

## Imbalance of Tumor Suppression Genes Expression Following Rat Tongue Carcinogenesis Induced by 4-Nitroquinoline 1-Oxide

DANIEL ARAKI RIBEIRO<sup>1,2</sup>, ANA CAROLINA CUZZUOL FRACALOSSO<sup>1</sup>, SILVIA AKEMI UATARI<sup>2</sup>, CELINA TIZUKO FUJIYAMA OSHIMA<sup>1</sup> and DAISY MARIA FAVERO SALVADORI<sup>3</sup>

<sup>1</sup>Departments of Pathology and <sup>2</sup>Biosciences, Federal University of Sao Paulo, UNIFESP, SP; <sup>3</sup>Department of Pathology, Botucatu Medical School, Sao Paulo State University, UNESP, SP, Brazil

**Abstract.** *This study was undertaken to investigate, by immunohistochemistry, the expression of some tumor suppressor genes such as p16, p21 and Retinoblastoma (Rb) during 4-Nitroquinoline 1-oxide induced rat tongue carcinogenesis. Male Wistar rats were distributed into three groups of 10 animals each and treated with 50 ppm 4NQO solution through their drinking water for 4, 12 or 20 weeks. Ten animals were used as negative control. Neither histopathological abnormalities were induced in the epithelium after 4 weeks of carcinogen exposure, nor statistically significant differences ( $p > 0.05$ ) in expression of all the tumor suppressor genes were found when compared to the negative control. However, the levels of Rb were increased ( $p < 0.05$ ) in pre-neoplastic lesions at 12 weeks following carcinogen exposure. In well-differentiated squamous cell carcinoma induced after 20 weeks of treatment with 4NQO, p16 and Rb were expressed in some tumor cells. Taken together, the results support the belief that the expression of Rb is closely event-related to malignant transformation and conversion of the oral mucosa, being a reliable biomarker linked to oral cancer pathogenesis.*

Carcinogenesis is a multi-step process, which is characterized by genetic, epigenetic, and phenotypic changes (1). Such changes involve genetic damage, mutation in critical genes related to the control of cell division, cell death and metastatic potential, and activation of signaling or metabolic pathways that give the cells favorable growth and survival characteristics (2). In particular, an important class of genes,

the so called tumor suppressor genes, is functionally recessive, inherited in an autosomal-dominant pattern and acts to suppress or regulate cell growth. Inactivation of these genes allows cells to proliferate unchecked, an important step in the progression of a cancer (3).

Retinoblastoma (Rb) and p16 gene products are part of the retinoblastoma pathway that controls the cell cycle. The Rb gene is located on the long arm of chromosome 13. The retinoblastoma protein is a nuclear phosphoprotein that is expressed in most normal cells. Rb functions during the G<sub>1</sub>-S transition within the cell cycle in that the hypophosphorylated form of the protein mediates G<sub>1</sub> arrest (4). Rb and p16 gene inactivations have been reported in many malignancies including those of oral cavity (5). 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 (6). Cellular DNA damage leads via p53-activation to an up-regulation of p21 causing cell-cycle arrest in the G1 phase with the cellular possibility of DNA-repair or the induction of apoptosis (7). In addition, p21 can be regulated independent of p53 by cellular growth factors (8).

Squamous cell carcinoma is the most common malignancy that affects the human oral cavity (9). Nowadays, oral cancer is the eighth most common cancer in the world, with epidemiological variations between different geographic regions. The World Health Organization predicts a rise in worldwide oral cancer incidence in the next decades (10). Smoking habits and alcohol intake are the most important risk factors involved during oral carcinogenesis (11, 12).

Despite recent advances in therapy, the prognosis of patients with oral squamous cell carcinoma has not been improved significantly in recent decades (13). It is desirable to examine the precise pathobiological mechanisms involved in oral tumorigenesis in order to identify reliable biomarkers

*Correspondence to:* Daniel Araki Ribeiro, DDS, Ph.D., Departamento de Biociências, Av. Ana Costa, 95, Vila Mathias, Santos-SP, 11060-001, Brazil. Tel: +55 1332218058, Fax: +55 1332232592, e-mail: daribeiro@unifesp.br, daribeiro@pesquisador.cnpq.br

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for the identification of oral squamous cell carcinomas, especially during neoplastic conversion. The most frequently used animal models in this line of research are the hamster buccal pouch with fat-soluble 7,12 dimethylbenzanthracene (DMBA), and the rat tongue with water-soluble 4-nitroquinoline 1-oxide (4NQO). Considering one of the most important routes of oral carcinogenesis is through liquid containing water-soluble carcinogens, 4NQO is well suited for examining the role of xenobiotics in experimental oral carcinogenesis (14). Based on the multi-step process of carcinogenesis characterized by initiation, promotion, and tumor progression, the chronic administration of 4NQO in drinking water simulates rat tongue carcinogenesis similar to its human counterpart (15-18).

