HUMAN PAPILLOMAVIRUS IN ORAL LESIONS

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Abstract Growing evidence suggests a role for human papillomavirus (HPV) in oral cancer; however its involvement is still controversial. This study evaluates the frequency of HPV DNA in a variety of oral lesions in patients from Argentina. A total of 77 oral tissue samples from 66 patients were selected (cases); the clinical-histopathological diagnoses corresponded to: 11 HPV-associated benign lesions, 8 non-HPV associated benign lesions, 33 premalignant lesions and 25 cancers. Sixty exfoliated cell samples from normal oral mucosa were used as controls. HPV detection and typing were performed by polymerase chain reaction (PCR) using primers MY09, 11, combined with RFLP or alternatively PCR using primers GP5+, 6+ combined with dot blot hybridization. HPV was detected in 91.0% of HPV-associated benign lesions, 14.3% of non-HPV associated benign lesions, 51.5% of preneoplasias and 60.0% of cancers. No control sample tested HPV positive. In benign HPV-associated lesions, 30.0% of HPV positive samples harbored high-risk types, while in preneoplastic lesions the value rose to 59.9%. In cancer lesions, HPV detection in verrucous carcinoma was 88.9% and in squamous cell carcinoma 43.8%, with high-risk type rates of 75.5% and 85.6%, respectively. The high HPV frequency detected in preneoplastic and neoplastic lesions supports an HPV etiological role in at least a subset of oral cancers.

Key words: human papillomavirus, oral cancer, oral mucosa, oral leukoplakia, oral lichen planus, HPV genotyping

Resumen Virus papiloma humano en lesiones orales. Crecientes evidencias sugieren que el virus Papiloma humano (HPV) tiene un rol en el cáncer oral; sin embargo su participación es todavía controvertida. Este estudio evalúa la frecuencia de ADN de HPV en una variedad de lesiones orales de pacientes de Argentina. Se seleccionaron 77 muestras de tejido oral de 66 pacientes (casos); el diagnóstico histo-patológico correspondió a: 11 lesiones benignas asociadas a HPV, 8 lesiones benignas no asociadas a HPV, 33 lesiones premalignas y 25 cánceres. Como controles se usaron 60 muestras de células exfoliadas de mucosa oral normal. La detección y tipificación de HPV se realizó por PCR empleando los primers MY09,11, seguida de RFLP, o PCR usando los primers GP5+, 6+ seguida de hibridación en dot blot. HPV fue detectado en 91% de las lesiones benignas asociadas a HPV, 14.3% de las lesiones benignas no asociadas a HPV, 51.5% de preneoplasias y 60% de cánceres. Ninguna muestra control resultó HPV positiva. En las lesiones benignas, 30% de las muestras HPV positivas correspondieron a tipos de alto riesgo, mientras que en las lesiones preneoplásicas la positividad ascendió a 59.9%. En cánceres, la detección de HPV en carcinomas verrugosos fue 88.9% y en carcinomas escamosos 43.8%, con 75.5% y 85.6% de tipos virales de alto riesgo, respectivamente. La alta frecuencia de HPV detectada en lesiones preneoplásicas y cánceres apoya un rol etiológico del HPV en, al menos, un subgrupo de cánceres orales.

Palabras clave: virus Papiloma humano, cáncer oral, mucosa oral, leucoplasia oral, liquen plano oral, tipificación de HPV

The causal role of “high-risk” human papillomaviruses (HPVs) in cancer of the cervix was established through the accumulation of epidemiological data and molecular studies1-3. To this date, 15 different HPVs have been included in the group of “high-risk” types (HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), being considered human carcinogens by the International Agency for Cancer Research (IARC)4-5. HPV infection has also been implicated as a risk factor for the development of oral cancer, which is suggested by histological similarities between lesions of the oral and genital mucosa6, and supported by in vitro studies showing that HPV can immortalize oral keratinocytes. However, understanding the role of HPV in oral carcinogenesis has been hampered because of HPV prevalence rates’ large variation between studies—from 10 to 80%—in oral preneoplastic and neoplastic lesions, even when polymerase chain reaction (PCR) methods are used6-13.
The reasons for these discrepancies are still unclear, albeit differences in sampling methods, patient profiles and detection systems could lead to inaccurate conclusions. Furthermore, the presence of HPV has also been shown in benign oral lesions and normal mucosa of patients without warts or tumors. Oral cancer is the sixth most common malignancy in developed countries, representing almost 3% of malignant tumors. In India and other regions of Southeast Asia, it is the predominant malignancy, accounting for up to 50% of all cancers.

In Argentina, the average oral cancer death rate between 1997 and 2000 was 1.15 per 100,000 inhabitants. The estimated incidence, from mortality data applying the system developed by the IARC ranges from 2.67 to 2.95 new cases per 100,000 inhabitants, per year.

