JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1996, p. 2635–2638 Vol. 34, No. 10
0095-1137/96/$04.00+0

Prevalence of Human Herpesvirus 8 DNA Sequences in Several Patient Populations

C. C. MARCHIOLI,[1] J. L. LOVE,[1] L. Z. ABBOTT,[1] Y. Q. HUANG,[2] S. C. REMICK,[3] N. SURTENTO-REODICA,[3] R. E. HUTCHISON,[4] D. MILDVAN,[5] A. E. FRIEDMAN-KIEN,[2] AND B. J. POIESZ[1]*

Division of Hematology and Oncology, Department of Internal Medicine,[1] and Department of Pathology,[4] SUNY Health Science Center, Syracuse, New York 13210; Departments of Dermatology and Microbiology, New York University Medical Center, New York, New York 10016[2]; Divisions of HIV Medicine and Medical Oncology, The Albany Medical College, Albany, New York 12208[3]; and Division of Infectious Diseases, Beth Israel Medical Center, New York, New York 10003[5]

Received 11 January 1996/Returned for modification 16 February 1996/Accepted 18 July 1996

We have undertaken a large-scale study of various tissues from normal controls and patients with Kaposi's sarcoma (KS) or other malignancies, both with and without human immunodeficiency virus infection, to determine the prevalence of human herpesvirus 8 (HHV-8) DNA. A total of 566 specimens were analyzed by PCR for the presence of HHV-8 DNA. Of the samples tested, 251 were obtained from patients with KS and 315 were obtained from patients without KS. HHV-8 DNA was detected in 103 (41%) of the 251 samples from patients with KS. In particular, 92% of KS tumor specimens were positive. None of the tissues from patients without KS showed evidence of HHV-8 DNA. Sequencing and phylogenetic analyses indicate a high degree of conservation (97.5 to 100%) among the HHV-8 strains tested.

Classic Kaposi's sarcoma (KS) was first described in 1872 by Moriz Kaposi (4). It is characterized as an indolent disease occurring in elderly men of Mediterranean or Jewish descent (20). Subsequently, a different form of KS termed endemic KS was identified in Africa by Kaminer and Murray in 1950 (15). In the 1980s, AIDS-associated KS, also referred to as epidemic KS, was shown to cause significant morbidity if not increased mortality in AIDS patients (9–11).

Recently, a putative gammaherpesvirus, termed KS-associated herpesvirus or human herpesvirus 8 (HHV-8), has been implicated as a possible etiologic agent for KS on the basis of the presence of discrete herpesvirus-like DNA sequences in the majority of tumor specimens obtained from KS patients with and without AIDS (6, 7, 12, 19, 24). We have previously reported on the presence of HHV-8 DNA sequences in tumor specimens from patients with classic, African endemic, and AIDS-associated (epidemic) KS (12). In addition, analysis of matched specimens of tumors, semen, and blood from patients with epidemic KS suggested that HHV-8 may be transmitted by semen and/or blood (13). Whitby et al. (26) provided further evidence as to the causal role of HHV-8 by showing that the detection of HHV-8 DNA sequences in peripheral blood mononuclear cells (PBMs) of human immunodeficiency virus (HIV)-infected individuals without KS predicted the development of KS lesions.

We have undertaken a large-scale analysis of specimens from KS patients, including sera and/or plasma, PBMs, tumor lesions, and semen, along with specimens from non-KS neoplasms and normal tissues. A total of 566 specimens from patients seen or referred to the authors' institutions were processed for PCR analysis. Included in this study were specimens analyzed and described in previous publications by the authors (11, 12). These specimens were 24 HIV-positive epidemic KS skin lesions, 2 HIV-negative homosexual KS skin lesions, 8 classic KS skin lesions, 10 African endemic KS skin lesions, 3 HIV-positive epidemic KS PBMs, and 3 HIV-positive KS semen samples. This study had Institutional Review Board approval, and all patients gave informed consent. DNA was organically extracted from sera and plasma. DNA from PBMs and semen was extracted with phenol-chloroform as previously outlined (17) or by a rapid lysis method (8). Fresh skin tissues were treated with proteinase K, and then the DNA was organically extracted. Paraffin-embedded tissues were processed as described by Slack et al. (22) and Miller et al. (18). Great care was taken to clean the microtome blade with HCl and NaOH between sections in order to eliminate possible contamination of samples. Sections of paraffin without tissue were cut and subjected to PCR amplification with HHV-8-specific primers as an additional control. DNA quantitation was accomplished by spectrophotometry for organically extracted tissue samples. PBM cell counts were made with a Coulter counter prior to rapid lysis, with 1.5×10^5 cells yielding approximately 1 μg of input DNA.

