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Kaposi's sarcoma herpesvirus load in biopsies of cutaneous and oral Kaposi's sarcoma lesions

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ABSTRACT

Objectives: To evaluate human herpesvirus 8/Kaposi's sarcoma associated herpesvirus (HHV-8/KSHV) viral load in diagnostic, (formalin fixed, paraffinised) biopsies and patient serum during tumour progression of oral and cutaneous AIDS-related Kaposi's sarcoma (AKS), and endemic Kaposi's sarcoma (EKS) by a sensitive and specific quantitative real time polymerase chain reaction (qRT-PCR) assay.

Study design: Eighty six biopsies of both AKS (oral and cutaneous AKS, 68) and EKS (cutaneous EKS, 18) were evaluated by qRT-PCR and immunohistochemistry (IHC). The viral load in human tumour tissue and serum of some individual patients were compared.

Results: Higher viral load as well as frequency of latency-associated nuclear antigen (LANA)+ tumour spindle cells (SC) and number of LANA granules per SC was found in oral AKS compared to cutaneous AKS. Although few cases were available, serum viral load appeared to decrease compared to tumour tissue during KS progression.

Conclusions: The higher viral load in oral rather than cutaneous AKS is consistent with the well recognised reservoir function of the oral mucosa. Decrease of serum HHV-8 load during KS progression may indicate decreased virus release and/or increased virus clearance.

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1. Introduction

Kaposi's sarcoma associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8) is a member of Rhadinovirus genus, gamma-2 herpesvirus known to infect humans and recognised as an etiologic and diagnostic agent for Kaposi's sarcoma[1] and certain forms of malignant lymphoma such as primary effusion lymphoma (PEL) or body cavity-based lymphoma (BCBL) and Multicentric Castleman's Disease (MCD).[2] The KS tumour spindle cells (SC) are mostly infected in a latent form but in MCD and PEL more cells also express lytic infection.[3] Kaposi's sarcoma starts as an inflammatory angioproliferative,

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tumour-like lesion primarily in the skin, but also in lymph nodes, viscera and oral cavity.[4,5] Histologically, all KS forms develop from early patch and plaque to late nodular lesions by an increase in tumour SC.[6] Although indistinguishable by histology, KS occurs in four different clinical forms, namely: endemic KS (EKS) in adults and children of tropical African countries, classic KS predominant in elderly Mediterranean men, Iatrogenic KS (IKS) seen mostly in transplant patients receiving immunosuppressive treatment, and AIDS-associated KS (AKS), which is the most aggressive and common tumour in AIDS patients globally.[1] Oral KS, previously rare, has become rather more common in association with HIV infection and is included in the staging of HIV (human immunodeficiency virus) disease progression.[7] The mechanisms of primary oral mucosal HHV-8 infection and KS development remain elusive.

The HHV-8 latency-associated nuclear antigen (LANA) is expressed in SC of all types of KS and not found in other vascular tumours, and is, therefore, a highly sensitive, pathognomonic marker for Kaposi sarcoma.[8,9] The demonstration by polymerase chain reaction (PCR) of HHV-8 DNA both in tumour lesions and blood cells, serum and saliva of KS patients indicates a systemic viral infection during KS oncogenesis. [10–12] PCR for the HHV-8 ORF 26 capsid (KS233) expressed in tumour SC of all clinical KS types and stages appears to be a sensitive and reproducible assay of infection[13,14] but few reports are documented on quantitative aspects of HHV-8 DNA load in KS tumour biopsies from different anatomical sites.[15,16] In this study we have, by real time PCR (qRT-PCR), quantified and compared the HHV-8 copy number in oral and cutaneous AKS and EKS during progression from early (patch/plaque) to late (nodular) stages of the tumour development.

2. Materials and methods

2.1. Biopsies and serum

Eighty six formalin fixed, paraffin embedded diagnostic biopsies (Department of Pathology, MUCHS, Dar Es Salaam, Tanzania) of cutaneous KS (60), representing patch/plaque AKS (13), nodular AKS (29), patch/plaque EKS (three), nodular EKS (15), and of oral KS (26), representing patch/plaque AKS (six) and oral nodular AKS (20), were included in the study (Table 1).

In a separate set of samples, serum from six KS patients and the corresponding tumour biopsies (three patch/plaque and three nodular AKS) were also studied. The sera were stored at −70 °C until processed for quantitative PCR (Immunopathology Lab and Department of Virology, SMI, Karolinska Institute, Sweden).

