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Human papillomavirus as a risk factor in oral carcinogenesis: a study using *in situ* hybridization with signal amplification

R. Acay, N. Rezende, A. Fontes, A. Aburad, F. Nunes, S. Sousa
Oral Pathology Department, School of Dentistry, University of São Paulo, São Paulo, Brazil

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Introduction: It is still controversial whether human papillomavirus (HPV) can be considered a risk factor in oral carcinogenesis. The aim of this study was to detect HPV DNA in 50 cases diagnosed as oral leukoplakias, with different degrees of epithelial dysplasia, and as oral squamous cell carcinomas, using in situ hybridization with signal amplification (CSA-ISH).
Methods: HPV DNA was assessed in paraffin sections using CSA-ISH with a wide-spectrum biotinylated DNA probe. In HPV-positive cases, genotyping with specific probes to HPV types 6/11, 16/18 and 31/33 was performed.
Results: The overall prevalence of HPV infection was 24%, markedly higher than that found in the control group. Results showed a discrete proportional relationship in the indices found in leukoplakia with no dysplasia, leukoplakia with dysplasia, and squamous cell carcinoma, but this was not statistically significant. When separating the group of leukoplakia by degrees of dysplasia, this relation of proportion was not observed. In genotyping, HPV types 16/18 were the most prevalent, and types 6/11 were only found in groups of mild or no dysplasia.
Conclusion: The results suggest that HPV is not likely to play a role in the progression of malignant transformation in oral lesions. Nevertheless, the increased prevalence of HPV infection compared to normal oral mucosa and the fact that high-risk HPV types were the most frequently identified do not allow the exclusion of HPV as a risk factor in oral carcinogenesis.

Key words: HPV; *in situ* hybridization; leukoplakia; oral cancer

Dr Renata Acay, Avenida Prof. Lineu Prestes, 2227 São Paulo 05508-000, Brazil
Tel.: +55 11 3091 7902;
fax: +55 11 3091 7894;
e-mail: acay@usp.br
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It is widely known that tobacco and alcohol or betel consumption are the major causes of the development of oral squamous cell carcinoma (OSCC). The fact that 15–20% of patients develop OSCC in the absence of exposure to these agents (10) or without any obvious predisposing genetic defect, however, strongly suggests the existence of other risk factors in oral carcinogenesis, such as the presence of infectious agents (19). Infection by human papillomavirus (HPV), especially types 16 and 18, of high oncogenic potential, is suggested as a possible factor associated with the development of OSCC (1).

Several studies have attempted to determine the prevalence of HPV infection in OSCC and premalignant lesions, reporting numbers that range from 0 to 100% (11). Despite the variation found, a recent meta-analysis on these studies concluded that there is quantitative evidence to indicate that HPV, especially high-risk types, is a risk factor in oral carcinogenesis (13). The issue is still controversial, although most of the recent review papers admit a relation between HPV and oral carcinogenesis (11, 22), indicating HPV as an independent risk factor (13, 19) or as an intensifier of the effects of tobacco and alcohol use (14, 15).

There are several methods used in studies to detect HPV in the oral cavity and in other anatomic sites and, probably

because of this, a great variety among HPV infection indices is reported in the literature. Polymerase chain reaction (PCR) is the most sensitive assay to detect HPV, having, in theory, the greatest reliability and least risk of false-negative results, hence its well-established use as a gold standard for HPV DNA identification (2, 5, 11, 12, 17). *In situ* hybridization with signal amplification (CSA-ISH), a novel signal amplification method based on the catalyzed amplification of positive hybridization signals using biotinyl–tyramide complexes, may be a useful alternative to PCR, especially when specimens are small and originate from archival paraffin-embedded material, because of the limitations of the PCR technique. CSA-ISH is performed on minimum amounts of material and is sensitive enough to detect one or two copies of HPV DNA, supposedly being as sensitive as PCR (2, 5, 8).

