

KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS DNA SEQUENCES IN PROSTATE TISSUE AND HUMAN SEMEN

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Abstract Background. Sequences of a novel herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV), have been identified in Kaposi's sarcoma tissue, but it is not known whether the virus is transmitted by sexual contact.

Methods. Using the polymerase chain reaction (PCR), we searched for KSHV DNA sequences in ejaculates from 43 healthy men and tissue from the urogenital tract or prostate of 100 immunocompetent adults.

Results. In an unblinded analysis, we identified KSHV DNA sequences in 2 of 20 tissue specimens from the urinary tract (10 percent; 15 men and 5 women), 3 of 46 specimens from the female genital tract (6.5 percent), 4 of 18 specimens from the glans or foreskin (22 percent), 7 of 16 specimens from the prostate (44 percent), and 30 of 33 ejaculates (91 percent). By contrast, such sequenc-

es were present in 1 of 18 samples of normal skin (5.5 percent) and 1 of 14 samples of peripheral-blood mononuclear cells (PBMCs; 7.1 percent). Ejaculates and PBMC samples from each of 10 study subjects were analyzed in a blinded, coded fashion, along with PBMCs and biopsy specimens of normal skin from 4 and 8 other patients, respectively. This analysis confirmed the presence of KSHV DNA sequences in semen. Viral DNA was not found in the sperm heads but was present in the fraction of the ejaculates that contained urothelial and other types of cells. Point mutations were found in PCR products amplified from both prostate tissue and sperm samples.

Conclusions. KSHV infects a large proportion of healthy adults and is probably transmitted by sexual contact. (N Engl J Med 1996;334:1168-72.)

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CHANG et al.¹ have found herpesvirus-like DNA sequences in Kaposi's sarcoma tissue from a patient with human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS). To date, these DNA sequences have been detected by the polymerase chain reaction (PCR) and Southern blot hybridization in nearly all biopsy specimens from patients with classic Kaposi's sarcoma (common in people of Mediterranean origin), endemic Kaposi's sarcoma (common in people in subtropical regions of Africa), and AIDS-associated Kaposi's sarcoma, as well as in body-cavity-based lymphomas from patients with AIDS.²⁻⁶ The DNA sequences of this novel virus, tentatively named Kaposi's sarcoma-associated herpesvirus (KSHV), showed a high degree of homology with those of Epstein-Barr virus (EBV) and herpesvirus saimiri, two lymphotropic herpesviruses belonging to the subfamily of the Gammaherpesvirinae.¹ Like all other herpesviruses, EBV is ubiquitous in humans, establishes latent infection in the host, and reactivates to spread in the population. KSHV DNA, however, was detected neither in normal tissues from immunocompetent persons nor in the majority of non-Kaposi's sarcoma tissues from patients with AIDS. As has been pointed out, the biologic and epidemiologic characteristics of KSHV should therefore differ from those of all other known human herpesviruses.⁷

The observation that Kaposi's sarcoma is 10 times more frequent among homosexual or bisexual men with

AIDS acquired sexually than it is among patients with AIDS transmitted in other ways has suggested that Kaposi's sarcoma may be caused by a sexually transmitted agent.^{8,9} This prompted us to analyze sperm and tissue specimens from the urogenital tract and prostate gland for KSHV DNA sequences.

