



## Short communication

## High prevalence of Human Herpes Virus 8 (HHV-8) in patients with Warthin's tumors of the salivary gland

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Received 8 August 2007; received in revised form 17 January 2008; accepted 31 January 2008

### Abstract

**Background:** Warthin's tumor is a common benign neoplasm of the salivary gland. Human Herpes Virus 8 (HHV-8) is the etiologic agent for all forms of Kaposi's sarcoma (KS), and HHV-8 DNA is present in saliva, suggesting that non-sexual transmission is associated with latent infection of the salivary gland.

**Objectives:** To provide insights into the HHV-8 cell tropism, the presence of HHV-8 was investigated in a series of Warthin's tumors of the salivary gland and corresponding adjacent normal tissue.

**Study design:** Forty-three patients with Warthin's tumors (cystadenolymphoma) were tested for the presence of HHV-8 DNA, and corresponding adjacent normal tissue samples were obtained from 15 patients. DNA was extracted from the paraffin-embedded tissues. A nested polymerase chain reaction (PCR) assay was applied, and the positive samples were confirmed by direct sequencing.

**Results:** HHV-8 DNA was detected in 19 out of 43 (44%) salivary gland tumor samples. Among the 15 cases with paired samples, 9 were HHV-8-positive for both samples, 4 were HHV-8-negative for both samples while in two cases HHV-8 was detected only in the tumor specimens.

**Conclusions:** HHV-8 is frequently detected in adenoid salivary neoplasms, suggesting a significant role of the virus in the etiopathogenesis of the disease. Larger studies are required to investigate the role of HHV-8 in the development or progression of Warthin's tumors.

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**Keywords:** HHV-8; Salivary glands; Nested PCR

### 1. Introduction

Warthin's tumor, which is the second most common benign salivary gland tumor, is located almost exclusively in

the parotid gland (Eneroth, 1971; Foote and Frazell, 1953; Warthin, 1996). It is also known as cystadenolymphoma, papillary cystadenoma or epitheliolymphoid cyst. Despite the detection of progesterone receptors (Teymoortash et al., 2001), evidence for the etiology and pathogenesis of the disease is still lacking (Teymoortash and Werner, 2005). As yet, risk factors for the presence of bilateral Warthin's tumor have not been determined. A small number of prior studies, mainly investigating the role of Epstein–Barr virus and cytomegalovirus, have been inconclusive (Laane et al., 2002; Ogata et al., 1997; Santucci et al., 1993).

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Human Herpes Virus 8 (HHV-8), a gamma-2 herpes virus (rhadinovirus) which naturally infects only humans, is the cause of several neoplastic disorders among immunocompromised individuals, especially Kaposi's sarcoma (KS) (Kaposi, 1872). Although a number of different salivary gland disorders are more common in HIV infection and AIDS specifically (severe and recurring protozoal, fungal, bacterial, and viral infections, lymphoepithelial cysts, diffuse interstitial lymphocytosis syndrome, malignant lymphoma), salivary gland KS is rare (Goedert et al., 1998).

Evidence for HHV-8 transmission other than between homosexual adults and during childhood – namely transmission through heterosexual contact or injection drug use – is growing, although these issues are still under study (Vieira et al., 1997). The discovery of HHV-8 DNA in saliva and oral mucosal shedding suggests an additional possible route of transmission (Boldogh et al., 1996; Koelle et al., 1997; LaDuca et al., 1998; Lampinen et al., 2000; Lucchini et al., 2001). Moreover, new findings have supported this idea (Ablashi et al., 2002; Lucht et al., 1998). Shedding of HHV-8 in saliva suggests a close association between this virus and salivary gland vascular or epithelial tissue (Chang et al., 1994; Yen et al., 2004).

In the present study, we investigated the incidence of HHV-8 DNA in a series of paraffin-embedded tissues from Greek patients with Warthin's tumors of the salivary gland. Our findings revealed a high prevalence of the HHV-8, suggesting a potential role for it in the pathogenesis of the disease.

## 2. Materials and methods

### 2.1. Patients

Samples were obtained from 43 non-immunocompromised patients with salivary gland neoplasm. The specimens were immediately fixed in buffered formalin and embedded in paraffin. Hematoxylin–eosin stained sections from all paraffin blocks were reviewed to confirm the diagnosis of Warthin's tumors (cystadenolymphomas). The corresponding, histologically examined, adjacent normal parotid tissues were also obtained from 15 patients, along with their tumor samples. Ethical approval was obtained from the local ethics board of the "Hellenic Red Cross" Hospital and informed consent was obtained from all patients.

### 2.2. Oligonucleotide primers and nested PCR amplification

All specimens were examined for the presence of HHV-8 DNA using a set of primers for the  $\beta$ -globin gene (forward: 5' TCCAACATCAACATCTTGGT 3'; reverse: 5' TCCCCAAATTCTAAGCAGA 3'). The DNA sequence within the open reading frame (Orf)-26 of the HHV-8 genome was amplified by nested PCR as

