

# Detection and quantification of Kaposi's sarcoma-associated herpesvirus to predict AIDS-associated Kaposi's sarcoma

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**Objective:** To identify immunologic and virologic predictors of AIDS-associated Kaposi's sarcoma (KS).

**Design:** Nested case-control analysis of KS risk in a cohort of 132 HIV-infected homosexual men in New York and Washington, DC, USA.

**Methods:** For each KS case, we selected two HIV-infected controls, matched for CD4 cell count and Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8; KSHV) serostatus (enzyme immunoassay for antibody to KSHV protein K8.1). Cell-associated KSHV and Epstein-Barr virus (EBV) viral loads were measured with quantitative real-time PCR assays on samples collected 1 year (median) before KS diagnosis.

**Results:** Thirty-one men developed AIDS-associated KS (incidence 3.1 per 100 person years). Among HIV-infected men, KS incidence was higher among those with K8.1 seropositivity (5.0 versus 1.4 per 100 person years;  $P = 0.004$ ), low CD4 cell count [hazard ratio (HR), 1.49; 95% confidence interval (CI), 1.24–1.79 per  $100 \times 10^6$  cells/l decline), or high HIV RNA level (HR, 3.96; 95% CI, 2.19–7.16 per  $\log_{10}$ ). In the case-control analysis, nine of 70 evaluated subjects had KSHV viremia, generally low level (median viral load 180 copies per  $1 \times 10^6$  cells). KSHV viremia was associated with increased KS risk (unadjusted odds ratio, 9.1; 95% CI, 1.7–48; odds ratio, 11.7; 95% CI, 1.8–76 after adjustment for K8.1 serostatus, CD4 cell count, and HIV RNA). Among K8.1-seropositive subjects, KS incidence was tenfold higher in those with KSHV viremia (30.3 per 100 person years versus 3.4 per 100 person years in those without viremia). Also, EBV viral loads were higher in cases than in controls ( $P = 0.07$ ).

**Conclusions:** Among individuals with HIV-KSHV coinfection, KSHV viremia identifies a subgroup with extremely high risk for developing KS.

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## Introduction

Persons with AIDS have a risk for Kaposi's sarcoma (KS) that is 100 000-fold higher than in the general population [1]. This extraordinarily increased risk is multifactorial. HIV-infected individuals, especially homosexual men, are frequently co-infected with KS-associated herpesvirus (KSHV, also known as human herpesvirus 8) [2,3], the viral cause of KS [4]. In HIV infection, KSHV coinfection, as identified either by antibodies or viremia, is associated with an elevated KS risk [2,5–9]. Additionally, measures of HIV-related immune suppression (e.g., low CD4 cell count, high circulating HIV RNA) or immune activation (e.g., high circulating neopterin or  $\beta_2$ -microglobulin) strongly predict KS [6,7,10].

Much remains unclear regarding the interplay of immunity and KSHV in KS pathogenesis. KSHV viremia is frequent in persons with AIDS-associated KS [5], suggesting that poor immune-mediated control of latent KSHV infection plays a role. To date, though, no study has measured the additional risk for KS associated with KSHV viremia, among HIV–KSHV coinfecting persons. Of related interest, persons with AIDS often exhibit high levels of circulating Epstein–Barr virus (EBV) [5,11], suggesting reactivation of latent infection with this prototype human  $\gamma$ -herpesvirus. However, it is unknown whether persons with AIDS-associated KS have especially high levels of EBV.

In the present case–control study of HIV-infected homosexual men, we examined multiple markers of KSHV and EBV infections as predictors of AIDS-associated KS. A strength of our study was the use of specimens collected before development of KS. In particular, we sought to identify whether cell-associated KSHV viremia predicts KS among HIV–KSHV coinfecting individuals, and to quantify that risk.

## Methods

### Subjects and study design

We studied subjects in the District of Columbia Gay (DCG) cohort study, which recruited homosexual men from primary care practices in Washington, DC and New York City in 1982 [12]. At subsequent yearly visits, clinical events were recorded, and biological specimens were obtained and shipped overnight for laboratory processing. KS, which occurred only in HIV-infected men, was identified by physical examination, reports by study participants on questionnaires, review of death certificates, and communications from

clinicians, and was pathologically confirmed when possible.

