Expression of p27, p21$^{WAF/Cip1}$, and p16^{INK4a} in Normal Oral Epithelium, Oral Squamous Papilloma, and Oral Squamous Cell Carcinoma

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Abstract. *Aim: To characterize the immunohistochemical expression of p27, p21$^{WAF/Cip1}$ and p16^{INK4a} in normal oral epithelium, oral squamous papilloma and oral squamous cell carcinoma. Materials and Methods: Immunohistochemical staining for p27, p21$^{WAF/Cip1}$ and p16^{INK4a} was evaluated in 32 samples of normal oral squamous epithelium, 30 samples of oral squamous papilloma, and 34 samples of oral squamous cell carcinoma. Results: Statistically significant differences ($p<0.05$) were found in p27 expression when comparing ordinary mucosa and oral squamous papilloma with the oral squamous cell carcinoma samples. Regarding p21$^{WAF/Cip1}$ expression, no statistically significant differences ($p>0.05$) were noticed. In the same way, no significant statistically differences ($p>0.05$) were observed for p16^{INK4a} among groups. Conclusion: Taken together, these findings indicate that p27 is closely involved in malignant transformation of oral mucosa cells, and may be a reliable biomarker for this purpose.*

The biological behavior of diverse affections and malignant phenotypes of various neoplasms have been investigated by a wide range of techniques, such as mitotic counts, nucleolar organizer region evaluation, bromodeoxyuridine and flow cytometry, as well as new techniques in genetic and molecular biology (1). Nowadays, immunohistochemistry is widely used for this purpose, with proliferating cell nuclear antigen (PCNA), Ki-67 and p53 being the most studied markers (2).

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In particular, an important class of genes, the so-called tumor suppressor genes, are functionally recessive, inherited in an autosomal-dominant pattern and act to suppress or regulate cell growth. Inactivation of these genes allows cells to proliferate unchecked, an important step in the progression of cancer (3). Cell cycle activities are commanded by cyclins, cyclin-dependent kinases (CDKs) and their inhibitors. During the cycle, the function of cyclins is to activate the CDKs, and their levels drop after they execute this function. The activity of cyclin–CDK complexes is regulated by CDK inhibitors. There are two main categories of inhibitors: the Cip/kip family and the INK-4/ARF family. In the Cip/Kip family, p21, p27, and p53 stand out as major regulators; in the INK-4/ARF family, p16 and p14 are the most prominent. These inhibitors work as tumor suppressors and are frequently altered in tumors.These inhibitors attach to the cyclin–CDK complexes and inactivate them. The p16^{INK4a} protein competes with cyclin D for connection to CDK-4 and inhibits the complex's capacity to phosphorylate pRb, causing the G_1 cycle to stop. The transcriptional activity of p21$^{WAF/Cip1}$ is controlled by the p53 protein. p21$^{WAF/Cip1}$ also competes with cyclin D in causing the cell cycle to stop. p27 responds to growth suppressors and inhibits the complex cyclin E/CDK-2, also causing cell cycle arrest at the G_1/S restriction point. p27 levels are normally increased in quiescent cells and drop rapidly after stimulation by mitogens. The down-regulation of p27 has been associated with a worse prognosis for patients with diverse carcinomas, including oral squamous cell carcinoma, being directly or indirectly related to abnormal cellular invasion and proliferation. It is still not clear, however, at which stage of oral carcinogenesis the down-regulation of this protein occur (4).

The most frequent histological type of oral cancer is squamous cell carcinoma (SCC), accounting for 90% to 95% of all cases (5). Oral squamous papilloma is a benign epithelial neoplasm, with exophytic growth, most frequently found on the tongue and palate, and normally associated with

infection by the human papilloma virus, mostly HPV-6 and HPV-11. It is the most common benign tumor in the oral cavity, and its biological potential for malignant transformation is still under research (6).

To date, the clinical significance of p21, p27 and p16 for oral papillomas and oral SCC is not yet clear. Consequently, the aim of this study was to investigate the expression of p16, p21, and p16 in ordinary mucosa, oral papillomas and oral SCC. To our knowledge, this is the first study in which the concomitant expression of these immunomarkers has been demonstrated in these oral lesions.

Materials and Methods

Cases. This was a retrospective study of tissue specimens from the oral cavity (tongue) in paraffin blocks from the archive of Federal University of Sao Paulo (UNIFESP), Department of Pathology, from 1996 to 2006. The use of these tissues for this research was approved by the UNIFESP/Human Ethical Committee. Comparisons were made between the following groups: 34 samples of oral squamous cell carcinoma; 30 samples of oral squamous papilloma; and 32 samples of oral normal epithelium obtained from adult patient necropsies. Diagnoses from histological typing of oral lesions and ordinary mucosa were made by two of the authors (ABQ and GF). All cases of oral squamous cell carcinomas were of moderately differentiated type.

