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RESEARCH ARTICLE

Oral squamous cell carcinoma versus oral verrucous carcinoma: an approach to cellular proliferation and negative relation to human papillomavirus (HPV)

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Abstract Human papillomavirus (HPV) has been cited as a possible initiating agent in the pathogenesis of oral cancer. However, the literature tends to be both controversial and inconclusive about the prevalence of HPV and its potential for proliferation in oral squamous cell carcinoma (SCC). The aim of this study was to investigate the cellular proliferation and the presence of HPV in SCC and verrucous carcinoma (VC). Forty-seven samples of SCC were selected and divided into three groups: 39 SCC, 8 VC, and 9 of normal mucosa (control-CT). Quantitative analyses of all groups showed a greater expression of PCNA, followed by Ki-67 and cyclin B1. A significant difference was observed in cyclin B1 expression in the SCC group compared with VC. PCNA, Ki-67, and cyclin B1 were statistically significant when comparing the SCC and CT groups. However, when SCC and VC were compared, there was no difference in Ki-67 expression. Our results showed that only cyclin B1 had an association with histological grade, and that poorly differentiated tumors presented a higher expression of cyclin B1. Therefore, considerable differences in the cellular proliferation between SCC and VC were observed, and no correlation with HPV was established, since all samples were negative for HPV.

Keywords Oral squamous cell carcinoma · Oral verrucous carcinoma · PCNA · Ki-67 · Cyclin B1 · HPV

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Introduction

The most common oral cancer of epithelial origin is squamous cell carcinoma (SCC) which can present certain histological variations [1]. Verrucous carcinoma (VC) is a low-grade variant of SCC and represent 0.3% to 10% of all oral cavity SCC [2–4]. It is considered to be a neoplasm of uncertain etiopathogenesis, and this makes its diagnosis difficult [2–5]. Squamous cell carcinoma is different from VC in its clinical and histopathological aspects, as well as in its prognosis [2, 6–11].

In the etiopathogenesis of oral cancer, there is no doubt that tobacco is the principal agent responsible for the development of the lesion, while alcohol consumption acts as a promoting agent [4, 6, 11–13]. In addition to these factors, human papillomavirus (HPV) has been cited by various authors as a possible initiating agent in the pathogenesis of oral cancer [6, 11, 13–16]. However, the

data in the literature tends to be both controversial and inconclusive about the prevalence of HPV in oral cancer and its real role in cancer pathogenesis [13, 15–21], as well as its potential for proliferation in SCC [22].

In recent times, many investigations of cellular proliferation in neoplastic lesions have been undertaken and several different methods have been developed to detect it in malignancies [23–33]. However, few studies have investigated the relationship between the cellular proliferation index and the presence of HPV [24, 25, 30, 31]. Huang et al. [22] postulated that HPV may contribute to the carcinogenesis of oral epithelial cells and that it may function to stimulate both the transformation and proliferation of SCC.

Cell proliferation is defined as an "increase in cell number resulting from completion of the cell cycle" [24–27]. Many different methods have been developed to detect cell proliferation in malignancies. In order to verify the proliferative activity in SCC, markers of cellular proliferation have been investigated by immunohistochemical techniques and the proliferative index has been shown to be relevant in determining the prognosis of oral SCC [24–28].

The antigen PCNA is a 36-kDa non-histonic nuclear protein associated with DNA polymerase-δ. It is synthesized in the late G1 and S phases of the cycle cell and has been proven to be a reliable marker for the detection of viable cells entering the cell cycle [27]. The monoclonal antibody Ki-67 recognizes a labile epitope on a nuclear antigen which is expressed in all active parts of the cycle (G1, S, G2, and M phases) [23]. Cyclin B1 plays an important role in G2–M transition, where a deregulation of this protein may promote cellular proliferation or cause uncontrolled cell growth [24].

This study set out to investigate the cellular proliferation index of these two neoplastic lesions by evaluating the expression of PCNA, Ki-67, and cyclin B1 proteins, using immunohistochemical analysis. It also sought to verify whether the presence of HPV would contribute to an increase in tumor cellular proliferation.