We conducted a nested case–control analysis to examine the association between laboratory markers and KS risk. For each KS case, eligible controls had not developed KS as of the calendar date (index date) when the case developed KS, were HIV-infected, and had the same KSHV antibody status as the case, as measured by K8.1 enzyme immunoassay (EIA; see below) on the most recent measurement before the index date. From this eligible pool, for each case we selected two controls whose CD4 cell counts were closest to the case's CD4 cell count.

### Laboratory assays

Serum or plasma samples (stored at  $-70^{\circ}\text{C}$ ) were tested for HIV RNA [13], using the HIV Monitor assay (Roche, Branchburg, New Jersey; detection limit, 200 copies/ml). CD4 lymphocyte counts were assayed using standard flow cytometry methods.

We previously performed serial KSHV antibody testing using an indirect immunofluorescence assay (IFA) for the KSHV protein latent nuclear antigen (encoded by orf73) [2]. In the present study, additional testing was performed using EIA for antibodies to recombinant K8.1 (a KSHV structural glycoprotein) and orf73. The K8.1 EIA was described previously [14]. The orf73 EIA was performed using a similar protocol (available on request) targeting a full-length baculovirus-expressed orf73 protein. For cases and controls, we measured fourfold IFA and EIA antibody titers on samples as closely preceding the index date as possible.

We measured cell-associated KSHV and EBV viral loads in cryopreserved peripheral blood mononuclear cell (PBMC) samples preceding the index date. DNA was extracted using the QIAmp DNA blood kit (Qiagen, Valencia, California, USA). DNA (150–200 ng per reaction) was used in quantitative TaqMan PCR assays (ABI Prism 7700, P.E. Biosystems, Foster City, California, USA) for KSHV (K6 primers [15]) and EBV (pol primers [16]). To standardize results to the number of circulating cells (i.e., viral load, defined as viral copies/ $1 \times 10^6$  PBMC), we used a quantitative PCR for ERV3, an endogenous human retrovirus present at two copies per cell [17].

### Statistical analyses

We used proportional hazards regression to compare KS incidence across subgroups of subjects. In the case–control analysis, we used logistic regression to identify KS risk factors. Specifically, we treated KS status as the dependent variable and tested for significant effects of

independent variables (i.e., laboratory markers) after controlling for the matching factors (K8.1 serostatus and CD4 cell count). In this analysis, we used log-transformed values of HIV RNA, KSHV antibody titers, and EBV copies and viral load.

## Results

### Baseline characteristics of study subjects and KS risk

The DCG cohort study included 132 men with HIV infection, either at the start of the follow-up (82 subjects) or as seroconverters during follow-up (50 subjects). On the first date known to be HIV-infected (i.e., baseline), subjects had median values for CD4 cell count and HIV RNA of  $472 \times 10^6$  cells/l [interquartile range (IQR),  $335\text{--}667 \times 10^6$  cells/l] and 4.35 log<sub>10</sub> copies/ml (IQR, 3.86–4.75 log<sub>10</sub> copies/ml), respectively. At baseline, 46 of 100 (46%) evaluated subjects were KSHV seropositive, as measured by K8.1.

We identified 31 KS cases arising during 994 person years when these subjects were known to be HIV infected (KS incidence, 3.1 per 100 person years). Among HIV-infected subjects, KS incidence was higher among K8.1-seropositive than K8.1-seronegative subjects, based on their most recent serologic results (5.0 versus 1.4 per 100 person years;  $P = 0.004$ ). KS incidence was also elevated in subjects with low CD4 cell counts [hazard ratio (HR), 1.49; 95% confidence interval (CI), 1.24–1.79 per  $100 \times 10^6$  cells/l decline]

or high HIV viral loads (HR, 3.96; 95% CI, 2.19–7.16 per log<sub>10</sub>).

### Case–control analysis

Two KS cases were excluded because samples were unavailable for K8.1 testing. The remaining 29 cases were matched by K8.1-serostatus and CD4 cell count to 57 controls (Table 1). Cases and controls were well matched for time between blood sampling and index date (Table 1).

