ESCOLA DE CIÊNCIAS DA SAÚDE
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JOÃO MATHEUS SCHERBAUM EIDT

RELAÇÃO DE FATORES CLÍNICOS, *Candida* spp., E-CADERINA E VIMENTINA COM ALTERAÇÕES DISPLÁSICAS NA LEUCOPLASIA ORAL

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RELATIONSHIP OF CLINICAL FEATURES, *Candida* spp. AND E-CADHERIN AND VIMENTIN EXPRESSION WITH DYSPLASTIC ALTERATIONS IN ORAL LEUKOPLAKIA

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Dissertação apresentada como requisito para obtenção do título de Mestre pelo Programa de Pós-Graduação em Odontologia, Área de Concentração: Estomatologia Clínica

Orientadora: Profª. Drª. Karen Cherubini

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Determinação, coragem e autoconfiança são fatores decisivos para o sucesso. Não importa quais sejam os obstáculos e as dificuldades, se estamos possuídos por uma inabalável determinação, conseguiremos superá-los. Independentemente das circunstâncias, devemos ser sempre humildes, recatados e despidos de orgulho.

Dalai Lama (1935 -)

Dedicatória

Dedico este trabalho, especialmente, aos meus pais, **João Antônio** e **Silvana**, ao meu irmão, **Gabriel**, e à minha noiva, **Mayara**. Obrigado pelo apoio incondicional durante esses dois anos. Amo muito vocês.

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Resumo

RESUMO

A leucoplasia é a lesão potencialmente maligna mais prevalente na cavidade oral. Embora alterações displásicas do epitélio sejam um indicador do potencial maligno da leucoplasia, a determinação exata do grau da displasia é uma tarefa difícil, o que compromete esse fator preditivo. Dessa forma, a predição da transformação maligna da leucoplasia oral é um desafio, e biomarcadores específicos são necessários para esse fim. O objetivo do presente estudo foi investigar a relação entre alterações displásicas da leucoplasia oral e fatores clínicos, *Candida* spp., e expressão de E-caderina e vimentina. Prontuários médicos e espécimes de biópsia emblocados em parafina pertencentes a 60 pacientes foram alocados em quatro grupos de acordo com as características histológicas da lesão: (1) sem-displasia: 15 casos de leucoplasia sem displasia epitelial; (2) displasia epitelial: 15 casos de leucoplasia com displasia epitelial (moderada ou severa); (3) carcinoma de células escamosas oral (OSCC): 15 casos de leucoplasia com diagnóstico histopatológico de OSCC; (4) grupo-controle: 15 casos de hiperplasia fibroepitelial da mucosa oral. Os prontuários foram revisados considerando-se os fatores idade e sexo dos pacientes, uso de álcool e/ou tabaco, sítio anatômico da lesão. Foi realizada análise imunoistoquímica para avaliar a expressão de E-caderina e vimentina, e a coloração de ácido periódico de Schiff (PAS) para detecção de *Candida* spp.. Sítios de alto risco exibiram associação com displasia epitelial e OSCC. Não houve diferença significativa entre os grupos para os demais fatores clínicos avaliados e para detecção de *Candida* spp. na coloração PAS. A avaliação quantitativa de expressão de E-caderina não diferiu significativamente entre os grupos avaliados, enquanto a expressão de vimentina foi significativamente maior na displasia epitelial e no OSCC do que nos demais grupos.

Conclusão: De acordo com os resultados do presente estudo, sítios de alto-risco (borda e ventre de língua e assoalho de boca) estão associados com o fenótipo de displasia epitelial da leucoplasia oral, enquanto idade, sexo, álcool, tabaco e *Candida* spp. não exibem essa associação. A expressão de vimentina está associada com o fenótipo de displasia epitelial e parece ser mais específica que a E-caderina para uso como marcador imunoistoquímico de detecção dessas alterações.

Palavras-chave: Câncer oral; transição epitélio-mesenquimal; leucoplasia oral; E-caderina; vimentina; *Candida spp.*

Summary

SUMMARY

Leukoplakia is the most prevalent potentially malignant lesion in the oral cavity, and histopathological examination is the gold standard for its diagnosis. Even though epithelial dysplastic features can be an indicator of malignant potential in oral leukoplakia, the exact determination of the grade of dysplasia is a hard task, which compromises this predictive factor. Therefore, predicting malignant transformation of oral leukoplakia is a challenge, and specific biomarkers are necessary for this purpose. The aim of the present study was to investigate the relationship of dysplastic changes in oral leukoplakia and clinical factors, *Candida* spp., and E-cadherin and vimentin expression. Medical records and paraffin blocks of biopsied specimens of 60 patients were distributed into 4 groups: (1) no-dysplasia: 15 cases of leukoplakia without epithelial dysplasia; (2) epithelial dysplasia: 15 cases of leukoplakia with epithelial dysplasia (moderate or severe); (3) oral squamous cell carcinoma (OSCC): 15 cases of leukoplakia with histopathological diagnosis of OSCC; and (4) control group: 15 cases of fibroepithelial hyperplasia. Medical records were reviewed regarding age, sex, alcohol and tobacco use, and anatomical site of the lesion. Immunohistochemical analysis was carried out for determination of E-cadherin and vimentin expression, and periodic acid of Schiff (PAS) staining for *Candida* spp. detection. High-risk sites showed association with the epithelial dysplasia and OSCC groups. There was no significant difference between the groups for the other clinical features analyzed and for *Candida* spp. positivity with PAS. Quantitative E-cadherin expression did not significantly differ between the groups analyzed. Vimentin expression was significantly greater in the epithelial dysplasia and OSCC groups than the others.

Conclusion: According to our results, high-risk sites (border/ventral surface of the tongue and floor of the mouth) are associated with the dysplastic phenotype of leukoplakia, whereas age, sex, alcohol, tobacco and *Candida* spp. do not show such association. Vimentin expression is associated with the oral dysplastic epithelial phenotype and it seems to be more specific than E-cadherin for use as an immunohistochemical marker to detect such alterations.

Key words: Oral cancer; epithelial-mesenchymal transition; oral leukoplakia; E-cadherin, vimentin; *Candida spp.*

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

O câncer de boca é o sexto tipo de câncer mais prevalente no mundo, tem elevado índice de mortalidade e é representado, em 90% dos casos, pelo carcinoma de células escamosas (Habiba *et al*., 2017; Warnakulasuriya, 2010). A maioria dos carcinomas orais é precedida por lesões potencialmente malignas, que sinalizam o risco de transformação carcinomatosa (Radhika *et al.*, 2016).

A leucoplasia é a lesão potencialmente maligna mais frequente na cavidade oral (Cheng *et al.*, 2016), e merece destaque em função de sua alta prevalência e elevado potencial de transformação maligna (von Zeidler *et al.*, 2014). Tabagismo e etilismo, localização e duração das lesões, idade avançada, ocorrência no sexo feminino e infecção por *Candida* spp., são considerados fatores de risco para a transformação maligna dessa lesão (Cheng *et al.*, 2016; Yardimci *et al.*, 2014). Entretanto, avaliar e estimar esse risco ainda é um desafio (Habiba *et al.*, 2017).

A infecção por *Candida* spp. em lesões leucoplásicas ocorre, principalmente, em adultos que fazem uso de tabaco e álcool (Dilhari *et al.*, 2016). A *Candida albicans* tem sido associada à progressão de leucoplasias orais desde 1960 (Bakri *et al.*, 2014). Em 1966, foi relatada na literatura, pela primeira vez, a possível influência da *Candida* spp. na progressão de lesões ceratóticas da mucosa oral para carcinoma (Cawson, 1966). Entretanto, ainda não está claro de que forma a infecção influenciaria o desenvolvimento ou a progressão da displasia epitelial (Hebbar *et al*., 2013).

A alteração da expressão de biomarcadores celulares durante a progressão do câncer oral tem sido estudada no intuito de identificar-se a gravidade e o potencial de transformação maligna dessas lesões (Dmello *et al.*, 2017; Lee *et al.*, 2015; Park *et al.*,

2016; Xu *et al.*, 2017). Durante a transformação carcinomatosa, as células epiteliais reorganizam seu citoesqueleto adquirindo um fenótipo mesenquimal, por meio do processo denominado transição epitélio-mesenquimal (EMT) (von Zeidler *et al.*, 2014). Esse processo está presente em displasias epiteliais orais e em sua progressão para o câncer (Theveneau; Mayor, 2012).

A E-caderina, considerada a principal caderina das células epiteliais, tem importante função nas junções de aderência epitelial, que estabelecem os contatos célula-célula (Rosado *et al.*, 2013). A redução de sua expressão está fortemente ligada à perda da diferenciação celular e acentuada invasividade (von Zeidler *et al.*, 2014). Outra proteína relacionada ao aumento da invasividade e capacidade migratória de células epiteliais é a vimentina. Essa proteína está, normalmente, presente em células mesenquimais. Entretanto, sua expressão pode ocorrer, fisiologicamente, em células epiteliais migratórias, como acontece na embriogênese e na cicatrização de feridas, o que confere a tais células maior mobilidade. Nas células epiteliais orais, a expressão de vimentina também está associada a tumores, favorecendo a invasão e a formação de metástases (Chaw *et al.*, 2012). A diminuição de E-caderina combinada ao aumento da expressão de vimentina torna essas proteínas importantes marcadores das alterações da EMT em células epiteliais (Chaw *et al.*, 2012).

A presente dissertação teve por objetivo investigar a relação de fatores clínicos, infecção por *Candida* spp., e expressão imunoistoquímica de E-caderina e vimentina com alterações displásicas em leucoplasias orais. O trabalho está estruturado sob a forma de dois artigos científicos. O primeiro consiste em uma revisão da literatura enfocando o papel da EMT no câncer oral, e o segundo apresenta o experimento desenvolvido.

Artigo 1

2 ARTIGO 1

O artigo a seguir intitula-se ***Epithelial mesenchymal transition: an overview focusing on oral squamous cell carcinoma*** e foi formatado de acordo com as normas do periódico *Archives of Oral Biology* (Anexo A).

Epithelial mesenchymal transition: an overview focusing on oral squamous cell carcinoma

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Key words: oral cancer; epithelial mesenchymal transition; E-cadherin; vimentin
Running title: *EMT and oral cancer*

Review Article

Highlights
-EMT is the best known process of malignant cell transformation.
-EMT is the first step in the metastatic invasion cascade.
-Changes in E-cadherin and vimentin mark EMT induction in oral cancer.