METHODS

Study Samples

The tissue samples studied consisted of 18 biopsy specimens of normal skin, 14 samples of peripheral-blood mononuclear cells (PBMCs) from immunocompetent persons, 2 biopsy specimens of normal kidney tissue, 8 kidney tumors, 1 biopsy specimen of normal bladder tissue, 5 bladder carcinomas, 1 biopsy specimen of normal ureteral tissue, 3 ureteral tumors, 9 biopsy specimens from normal uterine cervical tissue, 24 cervical carcinomas, 3 vaginal carcinomas, 3 biopsy specimens of normal vulvar tissue, 7 vulvar carcinomas, 4 aceto-white lesions (characterized by slight epithelial changes of various origins) of the glans, 14 samples of normal foreskin tissue, 8 hyperplastic lesions of the prostate, 8 prostate carcinomas, and 43 sperm ejaculates (Table 1). Six Kaposi's sarcoma skin lesions (two from patients with AIDS-associated Kaposi's sarcoma, two from Italian patients with classic Kaposi's sarcoma, and two from Ugandan patients with endemic Kaposi's sarcoma) and one lymph node from a patient with AIDS but no clinical evidence of Kaposi's sarcoma were also studied. Among these tissue specimens, ejaculates and PBMC samples from the same 10 donors, as well as 4 PBMC samples and 8 biopsy specimens of normal skin from other patients, were analyzed in a blinded, coded fashion. The samples of normal skin were obtained from immunocompetent persons undergoing various kinds of surgery. The sperm donors were Italian patients from the Po Valley who were undergoing surgery for varicocele and had no other apparent clinical condition.

PCR Assays

Twenty sperm samples were assayed by PCR for the presence of the HIV-1 *gag* gene, with negative results. The tissue samples were finely minced with disposable scalpels, suspended in a solution containing proteinase K (100 µg per milliliter of solution) and sodium dodecyl sulfate (0.2 percent wt/vol), and incubated at 37°C for 15 hours. The DNA samples were purified by three extractions with phenol-chloroform, two extractions with chloroform, and two extractions with diethyl ether and were precipitated in ethanol. The PBMCs were

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Table 1. KSHV DNA Sequences Detected in Urogenital Tissues, Prostate Tissue, and Sperm.

TYPE OF SAMPLE	NO. OF SAMPLES	NO. (%) POSITIVE FOR KSHV*
Unblinded analysis		
Kidney†		
Non-neoplastic	2	0
Neoplastic	8	2 (25)
All	10	2 (20)
Bladder‡		
Non-neoplastic	1	0
Neoplastic	5	0
All	6	0
Ureter§		
Non-neoplastic	1	0
Neoplastic	3	0
All	4	0
Uterine cervix		
Non-neoplastic	9	1 (11)
Neoplastic	24	1 (4)
All	33	2 (6)
Normal skin	10	0
Vagina (neoplastic)	3	0
Vulva		
Non-neoplastic	3	1 (33)
Neoplastic	7	0
All	10	1 (10)
Glans lesions	4	0
Normal foreskin	14	4 (29)
Prostate		
Non-neoplastic	8	5 (63)
Neoplastic	8	2 (25)
All	16	7 (44)
Sperm	33	30 (91)
Blinded analysis		
Sperm	10	5 (50)
Normal skin	8	1 (12)
PBMC	14	1 (7)

*Sequences were detected by both PCR and hybridization.

†Two of the 10 specimens were from women. Both KSHV-positive specimens were from men.

‡One of the six specimens was from a woman.

§Three of the four specimens were from women.

lysed according to a rapid DNA-extraction procedure; suspended in a solution containing 10 mM TRIS-hydrochloric acid (pH 8.00), 1 percent (vol/vol) polyoxyethylene lauryl ether, and proteinase K at a concentration of 100 µg per milliliter; incubated at 65°C for 2 hours; and boiled for 10 minutes. The equivalent of 75,000 cells (about 500 ng of DNA) was analyzed by PCR. DNA was extracted from the sperm specimens according to published procedures.¹⁰

