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p53 and p21$^{WAF1/CIP1}$ overexpression at the invasive front of lower lip squamous cell carcinoma

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BACKGROUND: Lower lip squamous cell carcinoma (LLSCC) is an oral cancer that has distinct epidemiology and etiopathogenesis. Although risk factors for this neoplasia are acknowledged, few studies have investigated the molecular basis of its development and behavior.

METHODS: Expression of p53 and p21$^{WAF1/CIP1}$ was examined by immunohistochemistry of archived tissue from 21 specimens of LLSCC. Differences in this expression between the whole tumor (WT) and the invasive front (IF) as well as correlation between p53 and p21$^{WAF1/CIP1}$ expression were analyzed.

RESULTS: p53 and p21$^{WAF1/CIP1}$ were overexpressed at the IF of LLSCC. The expression of both proteins was higher at IF than at WT. No correlation was observed between p53 and p21$^{WAF1/CIP1}$ expression.

CONCLUSIONS: Our results indicate that p53 and p21$^{WAF1/CIP1}$ overexpression is important in LLSCC pathogenesis, reinforce that IF is the most important area for tumor behavior, and support that p53-independent mechanisms should be involved in the expression of p21$^{WAF1/CIP1}$.

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Keywords: cyclin kinase inhibitor p21; lip cancer; squamous cell carcinoma; tumor suppressor protein p53

Introduction

The lower lip is the most common site of squamous cell carcinoma of the oral cavity (1). Lower lip squamous cell carcinoma (LLSCC) affects mainly white men, between the fifth and seventh decades of life (1–3). Although it is well known that the most relevant risk factor for LLSCC is exposure to ultraviolet radiation (2–4), its definitive pathogenic pathway remains unclear, and few studies have investigated the molecular basis of its development and behavior (3).

One of the most promising findings in prognostic factors in oral squamous cell carcinoma was the recognition that the invasive front (IF) may reflect prognosis better than other areas of the tumor (5–12). Even though the IF can be easily identified in LLSCC, no studies have evaluated the IF exclusively in this neoplasia.

p53 is a nuclear protein of 53 kDa, encoded by the tumor suppressor gene *TP53* (13–15). Abnormalities of the *p53* gene, which lead to the loss of p53 protein activity, are the most common molecular changes seen in human neoplasia (16). The cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$is one target of the transcriptionally active p53 and mediates cell cycle arrest after DNA damage (17–20). As p53 has been used as a prognostic marker for oral squamous cell carcinoma (21–23) and p21$^{WAF1/CIP1}$ is one its critical downstream mediators, p21$^{WAF1/CIP1}$ may also serve as a useful prognostic marker (19).

The aim of the present study was to assess the expression of p53 and p21$^{WAF1/CIP1}$ in LLSCC and to analyze this expression in the whole tumor (WT) compared with the IF of this neoplasia. The correlation between p53 and p21$^{WAF1/CIP1}$ expression in each region was also investigated.

Materials and methods

Tissues and samples

A total of 21 archival formalin-fixed, paraffin-embedded specimens of LLSCC were collected at the Mário Penna Hospital and the School of Medicine from the Federal University of Minas Gerais, Brazil, from 1995 to 2001. All tumor specimens were obtained by total surgical resection with free margins.

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Of the 21 patients whose tumors were evaluated, 14 were men and seven were women, ranging from 27 to 86 years of age (mean age, 61 years). Tumors were classified according to the International Union Against Cancer TNM classification. Classification included five stage I tumors, nine stage II tumors, five stage III tumors, one stage IV tumor, and one unclassified tumor.

Immunohistochemistry

For immunohistochemical detection, 4 μm sections from paraffin-embedded samples were used. Tissue sections were dewaxed with xylene, hydrated using graded alcohols and treated with 0.6% H_2O_2 in methanol for 10 min to eliminate endogenous peroxidase activity. Antigen retrieval was conducted by heating in a 0.01 M citrate buffer (pH 6.0) for 30 min. The following monoclonal antibodies were used: anti-p53 (clone DO-1, diluted 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-$p21^{WAF1/CIP1}$ (clone SX118, diluted 1:25; Dako Corporation, Glostrup, Denmark). The LSAB + kit (Dako Corporation) was used for application of the biotinylated link antibody and peroxidase-labeled streptavidin, according to the manufacturer's instructions. The reactive products were visualized by immersing the sections for 3 min in 0.03% diaminobenzidine solution, containing 2 mM H_2O_2. The sections were then counterstained with Mayer's hematoxylin, dehydrated and mounted. Sections of oral squamous cell carcinoma with known p53 and p21 expression were used as a positive control. Negative control was made by omission of each primary antibody.

Tumor cells with distinct brown nuclear staining were regarded as p53 and p21 positive. Tumor cell nuclei were independently counted at WT and at IF. Cell counts were made at ×400 magnification, using an eyepiece grid in light microscopy for at least 15 fields. The percentage of positive cells was than calculated to obtain one labeling index (LI) for each area. The pattern of p53 and $p21^{WAF1/CIP1}$ immunostaining at IF was also classified as overexpressed when LI > 10% (24–26).

