

Molecular Epidemiology of Kaposi's Sarcoma-Associated Herpes Virus, and Risk Factors in HIV-infected Patients in Tehran, 2014

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Abstract

Background: Kaposi's sarcoma (KS) remains the most common malignancy among HIV-infected patients. Human herpesvirus type-8 (HHV-8) is regarded as the infectious etiologic agent of Kaposi's sarcoma (KSHV). Diagnostic procedures associated with KSHV are not routinely performed in HIV-infected subjects.

Objectives: The main objective of this study is to obtain information on KSHV epidemiology in Iranian HIV-infected individuals.

Patients and Methods: In the present cross-sectional study, 109 patients with established HIV infection, who visited a governmental and referral center for HIV screening in Tehran (Tehran west health center (TWHC)) between May 2014 and July 2015 were enrolled according to the convenience sample strategy. After peripheral blood collection, isolation of plasma and peripheral blood mononuclear cell (PBMC) compartments, DNA extraction was performed. KSHV DNA was analyzed by nested polymerase chain reaction (nested PCR) using primers from ORF-26 (virus minor capsid).

Results: Among all 109 HIV-infected patients, 67 (61.5%) were male, with an age range of 2-64 years (mean \pm standard deviation 35.8 \pm 13.3). KSHV DNA was found in PBMC and plasma samples of six (5.5%) and four (3.6%) patients, respectively.

Conclusions: This study revealed a considerable prevalence of KSHV DNA, during latent and lytic phases, among HIV-infected patients. Risk factors for KSHV infection acquisition and concurrent. O+infection with HIV were also evaluated. Diagnosis of KSHV in the group could be helpful for prognosis of Kaposi's sarcoma and clinical management.

Keywords: Herpesvirus 8, Human, HIV, Sarcoma, Kaposi, Nested PCR

1. Background

Kaposi's sarcoma-associated herpes virus (KSHV), also known as human herpes virus-8 (HHV-8) was first discovered in 1994 (1). It is the infectious etiologic agent in all forms of endothelial-origin tumor involving blood and lymphatic vessels (Kaposi's sarcoma) and is consistently associated with lymphoproliferative diseases such as body-cavity-based lymphoma (BCBL) and multicentric Castleman's disease (2-4). Although, Kaposi's sarcoma (KS) remains an uncommon cancer in human populations, KSHV was affirmed as a class-one carcinogenic agent by the international agency for research on cancer (5).

The route of infection transmission is different. Saliva, blood or blood products, sexual contact, and organ transplantation may play an important role in KSHV transmission (6-8).

Following the occurrence of human immunodeficiency

virus (HIV) epidemics, the prevalence of KS gradually increased. Hence, previous reports indicated that immunosuppression, as in HIV-infected cases, was a significant cofactor for KS development (9, 10). Nowadays, KS remains the second most frequent malignancy in HIV-infected patients worldwide (11).

The pathogenesis of KSHV depends on several risk factors, including risky sexual behaviors (12); intravenous drug use (13); and immunocompromised patients such as HIV-infected patients, especially in patients with low CD4 cell count and those who are not enrolled in highly active antiretroviral therapy (HAART) (14). In the HAART era, the incidence of KSHV-AIDS-related malignancy, including Kaposi's sarcoma, seems to be dramatically decreased (15, 16).

2. Objectives

In Iran, there are few previous reports of KSHV among HIV-infected patients. Given the above introduction, the present study examines recent data on the molecular prevalence of KSHV (HHV-8) among HIV-infected patients. The findings may provide insights into significant future public health impacts of KSHV in the context of HIV co-infection in Iran.

3. Patients and Methods

3.1. Study Population

In the present cross-sectional investigation, blood samples were obtained from HIV-positive patients who attended a governmental and referral center for HIV screening in Tehran [HIV screening and monitoring center of Iran University of Medical Sciences, Tehran West health center (TWHC)] between May 2014 and July 2015. All of the admitted patients were assessed for inclusion in the present study, according to the convenience sampling strategy. In total, 151 HIV-positive cases who were referred during the study period were initially considered, of which 109 subjects met the following inclusion criteria: 1) agreed to take part in the current study, 2) Iranian nationality, 3) confirmed HIV infection, 4) negative for KS malignancy.

