High-risk human papillomavirus (HPV) is not associated with p53 and bcl-2 expression in oral squamous cell carcinomas

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Received 10 March 2008; accepted 10 October 2008
Available online 4 January 2009

Abstract

Objective: To determine the frequency and type of human papillomavirus (HPV) in oral squamous cell carcinomas (OSCC), as well as to identify a possible association between HPV infection and the expression pattern of p53 and bcl-2, and identify whether the oral HPV infection is a characteristic finding in our sample.

Methods: We performed polymerase chain reaction and dot blot hybridization for the detection of HPV DNA in paraffin sections as well as immunohistochemical analysis of p53 and bcl-2 in our sample.

Results: Twenty-six cases (29.5%) were positive for the virus by PCR. Dot blot hybridization identified HPV 18 in 21 (80.8%) cases, HPV 16 in one (3.8%) case and a combination of the two types in the four (15.4%) remaining cases. No other type of HPV was detected in the sample. Immunohistochemistry showed p53 in 26 (60.4%) cases and bcl-2 in 17 (39.5%) ones. No significant association was observed between the presence of HPV and the expression of the proteins studied (p = 0.988 and p = 0.748, respectively).

Conclusions: Although this investigation have detected only 29.5% of HR-HPV DNA in OSCC, it is possible that this virus contribute to the development of some case of this tumor. Furthermore, it seems that the immunohistochemical expression of p53 and bcl-2 and the presence of HPV DNA are independent events in OSCC.

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Keywords: HPV; Tumor suppressor proteins; Oncoproteins; PCR; Hybridization; Immunohistochemistry

1. Introduction

Oral squamous cell carcinoma (OSCC) is a malignant neoplasm affecting the lining epithelium of the oral mucosa. It represents the sixth most common malignancy in the world, and in developing countries this cancer accounts for the third most common malignancy. When malignancy of the mouth is included, OSCC accounts for 4% of all cancers, affecting mainly men [1–6].

Over the last two decades, biological agents have been implicated in the etiology of this tumor. Among these agents, human papillomavirus (HPV) is particularly important [7–11]. HPV is a DNA virus which encodes two oncoproteins that play an important role in carcinogenesis, i.e., E6 which binds, sequesters and degrades p53, and E7 which binds and degrades pRb; thus facilitating the development of tumors [7–9,12]. Although the relationship between this virus and malignant uterine
cervix tumors has been well established in the literature, with more than 90% of these tumors being positive for the virus, the same does not apply to oral carcinogenesis [13,14]. The HPV detection rates at this anatomical site is very variable, probably due to differences in sample size, in the population studied and in the sensitivity of the techniques used [15,16].

The functions of protein p53 are commonly altered in malignant tumors, including squamous cell carcinomas of the head and neck [1,7,8,17]. Abnormal expression of bcl-2 is a strong indicator of the blockade of apoptosis, which does not require the use of phenol–chloroform. This method was developed by the Laboratory of Molecular Biology, Dental School of the University of São Paulo (USP). First, the sections were deparaffinized and hydrated. Then, the tissue pellets were suspended in buffer solution and digested with proteinase K at 55 °C for 3–5 days at 24-h intervals. After, proteinase K was inactivated by incubation of the material at 95 °C for 10 min. Next, 200 μl of a 4 M ammonium acetate solution was added to each sample followed by 600 μl 100% isopropanol for DNA precipitation. Finally, the DNA pellets obtained were washed with 70% ethanol, dissolved in 50 μl TE buffer and stored at −20 °C.

2. Materials and methods

2.1. Study participants

After Institutional Ethics Committee approval, 88 cases of OSCC embedded in paraffin were studied. The clinical data were obtained from the medical records and histopathological reports.

2.2. Characterization of the sample

The present sample consisted of 88 cases of primary carcinomas obtained between July 1996 and June 2004. Of the 88 cases, 57 (64.7%) were males and 31 (35.3%) were females. Patient age ranged from 30 to 93 years, with a mean of 65.45 years. With respect to the anatomical location of the primary tumors in the oral cavity, they were located on the tongue, floor of the mouth, lower lip, buccal mucosa, palate, gingiva, and retromolar area. From each site mentioned was removed one tissue section as following: tongue (n = 20), floor of mouth (n = 17), lower lip (n = 14), buccal mucosa (n = 08), palate (n = 06), gingiva (n = 05), retromolar area (n = 05) and unspecified (n = 13). These data are described in Table 1.

