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## Human Herpesvirus 8 (HHV-8) in Non-HIV Associated Forms of Kaposi's Sarcoma from Turkey

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**Abstract:** Objective: Kaposi's sarcoma (KS), first described in 1872 by Moritz Kaposi, is a spindle cell, multifocal vascular tumor. Despite the intense work on the pathogenesis of KS, its etiology remains in doubt. The aim of this study was to determine the presence of HHV-8 sequences in non-HIV associated Kaposi's sarcoma patients, namely classical and immunosuppression-associated, and compare the results with normal healthy subjects.

**Materials and Methods:** Nineteen patients with classical Kaposi's sarcoma (CKS), and nine patients with immunosuppression-associated Kaposi's sarcoma (ISKS) (6 renal transplantation, 1 Behçet's disease, 1 high grade lymphoma, 1 ataxia telangiectasia) were included in this study. Skin samples of 29 healthy subjects who had elective plastic surgery were used as a control group. All samples were retrieved from the archives of the pathology department and studied in the microbiology department of a university hospital. Formalin fixed, paraffin embedded tissue samples of classical and immunosuppression-associated Kaposi's sarcoma patients were investigated by

polymerase chain reaction (PCR) and restriction enzyme analysis (REA) for the presence of HHV-8 DNA sequences.

**Results:** HHV-8 sequences were detected in 16 of 19 (84%) CKS patients, and 5 of 9 (55%) ISKS patients by PCR and REA. The prevalence of HHV-8 detection was lower in the ISKS group. In this group, five positive results belonged to renal transplant patients who received cyclosporine in addition to prednisolone. We did not detect HHV-8 sequences in the 29 healthy skin samples.

**Conclusions:** Our data suggest that HHV-8 probably plays an important role in the pathogenesis of non-HIV infection related forms of KS. Unlike other herpesviruses, HHV-8 is not widespread in the normal population. In immunosuppressed patients, HHV-8 expression may be due to cyclosporine treatment.

**Key Words:** Kaposi's sarcoma, Human herpesvirus-8

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### Introduction

Kaposi's sarcoma (KS), first described in 1872 by Moritz Kaposi, is a spindle cell, multifocal vascular tumor that occurs in the lymphoid and respiratory system and gastrointestinal tract as well as in the skin (1-3). Four different epidemiologic forms of Kaposi's sarcoma have been described: classical, African endemic, immunosuppression-associated, and human immunodeficiency virus infection-associated Kaposi's sarcoma (AIDS-KS) (4).

Despite the intense work on the pathogenesis of KS, its etiology remains in doubt. Formerly CMV, HBV, HAV, HHV-6, HIV-tat, and HPV were incriminated as etiologic agents in KS (5-9). Recently herpesvirus-like viral particles have been detected initially in HIV-associated

Kaposi's sarcoma by representational difference analysis (10). Thereafter these sequences were described in other forms of KS by PCR and other methods (11-18). To date, HHV-8 has been definitively associated with two more human diseases: primary effusion lymphoma and multicentric Castleman's disease (20,21). This virus, designated as KSHV and later renamed HHV-8, shows tegument and capsid homologies with the Gamma herpesvirus family (10).

The aim of this study was to determine the HHV-8 DNA in Turkish patients with two HIV-unrelated forms of Kaposi's sarcoma, namely CKS and ISKS, and the diagnostic utility of PCR in formaline fixed paraffin embedded skin samples. We also analyzed the prevalence of this new virus in a control group that consisted of 29 HIV seronegative healthy subjects.

**Materials and Methods**

Forty archival KS specimens, obtained between 1987 and 1997, were retrieved from the department of pathology of Hacettepe University, Faculty of Medicine, Ankara, Turkey. Twenty-eight of the specimens demonstrating active KS lesions were selected for the study. Patients were divided into two groups according to their immune status. Among the selected patients, 19 had CKS (Group 1) and 9 had ISKS (Group 2). In the second group, 6 of them were renal transplant patients receiving cyclosporine and prednisolone. Patients other than renal transplantation patients that were categorized as having ISKS were as follows: one patient with Behçet's disease taking prednisolone for two years, one patient with high grade lymphoma who developed KS after receiving radiotherapy and chemotherapy, and one patient with ataxia telangiectasia who developed KS at 12 years of age. Although they were immunocompromised by different causes they were included in the ISKS group. The duration between the initiation of immunosuppressive therapy and the development of Kaposi's sarcoma are shown in the Table. All patients were serologically HIV negative. Control skin samples were obtained from 29 healthy subjects undergoing elective plastic surgery.

