Activation of the Wnt/Beta-catenin Signaling Pathway during Oral Carcinogenesis Process Is Not Influenced by the Absence of Galectin-3 in Mice

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Abstract. *Background/Aim: Galectin-3 has been associated with activated Wnt pathway, translocating beta-catenin into the nucleus. However, it is still unknown whether this lectin drives the Wnt signaling activation in lesions from galectin-3-deficient ($Gal3^{-/-}$) mice. The purpose was to study beta-catenin expression in tongue lesions from $Gal3^{-/-}$ and wild-type ($Gal3^{+/+}$) mice and the status of Wnt signaling. Materials and Methods: Twenty $Gal3^{-/-}$ and $Gal3^{+/+}$ male mice were challenged with 4-nitroquinolin-1-oxide and killed at week 16 and 32. Tongues were processed and stained with H&E to detect dysplasias and carcinomas. An imunohistochemical assay was performed to evaluate beta-catenin expression. Results: Carcinomas were more evident in $Gal3^{+/+}$ than $Gal3^{-/-}$ mice (55.5% vs. 28.5%, respectively; $p>0.05$). Elevated expression of non-membranous beta-catenin was observed in dysplasias and carcinomas from both groups ($p>0.05$). Conclusion: Absence of galectin-3 does not interfere in the pattern of beta-catenin expression and therefore in the mediation of the Wnt signaling pathway.*

Squamous cell carcinoma of the oral cavity is one of the most common neoplasms in the world and it is characterized by poor prognosis and high mortality rate (1). Notwithstanding the recent advances made in the last decade regarding the biological aspects of this malignancy, the precise mechanism that is required for its development still deserves attention, especially in the identification of biomarkers which could permit an early diagnosis and at the same time improve overall outcomes (2). In this regard, the use of animal models of human cancer, in genetically modified animals, still continues to be useful for the discovery of biomarkers that might take place during the multistep process of carcinogenesis (3).

Galectins are mammary lectins that present high affinity to beta-galactoside residue on the cell surface. Among the 15 types of galectins recognized so far, galectin-3 is one of the most frequently investigated (4). Galectin-3, a protein of approximately 30 to 35 kDa, is involved in different physiological and pathophysiological processes, including cell growth, apoptosis, tumor transformation and metastasis, and angiogenesis, among others (5). However, little is known about the exact role played by galectin-3 in neoplastic transformation, tumor progression and metastasis (6). Although there is an enormous body of evidence showing the involvement of this lectin in the development of different kinds of cancer, including those affecting the head and neck region, the findings are still very contradictory (7-10).

The Wnt pathway is an important signal transduction pathway has a pivotal role during mammalian embryogenesis (11). The main signaling molecule of the pathway is beta-catenin, which in response to activated Wnt pathway, translocates into the nucleus resulting in the activation of the transcription of Wnt target genes, such as *c-MYC* and cyclin D1, and then cell growth (6). Consistent with this, several studies have shown that alteration of the pathway is responsible for tumorigenesis in many tissues and is apparently related to the different roles of beta-catenin inside cells, although the presence of mutation in other components of the same pathway, such as APC

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protein, may also contribute (11). Recent reports have shown galectin-3 to be a mediator of the Wnt pathway, binding and promoting beta-catenin translocation into the nucleus (12). More recently, it was shown that the level of galectin-3 is correlated with beta-catenin expression, as well as its nuclear accumulation in colon cancer cells (6). However, there are no studies addressing whether the absence of galectin-3 modulates beta-catenin expression as well as its distribution inside cells from dysplasia and carcinoma developed experimentally in mice.

Therefore, the aim of this work was to investigate beta-catenin expression comparatively by immunohistochemistry in samples of dysplasia and carcinoma induced by the carcinogen 4-nitroquinolin-1-oxide (4NQO) in tongue of wild-type (*Gal3*$^{+/+}$) and galectin-3-deficient (*Gal3*$^{-/-}$) mice in order to determine whether the absence of galectin-3 interferes in the localization of beta-catenin inside the cells, and therefore in the mediation of Wnt signaling during carcinogenesis.