Of the 109 patients, 67 (61.5%) were male, and 8 (7.3%) were children under ten years. The study protocol follows the ethical principles of the Declaration of Helsinki in addition to relevant local regulations. The study protocols were approved by the ethical committee of Iran University of Medical Sciences (code and date of ethical approval: 24466.IR.IUMS.REC.1394.), and written informed consent was obtained for each subject. Questionnaires were filled out by trained general practitioners who also interviewed the participants. Personal demographic information included: age, sex, homosexual or bisexual behavior, being an intravenous drug user, history of transfusion, hemophilia, and thalassemia. The privacy for research participants was respected in this study.

3.2. Collection and Preparation of the Samples

About 10 mL of peripheral blood was collected from each patient into sterile EDTA-containing vacutainer tubes. After separation of plasma from whole bloods by centrifugation, aliquots were transferred to sterile microtubes, and the samples were stored at -70°C until further molecular analysis. The PBMCs of the samples were isolated by a standard procedure of Ficoll-Hypaque (FH) gradient centrifugation (Lymphoprep, Oslo, Norway), following an established protocol (17). The PBMC pellet was washed more

than three times with phosphate-buffered saline (PBS) ($\text{pH} = 7.4 \pm 0.2$). The pellets were counted and then frozen at -70°C until use. Plasma and PBMC samples taken from a patient with established Kaposi's sarcoma, as well as a sample taken from a healthy blood donor, were used as positive and negative controls, respectively.

3.3. Confirmation of HIV Infection and Determination of HIV Viral Loads

HIV infection was confirmed by Western blot kit (Diaplus, San Francisco, USA) according to the manufacturer's protocol. For determination of HIV viral loads, viral RNA was extracted using a high pure viral nucleic acid kit (Roche Diagnostics, Germany). HIV viral loads were determined using a COBAS TaqMan 48 Analyzer (Roche Diagnostics, Germany) according to the manufacturer's instructions.

3.4. CD4 Cell Count

Although, for HIV-infected individuals, CD4 cells count by flow cytometry technique was performed in the Tehran west health center (TWHC) at the same time as viral loads were analyzed in Keyvan virology laboratory.

3.5. DNA Extraction

Specimens aliquots of 150 μL of plasma and approximately $2 - 3 \times 10^6$ PBMCs were thawed, and then pellets resuspended in 150 μL of phosphate-buffered saline (PBS) were used for DNA extraction and processed by NucleoSpin@ Dx Virus kit (Macherey-Nagel, Duren, Germany), following the manufacturer's instructions.

3.6. KSHV DNA Detection by Nested PCR

Detection of KSHV-DNA in plasma and PBMC specimens was performed by nested polymerase chain reaction (nested PCR) method.

A set of nested primers from the ORF26 coding region (KSHV minor capsid, VP23) was used, including an outer primer pair of 233-base span: forward primer HV1-1 (5'-AGC CGA AAG GAT TCC ACC AT-3'), and reverse primer HV1-2 (5'-TCC GTG TTG TCT ACG TCC AG-3'); and an inner primer pair of 138-base span: forward primer HV1-3 (5'-TAT TCT GCA GCA GCT GTT GG-3'), and reverse primer HV1-4 (5'-TCT ACG TCC AGA CGA TAT GTG C-3') (1, 18).

The first round PCR was performed in a 25 μL mixture containing 2.5 μL of each extracted sample, 1.5U of Taq DNA polymerase, and 2.5 μL of $10 \times$ PCR buffer (Roche Diagnostics, Germany), 10 pM of each outer primer, 200 μM mix dNTPs, and 1.5 mM MgCl_2 (CinnaGen, Alborz, Iran). Amplification was performed as follows: Initial denaturing for 5 minutes at 95°C and 35 cycles of 95°C for 40 seconds, 55°C

for 45 seconds, and 72°C for 40 seconds, followed by a final extension at 72°C for 8 minutes. After the first amplification, 2.5 μ L of PCR product was added to a second PCR amplification with the inner primer pair. For nested PCR, the same procedure was performed as described for the first PCR step. Amplification was performed for the second step as follows: Initial denaturing for 5 minutes at 95°C, and 35 cycles of 95°C for 35 seconds, 55°C for 40 seconds, and 72°C for 35 seconds, followed by a final extension at 72°C for 8 minutes. PCR products (138-bp) of specimens with positive and negative controls and DNA marker (ruler 100-bp) (Vivantis Technologies, Malaysia) were visualized by 2% agarose gel electrophoresis with ethidium-bromide staining (19). Positive results were verified by re-extracted and nested PCR re-tested on numerous PBMC and plasma new samples. Additionally, β -globin gene amplification was performed on each sample using primers PCO₃ and PCO₄ to ensure the accuracy of gene amplification (20, 21). To prevent contamination of samples and PCR products in each phase, standard circumspections on the basis of principles and regulations were carefully followed.