ABSTRACT

Background: Alterations in signaling pathways, transcription factors and cell biomarkers can trigger epithelial-mesenchymal transition (EMT), which represents the change in the phenotype of normal epithelial tissue.

Objective: We present here a literature review of EMT, with special focus on its role and specificities involved in oral cancer.

Method: The key words *epithelial-mesenchymal transition, oral cancer, E-cadherin, vimentin, transcription factor, signal pathway, metastasis* and their combinations were searched in MeSH in the PubMed database.

Results: EMT is a key mechanism of cancer cell invasion and an early event in the multistep process of invasion and metastasis. EMT markers are expressed at different patterns in normal oral tissue and oral cancer. Despite numerous studies in this field, there is still no ideal biomarker for identifying the initiation and progression of oral squamous cell carcinoma (OSCC).

Keywords: epithelial-mesenchymal transition, oral cancer, malignant transformation, metastasis, E-cadherin, vimentin, signaling pathways, transcription factors

INTRODUCTION

The conversion of epithelial cells into mesenchymal cells is essential for embryonic development and involves profound phenotypic alterations such as loss of cell adhesion and acquisition of migratory properties (Thiery, Acloque, Huang & Nieto, 2009). This process is called epithelial-mesenchymal transition (EMT), which participates in normal development allowing embryonic epithelial cells to become motile and capable of colonizing specific areas of the embryo (Mohd-Sarip *et al*., 2017). Besides taking part in embryonic development (Nieto, Huang, Jackson & Thiery, 2016), EMT is associated with

tissue healing and regeneration processes (Kalluri & Weinberg, 2009) and, paradoxically, has an important role in carcinomatous transformation (de Freitas Silva, Yamamoto-Silva, Pontes & Pinto Júnior Ddos, 2014; Huang & Zong, 2017).

Oral squamous cell carcinoma (OSCC), the most prevalent tumor in the oral cavity, has high rates of local invasiveness and regional lymph node metastases (Cheng & Schmidt, 2008). Cancer cell metastasis has a substantial impact on mortality (Kita *et al*., 2017), accounting for about 90% of cancer death causes (Dutton, Graham & Hoffman, 2002). Even though the oral cavity is easily accessed for clinical examination, most tumors are not diagnosed until they have grown extensively or have metastasized. This compromises the efficacy of treatment, either surgery, radiotherapy, or brachytherapy (Manikandan *et al.*, 2016), and their combinations, with or without chemotherapy and/or targeted therapy (Arunkumar *et al.*, 2018; Huang & O'Sullivan, 2013). The development of secondary tumors hinders treatment success, leading to poor prognosis with low rates of patient survival (Manikandan *et al.*, 2016). Therefore, understanding biological processes involved in the genesis of oral cancer and the identification of biomarkers capable of enhancing early diagnosis are critical factors for improving the clinical management of the disease. The present study reviewed, in the scientific literature, important aspects of EMT's role in the genesis of OSCC.

EMT

Normal oral mucosa consists of stratified squamous epithelium, whose primary cell type is the keratinocyte. Melanocytes, Langerhans cells, Merkel cells and transitory inflammatory cells also make part of this tissue. Structurally, these cells are organized in layers known as basal layer, spinous layer, granular layer and cornified layer in keratinized sites; and basal layer, intermediate layer and superficial layer in non-keratinized sites. Cell proliferation occurs in the basal layer, and cells undergo

differentiation as they move upwards through the strata (Rodini, Lopes, Lara & Mackenzie, 2017). Epithelial cells contact to each other very closely forming a structured barrier. These contacts are called intercellular junctions and work in the maintenance of epithelial tissue integrity. Epithelial cell layers are separated from the subjacent connective tissue by the basal lamina, and mesenchymal cells that form this connective tissue, in turn, are loosely arranged (Thiery *et al.*, 2009). An organized and balanced cell renewal is typical of normal oral mucosa but is progressively lost in cancer development (Rodini *et al.*, 2017).

EMT is a biological process where epithelial cells, which normally interact with the basal membrane through their basal surface, pass through various biochemical changes and acquire a mesenchymal cell phenotype (Fig.1). This phenotype gives these cells increased migratory and invasive capacity, high resistance to apoptosis and ability to produce extracellular matrix compounds (Kalluri & Weinberg, 2009).

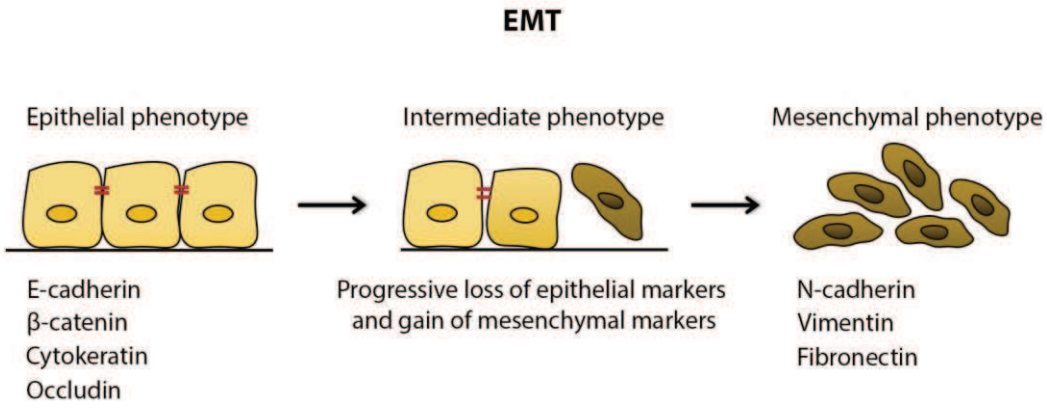

Figure 1 - Phases of epithelial-mesenchymal transition (EMT). Cells with epithelial phenotype positive for E-cadherin, β-catenin, cytokeratin and occluding acquire an intermediate phenotype with progressive loss of expression of those epithelial markers and new expression of mesenchymal ones (n-cadherin, vimentin, fibronectin) until a mesenchymal phenotype appears.

Many adult tissues and organs develop from a series of conversions of epithelial cells and mesenchymal cells, through EMT and its reverse process called mesenchymal-epithelial transition (MET) (Thiery *et al*., 2009). Along specific phases of embryogenesis and organ development, cells from some tissues show plasticity, which gives them the ability to display sometimes an epithelial phenotype and sometimes mesenchymal (Kalluri & Weinberg 2009; Lee, Dedhar, Kalluri & Thompson, 2006). Thus, many *rounds* of EMT and MET are needed for the final differentiation of specialized types of cells and acquisition of the complex tridimensional structure of internal organs (Thiery *et al*., 2009).

EMT occurs under three different biological forms (Fig.2), with their respective functions (Kalluri & Weinberg, 2009). EMT type I is related to embryonic development and implantation, and organ development as well, generating many types of cells that have a mesenchymal phenotype in common. This type of EMT can generate primary mesenchymal cells that have the potential to induce the reverse process (MET), to generate secondary epithelium (Kalluri & Weinberg, 2009). EMT is silenced in the adult body, but can be reactivated under pathological conditions such as wound healing, fibrosis and carcinomatous progression (Thiery *et al.*, 2009; Zidar *et al.*, 2018).

EMT type II is associated with healing, tissue regeneration and fibrosis. This process starts in situations of tissue trauma, where there is production of fibroblasts and inflammatory cells involved in tissue repair. As the inflammatory response diminishes, EMT type II ceases. But in organ fibrosis, EMT type II can persist in response to the ongoing inflammatory process, eventually leading to organ destruction (Kalluri & Weinberg, 2009).

EMT type III occurs in neoplastic cells that have previously undergone genetic and epigenetic alterations. Malignant cells undergoing this type of EMT can invade and

metastasize surrounding and distant tissues, which promotes cancer progression. Although the mechanism of induction of EMT type III in cancer cells has not yet been clarified (Kalluri & Weinberg, 2009), it is known as a process that plays a crucial role in malignant neoplasias (Ye & Weinberg, 2015). Substantial changes in the expression of epithelial and mesenchymal markers occur during EMT, particularly the epithelial markers E-cadherin (CDH1), β-catenin, occludin and cytokeratin, and the mesenchymal markers vimentin, fibronectin and N-cadherin (Kalluri & Winberg, 2009; Nieto *et al.*, 2016; Zhu *et al.*, 2012).

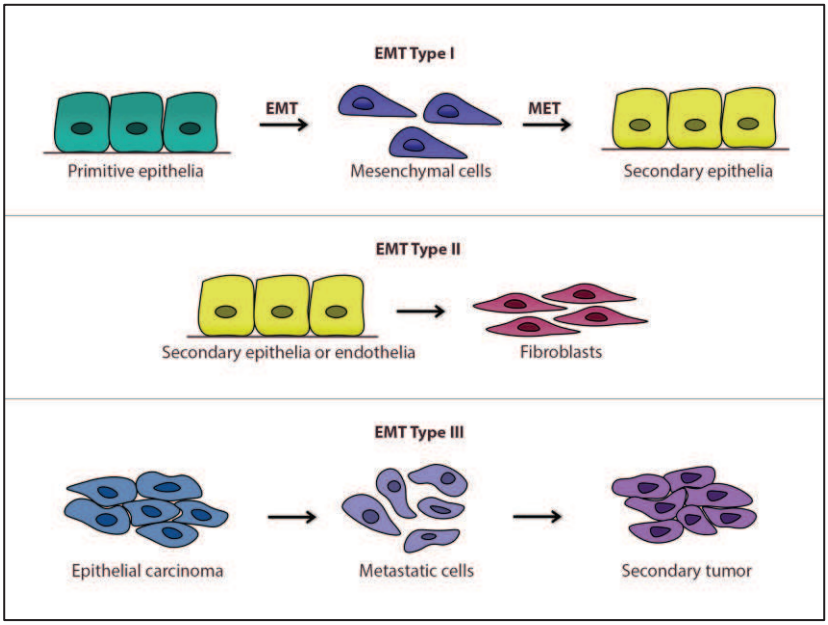

Figure 2 - Types of epithelial-mesenchymal transition (EMT): Type I (embryonic development), type II (tissue healing, regeneration and fibrosis) and type III (cancer progression and metastasis). MET=mesenchymal-epithelial transition

E-cadherin

E-cadherin belongs to the cadherin superfamily, which comprises single-pass transmembrane proteins. They were first identified in the early development of vertebrate embryos and epithelial tissue under the form of mediator glycoproteins of calcium-dependent cell-cell adhesion (Kemler, 1992; Sotomayor, Gaudet & Corey, 2014). Cadherins are classified into four major groups: classic cadherins (type I and type II), desmosomal cadherins (desmocollin and desmoglein), protocadherins (alpha, beta and

gamma) and atypical cadherins (Priest, Shafraz & Sivasankar, 2017; Sotomayor *et al*., 2014). Because of its early identification and complete characterization, classic E-cadherin type I (CDH1) is considered the prototype of cadherins, either in normal or pathological conditions (van Roy & Berx, 2008).