Materials and Methods

The experiment was approved by the Committee on Animal Experimentation of the Universidade Federal de Uberlândia (protocol number 038/09).

Animals. *Gal3*$^{-/-}$ mice generated through homology recombination and crossed with C57BL/6 mice were generously supplied for this experiment by Hsu's group (13). Twenty six-week-old male *Gal3*$^{-/-}$ mice, weighing approximately 23 g, were used in this study. Sex- and age-matched *Gal3*$^{+/+}$ mice with the same background were used as controls. Both *Gal3*$^{-/-}$ and *Gal3*$^{+/+}$ mice were divided into two groups according to killing point: at week 16, immediately after the 4NQO treatment (*Gal3*$^{-/-}$ n=10; *Gal3*$^{+/+}$ n=10) and at week 32, corresponding to 16 weeks after the end of 4NQO treatment (*Gal3*$^{-/-}$ n=10; *Gal3*$^{+/+}$ n=10). All mice were maintained under controlled conditions of temperature (22˚C), light-dark periods of 12 hours and with free access to commercial diet.

Experiment protocol. The treatment with 4NQO was based on a protocol described previously (14). The carcinogen 4NQO was previously diluted in propylene glycol (5 mg/ml) and thereafter in filtered water to achieve a concentration of 100 μg/ml. The solution was administered in the drinking bottles of the mice for an uninterrupted 16-week period to both groups of mice. During the entire treatment, the 4NQO solution was prepared and changed weekly. After 16 weeks, the treatment was interrupted and the animals received only filtered water, except for the *Gal3*$^{-/-}$ and *Gal3*$^{+/+}$ mice of week 16, which were sacrificed immediately at the end of the treatment.

Microscopic analysis. After deep ether anesthesia, all mice were killed by cervical dislocation. Tongues were removed and fixed immediately in 10% neutral-buffered formalin solution for 24 hours. After this period, a macroscopic investigation of the tongues was carried out in order to observe the alterations on the tongue surface. Next, tongues were cut transversally into five fragments. At this point, great care was taken when a visible lesion was found on the tongue surface. In this case, the lesion was carefully cut to

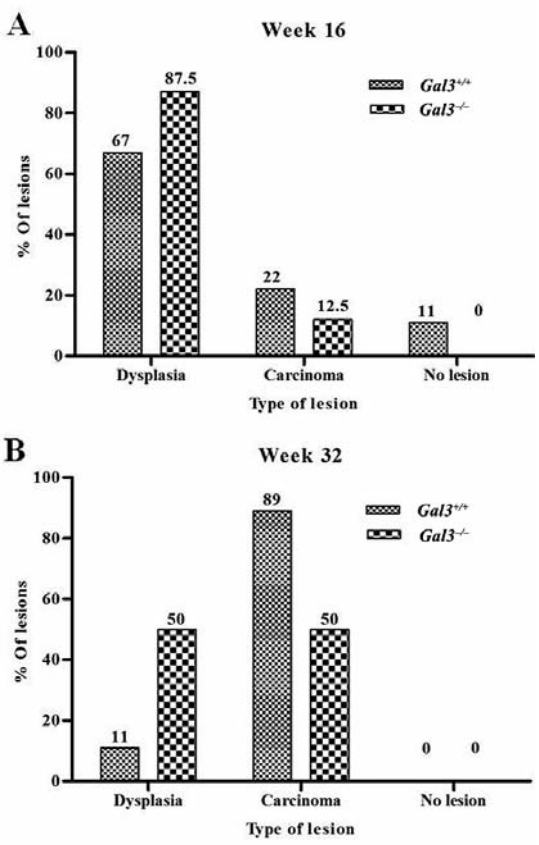

Figure 1. *Incidence of dysplasia and carcinoma developed in Gal3*$^{+/+}$ *and Gal3*$^{-/-}$ *mice at week 16 (A) and week 32 (B).*

