Human herpesvirus 8: virology and disease

Polstra, A.

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Chapter 5

Retrospective, longitudinal analysis of serum HHV8 viral DNA load in AIDS-related Kaposi’s sarcoma patients before and after diagnosis

Abeltje M. Polstra, Marion Cornelissen, Jaap Goudsmit, and Antoinette C. van der Kuyl
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Abstract

Human herpesvirus 8 (HHV8) has been implicated in at least three diseases: Kaposi's sarcoma (KS), multicentric Castleman's disease (MCD) and primary effusion lymphoma. In MCD, HHV8 viral load in both PBMC and serum is highly correlated with disease exacerbations and treatment response. HHV8 DNA is more often detected in patients progressing towards KS than in patients who do not develop the disease, suggesting that at a certain time point, the level of viremia might be indicative of KS development. So far no clear relationship between disease and viral load has been found. Therefore, we have analysed longitudinal serum samples from 19 AIDS-KS patients, ranging from 2 years before KS till 2 years after KS diagnosis. We found no correlation between viral load and progression to KS, or disease stage, but viral load was influenced by treatment. Especially, a decline of HHV8 DNA in serum was seen after therapy with protease inhibitors.

Introduction

Human herpesvirus 8 (HHV8) is a ?-herpesvirus that is implicated in all forms of Kaposi's sarcoma (KS), in about 50% of cases of multicentric Castleman's disease (MCD) cases, and in primary effusion lymphoma (PEL) (for a review see: 1). All of these lymphoproliferative conditions are strongly associated with concomitant HIV-1 infection. MCD in HIV-1 infected patients was found to be always associated with HHV8, and was postulated to represent a distinct, plasmablastic variant of MCD 2,3.

Detection of HHV8 DNA in various tissues is variable: in KS over 95% of the skin lesions test positive 4,5, while detection of viral DNA in blood varies between 41% and 88% 6-10. In HIV-1 associated MCD, where HHV8 infected cells are predominantly B-cells 3, detection of HHV8 DNA in blood is much more frequent during active disease than in KS: 86-100% of the samples are positive 11-13, and viral load in blood is much higher in MCD than in KS 14 Also, viral DNA load in MCD corresponds with clinical symptoms and treatment response 12,13,15,16. No studies on HHV8 DNA detection in blood have been done on patients with PEL, although viral copy numbers are high in all effusion fluids 17,18.

Although HHV8 DNA cannot consistently be found in the blood of all KS patients, it is more frequently detected in KS patients, or in patients subsequently progressing to KS, than in patients without KS, or those who do not develop KS 4,7,19-22, which suggested that detection of HHV8 DNA could be of clinical importance. In transplant patients, HHV8 viremia was found to be associated with KS progression in a multivariate analysis 23. But application of HHV8 specific real-time PCR assays did not show a straightforward correlation between viral load, either in PBMC or in plasma, and progression to KS in other studies 22,24, although a trend towards increased AIDS-related KS development in persons
with higher HHV8 loads was observed. In several studies, higher viral DNA loads were found in more advanced KS disease as compared with early stage KS, both in PBMC and in affected skin, although the results in PBMC could not always be replicated, and the conclusions regarding classical KS staging and viral load were found to be statistically unconvincing. A few studies have addressed the behaviour of the HHV8 DNA load in blood after drug treatment. HHV8 DNA plasma load in longitudinal samples from HIV-1 infected KS patients treated with HAART showed both increasing and decreasing trends while treatment with the antivirals ganciclovir and foscarnet did not affect HHV8 load in PBMC. Protease inhibitors, used in anti-HIV-1 triple therapy, have been shown to be very effective against KS (for a review see), and indeed a decrease or negation of the HHV8 DNA load in PBMC was seen in clinically responding KS patients. Up till now, all studies but three have quantified cell-associated virus. Yet, viremia is probably much better represented by the amount of cell-free virus, e.g. viral DNA in serum or plasma. Two studies that have measured HHV8 DNA in plasma in KS patients found that it could only be measured when the viral load in PBMC was high. Follow-up of HHV8 DNA load in plasma has been done after KS diagnosis and during treatment, but not yet before disease development. Studies done on other herpesviruses show that plasma cytomegalovirus (CMV) load is predictive of CMV disease and survival, and that peak levels of CMV DNA in plasma occur 4 months prior to clinical diagnosis of CMV disease. In contrast, a study on Epstein-Barr virus found that EBV DNA load is always high, and absolute levels of EBV in PBMC and serum cannot be correlated and are not predictive of subsequent development of AIDS-related non-Hodgkin lymphoma.