DNA was extracted from formalin fixed, paraffin embedded and fresh tissues. Paraffin embedded tissue blocks were cut into 50 mm sections. In order to prevent contamination and false positive results, the microtome knife was cleaned with xylene after each sample and sterile disposable pipettes and separate pairs of gloves were used. DNA on the contaminated surfaces was

destroyed using UV germicide lamps. The sections were deparaffinized twice by adding 1 ml xylene, mixed with a vortex for 5 seconds, shaken for 30 minutes, and then centrifuged for 5 minutes in a microcentrifuge at 12,000 g in order to precipitate tissues. Xylene was removed by adding 1 ml of 95% ethanol, and centrifugating and decanting the tubes in order to remove all of the paraffin which might inhibit the amplification reaction. The tissues were resuspended in 100 µl digestion buffer containing 50 mM Tris pH 8.5, 1 mM EDTA, 5% Tween 20, and proteinase K 200 µg/ml, and incubated overnight at 37°C. The next morning the tubes were incubated for 20 minutes on boiling water, and the debris was sedimented by spinning at 12,000 g for 5 minutes. The supernatant containing DNA was transferred to a clean tube and stored at -20°C until used for PCR. The primers used in this study were first described by Chang et al. and were specific for HHV-8. 5'TCCGTGTTGTCTACGTCCAG3' was used as the first primer, and 5'AGCCGAAAGGATTCCACCAT3' was used as the second (10). A positive control containing HHV-8 DNA obtained from a patient with HIV-associated KS was kindly provided by Dr. Friedman-Kien from NYU. Restriction enzyme analysis (REA) was used to verify that amplified DNA belonged to HHV-8. Restriction enzyme cutting sites were analyzed, and Pst-I restriction enzyme was selected. Amplified DNA was cut into two DNA fragments of 95 and 138 base pairs by this enzyme. All samples were studied in double blind fashion. After DNA amplification and restriction, digestion products were run on a polyacrilamide gel electrophoresis, stained by ethidium bromide, visualized and photographed over a UV transilluminator (Figures 1-2).

Table. Clinical features and results of HHV-8 DNA amplification by PCR and REA in ISKS patients.

GROUP II					
PN	S	A	PCR/REA	TI	T(Month)
1	F	40	+	RT	9
2	F	45	+	RT	12
3	F	15	+	RT	8
4	F	51	+	RT	48
5	F	31	-	RT	9
6	M	46	+	RT	10
7	M	51	-	HL	8
8	M	28	-	BD	24
9	F	12	-	AT	144

PN: Patient number, S: Sex, A: Age, TI: Type of immunosuppression, T: Duration before KS, RT: Renal transplantation, HL: High Grade Lymphoma, BD: Behcet's Disease, AT: Ataxia Telangiectasia.

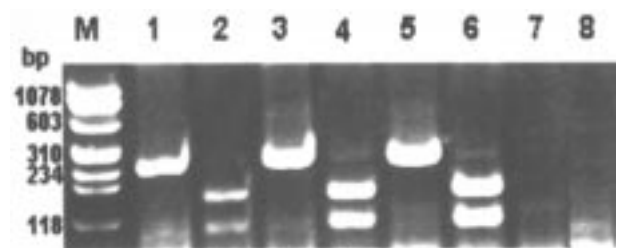


Figure 1. PCR amplification and REA of HHV-8 sequences from CKS patients. M: Molecular DNA weight marker. Lane 1: Positive control marker, Lane 2: REA of positive control. Lanes 3, 5: PCR amplification of the first and the second patients with CKS, Lanes 4, 6: REA of the same patients. Lanes 7, 8: Negative PCR and REA results of the third patient.