2.3. Extraction of DNA from the tissue samples

Ten 10-μm thick histological sections were obtained for DNA extraction, and one 3-μm section for each case was stained with hematoxylin/eosin and revised by a pathologist. Each tissue section used in the study corresponded to one case of OSCC. DNA was extracted using the ammonium acetate–isopropanol technique which does not require the use of phenol–chloroform. To evaluate the efficacy of DNA extraction, all samples were submitted to PCR (polymerase chain reaction) for amplification of the human β-globin gene. Then, a pair of primers designated PCO3+ and PCO4+ was used. It amplifies a fragment of about 110 bp of the β-globin gene [19]. PCR was carried out in an Eppendorf thermocycler in a reaction mixture containing the following components in a final volume of 50 μl: 0.5 μM of the PCO3 and PCO4 primers (Invitrogen, Life Technologies), 1.0 U Taq DNA polymerase (Biosystem), 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 200 μM dNTP (Amersham Bioscience), and 0.7 or 7 μl sample DNA. The PCR conditions for amplification of the β-globin gene were: initial denaturation at 95 °C for 4 min, followed by 40 cycles of amplification at 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products were analyzed by electrophoresis on 2.5% agarose gel (Nusieve) and visualized by ethidium bromide staining. C33 cell DNA was included as positive control and PCR without DNA negative control.

The samples positive for β-globin were analyzed by PCR regarding the presence of HPV DNA using pair of generic primers GP5+ and GP6+ [20], which flank a fragment of about 150 bp of the L1 gene. 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 μM GP5+/GP6+ (Invitrogen, Life Technologies), 1.0 U Taq DNA polymerase (Biosystem), 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 200 μM dNTP (Amersham Bioscience), and 0.7–7.0 μl DNA, in a final volume of 50 μl. The PCR conditions for the detection of HPV were: initial denaturation at 95 °C for 5 min, followed by 40 cycles of amplification at 95 °C for 1 min, 45 °C for 2 min and 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. HeLa cell DNA was included as positive control and PCR without DNA negative control. The amplified PCR products were typed by dot blot hybridization [21] using radioactive probes specific for the 19 HPV types: high-risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, and 58) and low risk (HPV 6, 11, 34, 40, 42, 43, and 44).

2.5. Immunohistochemistry for p53 and bcl-2

Of the 88 cases submitted to molecular analysis, 43 were selected due to the amount of available tissue for immunohistochemistry (13 HPV-positive and 30 HPV-negative cases). Three-micrometer sections from these specimens were deparaffinized, rehydrated and submitted to antigen retrieval in citrate buffer, pH 6.0, for p53, and in Tris–EDTA buffer, pH 9.0, in a pressure cooker for bcl-2. The sections were incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were then incubated overnight with the respective monoclonal antibodies: anti-p53 (clone DO-7, Dako A/S, Denmark) diluted 1:25, and anti-bcl-2 (clone mAb124, Dako A/S) diluted 1:40. The monoclonal antibody for p53 binds to both the normal and mutant form of the protein as described in previous studies. The reaction products were visualized with the Dako streptavidin-biotin-immunoperoxidase system as secondary antibody, which forms a complex with diaminobenzidine. The sections were counterstained with Mayer’s hematoxylin and mounted with glass coverslips and resin-based mountant. The slides were examined under a light microscope for the determination of immunoreactivity. Expression of p53 and bcl-2 was evaluated by counting 1000 cells in malignant epithelial tissue per specimen at a magnification of ×400 using a grid adapted to the eyepiece of the microscope, and the percentage of positive cells was calculated. Only nuclear staining was considered for p53 and cytoplasmic staining for bcl-2.

2.6. Data analysis

Data were analyzed statistically using the SPSS software. The Chi-Square ($\chi^2$) test was used to determine the association between the presence of HPV and patient gender as well as HPV and anatomical location. Possible associations between HPV and patient age and between HPV and p53 and bcl-2 expression were analyzed by the Student $t$ test. The level of significance was set at 95% ($p < 0.05$) for all tests. The correlation between the expression of p53 and bcl-2 was determined by nonparametric analysis using Pearson’s correlation test.

3. Results

3.1. Detection and typing of HPV DNA

Of the 88 cases positive for the human β-globin gene (Fig. 1), 26 (29.5%) were positive for HPV (Fig. 1). Of
these, 18 (69.2%) were males and 8 (30.8%) were females. Of the 62 HPV-negative cases, 39 (62.9%) were males and 23 (37.1%) were females. With respect to viral type, only the high-risk types HPV 16 and 18 were detected in the 26 samples. HPV 18 alone was identified in 21 (80.8%) cases, HPV 16 in one (3.8%) case, and double infection with both types was observed in 4 (15.4%) cases. No significant association was observed between the presence of HPV and patient gender ($\chi^2 = 0.140; p = 0.709$), or between HPV and patient age ($t = -0.629; \ p = 0.535$). These data are described in Tables 1 and 2. Furthermore, $\chi^2$ test showed no association between HPV infection and anatomical location.

