Epidemiology of Herpes Human Virus 6 and Epstein-Bar Virus Infections in Salivary Gland Neoplasms in Isfahan, Iran

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Abstract

Background: Viral infections were reported to be the cause of some human malignancies. The exclusive presence of EBV (Epstein-Bar virus) and HHV6 (Herpes Human Virus 6) has been investigated in previous studies. As such comparisons had never been carried out on salivary gland neoplasms, this study aimed to determine any relationship between these two viruses in salivary gland neoplasms.

Methods: Seventy eight formalin-fixed paraffin embedded tissue samples of salivary gland tumors were enrolled. The enrolled patients were those who referred to the Department of Oral Pathology of Isfahan University of Medical Sciences and to the state hospitals and private clinics in Isfahan, Iran from May 1995 to July 2005. The paraffin blocks were investigated for presence of HHV6 and EBV genomes by PCR.

Results: Out of the 78 samples, 15 were positive for both EBV and HHV6 infections while 6 were only positive for EBV, 21 were HHV6 positive but negative for EBV and 36 samples were reported negative for both viruses. A relationship was visible between EBV and HHV6 genomes.

Conclusion: The significant relationship between HHV6 and EBV genomes and salivary gland neoplasms denotes to the question that should be answered in the light of further research whether HHV6 infection in salivary gland tumors can increase the incidence of EBV infection.

Keywords: PCR; Herpes Human Virus 6; Epstein-bar virus

Introduction

Tumors of the salivary glands constitute an important area in the field of oral and maxillofacial pathology. The annual incidence of salivary gland tumors around the world ranges from about 1 to 6.5 cases per 100,000 people,1 while the incidence in Isfahan, Iran is reported far greater at 1.13%.2 The majority of salivary gland tumors (about 80%) arise in the parotid glands. The submandibular glands account for 10 to 15 % of tumors, and the remaining tumors develop in the sublingual or minor salivary glands.3 The presence of pleomorphic adenoma and adenoid cystic carcinoma of the salivary gland was shown by immunohistochemical markers in southern Iran.4

Persistent viral infections are estimated to be the cause of as many as 20% of human malignancies5 and the major risk factor second to tobacco for human carcinomas.6 A number of malignant and benign oral lesions have been identified to be related to Herpes viruses. Epstein Barr virus (EBV), for example, has been proved to be involved in leukoplakia, lymphoproliferative lesions, lymphoproliferative carcinoma, B-cell lymphoma, certain benign salivary gland tumors and salivary gland lymphoepithelial carcinoma.7,8

The most recent human Herpes virus as a potentially lymphotropic factor was isolated in 1986 in the leukocyte culture from patients with malignant
lymphoma and AIDS-dependent leukemia, which came to be designated as human Herpes virus type 6 (HHV6).\textsuperscript{9,10} Research findings have shown the possibility of HHV6 transfer via saliva and suggested that salivary glands may be a favorable site for the proliferation and survival of the virus.\textsuperscript{11,12}

The exclusive presence of the two EBV and HHV6 viruses has been investigated in some patients by a number of researchers. In one such study, HHV6 was only found in latently EBV-infected B-lymphocytes. The authors concluded that HHV6 activated other Herpes viruses such as EBV, CMV, and human papilloma viruses and that they may had a diminishing or accelerating effect on HIV proliferation.\textsuperscript{13} As such comparisons have never been carried out on salivary glands neoplasms, this study aims to investigate the presence of EBV and HHV6 genomes in a group of salivary glands neoplasm and to investigate any relationship that might hold between these two viruses.

**Materials and Methods**

This analytical descriptive study was performed in 78 formalin-fixed paraffin embedded tissue samples of salivary gland tumors (38 benign salivary gland tumors including pleomorphic adenoma and monomorphic adenoma and 40 malignant salivary gland tumors including mucoepidermoid carcinoma and adenoid cystic carcinoma).

The enrolled patients were those who referred to the Department of Oral Pathology of Isfahan University of Medical Sciences and to the state hospitals and private clinics in Isfahan, Iran from May 1995 to July 2005. The inclusion criteria were adequacy of tissue for H&E staining and polymerase chain reaction (PCR) from patients affected only by that specific neoplasm without suppressive conditions such as transplants or consumption of immune-suppressive drugs. Preparation of sections was accomplished under completely sterile conditions free from contamination. One 4μ sections were used for H&E lamella and 10μ sections for PCR tests. Two pathologists were consulted for the verification of the diagnosis of lesions using the H&E lamella.

