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ORIGINAL ARTICLE

Characterization of Wingless-Type signaling pathway proteins in odontogenic keratocyst and ameloblastoma

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Abstract:

Introduction: The Wnt signaling pathway is directly related to the development process of odontogenic keratocysts (OKC) and ameloblastomas (AM). However, the molecular mechanisms involved in the genesis and the activation process of the Wnt pathway of these tumors still need to be elucidated. **Objective:** To evaluate the immunohistochemical expression of Wingless-type (Wnt) proteins in OKC and AM. **Materials and methods:** Immunohistochemical staining was performed using antibodies against Wnt1, Wnt5a, SUFU and β-catenin proteins in cases of odontogenic keratocysts (n = 11) and ameloblastomas (n = 13). The analysis was performed by analysing staining intensity in both epithelial cells and fibroblasts. **Results:** Epithelial cells exhibited predominance of immunostaining in both lesions, with statistical association for SUFU markers in AM (p = 0.05) and β-catenin in OKC (p = 0.04). Wnt1 and Wnt5a had 100% immunostaining in epithelial cells and fibroblasts of AM and OKC, respectively. SUFU was significantly higher in OKC epithelial cells when compared to those in AM (p = 0.05). There was no statistical association in the immunosuppression of fibroblasts between AM and OKC, for all markers. **Conclusion:** The high intensity immunoexpression of Wnt1, β-catenin, SUFU and Wnt5a markers in OKC and AM epithelial cells suggests that Wnt signaling pathways, canonical and non-canonical, are activated and may contribute to the development, progression and aggressiveness of these lesions. On the other hand, it is suggested that the significant presence of SUFU in OKC epithelial cells confers a protective factor to this lesion, when compared to AM epithelial cells, indicating that the inactivation mechanisms of the canonical pathway may be functioning. However, further investigations should be conducted to confirm such hypotheses.

Keywords: Odontogenic Cysts; odontogenic Tumors; Wnt signaling pathway.

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INTRODUCTION

Odontogenic keratocyst (OKC) and ameloblastoma (AM) are locally invasive odontogenic lesions that present a high recurrence rate [1,2]. The Wnt signaling pathway has a direct influence on embryonic development and odontogenesis. In addition, it's possible that this pathway plays a fundamental role in the development of odontogenic lesions [3–8]. Wnts are signal transducing glycoproteins responsible for activating the Wnt signaling pathway [8,9]. There are three activation pathways for the Wnt signaling cascade; they are: canonical pathway or Wnt/β-catenin, non-canonical pathway or via Wnt/ planar cell polarity, or non-canonical pathway via Wnt/Ca2 +[7,8,10,11].

Wnt pathway activity is associated with the presence of complex proteins coordinated by β-catenin, its role as a transcriptional activator being well established[8,12]. In the absence of the Wnt signal, β-catenin plays an important role in cellular adhesion[7,9,10,13,14] and the cytoplasmic level of this protein is kept low, via degradation mechanisms regulated by the ubiquitin-proteasome pathway. On the other hand, signals from Wnt ligands, such as Wnt1 activate FZD family receptors and LRP-5/6 co-receptors[7,9,15]. This binding, in turn, recruits the Dvl (Disheveled) protein, whose role is to prevent the phosphorylation of GS3Kβ. Dephosphorylated GS3Kβ prevents ubiquitination and proteasomal degradation of β-catenin. As a consequence, there is an accumulation of β-catenin cytoplasm, which is translocated to the nucleus, where it combines with the TCF/LEF1 transcription factors and promotes the expression of genes that are involved in cell proliferation, inhibition of apoptosis, and invasion and migration of the tumor cell[7,9].

Wnt pathway activity in human tumors is associated with greater aggressiveness[8,9,16]. In contrast, tumor cells also express molecules that attenuate the activity of the Wnt pathway, e.g., SUFU and Wtn5a[17]. In this case, SUFU acts by translocating β-catenin from the nucleus to the cytoplasm, thereby preventing binding to the TCF/LEF1 transcription factors. Wnt5a, in turn, activates Wnt through mechanisms that inhibit the canonical pathway[18,19].

The objective of this study was to characterize proteins involved in the Wnt pathway, in OKC and AM, especially in regards to canonical activation (Wnt1 and β-catenin) and suppression (SUFU and Wnt5a) of the Wnt pathway in these lesions.

