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Hedgehog signaling pathway mediates tongue tumorigenesis in wild-type mice but not in Gal3-deficient mice

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is one of the most aggressive cancers of the oral cavity and an important cause of death worldwide. Currently, there are limited clinical tools aiding clinicians to establish its early diagnosis, and genetic and epigenetic events leading to the pathogenesis of OSCC remain unsolved. The use of carcinogen-induced knocked out mouse models would help to improve its early detection and also determine the role of proteins such as galectin-3 (Gal3) in this process. Here we used a mouse model of oral carcinogenesis employing two mouse genotypes: wild-type (Gal3 +/+) and galectin-3-deficient mice (Gal3 −/−) challenged by the carcinogen 4NQO for 16 weeks. After induction, the expression of Wnt1, Wnt3A, Shh and Gli3 proteins in tongue samples was evaluated using an immunohistochemistry approach. All samples of dysplasia and carcinoma were negative for Wnt1. Wnt3A expression was detected in both Gal3 +/+ and Gal3 −/− mice, at similar levels. Wnt3A expression did not predict tongue tumorigenesis in either genotype. Dysplastic- and carcinoma-expressing Shh was statistically significantly higher in Gal3 +/+ mice than Gal3 −/− mice ($p < 0.0001$), and was associated with tongue tumorigenesis only in the former. Gli3 expression decreased and increased from dysplasia to carcinoma in Gal3 +/+ and Gal3 −/− mice, respectively, although the difference was not significant. The results suggest that activated Wnt signaling is present in both mice, and that the Hh signaling pathway might play a role in tongue carcinoma development in Gal3 +/+ mice.

1. Introduction

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all malignant tumors arising in the oral epithelium surface and is a leading cause of cancer death worldwide (da Silva et al., 2011; Sant'ana et al., 2011). Although the complete mechanism responsible for its development has not yet been elucidated, it is known that genetic and epigenetic events affecting keratinocyte DNA may lead to OSCC formation. In this regard, the 4NQO-induced oral carcinogenesis model has been the gold-standard approach to identifying early changes in genes and their respective proteins as well as dysregulated signaling pathways that might be involved in the pathogenesis of OSCC (Kanojia and Vaidya, 2006; Reibel, 2003).

Galectins are carbohydrate-binding proteins that play crucial roles in different tissues and cells, with galectin-3 (Gal3) being a central member of this family (Liu and Rabinovich, 2005; Markowska et al., 2010). Gal3, a protein of 31 kDa, has been strongly linked to tumorigenesis in many tissues, but its role in OSCC remains to be determined (Sant'ana et al., 2011; Takenaka et al., 2004). To shed light on this matter, our group recently published an in vivo study showing that the incidence of tongue carcinoma did not differ significantly between wild-type (Gal3 +/+) and Gal3-deficient mice (Gal3 −/−), indicating that Gal3 does not seem to play any role in driving oral tumor formation (de Faria et al., 2011).

The Wnt signaling pathway has been implicated in a variety of normal cellular processes, including embryogenesis and differentiation (Giles et al., 2003). Studies have shown that aberrant Wnt signaling activation promotes tumorigenesis and may also be associated with OSCC development (Giles et al., 2003; Perez-Sayans et al., 2012). Following Wnt activation, beta-catenin is stabilized in the cytoplasm and then ferried to the nucleus via Gal3 mediation whereby Wnt target genes are upregulated (Sant'ana et al., 2011; Shimura et al., 2004; Song et al., 2009). Otherwise, glycogen synthase kinase-3beta (GSK-3b)-mediated beta-catenin phosphorylation leads it to be degraded via the ubiquitin–proteosome complex (Hagen and Vidal-Puig, 2002). We investigated this discovery

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regarding the Gal3–beta-catenin interaction, and hence the interaction with the Wnt signaling pathway, using the same mouse model (Sant'ana et al., 2011). In this study, we found a significant increase in non-membranous beta-catenin expression from dysplasia to carcinoma when compared with membranous expression in both Gal3 +/+ and Gal3 −/− mice, suggesting that Wnt signaling seems to be activated even in the absence of Gal3 (Sant'ana et al., 2011).

The Hedgehog signaling pathway (Hh) also plays a pivotal role in embryonic development (Beachy et al., 2004). In mammals, three Hh homologous genes are recognized: Sonic (Shh), Indian (Ihh), and Desert hedgehog (Dhh) (Mimeault and Batra, 2010). After binding of these proteins to the membrane receptor Patched and inducing Gli protein activation, a group of cell cycle-regulating genes are transcribed (Ruiz i Altaba, 1999; Tang et al., 2007; Xuan et al., 2006). As in the Wnt signaling pathway, GSK-3beta regulates this pathway by inhibiting the Gli family of proteins and it has been shown that this inhibition occurs mainly via a Sufu–GSK-3beta–Gli interaction, and in part due to a Sufu–Gal3 interaction (Briscoe and Therond, 2013; Kise et al., 2009; Mill et al., 2005; Paces-Fessy et al., 2004). Moreover, a recent report showed that augmented expression of inactive GSK-3beta was linked to tongue tumorigenesis in Gal3 +/+ mice but not in Gal3 −/− mice (Mendonca et al., 2012). These findings prompted us to address whether the Hh signaling pathway might be differentially activated in Gal3 +/+ mice compared to Gal3 −/− mice in this setting.