2.2. DNA extraction

DNA extraction was performed by a Chelex method.[17] Basically, three (10 μm) sections from each biopsy were deparaffinised in 100 μl 0.5% Tween 20, heated to 90 °C for 10 min, cooled to 55 °C, separated from the wax containing solution and digested in 2 μl of proteinase K (10 mg/ml) for 3 h at 55 °C with gentle agitation. Digested samples were heated to 99 °C for 10 min and 100 μl 5% Chelex-100 (Bio-Rad, Stockholm) in Tris-EDTA was added, gently shaken, spun at 10,500g for 15 min, cooled on ice and the remaining wax in supernatant was removed. The samples were heated to 45 °C, mixed with 100 μl chloroform, centrifuged for 15 min at 10,500g and the top phase with DNA was collected. The DNA concentration was measured by spectrophotometry and stored at −20 °C until use without further washing. 1 μgr DNA was used for each PCR reaction.

2.3. Total tissue DNA

For evaluation of total tissue DNA, RNase P (Applied Biosystems, USA), which is a single copy gene for the RNase P enzyme, was used to evaluate the number of cells corresponding to extracted DNA and the efficiency of real time PCR as previously described.[18,19]

Four ten-fold dilutions (1×10^1–10^4 copies/ml) of genomic human DNA (Applied Biosystem, USA, 1 ng = 333 RNase P genes) were included in the standard curve of each plate. HHV-8 and corresponding RNase P PCR measurements were performed in the same plate to minimise experimental errors.

2.4. HHV-8 plasmid

A standard DNA with a determined number of HHV-8 virus copies was prepared from DNA of HHV-8 positive BCBL cells

Table 1 – Comparison between HHV-8 load by qRT-PCR and frequency of immunostained LANA+ SC and number of LANA+ granules/SC nucleus in oral AKS (OAKS) and cutaneous (CAKS, CEKS)

Stages of KS	Tumour area/Section(%)	HHV-8 load[a]	Frequency (%) LANA+ SC[b]	LANA+ granules/SC nucleus
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Patch/Plaque CAKS (n = 13)	35 ± 2	100 ± 15	25 ± 1	2 ± 2
Nodular CAKS (n = 29)	80 ± 3	270 ± 25	56 ± 2	7 ± 1
Patch/Plaque CEKS (n = 3)	27 ± 0.3	90 ± 5	18 ± 0.3	2 ± 1
Nodular CEKS (n = 15)	70 ± 4	185 ± 20	55 ± 5	6 ± 2
Patch/Plaque OAKS (n = 6)	40 ± 2	125 ± 9	20 ± 0.5	2
Nodular OAKS (n = 20)	80 ± 2	400 ± 22	70 ± 2	12 ± 2

a Copy number/μl DNA sample determined by qRT-PCR.
b CD34+ cells.

by PCR amplification using primer KS330 for the HHV-8-ORF26, 233 base pairs (bp) amplicon and extraction from agarose gel with a QIAquick Gel Extraction Kit© (Qiagen® Helden, Germany). The extracted DNA was ligated into a pGEM-T Easy Vector©, transformed into competent *E. coli* cells (JM 109©, Promega® Madison, USA) and cultivated on LB-Ampicillin agar plates (100 μg/ml) which had been pretreated with IPTG and X-Gal. Five plasmid positive colonies were selected and re-cultivated. Plasmids were extracted with QIAprep Spin Miniprep kit© (Qiagen ®), and sequenced in an ABI PRISM 310© (Applied Biosystem®) with Sp6 and T7 as primers using Big Dye© reagents,[20] to confirm the sequence of the 233 bp amplicon. The amplicon was compared to sequences available at Gene Bank, matching HHV-8 (Accession No: U18551) and the final plasmid concentration was measured by optical density and the number of plasmid copies per litre was calculated with the formula:

(Plasmid concentration) g/L × (6.02×10^{23}) /Plasmid + Insert (bp) × 660.

Eight ten-fold dilutions (1×10^1–10^8 copies/well) of plasmids were included in each real-time PCR plate.

2.5. Quantitative real time polymerase chain reaction (qRT-PCR)

Extracted DNA was analysed by qRT-PCR as previously described.[21] Briefly, 25 μl PCR reaction mixture was prepared using 2.5 μl of 10× PCR buffer, 4 μl of 25 mM $MgCl_2$, 1.5 μl dNTPs (10 mM), 0.5 μl of HHV-8 primer (10 pmol) KS330 Forward-AGCCGAAAGGATTCCACCAT and Reverse-TCCGTGTTGTCTACGTCCAG[21,22] for the ORF26, 233-bp (capsid antigen) long fragment of HHV-8 (CyberGene, Stockholm, Sweden). An oligonucleotide probe with the reporter dye (FAM) was attached to the 5′ end and a rhodamine dye (TAMRA) quencher linked to the 3′ end (CyberGene, Stockholm, Sweden) and 0.2 μl of Taq gold DNA polymerase (2.5 U; Roche, Stockholm, Sweden) were used. The PCR solution (25 μl) was made to contain 10 μl of the extracted DNA sample. The qRT-PCR was performed as previously described and the DNA load was determined as the mean of triplicate sample values.[23,24]