Studies correlating HPV and oral carcinogenesis usually compare HPV detection indices in normal oral mucosa with premalignant and malignant lesions of the oral cavity and they, in general, find a higher prevalence of HPV in OSCC than in oral premalignant lesions, which in turn is higher than in normal oral mucosa, denoting a relation between the degree of malignancy and HPV infection (13). It is known, however, that premalignant lesions comprise a heterogeneous group regarding malignant progression, according to their clinical and histological features. Leukoplakia, the most common premalignant lesion of the oral cavity, may present histologically from simple hyperkeratosis to severe epithelial dysplasia (16), and such differences should be a reason not to analyze these lesions as a group. Hence, to contribute to the understanding of the role of HPV in oral carcinogenesis, the purpose of this study was to detect HPV DNA using CSA-ISH in oral leukoplakias and OSCC, dividing the group of premalignant lesions according to the degree of epithelial dysplasia observed within the lesion.

Material and methods

Sample

Cases diagnosed as oral leukoplakia and OSCC were retrieved from the archives of the Surgical Pathology Service at the School of Dentistry, University of São Paulo, as approved by its institutional Ethical Committee. Patients presenting with lesions in the oropharynx were excluded from the study. Hematoxylin and eosin-stained slides were reviewed by light microscopy to confirm the histological diagnosis and to classify lesions by degree of epithelial dysplasia, according to the World Health Organization 2005 criteria (1). The 50 selected lesions were then symmetrically divided into five groups: group 1, leukoplakia with no dysplasia; group 2, leukoplakia with mild dysplasia; group 3, leukoplakia with moderate dysplasia; group 4, leukoplakia with severe dysplasia; and group 5, OSCC. Also, 50 cases of oral mucosa with no, or very minor, histological alterations were included in this study, as a control group. From the formalin-fixed and paraffin-embedded oral biopsy specimens, 5-μm sections were obtained and placed on organosilane-pretreated slides to be submitted to *in situ* hybridization.

In situ hybridization with signal amplification

Sections were dewaxed in xylene and hydrated in ethanol series. Slides were then immersed in the Target Retrieval Solution (DakoCytomation, Carpinteria, CA) and given microwave treatment for four cycles of 4 min each for unmasking. After the solution was completely cooled to room temperature, background quenching was performed with a 0.9% hydrogen peroxide and methanol solution (volume/volume) for 20 min. About 10–15 μl wide-spectrum HPV DNA biotinylated probe (DakoCytomation) was applied to each section before covering with coverslips. Target and probe DNA were denatured by incubating the slides for 5 min on a hotplate at 95°C. Hybridization was performed in a humidified chamber at 37°C overnight. After washing, detection of hybridization was carried out using the catalyzed signal amplification system for *in situ* hybridization GenPoint™ (DakoCytomation), as follows: sections were incubated in stringent wash for 20 min at 55°C, then incubated with primary streptavidin–horseradish peroxidase (HRP) in a dilution of 1 : 200 for 15 min, incubated with biotinyl–tyramide solution for 15 min, followed by incubation with secondary streptavidin–HRP for 15 min and with diaminobenzidine chromogen for 7 min. Sections were counterstained with hematoxylin for 2 min, dehydrated and cleared in ethanol and xylene, before application of coverslips. In cases that were found to be positive using the wide-spectrum probe, the reaction was repeated using specific probes to HPV types 6/11, 16/18, and 31/33 (DakoCytomation) instead of the wide-spectrum probe, for genotyping. Samples from oral mucosa that had been previously tested by this method and with these probes and found to be HPV-positive were used as positive controls.

Analysis

Results were analyzed by qualitative evaluation of the presence of HPV-positive cells under light microscopy by two observers in a double-blind protocol. Dark-brown to black punctate or diffuse nuclear staining located within the lesional biopsy area, not in adjacent epithelium, was regarded as positive for HPV. Intensity of staining and number of positive cells were not considered. Fischer's exact test was performed for statistical analysis and $P < 0.05$ was considered significant.

Results

Wide-spectrum probe

Twelve (24%) out of the 50 selected cases presented a signal of hybridization to the wide-spectrum HPV probe, at different intensities and with varied numbers of cells, whereas only 2 (4%) of the 50 control cases were positive for the same probe. In groups 1–4, positive cells were predominantly observed in superficial layers of epithelium, but isolated cases also had positive cells in the spinous layer (Fig. 1).

