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Analysis of TIMP-1 expression in leukoplakia and oral squamous cell carcinoma

Análise da expressão de TIMP-1 em leucoplasia e carcinoma de boca de células escamosas

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ABSTRACT

Introduction: The current study about transition of oral epithelial dysplasia, present in lesions such as leukoplakia, for squamous cell carcinoma (SCC) involves not only the histopathological aspects, but also the analysis of the presence of biomarkers which influence the microenvironment where cells are embedded. **Objective:** To evaluate the tissue inhibitor of metalloproteinase-1 (TIMP-1) profile in cases of leukoplakia and SCC classified into different degrees of dysplasia and histological grading, respectively. The immunohistochemical findings were confronted with microscopic features adopted in the classification of each lesion. **Material and methods:** Cases of leukoplakia and SCC were recovered from files of The Oral Pathological Anatomy Service of the Dental School at the Universidade Federal do Espírito Santo (SAPB-UFES), between the years 2004 and 2010. New slides were obtained and submitted to immunohistochemical assay to determine TIMP-1 expression profile. Parenchyma, as well as the different layers of the epithelium and stroma was evaluated. **Results:** In all cases the presence of TIMP-1 was detected in the stroma and parenchyma. In mild leukoplakia, the basal layer with hyperplasia showed intense immunolabeling, whereas cells with loss of polarity presented weaker expression. In moderate leukoplakia, all epithelium layers, except the cornea, were labeled. Severe leukoplakia had the spinous layer most intensely labeled, with no variation in areas with pleomorphism. Stage I SCC showed the deepest islands with intense labeling in cells with pleomorphism and mitoses. In the tumor islands, less differentiated cells were weakly labeled, and in keratin pearl, labeling was weak or absent in central cells. In stage II SCC, labeling was observed in basal cell with hyperplasia and in cells of the spinous layer, however, the parabasal layer was not labeled. Also, on tumor islands, less differentiated cells did not express the protein and keratin pearls were not labeled. **Conclusion:** It was possible to detect TIMP-1 immunolabeling in all specimens, ranging in intensity and location. The absence of expression in less differentiated cell suggests that more aggressive lesions present reduced enzyme expression. The microenvironment is important for the various cellular activities, and TIMP is an enzyme that participates in matrix remodeling, therefore changes in its expression can be a valuable tool in the better understanding oral carcinogenesis.

Key words: oral leukoplakia; squamous cell carcinoma; oral cancer; tissue inhibitor of metalloproteinase-1.

RESUMO

Introdução: *O estudo atual da transição da displasia epitelial oral, presente em lesões como a leucoplasia, para o carcinoma de células escamosas (CCE), envolve não somente aspectos histopatológicos, como também a análise da presença de biomarcadores, os quais influenciam o microambiente em que as células estão inseridas.* ***Objetivo:*** *Avaliar o perfil da expressão do inibidor tecidual de metaloproteinase 1 (TIMP-1) em casos de leucoplasias e CCE classificados em diferentes graus de displasia e graus histopatológicos, respectivamente, e confrontar os achados imuno-histoquímicos com os aspectos microscópicos adotados na classificação das lesões.* ***Material e métodos:*** *Foram resgatados casos de leucoplasia e CCE do Serviço de Anatomia Patológica Bucal do curso de Odontologia da Universidade Federal do Espírito Santo (SAPB-UFES), entre os anos 2004-2010. Novas lâminas foram obtidas ao serem submetidas ao ensaio imuno-histoquímico para determinação do perfil de expressão de TIMP-1. Foram avaliados parênquima, bem como as diferentes camadas do epitélio e estroma.* ***Resultados:*** *Em todos os casos, foi detectada a presença de TIMP-1 no estroma e no parênquima. Na leucoplasia leve, a camada basal e com hiperplasia apresentou imunomarcação intensa;*