permit its complete study on microscopic as well as other fragments of the same tongue to exclude other microscopical changes. All fragments were embedded in paraffin blocks, cut, and stained with hematoxylin and eosin for microscopic analysis. Histopathological alteration of the tongue epithelium was graded as dysplasia or carcinoma according to criteria described previously (15, 16). All histological slides were blindly and independently examined by three well-trained pathologists (PRF, AML, and SVC). A consensus scoring was used to solve any discrepancies. When two or more areas of epithelial alterations were present in the same histological slide, the highest grade epithelial lesion was taken as representative to determine which pathologic alteration each mouse had. In addition, the frequency of tumors diagnosed microscopically in tongue for each animal was determined.

Immunohistochemistry. To identify beta-catenin expression by immunohistochemistry, we used the streptavidin-biotin-peroxidase method. Serial tongue sections of 3 μm were mounted on 3-aminopropyltriethoxy-silane-coated glass slides (Sigma Chemicol Co., St Louis, MI, USA), deparaffinized in xylene, dehydrated in graded ethanol, and then treated in a microwave with EDTA solution (1 mM, pH 8.0) for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 15 min, and the slides were

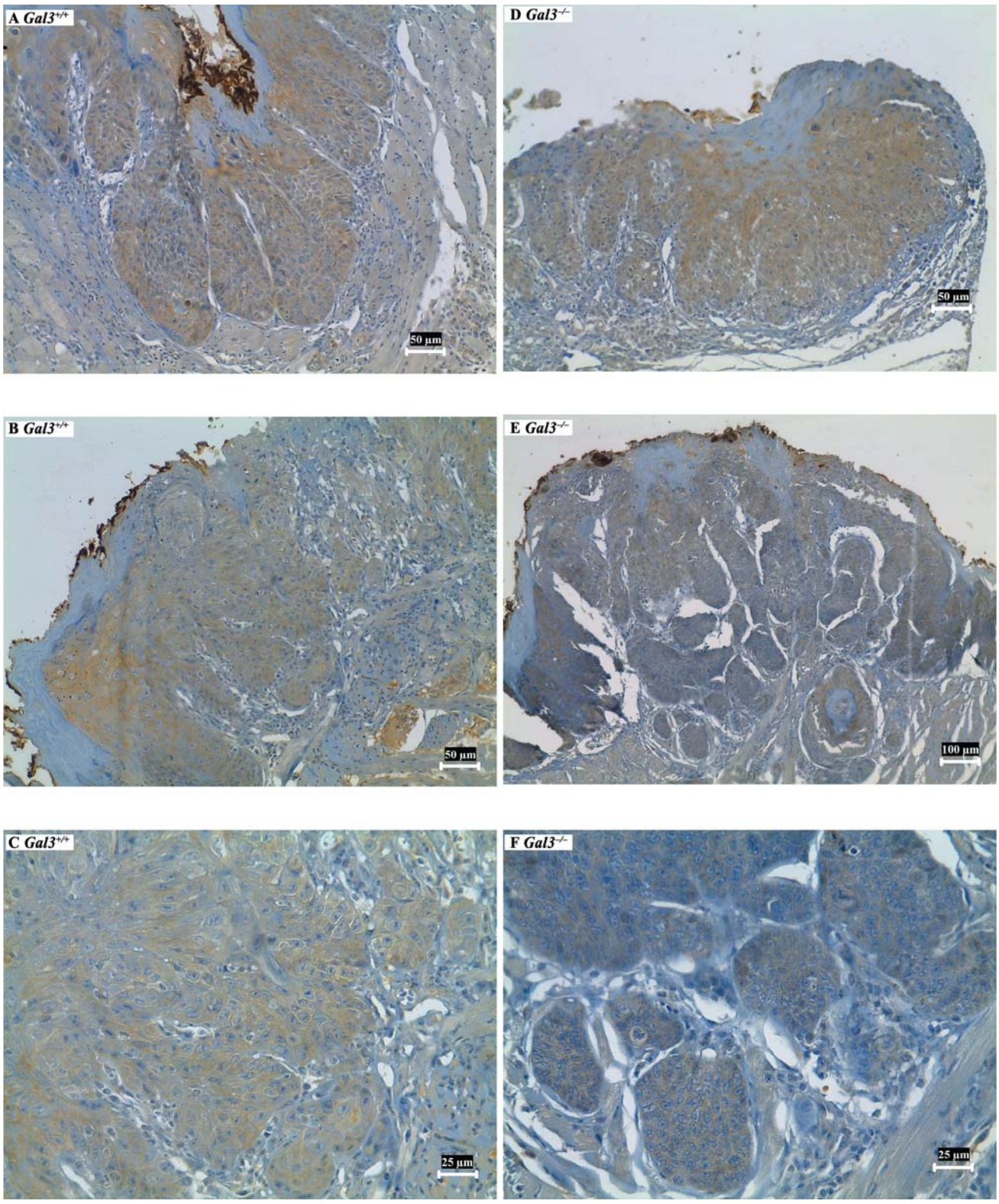

Figure 2. *Immunohistochemical staining of beta-catenin expression. A: Severe dysplasia from Gal3$^{+/+}$ mice. B: Invasive carcinoma from Gal3$^{+/+}$ mice. C: High power from B showing a predominance of non-membranous beta-catenin expression inside tumor cells. D: Moderate dysplasia from Gal3$^{-/-}$ mice. E: Invasive carcinoma from Gal3$^{-/-}$ mice. F: High power from E showing a predominance of non-membranous beta-catenin expression inside tumor cells.*