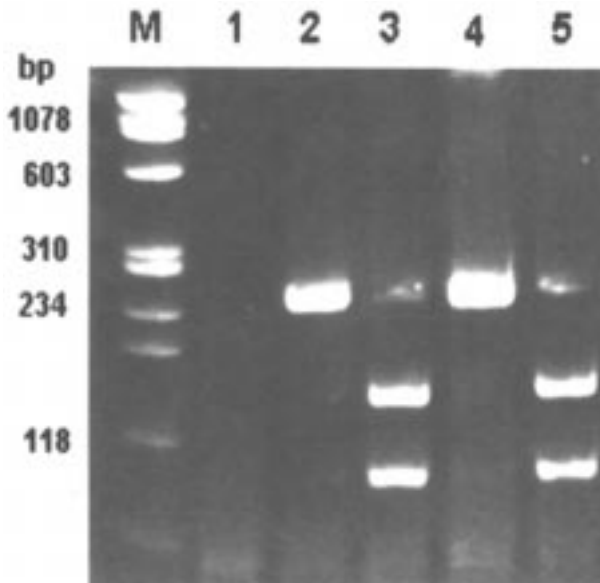


Figure 2. PCR amplification and REA of HHV-8 sequences from ISKS patients. M: Molecular DNA weight marker. Lane 1: Negative control sample (H<sub>2</sub>O). Lanes 2, 4: PCR amplification of the first and the second patients in ISKS group. Lanes 3, 5: REA of the same patients.

## Results

All biopsy specimens from patients with KS and healthy subjects were analyzed by PCR. In the first group there were 11 male and 8 female patients; the mean age was 54.89 (standard deviation 19.16, range 17-80). In the second group there were 6 female and 3 male patients; the mean age was 35.44 (standard deviation 14.79, range 12.51). PCR and subsequent REA revealed the presence of HHV-8 DNA in 16 of the 19 classical KS patients and 5 of the 9 immunosuppression associated KS patients. The prevalence of HHV-8 was 84% in the first group, and 55% in the second. In the second group, 5 positive results belonged to the renal transplant patients who received cyclosporine in addition to prednisolone. In only one patient with renal transplantation receiving cyclosporine plus prednisolone could we not demonstrate HHV-8 particles with PCR amplification. None of the control samples were positive for HHV-8 DNA by PCR. In all positive samples, REA with Pst-I showed expected restriction fragments as 138 and 95 base pairs.

## Discussion

After HHV-8 was described in HIV-associated KS, several authors have investigated the presence of this virus in other forms of KS. The great majority of KS samples were positive for the presence of this virus

whatever the epidemiologic group. So there is widespread consensus that this virus is universally present in KS lesions (10-20). Similar to other studies, we detected HHV-8 DNA in lesional tissue samples of 16 of the 19 classical KS (84%) and 5 of the 9 (55%) immunosuppression associated KS patients by PCR. Our data confirms the presence of HHV-8 in HIV-unrelated forms of KS, namely CKS and ISKS, in Turkish patients.

Lack of 100% positivity in CKS has been reported repeatedly by other researchers (22) as well in our study, and this sequence suggests the interplay of the underlying immunodeficiency in the expression of HHV-8 in CKS lesions. The relatively low detection rate of HHV-8 in these samples may be attributed to sequence variation, low copy number in the biopsy specimens or the absence of this virus in some of the lesions (12).

Herman et al., in a recent study, showed that paraffin embedded tissues are satisfactory for HHV-8 detection by PCR (22). Foreman et al. described distinct HHV-8 sequences in Saudi Arabian patients (23). Similarly, in our study, at least in some of the specimens, sequence variation seems to be responsible for the low detection rate rather than other factors.

ISKS was first recognized in 1950's with the introduction of immunosuppressive treatment for organ transplantation and other diseases such as autoimmune diseases, bullous diseases and atopic dermatitis (4,24-26). HHV-8 presence has been described in immunosuppression associated epithelial malignancies as well as ISKS (17). Rady et al. have investigated HHV-8 presence in 33 epithelial tumors of 4 organ transplant patients receiving cyclosporine, and showed the presence of HHV-8 in 82% by PCR (27). However, Wolf et al. could not show the presence of HHV-8 in premalignant and malignant skin lesions of patients receiving PUVA treatment. They proposed that HHV-8 is not a widespread latent virus and is activated by immunosuppression like other herpesviruses (28). Jensen et al. showed HHV-8 sequences in 11 of 14 patients, in three different forms of Kaposi's sarcoma, by PCR. Three of the positive results belonged to ISKS patients but the medications of these patients are not mentioned (29). Rady et al., in their recent study, investigated the presence of HHV-8 in 11 patients (7 with immunosuppression associated KS and 4 with organ transplantation associated KS). They found HHV-8 in 100% of specimens and confirmed it by Southern blot analysis (30).