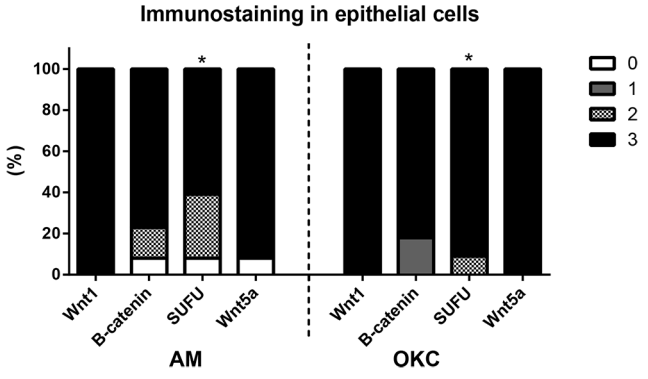
MATERIAL AND METHODS

After approval by the institutional ethics committee, samples were obtained from the archives of the Anatomical Pathology Laboratory of the Faculty of Dentistry at the Federal University of Bahia. Eleven samples of OKC and thirteen of AM were included in this study. All cases were reanalyzed by an experienced pathologist, in accordance with WHO criteria[20].

Histological sections of 3μm thickness were arranged on silanized sheets and used for the immunohistochemical reactions. Antigenic recovery was performed in a water bath with pH 6.0 citrate solution (Ready-to-use Dako solution) for 45' at 97 °C. Blocking of the endogenous peroxidase was performed with 3% H_2O_2 and for non-specific sites with Protein Blocking Solution™ (Dako, Carpinteria, USA) (Graph 1) and incubated overnight at 4 °C. This was followed by incubation with the reagents HRP Link and HRP Enzyme (Advance™, Dako Corporation, Carpinteria, USA) for 20' and with 3.3-diaminobenzidine (Dako, Carpinteria, USA) for 5' in the dark. Counterstaining was performed with Harris's haematoxylin. All of the reactions were accompanied by a positive control (Graph 1); the negative control was the normal serum of the same isotype for each antibody.

Immunohistochemical evaluation was performed by an observer using a light microscope coupled with a digital camera system (AxioStar®, Zeiss, Germany). Initially, a semi-quantitative analysis of the epithelial and fibroblasts cells was performed according to the intensity and proportion of the immunostaining ratio of the evaluated protein, according to the following scores: 0 (<5% positive cells); 1 (6-25% positive cells); 2 (26-75% positive cells); 3 (>75% positive cells)[21]. Figure 1 details the immunoexpression of all markers in both lesions.

For statistical analysis, the Statistical Package for the Social Sciences (SPSS), version 13.0, was used. The evaluation of possible associations between the groups examined was performed using Fisher's exact test and Pearson's Chi-Square test. The acceptable level for significance was $p<0.05$.



Graph 1. Distribution and analysis of immunostaining in epithelial cells of odontogenic keratocysts and ameloblastomas. * Pearson Chi-Square (p <0.05).

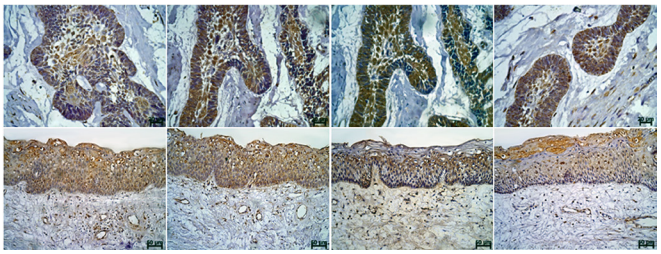


Figure 1. Photomicrographs of odontogenic keratocysts (OKC) and ameloblastomas (AM) samples demonstrating the pattern of immunoblotting with Wnt1, ß-catenin, SUFU and Wnt5a.