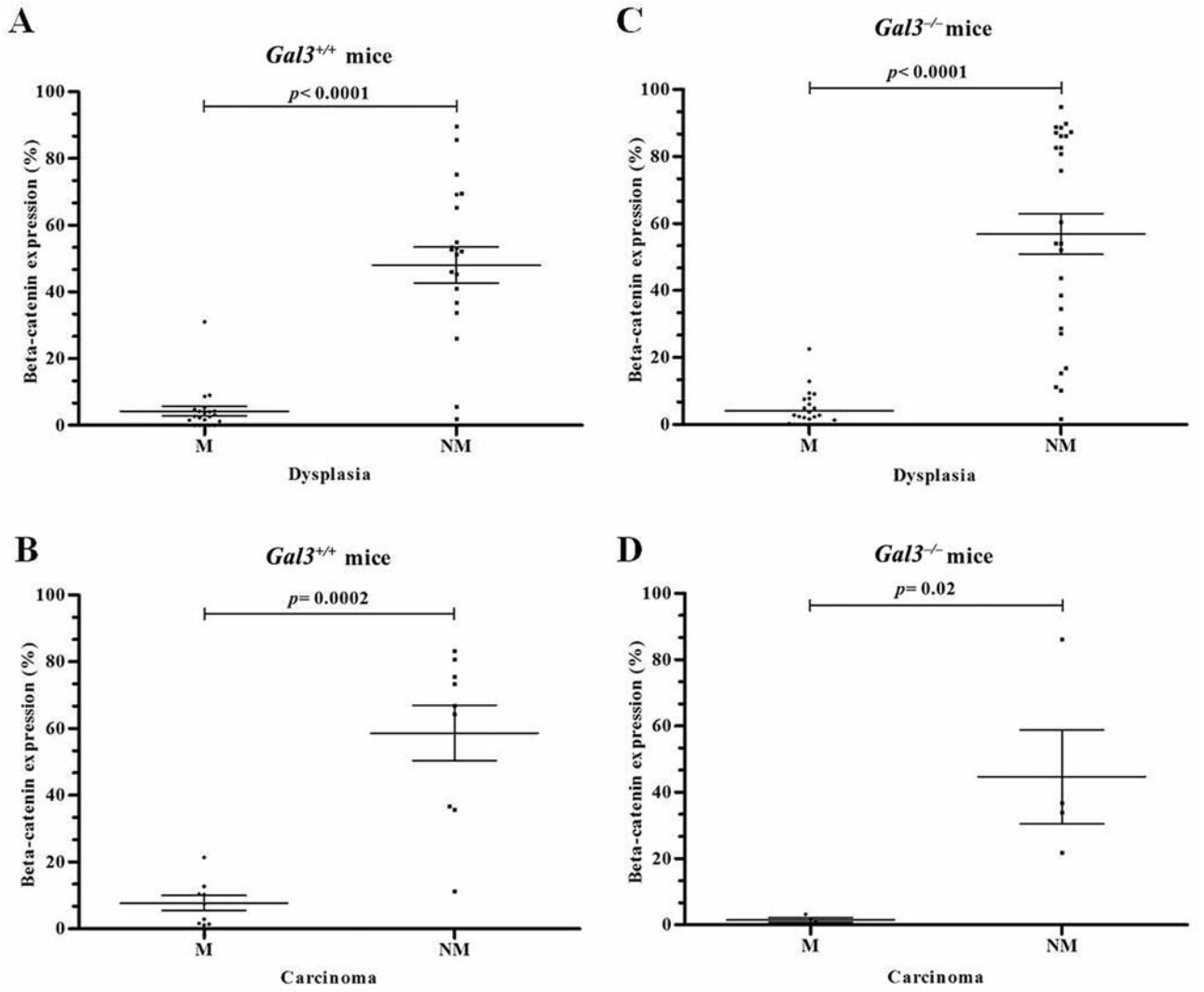

Figure 3. *Index for mean beta-catenin positivity. A and B: Index for membranous and non-membranous beta-catenin positivity in dysplasias and carcinomas from Gal3$^{+/+}$ mice. C and D: Index for membranous and non-membranous beta-catenin positivity in dysplasias and carcinomas from Gal3$^{-/-}$ mice. M: Index for membranous positivity. NM: Index for non-membranous positivity.*

preincubated with a protein block solution [1% skim milk, 0.05% Triton X, and phosphate-buffered saline (PBS)] for 20 min at room temperature to prevent non-specific binding. Sections were then incubated in a humid chamber overnight at 4˚C with primary anti-beta-catenin antibody (rabbit polyclonal, H-102; Santa Cruz Biotechnology, CA, USA), diluted at 1:200. The reaction was revealed with chromogen 3' 3'-diaminobenzidine tetrahydrochloride (Dako Co, Carpinteria, USA) and the sections were counterstained with Harris' hematoxylin. As positive control, we used palatine tonsil samples and as negative control, the diluted solution of the antibody.

Immunohistochemical evaluation. To evaluate beta-catenin expression, we established an index of immunopositive cells as being ratio of between the number of positive cells and the total cells counted in areas diagnosed as dysplasia and carcinoma. Due to the small size of the lesions obtained during the experiment, we considered the entire