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as células com perda de polaridade tiveram expressão menor. Na leucoplasia moderada, todas as camadas do epitélio, exceto a córnea, apresentaram marcação. A leucoplasia severa teve a camada espinhosa marcada mais intensamente, sem variação em áreas com pleomorfismo. O CCE grau I apresentou as ilhas mais profundas com marcação intensa em células com pleomorfismo e mitoses. Nas ilhas tumorais, células menos diferenciadas tiveram marcação menor, e em pérolas córneas a marcação foi fraca ou ausente nas células centrais. No CCE grau II, foi observada a marcação em células basais com hiperplasia e, em células da camada espinhosa, a camada parabasal não foi marcada. Também nas ilhas, células menos diferenciadas não expressaram a proteína e não houve marcação em pérolas córneas. ***Conclusão:*** *Foi possível detectar imunomarcação para TIMP-1 em todos os espécimes, com variação em intensidade e localização. A ausência de expressão em células menos diferenciadas sugere que lesões mais agressivas possuem redução da enzima. O microambiente é importante para as diversas atividades celulares, e TIMP é uma enzima que participa da remodelação da matriz. Portanto, alteração na sua expressão pode ser uma valiosa ferramenta para um melhor entendimento da carcinogênese da mucosa bucal.*

Unitermos: *leucoplasia oral; carcinoma de células escamosas; câncer oral; inibidor tecidual de metaloproteinase 1.*

RESUMEN

Introducción: *El estudio actual de la transición de displasia epitelial oral, presente en lesiones como la leucoplasia, hacia carcinoma epidermoide, implica no solo aspectos histopatológicos, sino también el análisis de la presencia de biomarcadores que influyen en el microambiente en el que se insertan las células.* ***Objetivo:*** *Evaluar el perfil del inhibidor tisular metaloproteinasa-1 (TIMP-1) en casos de leucoplasia y carcinoma de células escamosas (CCE) clasificados en diferentes grados de displasia y grados histopatológicos, respectivamente. Confrontar los hallazgos inmunohistoquímicos con los aspectos microscópicos adoptados en la clasificación de lesiones.* ***Material y métodos:*** *Casos de leucoplasia y CCE fueron recuperados del Servicio de Anatomía Patológica Bucal del Curso de Odontología de la Universidad Federal de Espírito Santo (SAPB-UFES), entre los años 2004-2010. Se obtuvieron nuevos portaobjetos y se los sometieron a ensayos inmunohistoquímicos para determinar el perfil de expresión de TIMP-1. Se evaluó el parénquima, así como las diferentes capas del epitelio y estroma.* ***Resultados:*** *En todos los casos se detectó TIMP-1 en estroma y parénquima. En la leucoplasia leve, la capa basal y con hiperplasia mostró inmunotinción intensa, mientras que las células con pérdida de polaridad tuvieron menos expresión. En la leucoplasia moderada, todas las capas del epitelio, excepto la córnea, mostraron inmunotinción. En la leucoplasia grave la capa espinosa tuvo inmunotinción más intensa, sin variación en áreas con pleomorfismo. El CCE grado I mostró las islas más profundas con tinción intensa en células con pleomorfismo y mitosis. En las islas tumorales, las células menos diferenciadas tuvieron tinción menor, y en las perlas de queratina la tinción fue débil o ausente en las células centrales. En el CCE grado II, se observó tinción en células basales con hiperplasia y, en células de la capa espinosa, la capa parabasal no fue marcada. También en las islas, las células menos diferenciadas no expresaron la proteína y no hubo tinción en las perlas de queratina.* ***Conclusión:*** *Fue posible detectar inmunotinción para TIMP-1 en todos los especímenes, con variación en intensidad y ubicación. La ausencia de expresión en células menos diferenciadas sugiere que las lesiones más agresivas tienen enzima reducida. El microambiente es importante para las diversas actividades celulares, y el TIMP es una enzima que participa en la remodelación de la matriz; por lo tanto, la alteración en su expresión puede ser una herramienta valiosa en el mejor entendimiento de la carcinogénesis de la mucosa oral.*

Palabras clave: *leucoplasia oral; carcinoma de células escamosas; cáncer de la boca; inhibidor tisular de metaloproteinasa-1.*

INTRODUCTION

Oral leukoplakia, the most frequent potentially malignant disorder in the oral cavity[1], and squamous cell carcinoma (SCC), the most common malignant neoplasm in the maxillofacial region, are among the most interesting lesions for understanding oral carcinogenesis. These lesions share similar clinical and microscopic characteristics and, at the same time, different and specific[2] that make them far apart on their impact on the health of the individual and the professional approach[3].

