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# Subcellular localization and expression of E-cadherin and SNAIL are relevant since early stages of oral carcinogenesis

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ABSTRACT

The biological process of epithelial-to-mesenchymal transition (EMT) has been studied in oral squamous cell carcinoma (OSCC) metastasis, but it is rarely evaluated at several stages of oral carcinogenesis. This study aimed to analyze the presence of SNAIL and E-cadherin proteins, markers of EMT, in the development and progression of OSCC, evaluating excised specimens of potentially malignant lesions (oral leukoplakia with and without dysplasia-OL and OLD, respectively), tumor tissues (OSCC), metastatic lymph nodes (LN), and normal oral mucosa (NOM) by immunohistochemistry, considering subcellular localization. Additionally, SNAIL and E-cadherin transcripts were evaluated *in vitro* by qPCR, using SCC-9 cell line in comparison to human keratinocytes (HPEC). There was a significant increase in nuclear expression of SNAIL from NOM to OLD followed by a noticeable decrease in nuclear expression accompanied by increased cytoplasmic expression in OSCC ($p < 0.05$). The E-cadherin cytoplasmic expression was remarkable and statistically significant higher in OSCC and LN, both compared to NOM ($p < 0.0001$), OL ($p < 0.01$) and OLD ($p < 0.0001$ and $p < 0.001$, respectively). *In vitro*, E-cadherin and SNAIL transcripts were lower in SCC-9 compared to HPEC cells, although only the decrease of E-cadherin was statistically significant ($p < 0.05$). Regarding the association of E-cadherin and SNAIL expression with the clinical findings, the analysis revealed an association between the cytoplasmic expression of SNAIL and the invasion pattern ($p = 0.05$) in OSCC. The increased nuclear SNAIL expression may be characteristic of OLD, and the presence of E-cadherin in cell cytoplasm a marker of transformation to malignancy of potentially malignant oral leukoplakias into OSCC.

## 1. Introduction

Oral squamous cell carcinoma (OSCC) is one of the most widely occurring cancers throughout the world and a major subtype of head and neck squamous cell carcinoma [1]. It accounts for approximately 90% of oral malignancies and mainly affects men over 40 years old, smokers and alcoholics [2,3]. OSCC is an aggressive neoplasm responsible for high morbidity and mortality rates with a low overall survival rate of less than 60% in five years [4,5]. The prognostic factor with the most significant impact in this disease is still the presence of metastases in cervical lymph nodes, present at the time of diagnosis in 50% of the cases [6].

In the pathogenesis of OSCC, a series of mutations resulting in the selected growth of mutated cells, which replace the normal cells in a specific region, occurs. This process is supposed to comprise a sequence of gradual architectural and cytological alterations (epithelial dysplasia) that presumably compromise the entire epithelial lining of a specific site within the oral cavity, and are followed by tumor establishment and later invasion of tumor cells to the underlying tissues [7,8]. OSCC often is preceded by a white or red mucosal change known as leukoplakia or erythroplakia, collectively classified as “potentially malignant disorders”, which may exhibit none or different degrees of epithelial dysplasia. The risk for dysplasia or carcinoma is higher for leukoplakia or erythroplakia from the lateral tongue and floor of mouth

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compared with those in other oral sites [9]. In this sense, the biological understanding of oral carcinogenesis is of key importance for the prediction of development as well as outcome of OSCC [8].

The mechanisms underlying the acquisition of the invasive phenotype and the subsequent systemic spread of the cancer cells have been studied and the activation of an epithelial-to-mesenchymal transition (EMT) program has been proposed as the critical mechanism for the acquisition of malignant phenotypes by some epithelial cancer cells [10,11]. EMT, a fundamental event in embryogenesis and wound healing, is a biological process by which cells lose their epithelial characteristics and come to present a phenotype similar to that of the mesenchymal cells [12,13]. This process is mediated by cell transcription factors and can orchestrate intracellular changes such as the down-regulation of epithelial markers in addition to the positive regulation of some mesenchymal markers [14–17].

During EMT, the transcription factor SNAIL binds to three E-boxes sequences present in the human E-cadherin promoter and represses transcription of E-cadherin, a cell membrane-associated protein involved in cell-cell adhesion, which is the key player in inducing cell polarity and organizing of the epithelium [11,18,19]. In cancer, EMT enables transitioned tumor cells to invade the underlying connective tissue and migrate to predetermined destinations, where they can revert back (though a mesenchymal-epithelial transition – MET) and establish metastasis regionally or at distant sites [11].

Much is known about the dynamics between SNAIL and E-cadherin in OSCC, but rarely these markers are evaluated from the early stages of OSCC carcinogenesis. The aim of this study was to analyze the presence of SNAIL and E-cadherin proteins in the development and progression of OSCC, evaluating potentially malignant disorders (leukoplakia), with and without different degrees of epithelial dysplasia, as well as tumor tissues and metastatic lymph nodes, in comparison to the morphologically healthy tissue.

