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Detection of human papillomaviruses of high oncogenic potential in oral squamous cell carcinoma in a Venezuelan population

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OBJECTIVE: The aim of this work was to detect and typify human papillomaviruses (HPV) in oral squamous cell carcinoma (OSCC) in a Venezuelan population.
MATERIAL(S) AND METHODS: Eighteen tissue samples were obtained from biopsies, formalin-fixed, and paraffin-embedded; 16 were diagnosed as SCC. We isolated DNA from paraffin-embedded tissue; two to three sections of 5 μm were obtained and resuspended in digestion buffer and proteinase K. Five microliters of the aqueous phase was used for polymerase chain reaction (PCR). The PCR for HPV amplification was carried out with consensus primers for L1 region (MY09 and MY11) and β-globin gene was used as internal control. The viral types were determined by molecular hybridization with a mix of probes for high/intermediate and low HPV oncogenic risk types.
RESULTS: The HPV-DNA was detected in 50% (eight of 16) of the SCC cases. Of these HPV-DNA-positive samples, 68% were histopathologically diagnosed as moderately differentiated SCC. The most common anatomical location was the alveolar ridge mucosa. All positive biopsies contained high oncogenic HPV types.
CONCLUSIONS: We observed a high prevalence of HPV infection of high oncogenic potential types in patients with SCC in our studied group. The moderately differentiated SCCs were more associated to HPV infection. These differences could be influenced by nutritional, environmental and genetical factors in our population but further studies should be carried out to determine these aspects.
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Introduction

Cancer of the oral cavity accounts worldwide for *c.* 220 000 new cases per year in men (5% of all cancers) and 90 000 in women (2% of all cancers) (Parkin, Pisani and Ferlay, 1999). Cancer in general represents the second cause of death in Venezuela and the sixth place is occupied by oral cancer.

Human papillomaviruses (HPV) are a group of DNA viruses, some of which have a remarkable host and target specificity (Pfister, 1984; Pfister and Fuchs, 1987), inducing hyperplastic, papillomatous and verrucous squamous epithelial lesions in the skin and at various mucosal sites, including anogenital tract, urethra, larynx, tracheobronchial mucosa, nasal and oral cavity (Syrjänen *et al*, 1987).

Human papillomaviruses comprise a subgroup with more than 100 members known as types, some of which are carcinogenic while the majority generally cause benign epithelial lesions or premalignant lesions (Lörincz, 1999). HPV have been associated with a range of clinicopathological changes, most notable in cervical cancer and its precursor lesions (Eversole and Laipis, 1988; Lörincz, 1999). The association of specific HPV types with anogenital malignancy and the increase in sexually transmitted diseases within the oral cavity are two factors that have prompted close examination of oral tumors for HPV (Syrjänen, Syrjänen and Laberg, 1986; Miller, Zeus and White, 1991).

Papillomaviruses are members of a very large family and their evolutionary relationship has been established by concerted sequencing of the viral genome. The viruses fall into two major groups: those that infect cutaneous epithelium and those that infect mucosal epithelium. These viruses are classified in high oncogenic risk and low oncogenic risk; the former being more consistently associated with cancer *in vivo* or increased incidence of cell transformation *in vitro*, while the latter mainly induce benign lesions. An etiologic role of HPV infection in the pathogenesis of oral precancer and

cancer lesions has been indicated by the discovery of HPV in oral cancer specimens, as well as by DNA hybridization studies disclosing HPV 11, 16 and 18 DNA in oral precancer lesions and SCC (Lindeberg *et al*, 1988; Chang *et al*, 1991; Cox, Eveson and Scully, 1991; Scully, 1993, 1996).

Among the HPV types, HPV 1,2, 4,6,7,11,13,16,18, 32,57 have been found in different types of oral lesions. Of these, HPV 13 and 32 seem to be specifically related to Multifocal Epithelial Hyperplasia (Scully, Prime and Maitland, 1985; De Villiers, 1989). Preliminary studies relating HPV to malignant cervical lesions from female Venezuelan patients have been carried out by Correnti *et al* (1997)), however, only few studies on HPV associated to oral malignancy have been reported in Venezuela (Premoli De Percoco, Ramirez and Galindo, 1998).

The aim of this study was to detect and typify HPV in oral squamous cell carcinomas (OSCC) in a Venezuelan population.