Carcinogenesis is described as a complex multistep and multifactorial process, through which a series of events results in loss of balance between activated proto-oncogenes and suppressor genes, which lead to cell transformation, autonomy, and uncontrolled growth. All of these events may occur due to influences of exposure to carcinogens and/or factors inherent to the host throughout life and in successive steps, leading to the transformation of multiple genes[4]. The loss of regulation of these genes, therefore, is the molecular basis for phenotypic changes that involve the development of cellular immortality, the ability to invade tissues, to produce metastasis, and induce angiogenesis[5].

The concept of oral cavity cancer development involves the mentioned changes, as well as, in a well-established way, the initial presence of a precursor lesion. Within this context, the most well-known lesion is leukoplakia[6].

However, among the phenotypic changes in the oral mucosa, it is important to highlight epithelial dysplasia, a fundamental field of study for understanding the malignant transformation of potential malignant disorders, as occurs with leukoplakia[7]. The analysis of tissue and cytological features recommended by the World Health Organization (WHO) (2005) guides the grading of precursor lesions in mild, moderate and severe, carcinoma *in situ*, and invasive carcinoma.

Although grading oral epithelial dysplasia is widely accepted and considered the most important tool to predict the risk of malignant transformation, it is still subjective and controversial[8].

Reibel (2003)[6] lists three major problems in this lesions association with oral epithelial dysplasia and its transformation: 1. subjective diagnosis; 2. not all lesions that exhibit epithelial dysplasia become malignant and some even revert; 3. carcinomas may develop from lesions without prior diagnosis of epithelial dysplasia on biopsies.

Based on the above, it is clear the need for other tools that can assist professionals in determining the risk of malignant transformation in the oral cavity. Therefore, the uses of biological and genomic markers have become good candidates in improving the prognostic evaluation of precursor lesions in oral cancer.

Biomarkers can assist in a more accurate diagnosis of epithelial dysplasia. In this regard, to assess the association of the histopathological features of oral epithelial dysplasia with biomarkers, Shimamaura *et al*. (2011)[9] compared the labeling for fascin and podoplanin in lesions with different grades of dysplasia, in carcinomas *in situ*, and in invasive carcinomas, considering them as key tools for the accurate diagnosis of this group of potentially malignant lesions.

Other studies using different biomarkers to clarify oral carcinogenesis have been published, such as perlecan, matrix metalloproteinases (MMP), and tissue inhibitors of metalloproteinase (TIMP)[8, 10-13].

Ikarashi *et al*., in 2004, demonstrated that dysplastic epithelial cells, or carcinoma in situ, can produce and deposite extracellular matrix (ECM) molecules before they acquire an invasive nature. Using 82 biopsies of normal, hyperplastic and dysplastic epithelium, and carcinoma, the authors assessed, immunohistochemically, perlecan and heparan sulfate proteoglycan and showed topographic differences in expression; they were overexpressed in dysplastic epithelial cells with reduced cell-cell adhesion.

Based on these findings, the same group of researchers assessed the differences in expression between MMP in the oral epithelial dysplasia progression to SCC in the attempt to recognize proliferation centers, as well as to confirm the intraepithelial ECM remodeling. They examined MMP-1, -2 e -7 in 20 cases of oral dysplasia, carcinoma *in situ*, and SCC and suggested that the MMP-7 increase would have an important role in cell proliferation and malignant transformation, becoming a valuable tool in this identification[8].

