

Expression Profiling of Cell Cycle Regulatory Proteins in Oropharyngeal Carcinomas Using Tissue Microarrays

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Abstract. *Aim: The aim of this study was to investigate the expressions of cell cycle regulatory proteins such as p53, p16, p21, and Rb in squamous cell carcinoma of the oropharynx and their relation to histological differentiation, staging of disease, and prognosis. Patients and Methods: Paraffin blocks from 21 primary tumors were obtained from archives of the Department of Pathology, Paulista Medical School, Federal University of São Paulo, UNIFESP/EPM. Immunohistochemistry was used to detect the expression of p53, p16, p21, and Rb by means of tissue microarrays. Results: Expression of p53, p21, p16 and Rb was not correlated with the stage of disease, histopathological grading or recurrence in squamous cell carcinoma of the oropharynx. Conclusion: Taken together, our results suggest that p53, p16, p21 and Rb are not reliable biomarkers for prognosis of the tumor severity or recurrence in squamous cell carcinoma of the oropharynx as depicted by tissue microarrays and immunohistochemistry.*

Squamous cell carcinoma of the head and neck arises from accumulated damage to genes that control cellular proliferation, invasion, motility and survival (1). These genetic alterations most often occur as the result of exposure to tobacco, alcohol, and other environmental carcinogens. In particular, prolonged exposure to these agents results in irreversible damage to the DNA of the oropharyngeal mucosa which leads to alterations in the coding and regulatory regions of cell cycle regulatory genes. To date, there are few studies demonstrating the expression of such genes in squamous cell carcinoma of the oropharynx, specially related to the stage of disease, histopathological grading and recurrence (1, 2).

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The tumor suppressors p53 and Rb play a critical role in regulating cell cycle control. The p53 gene regulates DNA repair, cell cycle, apoptosis, senescence, and genomic stability along with many other cellular functions. It is mutated in approximately half of all cases of human cancer. Particularly, in head and neck squamous cell carcinomas, p53 is mutated in approximately 33% to 45% of the cases (3, 4). Rb is known as anti-oncogenic protein and it has been correlated with the degree of tumor differentiation in human squamous cell carcinomas. Positive staining for Rb was observed in a number of well-differentiated squamous cell carcinomas (5), which synergistic effects result in a deregulated cell cycle control. Thus, it has been postulated that the Rb gene participates actively during carcinogenesis (6).

p16 is tumour suppressor protein whose expression is regulated by Rb protein, presumably through a negative feedback mechanism (7, 8). p16 itself also functions as a tumor suppressor gene by inhibiting cyclin-dependent kinases 4 and 6 which phosphorylate Rb, thus decelerating the cell cycle progression (9). Down-regulation of p16 expression has been seen in head and neck tumors; nevertheless, tumors of the larynx, pharynx, and oral cavity had significantly different incidences of decreased expression of p16 (10).

The cyclin-dependent kinase inhibitor p21^{WAF1/CIP1}, encoded by the WAF1/CIP1 gene, also plays an important role in the regulation of the G₁-S transition of the cell cycle. p21^{WAF1/CIP1} works as a main downstream effector of p53 (11, 12). The expression of p21^{WAF1/CIP1} was found to be associated with poor prognosis and tumor aggressiveness in squamous cell carcinomas of the head and neck region (13). To date, the clinical significance of p53, p16, p21 and Rb in squamous cell carcinoma of the oropharynx is not yet clear. Thus, the aim of this study was to investigate the expression of p53, p16, p21 and Rb in squamous cell carcinoma of the oropharynx. To our knowledge, this is the first study in which the concomitant expression of these immunomarkers has been demonstrated in squamous cell carcinomas of the oropharynx with respect to correlation to histological grading, stage of the disease or recurrence.

Patients and Methods

Cases. A total of twenty-one cases of squamous cell carcinoma of the oropharynx were obtained from the archives of the Department of Pathology, Federal University of Sao Paulo, UNIFESP/EPM. Ethical approval for this study was granted by the local Ethics Committee (resolution no. 196 of the National Health Council).

Tissue microarray (TMA). Histological sections of 4 μ m were cut from each block and stained by hematoxylin-eosin (H.E.). The slides were evaluated for diagnostic confirmation and re-evaluation of the histopathological findings, including the selection of sites for the removal of cylindrical cores used in TMA block construction. All tumors were classified according to U.I.C.C. (2000) classification (14).