previously reported (Boshoff and Weiss, 1998; Tohda et al., 2001). The sequences of the primers were as follows: Outer KS1, 5' AGCCGAAAGGATTCCACCAT 3'; KS2, 5' TCCGTGTTGTCTACGTCAG 3'; inner WH1, 5' GTGCTCGAATCCAACGGATT 3'; WH2, 5' ATGACA-CATTGGTGGTATAT 3'. DNA (100 ng) was added to a 25- $\mu$ l PCR mixture containing 2.5  $\mu$ l buffer solution, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 1.25 U Taq polymerase (Invitrogen Co.) and 25 pmol of each primer. PCR conditions were as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, 48 °C for 45 s, 72 °C for 60 s and a final 15-min elongation at 72 °C. One microliter of the 1st step product was added to the 2nd step mixture and amplified using primers WH1 and WH2. PCR conditions were as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 60 s, 44 °C for 60 s, 72 °C for 60 s and a final 15-min elongation at 72 °C. Both positive and negative PCRs were repeated at least three times. Positive controls were from an HHV-8 positive KS and a primary effusion lymphoma. To ensure that our PCR assay was sensitive enough to detect relatively low levels of viral DNA, a previously described (Panagiotakis et al., 2007) serial dilution assay was employed (data not shown).

### 2.3. Gel electrophoresis

The final nested PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Gels were exposed at UV to visualize the expected nested PCR amplification product of 172 bp. Each examination was performed multiple times to ascertain the reproducibility.

### 2.4. Sequencing

All PCR products were subjected to direct sequencing and the analysis to confirm the presence of HHV-8 DNA.

## 3. Results

The presence of HHV-8 DNA was determined in a series of paraffin-embedded Warthin's tumors along with adjacent normal tissues from 15 patients, applying a nested PCR assay and the results were highly reproducible. The quality and integrity of the extracted DNA were successfully tested in all samples, after amplification of the  $\beta$ 2-microglobin gene and all samples were found suitable for PCR analysis. The first round of PCR for the detection of HHV-8 was negative in all samples tested. However, the second PCR round revealed the presence of HHV-8 DNA in 19 out of 43 (44%) tumor samples. In 15 paired-sample cases, the presence of HHV-8 genomes was also tested in the corresponding adjacent normal salivary tissues of the patients. Among the 15 paired samples, HHV-8 was detected for both tumor and normal samples in 9 cases, 4 pairs were HHV-8-negative for both samples while in 2 cases HHV-8 was detected only in the Warthin's tumor (Table 1). HHV-8 was present in nor-

Table 1  
Cumulative results of the detection of HHV-8 in salivary gland carcinomas

PCR results	No. of samples	Percentage (%)
Positive	19	44
Negative	24	56
Total	43	100

  

No. of paired samples	PCR results	
	Tumor samples	Normal samples
9	Positive	Positive
2	Positive	Negative
4	Negative	Negative

mal salivary tissues only when the corresponding tissues also contained HHV-8 DNA.

#### 4. Discussion

A tropism for salivary gland tissue including latent infection of the salivary glands in healthy subjects is known for EBV, CMV, HHV-6 and HHV-7, but not established for HHV-8 in subjects without KS (Klussmann et al., 2000). Although HHV-8 has been found in salivary glands of both HIV-1-positive and -negative individuals, the route of infection is unknown.

We tested a variety of human salivary gland tissues for the presence of HHV-8 sequences to determine the relationship of this virus to Warthin's tumors. The detection of viral nucleic acids within tissue is valuable for evaluating any potential role of a virus in the etiology of human tumors. However, the detection of low levels of viral DNA in tissue requires the use of highly sensitive PCR methods that optimize the conditions of tissue acquisition, storage, and fixation. Considering several technical issues involved in the amplification of DNA extracted from formalin-fixed archival samples, a well-established PCR assay, suitable for both fresh tissue and paraffin-embedded tissues, was applied (Boshoff and Weiss, 1998; Klussmann et al., 2000). Furthermore, the direct sequencing of amplicons from the PCR-positive samples ensured the credibility of the results. A high prevalence of HHV-8 was revealed, with 44% of the salivary gland tumors containing HHV-8 DNA. Interestingly, PCR analysis revealed the presence of HHV-8 DNA not only in tumor but also, occasionally, in the corresponding adjacent normal tissue samples, suggesting these tissues can also be infected with HHV-8. The simultaneous presence of HHV-8 in the tumor specimen and absence of the virus in the normal tissue of several paired-samples served as an ideal internal control, reducing the possibility of virus detection because of its potential presence in the saliva. The PCR amplification of viral DNA, itself, from archival material, has several limitations, such as low levels of intact viral DNA, difficulty in extracting amplifiable DNA, and low and variable levels of amplification product. Despite these issues, HHV-8 DNA

was frequently detected in the samples tested, implying that our findings might underestimate the incidence of the virus in Warthin's tumors.

Our findings are consistent with previous studies in HIV-positive or AIDS patients but differ from a previous study where HHV-8 was detected only in one bilateral MALT-lymphoma of the parotid gland of an HHV-8-seropositive female patient suffering from Sjögren's syndrome (Klussmann et al., 2000). The incidence of HHV-8 in Greece ranges between 4% and 35% (Chatlynne and Ablashi, 1999; Schulz, 2000a,b; Zavos et al., 2005), while other studies suggest an average of 10% (Chatlynne and Ablashi, 1999; Lucht et al., 1998; Schulz, 2000a,b). In the patients of our study, the prevalence was higher (44%), suggesting that HHV-8 infection could play a role in a subset of adenoid salivary neoplasms. Further studies, including larger cohorts of samples are required to investigate the implication of HHV-8 in the development and progression of salivary gland tumors. Specific pathogenic mechanisms should be studied and further investigations should reveal the role of HHV-8 in salivary gland neoplasms.

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