J Bras Patol Med Lab, v. 52, n. 1, p. 43-49, February 2016

10.5935/1676-2444.20160005

HPV detection in floor of mouth squamous cell carcinoma by PCR amplification

Detecção do HPV em carcinoma espinocelular de assoalho de boca por amplificação da PCR

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ABSTRACT

Introduction: Studies conducted during the last years, using new technologies for viral detection, permit to consider human papillomavirus (HPV) an etiologic factor for cervical cancer. Besides the relation to genital regions, other anatomic sites have been associated with HPV, including head and neck regions. **Objectives:** To investigate the prevalence of HPV infection in 35 samples from paraffin-embedded tissues using polymerase chain reaction (PCR)-deoxyribonucleic acid (DNA) amplification, and correlate it with demographic, clinical, and morphological factors and prognosis. **Materials and methods:** All samples were first amplified with human β-globin gene primers. Samples with positive amplification were subjected to HPV-DNA detection with general GP5 and GP6 primers. **Results:** Only 30 samples were amplified for the β-globin gene. No floor of mouth squamous cell carcinoma cases showed amplification of HPV DNA. **Discussion:** The absence of HPV-DNA amplification does not suggest that this virus is absent from the process of oral carcinogenesis, since the selected sample is not in the risk group for the development of oral cancer associated with HPV infection. **Conclusions:** No correlation was found between HPV infection and floor of mouth carcinogenesis, however further studies are necessary.

Key words: squamous cell carcinoma; HPV-DNA tests; polymerase chain reaction; mouth neoplasms.

INTRODUCTION

Oral and oropharyngeal cancer represents a public health problem worldwide, standing out as one of the most common types of cancer and showing low chances of cure and five-year survival[1]. With approximately 300 thousand new cases every year, the mouth is considered the eighth anatomical site mostly affected by cancer[2]. For 2014, Instituto Nacional de Câncer (Inca)[3], estimated 11,280 new cases of oral cancer among men and 4,010 new cases among women in Brazil. Squamous cell carcinoma (SCC), which develops from epithelial cells, represents the most common neoplastic process in the mouth, being responsible for nearly 90% of all malignancies of the oral cavity[4].

Oral and oropharyngeal cancer is nowadays well correlated with tobacco and alcohol abuse, and some studies have also shown the prevalence of lower lip cancer due to chronic sun exposure[5-7]. Besides, other factors might be associated with the development of oral and oropharyngeal malignancies; among them, cytomegalovirus, herpes virus and human papillomavirus (HPV) have been indicated as probable oncogenic agents[8, 9].

Evidences that HPV could be related to carcinogenesis were first described by Syrjänen *et al*. (1983)[10], in 1983, when they noticed morphological alterations in cervical SCC samples suggesting the presence of HPV in 16 of 40 analyzed fragments. Since then, several studies have been carried out aiming at the detection of HPV in malignant lesions of genital and oral mucosas, with prevalence rates ranging from 0% to 100%. This wide variability reported in the literature might be due to the differences in populations, sample processing and HPV detection methodologies of each study[11-13].

Recently, over 200 HPV types have been identified in many different human lesions, being categorized as low- and high-risk HPVs, depending on their potential to lead the epithelium to carcinogenesis. In the oral cavity, low-risk HPV types 6 and 11 are

First submission on 21/06/15; last submission on 22/06/15; accepted for publication on 09/11/15; published on 20/02/16

the most prevalent in benign lesions, as the high-risk types 16 and 18 are respectively the most found in malignant ones. The viral genome of HPV encodes approximately eight open reading frames (ORFs), which can be sectioned into three parts: an early (E) region, which encodes proteins necessary for viral replication and transcription; a late (L) region, which encodes structural proteins of the viral capsid (L1 and L2); and a non-coding region segment, named long control region (LCR), which contains elements that regulates the viral deoxyribonucleic acid (DNA) replication and transcription[14, 15]. By definition, the nucleotide sequences of the E6, E7, and L1 ORFs of a new HPV type should be no more than 90% homologous to the corresponding sequences of known HPV types. HPVs have further been classified into subtypes, when they have 90% to 98% sequence similarity to the corresponding type; and variants, when they show more than 98% sequence homology to the prototype[15].