PCR primers KS1 and KS2, which have been previously described, amplify DNA sequences from nucleotide 987 to nucleotide 1218 in KS33Bam, a DNA fragment cloned from a Kaposi's sarcoma-tissue genomic-DNA library.¹ PCR reactions with these primers were performed as previously described,¹ with either 35 or 45 PCR cycles, as specified. Primers NS1 (5'ACGGATTTGACCCCGTGTTC3') and NS2 (5'AATGACACATTGGTGGTATA3') amplify DNA sequences from nucleotide 1021 to nucleotide 1180 and were used in a nested PCR analysis. In this analysis, 5 µl of the PCR products obtained with primers KS1 and KS2 were added to the PCR mixtures containing primers NS1 and NS2 and amplified as described as above for a total of 30 cycles at an annealing temperature of 56°C. The PCR products amplified with primers KS1 and KS2 were hybridized with ³²P-labeled-oligonucleotide DNA probes.¹

The products of the nested PCR reactions were labeled with phosphorus-32 (about 0.5 µg), digested with restriction enzymes, and fractionated on 5 percent nondenaturing polyacrylamide gels. Aliquots of nested-PCR products (about 1 µg) were gel-purified by absorption to a glass-powder matrix and sequenced by the dideoxy technique with a femtomole DNA-sequencing kit (Promega, Madi-

son, Wis.). Differential extraction of DNA from sperm heads and from the sperm fraction containing urothelial cells was performed as described previously.¹⁰ The DNA samples extracted from all the specimens and sperm fractions were positive for a β-globin DNA fragment on PCR amplification.

RESULTS

Tissue specimens from the urogenital tract, prostate tissue, and sperm samples were analyzed with primers KS1 and KS2, which amplify DNA sequences contained in KS33Bam, a DNA fragment isolated from a Kaposi's sarcoma-tissue genomic-DNA library.¹ In an unblinded analysis, viral sequences were detected by both PCR amplification and hybridization to an oligonucleotide probe in 2 of 20 tissue specimens from the urinary tract (10 percent), 3 of 46 specimens from the female genital tract (6.5 percent), 4 of 18 specimens of tissue from the glans and foreskin (22 percent), 7 of 16 specimens from the prostate (44 percent), and 30 of 33 sperm samples (91 percent) (Table 1). The samples testing positive were obtained from 2 infiltrating kidney adenocarcinomas, 1 adenocarcinoma of the uterine cervix, 1 sample of normal cervical tissue, 1 sample of normal vulvar tissue, 4 samples of normal foreskin tissue, 2 infiltrating adenocarcinomas of the prostate, 5 samples of prostate tissue with hyperplasia, and 30 ejaculates from patients undergoing surgery for varicocele. The prevalence of KSHV DNA was significantly higher in sperm than in the prostate ($P=0.007$). The prostate samples, in turn, had a significantly higher prevalence of viral sequences than the tissues from the urogenital tract ($P<0.005$).

In control reactions, PCR mixtures without the DNA template always tested negative, as did eight biopsy specimens of normal skin from other patients that were analyzed together with the sperm samples. Four control samples were created by processing aliquots of phosphate-buffered saline with the same procedures, reagents, and buffers used to extract DNA from the sperm samples. These control samples were prepared while a set of nine sperm samples was being processed, and the control samples tested negative on PCR amplification for both KSHV and β-globin DNA sequences.

These specimens were all subjected to 45 cycles of amplification. Four of the seven samples of prostate tissue that tested positive (57 percent) yielded signals clearly visualized on ethidium bromide staining (Fig. 1A and data not shown). All the other specimens, including the sperm samples, yielded positive signals only after hybridization to the oligonucleotide probe (Fig. 1B). Previous studies showed that KSHV DNA in Kaposi's sarcoma tissue is generally detected on ethidium bromide staining of PCR products amplified for only 35 cycles.¹ We analyzed the 6 skin lesions from patients with various types of Kaposi's sarcoma and the 16 prostate specimens by PCR amplification for 35 cycles. All six Kaposi's sarcoma specimens yielded clear signals on ethidium bromide staining (Fig. 1C), but none of the prostate specimens did so. Two biopsy specimens of normal skin and the lymph node from a patient with