The aim of this study was to evaluate the frequency of HPV infection in a variety of benign, preneoplastic and neoplastic oral lesions in patients from Argentina.

Materials and Methods

Samples were obtained from patients attending the Stomatology Department of the Faculty of Dentistry, Buenos Aires University.

A total of 66 patients (25 males and 32 females) were included. A total of 77 tissue samples (cases) were selected; the clinical-histopathological diagnoses corresponded to: 11 HPV-associated benign lesions (3 focal epithelial hyperplasia, 6 condyloma acuminatum, 2 verruca vulgaris), 8 non-HPV-associated benign lesions (2 benign erosive-ulcerative lesions, 5 reactive hyperplasia and 1 white sponge nevus); 33 premalignant lesions (11 leukoplakia and 22 lichen planus), and oral cancers (16 squamous cell carcinoma and 9 verrucous carcinoma). In 11 patients who presented HPV-associated lesions in different oral sites, more than one sample was analyzed.

Samples were archival formaldehydefixed and paraffin-embedded biopsies; 8 μm sections were cut with a microtome, using a new blade for each specimen to minimize block contamination.

Sixty exfoliated cell samples from patients (28 males and 32 females) without any sign of oral mucosa lesions were included (controls). Exfoliated cells were collected with cytobrush in sterile PBS (pH 7.4) from more than one oral cavity site (buccal mucosa, border of the tongue, and mouth floor) and transported to the laboratory in ice for virology processing within 48 hours.

Informal consent forms to use the specimens in viral testing were obtained from both, cases and controls. Fresh cells were processed by proteinase K digestion and phenol-chloroform purification standard protocols. The same protocol was used to process fixed and paraffin-embedded lesion tissues, after deparaffinizing specimens with n-octane and washing with ethanol.

DNAs were PCR tested for β-globin gene to confirm the presence of the adequate template in the samples. HPV DNA detection and typing was performed by PCR using MY09,11 primers, combined with restriction enzyme digestion of PCR products (RFLP analysis) according to Bernard et al.; these degenerate consensus primers target a region of approximately 450 bp of viral L1 gene. Briefly, aliquots of the PCR products were mixed with 10 U of 7 different restriction enzymes (Ban HI, Hae III, Hin f I Dde I, Pst I, Rsa I and Sau3A I) in separate reactions. Digestion products were separated by electrophoresis in 2.5% agarose gel and the pattern obtained was compared with published data. CaSki and HeLa cell DNAs (which harbor HPV 16 and 18 sequences, respectively) were used as positive controls; and water as negative control (without DNA template).

In the MY09,11 PCR negative samples, a PCR using G5+, 6+ generic HPV primers was performed. These primers target a region of approximately 140 bp in the same L1 viral region. The amplification products were analyzed in 1.5% agarose gel, visualized with ethidium bromide staining under UV light and photographed. GP-PCR positive samples were typed by dot-blot hybridization using type-specific biotinylated oligoprobes corresponding to HPV types 6, 11, 16, 18, 31, 33 and 45. Positive reactions were revealed by chemiluminescence, using ECL kit, according to manufacturer recommendations (Amersham).

Results

All DNA samples amplified the β-globin gene, and were therefore considered appropriate for the PCR study.

Viral detection and typing results are summarized in Table 1. HPV was detected in 55.8% (43/77) of the cases: 91.0% (10/11) corresponded to benign HPV-associated lesions, 12.5% (1/8) to benign non-HPV associated lesions, 51.5% (17/33) to preneoplasias and 60.0% (15/25) to cancers. All control samples tested HPV negative.

In benign HPV-associated lesions, 30% (3/10) of HPV positive samples harbored high-risk types, while in preneoplastic lesions the value rose to 58.8% (10/17). In cancer lesions, HPV was detected in 60% (15/25), being 88.9% (8/9) for verrucous carcinoma and 43.7% (7/16) for squamous cell carcinoma, with high-risk type rates of 75.5% (6/8) and 85.7% (6/7), respectively.

HPV 6 and 11 were the most frequent types in benign lesions, while HPV 16 was the most common type detected in preneoplastic and neoplastic lesions.

Mixed infections were proven in 27.9% (12/43) of the positive samples.

HPV type remained undetermined in 6.9% (3/43) of positive cases; the limited size of specimens prevented further characterization of the samples.

In 11 patients with multiple oral lesions, samples from 2 different sites were analyzed (Table 2). Two patients were HPV negative in both sites and 8 patients who tested HPV positive had at least one common genotype in both; in only one patient the HPV results from different locations did not match (HPV 11 and HPV negative).

