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**Research Article**

Recently, DNA sequences of what appear to be a unique human herpesvirus-like agent (HHV-8) have been detected in different types of Kaposi's sarcoma (KS) tumors (Chang, Y., E.C. Cesarman, M.S. Pessin, F. Lee, J.C. Culpepper, D.M. Knowles, and P.S. Moore. 1994. *Science* (Wash. DC). 266:1865-1869). To further elucidate the possibility that HHV-8 plays a role in the pathogenesis of KS, the expression of HHV-8 RNA was examined in fresh KS tissue specimens which were found to harbor HHV-8 DNA by polymerase chain reaction (PCR). The transcription of HHV-8 RNA was detected by RT-PCR in 26 of 29 specimens (89.7%) of the KS tumors including 2 of 3 CKS and 24 of 26 AIDS-KS. No positive signal was detected in eight biopsy specimens of normal skin from healthy donors. By Northern blot analysis, the expression of HHV-8 was detected in 2 of 10 KS tumors examined. Furthermore, the RNA transcripts were observed in endothelial cells lining the irregular vascular spaces and perivascular spindle-shaped cells histologically characteristic of KS in 2 out of 8 different KS specimens examined by in situ hybridization using an antisense probe specific of HHV-8. The detection of RNA expression of HHV-8 in KS tumors further supports the possible etiopathogenic role of this virus in the development of KS.

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# Transcription of Human Herpesvirus-like Agent (HHV-8) in Kaposi's Sarcoma

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## Abstract

Recently, DNA sequences of what appear to be a unique human herpesvirus-like agent (HHV-8) have been detected in different types of Kaposi's sarcoma (KS) tumors (Chang, Y., E.C. Cesarman, M.S. Pessin, F. Lee, J.C. Culpepper, D.M. Knowles, and P.S. Moore. 1994. *Science (Wash. DC)*. 266:1865–1869). To further elucidate the possibility that HHV-8 plays a role in the pathogenesis of KS, the expression of HHV-8 RNA was examined in fresh KS tissue specimens which were found to harbor HHV-8 DNA by polymerase chain reaction (PCR). The transcription of HHV-8 RNA was detected by RT-PCR in 26 of 29 specimens (89.7%) of the KS tumors including 2 of 3 CKS and 24 of 26 AIDS-KS. No positive signal was detected in eight biopsy specimens of normal skin from healthy donors. By Northern blot analysis, the expression of HHV-8 was detected in 2 of 10 KS tumors examined. Furthermore, the RNA transcripts were observed in endothelial cells lining the irregular vascular spaces and perivascular spindle-shaped cells histologically characteristic of KS in 2 out of 8 different KS specimens examined by *in situ* hybridization using an antisense probe specific for HHV-8. The detection of RNA expression of HHV-8 in KS tumors further supports the possible etiopathogenic role of this virus in the development of KS. (*J. Clin. Invest.* 1996. 97:2803–2806.) Key words: Kaposi's sarcoma • HHV-8 • RNA transcription • *in situ* hybridization • AIDS

## Introduction

A novel human herpesvirus-like agent (HHV-8)<sup>1</sup> has recently been identified in the various forms of Kaposi's sarcoma (KS) including the HIV-negative "classical" (Mediterranean type) of KS (CKS), and endemic African (KS), AIDS-associated KS (AIDS-KS), and in a cohort of HIV-negative immunocompetent homosexual men with KS (1–4). The detection of HHV-8 in KS has been demonstrated by polymerase chain reaction (PCR) and confirmed in certain specimens by genomic South-

ern blot analysis and *in situ* hybridization (2–6). HHV-8 has also been detected in a few biopsy samples of "normal" appearing skin distant from any tumors from patients with KS (3–5). These findings suggest that HHV-8 may play a role in the pathogenesis of KS occurring among the disparate populations in which this tumor is seen.

If HHV-8 plays a role in the pathogenesis of KS, the expression and replication of this virus should be demonstrable in KS tissue. To clarify whether the HHV-8 DNA detected in KS tumors in fact, plays a role in the pathogenesis of this neoplasm, RNA expression of HHV-8 in fresh KS tumor biopsy specimens was examined by reverse transcription combined with PCR (RT-PCR), Northern blot, and *in situ* hybridization techniques.