Concluding, as HHV8 viral DNA is more often found in patients subsequently progressing towards KS, it could be speculated that at some time before KS development, a rise in HHV8 viral copies in serum or plasma could be indicative of viremia and the onset KS. Therefore, we have analyzed HHV8 viral load in longitudinal serum samples from 19 HIV-1 infected individuals 24 months before diagnosis of AIDS-KS till 24 months after diagnosis in three-months intervals, with a real-time quantitative DNA PCR, to evaluate its prognostic value and behaviour over time.

Materials and Methods

Study population. This study was performed on longitudinal serum samples obtained from 19 homosexual men included in the Amsterdam Cohort Studies on HIV/AIDS (www.amsterdamcohortstudies.org). All patients were dually infected with HIV-1 and HHV8 and developed KS. The time-points were chosen at 24, 12, 6, and 3 months prior to, at the time of diagnosis, and 3, 6, 12, and 24 months after the onset of KS. All patients were HHV8 antibody-positive in either an ORF73 and/or an ORF65 ELISA before onset of KS. All subjects in this study
Longitudinal analysis of HHV8 DNA load

were sampled before the introduction of highly active antiretroviral therapy (HAART) in 1997, with the exception of a few samples from patients H8200, H6125 and H0157 after KS diagnosis. The samples taken after the introduction of HAART were not used in the calculations.

**DNA extraction.** PhHV, a type 1 seal herpesvirus, was added to the clinical samples as a control for both the isolation method as well as a control for the presence of PCR inhibitors encountered during the isolation as described. Aliquots of 150 μl serum were isolated using silica and guanidium thiocyanate with a final elution volume of 50 μl. All samples were tested in duplicate for both HHV8 and for PhHV.

**Real-time quantitative PCR assay.** The HHV8 DNA load in the samples was determined using an ORF65 TaqMan assay described earlier. In short, each PCR contained 10 μl of isolated DNA and 40 μl of PCR mixture consisting of Platinum Quantitative PCR Supermix UDG, 3.6 mM MgCl₂, 0.9 μM forward and reverse primer, 0.2 μM Taqman probe and 1 μl ROX reference Dye (50x concentrated) (Invitrogen/Life Technologies, Carlsbad, Calif.). Following the activation of UDG (2 min, 50°C) and activation of Platinum Taq DNA polymerase (10 min, 95 °C), 45 cycles (15 s, 95 °C and 1 min, 60 °C) were performed on an ABI 7700 sequence detection system (Perkin Elmer Applied Biosystems). The threshold cycle (Ct) for each sample of the standard curve was plotted against the input copy number. The value of Ct was determined by the first cycle number at which fluorescence was greater than the set threshold value. For accurate comparison of the samples the threshold was the same for all the experiments. Linear regression was used to determine the copy number of the experimental samples. The HHV8 copy number measured was converted to copies per ml sample. As a control for cross contamination samples consisting of distilled water were also subjected to the isolation method and the extracts were tested with both the ORF65 and the PhHV assay. The Ct for all these “no-template” samples was always >45 cycles. The mean Ct value for the PhHV assay was 24.9 ± 0.6 standard deviation, and was used to indicate which samples were truly negative and if no inhibition of amplification or loss of sample had occurred.

**Statistical analysis.** To compare changes in CD4+ cell counts a repeated measurements procedure was done using a generalised linear model (PROC MIXED of SAS software, SAS version 8.02, SAS Institute, Cary, NC, USA) entering the square root of the CD4+ cell count. Such an analysis takes into account that serial measurements of the outcome variable in one patient are correlated, and provides a valid statistical estimate of the main effect. Since HHV8-DNA load was undetectable in a considerable number of observations we used generalized estimating equations where HHV8 DNA was entered as a qualitative variable (present or absent). In addition the clinical relevance was evaluated by entering the severity of the KS lesions into this model.
Results