Statistical analysis

The data were analyzed by means of Biostat 3.0 software. Differences in p53 and $p21^{WAF1/CIP1}$ expression between the two regions were analyzed using the Wilcoxon test. The correlation between p53 and $p21^{WAF1/CIP1}$ expression in each region was analyzed using the Spearman test. $P < 0.05$ was considered significant.

Results

Overexpression of p53 (LI > 10% at the IF) was found in 18 of 21 specimens (85.7%) (Fig. 1). In 19 of the 21 specimens (90.4%), p53 LI was higher at IF than at WT. Statistically significant differences were found between p53 LI at IF (median: 44.0; range: 0–73.7) and at WT (median: 18.0; range: 0–51.6) ($P = 0.0001$) (Table 1).

Overexpression of $p21^{WAF1/CIP1}$ (LI > 10% at the IF) was found in 9 of 21 specimens (42.8%) (Fig. 1). In 17 of the 21 specimens (80.9%), $p21^{WAF1/CIP1}$ LI was higher at IF than at WT. Statistically significant differences

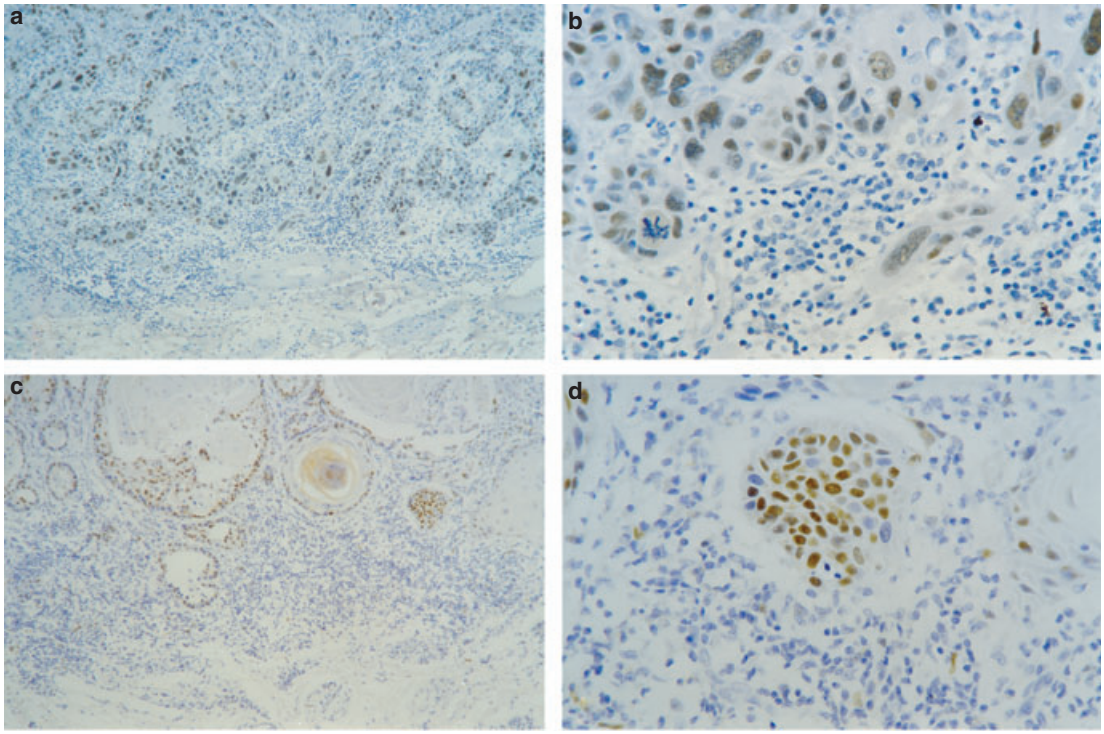

Figure 1 Immunohistochemical reactivity for p53 and $p21^{WAF1/CIP1}$. Invasive front area with tumor cells demonstrating nuclear immunostaining for p53 [original magnification: ×100 (a) and ×400 (b)] and $p21^{WAF1/CIP1}$ [original magnification: ×100 (c) and ×400 (d)].

Table 1 Median value and range of p53 and $p21^{WAF1/CIP1}$ LI at WT and at IF of the 21 specimens evaluated

	WT	*IF*	*P-value*[a]
p53 LI			
Median	18.0	44.0	0.0001
Range	0–51.6	0–73.7	
$p21^{WAF1/CIP1}$ LI			
Median	3.6	6.4	0.0025
Range	0–34.1	2.1–49.8	

LI, labeling index; WT, whole tumor; IF, invasive front.
[a]*P*-values were obtained from the Wilcoxon test.

were found when comparing $p21^{WAF1/CIP1}$ LI at IF (median: 6.4; range: 2.1–49.8) to that at WT (median: 3.6; range: 0–34.1) ($P = 0.0025$) (Table 1).