## Methods

**Samples.** 31 fresh KS skin biopsy specimens initially demonstrated to harbor HHV-8 DNA by PCR analysis were examined for the presence of HHV-8 RNA transcripts. Three of the KS tumor specimens were obtained from patients with CKS and 28 came from patients with AIDS-KS. A portion of each specimen was separately reserved for histopathological assessment. Eight samples of normal skin obtained from healthy patients undergoing plastic surgery were used as controls. Specimens were minced with a Tissue Mizer (Tekmar Co., Cincinnati, OH), followed by acid guanidinium thiocyanate/phenol/chloroform extraction of total RNA (7). 2 µg of each RNA sample was incubated with 4 U of ribonuclease-free deoxyribonuclease (Promega, Madison, WI) at 37°C for 30 min and then heated at 95°C for 5 min to inactivate the enzyme. One-third of each specimen was evaluated by PCR without the RT step in order to confirm that each RNA sample was free of DNA contamination (8). The remaining portion of each sample was incubated in 30 µl of a solution containing 100 ng random hexamer, 200 U of the Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, Maryland), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT at 37°C for 1 h. Each of the cDNA products were then equally divided into two aliquots for PCR amplification, one examined for HHV-8 and the other for β-actin to ascertain whether the DNA was amplifiable (9). The PCR solution contained 1 U of Taq DNA polymerase, Taq buffer (Perkin-Elmer Corp., Norwalk, CT), a primer pair either for HHV-8 (5'-AGC, CGA, AAG, GAT, TCC, ACC, ATT-3', and 5'-TCC, GTG, TTG, TCT, ACG, TCC, AGA-3') or for β-actin (5'-GAG, GAG, CAC, CCC, GTG, CTG, CTG, A-3'' and 5'-CTA, GAA, GCA, TTT, GCG, GTG, GAC, GAT, GGA, GGG, GCC-3'), and underwent 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C (1, 9). Upon completion of the amplification, the PCR products were analyzed by electrophoresis on a 1.5% agarose gel in Tris-borate-EDTA buffer (pH 8.0). Each of the DNA samples were then transferred to a Genescreen membrane (Dupont-NEN, Boston, MA). After prehybridization, the membranes were hybridized with a <sup>32</sup>P-dATP end-labeled probe for either HHV-8 (5'-TTG, GAG, TAG, ATG, TGG, TAC, ACC, AAC, A-3') or β-actin (5'-GAA, ATC, GTG, CGT, GAC, ATT, AAG, GAG, AAG-3'). The membranes were sequentially washed in 2× SSC, 1× SSC, and 0.5× SSC (1× SSC: 0.15M sodium chloride, 0.015 M sodium citrate) at 55°C for 30 min each wash, and then autoradiographed with Kodak x-ray film (Eastman Kodak Co., Rochester, NY).

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1. Abbreviations used in this paper: KS, Kaposi's sarcoma; AIDS-KS, AIDS related-KS; CKS, classical Kaposi's sarcoma; HHV-8, human herpesvirus-like 8; RT-PCR, reverse transcription polymerase chain reaction.

