**ABSTRACT:** Human Papillomaviruses (HPV) are a group of viruses associated with benign and malignant lesions of cutaneous and mucosal epithelia. Some “high risk” HPV types, especially HPV 16 and 18, are strongly correlated with cervical and anogenital cancers and are also related to the genesis of oral squamous cell carcinomas (OSCC). The aim of this work was to investigate the incidence of HPV infection in 40 paraffin-embedded or fresh specimens of OSCC, using PCR amplification of the viral DNA. Literature based primers (GP5+/GP6+) were used in order to amplify HPV DNA from the L1 gene, present in more than 22 types of HPV. A condyloma case with HPV 16 and 18 detected by *in situ* hybridization was used as a positive control. Amplification of HPV was observed only in the positive control. No squamous cell carcinoma cases showed DNA viral amplification. Absence of HPV DNA amplification by PCR in the analyzed specimens of OSCCs suggests that this virus not always plays a role in the carcinogenesis process. Discrepancy with some studies found in the literature may be related to methodology or population differences.

**DESCRIPTORS:** Carcinoma, squamous cell; Polymerase chain reaction; Papillomavirus, human; Oncogenic viruses.

**INTRODUCTION**

Human papillomaviruses (HPVs) are a family of icosahedral, non-enveloped viruses with a circular, double-stranded DNA genome of 7,500-8,000 base pairs (bp) and with a special affinity for epithelial cells. So far, more than 70 different HPV types have been defined by DNA sequence analysis. Specific types, most notably human papillomavirus (HPV) types 16, 18, and a few others, have been shown to cause the majority of cervical cancers and their high-grade precursor lesions. Infection with these HPV types are considered as “high risk” infections and the viral DNA is often integrated into the host genome. The viral oncoproteins E6 and E7 bind to the products of tumour suppressor genes p53 and Rb1, respectively, modifying or inactivating their normal functions, resulting in deregulation of the cell cycle with loss of control in crucial cellular events, such as DNA replication, DNA repair and apoptosis.

HPV 16, 18, and a number of additional HPV types have been found in about 95% of all speci-
mens of cervix cancer throughout the world. However, the association between HPV and oral squamous cell carcinomas (OSCCs) is still unclear and the results pertaining to that association have been contradictory. The reported prevalence of HPV DNA in oral cancer tissue has varied from 0 to 100%.\(^{15,16,29}\)

Diagnosis of HPV is based on the use of molecular tools. These methods can involve direct hybridization with complementary DNA probes, such as Southern blotting or in situ hybridization; signal amplification, such as the hybrid capture method; or target nucleic acid amplification by the polymerase chain reaction (PCR). The PCR is considered the most sensitive method for the detection of HPV DNA in clinical specimens.\(^{8,9,11}\) The PCR can be performed using type-specific PCR primers for individual HPV genome or general primers for different viruses DNA.\(^{2,8,9,22}\) The aim of this study was to investigate the incidence of HPV infection in OSCC by using the PCR technique with consensus primers designed to amplify HPV DNA from the L1 gene of multiple HPV genotypes.

**MATERIAL AND METHODS**

A total of 40 cases of OSCC were used in this study. A set of 23 specimens was taken from formalin-fixed, paraffin-embedded tissues from the files of the Oral Pathology Department, School of Dentistry, University of São Paulo (São Paulo, SP), and from the archives of the Luxemburgo Hospital (Belo Horizonte, MG) and Araújo Jorge Hospital (Goiânia, GO). The other 17 cases were obtained from fresh tissues of patients attending the Head and Neck surgery service, Clinical Hospital, University of São Paulo (São Paulo, SP). The tumor’s primary sites included lip (20 cases), tongue (14 cases), gingiva (3 cases), floor of mouth (2 cases) and palate (1 case). The mean patient age was 57 years (range 19–78), and comprised 32 men and 8 women.

**Extraction of DNA**

Before DNA extraction, sections of 10 µm were obtained from formalin-fixed, paraffin-embedded tissues (PET) and subjected to deparaffinization and rehydration.