A positive control of HHV-8 DNA extracted from fixed and paraffin embedded HHV-8 infected BCBL-1 cells and a template negative control (PCR mix only) were also included in each PCR run.[25] The standard curves were plotted by an automatic ABI detector system printing the Ct values against each known concentration of standards (HHV-8 plasmid or RNase P gene) and HHV-8 copy number/μl DNA sample was calculated by detector.

2.6. Normalisation of the results

The HHV-8 load differences between the KS cases were normalised for the same amount of RNase P as calculated from measured results of the machine (HHV-8 and RNase P were measured in the same reaction) since the equal amounts of DNA from biopsies of comparable stages of disease and area of tumour mass were used.

2.7. Immunohistochemical staining (IHC)

Immunostaining was performed as previously described.[26] Briefly, sections (4 μm) were deparaffinised and rehydrated and microwave heated (6 min in 0.1 M citrate buffer, pH 6.0) for antigen retrieval, cooled to room temperature (RT, 30 min) in tris buffer saline (TBS). After rinsing in TBS, the sections were blocked for non-specific antibody binding at room temperature with normal goat serum (1:20) for 30 min (DAKO, Glostrup, Denmark) (room temperature), followed by incubation with mouse antibody to CD34 (NOVOCASTRA, Newcastle, UK) 1:20 for 1 h at 37 °C or overnight at 4 °C. After rinsing with TBS, the sections were incubated with goat anti-mouse fluorescein isothiocyanate (FITC) 1:20 for 40 min at room temperature (Calbiochem, San Diego, CA, USA) and then processed on in the dark by washing with TBS and incubation with rat antibody to LANA (ABI, Colombia, MD, USA) 1: 200 at 37 °C for 2 h. After washings (TBS), sections were incubated with CY3-labelled goat anti-rat (Jackson, West Grove, PA, USA) 1:50 for 40 min in room temperature, washed (TBS) and counter-stained with 5 mM of 40-6-Diamidino-2-phenylindole-Hcl (DAPI) (Sigma-Aldrich, St Louis, MO, USA) in 500 mM of trisodium citrate and mounted in Vector shield (Vector, Burlingame, CA, USA) for evaluating with the help of fluorescence microscopy. All antibodies were diluted in TBS containing 1% bovine serum albumin (BSA) and 0.05% sodium azide. Formalin fixed and paraffinised BCBL-1 sections were used as positive LANA controls and TBS instead of primary antibody as nonspecific staining control which was also evaluated on tumour adjacent normal tissue.

2.8. Microscopic evaluation

A fluorescence microscope (Olympus BX60, Tokyo, Japan) equipped with a digital camera (Sony DKC-5000, Tokyo, Japan) and appropriate filters were used to document bright field as well as specific Cy3, and DAPI fluorescence. Images were edited and overlaid using Adobe Photoshop 6.0. Cells were evaluated on colour micrographs in eight adjacent non overlapping fields (256 × 190 μm each) corresponding to at least 800 cells for each specimen and the frequency of positive cells for LANA was calculated. The number of LANA granules per nucleus of SC was calculated in at least 400 nuclei for each biopsy.

2.9. Statistical analysis

Due to the limited number of biopsies in some group's, non-parametric tests or *t*-test were used for statistical analysis to determine the probability of differences between the study groups.

3. Results

3.1. HHV-8 load in cutaneous AKS/EKS and oral AKS

A significant higher viral load ($P < 0.005$) was found in nodular AKS (mean ± sem = 270 ± 25) than in patch/plaque AKS (mean ± sem = 100 ± 15) biopsies as well as in nodular EKS

Table 2 – P values for the parameters in Table 1

Stages of KS	HHV-8 load[a]	Frequency (%) LANA+SC	LANA+ granules/SC nucleus
Patch/Plaque versus Nodular OAKS	$P < 0.005$	$P < 0.005$	$P < 0.005$
Patch/Plaque versus Nodular CAKS	$P < 0.005$	$P < 0.005$	$P < 0.005$
Patch/Plaque versus Nodular CEKS	$P < 0.005$	$P < 0.005$	$P < 0.005$
Patch/PlaqueCAKS versus Patch/Plaque CEKS	$P < 0.2$	$P < 0.5$	$P < 0.1$
Nodular CAKS versus Nodular CEKS	$P < 0.1$	$P < 0.5$	$P < 0.5$
Nodular OAKS versus Nodular CAKS	$P < 0.04$	$P < 0.005$	$P < 0.001$

a Copy number/μl DNA sample determined by qRT-PCR.