Discussion

High risk HPV types are widely implicated in the pathogenesis of anogenital cancer, in contrast to their more speculative role in oral cancer. Tobacco smoking or chewing, and alcohol drinking are considered risk factors.
factors for oral cancer. However, these risk factors are absent in many cases, indicating other possible etiologic pathways that could include HPV or other infectious agents. This study investigated the presence of HPV DNA in benign (HPV associated or not associated), preneoplastic and neoplastic oral lesions (cases), and normal samples (controls) in patients from Argentina.

HPV was detected in almost 50% of the oral lesions included in this study. HPV 6 and 11 were the most prevalent in HPV-associated benign lesions, while in preneoplastic lesions and cancers HPV 16 was the most frequent viral type, similarly to the findings described for cervical lesions in Buenos Aires population. In leukoplakia and lichen planus, HPV16/18 were detected in the majority of samples, according to other reports. Other authors have shown the predominance of HPV18 and the relative absence of HPV16 in oral lesions in patients from some European regions. Considering the geographical influence on HPV type distribution, the World Health Organization has recommended further studies to investigate and acquire further knowledge on different HPV types' prevalence, not only in the anogenital tract but also in skin and the aerodigestive tract. This epidemiologic information may be considered for HPV prevention strategies applying prophylactic vaccines which are being licensed.

HPV was not detected in this series of control samples; this is consistent with other studies that revealed a very low HPV positivity in the control group. However, some groups have found a surprisingly high HPV infection rate in normal mucosa. Some reports consider that the use of exfoliated cells could underestimate viral detection, while others have obtained data similar to biopsy analyses. It should be born in mind that biopsy is an invasive procedure and normal mucosa samples may be ethically difficult to obtain; for this reason we used exfoliated cells as controls.

### TABLE 1.– HPV detection and typing in oral samples from Argentine patients

<table>
<thead>
<tr>
<th>Clinical histopathological diagnosis</th>
<th>Detected HPV types</th>
<th>%</th>
<th>HPV Neg.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 11 13 16 18 11/16 16/18 6/11/16 ND*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Cases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign lesions:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HPV Non-associated:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White sponge nevus n=1</td>
<td>1</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Benign erosive ulcerative n=2</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Reactional hyperplasia n=5</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>HPV- associated:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condyloma acuminatum n=6</td>
<td>1 3 1 1 0</td>
<td></td>
<td></td>
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<tr>
<td>Focal epithelial hyperplasia n=3</td>
<td>1 1 1 1 0</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Verruca vulgaris n=2</td>
<td></td>
<td>1</td>
<td></td>
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<tr>
<td>Preneoplastic lesions:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lichen planus n=22</td>
<td>1 2 3 3 1 1 1 5 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukoplakia n=11</td>
<td>1 1 1 1 1 1 5 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verrucous carcinoma n=9</td>
<td>1 1 4 1 1 1 1 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma n=16</td>
<td>1 2 3 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total n=77</td>
<td>5 8 1 13 1 9 2 1 3 34</td>
<td>(6.5) (10.4) (1.3) (16.9) (1.3) (11.7) (2.6) (1.3) (3.9) (44.15)</td>
<td></td>
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<tr>
<td>(%)</td>
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</table>

This table summarizes the results obtained by both PCR-RFLP and PCR-dot blot hybridization.

*ND: Non determined viral types: HPV positive samples analyzed by PCR-dot blot hybridization which its viral type could not be determined because they did not hybridized with none of the assayed probes (HPV 6, 11, 16, 18, 31, 33 and 45).
Nevertheless, HPV DNA has been demonstrated in healthy individuals' oral samples in a number of studies, making it clear that the oral mucosa may act as a reservoir for new HPV infections and/or a source of recurring HPV lesions\textsuperscript{12, 13, 36}. This study included some of the most common benign epithelial oral lesions; mucosal low-risk types were predominant, as already described\textsuperscript{8}. Our data showed that the viral types identified in oral locations were similar to those detected in the genital and laryngeal mucosa, with the exception of HPV13, which was only found in oral mucosa. Although verruca vulgaris has been associated to skin types, particularly HPV2\textsuperscript{37}, one case of verruca vulgaris was HPV negative, perhaps due to our technical approaches targeted on mucosotropic types which may have missed cutaneous cases.

HPV16 DNA was demonstrated in a white sponge nevus, a finding only previously described by Cox et al\textsuperscript{38}. This benign lesion was characterized many years ago as a hereditary dyskeratotic hyperplasia of the mucous membranes\textsuperscript{39}. Probably the nevus cells' differentiated state is compatible with the amplification of HPV DNA, which is thought to reside in a latent state in the basal epithelial cells; therefore the presence of HPV16 in these lesions appears to be random.