### Table 2

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>HR-HPV-negative, n (%)</th>
<th>HR-HPV-positive, n (%)</th>
<th>HR-HPV-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPV 18</td>
<td>HPV 16</td>
<td>HPV 16 and 18</td>
</tr>
<tr>
<td>Tongue</td>
<td>18 (29.0)</td>
<td>02 (7.7)</td>
<td>01</td>
</tr>
<tr>
<td>Mouth floor</td>
<td>13 (21.0)</td>
<td>04 (15.4)</td>
<td>03</td>
</tr>
<tr>
<td>Lower lip</td>
<td>11 (17.8)</td>
<td>03 (11.5)</td>
<td>02</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>05 (8.1)</td>
<td>03 (11.5)</td>
<td>03</td>
</tr>
<tr>
<td>Palate</td>
<td>03 (4.8)</td>
<td>03 (11.5)</td>
<td>03</td>
</tr>
<tr>
<td>Alveolar ridge</td>
<td>04 (6.4)</td>
<td>01 (3.9)</td>
<td>01</td>
</tr>
<tr>
<td>Retromolar region</td>
<td>03 (4.8)</td>
<td>02 (7.7)</td>
<td>02</td>
</tr>
<tr>
<td>No information</td>
<td>05 (8.1)</td>
<td>08 (30.8)</td>
<td>06</td>
</tr>
</tbody>
</table>

HR-HPV high-risk human papillomavirus; -, zero (0).

3.2. **Immunohistochemical expression of p53 and bcl-2 and its correlation with the presence of HPV**

Of the 43 cases, 26 (60.4%) were positive for p53. Nineteen (73%) of the 26 positive cases exhibited more than 70% of stained cells (Fig. 2) and 7 (27%) showed 10–70% of stained cells. The immunoexpression of bcl-2 exhibited a cytoplasmic location in tumor cells. Five to 10% of stained cells for bcl-2 were only observed in 4 of the 17 positive cases (Fig. 3), whereas labeling was scarce in the remaining cases ($n = 13$), with less than 5% of stained cells. No immunostaining for this protein was observed in most tumor specimens. Of the 43 cases submitted to immunohisto-

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**Fig. 1.** Amplification of a fragment of the L1 gene of HPV (150 bp) in the samples analyzed using primers GP5+/GP6+. PC: positive control (HeLa cell DNA); NC: negative control (PCR without DNA); MW: molecular weight marker (bp); lanes 1–7: samples analyzed.

**Fig. 2.** Overexpression of p53 in oral squamous cell carcinoma (Streptavidin–biotin, $\times 400$).

**Fig. 3.** Expression of bcl-2 in oral squamous cell carcinoma (Streptavidin–biotin, $\times 400$).
also observed in the present investigation [9, 15, 28, 29]. Some authors have found a higher prevalence of HPV 18, as typing available in the literature are also highly diverse. (15.4%). Similar to viral detection, the data regarding HPV method identified HPV 18 in 21 (80.8%) cases, HPV 16 in virus typing of the 26 HPV-positive cases performed by this using PCR analysis, as reported by Smith et al. [10]. Thus, copy of HPV per 1000 squamous cells in sample specimens because it is capable of identifying and typing less than one usefulness, although the sensitivity and specificity may vary. suggest that PCR method used for detection of HPV DNA is marked DNA fragmentation in some cases. Thus, we might paraffin-embedded tissues are used as they may show according to the present study, this aspect is important when amplifying a small fragment (150 bp) [20]. Furthermore, large amount of virus types. This occurs because it may amplify a small fragment (150 bp) [20]. Furthermore, according to the present study, this aspect is important when paraffin-embedded tissues are used as they may show marked DNA fragmentation in some cases. Thus, we might suggest that PCR method used for detection of HPV DNA is useful, although the sensitivity and specificity may vary. In this investigation, we used dot blot hybridization because it is capable of identifying and typing less than one copy of HPV per 1000 squamous cells in sample specimens using PCR analysis, as reported by Smith et al. [10]. Thus, viral typing of the 26 HPV-positive cases performed by this method identified HPV 18 in 21 (80.8%) cases, HPV 16 in one (3.8%) and double infection with types 16 and 18 in 4 (15.4%). Similar to viral detection, the data regarding HPV typing available in the literature are also highly diverse. Some authors have found a higher prevalence of HPV 18, as also observed in the present investigation [9, 15, 28, 29]. However, other studies report a predominance of HPV 16 in OSCC, with HPV 16 often being the only type detected [2, 8, 14, 30]. In these studies, Southern blot and dot blot hybridization, in addition to direct DNA sequencing, have been used for viral typing. Furthermore, some authors have detected HPV 16 DNA in oral dysplasias, a potentially malignant oral mucosal lesion [31]. A meta-analysis by Miller and Johnstone [13] demonstrated that HPV was two to three times more often detected in potentially malignant tumors and 4.7 times more frequently in oral carcinomas than in normal oral mucosa, with the probability of detection of high-risk HPV in oral carcinomas being 2.8 times higher than that of low-risk HPV. These findings provide quantitative evidence that oral infection with HPV, particularly high-risk types (HPV 16 and 18), represents a significant independent risk factor for oral carcinomas.