Amplification of extracted DNA was accomplished according to the protocol described by Huang et al. (12) with modifications based upon optimization for Mg^{2+} concentration, pH, and temperature. One microgram of target DNA was added to a final reaction mixture containing 50 pmol of each primer specific for herpesvirus-like DNA; 2.0 mM $MgCl_2$; 10 mM Tris-HCl (pH 9.0); 50 mM KCl; 180 μM (each) dATP, dCTP, dGTP, and dUTP; 2 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.); 17 μg of bovine serum albumin; and 1 U of uracil-*N*-glycosylase (Perkin-Elmer Cetus) in a total volume of 100 μl. Primer sequences for the $KS330_{233}$ amplicon were as described by Huang et al. (12). Each primer included a 21-bp linker attached to the 5′ end which contained restriction endonuclease sites (sense, 5′-ACAGGTACCTGC AGATCTAGA-3′; antisense, 5′-TACGAGCTCGCGAATTC ATGA-3′). Lack of PCR amplification of original sample

* Corresponding author. Mailing address: Division of Hematology and Oncology, Department of Internal Medicine, SUNY Health Science Center, Syracuse, NY 13210. Phone: (315) 464-8231. Fax: (315) 464-8255.

DNA with primers designed against only the 21-bp nonhuman, nonviral linker sequences was used to demonstrate an absence of carryover contamination (1). Prior to addition to the reaction mixture, the DNA polymerase was incubated for 10 min at room temperature with a 50-fold molar excess of anti-*Taq* antibody TP1 and a 5-fold molar excess of anti-*Taq* antibody TP4 (kindly provided by Johnson & Johnson Clinical Diagnostics Systems, Inc., Rochester, N.Y.) (21), which allowed for hot start PCR conditions. Also, the reaction mixture was incubated at room temperature for 5 min prior to PCR to allow for uracil-*N*-glycosylase sterilization (16). Samples were amplified for 35 cycles as described previously (12). All samples were also amplified with the human beta-globin gene primers PCO3 and PCO4 in a quantitative PCR assay (14) to confirm the presence of sufficient amounts of amplifiable human DNA. To prevent carryover contamination, all pre- and post-PCR reactions were conducted by separate personnel in different buildings.

Detection of amplified HHV-8 DNA sequences was accomplished by Southern blot hybridization as described by Huang et al. (12). The threshold of detection was approximately 10 copies of HHV-8 DNA in a background of 1 μg of total DNA input (data not shown). This was determined by amplifying replicate dilutions, in normal human DNA, of an HHV-8-positive KS tumor specimen whose copy number had previously been established by normalization with a cloned fragment of HHV-8 DNA. At a dilution of 10 HHV-8 copies per aliquot, the replicates were positive approximately 60% of the time. Amplified HHV-8 DNAs from a total of 26 KS samples were sequenced as previously described (11).

A total of 251 specimens from patients with KS were obtained and analyzed by PCR for the presence of HHV-8 DNA sequences (Table 1). HHV-8 DNA was detected in 103 of these 251 specimens (41.0%). Biopsies of KS skin lesions accounted for the highest percentage of samples reactive by PCR, with 44 of 48 (91.7%) being positive. HHV-8 DNA was detectable in a majority of KS skin lesions from patients with AIDS-associated (28 of 28, 100%), classic (7 of 8, 87.5%), and African endemic (7 of 10, 70%) KS, along with 2 of 2 specimens (100%) from homosexual HIV-negative KS patients. Six of 26 (23%) specimens of normal-appearing skin obtained from patients with KS were also noted to be positive for HHV-8 DNA. In addition, 46 of 101 (45.5%) PBMs from patients with KS were shown to be reactive. Semen and sera and/or plasma from KS patients also demonstrated detectable HHV-8 DNA, with 4 of 33 (12.1%) and 3 of 43 (7.0%) being positive, respectively.

A total of 315 specimens were available from patients with no evidence of KS (Table 1). All of these samples were noted to be negative for the presence of HHV-8 DNA. These specimens included samples from patients with and without HIV infection. Tissues analyzed included normal skin biopsies, PBMs, pediatric lymphomas, adult lymphomas (Hodgkin's and non-Hodgkin's), carcinomas (lung, anal, and skin), and PBMs from large granular lymphocyte leukemia patients (Table 1).