In our second group, we did not detect HHV-8 DNA by PCR in patients other than renal transplantation patients. This discrepancy may be due to different types of immunosuppression. On the other hand, in the ISKS group, patients other than renal transplantation patients developed KS without activation of HHV-8.

In the second group, one of the patients was diagnosed with ataxia telangiectasia and developed KS at 12 years of age. Although she did not receive immunosuppressive treatment, we considered this patient to be immunocompromised because AT is a congenital immunosuppressive disease. On the other hand, one patient with Behçet's disease developed KS within two years of the beginning of corticosteroid therapy. In both cases, we did not detect HHV-8 DNA.

Among immunosuppressive therapies, cyclosporine may have a peculiar effect, because in cyclosporine using renal transplant patients, development of KS is seen earlier than in patients using conventional therapies (4).

The prevalence of HHV-8 infection or latency in Turkey is not known, since no population based studies have been performed yet. If this virus contributes etiologically to KS, these sequences should be found in lesions across all geographic distributions. Dupin et al., in a recent study, showed that HHV-8 is not prevalent in non-KS skin diseases or in healthy control subjects and concluded that HHV-8 is not prevalent in France (31). Similarly, Herman et al. were unable to show HHV-8 sequences in non-KS dermatologic lesions of HIV negative patients by PCR (22). However, Monini et al. showed HHV-8 sequences in prostate and seminal tissue fluids of immunocompetent individuals and proposed that HHV-8, like other herpesviruses, is widespread in their population (32). In our control group, the absolute negativity of HHV-8 suggests that this virus does not appear to be widespread in our geographic region. Our results are in corroboration with Dupin and Herman et al. On the other hand, Cattani et al. found HHV-8 positivity in 23% of healthy control subjects in Italian patients and concluded that HHV-8 could be a widespread virus at least in the Mediterranean regions where KS is prevalent (33).

There are some reports about the distribution of this virus. Its presence in body cavity based lymphomas, multicentric Castleman's disease and genital tissues of non-HIV infected patients showed that this virus is not exclusively restricted to KS lesions, but is widespread in other tissues like other herpesviruses (20,21,32,34).

Whitby et al. showed that HHV-8 genomes are detectable in the blood of KS negative, HIV positive gay men and that their presence is strongly predictive of later development of KS (35). Geographically the detection of HHV-8 antibodies is associated with high incidence areas for KS (36). The prevalence of the virus is generally high in those human populations with a high incidence of KS, and the virus carries the tools to stimulate cell proliferation and to induce neovascularization (37).

A review of 21 published studies involving 549 patients, with all forms KS from various parts of the world, shows that HHV-8 DNA can be detected on average 95% of the time in KS lesions by PCR. It is now clearly established using a variety of techniques that HHV-8 is not only restricted to KS lesions, and viral DNA and RNA are detectable in most KS tumor spindle cells. To date, published assays show similar epidemiologic trends, despite important differences in their sensitivities and specificities. The relative HHV-8 seroprevalence measured by these assays matches the patients for KS risk groups among patients with AIDS and the patterns for non-HIV related KS incidence among patients from various countries (38).

For HHV-8 to be causally related to KS, infection must precede the onset of disease. This is an absolute criterion for causality and both seroconversion and DNA based detection studies provide evidence for KSHV infection among most AIDS-KS patients prior to disease onset (38).

Studies on HHV-8 now indicate that it is not a ubiquitous infection in most human populations, although high rates of infection in some populations may account for early studies suggesting widespread HHV-8 infection (38).

In conclusion, our results, like those of previous studies, suggest that this virus may play an important role either directly or indirectly in the pathogenesis of KS. But HHV-8 infection is not prerequisite for the development of KS; other factors, environmental or genetic, are required.

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