**Longitudinal analysis of serum HHV8 DNA load.** The HHV8 DNA load was measured in serum samples of 19 AIDS-KS patients to analyse the dynamics of HHV8 load before and after the onset of KS (Fig. 1). All patients were HHV8 DNA positive at one or more time points during the study. Table 1 summarizes patient characteristics. Frequency of detection of HHV8 DNA in this sample set was 58% at 24 months before diagnosis, 68% at the moment of KS diagnosis, and 61% 24 months after KS diagnosis. HHV8 DNA load in serum was generally low, ranging from undetectable (=<50 copies/ml) to a maximum of 14125 copies/ml, with most samples being below 1000 copies/ml. The results for all patients were combined and the median load was calculated at the different time intervals to investigate the behaviour of the HHV8 load over time (Fig. 2A). Median amounts of copies increased from 177 copies/ml (range, <50-4266) at 24 months before KS diagnosis till 260 copies/ml (range, <50-7244) at KS diagnosis, with a median of 263 copies/ml (range, <50-14125) 24 months after KS diagnosis. This increase was not statistically significant (p= 0.56), suggesting that the median HHV8 load merely fluctuates. Before KS diagnosis, 2 patients (H0157 and H6056) never had a detectable HHV8 load at any of the four time points, while 5 patients were always detectable. Of the remaining patients, 4 were only positive at a single time point before diagnosis. Examining the patients with at least 3 positive measurements before diagnosis (a total of 11 patients), a steady or slowly increasing viral load was observed. However, no consistent overall pattern was detected (Fig. 1). The viral load at KS diagnosis was not notably different from that at any other time point, while patterns varied: two patients (H0236 and H0546) showed a peak in viral load at diagnosis, patient H0591 had a barely detectable HHV8 load and five patients (H0479, H6056, H0157, H6125, and H0401) were HHV8 DNA negative at that time. Patient H6056 only became HHV8 DNA positive in serum two years after the manifestation of KS.

Serum HHV8 DNA levels 24 months after KS diagnosis were generally not much different per patient from levels measured at earlier time points. Only two patients, H0324 and H0546, showed steadily increasing HHV8 levels from diagnosis onwards with >5000 copies/ml at end-stage disease. A third patient, H6053, also reached a level >5000 copies/ml 24 months after diagnosis, but experienced a slight fall in HHV8 DNA viral load in the 3 months after KS diagnosis.

**Serum HHV8 DNA load and KS staging.** KS stage at moment of diagnosis, and after follow-up is shown in Table 1. All KS cases were diagnosed between 1987 and 1997. Medical information was not available for two patients, while of the remaining 17 patients three were diagnosed at presentation with mucocutaneous KS, 10 had developed a single cutaneous lesion, a single patient presented with an oral lesion, and 3 patients showed two or more cutaneous lesions. Progression to visceral KS was seen in two patients (H8003 and H0324), while 2 additional
patients developed mucocutaneous KS after presenting with a single cutaneous lesion. KS disease was more or less stable or regressed in the remaining patients. No correlation could be found between either the viral load before or at diagnosis and stage at diagnosis and progression of KS in this set of patients. Also, the presence or absence of detectable HHV8 DNA in serum was no indicator for the severity of KS at either moment (p=0.10).

<table>
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<tr>
<th>Patient no.</th>
<th>KS date</th>
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<th>KS follow-up</th>
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<th>Antiretroviral treatment</th>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>D4T, 3TC, Saquinavir,</td>
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</table>

Serum HHV8 DNA load and treatment: Seven patients in our study received a HIV-1 protease inhibitor (PI), indinavir, saquinavir, or ritonavir, between 3 to 18 months after KS diagnosis (Table 1). Individual treatment is also indicated in Fig.
1. The effect of these drugs was variable: three patients showed a decline in HHV8 DNA load, in two patients the load remained unchanged, one of them continuing to be undetectable, while in another patient (H6056) viral DNA could be measured for the first time 24 months after diagnosis during treatment with indinavir. Patient H0065 was treated with AZT alone after diagnosis, with an immediate decline in HHV8 load and KS burden. However, KS recurred, the viral load increased, and the patient was subsequently treated with radiotherapy. Patient H0236 was treated with radiotherapy after diagnosis, and showed a good clinical response, with a concomitant decline in viral load (Fig. 1). Chemotherapy (adriamycin/bleomycin/vincristine) was initiated in patients H6077 and H8003. Patient H6077 improved clinically at first, and viral load decreased, but the improvement and the viral load decrease were not permanent, and the patient died 2 years after diagnosis. Patient H8003 showed no clinical response to the chemotherapy, and no decrease in viral load. He died 30 months after KS diagnosis. Overall, HHV8 viral load in serum seems to be correlated to treatment, either with PI, AZT, radiation or chemotherapy, with a decrease in viral load corresponding to clinical improvement. However, patient numbers are low, as only 11 patients were treated with some form of therapy. Absolute changes in viral load after treatment were generally low due to the already low levels of detectable DNA before therapy. Of the patients treated with PI, six are still alive today. In three of these patients, a complete regression of KS lesions correlated with an undetectable HHV8 load 24 months after diagnosis. Patient H6056, the only one with a rise in HHV8 DNA load after PI treatment, died 5 years after KS diagnosis with a B-cell lymphoma.