3.7. Statistical Analysis

Statistical analysis was performed using SPSS software (version 16; SPSS Inc., Chicago, IL, USA). Chi-squared or Fisher's exact tests were used to examine the associations between categorical variables. To analyze the normality of variables the Kolmogorov-Smirnov test was utilized. The independent T-test and Mann-Whitney U-tests were applied to analyze the associations between continuous variables. Additionally, to confirm the reliability of results regarding the limited number of subjects, statistical analyses were performed by bootstrap method analysis with 1000 pseudoreplicates. P values less than 0.05 were considered statistically significant.

4. Results

In the current study, molecular analysis for KSHV DNA was conducted in 109 HIV-infected patients. Nested-PCR was performed in paired PBMC and plasma samples. Overall, KSHV DNA was found in PBMC samples of six patients (5.5%). Furthermore, KSHV DNA was detected only in four their plasma samples (3.6%). As shown in Table 1, KSHV infection was more prevalent in men; out of six positive results, 66.6% (4/6) patients were male and 33.3% (2/6) patients were female, with no significant differences. Obvious KSHV infection in different HIV-positive risk groups is detailed in Table 2. The mean age of HIV-infected individuals was 35.8 ± 13.3 years (range 2 - 64 years). The mean \pm SD age of patients was 32.16 ± 12.13 in KSHV-positive

cases, which is similar to that of 35.93 ± 13.91 among KSHV-negative patients (independent T-test, $P = 0.518$). Interestingly, one of the six KSHV infected patients was a child under ten years old.

Regarding the use of anti-retroviral therapy, out of 109 patients, 33% were receiving highly active anti-retroviral therapy (HAART), some subjects had recently received HAART, and some were receiving treatment at the time of HIV diagnosis for months or years. Fisher's exact test revealed significant differences between positivity and negativity of KSHV infection, and in the use of HAART among patients infected with HIV (P value = 0.042). The mean CD4 count was 377 ± 212 cells/ μ L (range 900 - 24 cells/ μ L). With respect to CD4 cell count, there was no significant difference between KSHV-positive and -negative cases (independent T-test, $P = 0.18$). In total, 24.8% of the patients had CD4+ T-cell count > 500 cells/ μ L, 54.1% had counts between 200 cells/ μ L and 500 cells/ μ L, and 21.1% counts of < 200 cells/ μ L. As described previously, low CD4 count and not being on anti-retroviral therapy are the main risk factors for Kaposi's sarcoma development among KSHV-positive patients with AIDS (22). Accordingly, in 50% (3/6) of patients positive for KSHV, the CD4 count was less than 200 cells/ μ L, and 67% (4/6) were not under HAART treatment.

Viral hepatitis co-infection with HIV was reported in 57% with hepatitis C virus and 3.5% with hepatitis B virus, respectively. None of the KSHV DNA-positive patients was co-infected with viral hepatitis, and also no-one had proven history of AIDS-defining illnesses, including Kaposi's sarcoma (KS); however, clinical examination revealed symptoms of skin lesions in some patients.

Among the HIV-infected patients, 42.2% (46/109) patients were male intravenous drug users (IVDU), and 24.7% (27/109) of patients were women whose spouse had a history of intravenous drug use. Nevertheless, we observed no significant difference between KSHV infection and being addictive. Among all patients, 38.3% (41/109) had a history of being in prison. Another major risk factor was homosexual and/or bisexual behavior among men: 10% of the 109 patients were homosexual and/or bisexual men with a history of multi-unprotected sexual behavior. The prevalence of KSHV infection among intravenous drug users and homosexual and/or bisexual subjects was the same in each group, at 33.3% (2/6).

HIV viral loads ranged between very low values, close to the assay detection limit, to more than 106 copies/mL. The median viral loads were $9.54 \text{ E}4$ and $1.25 \text{ E}3$ in KSHV-positive and KSHV-negative patients, respectively, and were not statistically significant (Mann-Whitney U-test, $P = 0.245$).

