Detecção do papilomavírus humano (HPV) em carcinoma espinocelular de lábio: correlação com aspectos clínicos e fatores de risco

Human papillomavirus (HPV) detection in lip squamous cell carcinoma: correlation with clinical aspects and risk factors

Resumo

O papilomavírus humano (HPV) está associado a um largo espectro de lesões em humanos e tem sido ligado à carcinogênese oral. O objetivo deste estudo foi investigar a presença do DNA do HPV em pacientes com carcinoma espinocelular de lábio e correlacioná-la com aspectos clínicos e fatores de risco. Foram estudados 33 pacientes com carcinoma espinocelular de lábio. Destes, 30 pacientes foram positivos para o gene da beta-globina humana e então foram testados para o DNA do HPV com uso da reação em cadeia de polimerase em duas etapas (PCR e nPCR) com os oligonucleotídeos iniciadores MY11/MY09 e GP5+/ GP6+. O DNA do HPV foi detectado em 43,33% dos 30 pacientes analisados. Não houve associação com os fatores de risco analisados.


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Financiamento: FUNDUNESP Processo nº 00149/07.
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Abstract

Human papillomavirus (HPV) is associated with a wide spectrum of lesions in humans, and it has been linked to oral carcinogenesis. The aim of this study was to investigate the presence of HPV DNA in patients with lip squamous cell carcinoma and to correlate it with clinical characteristics and risk factors. We studied 33 patients with lip squamous cell carcinomas. Of these, 30 were positive for human beta globin gene and tested for HPV DNA, using polymerase chain reaction in two steps (PCR and nPCR) with MY11/MY09 and GP5+/GP6+ primers. HPV DNA was detected in 43.33% of patients analyzed. There was no association with the risk factors analyzed.

Keywords: Epidemiology. Laboratory research. Squamous cell carcinoma. Papillomaviridae. Polimerase chain reaction. Oligonucleotides.

Introduction

According to the latest global estimate for 2008, cancer is responsible for approximately 7.6 million deaths per year. Head and neck cancer is a collective term that defines malignant tumors located in upper aerodigestive tract. This anatomical region includes oral cavity, pharynx and larynx. About 40% of the head and neck carcinomas occur in the oral cavity (lip, base of tongue, tongue, gums, oral floor and palate). Squamous cell carcinoma (SCC) represents approximately 90% of all oral malignancies and SCC of the lip comprising 25% to 30% of all oral sites.

Oral and oropharynx carcinomas accounting for 460,000 new cases and 250,900 deaths annually. In Brazil, the estimated incidence of oral cancer for 2010, including lip, is 10.64/100,000 inhabitants/year among men and 3.76/100,000 inhabitants/year among women. According to IARC’s lip cancer incidence in Brazil for 2007 ranging of 0.8/100,000 (Brasília) to 1.4/100,000 (São Paulo) for man and ranging 0.1/100,000 (Cuiabá) to 0.5/100,000 (Goiânia) for woman.

The highest prevalence of SCC occurs between the sixth and seventh decades of life. The lower lip is the oral site more affected by SCC and usually diagnosed in stage I. South of Australia is among the regions with the highest incidence of lip SCC, with 13.49 cases/100,000 inhabitants/year in men and 3.21 cases/100,000 inhabitants/year in women. In Europe, specifically in Spain, lip SCC represents a major impact on men (12.70 cases/100,000 inhabitants/year), and in Switzerland presents the highest incidence in women (0.83 cases/100,000 inhabitants/year). In France, the lip, mouth and pharynx cancer incidence reaches 38.5/100,000. In Brazil, according to the estimate of National Institute of Cancer for 2008, the incidence of oral cancer was 14.45/100,000 in men and 4.83/100,000 in women.