The detection, transmission and response to mechanical forces promoted by classic cadherins are responsible for tissue integrity (Priest *et al*., 2017) and have a key role in epithelial homeostasis (Kourtidis, Lu, Pence & Anastasiadis, 2017). Cadherins form adhesion complexes of mechanical support associated with the actin cytoskeleton and coupled to neighboring cells, transmitting mechanical forces from the extracellular environment to cytosol and triggering intracellular signaling events (LeckBand & Rooij, 2014; Priest *et al*., 2017).

There are reports of an association of reduced expression of E-cadherin with higher severity of epithelial dysplasia and phenotypic alterations of initial stages of oral cancer (von Zeidler, de Souza Botelho, Mendonça & Batista, 2014). The suppression of cell adhesion consequent to E-cadherin loss of function is believed to favor the onset of metastasis in various types of cancer (Priest *et al.*, 2017; van Roy & Berx, 2008). E-cadherin is considered a key molecule in cell adhesion, which binds to β-catenin, a cytoplasmic adapter protein. Besides participating in cell-cell adhesion, this association works in the transduction of signaling pathways that involve functions such as cell growth, differentiation and polarity (Angadi *et al.*, 2016). β-Catenin works as a transcriptional co-factor in the Wnt signaling pathway. As with E-cadherin, β-catenin can affect cell adhesion and contribute to tumorigenesis via EMT (González-Moles, Ruiz-Ávila, Gil-Montoya, Plaza-Campillo & Scully, 2014).

Vimentin

Intermediate filaments, microtubules and actin microfilaments are important components of the cytoskeleton (Lehtinen *et al*., 2013). Vimentin is a protein classified as a cytoplasmic intermediate type III filament, considered a biomarker of mesenchymal cells, found in several tissues during their stages of development. It is responsible for the maintenance of cell and tissue integrity and is involved in the EMT process (Coulombe & Wong 2004; Liu, Lin, Tang & Wang, 2015; Zhang *et al*., 2017). This protein is expressed in mesenchymal cells such as fibroblasts, endothelial cells and lymphocytes. Normal epithelial cells, in turn, do not express vimentin (Liu *et al*., 2016) and its high expression has been implicated in OSCC with poor clinicopathological features (Liu *et al.*, 2016; Sawant *et al.*, 2014).

Vimentin can be classified as a hallmark of EMT. During EMT, the cytoskeleton is rearranged, and vimentin overexpression occurs along with increased cell motility (Liu *et al*., 2016). Liu *et al*. (2015) analyzed the expression of vimentin as a mediator of cytoskeleton reorganization to maintain the mechanical integrity of breast cancer cells in the EMT process. Vimentin influences the organization of the cytoskeleton and the stability of focal adhesion, indicating that mechanical modulations generated by this protein intensify the malignant behavior of the cells. In addition, the intensity of vimentin expression is correlated with cancer progression, and its overexpression is linked to poor prognosis and high frequency of metastases in several cancers, including OSCC (Liu *et al*., 2010; Yang *et al*., 2017). Thus, overexpression of vimentin signals the possible onset of EMT and is capable of inducing cytoskeletal alteration favoring the migration of metastatic cells (Liu *et al.*, 2016; Liu *et al.*, 2015; Liu *et al.*, 2010).

EMT and oral cancer

Oral carcinogenesis occurs due to an imbalance and accumulation of genetic and epigenetic alterations (Eljabo *et al.*, 2018), as well as changes in the expression of coding and non-coding RNAs (Arunkumar *et al.*, 2018). miRNAs are a family of small non-coding RNAs, whose major function is to control gene expression (Hema, Smitha, Sheethal & Mirnalini, 2017). In general, they are located in genomic regions that are often prone to alterations in many types of cancer, including oral cancer (Manikandan *et al.*, 2016). The miR-200 family comprises miR-200a, miR-200b, miR-200c, miR-141 and miR-429, which share the same genetic sequence, and modulate EMT through the regulation of epithelial expression of E-cadherin (Park, Gaur, Lengyel & Peter, 2008). EMT is the best known process in cell motility acquisition during malignant cell transformation (Kalluri & Weinberg, 2009).

EMT and MET are not binary processes, and cancer cells can go through the transition at different levels. Some cells can acquire an epithelial/mesenchymal phenotype (E/M) (Kalluri & Weinberg, 2009), also called partial EMT phenotype (Nieto, 2013). Many carcinomatous cells can metastasize without losing their epithelial morphology at all or acquiring a complete mesenchymal phenotype (Jolly *et al.*, 2015; Klymkowsky & Savagner, 2009). These hybrid cells (E/M) show epithelial characteristics, such as cell adhesion, and mesenchymal ones, such as migratory capacity, allowing collective cell migration (Kalluri & Weinberg, 2009).

Metastasis consists of a sequence of events of neoplastic cell proliferation, neoangiogenesis, detachment of cancer cells from the primary site and their invasion into the bloodstream. This creates a new microenvironment, which comprises inflammatory cells and stroma with restricted oxygen and nutrients, and including attacks from the immune system (Pavithra *et al.*, 2017). These tissue microenvironment factors exert a

critical role, especially in the signaling pathways that regulate cell-cell and cell-matrix interactions (da Silva *et al.*, 2014; Howell & Grandis, 2005).

EMT marks the first step in the metastatic invasion cascade, since progression of OSCC from early to invasive stages is associated with morphological alterations of cancer cells that promote cell dissemination to distant organs (da Silva *et al.*, 2014). Epithelial cells of primary tumor lose cell adhesion and apical-basal polarity and, with mesenchymal phenotype acquisition, they gain the capacity of individual migration, penetrating the basal membrane and blood vessels (Fig.3). These cells stay in the bloodstream as circulating tumor cells, until they migrate to distant organs forming micrometastases. During this process, MET (reverse) also occurs, which is associated with cancer cell colonization at the metastatic site, and as mesenchymal cells achieve their destiny, epithelial characteristics are recovered and secondary tumors or macrometastases can be formed, completing the metastatic invasion cascade (Jolly *et al.*, 2015). EMT triggers the dissociation of cells from a primary carcinoma, which subsequently migrate and disseminate to distant sites. MET, in turn, ceases the migration of these cells inducing them to colonize and proliferate in the new tumor (Nieto *et al.*, 2016). EMT and MET then allow solid tumors, where 90% are carcinomas (Christiansen & Rajasekaran, 2006), to disseminate and colonize distant organs.

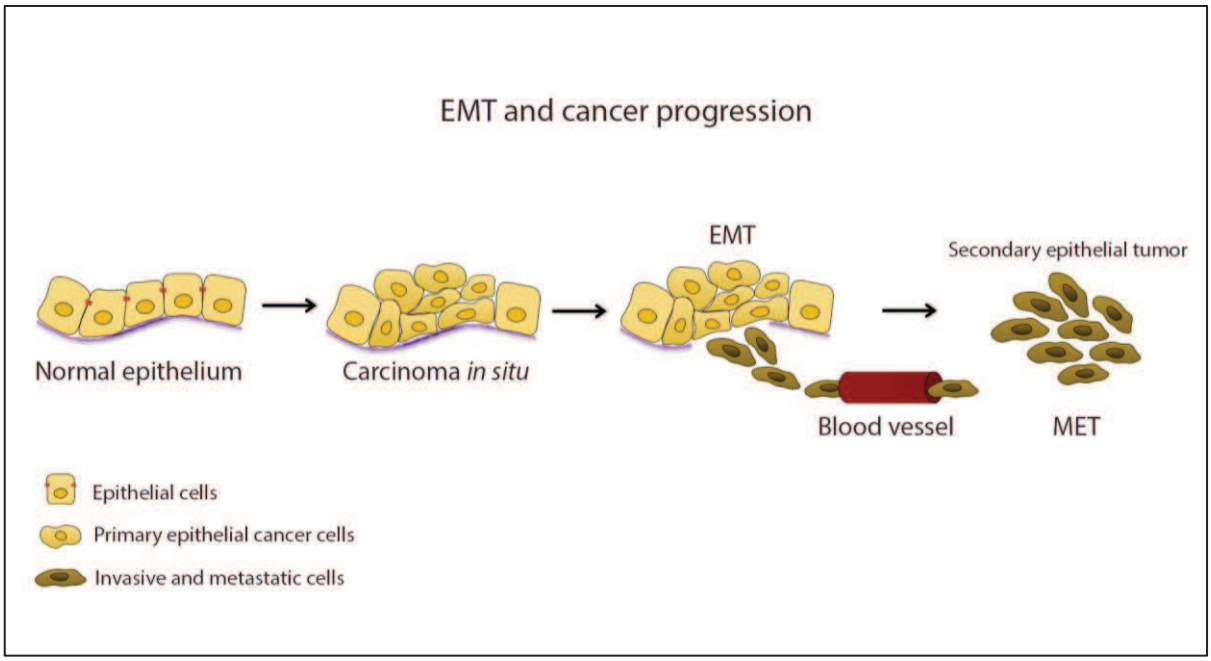

Figure 3 - Epithelial-mesenchymal transition (EMT) in cancer progression. Loss of cell adhesion and apico-basal polarity of epithelial cells and the acquisition of a mesenchymal phenotype contribute to carcinoma development. With breaching of the basement membrane, cancer cells invade the bloodstream and migrate to distant organs, being able to form micro- and macrometastases. In this process, mesenchymal-epithelial transition (MET) can be involved, causing reversion to the epithelial phenotype.