*HHV8 DNA viral load correlated with CD4 cell count.* Longitudinal CD4+ cell counts were analysed for all 19 KS patients described here, and are shown in Fig. 1 for each individual patient. Median CD4+ cell counts for each time point have been calculated and are shown in Fig. 2B. There was a statistically significant decrease in CD4+ cell counts in the course of KS development and beyond (p=0.0003), as has been described before 20.44.45.
Figure 1: Longitudinal evolution of HHV8 DNA copies in serum and CD4+ cell count in 19 HIV-1 infected patients with AIDS-KS before and after diagnosis. HHV8 DNA copies are indicated by closed squares (upper line) and open circles indicate CD4 cell counts (lower line). The limit of detection (<50 copies) of the ORF65 real-time PCR assay is indicated by a horizontal dotted line. A vertical line indicates time of KS diagnosis. Treatment after KS diagnosis is indicated with arrows.

Figure 2A: Mean HHV8 load measured in a total of 157 serum samples from 19 AIDS-KS patients over nine time points before and after KS diagnosis. The variation was not statistically significant (p=0.47). Figure 2B: Mean CD4+ cell counts over nine time points (total 151 counts) during 4 years of follow-up in 19 AIDS-KS patients. The CD4+ decline was statistically significant (p=0.0003).
Chapter 5

Discussion

In this study, we have retrospectively analysed the behaviour of the HHV8 DNA viral load by real-time PCR in longitudinal serum samples from AIDS-KS patients before, at, and after KS diagnosis. Three-month interval samples were available from a total of 48 months of follow-up (2 years before diagnosis till 2 years after diagnosis) for 19 patients. Frequency of detection of HHV8 DNA in these samples was around 62%, with the viral load ranging from undetectable (< 50 copies/ml) till a maximum of 14125 copies/ml. No correlation could be found between viral detection and/or copy numbers in serum and progression to KS, or with viral load and KS clinical stage. HHV8 viral load was associated with treatment response, where a decline in load was linked with clinical improvement, but patient numbers were low as only 9/19 patients received specific anti-KS therapy (PI, chemotherapy or radiotherapy). Especially treatment with antiretroviral protease inhibitors resulted in a strong decline in HHV8 load in serum, as was observed earlier for PBMC.

Clinical relevance of HHV8 load in KS has been questioned. Earlier studies have not been able to find a definite relation between HHV8 copy numbers in blood and progression to KS, although sometimes, but not always, a relation with KS disease stage was observed. Here, we have observed neither a relation with higher HHV8 DNA load and progression to KS nor a relation with higher HHV8 DNA load and advanced KS disease in 19 AIDS-KS patients. Therefore, the non-predictability of HHV8 load in KS resembles the situation with EBV, another gammaherpesvirus, whereby EBV load in PBMC is also not predictive of development of EBV-related non-Hodgkin lymphoma, whereas for CMV, a betaherpesvirus, load was found to predict CMV disease and to peak 4 months before onset of disease.

However, in contrast to the situation in KS, HHV8 load in blood in HHV8 positive MCD was found to be correlated with disease exacerbations and treatment response, whereby copy numbers were generally found to be higher than in KS. This suggests that for HHV8 the clinical relevance of viral load in blood is disease-specific. The infected cell type in HHV8+ MCD is mainly the B-cell, suggesting that viral copies measured in blood accurately represent ongoing viremia. In KS, the major HHV8 infected cell type expresses both endothelial and macrophage antigens, and is most likely of endothelial origin. The disease is mainly localised to the skin, suggesting that viral DNA detected in blood is not necessarily a direct representative of the disease process elsewhere, explaining the discrepancy between viral load in either PBMC or serum, and disease progression in KS.
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Chapter 5

References


