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Human papillomavirus in oral squamous cells carcinoma in a population of 75 Brazilian patients

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Abstract

Purpose: In the present study, we investigated the presence of human papillomavirus (HPV) DNA and viral types in 75 cases of oral squamous cells carcinoma from Brazil to obtain data that would contribute to a better understanding of the role of HPV in the pathogenesis of this tumor.

Materials and methods: DNA was extracted from paraffin-embedded tissue and amplified by polymerase chain reaction using a pair of primers designated PCO3+ and PCO4+ for the detection of a fragment of the human β-globin gene, followed by polymerase chain reaction for the detection of HPV DNA using a pair of generic primers, GP5+ and GP6+. Viral typing was performed by dot blot hybridization.

Results: Human papillomavirus DNA was detected in 18 (24%) of the 75 cases positive for the human β-globin gene. No significant association was observed between HPV and age, sex, or anatomical location of the tumor. The most prevalent viral type was HPV-18 (77,8%).

Conclusion: The low frequency of detection of HPV DNA in oral epidermoid carcinomas suggests a possible participation of the virus in the development and progression of only a subgroup of these tumors.

1. Introduction

Oral cancer is a severe and growing public health problem in Brazil. Its etiology is complex; and many factors, mainly smoking and alcohol, have been associated with this type of cancer [1]. In addition, biological agents such as viruses represent important factors able to induce neoplastic alterations. Among these agents, human papillomavirus (HPV) seems to play an important role in the induction of oral cancer.

The association between oncogenic HPVs and cancer has been well established for anogenital and uterine cervical carcinomas, but their relationship with oral squamous cells carcinoma (OSCC) is uncertain. Over the past years, various studies have investigated the role of HPVs in OSCC; but the results are highly controversial. So far, more than 130 different viral genotypes infecting the skin and mucosa have been described [2]. Most studies have indicated HPV-16 as the most prevalent HPV type in both oral and genital tumors [3].

In view of the importance of this virus in carcinogenesis, we investigated the presence of HPV DNA and the most frequent types in patients with OSCC.

2. Subjects and methods

Samples from 75 patients were collected from archived paraffin-embedded OSSC specimens from the Dr Luis Antônio Hospital Department of Pathology between 2000

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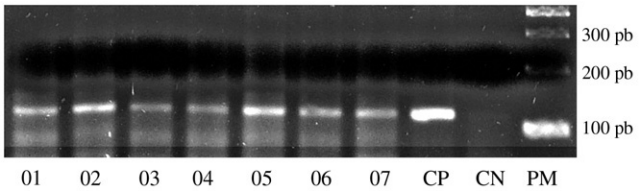

Fig. 1. Detection of β-globin gene DNA. Lanes 1 to 7, samples from patients; lane 8, C33 cell DNA without HPV infection; lane 9, negative control; lane 10, molecular marker (100 bp).

and 2003. Of the 75 patients investigated, 49 (65.3%) were men and 26 (34.7%) were women. Patient age ranged from 30 to 93 years, with a mean of 65.45 years. In the present study, all carcinomas were primary tumors located in the oral cavity, with 20 tumors involving the tongue, 17 the floor of the mouth, 14 the lower lip, 8 the jugal mucosa, 6 the palate, 5 the alveolar border, and 5 the retromolar trigone.

Ten 10-μm-thick histologic sections were obtained for DNA extraction, and two 3-μm sections were stained with hematoxylin/eosin for review by a pathologist.

DNA was extracted using the ammonium acetate-isopropanol technique, which does not require the use of phenol-chloroform. This method was developed by the research team of the Laboratory of Molecular Biology, Dental School of the University of São Paulo, and consisted of the initial removal of paraffin by baths in xylene heated to 65°C. Next, the tissues were hydrated in a decreasing ethanol series (absolute, 95%, 70%, and 50%). Afterward, 400-μL sterile lysis buffer (50 mmol/L NaCl, 5 mmol/L Tris-HCl, pH 8, 12.5 mmol/L EDTA, pH 8, and 0.25% sodium dodecyl sulfate) and proteinase K at a final concentration of 500 μg/mL were added to the tissue pellet of each sample; and the samples were incubated at 55°C for 3 to 5 days until complete dissolution of the material. Proteinase K (250 μg/mL) was added at 24-hour intervals. After this step, proteinase K was inactivated by incubation of the material at 95°C for 10 minutes. Next, 200 μL of a 4-mol/L ammonium acetate solution was added to each sample for protein precipitation, followed by 600 μL 100% isopropanol for DNA precipitation. Finally, the DNA pellets obtained were washed with 70% ethanol, dissolved in 50 μL Tris-EDTA buffer, and stored at −20°C.