Signaling pathways and transcription factors in EMT

Epithelial cells are capable of activating EMT through the influence of various signaling pathways, such as TGF-β (transformation growth factor β), EGF (epidermal growth factor), HGF (hepatocyte growth factor), Notch, FGF (fibroblastic growth factor), Wnt (wingless-related integration site, coined from *Drosophila melanogaster* wingless gene int-1) and IGF (insulin-like growth factor) (Thiery & Sleeman, 2006), and mechanical factors such as extracellular matrix density as well. These signaling pathways, in turn, activate transcription factors such as TWIST1 (twist family BHLH transcription factor 1), SNAI1/SNAIL (snail-related zinc-finger transcriptional repressor 1), SNAI2/SLUG (snail-related zinc-finger transcriptional repressor 2), ZEB1 (zinc finger E-box-binding homeobox 1) and ZEB2 (zinc finger E-box-binding homeobox 2) (Jolly *et al.*, 2015).

These transcription factors and downregulation of miR-200 family have been sufficient to trigger EMT (Nieto, 2013; Park *et al.*, 2008), repressing epithelial phenotype and intensifying mesenchymal features (Nieto, 2013). TWIST1, SNAI1, SNAI2, ZEB1 and ZEB2 suppress E-cadherin directly or indirectly (Jolly *et al.*, 2015[39]; Priest *et al.*, 2017).

Kong *et al.* (2015) analyzed the immunohistochemical expression pattern of E-cadherin, laminin subunit gamma-2 (LAMC2), SNAI1/2, TWIST1, ZEB1 and ZEB2) in the invasion zone of OSCC. They observed that TWIST1 and ZEB2 co-expression is associated with poor survival of patients, especially in cases with metastatic lymph nodes. In addition, there was a significant difference in expression of E-cadherin, LAMC2, SNAI1/2 and TWIST1 between OSCC and normal oral mucosa. Loss of E-cadherin was associated with Broder's grading, whereas diffuse expression of LAMC2 was associated with invasion and loss of cohesive pattern.

Various studies have confirmed the relation between the overexpression of SNAI1, SNAI2, ZEB1, ZEB2 and TWIST1 transcription factors and EMT induction in squamous cell carcinomas (Table 1) and alterations in E-cadherin and vimentin (Table 2), IL-1β (Lee *et al.*, 2015) and EGF as well (Xu *et al.*, 2017).

FINAL CONSIDERATIONS

EMT markers are differently expressed in normal oral mucosa and oral cancer (Rodini *et al.*, 2017; da Silva *et al.*, 2014). The exact mechanism that determines normal oral epithelium transformation into a potentially malignant lesion or its evolution to cancer is still unknown (de Freitas Silva *et al.*, 2014). However, it has already been established that potentially malignant lesions pass through various steps with occurrence of epigenetic and molecular alterations until becoming cancerous (Eljabo *et al.*, 2018; Hema *et al.*, 2017; Olinici *et al.*, 2018). Among these changes, we can highlight the behavior of E-cadherin

and vimentin, signaling pathways TGF-β, EGF, HGF, Notch, FGF, Wnt and IGF and transcription factors SNAI1, SNAI2, ZEB1, ZEB2 and TWIST1, which indicate the start of EMT.

Some investigations on oral cancer preventive therapies have focused on these targets. The anti-metastatic effect of black tea polyphenol extracts (BTE) was tested in oral squamous cell culture system (SCC-4). BTE repressed vimentin expression and increased E-cadherin expression in SCC-4 cells, suggesting that BTE may be useful as an effector for prevention of cancer metastasis, in addition to supporting the role of black tea as an oral cancer chemopreventive agent (Chang *et al.,* 2012). Tang *et al*. (2009) reported that S-allylcysteine (SAC) can modulate *in vitro* the expression of E-cadherin and inhibit malignant progression by suppression of the signal transduction pathways MAPK/ERK (mitogen-activated protein kinase/extracellular-signal-regulated kinase) and SLUG repressor protein. Kita *et al*. (2017) investigated the role of activin B in OSCC. Activin B knockdown cells showed higher expression of E-cadherin and Zo-1, where activin B is highly expressed in OSCC. The study provides new insight into a highly metastatic phenotype by controlling the expression of EMT-related genes and suggest that this multifunctional cytokine might be a potential therapeutic target for OSCC.

It seems that blocking EMT would be an interesting approach to improve OSCC management. Nonetheless, considering the great number of signaling pathways involved in this process, there is no consensus on which would be the best target (da Silva *et al.*, 2014; Wang *et al.*, 2017; Zhang *et al.*, 2017). Therefore, a deeper understanding of EMT and its biomolecular processes is crucial for the investigation of chemopreventive therapies and alternative strategies in oral cancer.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to this work.

Table 1 – Reports on expression of EMT-related transcription factors in squamous cell carcinoma

Type of study	EMT-TF	Method	Results	Reference
In vitro	SNAIL/SLUG	Cell culture OK (OKF4, OKF6); Cell culture SCC (SCC25, UMSCC1); RT-PCR	SLUG did not affect E-cadherin expression in SCC cells; SLUG promoted SCC progression	Joseph *et al*. (2009)[47]
In vitro	SNAIL/SLUG	Cell culture (SCC9); RT-PCR; W-B; IF; invasion assay	Overexpression of SNAIL related to EMT; cell lines with EMT phenotype showed higher potential for invasiveness and metastasis	Zhu *et al.* (2012)[17]
In vitro	ZEB1/ZEB2	Cell culture SCC (SCC9); RT-PCR; W-B;IF	Increased expression of ZEB family and E-cadherin suppression	Yang *et al.* (2013)[48]
In vitro OL [DL (n=10), DM (n=10), SD (n=10)]; SCC (n=20); NOM (n=10)]	TWIST1	Cell culture (FaDu, SCC-25); IHC; Western blot; double IF	IHC: NOM: higher expression of TWIST in basal and parabasal layers; DL and DM: TWIST expression more in the cytoplasm; SD: TWIST expression also in the upper epithelial compartment; SCC: express higher levels of TWIST	de Freitas Silva *et al.*, (2014)[5]
In vitro SCC, fresh samples (n=10); SCC; paraffin embedded samples (n=74)	TWIST1	Laser-capture microdissection; extraction and amplification of total RNA; complementary DNA microarray and probes; RT-PCR; IHC	TWIST1 associated with invasive SCC; overexpression of TWIST1 correlated with worse prognosis; TWIST1 suppression prevented oncogenic features of SCC invasive cells	da Silva *et al.* (2014)[42]
In vitro	SNAIL/SLUG	Cell culture SCC (WSU-HN6; CAL27); RT-PCR; IF; W-B; IHC; invasion assay	Overexpression of SNAIL related to nodal metastasis and observed in SCC tissue; SNAIL suppression reduced SCC cell migration and invasiveness	Li *et al.* (2014)[49]
In vitro	SNAIL/SLUG	Cell culture HNSCC (SAS, HSC-4); SNAIL transfection in SAS and HSC-4 cells; W-B; IF; wound-healing and invasion assays	SNAIL regulated EMT properties in HNSCC cells; SNAIL transfected cells showed greater invasiveness; SNAIL was capable of inducing EMT in HNSCC cells	Masui *et al.* (2014)[50]
In vitro	SNAIL/SLUG	Cell culture (HSC6, CAL33); qRT-PCR; IHC; IF; W-B	SLUG suppression prevented EMT in SCC cells	Wang *et al.* (2017)[51]
In vitro	ZEB1/ZEB2	Cell culture SCC (HSC-2, HSC-3, SQUU-A, SQUU-B, SQUU-BO, SQUU-BC, SAS); HaCaT culture (keratinocytes); RT-PCR; ICC;W-B	Strong expression of ZEB1 and ZEB2 in SQUU-B; ΔNp63β regulates miR-205, which contributes to EMT suppression by inhibiting ZEB1 and ZEB2 expression	Hashiguchi *et al.* (2018)[52]

EMT-FT=EMT transcription factor; DL=mild dysplasia; DM=moderate dysplasia; HNSCC=head and neck squamous cell carcinoma; ICC=immunocytochemistry; IF=immunofluorescence; IHC=immunohistochemistry; n= sample size; NOM=normal oral mucosa; OK=oral keratynocytes; OL=oral leukoplakia; SCC=squamous cell carcinoma; SD=severe dysplasia; SLUG=snail-related zinc-finger transcriptional repressor 2; SNAIL=snail-related zinc-finger transcriptional repressor 1; TWIST=twist family BHLH transcription factor 1; W-B=Western blot; ZEB=zinc finger E-box-binding homeobox

Table 2 – E-cadherin and vimentin profile in squamous cell carcinoma

Type of study/(n)	Marker	Method	Results	Reference
In vitro (n=28)	E-cadherin Vimentin	IHC of biopsies of SCC of the oral cavity, oropharynx, hypopharynx or larynx	E-cadherin expressed in cell membrane; cytoplasmic vimentin expression Low E-cadherin and high vimentin could identify tumors in which EMT has occurred	Nijkamp *et al.* (2011)[53]
In vitro OL (n=31); OCSCC N^+ (n=12); control (n=9)	E-cadherin	IHC	Moderate-severe dysplasia: reduced E-cadherin expression Epithelial dysplastic changes plus risk of malignant transformation increased: reduction in or loss of E-cadherin expression by keratinocytes	von Zeidler *et al.* (2014)[24]
In vitro NE (n=25); OL (n=25); OSCC (n=25)	E-cadherin	Cell culture (HSC-3), RNA extraction, cell lysates, W-B, IF, IHC	SD: reduced E-cadherin expression in epithelial cell membrane OSCC grade III: extreme loss of membrane expression and switch to weak cytoplasmic expression	Kyrodimou *et al.* (2014)[54]
In vitro OSCC(n=85)	Vimentin	Cell culture of OSCC cell lines (HN4. HN12), immunostaining and immunoblotting, RT-PCR, IHC	Vimentin expression is essential for the increased migration activity of OSCC cells Vimentin expression via IHC staining predicts poor survival rate of OSCC patients	Liu *et al.* (2016)[31]
In vitro	E-cadherin Vimentin	Cell culture of OSCC cell lines (SCC-4, SCC-9, SCC-15), W-B, RT-PCR, IF	Upregulation of vimentin; downregulation of E-cadherin OSCC cell lines exhibiting EMT signatures showed a decrease in mechanical stiffness compared with those without EMT signatures	Park *et al.* (2016)[55]
In vitro	Vimentin	Cell culture (AW13516, AW8507, DOK, HaCat and A431) qRT-PCR, RT-PCR, W-B, IF, IHC	Vimentin downregulation causes keratin profile alteration in OSCC cells; vimentin modulates the differentiation status and tumorigenic potential of epithelial cells	Dmello *et al.* (2017)[56]

n=sample size; IF=immunofluorescence; IHC=immunohistochemistry; W-B=Western blot; NE=normal epithelium; OSCC=oral squamous cell carcinoma; SD=severe dysplasia; SCC=squamous cell carcinoma; $OCSCCN^+$= oral cavity squamous cell carcinoma with cervical lymph node metastasis

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3 ARTIGO 2

O artigo a seguir intitula-se ***Relationship of clinical features, Candida spp. and expression of E-cadherin and vimentin with dysplastic alterations in oral leukoplakia*** e foi formatado de acordo com as normas do periódico ***Oral Oncology*** (Anexo B).