TMA blocks were constructed using Beecher™ equipment (Beecher Instruments, Silver Spring, MD, USA) according to the manufacturer's instructions, in the following stages: i) The selected area in the respective paraffin block was marked; ii) A cylindrical core was created in the receptor block using the apparatus; iii) One mm cylinder of tissue was extracted from the area of interest; iv) The cylindrical tissues obtained from the donating block were transferred to the core in the receptor block; v) New core positions were created in the receptor block, separated such that a collection of tissue samples was created following the matrix arrangement; vi) The quality of the block was assessed before storing. To guarantee adhesion of the TMA block slices on the slides, an adhesive tape system (Instrumedics Inc, Hackensack, NJ, USA) was used.

Immunohistochemistry. Conventional 4 μ m-thick sections were obtained and mounted on slides pretreated with 3-aminopropyl-triethoxysilane (Sigma, St. Louis, MO, USA). Sections were then deparaffinized, hydrated and processed as follows. For antigen retrieval, slides were placed in 0.01 M citrate buffer pH 6.0 and heated in a steamer for 30 minutes. Endogenous peroxidase was blocked by using 10% hydrogen peroxide for 20 minutes. After overnight incubation of sections with mouse monoclonal primary antibody to p16 (A-2506, 1:100; Santa Cruz); p21 (C-2006, 1:100; Santa Cruz); Rb (H-1204, 1:100; Santa Cruz) or p53 (C-1406, 1:100; Santa Cruz), all slides were allowed to react with secondary biotinylating antibody and streptavidin-biotin-peroxidase (LSAB; Dakocytomation) for 30 minutes each. Finally, the reaction was revealed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma) (0.7% diaminobenzidine in phosphate-buffered saline 0.05 M, pH 7.6, 0.01% hydrogen peroxide) counterstained with Harris's hematoxylin and coverslipped with Entellan (Sigma). Negative and positive controls were made to run simultaneously. Positive control composed breast carcinoma tissue; negative controls were made by eliminating the primary antibody.

Data analysis. The presence of tumor tissue was confirmed previously in each core. Immunostaining was scored by two trained independent observers without prior knowledge of the clinicopathological parameters. Discordant cases were reviewed and agreed upon before data were statistically analyzed. For this purpose, tumor sections stained using immunohistochemistry were analyzed for the percentage of immunopositive cells under optical microscopy. A total of 1000 epithelial cells were evaluated in 3-5 fields at $\times 400$ magnification. All values are given as labeling indices, *i.e.* number of positively stained cells per 1000 cells

counted (%). This protocol was established in previous studies conducted by our research group (15, 16).

Statistical analysis. Data from immunohistochemistry of p53, p16, p21, and Rb were evaluated by Kruskal-Wallis non-parametric test followed by Dunn's test. Statistical analyses were performed with SPSS 15.0 for Windows and a *p*-value less than 0.05 was considered statistically significant.

Results

In this study, the variables analyzed to assess their influence on cell cycle regulatory proteins were: size of tumour, presence of positive lymph nodes, stage (as given by the TNM system), grade of differentiation, and recurrence of disease.

A total of 21 cases were included in the present study. With respect to histological grading, six cases were categorized as well-differentiated (grade I), nine cases as moderately differentiated (grade II) and six as poorly differentiated (grade III). Regarding TNM staging, the majority (15) of cases were in an advanced stage, *i.e.* stage IV. A total of three cases were stage III and three cases were stage II. Finally, recurrence had occurred in eight cases, whereas thirteen cases have not yet shown recurrence (about four years and six months after initial diagnosis).

Immunohistochemical data revealed that p53 protein immunoreactivity (Figure 1) was considered high in squamous cell carcinomas of the oropharynx and was unrelated to histological grading, stage of the disease and recurrence (Figure 2). p53 expression was either nuclear or cytoplasmic. A similar pattern was found with Rb immunoreexpression (Figure 3), *i.e.* strong expression was found; however without significant statistical differences ($p > 0.05$) when the clinical parameters were considered (Figure 4).

Moderate immunoreexpression was detected for p21 proteins with a nuclear pattern (Figure 5). However, no significant statistical differences ($p > 0.05$) were observed either to histological grading, staging or recurrence (Figure 6).

Finally, we were able to evaluate the immunoreexpression for p16 protein. High expression of p16 was detected (Figure 7), but no significant statistical differences ($p > 0.05$) were noticed with respect to any of the parameters evaluated in this study (Figure 8).