To evaluate the efficacy of DNA extraction, all samples were submitted to polymerase chain reaction (PCR) for amplification of the human β-globin gene that is present in all human cells. For this, a pair of primers designated PCO3+ (5′CTTCTGACACAACTGTGTTCACTAGC3′) and PCO4+ (5′TCACCGCAAC TTCATCCACGTTCACC3′) was used, which amplifies a fragment of about 110 base pairs (bp) of the β-globin gene [4].

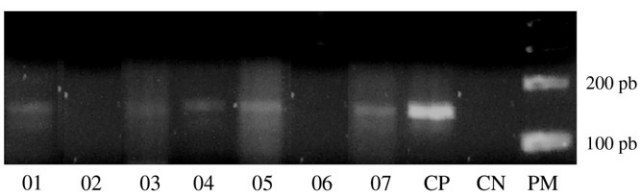

Fig. 2. Detection of HPV DNA in samples from patients with OSCC. Lanes 1 to 7, samples from patients; lane 8, HeLa cell DNA infected with HPV-18; lane 9, negative control; lane 10, molecular marker (100 bp).

Polymerase chain reaction was carried out in an Eppendorf thermocycler in a reaction mixture containing the following components in a final volume of 50 μL: 0.5 μmol/L of the PCO_3 and PCO_4 primers (Invitrogen, Life Technologies, Carlsbad, CA), 1.0 U Taq DNA polymerase (Biosystem, Campinas, Brazil), 20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP (Amersham Bioscience, Buckinghamshire, England), and 0.7 or 7 μL sample DNA. The PCR conditions for amplification of the β-globin gene were as follows: initial denaturation at 95°C for 4 minutes; followed by 40 cycles of amplification at 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute; and a final extension step at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis on 2.5% agarose gel (Nusieve) stained with ethidium bromide.

The samples positive for β-globin were analyzed by PCR regarding the presence of HPV DNA using pair of generic primers, GP5+ (5′TTTGTTACTGTGGTAGATACTAC3′) and GP6+ (5′GAAAAATA AACTGTAAATCAT ATTC3′) [5], which flanks a fragment of about 140 bp of the *L1* gene, a highly conserved sequence in the genome of mucosal (genital and oral) HPVs. The use of this primer pair permits the detection and amplification of this DNA segment from at least 23 individual mucosal (genital and oral) HPV types, including high-risk HPV types.

The reaction mixture contained 1.0 μmol/L GP5+/GP6+ (Invitrogen, Life Technologies), 1.0 U Taq DNA polymerase (Biosystem), 20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L deoxyribonucleotide triphosphate (Amersham Bioscience), and 0.7 to 7.0 μL DNA, in a final volume of 50 μL. The PCR conditions for the detection of HPV were as follows: initial denaturation at 95°C for 5 minutes; followed by 40 cycles of amplification at 95°C for 1 minute, 45°C for 2 minutes, and 72°C for 1.5 minutes; and a final extension step at 72°C for

Table 1
Comparison of sex and anatomical location between HPV-positive and HPV-negative cases (Natal/RN, 2005)

Clinical characteristics	Total	HPV positive		HPV negative		*P**
	n	n	%	n	%	
Sex						
Male	49	13	72.2	36	63.2	.747
Female	26	05	27.8	21	36.8	
Total	75	18	100	57	100	
Tumor site						
Tongue	20	02	11.1	18	31.6	.163
Mouth floor	18	04	22.2	14	24.6	
Others†	37	12	66.7	25	43.9	
Total	75	18	100	57	100	

* χ^2 test.
† Lip, jugal mucosa, palate, retromolar trigone, and alveolar border.

Table 2
Comparison of age between HPV-positive and HPV-negative cases (Natal/RN, 2005)

	Age				
HPV	n	Median	Standard deviation	Confidence interval (95%)	*P**
Negative	57	64.91	15.39	60.83-69.00	.859
Positive	18	65.67	16.53	57.45-73.89	

* Student *t* test.

10 minutes. The amplified PCR products were typed by dot blot hybridization [6] using radioactive probes specific for the 19 HPV types most common in mucosal (genital and oral) infections.

The results were analyzed statistically by the Student *t* test, Mann-Whitney test, and χ^2 test. The level of significance was set at 95% ($\alpha = .05$) for all tests. The research project was approved by the Ethics Committee of the Federal University of Rio Grande do Norte (no. 68/03).