Table 1. General Characteristics of Study Patients With Risk Factors: KSHV-Infected and Non-Infected Patients^a

| Variable | KSHV-Negative (n = 103) | KSHV-Positive (n = 6) | P Value |
|------------------------------------|-------------------------|-----------------------|---------|
| Gender | | | 0.57 |
| Male | 63 (61.1) | 4 (66.6) | |
| Female | 40 (38.9) | 2 (33.3) | |
| Marital status | | | 0.52 |
| Married | 48 (46.6) | 5 (83.3) | |
| Single | 37 (36) | 1 (16.7) | |
| Divorced | 10 (9.7) | - | |
| Widow | 8 (7.7) | - | |
| Level of education | | | 0.24 |
| Illiterate | 66 (64.1) | 5 (83.3) | |
| High school graduate | 29 (28.1) | - | |
| University graduate | 8 (7.8) | 1 (16.7) | |
| History of addiction | 43 (41.8) | 1 (16.7) | 0.51 |
| Being in prison | 38 (36.9) | 3 (50.0) | 0.42 |
| Routes of HIV infection | | | 0.43 |
| IVDUs | 44 (42.7) | 2 (33.3) | |
| Unprotected sex | 9 (8.8) | 2 (33.3) | |
| IVDUs wife | 26 (25.2) | 1 (16.7) | |
| Hemophilia and Thalassemia | 4 (3.9) | - | |
| Mother to child "vertical" | 9 (8.8) | 1 (16.7) | |
| Others | 11 (10.6) | - | |
| Under treatment with HAART3 | 79 (76.7) | 2 (33.3) | 0.042 |

Abbreviations: HAART3, highly active anti-retroviral therapies; IVDUs, intravenous drug users; others, Through needle stick, dentistry services, unsafe hairdressing salon, etc.

^aValues are expressed as No. (%).

Table 2. KSHV Infection in Six Patients With Concurrent HIV Infection, on Detailed Demographical Data

| Case | Sex | Age | Marital Status | Literacy | HIV Acquisition | HAART | CD4 Count, μL^{-1} | HIV Viral Load. Copies/mL |
|------|--------|-----|----------------|---------------------|-----------------|-------|-------------------------------|---------------------------|
| 1 | Female | 31 | Married | Illiterate | IVDU wife | No | 361 | 3900 |
| 2 | Female | 9 | Single | Illiterate | Vertical | Yes | 412 | 1786 |
| 3 | Male | 33 | Married | University graduate | Homosexual | No | 133 | 203337 |
| 4 | Male | 43 | Married | Illiterate | IVDU | Yes | 644 | 1211 |
| 5 | Male | 39 | Married | Illiterate | Homosexual | No | 24 | 319849 |
| 6 | Male | 38 | Married | Illiterate | IVDU | No | 89 | 186927 |

5. Discussion

The present study assessed concurrent KSHV infection in HIV-infected subjects, in addition to risk factors for KSHV acquisition among HIV-infected subjects in Tehran. PBMC and plasma samples from HIV-infected patients were an-

alyzed for molecular evaluation of KSHV. The results revealed that the overall prevalence of KSHV infection among HIV-positive patients was 5.5% (6/109). The findings suggest that the rate of infection among HIV-infected patients in Tehran, regardless of the presence of Kaposi's sarcoma (KS), is relatively high. Furthermore, as mentioned in pre-

vious reports, HIV/AIDS remains a public health crisis in Iran (23, 24), and KS is a common, life-threatening disease among HIV-infected patients.

We also analyzed risk factors for HIV acquisition, and found strong associations with intravenous drug users, having been in prison, low educational level, high-risk sexual behavior, and increased risk of transmission from infected mothers to children.

In the study, most of patients were introduced as infected patients with hepatitis C virus. In addition, HIV infected patients are specific populations for HCV as same as KSHV (25). Therefore, HCV patient's finding is very important issue in the HIV settings (26). These are the first data published on HIV-infected patients without Kaposi's sarcoma and KSHV co-infection molecular epidemiology in Tehran. However, previous limited seroepidemiological studies have been conducted among Iranian HIV-positive patients; one reported 45.7% KSHV seroprevalence among HIV-positive patients from Tehran, determined by IFT (27), whereas another showed that the frequency of KSHV antibodies among HIV-positive patients in Isfahan was only 18.2%, tested by ELISA (28). According to previous studies, among healthy global populations, KSHV infection is less prevalent in northern Europe, North America, and in most parts of Asia, but is more prevalent in most parts of South America, Mediterranean regions, and certainly in Sub-Saharan Africa (29). However, it is obvious that the epidemiological patterns in most parts of the world vary between HIV-infected populations. For example, in Turkey, which neighbors Iran, the prevalence of KSHV DNA in HIV-infected patients without KS was only 3.5% (30). Similar studies in Hungary and northern Germany reported prevalence of 6.1% and 7.8%, respectively (21, 31).