Discussion

In the current investigation, we evaluated the expression of some cell cycle regulatory proteins, such as p53, p16, p21 and Rb in squamous cell carcinomas of the oropharynx by means of TMA and immunohistochemistry in order to predict putative biomarkers for the disease. These immunomarkers were chosen because they play important roles in routine diagnostic pathology in our laboratory.

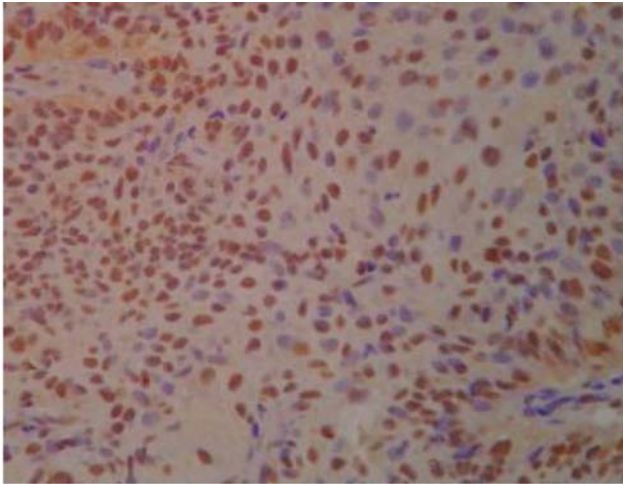


Figure 1. Immunohistochemical staining for p53 in a squamous cell carcinoma of the oropharynx ($\times 100$ magnification).

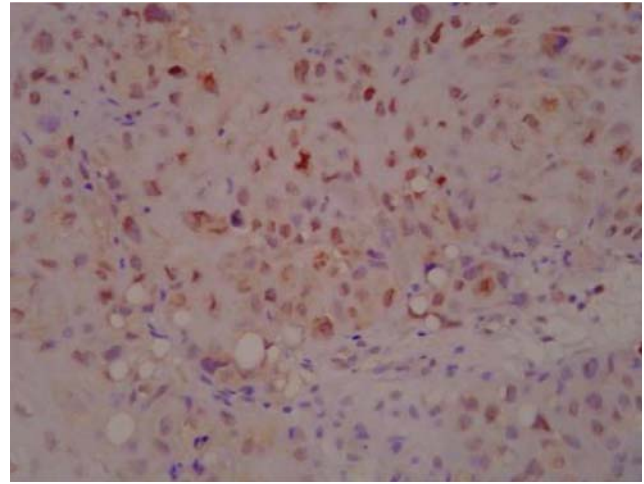


Figure 3. Immunohistochemical staining for Rb in a squamous cell carcinoma of the oropharynx ($\times 100$ magnification).

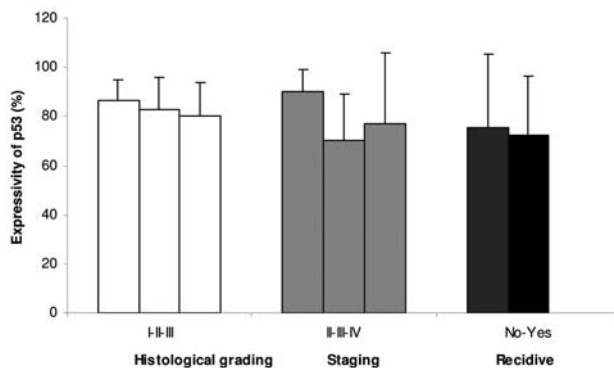


Figure 2. p53 labelling index in squamous cell carcinoma of the oropharynx with respect to histological grading, stage of the disease (TNM system) and recurrence. Data are shown as mean \pm S.D. $p > 0.05$.

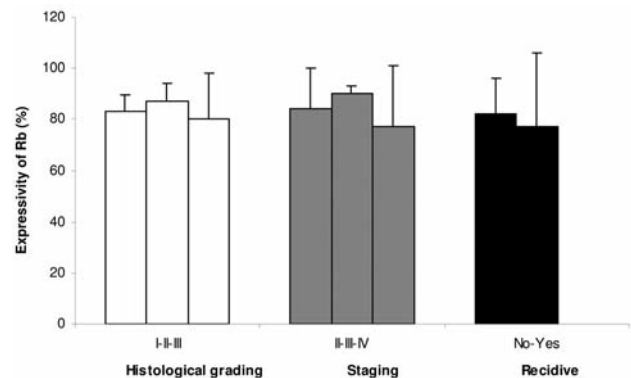


Figure 4. Rb labelling index in squamous cell carcinoma of the oropharynx with respect to histological grading, stage of the disease (TNM system) and recurrence. Data are shown as mean \pm S.D. $p > 0.05$.