The real biological significance of p21, p16 and Rb during the development of oral squamous cell carcinomas is not yet clear (20). Thus, the aim of this study was to investigate the expression of p16, p21 and Rb during rat tongue carcinogenesis induced by 4NQO using immunohistochemistry. To our knowledge, this is the first study in which the concomitant expression of these immunomarkers has been demonstrated in rat oral neoplasms.

## Materials and Methods

*Animals and experimental design.* All the experimental protocols involving animals conformed to procedures described in the Guiding Principles for the Use of Laboratory Animals and the study was approved by the Animal Committee of Botucatu Medical School, UNESP.

Forty male Wistar rats (8 weeks old) weighing approximately 250 g, were obtained from the Centro de Bioterismo (CEMIB), Universidade Estadual de Campinas, SP, Brazil. They were maintained under controlled conditions of temperature ( $24\pm 2^{\circ}\text{C}$ ), light-dark periods of 12 hours and with free access to water and commercial diet (Nuvital Curitiba, PR, Brazil). The animals were divided into 3 groups of 10 treated with 50 ppm 4NQO (Sigma Aldrich, St. Louis, MO, USA) solution in the drinking water for 4, 12 or 20 weeks and ten animals were used as negative control. At the end of the experimental period, the rats were sacrificed by 0.4% sodium pentobarbital (1 mL/kg, *i.p.*). The tongues were longitudinally bisected for histopathological examination. The tissues were fixed in 10% buffered formalin (Merck, Darmstadt, Germany), embedded in paraffin blocks and stained with hematoxylin and eosin (H.E., Merck).

*Histopathological analysis.* Histopathological evaluation was performed by light microscopy. Analyses of the tongue sections were graded as normal, hyperplasia, dysplasia, and carcinoma per animal according to Ribeiro *et al.* (18).

*Immunohistochemistry.* Serial longitudinal tongue sections of 4  $\mu\text{m}$  were deparaffinated in xylene and rehydrated in graded ethanol, then pretreated by microwave (Electrolux, Sao Carlos, SP, Brazil) with 10 mM citric acid buffer (pH=6) for 3 cycles of 5 min each at 850 W for antigen retrieval. They were pre-incubated with 0.3% hydrogen peroxide in PBS for 5 min for inactivation of endogenous

peroxidase, and then blocked with 5% normal goat serum in PBS for 10 min. The specimens were then incubated with anti-p16 antibody, anti-p21 antibody or anti-Rb antibody (Santa Cruz Biotechnology, San Diego, CA, USA) each at a concentration of 1:200. Incubation was carried out overnight at  $4^{\circ}\text{C}$  within the refrigerator. This was followed by two washes in PBS for 10 min. The sections were then incubated with biotin-conjugated secondary antibody anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1:200 in PBS for 1 h. The sections were washed twice with PBS followed by the application of horseradish peroxidase-labeled streptavidin (Dako, Copenhagen, Denmark) for 45 min. The bound complexes were visualized by the application of a 0.05% solution of 3-3'-diaminobenzidine solution and counterstained with Harris hematoxylin. For control studies of the antibodies, the serial sections were treated with rabbit IgG (Vector Laboratories) at a concentration of 1:200 in place of the primary antibody. Additionally, internal positive controls were performed with each staining batch.

*Quantification of immunohistochemistry.* The tongue sections stained by immunohistochemistry were analysed for the percentages of immunopositive cells in the areas diagnosed as normal, hyperplasia, dysplasia or carcinoma under optical microscopy. A total of 1000 epithelial cells were evaluated in 3-5 fields at  $\times 400$  magnification. All the values were used as labeling indices. This protocol was established in previous studies by our group (17, 18).