3. Results

Of the 75 cases positive for the human β-globin gene (Fig. 1), HPV was detected in 18 (24%) (Fig. 2), including 13 (72.2%) men and 5 (27.8%) women. The χ^2 test showed no significant association between the presence of HPV and sex (Table 1). With respect to anatomical location, the most frequent site affected was the tongue, followed by the floor of the mouth, lip, jugal mucosa, palate, retromolar trigone, alveolar border, and unidentified anatomical sites, with the χ^2d test showing no significant association between HPV and anatomical location of the tumor (Table 1). The mean age of HPV-positive patients was 64.91 years, whereas the mean age was 65.67 years in negative cases, with this difference not being significant by the Student *t* test (Table 2). The viral types found in the 18 positive cases were the high-risk strains HPV-16 and/or HPV-18. HPV-18 was identified in 14 (77.8%) cases, double infection with HPV-16 and HPV-18 was observed in 3 (16.7%) cases, and HPV-16 was identified in only 1 case.

4. Discussion

The present study investigated the presence of HPV DNA in paraffin-embedded OSCC specimens by PCR because this technique is the most sensitive for this purpose. The sensitivity and specificity of PCR-based methods may vary, mainly depending on the primer types, size of the PCR product, reaction conditions, performance of the DNA polymerase used, and capacity of detecting multiple virus types [7]. In the present study, we used the GP5+/GP6+ primer pair because, in addition to detecting a variety of virus types, it amplifies a small fragment (150 bp). This fact is important when evaluating archived paraffin-embedded tissue, as done in the present study, which may show marked DNA fragmentation in some cases.

There is currently no consensus regarding the best method to quantify HPV in biological specimens, but one of the most accurate and controlled assays available is real-time PCR. However, this technique requires expensive equipment and reagents [8]. Ha et al [8], using a quantitative PCR method, detected HPV DNA in only 1 (2.9%) of the 34 specimens analyzed. However, it should be emphasized that these authors used only primers specific for HPV-16, a fact that might have underestimated the number of positive cases if other virus types were present in the sample.

Human papillomavirus DNA was detected in 18 (24%) of the 75 cases evaluated. In the present investigation, only high-risk HPV-16 and/or HPV-18 was identified, in agreement with other studies [7]. In most cases (14/18, 77.8%), only HPV-18 alone was detected; in 3 cases (16.7%), both HPV-18 and HPV-16 were identified; and in 1 case, only HPV-16 was detected. The present results agree with those reported by Shima et al [9], Giovannelli et al [10], and Lo Muzio et al [11], who detected HPV-18 in most of their samples at frequencies of 54%, 87.5%, and 87%, respectively. However, HPV-16 was the most frequent type in other studies [11-13]. These divergent results suggest that the higher prevalence of HPV-18 observed here might be related to geographic and environmental factors or to race, peculiar aspects of our sample.

Regarding clinical variables, in the present study, no significant difference in HPV detection was observed between sexes. Human papillomavirus DNA was detected in 13 (26.5%) of the 49 male cases investigated, whereas 5 (19.2%) of the 26 female cases were positive for the virus, in agreement with other investigators [3,10,14-16]. In contrast, Zhang et al [13] observed a higher frequency of HPV infection in men (81.3%) compared with that in women (60%).

With respect to patient age, in the present study, no significant difference was observed between HPV-positive and HPV-negative patients ($P = .859$), in agreement with the results of other investigators who observed no direct relationship between these factors [3,4,15]. However, Giovannelli et al [10] found that HPV-positive patients with OSCC were older than patients not infected with the virus. These results may suggest that HPV acts independently of age; however, considering that HPV is a sexually transmitted virus, younger patients with OSCC would have a higher chance of being infected with the virus.

No association between the anatomical location of OSCC and HPV was observed in the present study, a fact also reported by Zhang et al [13]. However, D'Costa et al [14] and Schwartz et al [17] found a higher frequency of HPV in tongue OSCC, whereas Premoli-De-Percoco and Ramirez [18] and Sugiyama et al [19] observed higher rates in cases in which the tumor involved the floor of the mouth and jugal mucosa.

Several authors had investigated the association between HPV and the tumoral stages and the size of primary tumor, as well as between this oncovirus and the excessive

consumption of alcohol and/or tobacco, and had observed that there was no significant association between the presence of the HPV and these aspects [1,3,11,13,17,20-24]. Based on these results, we decided not to include these data in the study.

5. Conclusions

In the present study, HPV DNA was detected in a low percentage of OSCC cases (24%), suggesting that HPV plays an important role in the development and progression of OSCC in only a small subgroup of tumors. HPV-18 was the most prevalent type, suggesting that this type was specifically related to the development of OSCC in the present samples. No significant association was observed between the presence of HPV and age, sex, or anatomical location of the tumor, thus indicating that these factors do not interfere with the pathogenesis of HPV in OSCC.

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