MMP are involved in several processes, some physiological and also pathological, such as cancer[14]. The MMP activity is controlled on at least three levels: transcription, proteolytic activation, and inhibition of enzymatic activity by natural inhibitors, enzymes known as TIMP[15, 16]. The MMP network activity is a result of the balance between the levels of the active enzyme and the levels of TIMP, which together form a complex biological system that strictly controls the ECM degradation. TIMP also can form a complex with pro-MMPs, regulating the process of MMP activation. While TIMP have already been shown to block MMP activity, a key aspect in inhibiting tumorigenesis and, consequently, in disease progression, conversely, these enzymes can participate in tumorigenesis[17].

Therefore, TIMP, in tumorigenesis, are considered multifunctional molecules, since they act by regulating cell proliferation, apoptosis pro-MMP-2 activity, and angiogenesis; and, paradoxically, they can also facilitate the disease progression with these actions. The TIMP inhibitory effect on tumor growth and metastasis was suggested by the overexpression of the gene within neoplastic cells, however, it has been shown that TIMP has a growth-stimulating and anti-apoptotic effect[18].

Thus, it is believed that the anti-MMP function of TIMP may play an important inhibitory role during the late stage of tumor progression, but due to its growth promotion activities and anti-

apoptotic effect, it can act by stimulating formation in the initial stage of the lesion[17].

Among the molecules that are part of the TIMP family, the best-known is TIMP-1. Its production is induced by external stimuli, such as growth factors [basic fibroblast growth factor (b-FGF); platelet-derived growth factor (PDGF); epidermal growth factor (EGF)] and cytokines [interleukin 6 (IL-6), 1 (IL-1), and 1β (IL-1β)]. It is expressed by several cells, including fibroblasts, endothelial and epithelial cells, osteoblasts, chondrocytes, smooth muscle cells and many tumor cells, in addition to body fluid[18].

The stimulatory effect for TIMP-1 cell growth was initially recognized in erythroid activity; however, it is known that this effect is not limited to this cell type. In normal keratinocytes, fibroblasts, lung adenocarcinoma cells and melanoma cells, a mitogenic effect has also been demonstrated[17].

Increased expression has been reported as a feature paradoxically associated with poor prognosis in many solid tumors, such as breast cancer, colorectal cancer, stomach and lung cancer[17]. It was also related to regional and distant metastases. It is worth mentioning that the high level of TIMP-1 in the plasma of cancer patients suggests that the molecule is a marker of disease progression[19].

From the above, it is clear that the implementation of cell and molecular biology techniques, which allow a better understanding of the events that contribute to the onset, tumor growth, invasion and metastases, should be applied for the study of oral carcinogenesis. Some research has shown that the analysis of the expression, regulation and location of the TIMP-1 protein can be used as a possible marker for the prognosis of invasiveness in tumors of epithelial origin, such as oral SCC.

Thus, the present study evaluated the TIMP-1 expression profile in leukoplakia, considered a precursor lesion of SCC, as well as in the tumor itself, in different degrees of dysplasia and differentiation, respectively, with the purpose of outlining the profile of this important protein and its changes according to the severity of the lesion. Finally, the immunohistochemical findings were compared with the histopathological features adopted in the classification of each lesion to establish possible associations between TIMP-1 expression and tissue and cellular changes.

MATERIAL AND METHODS

The sample consisted of cases of leukoplakia and SCC registered at the of Oral Pathological Anatomy Service of the Dental School at the Federal University of Espírito Santo [Serviço de Anatomia Patológica Bucal do Curso de Odontologia da Universidade Federal do Espírito Santo (SAPB-UFES)], between the years 2004 and 2010. For histopathological and immunohistochemical studies, paraffined blocks from the SAPB-UFES collection were recovered.