Oral cancer etiology is multifatorial and the main risk factors are smoking and alcohol consumption. Moreover, about 10% to 20% of patients with oral cancer are
non-smokers and non-drinkers suggesting that other factors, including certain viruses, may have implications on the oral carcinogenesis\textsuperscript{11,12}. Among the viruses that appear to be related to oral cancer etiology is the human papillomavirus (HPV)\textsuperscript{11-13}. HPV role in the etiology of cervix carcinoma is clear, however its participation on carcinogenesis in oral cavity and other anatomical sites such skin, esophagus, paranasal conjunctiva, bronchus, larynx, oropharynx only is suggested\textsuperscript{13}. Syrjänen and cols. described in 1983\textsuperscript{14} a possible link between HPV and oral cancer due to the observation of cytopathic alterations in oral lesions similar to those typically induced by HPV previously found in cervix cancer. Regarding the significance of HPV DNA presence in prognosis of patients with oral SCC, literature data are controversial\textsuperscript{15-18}. However, its has been suggested differences between patients infected and no infected with HPV in relation to sex, age, tumor regions, histological grade, regional metastasis, rate of recurrence and survival\textsuperscript{18}. Due to the great controversy in the literature, further studies are needed to help clarify the remaining questions that still persist. The aim of this study was to investigate HPV DNA presence in biopsies of lip SCC and correlate with clinical aspects and risk factors.

**Materials and Methods**

This study was reviewed and approved by Research Ethics Committee of São Paulo State University – Dentistry School of Araçatuba. Thirty-three caucasian patients presenting lip SCC confirmed by histological diagnosis participated in the study.

A questionnaire was completed at the initial patient trial asking about age, sex, occupation, tobacco and alcohol consumption and sun exposure. If the patient had oral cancer and none of this risk factors above the risk factor is described as “other risk factors”. Smokers were considered those that smoked regularly during any time of life, even those who stopped. Non-smokers were those who never used tobacco. The same criteria were used for drinkers and non-drinkers classification. Information about TNM classification, location site of the primary tumour and degree of histological differentiation were obtained. The TNM classification of malignant tumours is a cancer staging system that describes the extent of cancer in a patient’s body: “T” describes the size of the tumor and whether it has invaded nearby tissue, “N” describes regional lymphnodes that are involved and “M” describes distant metastasis.

All biopsy specimens were graded by the same pathologist according to Broders\textsuperscript{19}.

**DNA extraction**

Sections were obtained from 33 paraffin blocks in order to obtain 25 mg of material. DNA extraction was performed with QIAamp DNA Mini Kit\textsuperscript{®} (QIAGEN Ltd, Crawley, UK) according to manufacture’s instructions. Purity of DNA was accessed spectrophotometrically (NanoDrop\textsuperscript{®} ND-1000 UV-Vis) and samples stored at -20°C.

**PCR amplification**

From thirty three cases, three did not show positivity for beta globin gene after PCR using primers GH20 and PC04 (Invitrogen Life Technologies\textsuperscript{®}, Brazil) as reported by Bell et al.\textsuperscript{20}. Therefore thirty cases were employed in this study. After confirmation of the presence and integrity of genomic human DNA, 150 to 300 ng of DNA were tested for HPV DNA by PCR. In first PCR round a mixture with 0,02 mM of each degenerate consensus primers\textsuperscript{21}. MY11 and MY09 (Invitrogen Life Technologies\textsuperscript{®}, Brazil), was used to amplify fragments of 450 bp from L1 late region of viral genome. The remaining reaction components were: 10.9 microlitres of ultra-pure water, 2.5 microlitres PCR buffer 10X (Invitrogen Life Technologies\textsuperscript{®}, Carlsbad, CA, EUA), 4mM MgCl2 (Invitrogen Life Technologies\textsuperscript{®}, Carlsbad, CA, EUA), 15 pmol dNTPs and 1 unit of Platinum Taq DNA polymerase (Invitrogen Life Technologies\textsuperscript{®}, Carlsbad, CA, EUA). Approximately 150-300
ng of genomic DNA from each sample were added to mixture. Same quantity of Hela cells, with up to 4 copies of HPV-18 per cell, was used as positive control for HPV infection. The negative control was composed by all PCR components except DNA. The mixture was allowed to 94°C for 10 min, 40 PCR cycles (94°C for 1 min; 55°C for 1 min; 72°C for 40) and 72°C for 4 minutes. Two microlitres of the product from the PCR reaction were used in all thirty samples for nested PCR (nPCR) reaction directly in a microtube containing: 0.02 mM of each primer GP5+ and GP6+ (Invitrogen Life Technologies®, Brazil), which produce a 150 pb DNA fragment\textsuperscript{22}. The remaining reaction components and conditions were as described for the first round of PCR, except the annealing temperature that was reduced to 43°C. Ten microlitres of the nPCR products were fractionated by electrophoresis in a 8% polyacrylamide gel, for 3 hours at 100 volts. Band visualization was performed by staining with silver nitrate solution. Samples were scored as either HPV DNA-positive or negative based on inspection of silver nitrate stained bands. PCR amplification was performed in triplicates for each sample. Samples were classified in positive or negative based on gel analysis. Presence or absence of HPV DNA were correlated with the following variables: gender, age, clinical staging, histologic grade (cell differentiation), survival, consumption of alcohol, smoking and solar radiation exposition.