Although HPV is the major etiologic factor for cervical cancer, the real role of this virus in oral carcinogenesis has not yet been clearly defined, as other etiologic factors might be associated with the development of malignant lesions in the mouth. Oral HPV infection is mainly acquired via sexual transmission, and increasing prevalence rates are attributed to changes in sexual behaviors of the global population; nevertheless, it has been suggested the HPV might be transmitted through kissing, as well as via intrapartum transmission[15-17]. Great differences between prevalence rates in studies found in the literature contribute to many doubts[12, 13]. On the other hand, a great majority of studies show HPV genome in at least about 20% of cases[18, 19].

Nowadays, many methods which can detect HPV in mucosal lesions are known (serologic, *in situ* hybridization, polymerase chain reaction (PCR), Southern blot, Northern blot, dot blot); however, every method has its strengths and weaknesses. *In situ* hybridization was the initial assay of choice for HPV detection before more sensitive molecular techniques were developed. The presence of HPV DNA in oral cavity samples is known to be inconsistent[20, 21]. PCR amplification has been used as a strongly sensitive assay for HPV-DNA detection in any given sample, and more recently it has been considered the gold standard for detecting HPV DNA in SCC samples, once this method presents strong advantages, like high sensitivity, type-specific and formalin-fixed paraffin-embedded (FFPE) tissue acceptance[22, 23].

OBJECTIVES

This study aimed to detect HPV DNA by the PCR method in floor of mouth SCC, and correlate it to pathologic, clinical and demographic factors, besides its influences on the prognosis of the affected patients.

MATERIALS AND METHODS

Ethical aspects

This research was performed after approval by the ethics committee of Universidade Estadual Paulista (Unesp), under process number 2005-00689.

Specimens

Thirty-five samples were obtained from patients with floor of mouth SCC treated in the Oral Oncology Center of Unesp during 1991-2005.

Demographic, clinical, pathological and therapeutic data

Data were obtained from individual records.

Preparation of samples

Each paraffin-embedded tissue was histologically analyzed. Five 10-µm sections were taken from each block for DNA extraction.

DNA extraction and quantification

All the samples were processed using the QIAamp DNA mini kit (QIAGEN Ltd., Crawley, UK). The method for nucleic acid extraction was employed in accordance to the manufacturer's protocol. DNA concentrations were estimated using NanoDrop® ND-1000 Spectrophotometer, and the processed samples were stored at -20ºC.

PCR of β-globin

A fragment of the human β-globin gene was amplified with primers PC04 and GH20 (**Table 1**). Positive β-globin amplification proved that the sample contained enough DNA and that no PCR inhibitors were present. If the result of the control gene amplification was negative, the sample was extracted and amplification was repeated. Each amplification reaction was carried out in a total volume of 25 µl and contained 1U Taq DNA polymerase (Invitrogen Life Technologies®, Brazil);

TABLE 1 – Primer pair DNA sequences

Primer*	Sequence (5'-3')	Fragment bp	Reference
PC04	CAACTTCATCCACGTTCACC	268	Bell *et al*. (1993)[24]
GH20	GAAGAGCCAAGGACAGGTAC		
GP5	TTTGTTACTGTGGTAGATAC	142	Snijders *et al*. (1990)[25]
GP6	AAAAATAAACTGTAAATCA		

**Invitrogen Life Technologies®, Brazil.*

2.5 µl reaction buffer 10× (10 mM Tris-HCl pH 8 and 50 mM KCl - Invitrogen Life Technologies®, Carlsbad, CA, USA); 4 mM $MgCl_2$ (Invitrogen Life Technologies®, Carlsbad, CA, USA); 1.5 µl deoxynucleotide (dNTP) (deoxyribonucleoside 5'-triphosphates – deoxyadenosine triphosphate [dATP], deoxycytidine triphosphate [dCTP], 2'-deoxyguanosine 5'-triphosphate [dGTP] and 2'-deoxythymidine 5'-triphosphate [dTTP] – Amersham Biosciences, Piscataway, NJ, USA); 15 pmol of each primer (PCO4/GH20 – Invitrogen Life Technologies®, Brazil) and 10.9 µl UltraPure™ Water (Invitrogen Life Technologies™, Carlsbad, CA, USA). The sensitivity assays were performed by amplification of human blood and biopsy of condyloma. Negative controls were carried out in the absence of template DNA. Special care was taken to avoid contamination. Conditions to control gene primer sets consisted of an initial denaturation step of 10 minutes at 94ºC, and 40 cycles of one minute of denaturation at 94ºC, one minute of primer annealing at 65ºC, two minutes of extension at 72ºC, and a final extension step of seven minutes at 72ºC. After amplification, 10 µl of the PCR product was analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide.