The inclusion criterion was the presence of sufficient material in the paraffin blocks to obtain the new slides. The leukoplakia and SCC paraffin-embedded specimen were obtained by incisional or excisional biopsy of patients referred to the Prevention and Early Diagnosis of Oral Cancer and Mouth Lesions Programme [Programa de Prevenção e Diagnóstico Precoce de Câncer de Boca e Lesões de Boca (SIEX/PROEX)], developed by the Stomatology and Oral and Maxillofacial Surgery II subjects of the Department of Dental Clinic of the UFES. The study was submitted to the Human Research Ethics Committee – Health Sciences Center/UFES under protocol and registration approval number 70/10.

Immunohistochemical processing for TIMP-1

Regarding the immunohistochemical assay, the paraffin blocks were subjected to manual microtomy to obtain 3 µm thick slices (Leica RM212 RT microtome, Leica Biosystems Nussioch GmbH, Germany). The cuts went through a 30% alcohol solution, with the purpose of stretch them, and then were immersed in a water bath at 45°C (Lupetec BH 05), and "fished" with silane coated slides (Immunoslide, EasyPath, Braszil) properly identified.

The histological sections were deparaffinized in an oven (FANEM 900 kw, model 310, Brazil) at 60°C for 120 minutes, and diaphonized in three xylol baths: xylol II; xylol I, and alcohol/xylol, each bath lasting 5 minutes. Then they were rehydrated in decreasing concentrations of ethanol (100%, 90%, and 80%) and water, each bath lasting 5 minutes. The removal of formolic pigment was performed with a solution of ammonium hydroxide (10%) diluted in ethanol (95%) for 10 minutes.

The cuts were washed with tryptic soy broth-tween (TBST) three times, 5 minutes each bath, then submitted to antigenic recovery, using a citrate buffer solution (10mM citric acid and 0.05% Tween 20 with pH 6), heated between 95 and 100°C, for 30 minutes. The polymer detection system was NovoLink Novocastra (Leica Microsystems, Nussioch GmbH, Germany). To neutralize endogenous peroxidase, peroxidase block was used for 5 minutes, followed by washing in TBST twice for 5 minutes. The sections were incubated with protein block for 5 minutes and again washed in TBST twice for 5 minutes.

The primary monoclonal anti-TIMP-1 antibody [Ms mAb to TIMP-1 (102D1), Abcam] diluted in TBS 1:100 for 60 minutes was used at room temperature and in a humid chamber. The cuts were again washed in TBST twice for 5 minutes and incubated with *post primary block* for 30 minutes, followed by washing in TBST, twice for 5 minutes. Then, the cuts were incubated with polymer for 30 minutes and washed in TBST buffer, twice for 5 minutes. We added 50 µl of chromogen diaminobenzidine (DAB) to 1 ml of DAB substrate buffer (polymer) to develop peroxidase activity for 5 minutes.

The slides were washed in running water for 10 minutes and then rinsed in TBST for 3 minutes and subjected to counter staining with Mayer's hematoxylin. Washing in water and ammonium hydroxide was carried out for 5 minutes; followed by another wash in running water for 10 minutes. The final step of the protocol was dehydration in a growing series of alcohol 70%, 85%, 90% absolute and xylol for 5 minutes each, and slides mounted with Ervmount (EasyPath, Brazil). The negative control was performed by omitting the primary antibody (data not shown).

Microscopic evaluation of the TIMP-1 expression profile

The analysis of immunohistochemical reactivity to TIMP-1 was performed by a single examiner, previously calibrated. The slides were examined at the Carlos Alberto Redins Laboratory for Cell Ultrastructure [Laboratório de Ultraestrutura Celular Carlos Alberto Redins (LUCCAR)] which has an optical microscope (Olympus AX70, Olympus America Inc., NY, USA).

To assess immunolabeling, the following parameters were determined:

- for leukoplakia – presence or absence of brown cytoplasmic staining in keratinocytes in the different layers of the epithelial tissue of the oral mucosa, considering cells in two areas of the lesions, parenchyma, and stroma;
- for SCC – presence or absence of brown cytoplasmic staining in neoplastic cells, tumor islands, and stroma.