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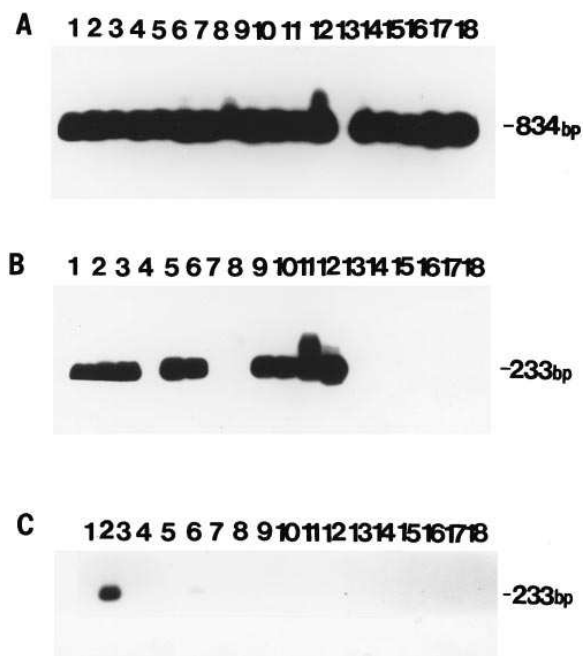
**Northern blot hybridization analysis.** The RNAs extracted from 10 KS samples and 2 specimens of normal skin were subjected to Northern blot hybridization analysis. 30  $\mu$ g of each RNA specimen was treated with formamide and formaldehyde, electrophoresed, and transferred to Genescreen membranes. A sequence-confirmed 233-bp PCR-amplified HHV-8 DNA fragment which is a portion of the putative minor capsid gene was radiolabeled by random priming, and then added to the prehybridized membranes in the solution containing 1% SDS, 1 M sodium chloride, 10% dextran sulfate, and salmon sperm DNA (100  $\mu$ g/ml). After the membranes were sequentially washed in 2 $\times$  SSC, 1 $\times$  SSC, and 0.2 $\times$  SSC at 60°C, they were exposed to Kodak x-ray film using an intensifying screen at -70°C for autoradiographic analysis (10).

**In situ hybridization.** To make the HHV-8 probe, a 233-bp fragment of the PCR product was cloned into pSPT 18 vector (Boehringer Mannheim). <sup>33</sup>P-UTP-labeled single stranded RNA probes were prepared by transcribing the fragment with either T7 or SP6 RNA polymerase (Boehringer Mannheim). In situ hybridization was performed to detect RNA as described previously (11). Briefly, serial 5- $\mu$ m thick cryostat sections of eight AIDS-KS tissues and three control normal skin tissues were mounted on silane-coated glass microscope slides, fixed in 4% paraformaldehyde and dehydrated through graded ethanol. The dehydrated tissue sections were then acetylated in 0.1 M trisethanolamine, 0.25% acetic anhydride for 10 min, washed in 0.2 $\times$  SSC and preincubated for 2 h at 45°C covered by a solution of

**Table I. Detection of HHV-8 Transcription in Kaposi's Sarcoma**

Case	Histologic features	HIV serology	PCR HHV-8	RT-PCR	Northern blot	In Situ hybridization
1*	KS nodular	-	+	+	-	ND
2*	KS plaque	-	+	+	ND	ND
3*	KS patch/plaque	-	+	-	ND	ND
4	KS patch/plaque	+	+	+	ND	ND
5	KS patch/plaque	+	+	+	ND	ND
6	KS patch/plaque	+	+	-	ND	ND
7	KS plaque	+	+	+	-	ND
8	KS plaque	+	+	+	+	+
9	KS nodular	+	+	+	ND	-
10	KS plaque	+	+	+	-	ND
11	KS nodular	+	+	+	+	ND
12	KS plaque	+	+	+	-	ND
13	KS plaque	+	+	-	ND	-
14	KS nodular	+	+	+	-	ND
15	KS plaque	+	+	+	ND	+
16	KS nodular	+	+	+	-	-
17	KS plaque	+	+	+	ND	-
18	KS nodular	+	+	+	-	-
19	KS plaque	+	+	+	-	ND
20	KS plaque	+	+	+	ND	-
21	KS plaque	+	+	+	ND	ND
22	KS plaque	+	+	+	ND	ND
23	KS plaque	+	+	+	ND	ND
24	KS plaque	+	+	+	ND	ND
25	KS plaque	+	+	+	ND	ND
26	KS plaque	+	+	+	ND	ND
27	KS nodular	+	+	+	ND	ND
28	KS nodular	+	+	+	ND	ND
29	KS plaque	+	+	+	ND	ND

\*CKS; (+) positive; (ND) not done; (-) negative.