Relationship of clinical features, *Candida* spp. and expression of E-cadherin and vimentin with dysplastic alterations in oral leukoplakia

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Key words: epithelial-mesenchymal transition; oral cancer; E-cadherin, protein, human; vimentin

Running title: *Candida* spp., E-cadherin and vimentin in oral leukoplakia

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ABSTRACT

Objective: To investigate the relationship of dysplastic changes in oral leukoplakia and clinical factors, *Candida* spp., and E-cadherin and vimentin expression.

Methods: Medical records and paraffin blocks of biopsied specimens of 60 patients were distributed into 4 groups: (1) no-dysplasia: 15 cases of leukoplakia without epithelial dysplasia; (2) epithelial dysplasia: 15 cases of leukoplakia with epithelial dysplasia (moderate or severe); (3) oral squamous cell carcinoma (OSCC): 15 cases of leukoplakia with histopathological diagnosis of OSCC; and (4) control group: 15 cases of fibroepithelial hyperplasia. Records were reviewed regarding age, sex, alcohol and tobacco use, and anatomical site of the lesion. Immunohistochemical analysis was carried out for determination of E-cadherin and vimentin expression, and periodic acid of Schiff (PAS) staining for *Candida* spp. detection.

Results: High-risk sites showed association with the epithelial dysplasia and OSCC groups. There was no significant difference between the groups for the other clinical features analyzed and for *Candida* spp. positivity with PAS. Quantitative E-cadherin expression did not significantly differ between the groups analyzed. Vimentin expression was significantly greater in the epithelial dysplasia and OSCC groups than the others.

Conclusion: According to our results, high-risk sites (border/ventral surface of the tongue and floor of the mouth) are associated with the dysplastic phenotype of leukoplakia, whereas age, sex, alcohol, tobacco and *Candida* spp. do not show such association. Vimentin expression is associated with the oral dysplastic epithelial phenotype and it seems to be more specific than E-cadherin for use as an immunohistochemical marker to detect such alterations.

INTRODUCTION

Oral cancer is the sixth most prevalent cancer in humans and one of the major causes of death worldwide [1,2]. Squamous cell carcinoma is the prototype of oral cancer, corresponding to about 90% of malignancies in the mouth. Frequently, it is preceded by easily identifiable oral lesions, which are called *potentially malignant lesions* [3]. Early diagnosis and clinical management of these lesions are crucial to reduce the morbidity and

mortality of oral cancer [1]. Leukoplakia is the most prevalent potentially malignant lesion in the oral cavity, with annual rates of malignant transformation between 2 and 3% [4]. Histopathological examination is the gold standard for its diagnosis [5], and even though dysplastic features can be an indicator of malignant potential in oral leukoplakia, the exact determination of the grade of dysplasia is a hard task, which compromises this predictive factor [1,6,7]. Therefore, predicting malignant transformation of oral leukoplakia is a challenge, and specific biomarkers are necessary for this purpose.

C. albicans is the most common species of *Candida* in the oral cavity of healthy individuals [8]. Alterations in the oral mucosa associated with trauma, atrophy, hyperplasia and dysplasia can compromise the mucosal barrier and predispose to this fungal infection [8,9]. *Candida* spp. have been implicated in malignant transformation of candidal leukoplakia, a type of oral leukoplakia associated with chronic infection with this fungus [10].

Carcinogenesis is related to epithelial-mesenchymal transition (EMT) [11]. During this process, epithelial differentiation is lost and a mesenchymal phenotype acquired. Embryogenesis, tissue repair and cancer cell metastasis are known as events that also develop through this process [12]. E-cadherin and vimentin are proteins associated with EMT, whose up-regulation or down-regulation can signal events of invasion and migration [11,12].

Considering that (1) some clinical features and *Candida* spp. infection can favor epithelial dysplasia in oral leukoplakia and that (2) changes in E-cadherin and vimentin expression are related to carcinogenesis, this study aimed to evaluate the relationship of clinical features, *Candida* spp. infection and immunohistochemical expression of E-cadherin and vimentin with dysplastic alterations in oral leukoplakia.

MATERIAL AND METHODS

We conducted a retrospective study, which was first approved by the Research Ethics Committee of Pontifical Catholic University of Rio Grande do Sul, protocol # 78767317.0.0000.5336. The sample was composed of medical records and paraffin blocks of specimens previously biopsied from patients with clinical diagnosis of oral leukoplakia and oral mucosa fibroepithelial hyperplasia. The sample was allocated into 4 groups according to histopathological diagnosis: (1) no-dysplasia group: 15 cases of leukoplakia without epithelial dysplasia; (2) epithelial dysplasia group: 15 cases of leukoplakia with epithelial dysplasia (only moderate or severe grades); (3) OSCC group: 15 cases of leukoplakia with histopathological diagnosis of oral squamous cell carcinoma; (4) control group: 15 cases of oral mucosa fibroepithelial hyperplasia. The sample comprised only the records with complete data and paraffin blocks with adequate specimens for histological analysis. Cases of lesions located in the vermillion border of the lips, as well as patients who had used antifungal agents within 14 days period prior to biopsy were excluded from the sample. Leukoplakias histopathologically diagnosed as mild epithelial dysplasia were also excluded; the group of epithelial dysplasia only comprised moderate and severe grades.

Data concerning age and sex of the patients, alcohol and tobacco use, and anatomical site of the lesions were collected from the records. Border and ventral surface of the tongue and floor of the mouth were considered high-risk sites; the other sites of oral mucosa were classified as low-risk sites [13].

Histological processing

Hematoxylin and eosin (H&E) slides were reviewed to confirm the histopathological diagnosis, according to World Health Organization (WHO) criteria [14]. Next, specimens

embedded in paraffin were subjected to periodic acid Schiff (PAS) staining and immunohistochemistry as follows.

PAS

Four-micrometer-thick histological sections were deparaffinized, re-hydrated in deionized water and immersed in 0.5% periodic acid solution for 20 min at room temperature (18 to 26ºC). The sections were washed and immersed in Schiff reagent for 20 min at room temperature; they were then washed again for 5 min and counterstained with Harris hematoxylin. The slides were mounted with xylene-based mounting media.

Immunohistochemistry processing

Three-micrometer-thick histological sections on silanized slides were deparaffinized with xylene at 59ºC and rehydrated in decreasing grades of ethanol. Processing was automated in Dako Autostainer Link 48 (Dako, Carpinteria, CA, USA). Antigens were retrieved using PT-Link (Dako) and EnVision Flex target retrieval solution (high pH). The sections were incubated with the antibodies FLEX monoclonal mouse anti-human E-cadherin (Clone NCH-38, Dako) and FLEX monoclonal mouse anti-vimentin (Clone V9, Dako). Sections were then counterstained with hematoxylin and coverslipped. The negative control comprised samples processed without the primary antibodies, and samples of breast and cecal appendix were used as positive controls for E-cadherin and vimentin, respectively.

Histological analysis

Images were captured by using a digital system with an Olympus BX-43 light microscope (Olympus, Tokyo, Japan), connected to a computer with an Olympus DP-73 digital camera (Olympus). Images were captured with a 20x objective and stored as uncompressed TIFF (true image format file). Three fields were captured in each slide (left side, middle and right side fields in the sections).

Images were analyzed in Image Pro Plus 4.5.0 (Media Cybernetics, Silver Spring, USA). E-cadherin and vimentin expression in the epithelial tissue was quantified by using the semiautomated segmentation technique [15] in the three fields captured for each slide (Fig. 1). PAS staining was classified as positive for *Candida* spp. considering stained structures morphologically compatible with the fungus [16]. Analysis was performed by a blinded and calibrated examiner. Calibration consisted in the evaluation of a series of 30 images for each marker (E-cadherin and vimentin) at two different moments. The results were subjected to the intraclass correlation test resulting in r=0.946 for E-cadherin and r=0.988 for vimentin.

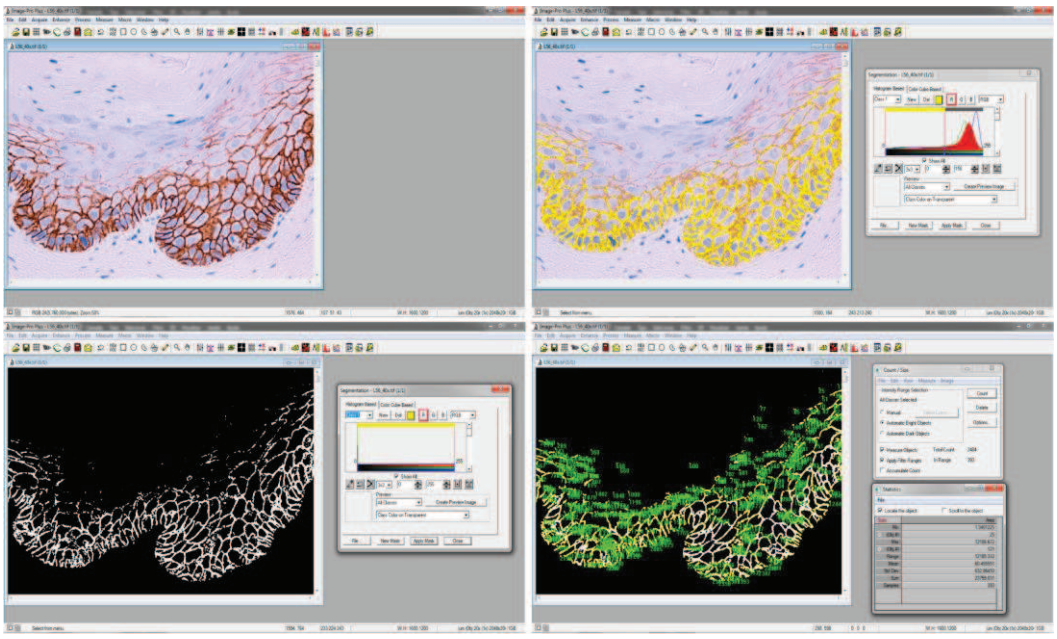

Figure 1 - Quantification of E-cadherin immunostaining by means of semiautomated segmentation technique in Image ProPlus software (Media Cybernetics, Bethesda, MD, USA)

Statistical analysis

Data were analyzed with descriptive and inferential statistics. Qualitative variables were expressed through absolute and relative frequency, whereas quantitative variables were analyzed with mean, standard deviation and median. Kolmogorov-Smirnov was used to test the normality of the data. Dichotomous variables were compared between the groups

with the chi-square test; ANOVA was used for age of the patients and immunostaining for E-cadherin and vimentin. Correlation of the variables was analyzed with Spearman correlation coefficient. Analysis was run in SPSS 17.0, at a significance level of 5%.