RESULTS

WNT1

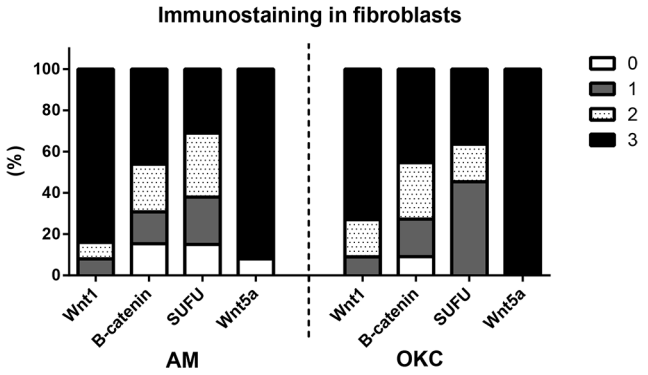
Wnt1 was detected in all OKC (n = 11) and AM cases (n = 13). The evaluation of the intensity score in epithelial cells did not show a statistical association between either lesion (p> 0.05), considering that all OKC and AM cases were classified as having high immunohistochemical expression (Graph 1). For both, OKC (n = 8, 73%) and AM (n = 11, 84%), the immunohistochemical analysis of fibroblasts showed a predominant staining intensity score of 3 (Graph 2). However, the analysis of the fibroblasts found no statistical significance in the immunostaining between AM and OKC (p = 0.5) (Graph 2), nor in the analysis of said relationship between epithelial cells and fibroblasts by lesion (p AM = 0.89 / p OKC = 1) (Graph 3 and 4).

β-catenin

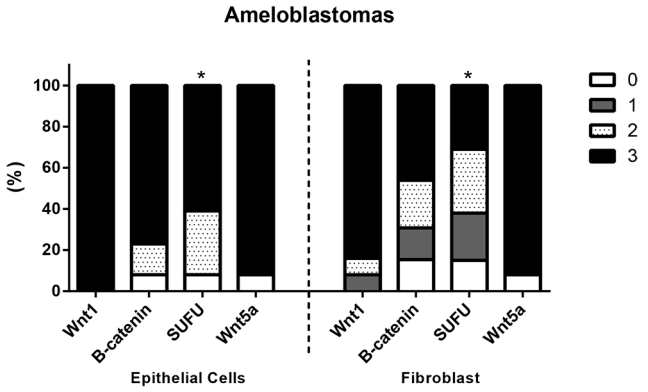
The analyses of immunostaining intensity in epithelial cells was found to be 100% (n = 11) positive for OKC, whereas 8% (n = 1) of AM cases were negative. An immunostaining score of 3 was observed in epithelial cells in 82% (n = 9) of OKC cases and 77% (n = 10) of AM cases. There was no immunostaining in the fibroblasts in 9% (n = 1) of OKC and 15% (n = 2) of AM cases. The analysis of immunostaining between ameloblastoma epithelial cells and fibroblasts found no statistical association (p = 0.08) (Graph 1). In contrast, the analyses of this same ratio in the OKC cases showed that the predominance of a staining intensity score of 3 in epithelial cells was higher (n = 9, 82%) when compared to the immunostaining seen in the fibroblasts (n = 5, 45.4%) (p = 0.04).

SUFU

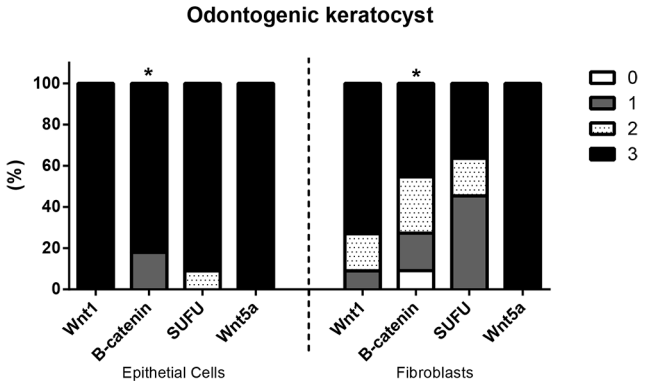
Immunostaining of SUFU was present in 100% (n = 11) of the OKC epithelial cell and fibroblast cases



Graph 2. Distribution and analysis of immunostaining in fibroblasts of odontogenic keratocysts and ameloblastomas. * Pearson Chi-Square (p <0.05).



Graph 3. Immunostaining analysis between fibroblasts and epithelial cells of ameloblastomas. * Pearson Chi-Square (p < 0.05).