Percentages of positive cases in groups 1–5 were respectively 20%, 40%, 10%, 20%, and 30%, therefore showing no

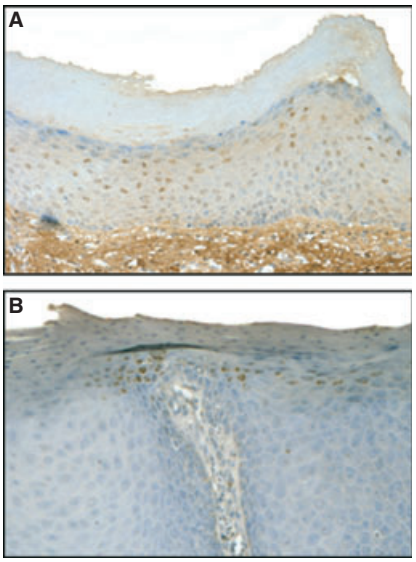

Fig. 1. Sections presenting the signal of hybridization for the wide-spectrum human papillomavirus (HPV) DNA probe for different intensities, positions, and number of cells.

correlation between degree of malignant progression and detection of HPV. If groups 2, 3, and 4, which have in commom the fact that they are all leukoplakias with epithelial dysplasia, are combined, the positivity to the wide-spectrum HPV probe increases according to the degree of malignant transformation of the group (20% in leukoplakias with no dysplasia, 26.7% in leukoplakias with dysplasia, and 30% in OSCC), but the difference among these indices is not statistically significant.

Specific probes

Within the 12 cases that were positive to the wide-spectrum HPV probe, six (50%) were positive to the types 16/18 probe, two (16.6%) were positive to the types 6/11 probe, and only one (8.3%) was positive to the types 31/33 probe. Two cases, one from group 2 and one from group 5, did not show a signal of hybridization to any of the specific probes used and one case, from group 5, was positive to two different probes (types 16/18 and 31/33). The distribution of these results among the groups is shown in Fig. 2. One case in the control group was positive to types 6/11 and the other, to types 16/18.

Discussion

It is still highly controversial whether HPV can be considered an etiological or risk factor for the development of premalignant and malignant lesions of the oral cavity. Despite all the variation found in HPV infection indices in normal mucosa, premalignant lesions, and OSCC, there is already evidence, at least quantitatively, of the relation between HPV and oral carcinogenesis.

Besides the variety of methods used to detect HPV, another fact that may contribute to the discrepancy in reported indices is the lack of standardization of the anatomic sites to be included in the study. Oropharyngeal and tonsillar carcinomas seem to exhibit higher HPV detection indices compared to those found in oral and laryngeal carcinomas (3, 6, 9). Studies considering head and neck carcinomas as a single entity or considering the oropharynx and/or tonsils as anatomic sites within the oral cavity may overestimate the actual prevalence of HPV infection related to oral carcinomas. For the present study, patients presenting with lesions in the oropharynx were excluded.

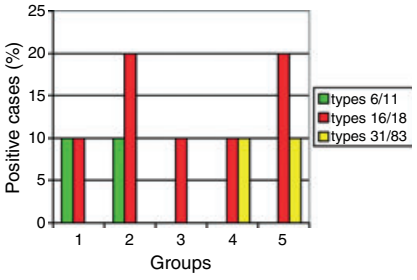

Fig. 2. Distribution of cases positive for the specific human papillomavirus (HPV) DNA probes within the analyzed groups.

In this study, the prevalence of HPV infection was 24%, which is markedly higher than was found in the control group. A discrete proportionality among indices found in leukoplakias with no dysplasia, leukoplakias with dysplasia, and OSCC was observed in our study, but it was not statistically significant. Separating the group of leukoplakias by degrees of dysplasia, it becomes more evident that there is no relation between the HPV infection index and the degree of malignant progression of each group of lesions, suggesting that HPV does not play a role in the progression of malignancy in oral lesions, although its participation, especially in early events of oral carcinogenesis, could be suggested because HPV detection in mild dysplasias was considerable. This is in accordance with a recent study that also found no relation of proportionality between the HPV infection indices in mild, moderate, and severe epithelial dysplasia (20). Another recent study, however, suggested the existence of this relation not by degree of dysplasia within the lesion, but by its malignant potential: lichen planus, which has little or no malignant potential, leukoplakia and actinic cheilitis, which has higher malignant potential, and OSCC were analyzed and high-risk HPV DNA was found in 9.2%, 16.7%, and 34.7% of cases, respectively (18).