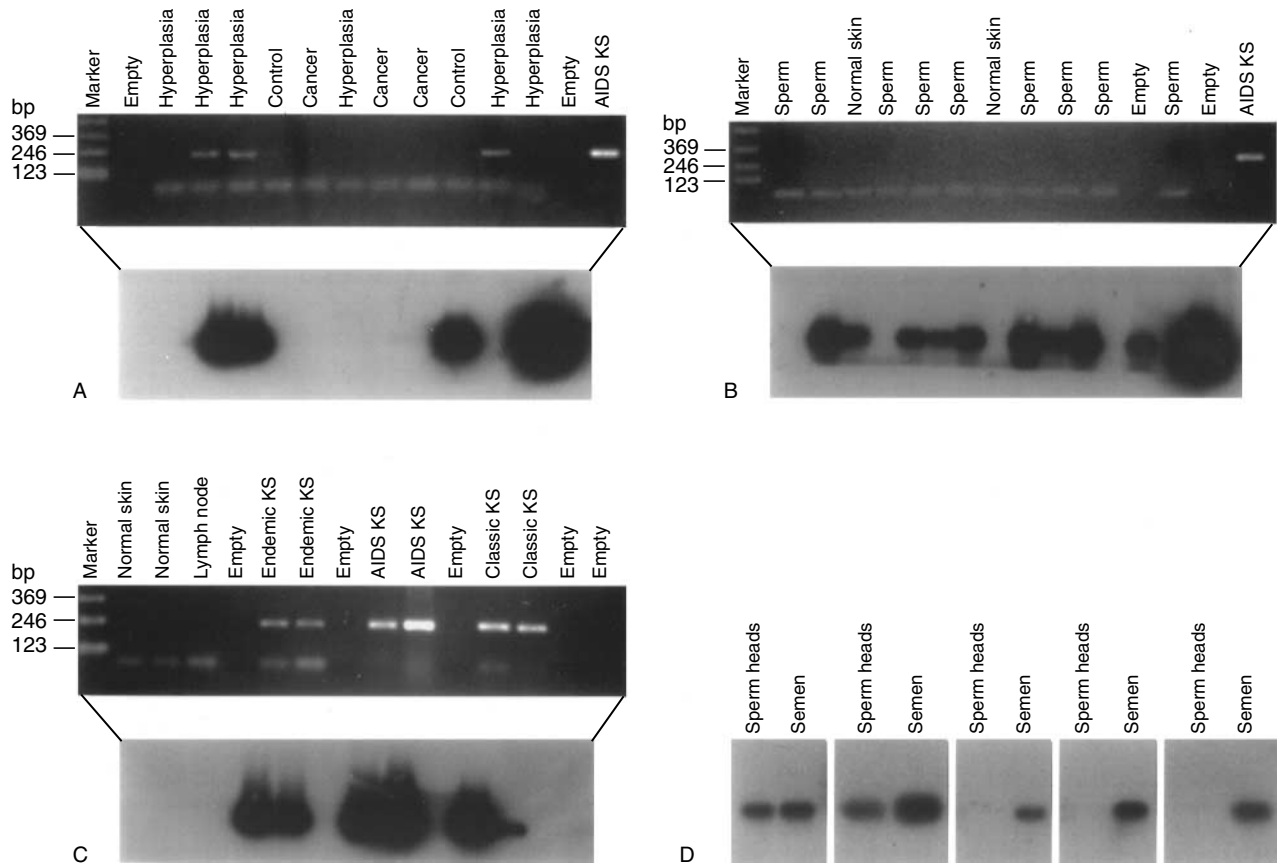


Figure 1. Amplification of KSHV DNA in Prostate Tissue, Sperm, and Kaposi's Sarcoma (KS) Lesions.