Graph 4. Immunostaining analysis between fibroblasts and epithelial cells of odontogenic keratocysts. * Pearson Chi-Square (p < 0.05).

analysed, and absent in 8% (n = 1) of AM epithelial cell and 15% (n = 2) of AM fibroblast cases (Graph 1 and 2). It was observed that epithelial cells showed a greater predominance of a staining intensity score of 3 in OKC (n = 10, 91%) when compared to AM cells (n = 8, 61%) (p = 0.05) (Graph 2). The ameloblastoma samples showed superiority in regards to an intensity score of

3, both for epithelial (n = 8, 61%) and fibroblastic (n = 4, 31%) analysis, with a statistical association found in the relation between these immunostaining patterns (p = 0.05) (Graph 3). For OKC, no statistical association was found between epithelial cells and fibroblasts when analyzing immunostaining (p = 0.42) (Graph 4).

Wnt5a

The results for Wnt5a immunostaining in epithelial cells (AM = 12, 92% / OKC = 11, 100%) and fibroblasts (AM = 12, 92% / OKC = 11%) were the same between lesions, with no statistical difference seen in any of the analysis (Graph 1, 2, 3 and 4). For both lesions, there was a predominant staining intensity score of 3 for both the epithelial cells and fibroblasts (AM = 12, 92% / OKC = 11, 100%), with the exception of AM which received an intensity score of 0 in 8% (n = 1) of the samples, in both the epithelium and the fibroblast.

DISCUSSION

This study demonstrates for the first time in the literature the immunostaining pattern of the proteins associated with canonical and non-canonical activation of the Wnt signaling pathway in epithelial cells and fibroblasts of ameloblastomas and keratocyst odontogenic cysts. The results revealed that Wnt1, β-catenin, SUFU and Wnt5a, in most cases analyzed, received a high score in both analyses. It should be noted that epithelial cell immunostaining was higher for all markers in both lesions and significantly higher for SUFU in ameloblastomas and β-catenin in OKC. In addition, SUFU has been shown to have greater activity in OKC epithelial cells when compared to ameloblastoma epithelial cells. Therefore, we can infer that wingless-type signaling pathway proteins may be involved in the development of odontogenic keratocysts and ameloblastomas.

The multiple interactions between stroma and epithelial cells play an essential role in the development, maintenance and progression of tumors[22]. In this context, studies have shown that mesenchymal cells are potentiators in the formation of odontogenic lesions[23–25]. In myofibroblasts, for example, there is an increase in the expression of smooth muscle actin[26–30], as well as a direct relation with the presence of proteins related to aggression, such as MMP- 2[27]. In contrast, other proteins linked to aggression and the production of myofibroblasts, such as TGF-β1 and IFN-γ[31], do not seem to be involved in this process. Although there are no studies that discuss the Wnt pathway in stromal odontogenic lesions, our results demonstrate that the proteins Wnt1, β-catenin and SUFU show greater activity in epithelial cells and such cells tend to play a significant role in the maintenance and development of these lesions in the canonical Wnt signaling pathway. In contrast, Wnt5a was shown to be highly immunoreactive and equally active in both cells and could contribute to both the inactivation of the canonical pathway and to the activation of the non-canonical pathway in fibroblasts associated with AM and OKC. However, further studies are recommended to better understand the participation of the Wnt signaling pathway in stromal odontogenic lesions.

The major ligands for activation of the Wnt signaling pathway, canonical and non-canonical, are the Wnt1 and Wnt5a molecules respectively[5,6,32,33]. According to our results, the expressive immunostaining of Wnt1 and Wnt5a in AM and OKC epithelial cells reveals that the Wnt signaling pathway may be activated by both pathways. However, there are caveats in regards to the function exerted by Wnt1 and Wnt5a that must still be explained.

The neoplastic transformation of Wnt1 is associated with inducing an increase in the cytoplasmic levels of β-catenin[8,9,34]. Although our results did not show the immunoexpression of the cellular compartment of this protein, such associations have previously been demonstrated in various articles, proving the proportional correlation between an increase in the complex WNT1 / β-catenin-cytoplasmic expression in AM and OKC epithelial cells[32,33] In addition, the varying results in intensity of β-catenin immunoexpression can be justified by the presence of SUFU, mainly in OKC, which in turn removes β-catenin from the nucleus and leaves it available in the cytoplasm for degradation of the ubiquitin proteasome pathway, thus avoiding canonical activation.