No statistically significant correlation was detected between p53 LI and $p21^{WAF1/CIP1}$ LI at WT or at IF ($P > 0.05$).

Discussion

Lower lip squamous cell carcinoma is a form of oral cancer that has distinct epidemiology and etiopathogenesis (2–4). Even if the risk factors for this neoplasia are well recognized, few studies have investigated the molecular basis of its development and behavior (3).

Although IF has been exhaustively studied in oral squamous cell carcinoma, few works assessed the expression of cell cycle regulatory proteins in this area (27, 28). Moreover, no study analyzed these proteins at the IF of LLSCC. The well-demarcated anatomic limits of the lower lip make the evaluation of the IF in LLSCC easier than in oral squamous cell carcinomas of other sites, mainly when the specimens were obtained by total surgical resection with free margins.

Mutations of *TP53* are the most common genetic abnormalities found in head and neck squamous cell carcinoma (16), and studies have demonstrated that *TP53* mutational spectra are different between squamous cell carcinomas of the lip and the oral cavity (29). While intra-oral tumors present changes characteristic of DNA damage caused by tobacco smoke, *TP53* mutations in lip tumors reveal a UV light signature (29). In the present study, overexpression of p53 was demonstrated in 85.7% of the specimens evaluated. This is in agreement with previous reports from lip squamous cell carcinoma (30–33). Our results support the importance of p53 pathway alterations in the development of lip squamous cell carcinoma, as p53 immunoreactivity is generally associated with the presence of *TP53* mutation (34, 35). However, it is important to emphasize that p53 immunoreactivity can occur in the absence of *TP53* mutation (15, 16). Moreover, mutations that result in deletion or truncation of the protein do not cause its accumulation and detection by immunohistochemistry (15, 16). Our study also demonstrates that p53 LI is higher at IF than at WT. Similar results from prior literature have been observed as regards oral squamous cell carcinoma (27). As the loss of the p53 function is related to genomic instability (36), these data suggest that, at IF, more tumor cells should be able to accumulate critical genetic alterations for invasion and metastasis, reinforcing the hypothesis that this region is responsible for tumor behavior. Furthermore, although studies on the correlation of p53 immunostaining and prognosis are controversial, p53 overexpression has been reported to be associated with worse prognosis in oral squamous cell carcinoma (22, 25, 37).

$p21^{WAF1/CIP1}$ works as a main downstream effector of the p53 protein, promoting cell cycle arrest in G1 in order to allow for the repair of damaged DNA (17–20). Therefore, $p21^{WAF1/CIP1}$ presents a tumor suppressor ability, demonstrated in oral squamous cell carcinoma xenograft model experiments, in that overexpression of $p21^{WAF1/CIP1}$ suppresses tumor growth (38). However, several functions of $p21^{WAF1/CIP1}$ are likely to promote carcinogenesis and tumor progression, as regards its ability: to inhibit apoptosis, to inhibit the expression of genes involved in the quality control of mitosis, and to promote the assembly of active cyclin-CDK complexes (39). Furthermore, it has been reported that $p21^{WAF1/CIP1}$ expression increases in oral squamous cell carcinoma (24–26, 40–44), suggesting that this protein alone may not be sufficient enough to arrest cell cycle and inhibit tumor cell growth. In the present study, overexpression of $p21^{WAF1/CIP1}$ was demonstrated in 42.8% of the specimens evaluated. This is in accordance with a study that demonstrated $p21^{WAF1/CIP1}$ overexpression in 9 of 29 (31%) cases of lip squamous cell carcinoma (26). In addition, $p21^{WAF1/CIP1}$ LI was also found to be higher at IF than at WT. These results reinforce the hypothesis that the IF is responsible for tumor behavior, as $p21^{WAF1/CIP1}$ overexpression has been reported to be associated with a worse prognosis in oral squamous cell carcinoma (25, 26, 40, 44). However, no association of $p21^{WAF1/CIP1}$ overexpression with prognosis (45) and association of $p21^{WAF1/CIP1}$ overexpression with a better prognosis (43) have also been demonstrated in oral squamous cell carcinoma.

Finally, while p53 and $p21^{WAF1/CIP1}$ are overexpressed at the IF of LLSCC, no correlation was found between p53 and $p21^{WAF1/CIP1}$ expression either at IF or at WT. These findings are in accordance with previous reports evaluating oral squamous cell carcinoma (24, 25, 34, 35, 41, 43) and support that p53-independent mechanisms are involved in the expression of $p21^{WAF1/CIP1}$.

In conclusion, p53 and $p21^{WAF1/CIP1}$ are overexpressed at the IF of LLSCC. It is important to emphasize that immunohistochemistry should, in its very nature, present false-negative and -positive results. Therefore, other studies concerning molecular analysis are necessary to clarify the role of these proteins in the pathogenesis of LLSCC.

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