HPV involvement in the etiology of potentially malignant oral lesions (e.g., leukoplakia and lichen planus) has been largely suspected albeit not clearly demonstrated\textsuperscript{6-8, 11-13, 32}. The HPV frequency here obtained for these lesions (51.5\%) is consistent with some previous data\textsuperscript{32, 40} in which HPV positivity was at least 50\%, but higher than the average obtained analyzing other reports (31\%)\textsuperscript{12, 41}. Independently of HPV type, in this study like in many others, HPV detection in potentially malignant oral lesions is undoubtedly greater than in normal controls.

Verrucous carcinoma is a locally invasive and non-metastatic variant of oral cavity squamous cell carcinoma\textsuperscript{42}. In both genital and oral verrucous carcinoma, low risk types HPV 6 and 11 have been reported as the most prevalent. In our limited series size, we noted a pre-

\begin{table}[h]
\centering
\begin{tabular}{llllll}
\hline
Case & Histopathological diagnosis & Detected HPV types & \multicolumn{4}{l}{HPV Site} \\
& & & 11 & 16 & 6/11/16 & 11/16 & 16/18 & Neg. & \\
\hline
1 & Lichen planus & Squamous cell carcinoma & 1 & & & & & Lateral side of tongue \\
2 & Lichen planus & Verrucous carcinoma & 1 & & & & & Lower edge \\
3 & Lichen planus & Verrucous carcinoma & 1 & & & & & Right buccal mucosa \\
4 & Lichen planus & Squamous cell carcinoma & 1 & & & & & Gingiva \\
5 & Leukoplakia & Squamous cell carcinoma & 1 & & & & & Ventrual aspect of the tongue \\
6 & Lichen planus & Squamous cell carcinoma & 1 & & & & & Ventrual aspect of the tongue \\
7 & Verrucous carcinoma & Verrucous carcinoma & 1 & & & & & Lower edge \\
8 & Lichen planus & Squamous cell carcinoma & 1 & & & & & Soft palate \\
9 & Leukoplakia & Squamous cell carcinoma & 1 & & & & & Buccal mucosa \\
10 & Leukoplakia & & 1 & & & & & Buccal mucosa and floor of the sulcus up to retromolar trigone \\
11 & Verrucosity & & 1 & & & & & Hard palate \\
\hline
\end{tabular}
\caption{HPV detection in multiple lesions from different oral sites}
\end{table}
dominance of HPV16; the level of HPV detection being markedly higher in verrucous carcinoma (88.9%) than in squamous cell carcinoma (43.8%), which is within the range reported in most PCR-based publications (25-75%)\textsuperscript{7, 40, 43}.

In this work, one squamous cell carcinoma harbored only HPV11. Although the evidence indicates the absence of carcinogenicity of HPV types 6 and 11\textsuperscript{3, 5}, these low risk types have been previously reported in oral cancer\textsuperscript{1} and penile carcinomas\textsuperscript{44-46} supporting their oncogenic potential.

In 11 patients carrying multiple lesions, samples from different sites were obtained. Most of them were pairs of samples of a preneoplastic lesion and a carcinoma, showing coincidental results. Although these lesions were located close but not in the same site, their origin could have been identical, and preneoplastic lesions could have acted as precursors.

Almost one half of the oral squamous cell carcinomas analyzed were HPV negative. A "hit and run" theory has been proposed to explain the HPV involvement in virus–negative tumors which could develop from HPV-containing precursors, not requiring the HPV to maintain the malignant state\textsuperscript{6, 13}. The method used can not rule out that these lesions harbor an undetectable number of viral copies. Oral carcinomas, like other HPV-related malignancies in the upper aerodigestive tract, appear to have low copy numbers of HPV, generally producing weaker PCR products than HPV-positive cervical specimens\textsuperscript{8, 12, 13, 25}. There is consensus on the need to develop a sensitive, validated laboratory test to detect HPV in oral exfoliated cells, that could reflect the high risk of HPV in head and neck tumors\textsuperscript{37, 40}.

Some authors have found that in a subset of head and neck tumors harboring HPV, the viral presence would be a marker for favorable outcome\textsuperscript{46, 49}. A retrospective oral epithelial dysplasia case-control study suggested that the prevalence of HPV16 is higher in dysplasias progressing to oral SCC than in those that do not, although the association was based on a small sample size and not significant\textsuperscript{50}. Research will be required to determine the use of HPV testing with respect to prevention, therapy, outcome and surveillance of oral lesions' recurrence.

This study contributes the first data on HPV diversity in a variety of oral lesions from Argentine patients. They contribute further evidence that oral infection with HPV, particularly carcinogenic types, is a risk factor for, at least, a subset of preneoplastic and neoplastic oral lesions. On the other hand, it has been pointed out that, in addition to the viral role, other factors should be taken into account in the progression of HPV induced lesions, like immune compromise, genetic background and exposure to chemical or physical carcinogens. A long-term follow-up of potentially malignant lesions could help define the effective role of HPV in their etiology.

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