Materials and methods

Samples

Thirty-nine surgically excised specimens of primary SCC and eight of VC were obtained from the archives of the Anatomopathology and Cytopathology Division of the Araújo Jorge Hospital and the Combat Cancer Association, Goiânia, Brazil. All patients were submitted to surgical treatment, and none received radiotherapy, chemotherapy, or any other treatment prior to surgery. Clinical data (gender, age, ethnic group, tobacco, and alcohol consumption, tumor location, extension and T and N stages) and follow-up information (recurrence and death) were all obtained from medical records. Nine samples of human clinically healthy oral mucosa (control) were obtained from the archives of the Oral Pathology Laboratory of the Dental School of the Federal University of Goiás, Brazil. The patients of the control group had no history of tobacco consumption, and microscopically examined samples showed mild or absent inflammatory infiltrates. This study was approved by the Institutional Ethics Committee for Human Subjects (Protocol CEPMHA/HC/UFG Number 121/04).

Light microscopy

All specimens were fixed in 10% buffered formalin (pH 7.4) and paraffin embedded. The microscopic features were evaluated from an analysis of a 5-μm section of each sample, routinely stained with hematoxylin and eosin. The microscopic features were analyzed by two independent examiners according to the World Health Organization (WHO) classification of tumors [34].

Immunohistochemistry

For an assessment of the growth fractions of the SCC, VC, and CT cases, an immunohistochemical study of the expression of PCNA (clone PC10, DAKO, Glostrup-Denmark) diluted 1:2,000, Ki-67 (clone MM1, Novocastra, Newcastle, UK) diluted 1:100, and cyclin B1 (clone 7A9, Novocastra, Newcastle, UK) 1:40 was carried out. Then, paraffin-embedded tissues were sectioned (3 μm) and collected in serial sections on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma Chemicals, St. Louis, MO, USA). The sections were deparaffinized by immersion in xylene, and this was followed by immersion in alcohol and then incubation with 3% hydrogen peroxide diluted in Tris-buffered saline (TBS) (pH 7.4) for 40 min. The sections were then immersed in a citrate buffer (pH 6.0; SIGMA, P4809, St. Louis-USA) for 20 min at 95°C for antigen retrieval. Soon afterwards, the sections were blocked by incubation with 3% normal goat serum diluted in distilled water, at room temperature, for 20 min. The slides were then incubated overnight with the primary antibodies at 4°C in a humidified chamber.

After washing in TBS, the sections were treated with labeled streptavidin–biotin–peroxidase (LSAB) kits (K0492, DAKO, Denmark). The sections were then incubated in 3.3′ diaminobenzidine in a chromogen solution (DAKO, K3468) for 2 to 5 min at room temperature. Finally, the sections were stained with Mayer's hematoxylin and covered. Negative controls were obtained by the omission of primary antibodies, which were substituted by 1% PBS-BSA and by non-immune mouse (DAKO, X0910) serum.

Qualitative and quantitative analysis

The location and distribution of $PCNA^+$, Ki-67^+ and cyclin $B1^+$ neoplastic/epithelial cells were also analyzed in basal and parabasal layers VC and CT. In SCC, all the layers were analyzed. The positive cells were determined by morphometric analysis. In each sample, epithelial cells in basal and parabasal layers were quantified into ten representative and consecutive microscopic high-power fields (×400) using an integration graticule (CARL ZEISS-4740680000000-Netzmikrometer ×12.5), each field had an area of 0.0961. All the images analyzed were counter-checked by the same person, and the percentage of positive cells in the total sample was determined. Descriptive analyses were expressed as means±standard deviations (SD) of *n* observations considering the number of cells showing staining for PCNA, Ki-67, and cyclin B1 as a proportion of the total epithelial cell population in SCC, VC, and CT samples.

Statistical analysis

A descriptive and comparative analysis was performed using the SPSS® 14.0.0 Program for Windows® (Microsoft Corporation, Chicago, IL, USA). Comparative analyses between the immunoexpression of proliferative markers in the SCC, VC, and CT groups were performed using the non-parametric Mann–Whitney test and the Kruskal–Wallis test to analyze the expression of each marker with histological gradation. Correlations tests were used to check the association among PCNA, Ki-67, and cyclin B1 immunoexpressions and the association between these expressions and histological grade. Significance was set at 0.05.