As shown, after matching, cases and controls did not differ on demographic factors. HIV RNA levels were higher in cases ( $P = 0.02$ ). Although cases and controls were matched on K8.1 serostatus, cases still tended to have higher KSHV antibody titers than controls, as determined by IFA ( $P = 0.08$ ) and orf73 EIA ( $P = 0.09$ , Table 1).

Detectable cell-associated KSHV viremia was strongly associated with KS (unadjusted odds ratio, 9.1; 95% CI, 1.7–48, based on seven cases and two controls with viremia). This association was strengthened after adjustment for matching factors (K8.1 serostatus, CD4 cell count) and HIV RNA (adjusted odds ratio, 11.7; 95% CI, 1.8–76). Levels of circulating KSHV were generally low (median 5 copies detected, median viral load 180 copies/ $1 \times 10^6$  cells). With only nine KSHV-viremic subjects, we could not test whether KSHV viral loads differed between cases and controls.

All KSHV-viremic subjects were K8.1-seropositive. We calculated KS incidence in K8.1-seropositive sub-

**Table 1. Case–control analysis of risk factors for Kaposi's sarcoma.** Values shown are number of patients with trait as a percentage of the total number tested for categorical traits and median (interquartile range) for continuous measures, except for KSHV copy number and viral load, for which individual values are listed. Subjects with missing values are excluded. Results with missing data (number missing) were: IFA (11), K8.1 titer (12), IFA titer (13), orf73 titer (24), HIV RNA (7), KSHV copy (16), and EBV copy (17).

Characteristic	Cases (n = 29)	Controls (n = 57)	<i>P</i> <sup>a</sup>
K8.1 EIA seropositive [n with trait/total (%)]	22/29 (76)	43/57 (75)	Matching factor
CD4 cell count ( $\times 10^6$ /l) [median (IQR)]	357 (241–459)	396 (264–554)	Matching factor
Days from serum draw to index <sup>b</sup> [median (IQR)]	328 (224–540)	374 (259–581)	Matching factor
Days from PBMC draw to index <sup>b</sup> [median (IQR)]	320 (230–451)	338 (232–465)	Matching factor
HIV RNA, log <sub>10</sub> copies/ml [median (IQR)]	4.81 (4.59–5.26)	4.38 (3.75–5.09)	0.02
K8.1 EIA titer [median (IQR)]	290 (140–1600)	250 (80–600)	0.29
IFA seropositive	16/25 (64)	29/50 (58)	0.78
IFA titer [median (IQR)]	4800 (800–11 200)	1600 (400–4800)	0.08
Orf73 EIA seropositive	22/29 (76)	41/57 (72)	0.55
Orf73 EIA titer [median (IQR)]	33 000 (4500–66 000)	10 000 (4700–34 000)	0.09
ERV3 copy number [median (IQR)]	54 000 (44 000–73 250)	52 500 (43 250–68 750)	0.97
KSHV DNA positive	7/24 (29)	2/46 (4)	0.01
KSHV copy number	1, 3, 4, 5, 8, 13, 14	1, 270	–
KSHV viral load (copies/ $1 \times 10^6$ cells)	24, 140, 170, 180, 320, 340, 550	27, 17 000	–
EBV DNA positive	20/23 (87)	35/46 (76)	0.40
EBV copy number [median (IQR)]	230 (117–425)	85 (33–310)	0.04
EBV load (copies/ $1 \times 10^6$ cells) [median (IQR)]	8700 (4000–16 000)	3700 (1100–11 000)	0.07

<sup>a</sup>*P* values were calculated using logistic regression, adjusting for K8.1 serostatus and CD4 cell count. No *P* value was calculated for Kaposi's sarcoma-associated herpesvirus (KSHV) copy number or viral load, given the small number of viremic subjects. <sup>b</sup>Index date was the date of Kaposi's sarcoma diagnosis (cases) or the corresponding date for the matched controls. EIA, enzyme immunoassay; PBMC, peripheral blood mononuclear cells; IQR, interquartile range; IFA, immunofluorescence assay; EBV, Epstein–Barr virus.