Immunohistochemistry. Paraffin-embedded tissue blocks were used to cut 3 μm-thick sections. Hematoxylin and eosin staining was carried out and serial sections were used for immunostaining of p27, $p21^{WAF/Cip1}$ and $p16^{INK4a}$ proteins. Immunohistochemical staining was performed using the avidin-biotin method. Briefly, slides were deparaffinized in xylene and hydrated in ethanol. For antigen retrieval, the sections were boiled in citrate buffer (2.94 g/l sodium citrate, pH 6.0) for 30 minutes and subsequently cooled to 30˚C. Endogenous peroxidase activity was blocked by incubating the slides in methanol with 3% H_2O_2 for 20 minutes, followed by washing in phosphate-buffered saline (PBS; pH 7.4). The primary antibodies were diluted 1:100 for p27 ($p27^{Kip1}$ monoclonal mouse anti-human SX53G8; Santa Cruz, CA, USA); 1:50 for $p21^{Waf/Cip1}$ ($p21^{WAF/Cip1}$ monoclonal mouse anti-human SX118; Santa Cruz) and 1:50 for p16INK4a (clone ab716PO7; Neomarkers, Fremont, CA, USA) in 1% bovine serum albumin (BSA), and sections were incubated for 16 hours at 4˚C. After washing in PBS, the sections were incubated with secondary biotinylated antibody for 30 minutes with peroxidase-streptavidin conjugate (LSAB-HRP, DAKO, Denmark). The sections were washed in PBS (pH 7.4) and the proteins were visualized for light microscopy with DAB reagent 0.06% 3,3-diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 in phosphate-citrate buffer (Sigma, USA). Sections were counterstained with hematoxylin for 3 minutes. Positive controls were represented by mammary tissue. Negative controls were made by eliminating the primary antibody as established in previous studies conducted by our group (7).

Quantification of immunohistochemistry. The sections were examined blindly by two of the authors (ABQ and CD) in randomly selected microscopic fields at a magnification of ×400. The percentage of positive cells was classified as follows: negative, no stained cells; focally positive, fewer than 25% of stained cells (+); moderately positive, more than 25% and fewer than 50% of stained cells (++); diffusely positive, more than 50% of stained cells (+++) (7). Occasional disagreements regarding the classification were discussed and a consensus reached.

Statistical analysis. The relationship between oral squamous cell carcinoma, oral squamous papilloma and oral normal epithelium according to immunohistochemical expression of p27, $p21^{WAF/Cip1}$ and $p16^{INK4a}$ was tested by χ^2 or the Fisher exact test. A *p*-value of <0.05 was considered statistically significant.

Table I. *Immunohistochemical expression of p27 in normal epithelium, squamous papilloma and squamous cell carcinoma.*

p27	Normal		Papilloma		SCC		Total	
	N	%	N	%	N	%	N	%
Focal positive	6	18.75	10	33.33*	33	97.06*	49	51.04
Diffuse positive	26	81.25	20	66.67*	1	2.94*	47	48.96
Total	32	100.00	30	100.00	34	100.00	96	100.00

N, Number; *$p<0.05$ when compared to control.

Table II. *Immunohistochemical expression of $p21^{WAF/Cip1}$ in normal epithelium, squamous papilloma and squamous cell carcinoma.*

$p21^{WAF/Cip1}$	Normal		Papilloma		SCC		Total	
	N	%	N	%	N	%	N	%
Focal positive	32	100.00	27	90.00	34	100.00	93	96.88
Diffuse positive	-	-	3	10.00	-	-	3	3.13
Total	32	100.00	30	100.00	34	100.00	96	100.00

N, Number; $p>0.05$.

Table III. *Immunohistochemical expression of $p16^{INK4a}$ in normal squamous epithelium, squamous papilloma and squamous cell carcinoma.*

$p16^{INK4a}$	Normal		Papilloma		SCC		Total	
	N	%	N	%	N	%	N	%
Focal positive	32	100.00	30	100.00	32	94.00	94	98.00
Diffuse positive	-	-	-	-	2	6.00	2	2.00
Total	32	100.00	30	100.00	34	100.00	96	100.00

N, Number; $p>0.05$.