Polymerase chain reaction

First, DNA was isolated from the 5-μm sections of formalin-fixed, paraffin-embedded tissue specimens. The samples were deparaffinized in microcentrifuge tubes, then digested by 400 mg/ml proteinase-K in 200 ml TE10E1 buffer at 55°C for 24–48 h [35]. After heat inactivation of the enzyme, DNA was isolated by using the PureGene Extraction kit (Gentra Systems, Inc., Minneapolis, Minn.) according to the manufacture's protocol. Polymerase chain reaction (PCR) amplification with the L1 consensus primers Gp5+/Gp6+ (de Roda Husman et al. [36]) gave an expected PCR product with approximately 140 bp. These primers allow the detection of a broad spectrum of mucosotropic HPV genotypes (6, 11, 13, 16, 18, 30–35, 39, 40, 42, 45, 51–53, 56, 58, 61, 66). The PCR reactions were carried out in a 50-μl reaction, containing 2.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase (Invitrogen, Carlsbad, Califórnia, USA), 250 μM of each dNTP (dATP, dCTP, dGTP, e dTTP), 50 pmol of each primer (GP5+ or GP6+), and 100 ng DNA in 1× buffer (Taq DNA Polymerase Buffer—Invitrogen). Each amplification cycle consisted of 1 min denaturation at 94°C, 1 min annealing at 45°C, and 1 min elongation at 72°C. The first cycle was preceded by 7 min denaturation at 94°C, and the last cycle was followed by a 7-min elongation step at 72°C. The presence and the correct sizes of PCR amplicons were checked by 8% polyacrylamide gel electrophoresis. To avoid contamination and false-positive results, all reagents were previously aliquoted, pre-and post-PCR reactions were handled separately in different physical settings, all PCR products were handled in a hood, gloves were worn during all steps and filter-equipped pipette tips were used. The reactions were performed in parallel with positive and negative controls. The negative controls included all reagents but with water instead of DNA. The DNA obtained from cervical tumor cell lines (HeLa) containing recognized HPV sequences were used as the positive control. In order to confirm the results obtained, the PCR reactions were repeated at least twice for each sample. The HPV-negative samples were submitted to at least two amplifications of approximately 92 bp fragments corresponding to the D8S135 microsatellite region located at 8p.11. D8S135 microsatellite amplification was carried out in order to confirm the presence and the quality of purified DNA samples.

Results

The main clinical features of our series of 47 patients with oral SCC and VC are summarized in Table 1. The analysis of patients with oral carcinomas (39 SCC and 8 VC) revealed differences (tumor site, gender, T stage) between the groups investigated. A bivariate analysis showed that there were no differences found ($p>0.05$) between cellular proliferation values (PCNA, Ki-67, and cyclin B1) when considering patients' characteristics (age, gender, ethnic group, smoking, alcohol consumption, and T stage).

The qualitative analysis of PCNA, Ki-67, and cyclin B1 protein expressions shows that there was a variation in the location of the positive cells in the epithelial layer (Fig. 1). The PCNA and Ki-67 proteins were expressed in the nucleus of the positive cells and cyclin B1 in the cytoplasm of SCC, VC, and in the normal oral mucosa in the nucleus [37].

Another difference found between the SCC and CT group was that the expression of proliferative markers in SCC was distributed over the superficial layers and in the invasive tumor front. In the VC group, there was a strong expression of Ki-67 and cyclin B1 proteins in the basal and

Table 1 The main clinical characteristics of patients with SCC (n=39) and VC (n=8) expressed in percentages

Clinical features		SCC	VC
Age	≤65 years	48.7	25.0
	>65 years	51.3	75.0
Gender	Male	77.0	50.0
	Female	23.0	50.0
Ethnic group	Caucasian	31.9	50.0
	Non-Caucasian	68.1	50.0
Location	Tongue	33.3	25.0
	Floor of the mouth	28.3	–
	Retromolar area	12.8	–
	Hard palate	10.2	25.0
	Alveolar ridge	7.7	–
	Buccal mucosa	5.2	50.0
	Gingiva	2.5	–
Tobacco	Yes	77.0	50.0
	No	23.0	50.0
Alcohol	Yes	61.5	62.5
	No	38.5	37.5
T stage	TX–Tis	2.56	62.5
	T1–T2	41.0	–
	T3–T4	56.44	37.5
Follow-up	Recurrence	–	–
	Death	25.64	12.5

parabasal layers, while PCNA expression was detected in all epithelial layers and approximately 100% of the cells were PCNA positive. The same percentage of $PCNA^{+}$ cells was detected in the CT group. The expression of Ki-67 in the CT group was also concentrated in the basal and parabasal layers, as in the VC group, although this expression was more significant in the VC group.