length of each lesion on microscopic view in the analysis of beta-catenin-positive cells. In addition, as beta-catenin exerts dual functions depending on its location in the cell, two patterns of beta-catenin immunostaining were considered: membranous (positivity only at the membrane) and non-membranous (positivity in the cytoplasm and/or nucleus).

Statistical analysis. Fisher's exact probability test was used to evaluate the incidence of dysplasia and carcinomas among *Gal3*$^{+/+}$ and *Gal3*$^{-/-}$ mice. For the evaluation of non-membranous and membranous beta-catenin expression in dysplasia and carcinoma from both group of mice, the Mann-Whitney non-parametric test was used. The values are expressed as the mean±SD. $P<0.05$ was considered to be statistically significant.

Results

During the study, two *Gal3*$^{+/+}$ and six *Gal3*$^{-/-}$ mice died. Oral carcinogenesis occurred in both groups. Dysplastic alterations with various degrees of atypia were found in the tongue. In summary, 10 (55.5%) *Gal3*$^{+/+}$ and 4 (28.5%) *Gal3*$^{-/-}$ mice were diagnosed with carcinoma. As shown in Figure 1, the incidence of carcinomas increased from week 16 to week 32 in both groups of mice, especially in *Gal3*$^{+/+}$ mice at week 32. However, no statistical difference was reached. Moreover, all carcinomas developed during the experiment were histopathologically classified as well-differentiated.

