Altered β-catenin expression in oral mucosal dysplasia: a comparative study

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ABSTRACT

Objective: The current study aimed to investigate the β-catenin expression in oral leukoplakia (OL) with different degrees of epithelial dysplasia and normal oral mucosa. Material and Methods: Formalin-fixed, paraffin-embedded tissue samples of 39 OL (mild dysplasia n=19, moderate dysplasia n=13, and severe dysplasia n=7), and 10 normal oral mucosa (control group) were submitted to immunohistochemical reactions to anti-β-catenin primary antibody. A qualitative β-catenin analysis was performed based on the percentage of positive cells. The cellular location and the epithelial layer were also considered. The Chi-square test and the Fisher’s exact test were used to verify possible differences in the β-catenin expression among the OL groups. A p-value of <0.05 was considered statistically significant. Results: Membranous expression of β-catenin in parabasal and basal layers was gradually lost in the higher degrees of epithelial dysplasia. In normal oral mucosa, β-catenin was detected only in the cytoplasmic membrane. However, a significant increase in cytoplasmic β-catenin could be observed between mild and moderate dysplasia (Fisher Exact test - p<0.001) and between mild and severe dysplasia (p<0.001). Conclusions: The β-catenin cytoplasmic expression observed in this study may represent the initial stage of modifications in the E-cadherin-catenin complex, along with morphological cellular changes.

Keywords: Beta-catenin. Oral dysplasia. Oral cancer. Oral leukoplakia.

INTRODUCTION

Disturbances in cell adhesion and cytoskeleton dynamics could signal an early event in oral carcinogenesis8. These alterations, including loss of cell-cell adhesion and increase in cell motility, occur in the epithelial-mesenchymal transition (EMT) process20. EMT transition is a biological process characterized by changes in cellular phenotype and function, undergone by cells from epithelial phenotype to motile mesenchymal phenotype13. However, this transition is also seen in tumor progression and metastasis10. EMT is activated by wide-ranging stimuli provided by growing factors and hypoxia, as well as by transcription factors such as Snail, Slug and Twist15. Twist is an important negative regulator of the transmembrane adhesion protein E-cadherin; it is believed that the downregulation of E-cadherin is the central event of EMT29. E-cadherin is located in the epithelial cell regions of cell-cell adhesion known as adherens junctions4. E-cadherin loss leads to the disassembly of adherens junctions, increased tumor cell mobility and invasiveness23. β-catenin is a bifunctional protein that acts in cell adhesion and as a transcription factor activated by the Wnt pathway4. E-cadherin interacts with β-catenin through intercellular connections to form stable adhesions; the dissociation of this complex is involved in malignant progression5. Downregulation of membranous E-cadherin and cytoplasmic or nuclear accumulation of β-catenin...
have been previously implicated in the loss of differentiation and more aggressive phenotypes, in a variety of different cancers.\(^7\,18\).

Recently, it was suggested that the altered expression of E-cadherin/\(\beta\)-catenin complex is involved in growth regulation and phenotype changes of both dysplastic oral epithelia and malignant oral epithelia.\(^17\) According to Kaur, et al.\(^15\) (2013), losses of E-cadherin and \(\beta\)-catenin altered expressions are considered early events in oral carcinogenesis. Moreover, an aberrant expression of \(\beta\)-catenin was also found in actinic cheilitis cases and in OSCC of the lip, indicating that altered patterns of this protein are also present in oral lesions with actinic etiology.\(^26\) Lo Muzio, et al.\(^19\) (2009) observed an altered expression of \(\gamma\)-catenin in a large group of dysplastic lesions of the oral mucosa. However, they suggest that the role of \(\gamma\)-catenin expression as a prognostic marker in dysplastic oral lesions seems to be restricted.

In our previous study, we observed that the downregulation of E-cadherin promoted by Twist could be involved in oral carcinogenesis through the Wnt pathway.\(^9\) Accordingly, the current study aimed at testing this hypothesis by investigating the \(\beta\)-catenin immunohistochemical expression in oral leukoplakia (OL) with different degrees of epithelial dysplasia and normal oral mucosa.

### MATERIAL AND METHODS

This study was approved by the Human Ethics Committee of the Institutional Review Board (Committee approval no. 015/2010).

#### Specimens

Formalin-fixed, paraffin-embedded tissue samples of 39 OL (mild dysplasia \(n=19\), moderate dysplasia \(n=13\), and severe dysplasia \(n=7\)) and 10 normal oral mucosa from 18 males and 21 females were collected from the Laboratory of Oral Pathology of the Federal University of Goiás and from the Laboratory of Oral Pathology of the University of São Paulo. All the biopsed specimens were collected in the period of 1998-2012. The clinical pathological data from all studied cases were described in Figure 1. The samples were submitted to 5 \(\mu\)m histological sections for routine staining with hematoxylin eosin (H&E) and analyzed under light microscopy. The histological grades of oral dysplasia were reviewed by two independent oral pathologists in blind fashion, according to the World Health Organization (WHO).\(^7\) Any disagreement in the findings was discussed among the pathologists to render a final evaluation.