RESULTS

Sex and age of the patients

The sample was composed of 31 (51.7%) male patients and 29 (48.3%) female patients, with a mean age of 57.23 (±15.08) years. There was significant difference in neither age (*P*= 0.541) nor sex prevalence (*P*=0.333) between the no-dysplasia, epithelial dysplasia, OSCC and control groups (Table 1, chi-square and ANOVA, α=0.05).

Table 1 – Distribution of the sample in the groups according to sex and age of the patients

Group	Sex				Age (years)	
	Male		Female			
	n	%	n	%	Mean	SD
No-dysplasia	8	53.3	7	46.7	57.07	11.59
Epithelial dysplasia	8	53.3	7	46.7	60.80	13.03
OSCC	10	66.7	5	33.3	52.80	11.68
Control	5	33.3	10	66.7	58.27	21.96
Total	31	51.7	29	48.3	57.23	15.08
P	0.333*				0.541**	

**P* value for chi-square, α=0.05
** *P* value for ANOVA, α=0.05
OSCC=oral squamous cell carcinoma; SD=standard deviation

Tobacco and alcohol use

The prevalence of tobacco use was significantly higher in the no-dysplasia group, whereas the control group was associated with absence of tobacco. The groups epithelial dysplasia and OSCC showed similar prevalence for this variable (Table 2, chi-square, adjusted residual analysis, α=0.05). The prevalence of alcohol use did not show any

significant difference between the groups, where most of the sample (93.3%) was characterized by the absence of this variable (Table 2, chi-square, *P*=0.543).

Table 2 – Sample distribution according to prevalence of tobacco and alcohol use

Group	Tobacco				Alcohol			
	Presence		Absence		Presence		Absence	
	n	%	n	%	n	%	n	%
No-dysplasia	**11**	73.3	4	26.7	1	6.7	14	93.3
Epithelial dysplasia	5	33.3	10	66.7	1	6.7	14	93.3
OSCC	7	46.7	8	53.3	2	13.3	13	86.7
Control	0	0.0	**15**	100	0	0.0	15	100
Total	23	38.3	37	61.37	4	6.7	56	93.3
*P**	0.001				0.543			

**P* value for chi-square test, adjusted residual analysis, α=0.05
Bold values showed significant difference

Anatomic site of the lesion and positivity for *Candida* spp. with PAS

The groups epithelial dysplasia and OSCC showed significantly higher prevalence of high-risk sites; the group no-dysplasia had no significant results for this variable, and the control group was associated with low-risk sites (Table 3, chi-square, adjusted residual analysis, α=0.05). There was no significant difference in positivity for *Candida* spp. with PAS between the groups, with most of the sample showing absence of this variable (Table 3, chi-square, adjusted residual analysis, *P*=0.295).

Table 3 – Sample distribution according to anatomic site of the lesion and positivity for *Candida* spp. with PAS

Group	Low risk		High risk		*Candida* spp.			
					Presence		Absence	
	n	%	n	%	n	%	n	%
No-dysplasia	11	73.3	4	26.7	1	6.7	14	93.3
Dysplasia	5	33.3	**10**	66.7	3	20.0	12	80.0
Oral squamous cell carcinoma	4	26.7	**11**	73.3	2	13.3	13	86.7
Control	**14**	93.3	1	6.7	0	0.0	15	100
Total	34	56.7	26	43.3	6	10.0	54	90.0
*P**	0.000				0.295			

**P* value for chi-square, adjusted residual analysis, α=0.05; bold values showed significant difference
High risk=border of the tongue; ventral surface of the tongue; floor of the mouth; low risk= the other sites of oral mucosa

Immunoexpression of E-cadherin and vimentin

Quantitative analysis

E-cadherin expression did not significantly differ between the groups analyzed (Table 4, ANOVA, *P*=0.245). On the other hand, vimentin expression was significantly greater in the OSCC and epithelial dysplasia groups than in the other groups; when OSCC and epithelial dysplasia were compared to each other, the former had greater values. The groups no-dysplasia and control did not differ significantly from each other for this variable (Table 4, ANOVA, Tukey test, *P*=0.000).

Table 4 – Immunohistochemical expression of E-cadherin and vimentin in the no-dysplasia, epithelial dysplasia, oral squamous cell carcinoma (OSCC) and control groups

Group	E-cadherin (%)			Vimentin (%)		
	Mean	SD	MD	Mean	SD	MD
No-dysplasia	17.546 ^{A}	8.489	17.460	1.775^{A}	1.324	1.560
Epithelial dysplasia	16.278 ^{A}	7.827	14.059	**4.734^{B}**	3.833	3.693
OSCC	20.417 ^{A}	11.282	22.379	**7.003^{C}**	6.590	4.494
Control	18.050 ^{A}	11.211	18.424	2.038^{A}	2.268	1.053
*P**		0.245			0.000	

**P* value for ANOVA, complemented by Tukey's multiple comparisons test, α=0.05
Means followed by different letters in the column showed significant difference between the groups
OSCC=oral squamous cell carcinoma

Correlations

PAS staining was positively correlated with vimentin (r=0.159) and negatively correlated with E-cadherin (r =-0.217; Table 5).

Table 5 – "*r*" values for Spearman correlation

Variable	E-cadherin	Vimentin	PAS
E-cadherin	1		
Vimentin	0.003	1	
PAS	-0.217**	0.159*	1

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

Qualitative analysis

E-cadherin immunostaining in the group no-dysplasia was stronger in the basal and parabasal epithelial layers and, in general, restricted to the cytoplasmic membrane. Comparing no-dysplasia and epithelial dysplasia groups, the latter showed less expression in the basal and parabasal layers, and in some cases, membrane staining was partially switched to sparse cytoplasmic staining. The OSCC group had weak staining or loss of

staining in the basal and parabasal layers; partial switching of membrane to cytoplasmic staining also occurred. The control group showed a similar staining pattern as the no-dysplasia group (Fig.2).

The group no-dysplasia showed no vimentin staining in the cytoplasm in the epithelium. In the epithelial dysplasia group, we noted strong cytoplasmic staining spread throughout the epithelial layers. In the OSCC group, there was an increase in the number of stained cells, and in some cases, there was a switching in the pattern of cytoplasmic staining from a strong brown color to weak brown. The control group displayed a similar behavior as the no-dysplasia group (Fig.2).

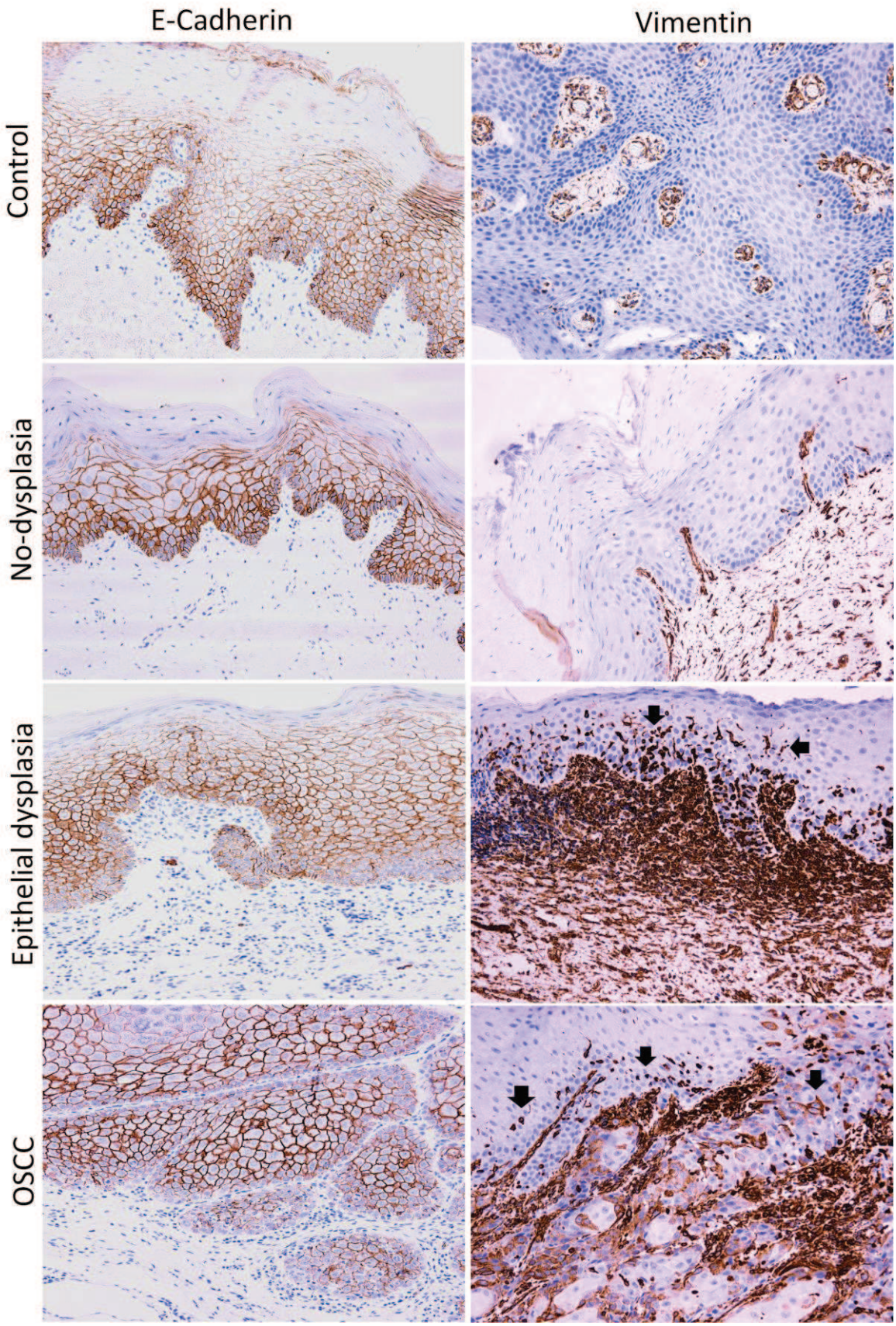

Figure 2 - Immunohistochemical staining for E-cadherin and vimentin in the control, no-dysplasia, epithelial dysplasia and oral squamous cell carcinoma (OSCC) groups.