In regards to the quantitative analysis, Table 2 shows the mean scores for the nuclear expression of PCNA and Ki-67 and the cytoplasmatic expression of cyclin B1 for the SCC, VC, and nuclear in CT groups (Fig. 1). A significant difference was found between the SCC and the CT groups in the expression of all proliferative markers studied ($p<0.05$). In our study, there was an increase of cellular proliferation in the SCC group when compared to the CT group.

When comparing the PCNA, Ki-67, and cyclin B1 immunoexpressions between the SCC and VC groups, a significant difference was noted in relation to PCNA and cyclin B1 ($p<0.05$). However, this was not seen for Ki-67 (Table 2 and Fig. 2).

Our results showed that only cyclin B1 had an association with histological grade ($p=0.04$, Kruskal–Wallis test) and verified that poorly differentiated tumors had a higher expression of cyclin B1($r=0.439$, Spearman's test). In addition, our findings showed a positive correlation between cyclin B1 and Ki-67 ($r=0.557$), although this correlation was not strong. There was a poor correlation between PCNA and cyclin B1 scores ($r=-0.44$): when cyclin B1 increased, PCNA decreased.

Human papillomavirus was amplified in the positive control in all reactions, where DNA obtained from cervical tumor samples containing recognized HPV sequences was used as a positive control. However, HPV amplification was not detected in any of the 39 SCC samples. As our results were negative for HPV, all of the specimens underwent a new PCR with primer D8135S to confirm the presence of extracted human DNA.

Discussion

The present study was carried out in order to investigate the potential of the presence of HPV in samples of SCC and its relation with cellular proliferation. Our hypothesis was that the presence of HPV would have a role in the promotion of tumor proliferation.

Regarding the clinical characteristics, in our study, the anatomical site where VC occurred most frequently was the buccal mucosa. This data was similar to that found by Rajendran et al. [7]. Another finding was that tobacco (78.7%) and alcohol (57.4%) consumption were the principal risk factors in our study which is in concordance with other research [11–13].

Concerning the locoregional and distance metastasis, this type of occurrence was not present in any of the VC cases, a fact which corresponds to results observed by other authors [3]. Surgery was the principal therapeutic modality used for treatment in both groups. Radiotherapy was used as a support treatment in 56.4% of patients in the SCC group while one patient in the VC group received radiotherapy as an adjuvant treatment, even though there are reports in the literature of the possibility of radiotherapy transforming VC into a more aggressive lesion [9, 38–40].

Regarding expression of the markers in the epithelial layers, it was observed that in the SCC group, the expression of all markers was distributed over the more superficial layers and invasive tumor front. These results were also observed by Steinbeck et al. [27] and Costa et al. [28]. In the VC group, we only detected a strong expression of Ki-67 and cyclin B1 proteins in the basal and parabasal layers of the epithelium, whereas the expression of PCNA was seen in all of the epithelial layers. This data is in agreement with data in the literature [26]. We believe that the higher proliferation index for PCNA was due to the similarity between the VC and CT groups. For this reason, PCNA is no longer considered as an adequate marker for studies investigating cellular proliferation in oral cancer [41].