Immunohistochemistry. The pattern of beta-catenin expression from *Gal3*$^{+/+}$ and *Gal3*$^{-/-}$ can be seen in Figure 2.

The mean index of immunoreactivity for membranous beta-catenin was low and closely similar in the areas of dysplasia from *Gal3*$^{-/-}$ and *Gal3*$^{+/+}$ mice (4±5.2% *vs*. 4.3±6.6%, respectively). On the other hand, an increase of non-membranous beta-catenin-positive cells in the same area was found in both groups, being more evident in *Gal3*$^{-/-}$ mice (mean index=56.9±30.8%) than *Gal3*$^{+/+}$ mice (mean index=48±24.9%). However, this difference was not statistically significant. Among carcinomas from *Gal3*$^{+/+}$ and *Gal3*$^{-/-}$ mice, the mean index of membranous beta-catenin-positive cells was also low compared to the non-membranous expression pattern. In this sense, the mean index of cells positive for membranous beta-catenin in carcinomas from *Gal3*$^{-/-}$ mice was 1.6±1.3% compared to 7.7±6.8% in *Gal3*$^{+/+}$ mice. With regard to non-membranous immunostaining pattern in these lesions, an increase in the mean index of positivity was observed in both groups, especially in *Gal3*$^{+/+}$ mice. In this group, a mean index of 58.7±24.9% was found, while in *Gal3*$^{-/-}$ mice a mean index of 44.7±28.4% was reached (Figure 3). However, no difference statistically significant was found, although our results indicate a trend for higher Wnt signaling activation in *Gal3*$^{+/+}$ than *Gal3*$^{-/-}$ mice.

Discussion

Countless animal models are used for oral carcinoma development experimentally, varying according to the animal type employed, the carcinogen used and time of tumor induction (14, 17). Most studies have used the carcinogens 7,12-dimethylbenz(a)anthracene (DMBA) and 4NQO. Although both these carcinogens induce tumors in the oral cavity, recent studies have shown that 4NQO presents advantages in relation to DMBA, mainly as regards the development of histopathological and genetic changes in a manner similar to its human tumor counterpart (18, 19). 4NQO mediates experimental carcinogenesis in the oral cavity and aerodigestive track *via* oxidative stress and formation of DNA adduct by 4-hydroxyaminoquinoline-1-oxide, an intermediate compound produced by the enzyme 4NQO reductase (20-22). It has also been showed that 4NQO has specificity for carcinoma induction in the esophagus and posterior dorsal region of the tongue, which in turn seems to be related to a high concentration of the 4NQO reductase (20, 22). Hence, both regions demonstrate high susceptibility to tumor formation after 4NQO insult, as recently observed (14). Using the same experimental approach, we were able to induce tongue carcinomas in both groups of mice that, in general, presented histopathological aspects similar to those in other 4NQO-induced oral carcinogenesis studies and the majority of their human tumor counterparts.

Overall, the incidence of carcinomas was higher in *Gal3*$^{+/+}$ than in *Gal3*$^{-/-}$ mice (55.5% and 28.5% respectively). As shown in Figure 1, the incidence of carcinomas increased from week 16 to week 32 in both groups, this increment being more evident in *Gal3*$^{+/+}$ mice at week 32. However, no statistical significance in difference was reached. Similar to the results previously found by us using the same mouse model (10), the data showed here corroborate the evidence that the absence of galectin-3 does not affect the development of tongue carcinoma and then the oral carcinogenesis, even though the higher incidence of carcinomas in *Gal3*$^{+/+}$ than *Gal3*$^{-/-}$ mice at week 32. On the other hand, our data are different from those found in a previous study (8), which used 4-methylnitrosamino-1-3-pyridyl-1-butanone-induced lung carcinogenesis in Gal3$^{+/+}$ and Gal3$^{-/-}$ mice, which found a significant high incidence of lung tumors in Gal3$^{+/+}$ mice at week 32. Notwithstanding this, one might state that the lack of significance of our findings could be ascribed to the real absence of functional effects of galectin-3 in oral carcinogenesis. In fact, it is well established that galectin-3 exerts a multitude of biological effects, which sometimes include opposing functions, in a cell- and tissue-dependent manner (5). Indeed, a recent study using galectin-3 null mice revealed that galectin-3 did not affect the development of lung tumor or tumor metastasis (9). However, there are no studies evaluating the functional relevance of galectin-3 in tongue epithelium under physiological and pathological conditions. It is probable that the mechanism of action of galectin-3 in the tongue is different to that in other regions, such as lung, and other studies are warranted to determine whether this lectin is truly important for the development of tongue carcinomas in mice.