DNA extraction was performed according to Neves et al.\(^{17}\) (2002). PET samples were first digested with proteinase K during 3-5 days at 55-60°C; fresh tissues were digested during one day. After proteinase K inactivation, the microtubes containing lysed OSCC tissues received 200 µl of an ammonium acetate solution (4 M) for protein precipitation. Additionally, tubes were vortexed for 20 seconds under high speed, incubated on ice for 5 minutes, and centrifuged at 13,000 x g for 3 minutes. The supernatant containing the DNA was transferred to another tube, where 600 µl of isopropanol were added, and centrifuged at 16,000 x g for 5 minutes. The DNA pellet was washed in 70% ethanol and centrifuged at 16,000 x g for 1 minute. The supernatant was discarded. Samples were dried and the DNA was dissolved in 30-50 µl of TE solution (Tris-HCl 10 mM, pH 7.4 and EDTA 1 mM, pH 8).

**Polymerase chain reaction**

DNA integrity and absence of PCR inhibitors were tested by amplification of exon 15 (166 bp amplicon) of the human adenomatous polyposi coli (APC) gene. Only samples with a visible APC band in the gel were included in this study.

PCR for HPV DNA amplification was performed with a set of general primers, GP5+/GP6+, aligned with corresponding sequences of the L1 region of 23 mucosotropic HPV genotypes (including HPV 6, 11, 16, 18, 31 and 33). A PCR product of approximately 150 bp was produced. Amplifications were carried out in a total volume of 25 µl, with a final concentration of 2 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 10 x PCR buffer (Tris-HCL 200 mM, pH 8.4; KCl 500 mM), 0.3 mM of dNTP (2’-deoxynucleotilde 5’-triphosphate, dATP, dTTP, dCTP, dGTP) (Invitrogen, Carlsbad, CA, USA), 4 mM of MgCl\(_2\), and 500 pM of each primer. The PCR cycling conditions were pretreatment at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing for 50 seconds at 42°C, and extension at 72°C during 1 minute followed by an additional 7 minutes at 72°C.

Each PCR experiment was run with a negative control (sterile water in place of DNA) and a positive control, a case of condyloma, obtained from PET, which was positive for the HPV 16 and 18 as demonstrated by in situ hybridization, and by the PCR. Positive controls always produced amplimers of the correct size.

The PCR products were analyzed by running a 2% agarose electrophoretic gel containing ethidium bromide (0.5 g/ml) and visualized under ultraviolet illumination. A low DNA mass ladder was used as a base-pair molecular weight pattern (Low DNA Mass Ladder, Invitrogen).
RESULTS

HPV was amplified in the positive-control in all reactions (viral DNA of a condyloma case). However, HPV amplification was not observed in any of the 40 OSCC specimens. All samples presented exon 15 (166 bp amplicon) amplification of the human adenomatous polyposi coli (APC) gene.

DISCUSSION

The pathogenesis of OSCC involves recognized carcinogens as tobacco and/or alcohol in intra-oral cancers, and sun exposure in lip cancers. Other factors, including viruses, may nevertheless be implicated in oral carcinogenesis, mainly when in conjunction with the carcinogens present in tobacco. Recent reports have shown that a specific subset of head and neck squamous cell carcinomas, the oropharyngeal carcinoma, is highly related to “high risk” HPVs.

The role of HPV in oral carcinogenesis has been extensively investigated. In the present work the complete absence of DNA HPV in OSCC was not expected. To ascertain the presence or absence of HPV DNA, we chose the PCR assay, which is considered the most sensitive method for the detection of HPV DNA in clinical specimens, using a consensus primer, Gp5+/Gp6+, described by de Roda Husman et al. (1995). General or consensus primer-mediated PCR assay have been developed to screen for a broad spectrum of HPV types using a single PCR reaction. The Gp5+/Gp6+ primer amplifies a region of 150 bp from the conserved L1 open reading frame (ORF). The L1 region is common to 23 mucosotropic HPV genotypes, including HPV 16 and 18, the most commonly detected in oral lesions. Inclusion of a positive control in the PCR reaction is very important to rule out the possibility of false negatives; so is the negative control, to detect any possibility of false positive results by contamination during the PCR procedure. In the present work a case of condyloma, positive for HPV 16 and 18 by in situ hybridization, was used as a positive control, and all experiments produced amplimers of the correct size. Finally, to rule out any non-amplifiable samples, an additional PCR for the APC gene was carried out, and all cases showed amplification.

Absence of HPV DNA amplification by PCR in the OSCCs studied cases suggests that this virus not always plays a role in the carcinogenesis process. Discrepant results found in the literature can be explained by methodological and technical differences in detecting and typing of HPV, as well as by geographic and ethnical differences that certainly influence the carcinogenesis process.

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