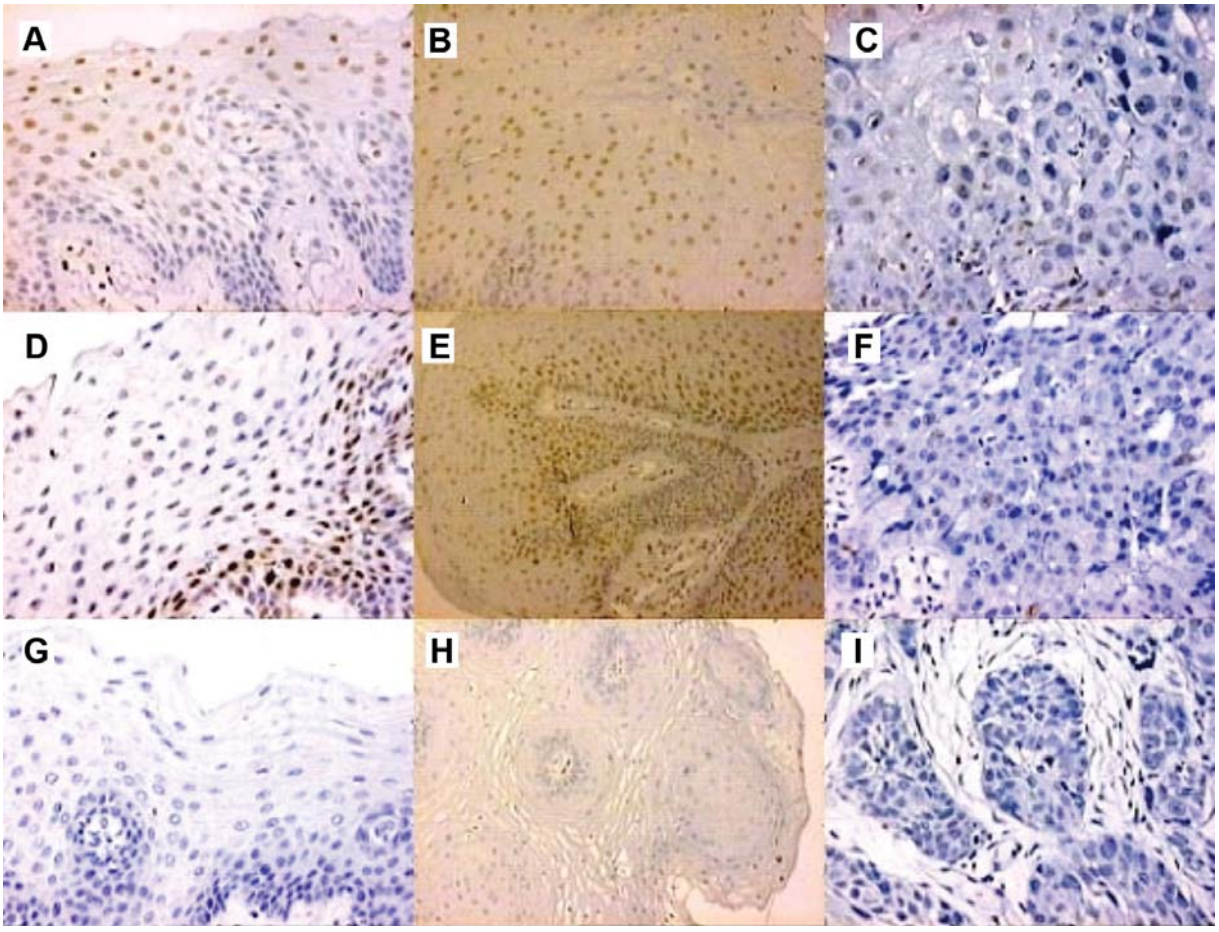

Figure 1. *Representative immunostaining of p27, p21 and p16. A: Immunohistochemical expression of p27 in normal oral squamous epithelium. Note nuclear positivity in the superficial and intermediary thirds, associated with epithelial differentiation and maturation. B: Immunohistochemical expression of p27 in oral squamous papilloma. Note nuclear positivity in mature cells, associated with epithelial differentiation and maturation. C: Immunohistochemical expression of p27 in oral squamous cell carcinoma. Note nuclear positivity in differentiated cells. D: Immunohistochemical expression of p21 in normal oral squamous epithelium. Note nuclear positivity in suprabasal immature cells of the deep third of the epithelium. E: Immunohistochemical expression of p21 in oral squamous papilloma. Note nuclear positivity in suprabasal immature cells of the deep third of the epithelium. F: Immunohistochemical expression of p21 in oral squamous cell carcinoma. Note the random distribution of nuclear positivity, in differentiated and undifferentiated cells. G: Immunohistochemical expression of p16 in normal oral squamous epithelium. Note the lack of staining throughout the epithelium. H: Immunohistochemical expression of p21 in oral squamous papilloma. Note the lack of staining throughout the epithelium. I: Immunohistochemical expression of p16 in oral squamous cell carcinoma. Note the lack of immunopositive neoplastic cells. A-I, Original magnification ×200.*

Results

The results of the immunohistochemical expression of p27, $p21^{WAF/Cip1}$ and $p16^{INK4a}$ are shown in Tables I, II and III, respectively and Figure 1. Immunohistochemical analysis demonstrated 81.25% of the cases having diffuse immunopositivity for p27 in the control group, and 66.67% of the cases in the squamous papilloma group. On the other hand, in the SCC, 97.06% of cases showed focal immunopositivity (Table I). Statistically significant differences ($p<0.05$) were found for oral SCC and oral papillomas when compared to ordinary oral mucosa.