DISCUSSION

It is believed that female sex, advanced age, tobacco and alcohol use, anatomical site and duration of the lesion, and *Candida* spp. infection as well, are risk factors for carcinomatous transformation of leukoplakia [10,13]. In the present study, only lesion site showed a significant difference between the groups. There were no significances for sex, age, alcohol and tobacco use and *Candida* spp. infection, which meant that there was no association of these factors with leukoplakia showing dysplasia and/or malignant transformation. Accordingly, it is important to emphasize that the criteria for the selection and allocation of leukoplakias into the study groups were not the clinical features, but the histopathological ones. Considering the presence or absence of epithelial dysplasia or carcinoma in the histopathological examination, the groups were determined, and afterwards, clinical features of the patients (sex, age, alcohol and tobacco use, site of the lesions) were evaluated. Therefore, our findings suggest that, regardless of age and sex of the patients and alcohol or tobacco habits, leukoplakia has *per se* the potential for malignant transformation [13,17]. This is in regard especially to the idiopathic cases, which in fact show reportedly higher malignant transformation rates [13]. Likewise, even though *Candida* has been reported as a risk factor for carcinomatous transformation of leukoplakia [10,18,19], its detection with PAS showed no significant difference between the groups. At first, as happened with the clinical factors previously discussed, this suggested that leukoplakia did not depend on *Candida* infection to undergo dysplastic alterations. However, this finding does not rule out the possibility of *Candida* contributing to such alterations. In fact, we found only a few cases with positive PAS staining for *Candida*. Although we tried to control biases, including in our sample only patients without history of use of antifungal agents within 14 days prior to biopsy, this was a retrospective study and it is possible that patients had previously used antifungal agents at

some time. Further prospective studies comprising a larger standardized sample and more specific techniques such as culture and PCR could better determine *Candida* at dysplastic alterations of the oral epithelium. Interestingly, both the epithelial dysplasia and OSCC groups were associated with high-risk anatomical sites, whereas the no-dysplasia group did not show such association. Accordingly, some authors report that, regardless of the clinical feature of being homogeneous, leukoplakias on the ventral surface and border of the tongue and floor of the mouth do show high risk for malignant transformation [13].

In the quantitative analysis, we found no significant difference in E-cadherin expression between the groups. This was not expected, since the literature reports E-cadherin down-regulation in EMT [7,20,21]. Anyway, there are reports of similar results as ours, indicating that E-cadherin use as a prognostic indicator needs further evaluation, where E-cadherin had a heterogeneous expression with 54% of OSCC specimens showing strong expression [22]. Qualitative analysis, on the other hand, showed some variation in the expression pattern of this marker in the groups, characterized by some tendency for switching to weaker expression in cytoplasmic membrane with some sparse expression in the cytoplasm in the epithelial dysplasia and OSCC groups. Such profile was already reported by Kyrodimou *et al.* [21]. At first, we could point out that our sample was small and, for this reason, significant differences in the E-cadherin quantitative analysis could not be determined. Nevertheless, for vimentin, this did not happen, as vimentin did show significant differences between the groups, with higher expression in the groups with dysplastic changes (epithelial dysplasia and OSCC). Thus, some considerations regarding specificities of these markers and their relation with the tissue analyzed should be raised. E-cadherin normally occurs at high levels with uniform distribution in the normal epithelium [21-23], and therefore progressive alterations in its expression during initial phases of cancer may not be so evident, especially in a quantitative approach. Our groups

corresponded to lesions clinically diagnosed as leukoplakias and, when carcinomatous transformation occurred in these lesions, it really represented early stages of OSCC. This fact could explain our quantitative results for E-cadherin, suggesting that, if used as a marker for EMT, it should be analyzed in a qualitative manner considering switching expression between membrane and cytoplasm, especially in the lower layers of the epithelium, instead of quantitative one.

The vimentin profile was different from that of E-cadherin, showing important differences in the quantitative analysis. The epithelial dysplasia and OSCC groups showed greater vimentin expression compared to the other groups and also differed from each other for this variable, with greater values in OSCC. Unlike E-cadherin, vimentin is not normally expressed in epithelial cells [24], which seems to make it a better marker for dysplastic alterations since it would be easier to identify its abnormal behavior either quantitative- or qualitatively. Accordingly, the literature has reported increased expression of this protein in advanced dysplastic oral lesions and OSCC [25].

CONCLUSION

According to our results, high-risk sites (border and ventral surface of the tongue and floor of the mouth) were associated with the dysplastic phenotype of leukoplakia, whereas age, sex, alcohol, tobacco and *Candida* spp. did not show such association. Vimentin expression was indeed involved in the oral epithelial dysplastic phenotype of leukoplakia, and it seemed to be more specific than E-cadherin for use as an immunohistochemical marker to detect such alterations. Further prospective and controlled studies investigating these biomarkers and genetic/epigenetic alterations are needed to establish new strategies for determining risk of carcinomatous transformation in leukoplakia.

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ETHICS STATEMENT

This study was approved by the Research Ethics Committee of Pontifical Catholic University of Rio Grande do Sul, protocol # 78767317.0.0000.5336.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to this work.

HIGHLIGHTS

-In some leukoplakia cases, there is paradoxical upregulation of E-cadherin.

-Vimentin is shown to be relevant when leukoplakia undergoes dysplastic changes.

-*Candida* may help induce epithelial dysplastic alterations.

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Discussão Geral

4 DISCUSSÃO GERAL

A leucoplasia é a lesão potencialmente maligna mais prevalente na cavidade oral, e seu diagnóstico e manejo clínico constituem um processo complexo e desafiador, principalmente em função do amplo espectro de alterações histopatológicas que essa lesão contempla. Leucoplasia é um termo clínico, que se refere a uma placa branca que, ao exame histopatológico, pode variar de hiperceratose, acantose e hiperplasia epitelial até displasia epitelial, carcinoma *in situ* ou mesmo carcinoma invasivo (van der Waal, 2018; Villa; Woo, 2017).

O principal fator indicador de risco de transformação maligna da leucoplasia oral é a presença de displasia epitelial (Warnakulasuriya *et al.*, 2007). Lesões que se apresentem, ao exame clínico, espessas e não-homogêneas têm maior probabilidade de ocorrência de displasia, entretanto, uma lesão leucoplásica homogênea pode exibir atipias epiteliais sem que seu aspecto clínico seja alterado (Lee *et al.*, 2006). Também ao exame histopatológico, é difícil precisar o grau das alterações displásicas. Tendo em vista a subjetividade em questão, diversos biomarcadores têm sido investigados com o objetivo de se otimizarem os processos de diagnóstico e avaliação de risco de transformação carcinomatosa da leucoplasia oral e, assim, favorecer o desfecho dos casos e aumentar as taxas de cura e sobrevida dos pacientes. Embora os marcadores p53 e Ki67 sejam apontados como ferramentas úteis (Gissi *et al.*, 2015), até o momento, não existe um marcador específico e preciso para cumprir essa função.

A transição epitélio-mesenquimal (EMT), considerada o processo biológico mais conhecido durante a transformação carcinomatosa (Kalluri; Weinberg, 2009), tem sido amplamente estudada nesse sentido, evidenciando diferentes comportamentos de diversos biomarcadores. Via de regra, a supressão de E-caderina e o aumento da

expressão de vimentina, sinalizam o início do processo de EMT (Chaw *et al.*, 2012; Webber *et al.*, 2017).

O presente estudo investigou a relação entre displasia epitelial em leucoplasias orais e fatores clínicos, *Candida* spp. e expressão de E-caderina e vimentina. Entre os fatores clínicos investigados (sexo, idade, álcool, tabaco e sítio anatômico das lesões), somente o sítio anatômico das lesões exibiu associação com displasia epitelial. Tal achado está de acordo com os relatos da literatura (Warnakulasuriya; Ariyawardana, 2016), segundo os quais borda/ventre de língua e assoalho de boca são considerados sítios de alto risco para transformação carcinomatosa da leucoplasia.

Os resultados obtidos no presente estudo para expressão imunoistoquímica de E-caderina foram um pouco paradoxais, se comparados aos relatos da literatura. Na avaliação quantitativa, a expressão de E-caderina não diferiu significativamente entre os grupos *sem displasia*, *com displasia*, *OSCC* e controle. Essa proteína é considerada proteína-chave de adesão das células epiteliais (Adams; Nelson, 1998), o que pode prevenir a mobilidade celular e disseminação metastática. Entretanto, segundo Maeda *et al*. (2005), a alteração da expressão das caderinas, apesar de essencial, não é indispensável para ocorrerem alterações celulares morfológicas que acompanham a EMT. Além disso, alguns autores consideram a expressão de N-caderina mais importante do que a E-caderina, quando se trata de metástase (Nakajima *et al.*, 2004).

A expressão de vimentina, por outro lado, exibiu resultados que corroboram os relatos da literatura. O aumento de expressão dessa proteína tem sido associado a um pior prognóstico em vários tipos de carcinoma, incluindo o OSCC (Liu *et al.*, 2010). Também tem sido proposto que a localização de marcadores moleculares, especialmente na zona de invasão do tumor, tem importante valor prognóstico (Schliephake, 2003). É

possível que, pelo fato de a vimentina não ser normalmente expressa no epitélio, a alteração de seu padrão de comportamento e sua identificação neste tecido sejam mais evidentes por ocasião da avaliação.