Table 3 – Comparison of HHV-8 load in individual biopsies[a] and sera (copy/ml) of patch/plaque and nodular AKS stage

Cases	Diagnosis	qRT-PCR HHV-8 load[b] in biopsy Mean ± SEM	qRT-PCR HHV-8 load[c] in serum Mean
1	Patch/PlaqueAKS	100 ± 10	5400
2	Patch/PlaqueAKS	97 ± 15	4600
3	Patch/PlaqueAKS	85 ± 9	3400
4	Nodular AKS	450 ± 5	240
5	Nodular AKS	200 ± 22	Undetectable
6	Nodular AKS	230 ± 15	Undetectable

a Tissue origin not known, not the same cases as in Table 1.
b Copy number/μl DNA sample.
c Copy number/ml serum.

(mean ± sem = 185 ± 20) and patch/plaque EKS (mean ± sem = 90 ± 5), $P < 0.005$ cases (Tables 1 and 2).

Comparison of patch/plaque AKS (mean ± sem = 100 ± 15) to EKS (mean ± sem = 90 ± 5) showed approximately the same viral load, but was higher in nodular AKS (mean ± sem = 270 ± 25) than EKS (mean ± sem = 185 ± 20), although the difference was not significant (Tables 1 and 2).

In oral (OAKS, mean ± sem = 125 ± 9) and cutaneous AKS (CAKS, mean ± sem = 100 ± 15), no significant difference was seen in viral load in early stage (patch/plaque) but a significant difference was seen in late nodular lesions with a median viral load of 400 and 270 ($P < 0.04$), respectively (Tables 1 and 2).

3.2. *HHV-8 load in biopsies and serum of early and late AKS*

Comparison of biopsy (tissue origin not known) and serum in individual AKS patients showed a range of viral load in biopsy from 85 to 100 in early (patch/plaque AKS) and 200 to 450 in nodular stages (Table 3). By real time PCR the range of virus per ml serum ranged from 3400 to 5400 in patch/plaque and from 0 (undetectable) to 240 in nodular AKS cases. Thus the biopsy viral load during progression from patch to nodular AKS is increasing but appeared to decrease in the serum of these patients. The differences in viral load of patch/plaque

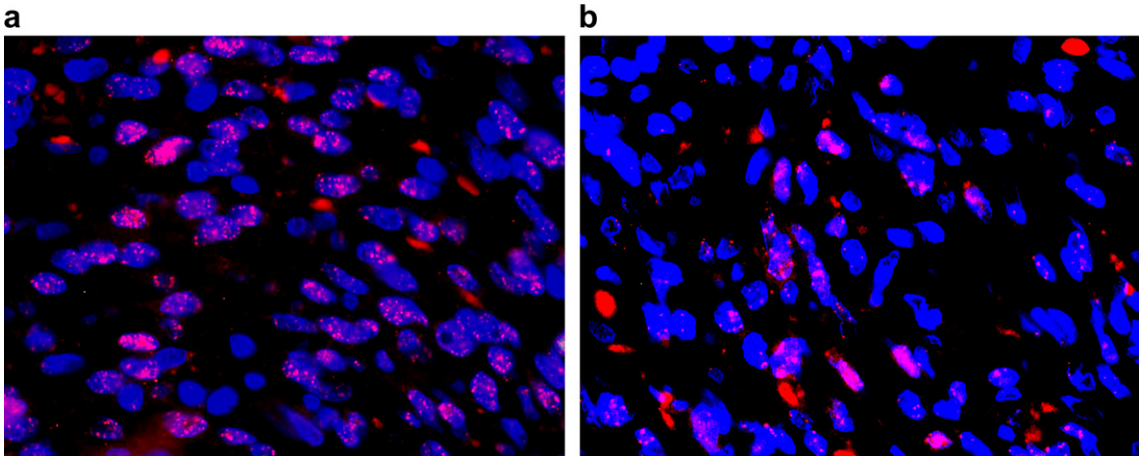

Fig. 1 – Immunofluorescence assay (IFA) micrographs from oral (a) and cutaneous (b) cases showing LANA immunoreactivity. Note that the mean (i) LANA+ cell count and (ii) LANA+ granules per SC nucleus was higher in oral (a) than in cutaneous AKS (b). The average was ten granules /LANA+ cell nucleus in oral case and five granules/LANA+ cell nucleus in cutaneous case (see Table 1).

versus nodular AKS biopsies and the corresponding serum viral loads were significant, $P < 0.005$ (Table 3).