Materials and methods

Subjects

The present study comprises 18 patients with oral mucosal lesions with a clinical presumptive diagnosis of oral cancer, who were biopsied at the Oral Surgery Department and 16 were histopathologically diagnosed as SCC. Two cases were reported as hyperkeratosis and acanthosis and were used as negative controls. The mean age of the patient was 54 years and the female to male ratio was 9:7. The patients represent an unselected series collected from the files of the Oral Pathology Laboratory, Faculty of Dentistry. Central University of Venezuela, Caracas.

Methodology

Eighteen tissue samples were fixed in 10% neutral formalin and paraffin-embedded and processed to obtain 5 μm thick paraffin sections and routinely stained with H&E. We obtained DNA from paraffin-embedded tissue and two to three sections of 5 μm were performed, resuspended in digested buffer and proteinase K. After centrifugation at 12.0549 ***g*** for 5 min, 5 μl of aqueous phase were used for polymerase chain reaction (PCR). HPV-L1 region consensus primers MYO9 (3′ biotinilated sequence)/MY11 which amplify a fragment of about 450 bp and β-globin gene (184 bp fragment) was used as internal controls for quality DNA preparation and contamination by HPV-positive samples (Manos *et al*, 1989). A total of 35 PCR amplification cycles were performed using thermocycler step parameters of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. In all amplifications, DNA purified cultures of HeLa cells infected with HPV 18 were included as a positive controls. The amplification products were analyzed from electrophoresis in agarose gels at 2% in buffer Tris Borate ethylenediaminetetraacetic acid (EDTA, 1X); visualized by ethidium bromide tinting. The viral types were determined using the sharp signal assay (Digene system, Digene Diagnostics Inc, Bellsville, MD, USA). This methodology employs a mixture of RNA probes of low oncogenic risk (6,11,42–44) and intermediate/high oncogenic risk (16,18,31,33,35,45 51–53,56,58). The biotinilated-amplified samples were placed in polyvinilesteren plates covered with streptoavidin and were incubated with RNA probes respectively. The detection was made using an antibody conjugated with alkaline phosphatase that recognized DNA/RNA hybrid molecules. The plates were read at 405 nm.

Statistical analysis was performed using Fisher's test (INSTAT 4.0, GraphPad Software Inc, San Diego, CA, USA).

Results

In the present study, the most common anatomical location was the alveolar ridge mucosa followed by lateral border of the tongue. Eleven of 16 cases (68%) were histopathologically diagnosed as moderately differentiated SCC, followed by well differentiated with two cases (12.5%), one case poorly differentiated (6.2%), one carcinoma *in situ* case and a superficially invasive SCC. In this study, the predominant age was between 41–86 years and only two cases were in the group of 20–40 years (Table 1). In the studied population there was a slight female predominance, nine of 16 cases (56%).

In 50% (eight of 16) of the patients, the presence of HPV-DNA was observed (Figure 1). The cases of hyperkeratosis and acanthosis were negative for HPV. According to anatomical location and HPV positivity, four cases were located on the alveolar ridge mucosa, followed by two cases on lateral border of the tongue, one case on lingual aspect of the gingiva and one case on soft palate. We found a high proportion of HPV infection in the female population, six of eight (66%) compared with two of eight (25%) of the male group ($P < 0.0001$). The results of the hybridization assay showed that all PCR-positive cases had HPV intermediate/high oncogenic risk types. Of these HPV-DNA-positive samples, 62.5% were diagnosed histopathologically as moderately differentiated SCC and 12.5% as poorly differentiated (Table 2).

Discussion

Oral cancer continues to be a major source of morbidity and mortality in developing countries; although a causative association between HPV and oral cancer has not yet been proved, evidence is accumulating that infection with HPV may play more than a transient role in oral carcinogenesis (Chang *et al*, 1991; Al-Bakkal

Table 1 Distribution of oral squamous cell carcinoma (SSC) by age groups

	Age groups		
	20–40	*41–60*	*>60*
Number of SSC cases	2/16 (12%)	7/16 (44%)	7/16 (44%)