Phylogenetic analysis was performed on 193 bp of HHV-8 DNA from various isolates, including those obtained from AIDS-associated KS, African endemic KS, and classic KS samples (6, 12). Homologous Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) (subgroup A) sequences were used as outgroup roots (2, 3). A heuristic search utilizing the parsimony method (25) yielded a phylogram (Fig. 1) in which individual HHV-8 DNA isolates were evident as unique sequences but did not cluster according to KS sample type. The divergence among HHV-8 strains ranged from 0 to 2.5%, and as expected, the HHV-8 sequences were more closely related to

TABLE 1. PCR analysis of specimens for HHV-8 DNA sequences

Sample[a]	No. HHV-8 positive	% HHV-8 positive
Patients with KS		
KS skin lesion		
HIV$^+$ epidemic	28/28	100
Classic	7/8	87.5
African endemic	7/10	70
Homosexual HIV$^-$	2/2	100
Subtotal	44/48	91.7
Normal-appearing skin	6/26	23
Subtotal	6/26	23
PBM		
HIV$^+$ epidemic	46/99	46.5
Homosexual HIV$^-$	0/2	0
Subtotal	46/101	45.5
Semen		
HIV$^+$ epidemic	4/31	12.9
Homosexual HIV$^-$	0/2	0
Subtotal	4/33	12.1
Serum		
HIV$^+$ epidemic	3/28	10.7
Homosexual HIV$^-$	0/2	0
Subtotal	3/30	10
Plasma, HIV$^+$ epidemic	0/13	0
Subtotal	0/13	0
Total	103/251	41
Patients without KS		
Normal skin	0/10	0
Subtotal	0/10	0
PBM		
HIV$^+$	0/64	0
HIV$^-$ (normal controls)	0/163	0
Subtotal	0/227	0
Pediatric lymphoma	0/8	0
Subtotal	0/8	0
Adult lymphoma		
HIV$^+$	0/28	0
HIV$^-$	0/9	0
Subtotal	0/37	0
Carcinoma		
HIV$^+$	0/10	0
HIV$^-$	0/2	0
Subtotal	0/12	0
LGL leukemia (PBM)	0/21	0
Subtotal	0/21	0
Total	0/315	0

[a] HIV$^+$, HIV positive; HIV$^-$, HIV negative; LGL, large granular lymphocyte.

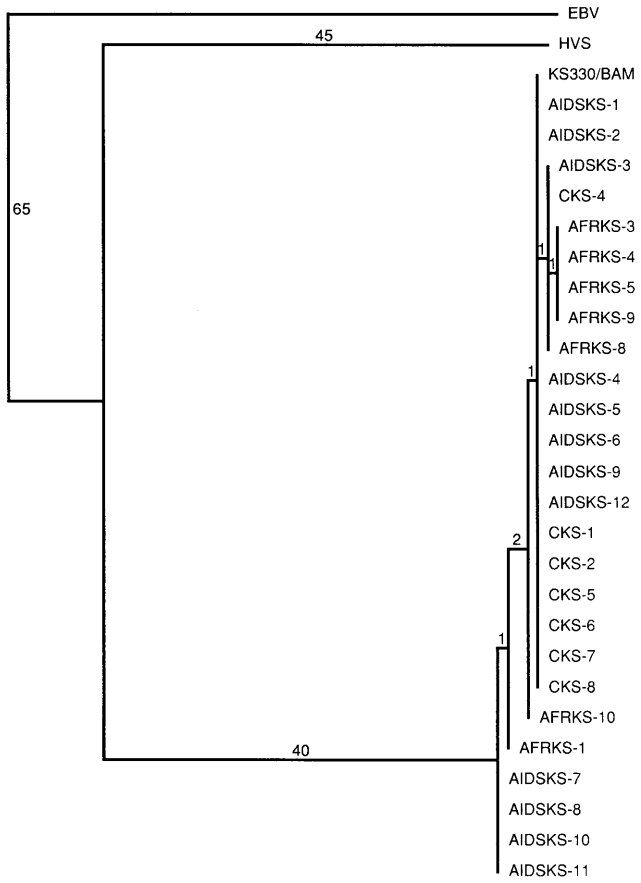

FIG. 1. A phylogram based on 193 bp of HHV-8 DNA sequence, generated by the parsimony method. Homologous EBV and HVS (subgroup A) sequences were used as outgroup roots. The number of unique bases on each branch is shown. The 26 HHV-8 strains sequenced from samples tested in this report are those from AIDSKS-1 on down (GenBank numbers U61113 to U61138). Among these 26 strains, there are five unique HHV-8 sequences, but they do not cluster according to KS sample type. The bootstrap values between EBV and HVS and HHV-8 and between HVS and HHV-8 are 100%. However, the bootstrap values among the HHV-8 strains are all below 50%. AIDSKS, AIDS-associated KS; CKS, classical HIV-1-negative KS; AFRKS, African HIV-1-negative KS. KS330/BAM is a previously reported AIDS-associated KS HHV-8 strain (6).