HPV detection by PCR

Samples with positive amplification of the control gene were subjected to HPV-DNA detection with general primers GP5 and GP6, which generate a 142-bp long fragment of the L1 gene[24, 25] (Table 1). Each amplification reaction was carried out in a total volume of 25 µl and contained 1U Taq DNA polymerase (Invitrogen Life Technologies®, Brazil); 2.5 µl reaction buffer 10× (10 mM Tris-HCl pH 8 and 50 mM KCl – Invitrogen Life Technologies®, Carlsbad, CA, USA), 4 mM $MgCl_2$ (Invitrogen Life Technologies®, Carlsbad, CA, USA); 1.5 µl dNTP (deoxyribonucleoside 5'-triphosphates – dATP, dCTP, dGTP and dTTP – Amersham Biosciences, Piscataway, NJ, USA); 15 pmol of each primer (PCO4/GH20 – Invitrogen Life Technologies®, Brazil) and 10.9 µl UltraPure™ Water (Invitrogen Life Technologies™, Carlsbad, CA, USA). DNA from biopsy of condyloma and HeLa, a human cervical cancer cell line containing one or two copies of the HPV 18 genome per cell, were included in every run as positive controls. Blank control was used as negative control. Conditions for HPV primer sets consisted of an initial denaturation step of 10 minutes at 94ºC, and 40 cycles of one minute of denaturation at 94ºC, one minute of primer annealing at 53ºC, 30 seconds of extension at 72ºC, and a final extension step of seven minutes at 72ºC. Each PCR product was analyzed by electrophoresis with 8% polyacrylamide gels.

Statistics analysis

The results were studied using chi-square (χ^2) and Fisher tests.

RESULTS

General characteristics of samples are displayed in **Table 2**.

TABLE 2 – General characteristics in 35 patients with floor of mouth SCC

Variables	Number of cases (%)
Sex	
Male	33 (94.3)
Female	2 (5.7)
Age	
< 60	11 (31.5)
≥ 60	24 (68.5)
Clinical staging	
0	1 (2.9)
I	5 (14.3)
II	16 (45.7)
III	8 (22.8)
IV-A	5 (14.3)
Histological grade*	
I	10 (28.6)
II	19 (54.2)
III	5 (14.3)
Neck dissection	
Yes	26 (74.3)
No	9 (25.7)
Tobacco consumption*	
Yes	31 (88.5)
No	3 (8.6)
Alcohol consumption*	
Yes	24 (68.5)
No	10 (28.6)

**1 (2.9%) no information in the records; SCC: squamous cell carcinoma.*

β-globin gene amplification

Initially, the 35 samples of floor of mouth SCC were amplified for the β-globin gene, a constitutive gene. There was amplification in 30 (85.7%) of the 35 samples; the others were excluded from the study (**Figure 1**).

HPV-DNA amplification

PCR amplification for HPV was carried out in 30 samples positive for β-globin. Only positive controls amplified HPV DNA; none of the SCC samples showed HPV DNA (**Figure 2**).

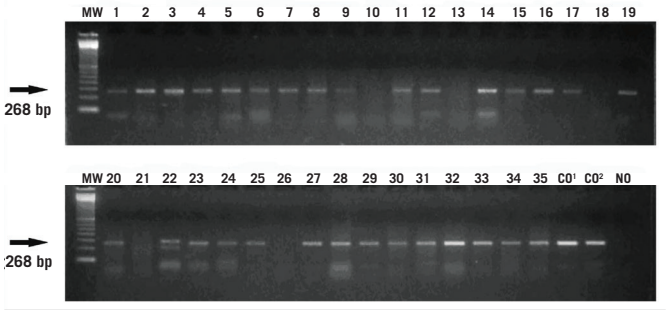

FIGURE 1 – *Electrophoresis in 2% agarose gel stained with ethidium bromide. β-globin DNA amplification (268 bp) from the 35 floor of mouth squamous cell carcinoma samples*

DNA: deoxyribonucleic acid; MW: 100 bp DNA ladder; CO¹: human genomic DNA; CO²: condyloma DNA; NO: no DNA control; MW: molecular weight.