## 2. Material and methods

### 2.1. Study design

SNAIL and E-cadherin were evaluated at protein level by means of immunohistochemistry and at the molecular level by quantitative real-time polymerase chain reactions (qRT-PCR). Immunoexpression was performed in formalin-fixed paraffin-embedded human tissue specimens corresponding to different stages of oral carcinogenesis as well as normal oral mucosa, while gene transcripts were assessed in SCC-9 OSCC cell line in comparison to a human keratinocytes cell line.

### 2.2. Patients and clinical specimens

Tissue samples consisted of 26 cases of oral leukoplakia – 10 cases without epithelial dysplasia (OL) and 16 cases with epithelial dysplasia (OLD) -, 15 cases of OSCC and 9 metastatic lymph nodes (LN), referring to incisional biopsies and/or surgical specimens included in paraffin and selected retrospectively from the anatomic-pathological reports of the archives of the Oral Pathology Laboratories from both School of Dentistry of Bauru, University of São Paulo (FOB-USP), and School of Dentistry of the Federal University of Goiás (FO-UFG). As a control group, morphologically healthy tissue samples were obtained from gingival tissue covering impacted mandibular third molars with indication of surgical removal, and consisted of ten cases of normal oral mucosa (NOM) retrieved from the archives of the Laboratory of Pathological Anatomy of the Faculty of Dentistry of the Federal University of Bahia (FO-UFBA). Clinical and histopathological data were also retrieved from the archives. The present study was approved by the Human Ethics Committee of Bauru School of Dentistry (CAEE 25465913.0.0000.5417).

### 2.3. Light microscopy

All histological slides were stained with hematoxylin-eosin (HE) and previously reviewed under the light microscope for diagnostic confirmation and classification. OLD slides were graded by two oral pathologists according first to the World Health Organization (WHO) criteria [20] and later to the binary classification system in high and low risk, based on the morphological criteria for prediction of malignant transformation defined by Kujan et al. [21]. OSCC were classified solely according to the WHO criteria [20].

### 2.4. Immunohistochemistry

The paraffin-embedded tissues were sectioned (3 µm) and collected sequentially on silane-coated glass slides. Following deparaffinization with xylene and rehydration with ethanol, the sections were immersed in citrate buffer for 20 min to antigen retrieval and incubated with 3% hydrogen peroxide at 95 °C for 30 min. Afterwards, the slides were incubated at room temperature for 2 h with the primary antibody anti-SNAIL (#ab53519, polyclonal, dilution 1:75; Abcam, Cambridge/UK) and anti-E-Cadherin (#ab22585, monoclonal, dilution 1:200; Abcam, Cambridge/UK). After washing with Phophate Buffered Saline (PBS), the sections were incubated for 30 min with N-Histofine® Simple Stain™ MAX PO polymer (#414151 F; Nichirei Biosciences Inc., Tokyo/Japan) and then labeled with 3,3′- diaminobenzidine (#K3468, Liquid DAB Plus; Dako, Glostrup/Denmark) for 2–3 min at room temperature and counterstained with Harris' hematoxylin. Breast or lung tissue samples with known positive reactivity were included as positive controls, and negative controls were obtained through the omission of primary antibody.

### 2.5. Evaluation of immunoreactivity

Qualitative and semi-quantitative assessment of the immunostaining were performed under 400x magnification (DM500; Leica Microsystems, Wetzlar/Germany) and immunoreactivity was considered specific if the specimens had no background staining. Semi-quantitative analysis consisted of a scoring system (Table 1) based on proportion of positive cells (E-cadherin) and intensity of the immunostaining (SNAIL) within cell compartments (cell membrane, cytoplasm and nucleus) of the epithelial cells (NOM, OL, OLD), tumor cells of invasive tumor front (OSCC) and tumor cells close to capsular region of metastatic lymph nodes (LN). The proportion of SNAIL immunoreactivity was not evaluated because all epithelial cells showed nuclear and cytoplasmic labeling in every sample. For final analyzes, SNAIL and E-cadherin immunoexpression were dichotomized (Table 1) according to protein expression as low expression (scores 0 and 1) and high expression (scores 2 and 3).

### 2.6. Cell culture

SCC-9 cells (ATCC® CRL-1629™; Manassas/USA), derived from a

**Table 1**
Scoring system used for immunoreactivity analysis. E-cadherin immunoreactivity was analyzed semi-quantitatively as to the proportion of labeled cells, while SNAIL immunoreactivity was analyzed regarding the intensity of labeling. Both analyzes received a score of 0 to 3, which was also dichotomized in low and high expression for some statistical analyzes. Modified from [40] Yuen et al.

| Score | Dichotomization | E-cadherin | SNAIL |
|---|---|---|---|
| 0 | Low expression | < 1% | Negative |
| 1 | | 1% to 39% | Weak |
| 2 | High expression | 40% to 79% | Moderate |
| 3 | | 80% to 100% | Strong |