Ainda não está claro de que forma a infecção oral por *Candida* spp. influenciaria a progressão da displasia epitelial (Cheng *et al.*, 2016). A ideia do presente estudo foi de relacionar a presença do fungo com as alterações de EMT, mais especificamente, com a diminuição de expressão da E-caderina e expressão aberrante de vimentina. Mesmo tendo identificado hifas de *Candida* spp. em espécimes de leucoplasia oral com displasia epitelial e, até mesmo, em espécimes com diagnóstico de OSCC, não foi possível estabelecer a associação desse fungo com o desenvolvimento de displasia, já que sua ocorrência não exibiu diferenças significativas entre os grupos avaliados. Aspectos metodológicos como técnica de identificação, que empregou espécimes em parafina, e o caráter de estudo retrospectivo, em que não é possível ter a certeza de que os pacientes não usaram algum antimicrobiano, entre outros, podem ter operado como vieses nessa avaliação. Portanto, novos estudos prospectivos e controlados seriam de valia para definir o real papel da *Candida* spp. no desenvolvimento e na progressão da displasia epitelial em leucoplasias orais.

De acordo com os resultados do presente estudo, os sítios anatômicos de alto risco, borda/ventre de língua e assoalho de boca, bem como a expressão de vimentina, são fatores associados à ocorrência de displasia epitelial na leucoplasia oral. Dessa forma, é importante que se atente à localização anatômica das lesões e, por ora, a expressão imunoistoquímica de vimentina pode ser uma alternativa para complementar a avaliação histológica de rotina (H&E) como fator preditivo de transformação carcinomatosa. Com relação ao comportamento da E-caderina, a avaliação quantitativa,

embora diminua significativamente a subjetividade do observador, não se mostrou uma ferramenta eficaz, estando mais indicada, no caso de seu emprego, a interpretação qualitativa.

A trajetória na busca de novos biomarcadores que auxiliem na predição da transformação maligna de lesões epiteliais, bem como o desenvolvimento de novos métodos de diagnóstico precoce e predição prognóstica confiável, deve ser permanente. Até o momento, o manejo das lesões leucoplásicas baseia-se em controle clínico e excisão cirúrgica, esta com elevado risco de recidiva ou mesmo agravamento das lesões (Starzyńska *et al.*, 2015). A identificação de mediadores do processo de EMT que possam ser alvo de imunoterapia pode ser uma perspectiva de otimização do manejo desses pacientes.

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Anexos

ANEXO A

Normas para submissão de artigos ao periódico *Archives of Oral Biology*

https://www.elsevier.com/wps/find/journaldescription.cws_home/203?generatepdf=true

ANEXO B

Normas para submissão de artigos ao periódico *Oral Oncology*

https://www.elsevier.com/journals/oral-oncology/1368-8375?generatepdf=true

ANEXO C

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 8316

Porto Alegre, 4 de outubro de 2017.

Prezado(a) Pesquisador(a),

A Comissão Científica da FACULDADE DE ODONTOLOGIA da PUCRS apreciou e aprovou o Projeto de Pesquisa "Inter-relação de fatores clínicos, Candida sp., E-caderina, vimentina e alterações displásicas na leucoplasia oral". Este projeto necessita da apreciação do Comitê de Ética em Pesquisa (CEP). Toda a documentação anexa deve ser idêntica à documentação enviada ao CEP, juntamente com o Documento Unificado gerado pelo SIPESQ.

Atenciosamente,

Comissão Científica da FACULDADE DE ODONTOLOGIA

ANEXO D

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Inter-relação de fatores clínicos, Candida sp., E-caderina, vimentina e alterações displásicas na leucoplasia oral

Pesquisador: Karen Cherubini

Área Temática:

Versão: 1

CAAE: 78767317.0.0000.5336

Instituição Proponente: UNIAO BRASILEIRA DE EDUCACAO E ASSISTENCIA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.357.893

Apresentação do Projeto:

A leucoplasia oral é a lesão potencialmente maligna mais prevalente na cavidade oral, com uma taxa anual de transformação carcinomatosa entre 2% e 3%. A avaliação exata do grau de displasia é um processo difícil, o que compromete a previsão do potencial maligno desta lesão, fazendo-se necessária a utilização de biomarcadores específicos para tal fim. Fatores de risco como tabagismo, etilismo e presença de infecção por Candida sp estão associados à transformação carcinomatosa da leucoplasia oral, assim como a alteração da expressão dos biomarcadores E-caderina e vimentina. O presente estudo tem por objetivo avaliar a inter-relação de fatores clínicos, infecção por Candida sp. e da expressão imunoistoquímica de E-caderina e vimentina em leucoplasias orais com e sem alterações displásicas, quantificar a expressão imunoistoquímica de E-caderina e vimentina em leucoplasia oral com e sem displasia epitelial ou transformação carcinomatosa, identificar hifas de Candida sp. em leucoplasia oral com e sem displasia epitelial ou transformação carcinomatosa e correlacionar as variáveis expressão imunoistoquímica de E-caderina e vimentina, frequência de Candida sp., displasia epitelial, localização anatômica das lesões, tabagismo, etilismo e sexo dos pacientes

Objetivo da Pesquisa:

Objetivo Primário: Avaliar a inter-relação de fatores clínicos, infecção por Candida sp. e da expressão imunoistoquímica de E-caderina e vimentina em leucoplasias orais com e sem

Endereço: Av.Ipiranga, 6681, prédio 50, sala 703
Bairro: Partenon **CEP:** 90.619-900
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3320-3345 **Fax:** (51)3320-3345 **E-mail:** cep@pucrs.br

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

Continuação do Parecer: 2.357.893

alterações displásicas. Objetivo Secundário: 1) Quantificar a expressão imunoistoquímica de E-caderina e vimentina em leucoplasia oral com e sem displasia epitelial ou transformação carcinomatosa.2) Identificar hifas de Candida sp. em leucoplasia oral com e sem displasia epitelial ou transformação carcinomatosa.3) Correlacionar as variáveis expressão imunoistoquímica de E-caderina e vimentina, frequência de Candida sp., displasia epitelial, localização anatômica das lesões, tabagismo, etilismo e sexo dos pacientes

Avaliação dos Riscos e Benefícios:

Estudo basicamente retrospectivo com riscos mínimos. O risco de identificação pelas imagens das lesões provavelmente também é pequeno.

Sem benefícios para os participantes.

Comentários e Considerações sobre a Pesquisa:

Pesquisa de relevância para o conhecimento científico

Considerações sobre os Termos de apresentação obrigatória:

Termos apresentados estão adequados. TCLE para os pacientes que ainda se encontram em tratamento. Apresenta termo de compromisso para uso de dados.

Conclusões ou Pendências e Lista de Inadequações:

Aprovado sem pendências

Considerações Finais a critério do CEP:

Diante do exposto, o CEP-PUCRS, de acordo com suas atribuições definidas nas Resoluções n° 466 de 2012 e Norma Operacional n° 001 de 2013 do Conselho Nacional de Saúde, manifesta-se pela aprovação do estudo.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1009895.pdf	06/10/2017 07:34:59		Aceito
Outros	Carta_de_Aprovacao_da_Comissao_Cientifica_1507117245157.pdf	05/10/2017 20:41:51	Karen Cherubini	Aceito
Cronograma	Cronograma.pdf	05/10/2017 20:39:04	Karen Cherubini	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Termo_de_Consentimento_Livre_e_Esclarecido.pdf	05/10/2017 20:37:38	Karen Cherubini	Aceito

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Bairro: Partenon **CEP:** 90.619-900
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3320-3345 **Fax:** (51)3320-3345 **E-mail:** cep@pucrs.br

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

Continuação do Parecer: 2.357.893

Outros	Carta_Chefe_Laboratorio_modelo_CEP.pdf	05/10/2017 20:36:36	Karen Cherubini	Aceito
Orçamento	Orcamento_CPC.pdf	05/10/2017 20:35:38	Karen Cherubini	Aceito
Outros	Link_para_Curriculo_Lattes.docx	05/10/2017 20:34:41	Karen Cherubini	Aceito
Outros	Termo_de_Compromisso_modelo_CEP.pdf	05/10/2017 20:32:50	Karen Cherubini	Aceito
Outros	Carta_Chefe_do_Servico_modelo_CEP.pdf	05/10/2017 20:30:57	Karen Cherubini	Aceito
Outros	Carta_de_Apresentacao.pdf	05/10/2017 20:20:03	Karen Cherubini	Aceito
Projeto Detalhado / Brochura Investigador	Documento_Unificado_do_Projeto_de_Pesquisa_1507117245157.pdf	05/10/2017 20:17:57	Karen Cherubini	Aceito
Folha de Rosto	Folha_de_Rosto_Assinada.pdf	05/10/2017 20:01:07	Karen Cherubini	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 30 de Outubro de 2017

Assinado por:
Denise Cantarelli Machado
(Coordenador)

ANEXO E

Pontifícia Universidade Católica do Rio Grande do Sul
FACULDADE DE ODONTOLOGIA
PÓS-GRADUAÇÃO

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO: ESTOMATOLOGIA CLÍNICA
NÍVEL: MESTRADO
EXAME DE QUALIFICAÇÃO – ATA 16/17

Data: 27/09/2017 Horário: 15h

Aluno: João Matheus Scherbaum Eidt

Orientadora: Profa. Dra. Karen Cherubini

Título da pesquisa:
"INTER-RELAÇÃO DE FATORES CLÍNICOS, *Candida* sp., E-CADERINA, VIMENTINA E ALTERAÇÕES DISPLÁSICAS NA LEUCOPLASIA ORAL"

Comissão Examinadora: Profa. Dra. Fernanda Gonçalves Salum (PUCRS)
Profa. Dra. Maria Antonia Z. de Figueiredo (PUCRS)

(X) Aprovado

( ) Aprovado com projeto pendente

( ) Reprovado

Ass.: _____
João Matheus Scherbaum Eidt
Aluno

Ass.: _____
Profa. Dra. Karen Cherubini
Orientadora

Ass.: _____
Profa. Dra. Fernanda Gonçalves Salum
Professora Avaliadora

Ass.: _____
Profa. Dra. Maria Antonia Z. de Figueiredo
Professora Avaliadora

Ass.: _____
Profa. Dra. Maria Martha Campos
Coordenadora do Programa de Pós-Graduação em Odontologia