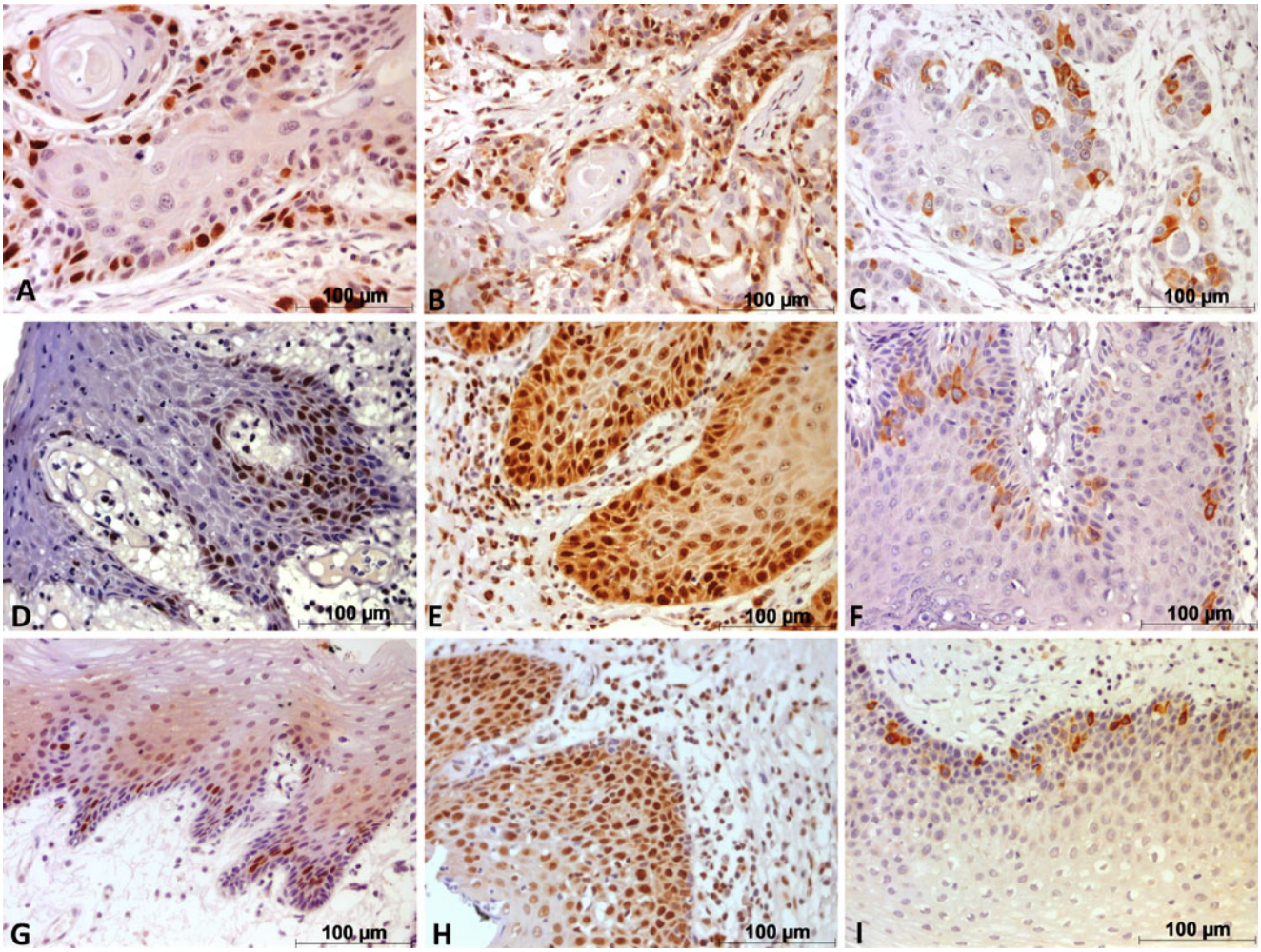

Fig. 1 Immunoexpression of Ki-67, PCNA, and cyclin B1 in all groups. Ki-67$^+$ cells in all epithelial layers of SCC (**a**) and in the basal and parabasal layers of VC (**d**) and CT (**g**). Predominantly positive nuclear staining of PCNA in all epithelial layers of the three groups (**b**, **e**, **h**). Cyclin B1+ cells in all epithelial layers of SCC (**c**) and in the basal and parabasal layers of VC (**f**) and CT (**i**). Immunohistochemical staining, original magnification ×400

The Ki-67 expression in CT group was also concentrated in the basal and parabasal layers, as in the VC group, although this expression was higher in the VC group. We believe that the concentration in these layers was directly related to cell division, which is more specific in these sites. This information is significant when making a differential diagnosis between a benign or a malignant neoplasm, mainly in the diagnosis of VC, and other authors have also observed this [27, 29].