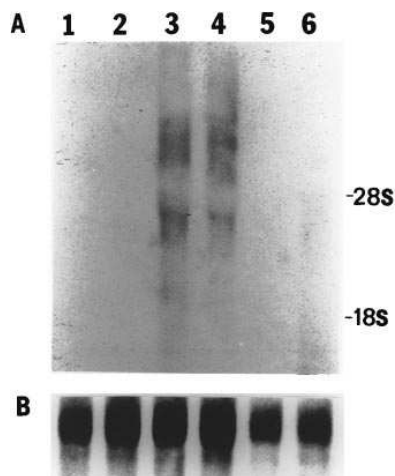


**Figure 1.** Representative result of the detection of HHV-8 transcription in KS tumors by RT-PCR. Southern blot hybridization was performed on amplified products for  $\beta$ -actin RNA (A), HHV-8 RNA (B), and HHV-8 DNA (C). Lanes 1-12, RNA extracted from KS tumors; lanes 13-18, samples of normal skin. An amplified 834-bp band is not observed in lane 13 of A which indicates that RNA from this sample is not amplifiable. An amplified 233-bp band is observed in lane 2 and 6 of C which indicates that residual DNA exists in these two samples of RNA. Therefore, KS samples in lanes 1, 3, 5, and 9-12 are positive, in lanes 4, 7, and 8 are negative, and in lane 2 and 6 are interpreted as false positive. Specimens of normal skin in lanes 14 to 18 are negative.

50% formamide, 0.6 M NaCl, 2.5 $\times$  Denhardt's solution, 10% dextran sulfate, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% SDS and 0.15 mg/ml t-RNA. The tissue sections were then incubated with <sup>33</sup>P-labeled anti-sense probe in the same solution at 45°C for 16 h. The slides were washed in 2 $\times$  SSC, treated with RNase (5  $\mu$ g/ml), sequentially washed for 30 min each in 1 $\times$  SSC, 0.5 $\times$  SSC at 60°C, dehydrated, and finally coated with Kodak NTB-2 emulsion. After 7-14 d, the slides were developed and counterstained with hematoxylin. A <sup>33</sup>P-labeled sense probe was used as a control.

## Results

31 KS specimens shown to contain HHV-8 DNA by PCR were further examined for the presence of viral RNA transcripts using RT-PCR. The results are summarized in Table I with representative examples of the amplification products shown in Fig. 1. To ensure that the RNA extracted from each specimen was amplifiable, RT-PCR was also performed with a primer pair specific for  $\beta$ -actin. A 834-bp amplified band was detected in all RNA specimens with the exception of one sample extracted from a normal skin specimen (Fig. 1 A). Direct PCR was also performed on all RNase-free DNase treated RNA samples to determine whether any residual HHV-8 template DNA was present. The amplified 233 bp fragment was detected in two of the AIDS-KS specimens (Fig. 1 C); therefore, positive results from the corresponding RT-PCR of these two samples were not counted. The positive results of RT-PCR



**Figure 2.** Northern blot analysis for detection of the expression of HHV-8 in KS tumors. (A) Examination of HHV-8 transcription by  $^{32}\text{P}$ -dCTP end-labeled probe specific for HHV-8. Positive bands were detected in two KS tumor samples (lanes 3 and 4). (B) Membrane probed with  $\beta$ -actin. Lanes 1–5, RNA samples extracted from AIDS-KS lesions. Lane 6, RNA obtained from normal skin.

ern blot hybridization. The HHV-8 RNA transcripts were found in two specimens from AIDS-KS patients (Fig. 2). No positive signals were observed by Northern blot in the remaining eight KS samples (1 CKS, 7 AIDS-KS), although HHV-8 RNA transcripts had been previously detected by RT-PCR in all of these specimens. In the two control samples of skin, HHV-8 RNA expression was not detected by Northern blot analysis.