*Statistical methods.* The immunohistochemistry data were assessed by Kruskal Wallis non-parametric test followed by *post-hoc* analysis (Dunn's test) using SPSS software pack (Chicago, IL, USA, version 1.0). Relationships between p16 and Rb expression during the development of oral squamous cell carcinomas were evaluated with Spearman's correlation coefficient. A *p*-value  $< 0.05$  was considered statistically significant.

## Results

*Histopathological evaluation.* No histopathological changes in the tongue epithelia were observed in the control group (Figure 1A) or after treatment for 4 weeks with 4NQO. The primary histopathological change, *i.e.* hyperplasia and hyperkeratosis with the spinous cell layer gradually thickened was observed after 12 weeks of treatment (Figure 1B). In this period, epithelial dysplasia was also found in mild and moderate forms (Figure 1C). At 20 weeks, moderate and/or severe oral dysplasia and squamous cell carcinoma in the tongue (Figure 1D) were found, being that in the majority of animals having squamous cell carcinoma. The histopathological grade was usually of a well-differentiated type. The tumors spread into the submucosa and underlying muscle layer, forming small nests with typical keratin pearl formation. In advanced cases, severe atypia was frequently found. The histopathological findings are summarized in the Table I.

*Immunohistochemistry.* Regarding the p16 immunomarker, positive expression was detected in the nucleus of the oral mucosa cells in the negative control group (Figure 2A) as well as after 4 weeks of carcinogen exposure, without any

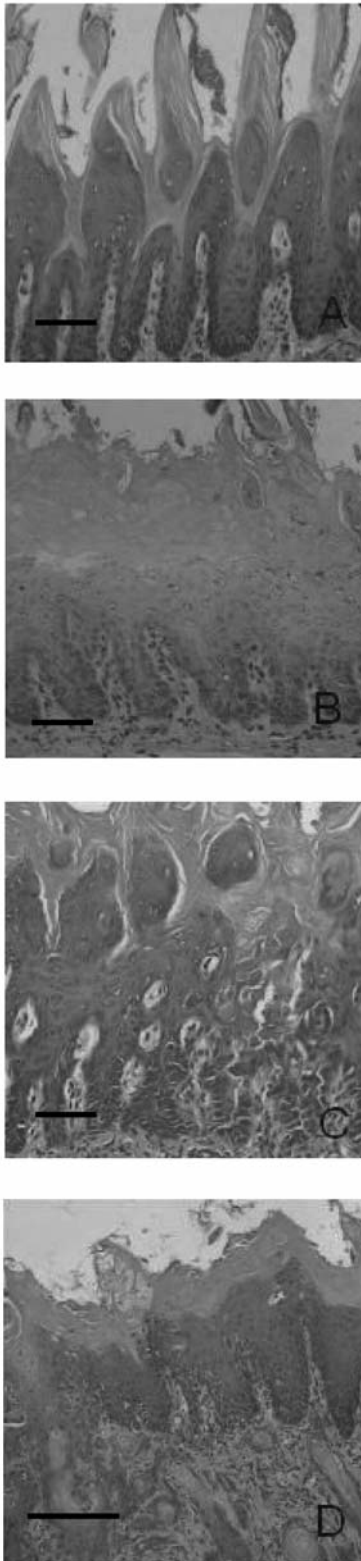


Figure 1. Stages of the multi-step process of rat tongue carcinogenesis. (A) no histopathological change (control), (B) hyperplasia and hyperkeratosis, (C) epithelial dysplasia, (D) squamous cell carcinoma of well-differentiated type. (Hematoxylin and eosin stain; Bar=30 µm).