Panels A, B, and C show agarose gels stained with ethidium bromide (above) and blots indicating hybridization of PCR products (below). The marker is a 123-bp DNA-ladder molecular-weight marker. The band below the 123-bp marker in the ethidium bromide-stained agarose gels consists of PCR primers. Panel A shows an analysis of DNA from prostate tissue with cancer (adenocarcinoma) or hyperplasia after 45 cycles of PCR. The controls were PCR reactions with no DNA template. DNA from an AIDS-associated Kaposi's sarcoma skin lesion (AIDS KS) was amplified for 35 PCR cycles. Panel B shows an analysis of DNA extracted from sperm samples and biopsy specimens of normal skin after 45 cycles of PCR. Panel C shows an analysis of various Kaposi's sarcoma skin lesions after 35 PCR cycles, with two biopsy specimens of normal skin and a lymph node from a patient with AIDS. Panel D shows an analysis of DNA from five KSHV-positive sperm specimens after 45 cycles of PCR. For each specimen, DNA extracted from the fraction containing sperm heads and DNA extracted from semen (the sperm fraction containing urothelial cells) were analyzed separately.

AIDS but no clinical evidence of Kaposi's sarcoma all tested negative (Fig. 1C).

To confirm the association of KSHV DNA sequences with sperm, we performed a blinded analysis of ejaculates and samples of PBMCs from each of 10 donors, as well as 4 PBMC samples and 8 biopsy specimens of normal skin from other patients undergoing various kinds of surgery. We prepared eight control samples while the DNA was being extracted from the PBMC and sperm samples. The samples of purified DNA were quantitated and diluted to a final concentration of 50 ng per microliter; 10- μ l aliquots of the DNA and control samples were analyzed in coded, blinded fashion with primers KS1 and KS2 (for 45 cycles) and primers for the β -globin gene. In these experiments, five sperm specimens, one PBMC sample, and one skin-biopsy specimen tested positive for KSHV DNA sequences. The positive PBMC sample was from a patient whose sperm was positive for KSHV DNA sequences. None of the control

samples or the PCR control reactions that did not have DNA templates were positive. All the specimens except the control samples were positive by PCR for the DNA fragment of the β -globin gene.

Five KSHV-positive sperm specimens were lysed and digested with proteinase K under nonreducing conditions, a procedure that leaves the sperm heads intact but results in the lysis of the other types of cells present in the sperm.¹⁰ These cells consist mainly of urothelial cells.¹¹ After the lysis of the sperm fraction containing urothelial cells, the intact sperm heads were centrifuged, washed extensively, and lysed under reducing conditions. The DNA samples extracted from both cell fractions were subjected to PCR amplification with primers KS1 and KS2 (Fig. 1D). In three cases, the sperm-head fraction tested negative for viral DNA on PCR amplification, whereas the fraction containing urothelial cells tested positive. In the other two cases, the signal obtained from the sperm-head fraction was less intense

than that associated with the sperm fraction containing urothelial cells (Fig. 1D). DNA samples extracted from both sperm-head fractions were positive for β -globin-gene DNA sequences on PCR amplification (data not shown).

The specimens positive on DNA amplification with primers KS1 and KS2 were subjected to a nested PCR. After 30 cycles, they all yielded clear signals on ethidium bromide staining. Four nested-PCR products from samples of prostate tissue and five from sperm specimens were analyzed with restriction endonucleases *Nco*I and *Pst*I, cutting once within the viral sequences amplified with primers KS1 and KS2. All nine specimens showed the restriction pattern expected on the basis of the published DNA sequences¹ (data not shown). The bulk of the nested-PCR products from three prostate-tissue specimens and three sperm samples were sequenced. Point mutations were found in the PCR products from both types of samples (C→T at position 1086 and A→C at position 1139). These mutations are contained within a putative viral gene¹ but do not determine any amino acid substitution in the corresponding polypeptide. Identical mutations have been detected by other investigators in Kaposi's sarcoma tissue.³