#### Immunohistochemistry

The immunohistochemical reactions were performed using 3 \(\mu\)m 4% formalin-fixed slides followed by dewaxing and rehydration in an ethanol series. Antigen retrieval consisted of immersing the sections in a solution of 10 mM monohydrated citrate buffer solution (pH 6.0) and heating them in a water bath at 95°C for 30 minutes. Endogenous peroxidase activity was blocked with 6% hydrogen peroxide and a methanol solution, in two baths of 15 minutes each at room temperature. After washing with Tris buffer (pH 7.4), the slides were incubated with anti-\(\beta\)-catenin (E-5; Santa Cruz Biotechnology, Santa Cruz, CA, US, dilution 1:100) primary antibody overnight at 4°C. The negative control was obtained by omitting the specific primary antibody during the reaction. The slides were then exposed to avidin-biotin complex (LSAB-Kit + HRP; DakoCytomation, Carpinteria, CA, USA) and to 3,3'-diaminobenzidine chromogen (DAB+; DakoCytomation, Carpinteria, CA, USA). The sections were counterstained with Meyer hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted.

A qualitative \(\beta\)-catenin analysis was performed based on the percentage of positive cells, considering positive only those specimens that presented 25% or more cells presenting \(\beta\)-catenin expression. The cellular location (cytoplasmic membrane, cytoplasm and nucleus) and the epithelial layer (basal, parabasal and spinous cell layers) were also considered. \(\beta\)-catenin immunohistochemical expressions were analyzed by two independent blind and calibrated observers under light microscopy at 200-fold magnification.

#### Statistical analysis

The data was tabulated with Microsoft Excel software, and statistical analysis was performed by Statistical Package for Social Sciences software (SPSS 16, Headquarters, USA). The Chi-square test and the Fisher’s exact test were used to verify possible differences in the \(\beta\)-catenin expression among the OL groups. A \(p\)-value of <0.05 was considered statistically significant.

#### RESULTS

In general, \(\beta\)-catenin immunohistochemical staining was observed in the cytoplasmic membrane in all OL dysplastic groups (\(n=39\); mild, moderate and severe dysplasia) (Figure 2). In mild, moderate and severe dysplasia, \(\beta\)-catenin staining was also detected in the cytoplasm in 51.2% (\(n=20\)) of OL cases (Figure 2, A-F). In addition, membranous expression of \(\beta\)-catenin in parabasal and basal layers was gradually lost in the higher degrees of epithelial dysplasia (Figure 2, B, D, F, H).
Table 1- Differences in β-catenin expression in mild, moderate and severe oral dysplasia, and normal oral mucosa (Control)\textsuperscript{a,b}

| Cytoplasmic localization | Mild | Moderate | Severe | Control |
|-------------------------|------|----------|--------|---------|
|                         | n    | %        | n      | %       | n      | %       |
| Basal layer             |      |          |        |         |        |         |
| Negative                | 17   | 89.5     | 2      | 15.4    | 0      | 10      | 100     |
| Positive                | 2    | 10.5     | 11     | 84.6    | 7      | 100     | <0.001  |
| Total                   | 19   | 100      | 13     | 100     | 7      | 100     |         |
| Basal and Parabasal layers |      |          |        |         |        |         |
| Negative                | 19   | 100      | 4      | 30.8    | 1      | 14.3    | 10      | 100     |
| Positive                | —    | 0        | 9      | 69.2    | 6      | 85.7    | —       | <0.001  |
| Total                   | 19   | 100      | 13     | 100     | 7      | 100     | 10      | 100     |

\textsuperscript{a} Chi-square test
\textsuperscript{b} Equal letters indicate statistical difference by Fisher’s Exact Test.
Figure 2- β-catenin immunohistochemical expression in oral leukoplakia (OL) (mild, moderate and severe dysplasia) and normal oral mucosa. (A) β-catenin cytoplasmic membrane staining, in all three layers of mild dysplasia (x100), and (B) in the cytoplasm of basal and parabasal cells. (C, D) Illustrations of the β-catenin cytoplasmic membrane (CM) expression in the upper layers of moderate dysplasia, showing a decrease in cytoplasmic membrane expression, with more evident cytoplasmic localization in basal and parabasal cells (arrows head) (x100 - x400). (E, F) Weak membranous expression of β-catenin in parabasal and basal layers of severe dysplasia, with a significant increase in its expression, in the cytoplasm (arrows head) of cells of basaloïd appearance (x100 - x400). (G, H) β-catenin in normal oral mucosa (x100 - x400).
In normal oral mucosa, β-catenin was detected only in the cytoplasmic membrane (Figure 2, G-H). On the other hand, a significant difference in β-catenin cytoplasmic staining was identified among all the OL groups (Chi Square test - p<0.001) (Table 1). When specifying these differences in the β-catenin expression, a significant increase in cytoplasmic β-catenin could be observed between mild and moderate dysplasia (Fisher Exact test - p<0.001), mild and severe dysplasia (p<0.001), normal oral mucosa and moderate dysplasia (p<0.001), and oral normal mucosa and severe dysplasia (p<0.001) (Table 1). A cytoplasmic β-catenin expression was clearly detected in cells with a basaloid appearance.