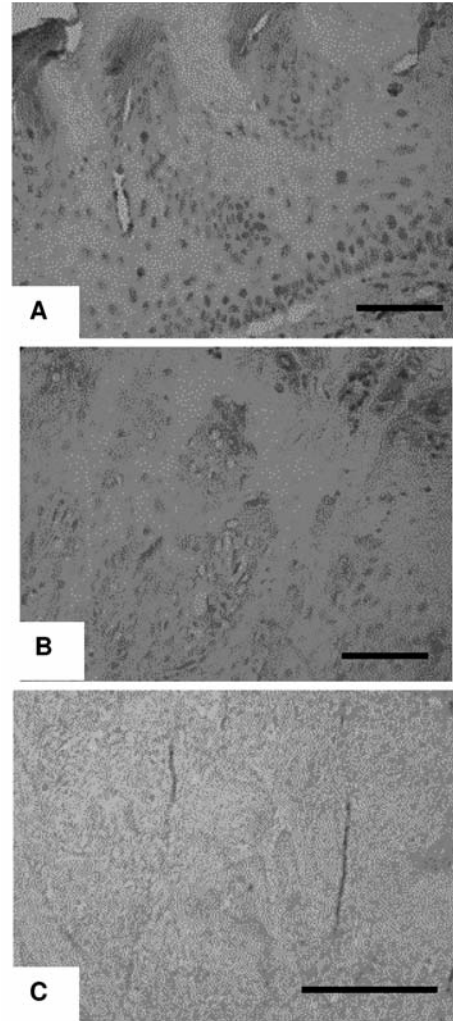


Figure 2. Immunohistochemical staining for p16. (A) rat control epithelium; (B) pre-neoplastic lesion after 12 weeks of carcinogen administration and (C) squamous cell carcinoma of well-differentiated type (Bar=30 µm).

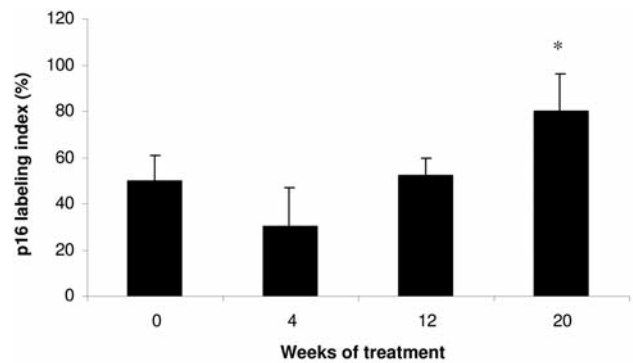


Figure 3. P16 labeling index in the negative control (zero) and those exposed to 4-nitroquinoline 1-oxide for 4, 12 or 20 weeks. Values are means±S.D. \* $p < 0.05$  when compared to negative control group.

Table I. Incidence of histopathological lesions in tongue of rats in the 4-nitroquinoline 1-oxide (4NQO)<sup>a</sup> model of oral carcinogenesis.

Groups (week)	No. of animals	Lesions			
		Normal	Hyperplasia	Dysplasia	Carcinoma
0 (Control)	10	10	0	0	0
4	10	10	0	0	0
12	10	0	7	3	0
20	10	0	0	3	7

<sup>a</sup>4NQO – 50 ppm in the drinking water.

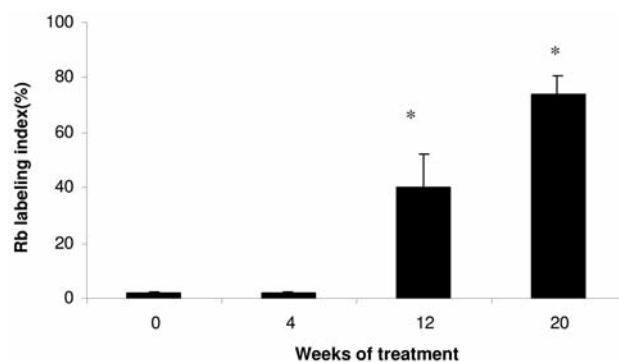


Figure 5. Rb labeling index in the negative control (zero) and those exposed to 4-nitroquinoline 1-oxide for 4, 12 or 20 weeks. Values are  $\pm$ S.D. \* $p < 0.05$  when compared to negative control group.