The HPV detection rates observed in the present study, as well as the viral types identified which are considered to be high-risk types, agree with previous studies [6]. This suggests a role of HPV in the etiology of at least some cases of squamous cell carcinomas affecting the oral cavity in our sample, or even attribute to the virus a synergistic action with other important carcinogens such as smoking and alcohol; thus potentiating the effects of these agents and significantly contributing to etiology of some malignant tumors of the mouth. Some studies using other oncogenic markers as those involved in the pRb pathway support this evidence [4, 26, 31, 32].

In the present study, no association was observed between the presence of HPV and patient age or gender, as also reported by Cortezzi et al. and Soares et al. [8, 33]. In contrast, Zhang et al. [24] have found a correlation between these variables and HPV. According to Ritchie et al. [23] HPV infected males show better prognosis for OSCC than HPV-negative males, however this is not the case among females. Furthermore, we found a lack of correlation between the presence of HPV-positive cases and anatomical site of OSCC. This finding has also been reported by other authors [4, 24]. It is important to state that although we could not assess the clinical appearance of the tumors studied, Sugiyama et al. [14] observed that the HPV 16 DNA is frequent in verrucous, papillomatous and leukoplakia-like OSCC. According to Termine et al. [34], a correct distinction of head and neck squamous cell carcinoma by site, together with the use of more sensitive HPV DNA detection methods, should be regarded as key prerogatives in

### Table 3

Relation between p53/bcl-2 immunohistochemical phenotype and HR-HPV-positivity/negativity.

<table>
<thead>
<tr>
<th>Immunohistochemical phenotype</th>
<th>HR-HPV-positive, n (%)</th>
<th>HR-HPV-negative, n (%)</th>
<th>Median</th>
<th>Standard deviation</th>
<th>“r” Value</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 immunopositive cases</td>
<td>8/13 (61.5)</td>
<td>18/30 (60.0)</td>
<td>45.900</td>
<td>39.767</td>
<td>0.015</td>
<td>0.988</td>
</tr>
<tr>
<td>p53 immunonegative cases</td>
<td>5/13 (38.5)</td>
<td>12/30 (40.0)</td>
<td>46.097</td>
<td>40.924</td>
<td>0.710</td>
<td>0.748</td>
</tr>
<tr>
<td>bcl-2 immunopositive cases</td>
<td>2/13 (15.4)</td>
<td>15/30 (50.0)</td>
<td>0.68</td>
<td>1.72</td>
<td>0.015</td>
<td>0.988</td>
</tr>
<tr>
<td>bcl-2 immunonegative cases</td>
<td>11/13 (84.6)</td>
<td>15/30 (50.0)</td>
<td>1.08</td>
<td>1.68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HR-HPV high-risk human papillomavirus.

t and p values were obtained from Student t test.

4. Discussion

Our results showed that the presence of HPV DNA by PCR was detected in only 29.5% of OSCC cases, 80.8% of which were identified for HPV 18. This frequency is closely similar to those demonstrated by Badaracco et al. [7], Sugiyama et al. [14], Smith et al. [10] and Syrjänen [2]. However, other investigators have reported lower and higher detection rates than those observed in our sample [8, 11, 16, 22–27]. The wide variation in the HPV detection rates found in the studies using this same technique may be due to differences between the populations and samples studied for each study. In addition, different methods of detection have also been considered [15, 16, 24]. In this study, we used the GP5+/+GP6+ primer pair which increases sensitivity in epidemiological studies [12] and detects a large amount of virus types. This occurs because it may amplify a small fragment (150 bp) [20]. Furthermore, according to the present study, this aspect is important when paraffin-embedded tissues are used as they may show marked DNA fragmentation in some cases. Thus, we might suggest that PCR method used for detection of HPV DNA is useful, although the sensitivity and specificity may vary.