Statistical analysis

The data were analyzed using Qui-square test and Fisher's exact test with Epi Info 6 Statistical Analysis System Software (1997, Centers for Disease Control and Prevention, USA). Differences were considered to be statistically significant when $P$ values were less than 0.05. Survival rate was measured in years, comprehending the period from diagnosis date until death, confirmed by death certificate, or the last contact for alive patients. Kaplan Meyer was employed for global and free disease survival.

Results

In this study thirty three patients were selected, but three were not positive for β-globin (without DNA), then thirty samples have been analyzed by PCR and re-analyzed by nPCR. Overall, HPV DNA sequences were not amplified by PCR, but were amplified by nPCR in 43.33% of the samples (Figure 1). There was not statistical significance between demographic characteristics of patients and HPV DNA presence (Table 1). Disease-free survival average was 9.3 years. The survival rate of 5 years was 89.6% and showed 9.54 and 9.14 years for HPV DNA positive and negative groups, respectively (Figure 2). There was only one death by lip SCC. There was not statistical difference between mean survival rate of HPV DNA positive and negative groups.

Discussion

Head and neck SCC are correlated with broadly varying rates of HPV DNA incidence worldwide\textsuperscript{23}. These differences in the detection of HPV DNA in oral SCC suggest a potential difference of PCR based methodology in the ability to amplify fragments of different sizes and specific types of HPV, according to the DNA detection method used. They can also be the result of different material sources (smears, fresh, frozen, paraffin embedded), anatomic site, diverse population, PCR conditions, design of primers and number of samples analyzed. PCR method used in this study is one of the most sensitive to detect HPV DNA\textsuperscript{3}.

The primers generally used in HPV DNA researches are MY09/MY11\textsuperscript{21}, GP5/GP6\textsuperscript{24} or GP5+ / GP6+\textsuperscript{22}. PCR with consensus primers M09/MY11 has shown to be less sensitive compared to the PCR in two stages (nPCR)\textsuperscript{25-27}, employed in the present study.

PCR studies with paraffin-embedded tissues have showed worst results when compared with nPCR\textsuperscript{28-30}, fact ratified in this study.

Differences between populations with oral SCC were reported in a systematic review.
of studies that evaluated HPV DNA detection methods used in several continents\textsuperscript{23}. For European and Asian countries the rates ranged between 16% and 33%\textsuperscript{23}, but when considered the Latin American population the rates ranged between 50% and 60% for oral SCC\textsuperscript{31,32}. We agree with Cañadas\textsuperscript{33} that reports that infection with HPV DNA is directly linked to early sexual experience, number of sexual partners and sexual contact with multiple partners, referring that these facts can increase the rates of HPV infection.

We believe that lip SCC is an oral pathology that has its own specific behavior, therefore has a particular HPV infection rate. Few studies evaluated exclusively lip SCC, including this location among all oral SCC, were most of them ranged from 1 to 7 cases with HPV DNA detection range from 0% to 33.34\%\textsuperscript{17,28,35,36}. The largest studies evaluating HPV DNA detection of in lip SCC was a multicenter study held in Sweden, Norway and Finland, which included 57 cases and found 4% DNA HPV positive, in a population greatly different from of latin american\textsuperscript{35}.

Detection rate of HPV DNA similar to present work was found by Riethdorf\textsuperscript{15} that evaluated 18 cases of lip SCC by PCR using frozen tissues and found 50\% of positivity.