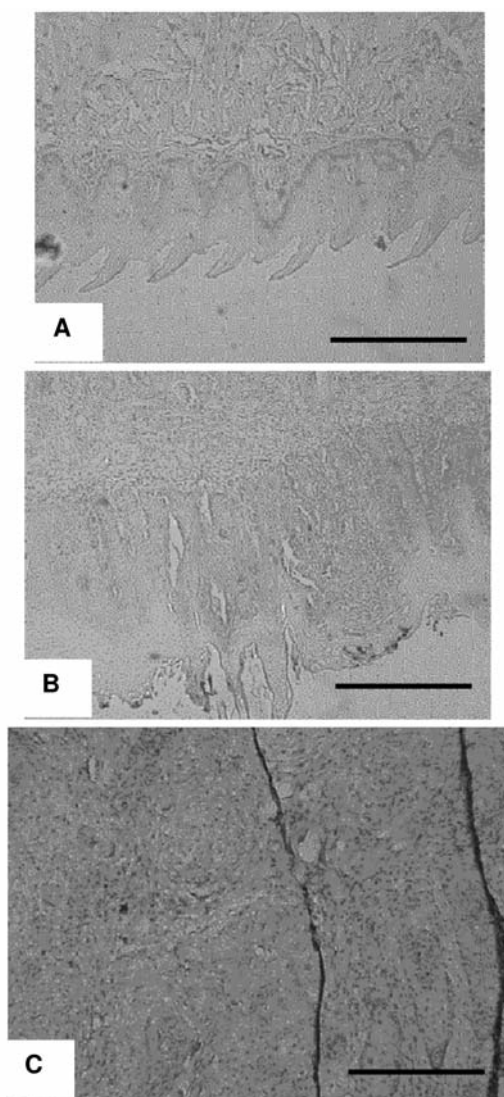


Figure 4. Immunohistochemical staining for pRb. (A) rat control epithelium; (B) pre-neoplastic lesion after 12 weeks of carcinogen administration and (C) squamous cell carcinoma of well-differentiated type (Bar=30  $\mu$ m).

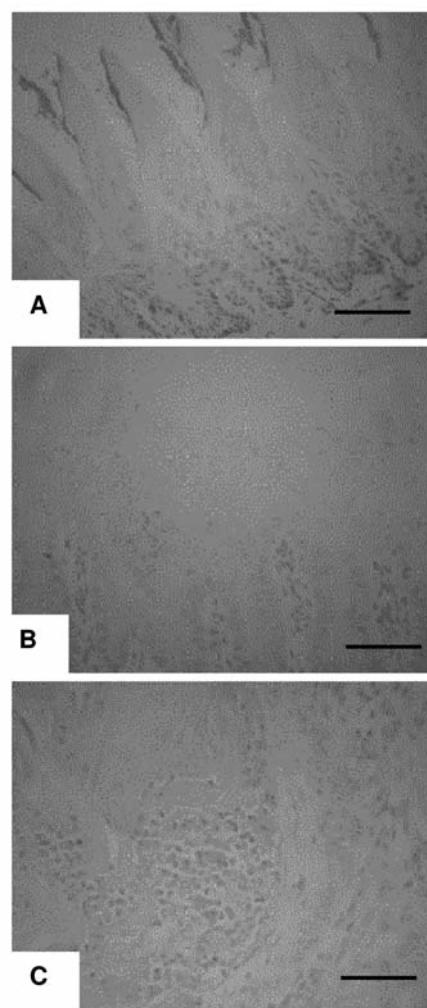


Figure 6. Immunohistochemical staining for p21. (A) rat control epithelium, (B) pre-neoplastic lesion after 12 weeks of carcinogen administration and (C) squamous cell carcinoma of well-differentiated type (Bar=30  $\mu$ m).

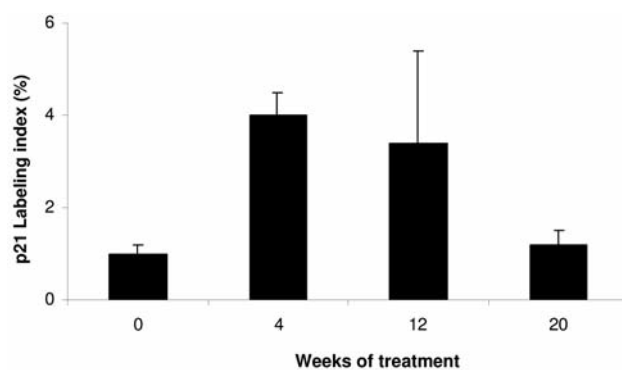


Figure 7. P21 labeling index in the negative control (zero) and those exposed to 4-nitroquinoline 1-oxide for 4, 12 or 20 weeks. Values are means $\pm$ S.D.  $p>0.05$ , not detected.

statistically significant differences ( $p>0.05$ ) between these groups. P16 immunoreactivity was found in the dysplastic cells following 12 weeks of 4NQO-exposure (Figure 2B). Nevertheless, no statistically significant difference was noted between this group and the negative control (Figure 3). In the well-differentiated squamous cell carcinomas, strong expression was observed (Figure 2C). As expected, significant statistically difference ( $p<0.05$ ) was detected in this group (Figure 3).