3.3. *LANA reactivity in oral and cutaneous AKS*

Immunostaining showed a significant ($P < 0.005$) higher percentage of LANA+ cells in nodular oral (70%) as compared to cutaneous (50%) AKS (Tables 1 and 2). Likewise, the average number of LANA+ granules per SC nucleus in nodular oral (12 ± 2) was significantly ($P < 0.001$) higher than in nodular cutaneous AKS (5 ± 1) (Tables 1 and 2; Fig. 1a and b).

These results thus indicate that in the tumour, HHV-8 viral load increases concordantly with the number/frequency of LANA+ SC during KS progression and that the number of LANA+ SC and LANA+ granules per nucleus is higher in oral nodular compared to cutaneous nodular AKS. In contrast, during the AKS tumour progression the serum viral load in the patients decreased.

4. Discussion

In this study we have compared HHV-8 viral load in early and late stages of AKS and EKS tumour, in cutaneous and oral AKS and in biopsies versus serum of individual patients.

The HHV-8 load and Rnase-P (single copy gene as internal control) were measured by qRT-PCR in the same amount of DNA extracted from biopsy sections, selected by stage (patch/plaque or nodular) and for approximately the same amount of tumour mass per section. HHV-8 load was normalised for the same amount of RNase P.

The observed increase of HHV-8/KSHV load by PCR in lesions of AKS (oral/cutaneous) and EKS during progression from early (patch/plaque) to late (nodular) stages[4,27] is in accordance with our immunohistochemistry findings for AKS and EKS lesions showing an increase in the frequency of infected SC with KS tumour progression, indicating that the virus infection (latent) is not aborted during KS progression.[10,26] The qRT-PCR and immunohistochemistry results also show that HHV-8/KSHV load is not significantly different between AKS and EKS biopsies at the same stage of development which is concordant with our previous findings[10,26] and consistent with the notion of basically similar pathogenic mechanisms for these clinically distinct lesions.[6] Thus the higher clinical aggressiveness of AKS compared to EKS probably reflects effects of HIV-related factors such as immunodeficiency and angiogenic HIV-tat.

The observed higher viral load in oral AKS compared to cutaneous AKS is consistent with the previously documented importance of oral virus transmission[13] and with our findings that mucosal SC are more infected with HHV-8 and harbour more virus per cell compared to cutaneous SC. Furthermore, SC in oral AKS showed a range of 68% to 72% LANA+ indicating a rather uniform infection rate of oral tumour cells in all cases.

No comparison like the present study of viral load examined both by PCR and immunostaining in oral and cutaneous lesions has previously been documented.

The higher cell proliferation rate (Ki67+frequency) of LANA+ cells found in this study (results are not shown) and other studies by us,[28] of oral compared to cutaneous KS tissue, validates that HHV-8 can induce cell proliferation by P53 modulation.[4,13,29]

Our observations by qRT-PCR are consistent with the LANA immunohistochemistry findings of higher frequencies of HHV-8 infected cells in oral nodular compared to cutaneous AKS and with the observed rapid progression of oral AKS[5] since LANA can modulate p53, promote cell cycle progression and proliferation and decrease apoptosis by its effect on Bcl-2 and v-FLIP.[6,30,31]

The previously documented upregulation of cell proliferation promoting factors such as inflammatory chemokine/cytokines in mucosal tissues of HIV infected patients[29,32] may also contribute to the higher HHV-8 viral load and the number of LANA+ SC in oral versus cutaneous lesions observed in this study.[4]

Some previous studies report an increase in KSHV/HHV-8 DNA load of patient serum with KS clinical stage[12,24] which was not found in this and other studies.[33–35] Rather, our observations indicate a lack of HHV-8 DNA load correlation between late AKS lesions and sera in individual patients. Considering that viremia was higher in early stages than later AKS/EKS stages,[36] this seems to indicate an early virus release to serum from tissue reservoirs rather than from the developing KS lesion and possibly also suppression of HHV-8 release and/or immunosegregation of virus during later stages of KS.[11,31,37]

In conclusion, using a sensitive real-time PCR for HHV-8/KSHV, an inverted correlation of viral load in serum and KS biopsies was observed. Furthermore, significantly more virus was found in oral than cutaneous KS lesions which is consistent with the recognised high risk of HHV-8/KSHV horizontal transmission.

Conflict of interest statement

None declared.

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