During the entire study stage and description of the TIMP-1 expression profile, according to the criteria mentioned above, the slides were examined directly under the microscope. Objectives lenses with different magnifications were used, allowing an initial panoramic analysis, with subsequent observation of the labeling details in the layers, islands, and cells. The microscopic findings have been compiled and are presented descriptively.

RESULTS

The immunohistochemical analysis sample was represented by surgical specimens embedded with enough material to obtain the new slides and composed of two cases of each degree of leukoplakia (mild, moderate and severe), and two cases of each type of SCC (stage I and stage II).

Immunolabeling for TIMP-1 was observed in all specimens, intensity and location varied between lesions and at different locations in the same lesion.

TIMP-1 expression in leukoplakia lesions

In mild leukoplakia sections, TIMP-1 expression was detected in different tissues and cells analyzed, including lining epithelium, fibroblasts, endothelial cells in the underlying connective tissue, skeletal striated muscle cells, and cytoplasm of mucous gland ducts. Regarding the epithelial tissue, labeling was observed in all thickness, however, when the layers were analyzed, it was possible to identify that keratinocytes into the basal layer showed more intense immunolabeling and presence of cytoplasmic granules containing the studied protein, highlighted in regions with basal cells hyperplasia. It was not possible to establish a nuclear labeling pattern; some epithelial cells presented a blue stained nucleus, while others, a brownish immunolabeling nucleus. This variation was more apparent in cells with loss of polarity (**Figure 1A** and **B**).

In moderate leukoplakia, immunolabeling was detected in all layers of the lining epithelial tissue, except for the cornea. However, differently from what was observed in mild leukoplakia, no variation in the intensity of expression was identified between the layers of the epithelium, as well as there was no predominance of TIMP-1 in relation to any dysplastic factor (**Figure 1C** and **D**).

In severe leukoplakia, all layers of epithelial tissue showed TIMP-1 expression, however in a heterogeneous way. The cells of the spinous layer were the ones that showed more intense immunolabeling when compared to other strata of the epithelium. However, keratinocytes in this layer also showed variation in protein expression and are between strong and weakly labeled. Despite the presence of extensive areas with cellular and nuclear pleomorphism, in these regions there was no variation in TIMP-1 expression (**Figure 1E** and **F**).

TIMP-1 expression in SCC lesions

When analyzing stage I SCC, it was possible to observe TIMP-1 expression both in the tumor parenchyma and stroma, however,