It is worth repeating that, in addition to activating the non-canonical pathway, Wnt5a is also recognized for inhibiting and activating the canonical pathway. The mechanism for inhibition may occur from the interactions of this molecule with calcium/calmodulin-dependent protein kinase II (CaMKII) or with the Ro2 receptor, which prevents the binding of β-catenin with the TCF/LEF1 transcription factors. This consequently inhibits the transcription of target genes linked to the activation of the canonical Wnt signaling pathway. On the other hand, Wnt5a can activate the canonical pathway in a

manner similar to Wnt1[18,35], binding to the Fz receptor and its LPR5/6 co-receptor, which recruits and activates Dvl, in such a way that the enzyme GSK3β is inhibited. Such interactions compromise the phosphorylation of β-catenin and, consequently, its degradation via the ubiquitin proteasome[9]. Thus, excess β-catenin is stabilized in the cytoplasm and translocates to the nucleus, where it activates target gene transcription via TCF/LEF1. Activation of these transcription factors may also occur secondary to the activation of protein kinase A (PKA). Given that, in the presence of Wnt5a, PKA inhibits the enzyme GSK3β, thus leading to the stabilization and nuclear accumulation of β-catenin and, concomitantly, to an increase in the transcription of the target genes[18,35]. In light of our results and the broad influence of Wnt5a, we can only infer that this protein may be involved in the Wnt signaling pathway. However, the exact definition of the mechanism can be only be confirmed via molecular studies.

In our comparative analysis between the lesions, we observed that Wnt1, β-catenin and Wnt5a, are equally distributed among the lesions, with the exception of the SUFU marker, which is found to be significantly higher in OKC epithelial cells. Considering that SUFU translocates β-catenin from the nucleus to the cytoplasm and inhibits the activation of the canonical pathway[8,36], this result denotes a protective factor for OKC in relation to AM. Thus, we could infer that the signaling pathway Wnt may be more active in ameloblastoma and therefore could possess more aggressive characteristics, such as increased cell proliferation motility. However, in the literature there is some controversy when comparing the proliferation potential of these lesions. There are studies that show that AM present a greater potential for proliferation[26-28], whereas there are other studies which show that this profile is more common in OKC[29,30] and others which do not show any statistical differences[31]. In studies analyzing the homeostatic balance of the tissue, by means of apoptotic index, one observes in the discussions the same imprecision in results[32-34]. Recently OKC was reclassified as an odontogenic cyst[20]. However, the comparison between the proliferative profile of the benign AM tumor and OKC needs to be better elucidated so as to give a better understanding of the pathogenesis of these lesions.

CONCLUSION

Studies that have evaluated the Wnt signaling pathway, comparing the expression of the canonical and non-canonical signaling pathway molecules in OKC and AM, are still limited. However, the literature has shown that disorders in the expression of molecules involved in cell signaling, such as Wnt1[5,32,34], Wnt5a[5,33,37], Wnt10a[5,32], β-catenin[3,12,19], APC[3], Axina1[4] and Axina2[12] have been observed in OKC and AM. Regarding SUFU, only one study found immunostaining in OKC, with its association with the AM[36]. Therefore, according to our results, we can infer that Wnt1, Wnt5, SUFU and β-catenin proteins play a representative role in the pathogenic process of ameloblastomas and odontogenic keratocysts. However, in the literature there is a shortage of studies discussing this subject in regards to these lesions, which makes it difficult to better understand and gauge our results. It should be pointed out that an in-depth study of the Wnt pathway is important to clarify the role of Wnt signaling molecules in the genesis and cytodifferentiation of odontogenic lesions[42,43]. Elucidation of this pathway will hopefully provide the elements necessary to better understand the pathogenesis of odontogenic lesions. This will in turn help in the search for diagnostic and prognostic markers, as well as the development of new therapeutic approaches that prevent relapse and guarantee better quality of life to the patients affected by these pathologies[4,6-8,13,19,22,24,42]. For these reasons it is important to deepen the biological knowledge of the Wnt signaling pathway via further studies of odontogenic lesions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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