DISCUSSION

Considerable evidence suggests that Kaposi's sarcoma may be caused by an infectious agent transmitted by sexual contact. Kaposi's sarcoma develops in homosexual men with AIDS 10 to 20 times more frequently than it develops in other patients with AIDS,^{8,9} and there is evidence that the presence of Kaposi's sarcoma may be related to specific homosexual practices.¹² It has been observed that the sexual partners of bisexual men with AIDS are at high risk for the development of the sarcoma.⁸ In this study, KSHV DNA was detected by PCR in 12 percent of tissue specimens from the urogenital tract, 44 percent of samples from prostate tissue, and 81 percent of ejaculates from immunocompetent persons. The high frequency of positive semen samples supports the hypothesis that KSHV may be transmitted sexually. The fact that KSHV DNA was detected less frequently in the prostate than in semen suggests that it may be present in only some areas of prostate tissue. Alternatively, the high prevalence of KSHV DNA in sperm may be attributable in part to the infection of other spermatogenic organs.

The ejaculates analyzed in this study were obtained from patients who had no apparent clinical condition besides varicocele. KSHV, therefore, appears to be a ubiquitous virus that produces occult infection in a large proportion of the general population. This finding makes KSHV similar to the other known human herpesviruses, which are frequently present in the population and produce persistent, latent infection in otherwise healthy persons.⁷ The presence of KSHV DNA sequences in the prostate tissue and sperm of immunocompetent men suggests that the spermatogenic organs may be sites of latent KSHV infection in healthy people.

The pathogenesis of Kaposi's sarcoma has long been

controversial. The disease has appeared to be associated with immunosuppression, but the role of immunologic deficits in Kaposi's sarcoma has never been well defined. Two forms of the disease (AIDS-associated Kaposi's sarcoma and iatrogenic Kaposi's sarcoma) develop in immunosuppressed people, but it is not clear whether immunosuppression plays a part in the endemic form of Kaposi's sarcoma. Kaposi's sarcoma lesions can regress in transplant recipients after the immunosuppressive therapy is discontinued,¹³ but regression and even complete remission have also been described in severely immunocompromised people, such as patients with AIDS.¹⁴

Kaposi's sarcoma is particularly prevalent in some population groups from specific geographic areas, such as elderly people from the Mediterranean region, young males from subequatorial Africa, and immigrants to the United States from the Caribbean.¹⁵ There is no obvious explanation for this geographic segregation. There is evidence that Kaposi's sarcoma is a cytokine-mediated disease, because Kaposi's sarcoma-like lesions are induced in nude mice injected with Kaposi's sarcoma cells from humans, which recruit host cells by secreting cytokines with autocrine and paracrine growth effects.¹⁶ The HIV Tat protein interacts synergistically with one of these cytokines, basic fibroblast growth factor, in inducing such lesions.¹⁷ Homosexual men, the group at highest risk for Kaposi's sarcoma, have abnormal cytokine levels, suggesting that the pathogenesis of Kaposi's sarcoma may be related to both an early phase of immunostimulation and a subsequent phase of immunosuppression.¹⁸

All these observations are difficult to reconcile with a simple model of the pathogenesis of Kaposi's sarcoma. The finding that KSHV is ubiquitous, however, may provide insights useful in studying the pathogenetic mechanisms of Kaposi's sarcoma. Since the virus is frequently present in otherwise healthy people, it appears that these mechanisms would be related to viral reactivation rather than primary infection. As in the case of other human herpesviruses, a number of factors may cooperate to promote viral reactivation. Immunostimulation, immunosuppression, contact with other infectious agents, and genetic, behavioral, or environmental conditions associated with populations from specific geographic areas may be among these factors. Contact between the rectal mucosa and infected semen may contribute to viral reinfection and reactivation in homosexual men, accounting for the higher prevalence of Kaposi's sarcoma in this risk group. There is hope that studies of the latency and reactivation of KSHV will unveil some of the factors in the pathogenesis of Kaposi's sarcoma and other KSHV-related diseases.

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