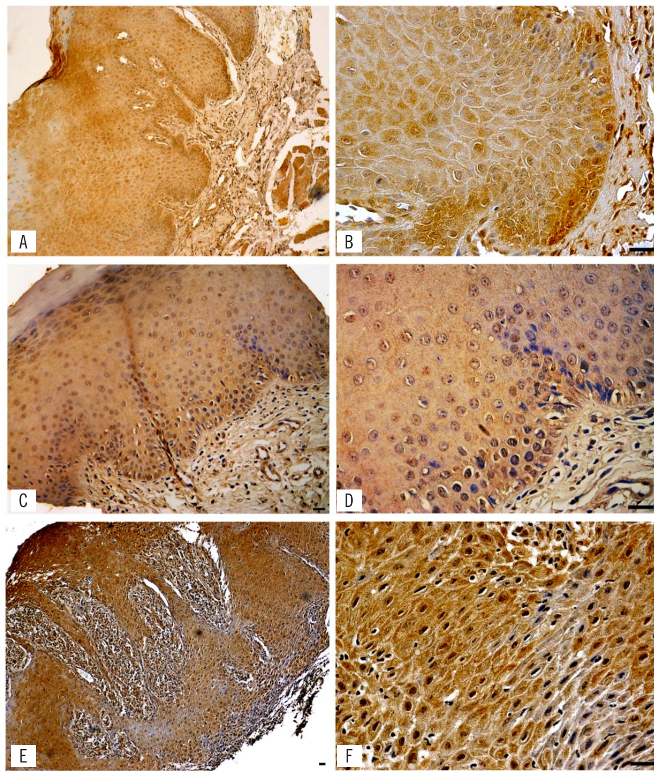

FIGURE 1 – *Immunolabeling photomicrographs for TIMP-1 in leukoplakia*

A) mild leukoplakia with protein expression in connective epithelium, vessels, and skeletal striated muscle; B) immunolabeling in all epithelial layers, more intense in the basal layer and in the area of hyperplasia; C and D) moderate leukoplakia with TIMP-1 expression in all epithelial layers, with no variation of intensity in each strata; E) severe leukoplakia with heterogeneous immunolabeling, present in all epithelial layers; F) stronger intensity in the spinous layer, with pleomorphic keratinocytes and variations between strong and weak labeling; scale bar = 20 µm.
TIMP-1: tissue inhibitor of metalloproteinase-1.

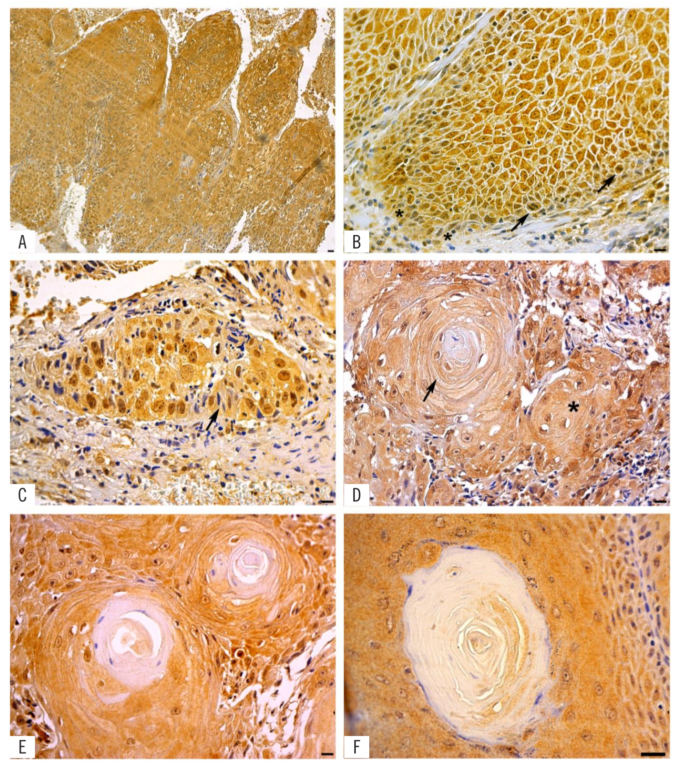

FIGURE 2 – *Immunolabeling photomicrographs for TIMP-1 in stage I SCC*

A) panoramic view with molecule expression in the stroma and parenchyma, more intense in the latter; B) deeper layers of the lesion with intense labeling on pleomorphic cells and mitosis (arrows); cells with loss of polarity showed weaker labeling (*); C) tumor island with cells showing variation in TIMP-1 expression – the less differentiated ones presented weaker labeling (seta); stromal cells around the island weakly labeled; D) keratin pearl formation (*) and the actual structure (arrow), showing different TIMP-1 expression patterns; E and F) presence of well-organized keratin pearls, with intense labeling on peripheral keratinocytes and very weak or absent labeling on central cells; scale bar = 20 µm.
TIMP-1: tissue inhibitor of metalloproteinase-1; SCC: squamous cell carcinoma.

stromal cells showed weaker labeling intensity when compared to epithelial cells (**Figure 2A**). In parenchyma, the labeling was not uniform. The deeper layers of the lesion showed intense labeling in cells with pleomorphism, and in areas with mitosis (arrows), as to the cells with loss of polarity displayed weaker labeling (*) (**Figure 2B**). In invasive regions, such as the tumor islands, there was variation in the immunolabeling, the less differentiated cells displayed weaker labeling (**Figure 2C** – arrow), in turn, keratinocytes with increased nucleoli and changes in the nucleus-cytoplasm ratio showed greater expression. In areas with well-arranged keratin pearls, intense expression was observed in peripheral keratinocytes and weak or absent labeling in central cells (**Figures 2D-F**).