In geographic areas where KSHV is comparatively common, KSHV prevalence displays the same pattern among IVDUs and the general populations; nevertheless, it seems that most KSHV-positive patients in the present study are injecting drug users; furthermore, as expected, syringe sharing, in addition to increasing the risk of hepatitis B and C, is also a risk factor for KSHV infection (32). As in previous reports, most HIV-positive subjects were intravenous drug users, therefore monitoring of the patients is very important for prevention of related disease (33-35).

This study showed that the incidence of infection can also occur in other ways in addition to injection in Tehran: KSHV might occur occasionally among individuals with multiple sexual partners, particularly among those who had sex with homosexual or bisexual men. It should be mentioned that mother-to-child transmission during child delivery or through breastfeeding is very unlikely to occur; nevertheless, further investigation is required on this issue (36-38).

PCR-based analysis of PBMC and plasma as two major blood compartments showed latent and lytic infections in this patient group, respectively. Four patients out of six (67%) were positive for KSHV DNA in plasma. Detection of DNA in plasma would be expected, mainly during viremia phases.

KSHV is an uncommon infection in healthy populations, but has been seen in immunocompromised groups, particularly in HIV-infected patients. In addition, according to a study by Zong, KSHV is a highly host-adapted virus, and infectivity of the virus only occurs under conditions of immunosuppression (39). Therefore, the patients most affected are those with suppressed immune system suppressed, e.g., HIV-positive patients, especially those with low CD4 count and who are not under treatment with HAART. The main treatment for this infection is the use of effective anti-retroviral therapy; therefore, Kaposi's sarcoma is comparatively rare in countries that have universal access to HAART. The results of this study demonstrate a significant difference for HAART and KSHV infectivity (P value = 0.042). Hence, it should be pointed out that HIV-positive patients in Iran have convenient access to HAART, which is perhaps a major reason why Kaposi's sarcoma is the rarest malignancy among HIV-infected subjects in Iran.

In comparison with the similar investigations in Iran with some weaknesses including exclusively antibodies evaluations, failure to molecular analysis and assessments of mainly risk factors in HIV-positive patients (25, 26, 40), the current study was evaluated a multi risk factors for KSHV infection in HIV-positive patients and evaluated molecular epidemiology in paired PBMC and plasma specimens of HIV-positive subjects and consequently investigation of KSHV latent and lytic infections in the subjects group provides valuable data on the epidemiology of KSHV infection in Iran. However, this study has some limitations, including its cross-sectional design, and the narrow sample size selected for this investigation, in addition to lack of representation of the general population. It is necessary to consider that access to HIV-positive patients in Iran is simply not possible. Additionally, it should be mentioned that the one of the main reasons for the statistical insignificance of most positive and negative results between the HIV-infected patients group was the small number of available patients.

5.1. Conclusion

The association of KSHV with addiction and unsafe sexual behavior, found in our study, confirms that this virus may be transmitted through multiple routes. The main factors for the pathogenesis of the virus, particularly progress toward Kaposi's sarcoma in patients infected

with HIV, are low CD4 count and lack of HAART. Accumulating evidence suggests that HAART plays a significant role in protecting against KSHV-related malignancies; therefore, KSHV infection might have been resolved during successful HIV therapy. In Iran, the diagnosis of KSHV is not a routine procedure. According to data from the study, the molecular prevalence of KSHV among HIV-infected patients in Tehran is relatively high, and therefore the screening of HIV cohorts for KSHV may be beneficial. Further research will be necessary to define epidemiology, risk factors, and related malignancies associated with KSHV infection in different high-risk groups in Iran.

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Footnotes

Authors' Contribution: Study concept, design, and supervision: Maryam Esghaei; administrative, technical, and material support: Maryam Esghaei, Farah Bokharaei-Salim, Seyed Hamidreza Monavari, and Hossein Keyvani; data acquisition and statistical analysis: Farah Bokharaei-Salim, Khashayar Hesamizadeh; interpretation of data: Farah Bokharaei-Salim, Khashayar Hesamizadeh, Hossein Keyvani, Seyed Hamidreza Monavari, and Maryam Esghaei; drafting of the manuscript: Hesamizadeh; critical revision of the manuscript: Farah Bokharaei-Salim and Maryam Esghaei.

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