One of the most important genes in the regulation of apoptosis is *p53*. This gene encodes a protein, p53, of molecular weight 53 kDa (17). The protein product of the *p53* gene restrains cellular proliferation by binding to specific regions of DNA, where it may regulate expression of other genes. It also suppresses cell growth by controlling entry into the S phase of the cell cycle. Normally, wild-type p53 protein has a very short half-life (6-20 min) and, thus, cannot be detected by standard immunohistochemical methods. Therefore, positive staining for p53 protein has been proposed as an indicator of *p53* gene mutations (18). Nevertheless, regulatory defects of the *p53* gene may, in some cases, result in overexpression or stabilization of wild-type p53 protein (19). In this study, stained p53-positive cells (wild and/or mutant type) were found in the epithelium of squamous cell carcinoma of the oropharynx. However, our

results demonstrated that p53 expressivity was not related to histological grading, stage of the disease or prognosis. Previous studies have reported that p53 protein was not significantly predictive in squamous cell carcinomas of the oropharynx (20). By contrast, positive p53 expression has been related to more aggressive features and unfavorable outcome in pharyngeal squamous cell carcinoma (21). However, the same authors state that unlike more traditional variables, p53 expression is not an independent predictor of disease outcome in pharyngeal squamous cell carcinoma (21). Grabenbauer *et al.* (22) have postulated that a weak p53 nuclear intensity is able to identify patients with squamous cell carcinoma of the oropharynx at high risk for local recurrence after surgery and postoperative radiotherapy. In our study, strong p53 expression was noticed in all cases, regardless of clinical stage.

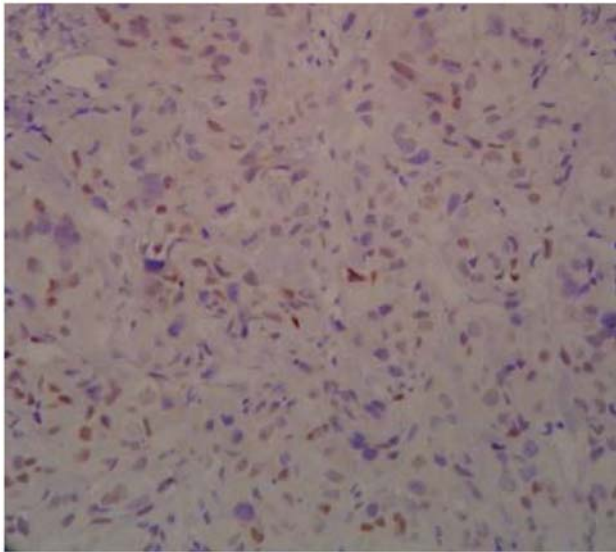


Figure 5. Immunohistochemical staining for p21 in a squamous cell carcinoma of the oropharynx ($\times 100$ magnification).

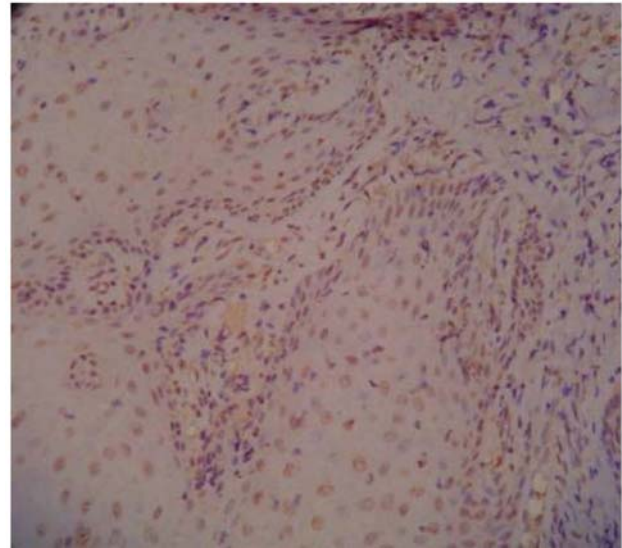


Figure 7. Immunohistochemical staining for p16 in a squamous cell carcinoma of the oropharynx ($\times 100$ magnification).