With respect to beta-catenin expression, the results found here revealed a predominance of non-membranous beta-catenin-positive cells in dysplasias and carcinomas from *Gal3*$^{+/+}$ and *Gal3*$^{-/-}$ mice compared to the membranous pattern, which was much lower in both groups as well. A reduced membranous beta-catenin expression and increase in its extension in the cytoplasm of oral squamous cell carcinomas has been described (23). It has also been showed that the accumulation of beta-catenin in the cytoplasm and/or

nucleus is one of the hallmarks of an activated Wnt pathway (24). In addition, galectin-3 has been recently considered an important mediator of the Wnt signaling pathway, a signal transduction that is frequently mutated in different tumors and leads to uncontrolled proliferation *via* transcription of *c-MYC* and cyclin D1 genes (12, 25, 26). Corroborating these data, an *in vivo* study recently showed that development of lung tumor was associated with activated Wnt signaling in *Gal3*$^{+/+}$ mice (8). However, the mechanism by which galectin-3 may modulate Wnt signaling and beta-catenin level is still unclear. Previously, it was shown galectin-3 mediated activation of PI3K/Akt signaling in J82 cells overexpressing galectin-3 (27). More recently, it was showed that galectin-3 positively mediates the Wnt pathway, as well as the beta-catenin level, inside cells through regulation of GSK3-beta *via* Akt activation in colon cancer cells (6). Furthermore, they also showed a positive correlation between high galectin-3 level and nuclear accumulation of beta-catenin, which in turn was accomplished by cyclin D1 expression. Here, we did not discriminate the subcellular distribution of beta-catenin between the cytoplasm and nucleus in dysplasias and carcinomas from both groups. Instead, we categorized its presence in both compartments or only in a unique compartment, *i.e.* the cytoplasm or nucleus, as a non-membranous pattern. Based on this, the high positivity index for non-membranous beta-catenin in dysplasias and carcinomas from *Gal3*$^{-/-}$, as observed in *Gal3*$^{+/+}$ mice, may not truly reflect the occurrence of activated Wnt signaling in this group of mice. It is widely known that the presence of beta-catenin in the nucleus leads to the transcription of Wnt target genes, the hallmark of activated Wnt signaling. So, it is possible to suppose that the high expression of non-membranous beta-catenin in dysplasias and carcinomas from *Gal3*$^{-/-}$ may be associated with its accumulation mainly in the cytoplasm and rarely in the nucleus of cells. However, other studies are warranted to confirm its distribution inside cells from oral premalignant and malignant lesions developed in *Gal3*$^{-/-}$ mice.

Our results indicate that the absence of galectin-3 does not seem to affect the localization of beta-catenin in oral dysplastic lesions and carcinomas from *Gal3*$^{-/-}$ when compared to *Gal3*$^{+/+}$ mice. Furthermore, we showed that in both groups of mice, the Wnt signaling pathway is activated, which could explain the absence of significance in respect to the number of *Gal3*$^{+/+}$ and *Gal3*$^{-/-}$ mice affected by carcinoma. Other studies remain to be developed to provide new information about the biological properties of galectin-3 that may be associated with tongue tumorigenesis in mice.

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