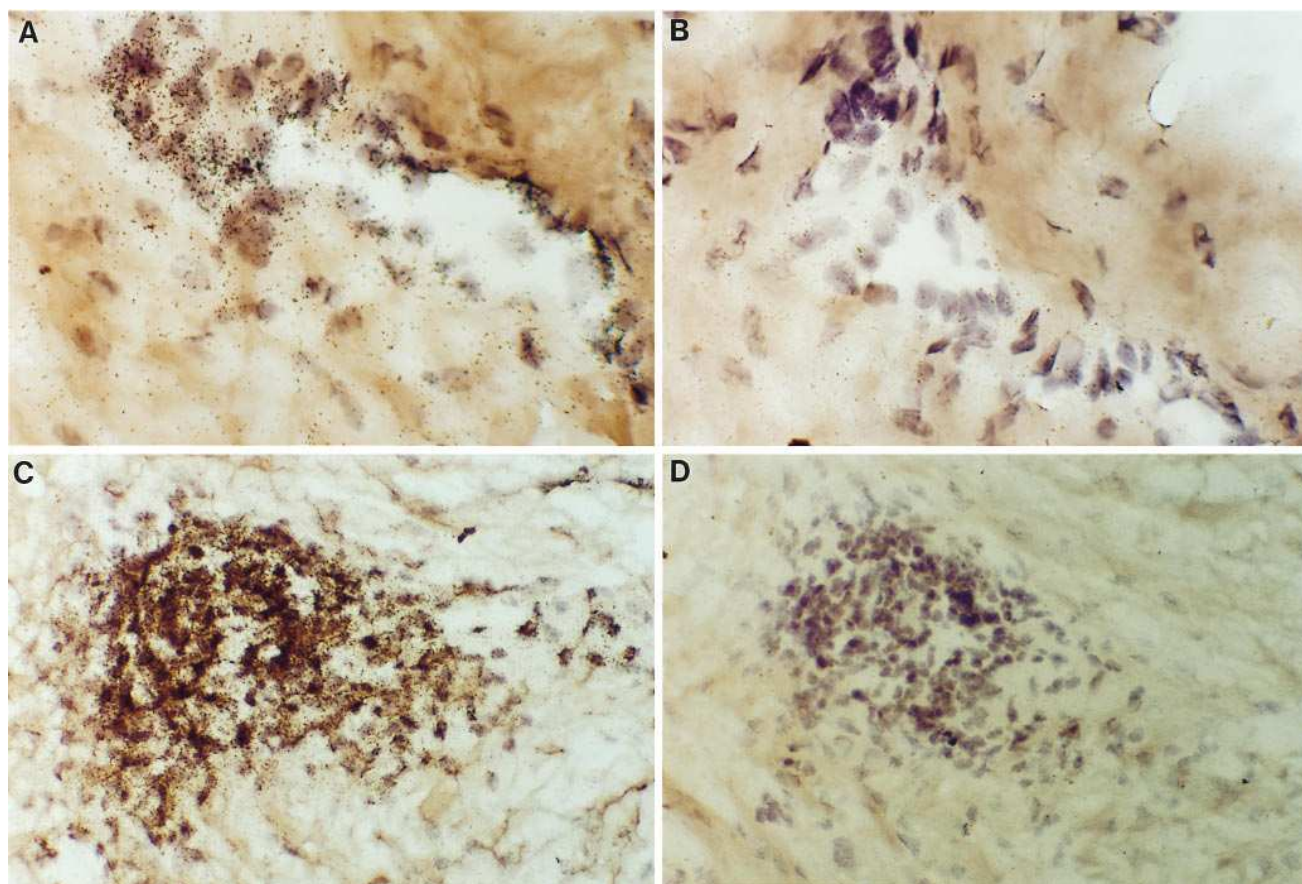
To attempt to localize the expression of HHV-8 RNA to specific cells within KS tumor tissues, in situ hybridization was performed on 5- $\mu\text{m}$  frozen sections of KS specimens. In 2 of 8 AIDS-KS samples examined, HHV-8 RNA transcripts were detected and localized to the endothelial cells lining the irregular vascular slits and to perivascular spindle-shaped cells histologically characteristic of KS (Fig. 3). The specific detection of HHV-8 RNA in these cells by in situ hybridization was further established by using a sense probe on the same tissue sections; no positive signal was detected in the controls (12).

amplified originally from viral RNA were identified in 26 of 29 KS specimens examined, including 2 of 3 CKS and 24 of 26 KS specimens from AIDS patients. No positive signals were elicited from the normal control skin specimens (Fig. 1, B and C).