Table 2 Mean PCNA, Ki-67, and cyclin B1 scores and standard deviation (SD) in oral carcinomas and the control group

Diagnosis (*n*—sample)	PCNA %	Ki-67 %	Cyclin B1 %
SCC group (39)	76.2±19.4	26.9±12.4	10.4±4.6
VC group (8)	93.4±6.4	30.5±14.3	5.5±2.6
CT group (9)	100	8.9±3.4	1.99±0.8

% Positive cells of neoplastic cells

SCC squamous cell carcinoma, *VC* verrucous carcinoma, *CT* control

Considering the quantitative analyses, there was a prominent increase of cellular proliferation in the SCC group compared with the CT group. Our findings were similar to results described by Kushner et al. [25], who investigated cyclin B1 and Ki-67 in SCC.

A high proliferation index percentage of PCNA was found in the CT group (100%) when compared to the SCC group, and this result was also detected by the study of Merne et al. [26]. We believe that in the CT group, the cell cycle was working under natural conditions and since PCNA is characterized by a longer half-life, it was thus detectable in many cells that had already passed through the

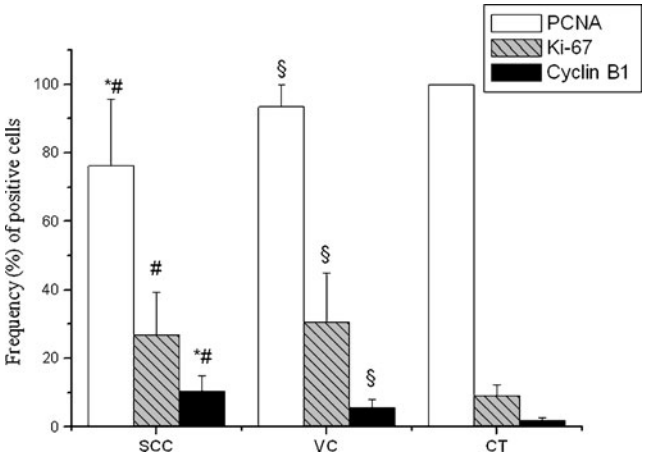

Fig. 2 Percentages of PCNA+, Ki67+, and cyclin B1+ cells in all three groups: squamous cell carcinoma (SCC), verrucous carcinoma (VC), and the control group (CT). Results are expressed as means±standard deviations.* $p<0.05$ when compared SCC and VC, significant difference between the SCC and CT groups with #$p<0.05$ and §$p<0.05$ between the VC and CT groups (Mann–Whitney test)

cycle. Our hypothesis is that the majority of the PCNA-positive cells were in the resting stage. In simple terms, the expression of PCNA occurred, but it was not indicative of an increase or a decrease in cellular proliferation [28].

A significant difference in PCNA and cyclin B1 was found between SCC and VC groups; however, this was not seen for Ki-67. This result for Ki-67 contradicts the findings of Saito et al. [29]. However, this discrepancy between the two results does not invalidate their result because the samples were different. It is worth noting that other authors also found Ki-67 averages in the SCC group similar to those of this study, as was the case of Kurokawa et al. [30].

The quantitative analyses of the VC and CT groups showed that the immunoexpression of Ki-67 and cyclin B1 were higher in the VC than in the CT group. The greater expression of Ki-67 and cyclin B1 when compared to normal mucosa was indicative of the proliferative potential of the VC. Other authors investigating Ki-67 [29] and cyclin B1 [25] found similar results.

In terms of the histological grade of malignancy (HGM), it was seen that the results for PCNA and Ki-67 were not significant, while cyclin B1 expression was associated with the histological grade. In our study, all of the samples were reclassified using the criterion for HGM as proposed by World Health Organization (WHO) [34]. According to our data, cyclin B1 showed a major expression when the tumor was poorly differentiated. This data is indicative that cyclin B1 can be considered as a valuable marker for prognosis, as was observed by other authors [24, 25]. Similarly, Hassan et al. [24] reported that cyclin B1 expression was more intense in poorly differentiated cells. Cyclin B1 has a direct effect on mitosis, and overexpression may lead to uncontrolled cell proliferation, which is characteristic of the oral cavity [42, 43]. In head and neck SCC, several data suggest that cell proliferation indices are reliable and reproducible indicators of tumor aggressiveness [44].