Low Rb positivity was found in the negative control group (Figure 4A). In a similar manner, some epithelial cells showed Rb immunoreactivity after 4 weeks of carcinogen exposure. By contrast, Rb positivity increased in the preneoplastic lesions after 12 weeks (Figure 4B). With respect to squamous cell carcinomas induced after 20 weeks of 4NQO exposure, Rb was evident in some tumor cells (Figure 4C). Data analysis demonstrated statistically significant differences ( $p<0.05$ ) between the control and the intermediate and final experimental periods (12 and 20 weeks, respectively). These data are summarized in Figure 5.

Mild staining was noticed for the p21 immunomarker in the negative control and all the experimental periods (Figures 6A-C). No statistical differences ( $p>0.05$ ) were detected between the groups (Figure 7). In addition, no staining was seen for any of the antibodies in the negative immunohistochemical controls.

Finally, this study confirmed the hypothesis of a significant relationship between increased Rb and p16 expression during the development of oral squamous cell carcinomas ( $p<0.05$ ,  $r>0.5$ ) (Table II).

## Discussion

The aim of this study was to investigate the expression of some tumor suppressor genes during experimental oral carcinogenesis using rats in an attempt to establish the significance of a battery of molecular alterations and thereby identify risk predictors in oral carcinogenesis.

Table II. Spearman's correlation coefficient between tumor suppressor genes expression after the administration of 4-nitroquinoline 1-oxide (4NQO) in rats.

Immunomarkers	Correlation coefficient	P-value
RbX p16	0.55	<0.05

For this purpose, we evaluated the expressivity of p16 and Rb following 4NQO administration in order to determine its role during oral tumorigenesis phase by phase. In humans, only a small portion of initial lesions develop into oral carcinomas (18). Therefore, the challenge is to identify which lesions have real malignant potential. In this study, positive expression of Rb was found in the pre-malignant lesions and the squamous cell carcinomas following 12 or 20 weeks of 4NQO exposure, respectively. Consistent with these data, Rb expression has been shown either in pre-neoplastic lesions or in oral squamous cell carcinomas induced by 4NQO (19). In contrast, the lack of a detectable level of Rb expression has been reported in carcinomas of the lung, bladder, breast, esophagus or even the oral mucosa (19). The same findings were observed to p16, in which positive expression was mainly detected in the oral squamous cell carcinomas following 20 weeks of carcinogen administration. Bradley *et al.* (20) have 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 the early stage of oral mucosal dysplasia in the multistep process of oral tumorigenesis. However, p16 may be considered as a useful prognostic marker for the progression of oral cancer (21). One study also demonstrated that the Rb pathway proteins are comparatively more important than the p53 pathway proteins for the prognostication of oral carcinoma patients (22). In a recent study, deregulation of the p16/pRb/p21 pathway was considered as an early event in the acquisition of dysplasia, but deregulation of both pRb and p53 pathways was associated with malignant transformation and adverse prognosis in oral tumorigenesis (23). These findings are fully in line with the present data since a positive correlation was detected between p53 and Rb expression and Rb and p16 expression.

To the best of our knowledge, no study using p21 following 4NQO-induced rat tongue carcinogenesis has been performed up to now. Interestingly, the present results demonstrated no p21 expression in the all experimental groups or in the negative control. It is probable that p21 protein expression in tumor cells is associated with the methylation process in DNA, a known epigenetic mechanism (24). Thus, the mode of action of the 4NQO carcinogen merits discussion. 4NQO is converted intracellularly into 4-hydroxyaminoquinoline 1-

oxide (4HAQO). As a result, DNA damage is extensively induced by combination with the purine body of DNA within the nucleus to form 4HAQO-DNA adducts through reactive oxygen species (19). In addition, 4HAQO is able to promote methylation in the promoter regions of genes (19). Probably, this might explain the lack of p21 expression during rat tongue carcinogenesis induced by 4NQO.

The expression of Rb may be an important event in the malignant conversion and their expression in pre-malignant lesions is a hallmark of malignancy. The applicability to clinical practice for persons at high risk of oral cancer, such as smokers or alcoholics, as well as patients diagnosed with oral dysplasia or carcinoma, remains to be developed.

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