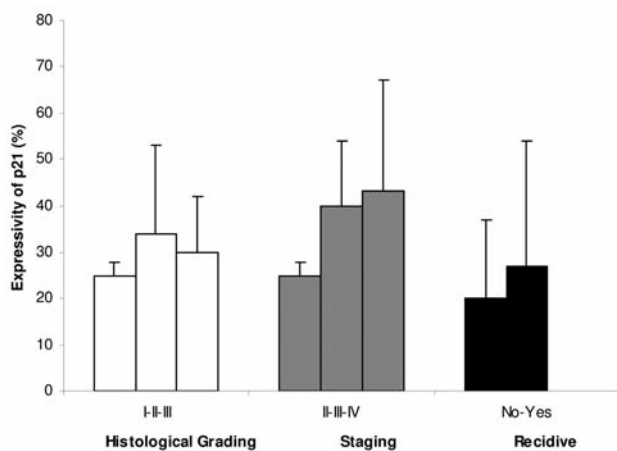


Figure 6. p21 labelling index in a squamous cell carcinoma of the oropharynx with respect to histological grading, stage of the disease (TNM system) and recurrence. Data are shown as mean \pm S.D. $p > 0.05$.

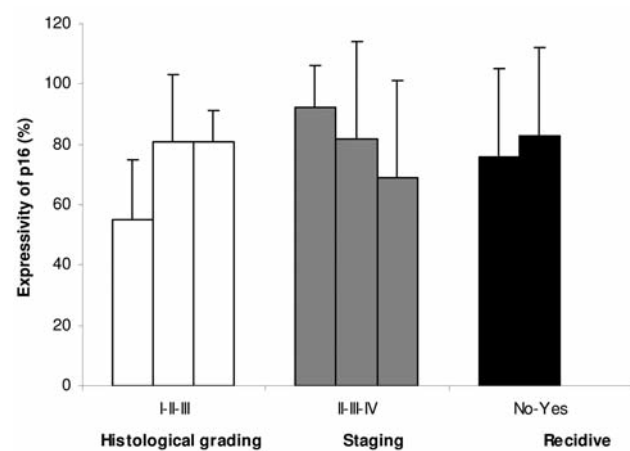


Figure 8. p16 labelling index in squamous cell carcinoma of the oropharynx with respect to histological grading, stage of the disease (TNM system) and recurrence. Data are shown as mean \pm S.D. $p > 0.05$.

Retinoblastoma (*Rb*) and *p16* gene products are part of the retinoblastoma pathway that controls the cell cycle. We also evaluated the expression of p16 and Rb in squamous cell carcinomas of the oropharynx. Positive expression for both immunomarkers was found in these neoplasm without any predictor for disease behavior. Consistent with these data, Rb and p16 expression have not been associated with any of the markers nor the remission rate, T and N stages, rate of loco-regional recurrence, nor distant metastases (23). By comparison, Bradley *et al.* (24) reported that p16 immunoreactivity is not helpful in differentiating dysplastic from non-dysplastic mucosa in oral cavity biopsies, and thus

is not a reliable biomarker for use in routine clinical practice. Additionally, some authors have assumed that inactivation of p16 occurs at an early stage of dysplasia in the multistep process of tumorigenesis in the head and neck region (25). Taken together, we feel that Rb and p16 expressions are not reliable biomarkers for squamous cell carcinomas of the oropharynx.

p21 is a cyclin-dependent kinase inhibitor protein essential for cellular growth, differentiation and apoptosis (26). It has been established that p21 expression in invasive fronts is a significant indicator for survival (27). Moreover, p21 is one of the important factors regulating the

progression of malignant cells in squamous cell carcinomas such as those in the oral cavity (27). Loss of p21 expression in invasive fronts was found to be associated with clinicopathological factors of tumor progression and poor prognosis. P21 protein expression in squamous cell carcinomas tumors is affected by *p21^{WAF1}* genotype (28). Our results demonstrated a moderate expression for this immunomarker. Nevertheless, p21 expression was not affected by histological grading, stage of the disease, or recurrence in this study. In the same way, Kuropkat *et al*. (20) showed that p21 expression was not significantly predictive in carcinoma of the oropharynx.

Taken as a whole, our results support the notion that the protein expressions of p53, p16, Rb and p21 are not suitable biomarkers for histological grading, disease stage or recurrence in carcinoma of the oropharynx.

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