Analyzing the cytoplasmic β-catenin distribution among epithelial layers, a predominance of basal and parabasal localization of this protein was noted in the highest grades of dysplasia (moderate and severe dysplasia) (Figure 2, C-F). In mild dysplasia, cytoplasmic β-catenin staining was found only in the basal layer (Figure 2, A-B; Table 1).

**DISCUSSION**

β-catenin is an adhesion molecule that interacts with E-cadherin through its cytoplasmic domain. The E-cadherin-β-catenin complex disruption from cell membrane seems to be important in malignant transformation9,14-16,22,25,27-29, and interferences in this complex could lead to β-catenin cytoplasmic accumulation and consequent nuclear translocation21,27. This altered localization is related to the activation of some genes involved in cell proliferative activities3, malignant transformation and tumor progression24. Accordingly, the Twist protein seems to play an important role in E-cadherin repression, also linked to β-catenin altered expression8,11,24 through the Wnt pathway30-32. The altered expression of E-cadherin immunoexpression found in epithelial dysplasia, and the possible role of Twist in oral malignant transformation, found in our previous reports8,26,30, led us to investigate the β-catenin immunoexpression in oral leukoplakia (OL) with different degrees of epithelial dysplasia.

In this study, we observed an altered (cytoplasmic) β-catenin immunoexpression among OL dysplastic groups. In our previous report, we showed a gradual decrease in E-cadherin immunoexpression in the basal and parabasal cell layers, as the degree of dysplasia increased8. Interestingly, in the present investigation we found a similar inverse pattern, with a predominance of basal and parabasal localization of cytoplasmic β-catenin, and with an intensification of its expression concomitantly with OL progression to a more severe grade of dysplasia. This could indicate the presence of disturbances in the E-cadherin-β-catenin complex in OL, thus indicating that losses of E-cadherin and β-catenin abnormal expressions are early events of oral carcinogenesis.

In this case, it is plausible to assume that cytoplasmic β-catenin could participate in oral malignant transformation, insofar as its cytoplasmic accumulation has been demonstrated in different tumors19 and also in oral epithelium with dysplastic changes15,25. Although nuclear β-catenin is considered a solid sign of pathway deregulation12-25, it is reasonable to presume that the cytoplasmic β-catenin localization found in the present study is an event that precedes β-catenin translocation to the nucleus. Theoretically, this may be attributed to activation of the Wnt pathway that inhibits phosphorylation and degradation of β-catenin, and that induces its cytoplasmic accumulation2. Additionally, downregulation of membranous E-cadherin with cytoplasmic accumulation of β-catenin has been previously reported in several tumors6.

Recently, a few studies have also found increased β-catenin cytoplasmic accumulation in oral dysplasia15,19,25. Nevertheless, the exact role of catenin expression in oral malignant transformation is still uncertain. Ishida, et al.12 (2007) observed β-catenin expression in the cell membrane and the cytoplasm of 24% of basal and spinous cells of oral leukoplakia without dysplasia. However, they also found significant nuclear β-catenin expression in more than 80% of epithelial cells of oral leukoplakia with dysplasia. Additionally, it was noted that the nuclear expression of β-catenin in epithelia increased depending on the grade of dysplasia.

Lo Muzio, et al.19 (2009) evaluated the immunohistochemical staining of β-catenin and β-catenin in 49 cases of oral epithelial dysplasia and in 10 samples of normal oral mucosa. As in the present study, they described that when the catenin expression was lost, a cytoplasmic delocalization occurred. However, they were not able to find significant differences in the catenin expression between cases that progressed to oral squamous cell carcinoma (OSCC) and cases that did not.

In cases of carcinoma in situ, Alvarado, et al.1 (2011) observed β-catenin in the nucleus of cells with a basaloid appearance and a diffusely β-catenin expression in the cytoplasm of lower-half basaloid cells. Although they considered the nuclear expression of β-catenin a sign of its nuclear translocation, they also mentioned that β-catenin had been previously found restricted to the cytoplasm in OSCC samples. In the present investigation, we found a similar cytoplasmic expression in basaloid cells in lower-half of OL.
dysplastic epithelia. This could indicate that cytoplasmic accumulation of \( \beta \)-catenin participates in the acquisition of a more motile phenotype. In a recent study, Schussel, et al.\(^{25}\) (2011) evaluated cases of actinic cheilitis (AC) and squamous cell carcinoma (SCC) of the lip, and observed that most cases of AC showed both membrane and cytoplasmic expression of \( \beta \)-catenin, with only 22% expressing \( \beta \)-catenin in the nucleus.

Our data showed that membranous expression of \( \beta \)-catenin in parabasal and basal layers was gradually lost in the higher degrees of epithelial dysplasia. In addition, a significant difference in \( \beta \)-catenin cytoplasmatic staining was identified among all the OG groups.

CONCLUSIONS

The \( \beta \)-catenin cytoplasmic expression observed in this study may represent the initial stage of modifications in the E-cadherin-catenin complex, along with morphological cellular changes. We suggest that this cellular mechanism could be associated with Twist overexpression through the Wnt pathway. Further studies are needed to test this hypothesis.

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