As can be seen from Table II, no differences existed between the three groups regarding the immunoexpression of $p21^{WAF/Cip1}$. All of the cases from the control and the SCC groups were 100% focally immunopositive, and 10% of the squamous papilloma group were diffusely immunopositive for $p21^{WAF/Cip1}$ only (Table II).

In Table III, 100% of the cases of the control and the squamous papilloma groups were focally immunopositive for $p16^{INK4a}$. For the oral SCC group, 94% of the cases demonstrated focal immunopositivity, and 6% diffuse immunopositivity. In the same way, no statistically significant differences ($p>0.05$) were noticed among the groups.

Discussion

The aim of this study was to characterize the protein expression of some tumor suppressor genes such as *p21, p16* and *p27* in normal oral mucosa, oral papillomas and oral SCC to better understand the pathogenesis of these oral lesions. To the best of our knowledge, this approach is new.

Cyclin-dependent kinase inhibitors (CDKIs), such as p21, exert a direct control on the cell cycle. p21 is a negative regulator of cyclin-dependent kinases and in this function is a negative check-point regulator of the cell cycle. Some studies have suggested that p21 in carcinoma of the oral cavity seems to be a predictive parameter in the regulation and prognosis of squamous cell carcinomas (8). Cellular DNA damage *via* p53-activation, leads to an up-regulation of p21 causing cell-cycle arrest in the G_1 phase, with the possibility of DNA repair or the induction of apoptosis (9). In addition, p21 can be regulated independently of p53 by cellular growth factors (10). p21 expression in histologically normal squamous epithelium is described in mature, basal and suprabasal non-differentiated cells (11). Our study showed p21 expression in the basal third of the normal epithelium, *i.e.* higher expression in immature and non-differentiated cells. p21 was not found in mature superficial cells. Previous studies have postulated that increases in p21 expression are not found in normal epithelium (12-14). According to Freer *et al.* (12), p21 expression is generally restricted to intermediate oral mucosa cells and well-differentiated carcinomas, not being detected in severely dysplastic lesions and poorly differentiated SCC. Our results demonstrated the lack of statistical significance for differences in p21 immunoexpression between SCC and oral papillomas when compared to normal oral mucosa. These results suggest that p21 expression is common to differentiated tissue, indicating that p21 is not a useful predictor of cell proliferation or malignant potential. By comparison, Hogmo *et al.* (15) demonstrated that p21 expression was not different between the SCC and the control group. Our findings are in agreement with these results.

Regarding p16, normal epithelium was negative, while atypical epithelium showed irregular staining. In the control group, none of the cases showed any immunopositivity. Mutation in the *p16* gene has been detected with a relatively high frequency in oral SCC (16-19). Hypermethylation, leading to inactivation of tumor suppressor genes such as *p16*, has been shown to be an early event in head and neck cancer (20-22). Further studies are necessary to clarify the issue.

The high levels of p27 expression in normal oral squamous epithelium are a clear indication of its role in maintaining normal tissue cell cycle mechanisms. Normal epithelium shows increased nuclear p27 expression (23), which is closely related to differentiation of superficial mature cells (11). In this study, 81.25% of the control group cases had p27 expression. By contrast, we observed lower p27 expression (fewer than 50% of the cells) in 97.06% of the oral SCC. There was immunoexpression of p27 in more than 50% of the cells and 2.94% of the cases only. Reduction of p27 expression, due to the loss of cell cycle regulation, has been shown in the early invasion phase in oral SCC, showing its important role in abnormal proliferation in which its reduction may relate to the ability for tissue invasion by neoplastic cells (23). Poorly differentiated SCC did not show p27 expression, probably due to the lack of mature cells in that kind of lesion. In moderately and well-differentiated carcinomas, however, there was considerable expression of that protein, related to the growing presence of mature cells. Moreover, p27 expression can be related to a better prognosis in patients with SCC (4). Our results are in fully in line with these findings. In our study, papillomas showed diffuse expression for p27 protein in most cases. This indicates that squamous papilloma is similar to ordinary oral mucosa regarding this immunomarker.

In conclusion, the immunohistochemical findings in the lesions investigated in the present study suggest that the biological behavior of SCC of the mouth may be associated with deregulation of cell proliferation and/or death, as indicated by lowered expression of p27, while p21 and p16 status do not seem to play a significant role. Therefore, we conclude that p27 is a suitable immunomarker for predicting malignant transformation of oral mucosa cells.

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