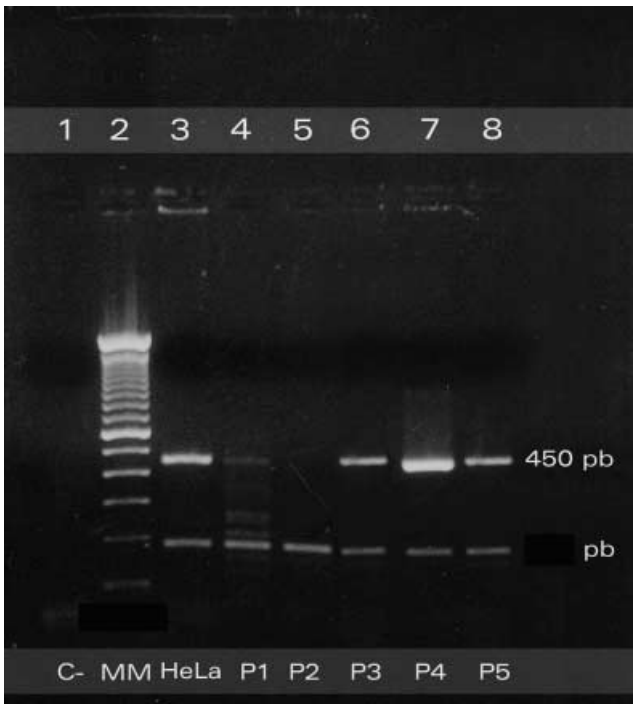

Figure 1 Detection of human papillomaviruses (HPV)-DNA in samples from patients with squamous cell carcinoma (SCC) lesions. Lane 1, negative control; lane 2, molecular marker (lambda leader, 100 pb); lane 3, HeLa-DNA infected with HPV 18; lanes 4–6, patients with moderate differentiated SCC; lane 7, patients with poorly differentiated SCC; lane 8, cancer *in situ*

Table 2 Squamous cell carcinoma (SCC) histological grading according to human papillomaviruses (HPV) infection

Total HPV + patient	*Moderate differentiated*	*Poorly differentiated*	*Carcinoma in situ*	*Superficial invasive*
N = 8	62.5% (5/8)	12.5% (1/8)	12.5% (1/8)	12.5% (1/8)

et al, 1999). The HPV have been inconsistently implicated in several oral lesions, whereas epidemiological studies have established a strong association between specific high risk HPV genotype and anogenital and cervical cancer. In view of the oncogenic potential of some HPV, the histological similarities between oral an genital mucosa, and the preference of high risk HPV genotype for these tissues, a possible link between HPV infection and oral cancer has been suggested (Loning *et al*, 1985; Yeudall, 1992).

Previous studies from the USA including 284 cases of oral and pharyngeal cancer, have been reported where 16-HPV-DNA was more frequently detected in tonsillar carcinomas (34%) and oropharyngeal carcinomas (36%) compared with other sites that presented $<15\%$ (Franceschi *et al*, 2000). Bouda *et al* (2000), analyzed the presence of HPV in 59 potentially neoplastic lesions and neoplastic cases, and they found that 91% were positive for the HPV infection; HPV type 16 was observed in 71% of the positive cases. The physical status of HPV in these cases was evaluated by non-isotopic *in situ* hybridization. Bouda *et al* (2000) demonstrated the episomal and integrative pattern of HPV infection and their findings are suggestive of an early involvement of high risk HPV types in oral carcinogenesis.

The reported incidence and prevalence of HPV types 16 and 18 in oral tissues varies widely according to the sensitivity of the methods used (Ostwald *et al*, 1994). Recently, Miller and Johnstone (2001) published a meta-analysis study indicating that the probability of detecting high risk HPVs in OSCCs was 2.8 times greater than that of low risk HPVs.

In the present report, the detection of HPV-DNA in 50% of OSCC demonstrated a high prevalence of HPV infection in our patients and interestingly all our cases were positive for high oncogenic risk HPV types. SCC is the most frequent oral epithelial malignancy found in patients during the fifth and sixth decades of life. The most common anatomical location is lateral border of the tongue and floor of the mouth. However, in our study the alveolar ridge mucosa and lateral border of the tongue represented the commonest sites in females during the fourth decade of life. This location on alveolar ridge mucosa is relatively infrequent for OSCC, as well as the age of presentation. In the present work, it is noteworthy that 16 cases of OSCC were moderately differentiated. Of these, 62.5% was positive for HPV-DNA whereas 12.5% was only detected in poorly differentiated SCC, all associated to high oncogenic risk types. In contrast, with other related studies HPV-16 DNA has been detected in 38% of well differentiated SCC and in 24% of moderately differentiated SCC, while 60% was detected in poorly differentiated SCC in a Japanese population (Sugiyama *et al*, 2003). In addition, Higa *et al* (2003) reported that HPV was more frequently detected in well-differentiated SCC cases than the moderately and poorly differentiated SCC. We may speculate that these contradictory results maybe influenced by small sample size, geographical location, nutritional, oral hygiene, habits and finally a genetical predisposition in those individuals. Our results represent an important preliminary baseline data on HPV-related to OSCC in a Venezuelan population. Further studies in this particular area are required.

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