The extraction of DNA from formalin fixed paraffin embedded tissues (taken from patients and provided from normal oral tissue samples) was performed by 5% chelex as described below: Briefly, 25-50 mg of tissue samples were deparaffinized in 800 μL xylol for 30 minutes and pelleted by centrifugation.\textsuperscript{14} Xylol was removed and cleaned from tissue by adding 1 mL of 100% ethanol. For isolating DNA, tissue sections were incubated overnight at 37°C (chelating resin) in 10 mg/ml proteinase k, then boiling for 5-10 minutes. 5% chelex and 1.00 NL tris-EDTA were added to the lysis solution and heated at 99°C for 10 minutes followed by centrifugation at 10500 g for 15 minutes. Finally, the supernatant was used directly for amplification.

DNA quality and quantity control were performed. The PCR mixture contained 50 Mm Kcl, 10 Mm Tris-Hcl (pH =8.3) and 2.25 M Mgcl\textsubscript{2} as a 1X reaction buffer, 200 mM of each DNtp, 10 μm of each of primers and 1 U taq polymerase (Roche, Germany). PCR was performed with EBV primers including: 5'- GTC, GTC, CGC, ATG, TTT, TGA, TC, 3' and 5'- GCA, ACG, GCT, CTG, TTT, GA-3', and HHV6 primers of: 5'-CAT, TGC, CAA, ATT, ATC, CTG, AGC,G- 3' and 5'- CGC, ATG, CTT, GAC, GAT, GTA, GCA-3' in a total reaction volume of 50 μl of sample DNA.

To confirm the specificity of PCR products, PCR with control DNAs and PCR without DNA were used as EBV and HHV6-Positive and negative controls respectively. All samples were co-amplified with β-globin genomic sequences to ensure their adequacy for PCR. PCR amplification consisted of denaturation at 95°C for 15 minutes followed by 40 cycles of denaturation as 95°C for 15seconds and annealing and extension at 58°C for 60 seconds.

The amplified reaction mixture was analyzed by electrophoresis on 2% agarose gel in 1XTAE stained with 0.05 μgr/µL of ethidium bromide that photographed under UV light. The bands with correct location and molecular weight and markers with positive and negative control were selected. We used positive and negative controls in two stages of extraction and PCR.

The relationship of two groups was analyzed through Mc- Nemar test by SPSS software (version 11, Chicago, IL, USA). A p value less than 0.05 was considered significant.

**Results**

Eighty seven paraffin blocks of malignant and benign neoplasms of both major and minor salivary glands with equal chances of presence of EBV and HHV6 in the samples were compared. Out of the 78 samples, 15 were positive for both EBV and HHV6 while 6
were only positive for EBV, 21 samples were positive for HHV6 but negative for EBV, and 36 samples were reported negative for both HHV6 and EBV. A relationship was noticed between EBV and HHV6 genomes ($p=0.007$). It can, therefore, be suggested that a relationship is likely to exist between these two viruses in salivary glands neoplasms.

Discussion

Salivary glands tumors are of great importance in maxillofacial pathology. Recent studies have been focused on the role of viruses in salivary glands neoplasms. The EBV and the HHV have been identified as one of the most important oncogenic viruses capable of producing a number of oncogenic factors such as Bcl$_2$, Bcl$_{10}$, Clgr, Jun and fas. It has also been found that a number of EBV-related nuclear antigens (e.g., LMP1, EBNA1, and EBNA2) have oncogenic effects.

The HHV6 has also been identified in salivary glands neoplasms. A number of studies have suggested the presence of numerous oncogenic zones in the genome of this virus, but to speak of this virus as an etiological agent in cancer requires further research for validation. Diluca et al. (1995) reported the presence of HHV6 genome in 63% of their healthy salivary glands and in only 3% of their saliva samples. Levy et al. (1997) observed HHV6 genome in only latently EBV-infected T-lymphocytes. He asserted that HHV6 activated other Herpes viruses such as EBV, CMV, and HPV and that it might have a diminishing or accelerating effect on HIV proliferation. The findings from our study are in agreement with those of Levy’s, suggesting that the presence of HHV6 might be a precursor to the later presence of EBV. It should be noted, however, that the sample tissues used in the present study were different from those of Levy’s; hence, further research is required to validate this claim.

A number of studies have confirmed the presence of HHV6 genome in normal salivary gland tissues. Fox (1990), for instance, identified normal salivary gland tissues as the proliferation site for HHV6 genome. Zhao et al. also observed HHV6 genome in 14 of their 18 salivary gland specimens. In the light of these studies, the high number of HHV6-positive samples compared to EBV-positive in our study might be due to the presence of HHV6 in normal salivary tissues. Based on the findings of the present study, however, the likelihood of a relationship existing between the presence of EBV and HHV6 in salivary gland neoplasms may not seem far from reality. This study suggests that the presence of HHV6 might have in some way accelerated subsequent EBV infection in the studied samples. Further research with a greater number of samples, especially samples from normal salivary gland tissues, and comparison of the findings with those from similar studies may shed light on the issue, confirming or refuting our hypothesis.

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Conflict of interest: None declared.
References