosarcomas examined was not specified in that study. Detection of HHV8 as a "passenger" in nonneoplastic cells in HHV8-positive patients is another possibility. Finally, minimal random contamination cannot be fully dismissed. However, McDonagh et al. [23] used standard PCR amplification, which has less potential for contamination than a nested PCR assay. Nested PCR assay, based on re-amplification of already existing PCR products, carries an enhanced potential for minimal random contamination compared with standard PCR. However, false-positive results caused by contamination may occur in standard PCR assays as well.

The presence of low levels of HHV8 in urogenital tissues and in 91% of human semen from HIV-negative donors was reported by Monini et al. [24]. A later study by Viviano et al. [33] detected HHV8 in 13% of semen samples. However, other investigators did not find HHV8 sequences in normal prostatic tissue and pointed out the possibility of false-positive results caused by random contamination during a nested PCR procedure [32]. Original HHV8-positive findings in semen [19] have recently been retracted by the authour and ascribed to minimal random contamination [20].

Some of the HHV8 positivity in cutaneous postransplant squamous cell carcinomas has been reinvestigated and reinterpreted. Originally, Rady et al. analysed 33 such lesions and detected HHV8 sequences in 82% of the cases [27]. This observation was initially confirmed by Boshoff et al. [3], who showed that 38% (14/37) of cutaneous squamous cell carcinomas from immunosuppressed patients were HHV8 positive. However, later experiments by Boshoff et al. [4] could not confirm the presence of HHV8 in posttransplant squamous cell lesions of the skin, and they ascribed their earlier positive results to PCR amplification of low levels of HHV8 laboratory contamination. Extensive studies by Cathomas et al. [6] did not reveal HHV8 sequences in transplant-related tumors.

Whitby et al. [34] showed HHV8 sequences in peripheral blood of Kaposi's sarcoma patients, and Viviano et al. [33] detected HHV8 sequences in 11% of PBMC from healthy HIV-negative individuals. However, our study showed no evidence of reproducibly HHV8-positive results in peripheral blood samples of healthy individuals.

Our results also suggest that the possibility of minimal random contamination has to be seriously considered and excluded when nested PCR yields positive results. Analysis of sequence variation between the cases

may help to identify random contamination. However, the sequence variation in the region amplified from HHV8 appears to be minimal [9, 24, 25] and the search for random contamination using the sequencing approach is problematic. The use of multiple negative controls flanking the sample can help to monitor minimal random contamination, and the use of additional PCR assays employing primers designed outside the previously amplified sequences helps to distinguish truly positive results from random contamination [4]. Using the "outer primer strategy" we have reinvestigated our primary findings and concluded that the previously detected low levels of HHV8 represented minimal contamination.

It is not known how old HHV8 infection is in the history of mankind. An early ultrastructural study from the 1970s showed the presence of viral particles in Kaposi's sarcoma, which at that time were believed to be CMV [13]. The virions seen by electron microscopy in Kaposi's sarcomas were first identified as HHV8 particles by Renne et al. in 1996 [28]. To put our study in a broader historical perspective, Kaposi's sarcomas collected approximately 40–50 years ago were analysed. Despite the poor quality of the DNA templates, HHV8 sequences were detected in 3 of 5 cases. Detection of HHV8 sequences in the specimens of Kaposi's sarcoma from 50 years ago indicates that HHV8 antedates the association of epidemies of HIV and HHV8.

We have evaluated a large group of angiosarcomas for HHV8 sequences and found all cases negative in sensitive PCR assays. The absence of HHV8 sequences suggests that this virus is not commonly involved in angiosarcomas and is not a significant candidate for their pathogenesis. In contrast, Kaposi's sarcomas, including historical cases, are HHV8 positive, suggesting that the presence of HHV8 sequences is a molecular marker for Kaposi's sarcoma.

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