HVS than to EBV. Distinct clades among the HHV-8 strains were not supported by significant bootstrap values. The existence of unique HHV-8 DNA sequences substantiates the point that these sequences represent real HHV-8 strains and do not represent PCR carryover contamination with a single laboratory amplicon. *Taq* DNA polymerase has been shown to have a fidelity greater than a 1-bp error rate per 193 bp in our laboratory, with as many as 20 clones from the same PCR-amplified target showing identical DNA sequences (data not shown).

In this study, we have substantiated, with a large-scale analysis, the prevalence of HHV-8 DNA in various tissue specimens from KS patients. Of particular note is the presence of HHV-8 DNA in semen and blood components of individuals with KS, again suggesting a possible mode of transmission via body secretions. Whitby et al. (26) reported that HHV-8 detection in peripheral blood cells of HIV-positive patients without KS predicted the subsequent appearance of KS lesions. Their patient population included 189 HIV-infected individuals, 145 of whom were homosexual and 7 of whom were bisexual. In our study, virtually all of the 64 HIV-positive patients without KS had intravenous drug abuse as the primary risk factor as opposed to homosexuality. This factor is a possible explanation for the lack of detectable HHV-8 DNA in our subgroup.

HHV-8 DNA has also been found in tissue specimens from patients with diseases other than KS. Soulier et al. (23) reported the association of HHV-8 DNA sequences with multicentric Castleman's disease, and Cesarman et al. (5) reported the presence of HHV-8 DNA sequences in AIDS-related body-cavity-based lymphomas but not in any other type of lymphoid neoplasm. In our analysis, we were unable to detect HHV-8 DNA in any of the adult lymphoma specimens that we tested, including 28 from HIV type 1 (HIV-1)-positive individuals. However, none of our HIV-1-infected lymphoma patients seem to meet the clinical criteria for Castleman's disease or body-cavity-based lymphoma (5, 23). In addition, HHV-8 DNA sequences were not detected in pediatric lymphomas, adult carcinomas, or large granular lymphocyte leukemias.

The results of our large-scale analysis support previous implications of an association between HHV-8 and KS and the potential role of body fluids in the transmission of the putative viral agent. Why only 92% and not 100% of KS tumor lesions were positive for HHV-8 is unclear. Possible explanations include sequence differences relative to our primers and probes among some HHV-8 strains, low HHV-8 copy number in the negative tumor samples, or the presence of low-level inhibitors of PCR. In addition, the absence of detectable HHV-8 DNA in 315 specimens from patients without KS lends further credence to the specificity of the HHV-8–KS association.

This work was supported by PHS grants HB-67021 and V01-AI-27658 and by NIH grants AL#32670-03 and CA62782-01A2.

REFERENCES

1. **Abbott, L. Z., T. Spicer, V. Bryz-Gornia, S. Kwok, J. Sninsky, and B. J. Poiesz.** 1994. Design and use of signature primers to detect carryover of amplified material. J. Virol. Methods **46:**51–59.
2. **Albrecht, J.-C., J. Nicholas, D. Biller, K. R. Cameron, B. Biesinger, C. Newman, S. Wittman, M. A. Craxton, H. Coleman, B. Fleckenstein, and R. W. Honess.** 1992. Primary structure of the herpesvirus saimiri genome. J. Virol. **66:**5047–5058.
3. **Baer, R. J., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. F. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310:**207–211.
4. **Braun, M.** 1982. Classics in oncology: idiopathic multiple pigmented sarcoma of the skin (by M. Kaposi). CA—Cancer J. Clin. **32:**340–347. (Translated from the original German from Arch. Dermatol. Syphilis [1872] **4:**265–273.)
5. **Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N. Engl. J. Med. **332:**1186–1191.
6. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865–1869.
7. **Dupin, N., M. Grandadam, V. Calvez, I. Gorin, J. T. Aubin, S. Havard, F. Lamy, M. Leibowitch, J. M. Huraux, J. P. Escande, and H. Agut.** 1995. Herpesvirus-like DNA sequences in patients with Mediterranean Kaposi's sarcoma. Lancet **345:**761–762.
8. **Dyster, L. M., L. Abbott, V. Bryz-Gornia, B. J. Poiesz, and L. D. Papsidero.** 1994. Microplate-based DNA hybridization assays for detection of human retroviral gene sequences. J. Clin. Microbiol. **32:**547–550.
9. **Friedman-Kien, A. E.** 1981. Disseminated Kaposi's sarcoma syndrome in young homosexual men. J. Am. Acad. Dermatol. **5:**468–471.
10. **Friedman-Kien, A. E.** 1983. Epidemic Kaposi's sarcoma: a manifestation of the acquired immune deficiency syndrome. J. Dermatol. Surg. Oncol. **9:**637–640.
11. **Gottlieb, G. J., A. Ragaz, J. V. Vogel, A. Friedman-Kien, A. M. Rywlin, E. A. Weiner, and A. B. Ackerman.** 1981. A preliminary communication on extensively disseminated Kaposi's sarcoma in young homosexual men. Am. J. Dermatopathol. **3:**111–114.
12. **Huang, Y. Q., J. J. Li, M. H. Kaplan, B. J. Poiesz, E. Katabira, W. C. Zhang, D. Feiner, and A. E. Friedman-Kien.** 1995. Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. Lancet **345:**759–761.