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The HPV detection rates observed in the present study, as well as the viral types identified which are considered to be high-risk types, agree with previous studies [6]. This suggests a role of HPV in the etiology of at least some cases of squamous cell carcinomas affecting the oral cavity in our sample, or even attribute to the virus a synergistic action with other important carcinogens such as smoking and alcohol; thus potentiating the effects of these agents and significantly contributing to etiology of some malignant tumors of the mouth. Some studies using other oncogenic markers as those involved in the pRb pathway support this evidence [4, 26, 31, 32].

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performing future investigations into viral prevalence in head and neck tumors.

With respect to immunohistochemical assessment and viral infection, no association was observed between the expression of p53 and the presence of HPV in the OSCC cases studied ($t = 0.015; \ p = 0.988$). This finding is in accordance with those obtained by Badaracco et al. [7], Cortezzi et al. [8] and Nemes et al. [4]. However, van Houten et al. [30] showed 36% of head and neck squamous cell carcinomas positives for HPV DNA with mutations in the p53 gene. A highly significant association between HPV infection and overexpression of p53 protein have been observed in squamous cell carcinoma of the uterine cervix [17,22]. Similar to p53, no association was observed between the expression of bcl-2 and the presence of HPV in the oral carcinoma cases studied ($t = 0.710; \ p = 0.482$). These findings disagree with those reported by Badaracco et al. [7], Rihakova et al. [35], Grace et al. [17], Du et al. [18] and Campisi et al. [28]. Probably, a high number of cases should be analyzed to clarify this matter. It is important to state that mutations on p53 gene may stabilize the mutant p53 protein, resulting an increase its half-life. Due to this, the mutant form may be detected by immunohistochemistry in contrast to wild one.

Despite the marked presence of high-risk HPVs in oral carcinomas as widely discussed in the literature and in this study, the presence of this virus alone does not seem to be sufficient for the process of tumorigenesis. This suggests the concomitant occurrence of other additional cellular events [27]. Protein E6 of high-risk HPVs binds to p53 and causes rapid degradation of this protein. However, epidemiological data do not support the hypothesis that the loss of p53's normal functions induced by viral oncproteins is the only oncogenic mechanism. HPV may also act on oral carcinogenesis through a p53-independent route. In the present study, an accumulation of p53 was detected without the observation of a direct association with the presence or absence of viral sequences as described previously. In addition, the mucosal surfaces in the head and neck region may be exposed to exogenous mutagens, such as metabolites derived from the consumption of tobacco and alcohol. These factors may synergistically or additionally interfere with the p53 and bcl-2 control mechanisms and, consequently, with the mechanisms that control cell proliferation.

Studies analyzing the overexpression of p53 and bcl-2 have found no clear relationship between these biological markers, suggesting that carcinogenesis is more complex and probably involves other genes [7]. Ravi et al. [1] proposed that overexpression of bcl-2 in tumors positive for p53 is due to the presence of a nonfunctional p53. In contrast, Grace et al. [17] believe that the inactivation of p53 by oncoprotein E6 of high-risk HPVs reverses the repression of bcl-2, causing overexpression of both proteins in uterine cervix carcinomas. It is also possible that other genes, such as cyclin D1 and p16 which are frequently altered in head and neck tumors, and HPV oncoproteins cooperate in the dysregulation of the cell cycle in some tumors and act independently on others [4,7,32]. Thus, these findings suggest that p53 inactivation by mutation and by HPV infection and the altered expression of oncoprotein bcl-2 are important genetic events in the development of oral carcinomas, but are not mutually exclusive events, which can coexist in these tumors.

In conclusion, it is possible that other techniques of HPV detection could provide a novel insight into this study. In addition, although this investigation have detected only 29.5% of HPV DNA in OSCC, it is possible that this virus contribute to the development of some cases of the tumor. Furthermore, it seems that the immunohistochemical expression of p53 and bcl-2 and the presence of HPV DNA are independent events in OSCC.

Acknowledgements

We are so grateful to Dr. Carlos César Formiga Ramos (in memorian) who provided part of the cases of cancer and Dr. Jean Nunes dos Santos from Federal University of Bahia and Consultant in Oral Pathology at State University of Feira de Santana. This study was supported by CAPES.

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