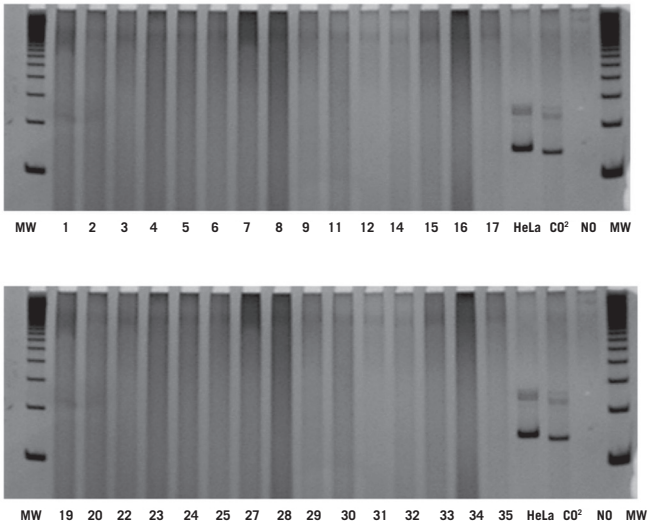

FIGURE 2 – *Electrophoresis in 8% polyacrylamide gel stained with silver nitrate. HPV amplification (142 bp)*

HPV: human papillomavirus; MW: 100 bp DNA ladder; HeLa: DNA from HPV 18-infected cervical carcinoma cell line; CO²: condyloma DNA; NO: no DNA control; DNA: deoxyribonucleic acid; MW: molecular weight.

Statistics analysis

X^2 test was used and none of the variables showed correlation with HPV DNA presence.

DISCUSSION

Recent works have shown that about 99.7% of the women affected by cervical cancer have been exposed to HPV once in their lives, leading this infection to be widely considered the main etiologic factor for cervical cancer development[26]. Although the relationship between cervical cancer and HPV infection is well established, the real role of this virus in oral carcinogenesis remains controversial, once, despite several studies have demonstrated the presence of the HPV in oral SCC (OSCC), in most cases of HPV-related OSCC, patients are exposed to etiologic factors that are strongly associated with oral carcinogenesis[6, 27].

Prevalence rates of HPV in oral malignant lesions range from 0% to 100% due to many factors, including the type of tissue used for analysis. Most studies in the literature present this prevalence in at least 18.9%[18]. PCR amplification is a method that consists of multiplying DNA sequences exponentially, making the detection of HPV DNA in human tissues easier and more sensitive than other methods[15, 21]. With this method, greater accuracy can be achieved by using fresh frozen tissues as samples instead of formalin-fixed paraffin-embedded (FFPE) ones[28]. On the other hand, studies have shown FFPE samples are adequate and might be used for HPV DNA detection[22]. We chose paraffinized tissues to perform the PCR amplification, because this is a retrospective study, and the available time is important for choosing the sample processing method[23]. DNA extraction was performed using the QIAamp DNA mini kit, recommended for paraffinized tissue, inasmuch as it gives higher quality DNA detection when analyzed by spectrofotometry[29]. The use of different primer pairs designed to differentiate regions of HPV genome is another factor that changes HPV detection rate. The GP5/GP6 primer pair, used in this study, was described by Snijders *et al*. (1990)[25] and derived from the L1 (late) region of the HPV genome. Although this region is more suitable for PCR amplification and HPV detection[30], in a review conducted by Syrjänen *et al*. (2011)[31], the authors reported no distinction between the PCR primers used in HPV detection. The use of PCR primers specific for the E2 gene is not recommended, since this gene is a common point of alteration in integrated HPV[21].