13. **Huang, Y. Q., J. J. Li, B. J. Poiesz, M. H. Kaplan, and A. E. Friedman-Kien.** Occurrence of the human gammaherpesvirus-like agent (HHV-8) in matched specimens of tumors, uninvolved skin, semen, and blood from patients with epidemic Kaposi's sarcoma. Am. J. Pathol., in press.
14. **Iannone, R., M. P. Sherman, P. E. B. Rodgers-Johnson, M. A. Beilke, C. A. Mora, R. M. Amin, S. R. Tinsley, L. D. Papsidero, B. J. Poiesz, and C. J. Gibbs, Jr.** 1992. HTLV-I DNA sequences in CNS tissue of a patient with tropical spastic paraparesis and HTLV-I associated myelopathy. J. Acquired Immune Defic. Syndr. **5:**810–816.
15. **Kaminer, B., and J. F. Murray.** 1950. Sarcoma idiopathicum multiplex haemorrhagicum of Kaposi, with special reference to its incidence in the South African Negro, and two case reports. S. Afr. J. Clin. Sci. **1:**1–25.
16. **Longo, M. C., M. S. Berninger, and J. L. Hartley.** 1990. Use of uracil DNA glycosylase to control carryover contamination in polymerase chain reaction. Gene **93:**125–128.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Miller, S. A., D. D. Dykes, and H. F. Polesky.** 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. **16:**1215.
19. **Moore, P. S., and Y. Chang.** 1995. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and those without HIV infection. N. Engl. J. Med. **332:**1181–1185.
20. **Reynolds, W. A., R. K. Winkelmann, and E. H. Soule.** 1965. Kaposi's sarcoma: a clinicopathologic study with particular reference to its relationship to the reticuloendothelial system. Medicine (Baltimore) **44:**419–443.
21. **Sharkey, D. J., E. R. Scalice, K. G. Christy, Jr., S. M. Atwood, and J. L. Daiss.** 1994. Antibodies as thermolabile switches: high temperature triggering for the polymerase chain reaction. Bio/Technology **12:**506–509.
22. **Slack, D. N., K. P. McCarthy, L. M. Wiedemann, and J. P. Sloane.** 1993. Evaluation of sensitivity, specificity, and reproducibility of an optimized method for detecting clonal rearrangement of immunoglobulin and T-cell receptor genes in formalin-fixed, paraffin-embedded sections. Diagn. Mol. Pathol. **2:**223–232.
23. **Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M. F. d'Agay, J. P. Clauvel, M. Raphael, L. Degos, and F. Sigaux.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood **86:**1276–1280.
24. **Su, I. J., Y. S. Hsu, Y. C. Chang, and I. W. Wang.** 1995. Herpesvirus-like DNA sequence in Kaposi's sarcoma from AIDS and non-AIDS patients in Taiwan. Lancet **345:**722–723.
25. **Swofford, D. L.** 1991. PAUP: phylogenetic analysis using parsimony, version 3.05. Computer program distributed by the Illinois National History Survey, Champaign.
26. **Whitby, D., M. R. Howard, M. Tenant-Flowers, N. S. Brink, A. Copas, C. Boshoff, T. Hatzioannou, F. E. A. Suggett, D. M. Aldam, A. S. Denton, R. F. Miller, I. V. D. Weller, R. A. Weiss, R. S. Tedder, and T. F. Schulz.** 1995. Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet **346:** 799–802.