Although the results for Ki-67 were not statistically significant in our research, there are several studies which associated Ki-67 with HGM and which attributed a prognostic value to it [25, 29–31].

It was interesting to observe that there were cyclin B1-positive cells in both SCC and VC, with average percentages of 10.4% and 5.5%, respectively. This data probably indicates the higher proliferative activity of SCC when compared to the lower proliferation index of the VC group. Similar results were found by Harada et al. [37] and Watanabe et al. [45] when analyzing cyclin B1 in normal squamous epithelium and tumor tissue.

Our results showed a positive correlation between cyclin B1 and Ki-67 expressions, as did those of Kushner et al. [25]. Although this correlation between Ki-67 and cyclin B1 was not strong, it was an indication that both markers have the potential for identifying uncontrolled cellular proliferation, and this correlation is dependent on the expression of these markers in the cell cycle.

In our study for the detection of HPV in the SCC and VC groups, the result was negative. Although the technique for DNA extraction from paraffin-embedded tissue is considered difficult, the method used in this investigation was proven to be efficient, since a constitutive DNA sequence was amplified from all tumor samples [35]. Positive controls using cervical cancer tissue with proven HPV and human DNA also supported the success of both the DNA extraction and PCR technique employed in this analysis.

The generic primers GP5+/GP6+ and MY09/MY11, which are capable of amplify various HPV types, are the most widely used primers sets in various studies [46–48]. In our study, the primer set GP5+/6+ was chosen because in addition to detecting a variety of HPV genotypes, including HPV 16 and 18, it is the most commonly detected in oral lesions. The GP5+/GP6+ primer set amplifies a small DNA fragment (150 bp), thereby making it more appropriate for the analysis of DNA samples obtained from archived paraffin-embedded tissue [36, 46–48]. The credibility of the results found in the PCR performed in this study was due to the use of negative and positive controls in the reactions, as well as to the use of GP5+/6+ primers, which have been proven to be superior for the detection of HPV in paraffin-embedded tissue samples [36].

The presence of HPV in oral cancers as an etiological factor is controversial [49]. Several studies indicate that HPV has this potential, and research has been carried out in order to verify the implications of the presence of HPV in

oral cancer [15–18]. Although HPV genomic sequences have been identified in SCC, the wide range of viral prevalence (0–100%) reported in the literature does not contribute to the clarification of the relationship between HPV and oral carcinogenesis [17, 50]. In addition, the majority of studies have reported a small to medium-sized sample (<100 patients) presenting a wide variation in HPV prevalence. In general, larger studies tend to show lower HPV prevalence (varying between 1.4% and 48.8%) than smaller studies (varying between 0% and 100%). According to Termine et al. [50], there is a publication bias with a preponderance of published studies reporting positive (rather than negative) findings.

In our study, it was impossible to verify the influence of the presence HPV in the cellular proliferation and carcinogenesis. Several factors can influence this process, such as variegated sample sizes, differing age and patient groups with various social habits, genetic and geographical risk factors, technical bias related to specimen collection, and laboratory procedures carried out by different operators, implying various degrees of sensitivity and specificity [50–52].

We believe that the anatomical site investigated in our study, namely the oral cavity exclusively, was one of the factors responsible for our negative results, since other studies have showed major evidence of HPV infection in oropharyngeal squamous cells carcinoma sites [17, 18, 51, 53]. In the literature, the lower index of HPV infection in oral squamous cells carcinoma and its more frequent occurrence in oropharyngeal and laryngeal sites could explain our result.

In conclusion, our findings suggest that HPV infection in this area plays a marginal role in oral carcinogenesis and that different cofactors participate in the development of the SCC. Immunohistochemical study indicated the expression status of various cell cycle-associated proteins, mainly the cyclin B1 in typical SCC and VC and corroborated the differential diagnosis between the two entities of SCC. It was possible to verify that there are considerable differences between SCC and VC in terms of cellular proliferation. Further studies of large sample sizes with similar tumor sites should be undertaken to investigate this possible correlation between HPV and cellular proliferation.

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