So, the aim of the current study was to investigate whether the Wnt and Hh signaling pathways influence tongue carcinogenesis in Gal3 +/+ and Gal3 −/− mice. The results suggest that the Hh signaling pathway plays an essential role in driving tongue tumorigenesis in Gal3 +/+ mice and also confirm our previous report that activation of the Wnt signaling pathway occurs even in the absence of Gal3 and seems to be mediated by the Wnt3 signal.

2. Material and methods

2.1. Experimental protocol

To study the expression of Wnt-1, Wnt-3A, Shh, and Gli-3 proteins in dysplasias and carcinomas developed experimentally in mouse tongue, a standardized protocol for the 4NQO-induced experimental mouse tongue carcinogenesis model was employed. For this, 38 Gal-3 −/− male mice aged 6 weeks and weighing 21–23 g, kindly bred and supplied by Hsu's group (Hsu et al., 2000), and 36 age-matched Gal3 +/+ mice (control group) were challenged with 4NQO after an acclimatization period of 2 weeks. All animals were housed in the Center for Bioterism and Animal Experimentation at the Universidade Federal de Uberlândia (UFU), and maintained in controlled conditions of humidity, temperature, and a 12-h light–dark cycle. The animal study was managed following an animal protocol approved by the Committee on Animal Experimentation of the UFU.

The carcinogen 4NQO was prepared based on the protocol established by Tang et al. (2004), with some modifications. In short, 4NQO was diluted in filtered water to a final concentration of 100 μg/ml. Every week a fresh 4NQO solution was prepared and given to the mice in their drinking water ad libitum. For all groups, 4NQO administration terminated after 16 weeks of carcinogen intake. After this period of induction, groups of five Gal3 +/+ and Gal3 −/− mice were killed at specific time points, as follows: immediately after the end of treatment (at week 16), 4 weeks later (at week 20), 8 weeks later (at week 24), 12 weeks later (at week 28), and 16 weeks later (at week 32). Before killing the mice by cervical dislocation, all of them were anesthetized with xylazine and ketamine via intraperitoneal injection. After that, their tongues were removed, fixed in buffered formalin (4%) for 24 h, routinely processed, and embedded in paraffin. The first fragment of each paraffin block was cut into 5-μm thick sections using a microtome and stained with hematoxylin and eosin (HE) for histopathological examination focusing on the detection of dysplasia (mild, moderate and severe) and carcinoma. To this end, both tongue-developing lesions were diagnosed in accordance with criteria reported by Lumerman et al. (1995) and Cardesa et al. (2005), respectively. All lingual tissue samples were analyzed independently by three oral pathologists (PRF, SVC, and AML) and cases with controversial diagnoses were decided by consensus. In the meantime, serial sections of 3-μm thickness were taken from the same paraffin blocks with the aim of studying the expression of each antibody using a standardized immunohistochemical tool.

2.2. Immunohistochemistry

The streptavidin–biotin peroxidase method was used to evaluate the expression of Wnt-1, Wnt-3A, Shh, and Gli-3 proteins in dysplasias and carcinomas in each group of mice. All information about the antibodies employed in the present study and their respective dilutions is depicted in Table 1.

Initially, the fragments were mounted on glass slides previously treated with 3-aminopropyltrietoxy silane (Sigma, Chemical Co., St. Louis, USA), dewaxed in xylene and dehydrated in decreasing ethanol solutions. After this, the tissue sections were immersed in a solution of ammonium hydroxide (10%) and ethanol (95%) for 15 min to remove the formolic pigment and then treated with H_2O_2 (10 V) to block endogenous peroxidase activity. Next, these tissue sections were boiled in 1 mM EDTA solution, pH 8.0, with three cycles of 5 min each in a microwave, for antigen retrieval. Then, the slides were washed in Tris–HCl buffer solution (20 mM, pH 7.8) and incubated with the primary antibodies overnight at 4 °C. Following incubation, the fragments were washed in Tris–HCl buffer solution and then incubated with the biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase for 30 min each (LSAB, Dako, Carpinteria, CA, USA). The antibody reaction was detected using the diaminobenzidine chromogen and the tissue samples then counterstained with Harris's hematoxylin. A human breast carcinoma sample was used as a positive control for Wnt-1 and Wnt-3A, and the stomach and testis for Shh and Gli-3, respectively. Omission of primary antibody was used as the negative control.

2.3. Immunohistochemical evaluation

A semi-quantitative analysis was used to evaluate the immunoreactivity of each antibody employed in the present study. To this end, both the intensity and percentage of positive cells were considered in the analysis (Sinicrope et al., 1995). Immunostaining intensity was scored in three categories: weak (1), moderate (2), and intense (3). The percentage of positive cells was classified in four categories: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (>75%). To determine the mean score/lesion value, the percentage and immunostaining intensity of positive cells were established for each lesion's field as mentioned above, and then the values multiplied to obtain a preliminary score/field. Lastly, the sum of each field's score was divided by the number of fields took from the lesion to give the mean score/lesion value. All lesions were independently evaluated by two observers (DOS and PRF), and those ones were differentially scored were reanalyzed together until achieving a consensus. Moreover, due to the small size

Table 1
Antibodies used in immunohistochemical analysis.

Antibody	Source	Catalog number	Dilution
Wnt-1	Santa Cruz[a]	6280	1:200
Wnt-3A	Milipore[b]	09-162	1:200
Gli-3	Santa Cruz[a]	20688	1:50
Shh	Santa Cruz[a]	9024	1:100

[a] Santa Cruz Biotechnology, Santa Cruz, CA, USA.
[b] Millipore Corporation, Billerica, MA, USA.