jects, stratified by the presence or absence of KSHV viremia. Overall in the DCG cohort, there were 22 KS cases arising in HIV-infected K8.1-seropositive men (total follow-up 442 person years). In the case-control analysis, KSHV viremia was present in seven of 19 evaluated K8.1-seropositive cases and two of 33 evaluated K8.1-seropositive controls. Assuming that these cases and controls were representative of other K8.1-seropositive DCG subjects, we calculate that KS incidence was  $[22 \times (7/19)] / [442 \times (2/33)] = 30.3$  per 100 person years among K8.1-seropositive subjects with viremia and  $[22 \times (12/19)] / [442 \times (31/33)] = 3.4$  per 100 person years among K8.1-seropositive subjects without viremia. By comparison, among K8.1-seropositive subjects, KS incidence was 13.2 per 100 person years in those with CD4 cell counts  $< 250 \times 10^6$  cells/l and 12.5 per 100 person years in those with HIV RNA  $\geq 5.00 \log_{10}$  copies/ml.

Cell-associated EBV viremia was common and occurred similarly in cases and controls (Table 1). Interestingly, EBV levels were higher in cases than in controls (median copies 230 versus 85;  $P = 0.04$ ; median viral load 8700 versus 3700 copies/ $1 \times 10^6$  cells;  $P = 0.07$ ).

## Discussion

We analyzed risk factors for AIDS-associated KS and found, as expected, strong associations with CD4 cell count, HIV RNA, and KSHV infection, based on antibody testing. An important aspect of our study was the evaluation of KSHV-seropositive subjects for cell-associated KSHV viremia. This allowed us to measure the very large increase in KS risk among HIV-KSHV coinfecting individuals that was independently associated with viremia (adjusted odds ratio, 11.7; 95% CI, 1.8–76). This result is similar to that recently reported by Cannon *et al.* from a cross-sectional study [18], wherein KS patients were more likely than other HIV-KSHV coinfecting individuals to have detectable KSHV viremia (odds ratio 8.1 after adjustment for CD4 cell count, 7.9 after adjustment for HIV RNA).

Importantly, because we assessed KSHV viremia, on average, 1 year before KS onset, our study demonstrates that viremia can be a relatively early manifestation of the pathophysiologic events leading up to KS. Among individuals at risk for KS, the presence of detectable KSHV in PBMC (primarily CD19-positive B lymphocytes [19]) probably reflects an expansion of the pool of latently infected B lymphocytes, which could seed KSHV infection to cells capable of developing into KS. Alternatively, KSHV viremia may be a marker for impaired KSHV-specific immunity, which may allow progression to overt KS in precursor cells

already infected with KSHV [20]. Among K8.1-seropositive individuals, KSHV viremia identified an extremely high KS risk (incidence 30.3 per 100 person years), suggesting that this group might be optimal candidates for investigation of the safety and efficacy of prophylactic ganciclovir [21] or other drugs. Among persons who already have KS, KSHV viremia may also be a marker for risk of tumor progression [18,22].

We were intrigued to find that levels of circulating EBV, the other human  $\gamma$ -herpesvirus, were elevated in persons at risk for KS. EBV infection is ubiquitous, but EBV viremia is relatively uncommon in healthy individuals [5]. As for KSHV, high circulating levels of EBV may be caused by specific immune deficits or, conceivably, by a broader deficit in control of  $\gamma$ -herpesviruses. Interestingly, individuals with AIDS-associated KS have an increased risk for immunoblastic lymphoma, compared to others with AIDS [23]. EBV is found frequently in malignant cells of this lymphoma type [24]. We speculate that poor immune-mediated control of EBV in persons with KS might partly explain their heightened risk for immunoblastic lymphoma.

Our study has several limitations. First, with only 31 KS cases, our statistical power was modest. Second, not all KS cases were histologically confirmed. Although these considerations might have lessened our ability to find associations with KS, they would not have jeopardized the validity of associations (e.g., with KSHV viremia) that we did detect. Additionally, overnight delays in processing samples obtained from study subjects may have decreased our sensitivity in detecting KSHV viremia or reduced the measured viral load. Given small numbers, we could not evaluate factors associated with KSHV viremia.

In summary, among HIV-KSHV-infected individuals, cell-associated KSHV viremia was strongly associated with the subsequent development of KS. The presence of KSHV viremia identifies a population at highest risk of this disease. Our study provides evidence that AIDS-associated KS arises in the setting of poor immune-mediated control of this  $\gamma$ -herpesvirus.

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