Regarding the survival rate of 5 years in

\begin{table}[h]
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\begin{tabular}{ l c c c c }
\hline
\textbf{Variáveis} & \textbf{Pacientes} & \textbf{HPV +} & \textbf{HPV -} & \textbf{P*} \\
 & (n=30) & (n=13) & (n=17) & \\
\hline
Gênero & & & & \\
Masculino & 26(86,7) & 10(76,9) & 16(94,1) & \\
Feminino & 04(13,3) & 03(23,1) & 01(5,9) & 0,20 \\
Idade & & & & \\
<60 & 14(46,7) & 06(46,2) & 08(47,1) & \\
\geq60 & 16(53,3) & 07(53,8) & 09(52,9) & 0,96 \\
Estadiamento & & & & \\
I & 18(60,0) & 07(53,8) & 11(64,7) & \\
II & 05(16,7) & 04(30,8) & 01(5,9) & 0,27 \\
III/IV & 07(23,3) & 02(15,4) & 05(29,4) & \\
Grau histológico & & & & \\
Bem diferenciado & 09(30,0) & 05(38,5) & 04(23,5) & \\
Moderadamente diferenciado & 20(66,7) & 08(61,5) & 12(70,6) & 0,49 \\
Pouco diferenciado & 01(3,3) & -- & 01(5,9) & \\
Exposição solar & & & & \\
Sim & 23(76,7) & 08(61,5) & 15(88,2) & 0,10 \\
Não & 07(23,3) & 05(38,5) & 02(11,8) & \\
Tabagismo & & & & \\
Sim & 19(63,3) & 07(61,5) & 12(64,7) & \\
Não & 11(36,7) & 04(38,5) & 07(35,3) & 0,86 \\
Etilismo & & & & \\
Sim & 15(50,0) & 09(69,2) & 06(35,3) & \\
Não & 15(50,0) & 04(30,8) & 11(64,7) & 0,06 \\
Outros fatores de risco & & & & \\
\chi^2 ou Teste de Fisher & 02(6,67) & 02(100) & 0(0) & \\
\hline
\end{tabular}
\caption{Tabela 1 – Relação entre a presença do DNA do HPV, variáveis clínico-patológicas e fatores de risco em pacientes com carcinoma espinocelular de lábio.}
\end{table}

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Pouco diferenciado & 01(3,3) & -- & 01(5,9) & \\
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Tabagismo & & & & \\
Sim & 19(63,3) & 07(61,5) & 12(64,7) & \\
Não & 11(36,7) & 04(38,5) & 07(35,3) & 0,86 \\
Etilismo & & & & \\
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Não & 15(50,0) & 04(30,8) & 11(64,7) & 0,06 \\
Outros fatores de risco & & & & \\
\chi^2 ou Teste de Fisher & 02(6,67) & 02(100) & 0(0) & \\
\hline
\end{tabular}
\caption{Table 1 – Relationship between presence of HPV DNA, clinical-pathological variables and risk factors for patients with lip squamous carcinoma.}
\end{table}
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lip SCC, Ogura\textsuperscript{16} presented 82.5%, despite the fact that his study had 75% of the lesions with clinical staging I and about 43% were well differentiated. In our study 60% of the lesions were in stage I and 30% were well differentiated, despite that survival rate was higher (89.6%).

As for the other variables (gender, age, clinical staging, histologic grade, tobacco and alcohol) there was no statistically significant difference in the association of any of these variables and the presence of HPV. This lack of association between HPV infections and these variables was also observed in other studies\textsuperscript{15,36}. We believe that the lip can be only a reservoir for the human papillomavirus.

The association nearest to be statistically significant was between the presence of HPV in patients alcoholism (p=0.06544). It is possible that with a higher patient number we could find a significant correlation.

An interesting finding in this study was that two HPV positive cases had no exposure to any of the etiological factors evaluated (solar radiation, use of tobacco, and alcohol), justifying further investigation to elucidate the role of HPV.

Our findings showed that the presence of HPV DNA was detected in 43.33% of samples of lip SCC samples studied. The presence of viral particles of HPV DNA had no correlation with age, sex, smoking and consumption of alcohol, solar radiation, clinical staging, histological grade or survival, nevertheless more studies are necessary to understand the real HPV role in lip carcinogenesis.
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Rev Bras Epidemiol 2011; 14(1): 98-105

References