None of the cases of floor of mouth SCC analyzed in the present work was positive for HPV DNA. This data must not be attributed only to the HPV DNA detection method utilized: previous studies have shown patients affected by HPV-negative head and neck SCC present a stronger association with other risk factors, as tobacco

and alcohol abuse, and poor oral hygiene[18, 19]. This corroborates our results, once 88.5% and 68.5% of the patients were, respectively, current tobacco and alcohol users, which, as said before, are the greatest etiologic factors related to OSCC[32]. Most patients affected by OSCC in this study were older than 60 years, age which, according to previous works, is not the most likely to oral cancer development due to HPV infection. This characteristic is widely more common in young patients, due to changes in sexual behavior through the last years, which increased HPV infection rates among young people[16, 17, 19]. Furthermore, in the present study, we selected SCC of the floor of mouth as samples, since this is not the most likely anatomical site for development of OSCC associated with HPV infection – according to the literature, the most commonly affected site by HPV-positive OSCC is the tongue base, followed by the palatine tonsils[15, 20, 23, 28].

Considering the occurrence of no positive results for viral DNA amplification in this study, there was no association of demographic, pathological and clinical characteristics with the presence of HPV DNA. Prognosis did not correlate with the presence of HPV DNA like in previous studies, which show a better prognosis of HPV-positive OSCC than HPV-negative OSCC, suggesting that other studies must be performed aiming to evaluate the prognosis of OSCC associated with HPV infection[12]. Whereas some authors have stated that HPV-DNA detection by PCR amplification using FFPE samples is not a reliable technique, our results must not be only attributed to our HPV-DNA detection method, but also, and especially, to sample selection. Even so, further studies are required to confirm this data, testing the several HPV detection methods available, as well as sample selection, sample size and sample processing.

According to Zur Hausen (1991)[33], HPV infection alone is not able to induce carcinogenesis, however the HPV oncogenic potential might be considered in association with some conditions, such as exposure to physical agents (sun light), chemical agents (tobacco, alcohol) and other viruses (herpes virus, cytomegalovirus). Current literature shows many works using different HPV detection techniques producing different results, what is consistent with the concept of multifactorial etiology of oral cancer.

CONCLUSION

Based on epidemiological studies, the absence of HPV-DNA amplification in floor of mouth OSCC found in this study does not suggest that this virus is absent from the carcinogenesis process in the oral cavity. Therefore, further studies are necessary, considering the large number of factors that may influence this clinical condition.

CONFLICTS OF INTERESTS

The authors declare the absence of any conflict of interest.

RESUMO

Introdução: *Estudos realizados durante os últimos anos permitem considerar a infecção pelo papilomavírus humano (HPV) um fator etiológico para o câncer cervical. Apesar da íntima relação desse vírus com as regiões genitais, outras localizações anatômicas têm sido associadas a tal infecção, inclusive as regiões de cabeça e pescoço.* ***Objetivos:*** *Investigar a prevalência da infecção pelo HPV em 35 amostras parafinadas de carcinoma espinocelular de assoalho de boca, utilizando a amplificação da reação em cadeia da polimerase (PCR) como método de detecção do ácido desoxirribonucleico (DNA) viral, bem como correlacionar aspectos demográficos, clínicos e morfológicos com o prognóstico da doença.* ***Materiais e métodos:*** *Todas as amostras foram inicialmente amplificadas com o* primer *para detecção do gene da β-globina humana. As que tiveram amplificação positiva para o gene da β-globina foram então submetidas à detecção do DNA viral com os* primers *GP5 e GP6.* ***Resultados:*** *Apenas 30 amostras foram amplificadas para o gene β-globin. Nenhuma das amostras de carcinoma de assoalho de boca demonstrou resultado positivo para amplificação do DNA viral.* ***Discussão:*** *Apesar de a influência do vírus na carcinogênese oral não ter sido comprovada devido à ausência de DNA viral nas amostras, a relação não pode ser descartada, uma vez que as amostras selecionadas não se encontravam em grupo de risco para o desenvolvimento de carcinoma espinocelular de boca associado à infecção pelo HPV.* ***Conclusão:*** *Não foi detectada relação entre a infecção pelo HPV e o carcinoma de assoalho de boca, no entanto mais estudos são necessários sobre o tema.*

Unitermos: *carcinoma de células escamosas; testes de DNA para HPV; reação em cadeia da polimerase; neoplasias bucais.*

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