To further confirm the expression of HHV-8 RNA in KS tumors, the available RNAs from nine AIDS-KS lesions and one non-HIV-related CKS specimen were analyzed by North-

## Discussion

Using PCR and in situ hybridization we have recently demonstrated the presence of HHV-8 in the endothelial cells and perivascular spindle cells typically seen in KS tissue specimens suggesting that this herpesvirus may be involved in the pathogenesis of KS (6). However, HHV-8 DNA has also been de-



**Figure 3.** In situ hybridization with HHV-8 specific probe for detection of HHV-8 transcripts in AIDS-KS tumors. (A) Localization of HHV-8 RNA (silver grains) to the nuclei of vascular endothelial cells and perivascular spindle-shaped cells of a KS tumor ( $\times 200$ ). (B) Negative control using sense RNA probe on the same specimen; only background levels of grains are present. (C) Strong labeling of the vascular endothelial cells and perivascular spindle-shaped cells in another KS lesion is observed ( $\times 125$ ). (D) No labeling is seen after hybridization with a sense RNA probe ( $\times 125$ ).

tected in normal appearing skin from patients with KS distant from any KS lesions, as well as in some AIDS-associated body cavity lymphomas BCBL. Recently, lytic growth of HHV-8 has been observed in cells derived from an AIDS-related BCBL-based (14). To date, the virus has not been observed by electron microscopy in either KS tumors or KS-derived cultured cells. The present study was designed to ascertain whether HHV-8 is expressed in KS tumors and to determine in which cells of the tumor HHV-8 transcripts are localized.

Using the RT-PCR techniques, RNA expression of HHV-8 was detected in 89.7% (26/29) of KS specimens examined. The transcription of HHV-8 was not detected in 3 KS samples which were only positive for HHV-8 by PCR. There are several possible explanations for this observation; the detection of mRNA by the RT-PCR method has technical limitations (15). The absence of RNA detection in some of the specimens could be related to insufficient or very low levels of transcription. Alternatively, despite the precautions taken, RNA degradation due to instability may have yielded negative results, especially in the circumstance of low level RNA expression, or some of the KS specimens may harbor HHV-8 in the absence of viral expression. The expression of HHV-8 in some of the fresh KS specimens examined was further confirmed by Northern blot hybridization analysis.

The histopathological features of KS include a proliferation of spindle-shaped cells forming fascicles or bundles that surround and eventually compress the irregular vascular spaces lined with endothelial cells, and an inflammatory cell infiltrate composed of plasma cells, lymphocytes, and monocytes; dermal dendrocytes and extravasated erythrocytes, are also present. Although the progenitor cell of KS remains undetermined, vascular or lymphatic endothelial cells, dermal dendrocytes, and vascular smooth muscle cells have been proposed to be the cell of origin of KS (16). The present study demonstrates by *in situ* hybridization that the RNA expression of HHV-8 is localized to the endothelial cells that line the bizarre-shaped vascular structures and to some of the perivascular spindle-shaped cells seen in KS lesions. These observations are consistent with, and confirm, our recent *in situ* hybridization studies which demonstrate that HHV-8 DNA is localized to the vascular endothelial cells and perivascular spindle-shaped cells in KS tissues (6).

RNA expression of HHV-8 in KS tumors was detected by three different techniques. Our data show that the positive ratio of HHV-8 transcripts detected by RT-PCR is much higher than that detected by Northern blot or *in situ* hybridization. These results could be explained by the high sensitivity of the RT-PCR method; the present study indicated that the level of RNA expression of HHV-8 is not high enough in all KS tumor samples for detection by less sensitive methods such as Northern blot and *in situ* hybridization. Therefore, the relationship between the level of expression of HHV-8 and the stages of KS development needs to be further studied by establishing a

quantitative RT-PCR method. Nevertheless, the detection of RNA expression of HHV-8 in a high percentage of KS tumors reported here suggests that HHV-8 may play a role in the pathogenesis of KS.

## Acknowledgments

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