Besides detection of HPV, its identification by genotyping is also crucial in attempting to relate HPV and oral carcinogenesis, as different types of HPV have different risks of causing malignant transformation in a tissue. HPV can transform oral keratinocytes, especially with chemical carcinogens, but only the high-risk HPV types are able to immortalize these cells. Oncoproteins produced by high-risk HPV types bind to and interfere more effectively with p53 and retinoblastoma protein, known tumor suppressor proteins, than the oncoproteins produced by low-risk types (19). Types 16 and 18 are the most frequently identified in studies concerning OSCC and its precursor lesions, besides other high-risk types, such as 31, 33, 39, 45, 52, 58, and 69 (11) and low-risk types, such as 6 and 11 (21), and this contributes to the suggestion that HPV has a role in oral carcinogenesis.

In our cases, types 6 and 11, known low-risk types, were identified only in groups 1 and 2, with no dysplasia or low degree of dysplasia. Considering all the cases that proved positive to the wide-spectrum probe, types 16 and 18, known high-risk types, were the most prevalent. These two findings suggest that there is a relationship between the presence of high-risk HPV types and premalignant and malignant lesions of the oral cavity, indicating high-risk HPVs as, at least, co-factors in oral carcinogenesis. This is in accordance with the majority of studies genotyping HPV in oral lesions, including a recent study, which also used the CSA-ISH method, that verified a marked higher prevalence of HPV types 16 and 18 in OSCC and its precursor lesions, whereas in oral squamous papillomas, benign lesions associated with HPV, the authors found a higher prevalence of types 6 and 11 (8).

Only one of our cases, belonging to the OSCC group, was positive simultaneously for two different specific probes, types 16/18 and 31/33. This has been observed before in oral lesions (5, 21). Infection with multiple types of HPV within the same lesion may be associated with an increased risk of development of epithelial dysplasia in cervical lesions. However, it is not known accurately whether certain types of HPV favor infection by other types of HPV, or whether a superinfection with multiple types of HPV can enhance malignant transformation (12).

Although PCR is the gold standard method for assessing HPV DNA, we decided to use the CSA-ISH method for the following reasons. First, in our service, we perform most of our studies on paraffin-embedded material retrieved from archives and this may result in poor material for PCR, mainly because of the size of the biopsy material embedded in paraffin and because the case has been used in a previous study. It is known that PCR requires a considerable amount of tissue with good-quality DNA to be performed properly. In most of our selected cases the size of the specimen included in paraffin was not sufficient to obtain satisfactory amplification and reliable results to detect this specific virus. This may be why some studies, even using this very sensitive method, find an extremely low prevalence

of HPV in premalignant and malignant lesions of the oral cavity. Second, another limitation of PCR is the impossibility of verifying the relation of the virus with the infected cells, to determine how much damage the virus is causing or to determine the position of the infected cells within the epithelium – these parameters may contribute to describing a causal relationship between HPV infection and disease (4).

In situ hybridization is a good alternative when PCR has limitations, because it can be performed on only one tissue section and with preservation of tissue morphology. However, conventional *in situ* hybridization is not very sensitive – it cannot detect <10 to 50 viral copies per cell. This is particularly unsatisfactory for research on HPV and carcinogenesis because most cervical carcinomas harbor fewer than 50 HPV copies per cell (5) as do OSCC (13). Our option then was to use CSA-ISH, which is a novel signal amplification method based on the catalyzed amplification of positive hybridization signals using biotinyl-tyramide complexes. This system is sensitive enough to detect one or two copies of HPV DNA, supposedly being as sensitive as PCR (5, 8). It is difficult to optimize this method and background staining can be misinterpreted as punctate HPV-positive staining (2, 7). However, there is an agreement that CSA-ISH is a reliable method, better than conventional in situ hybridization, to detect HPV DNA preserving tissue morphology, although ideally, it would be better to use it combined with a PCR technique (2, 5).

Our results showed a lack of correlation between HPV infection and degree of dysplasia in the oral epithelia of the analyzed groups. Nevertheless, the fact that the overall prevalence in these premalignant and malignant lesions was markedly higher than in the control group, and that high-risk types were the most frequently found within HPV-positive cases, strongly suggests that HPV is likely to play a role in oral carcinogenesis. Further studies with larger sample size, using different concomitant assays for viral detection, assessing different factors in viral infection and correlating them with biological markers are necessary to elucidate the relation between HPV and development of premalignant and malignant lesions of the oral cavity.

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