In stage II SCC, stroma and parenchyma showed heterogeneous labeling (**Figure 3A**). In the stroma, fibroblast labeling was observed (**Figure 3B**). Regarding the tumor parenchyma, there was variation in molecule expression according to the layer. Basal cells with hyperplasia showed intense labeling, as did the cells of the spinous layer. However, between these two regions, a band of keratinocytes was detected, corresponding to the parabasal layer with unlabeled keratinocytes (**Figure 3B-D**). As described for stage I SCC, in the tumor islands, there was variation in labeling. Cells with prominent nucleoli had intense cytoplasmic labeling, while those less differentiated failed to express the protein (**Figure 3E**). Another aspect observed was the absence of labeling on keratin pearls, which were in smaller number and size when compared to pearls in stage I SCC lesions (data not shown). The inversion in the molecule expression was also observed in areas of the lesion in which the structure of the epithelium was preserved; the presence of strongly labeled spinous layer cells was detected, while keratinocytes in the basal layer and with loss of polarity were weakly labeled (**Figure 3F**).

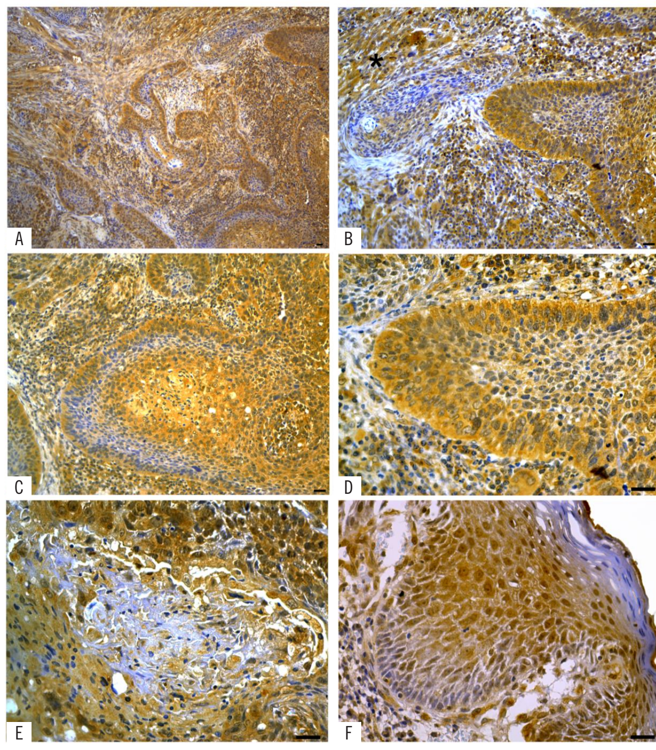

FIGURE 3 – *Immunolabeling photomicrographs for TIMP-1 in stage II SCC*

A) panoramic image showing invasive areas and loss of epithelial architecture. Labeling present in the parenchyma and stroma heterogeneously; B) fibroblasts expressing TIMP-1 (*) and hyperplastic basal epithelial layer with intense labeling; C and D) detail of the epithelium showing variation in the molecule expression among strata, strong labeled keratinocytes of the basal layer and spinous layer, and cells of the parabasal layer not labeled; E) tumor island with variation in TIMP-1 expression, most of the cells are poorly differentiated and weakly labeled; F) area of the lesion in which the epithelium structure is preserved, presence of strong labeled cells in the spinous layer, while the keratinocytes in the basal layer and with loss of polarity are weakly labeled; scale bar = 20 µm.
TIMP-1: tissue inhibitor of metalloproteinase-1; SCC: squamous cell carcinoma.

Figure 4 presents the lesions evaluated in the study, allowing a panoramic observation of both the histopathological features (A-E) and the immunolabeling profile (F-J). It is possible to observe that TIMP-1 was expressed in all cases analyzed and in a heterogeneous way.

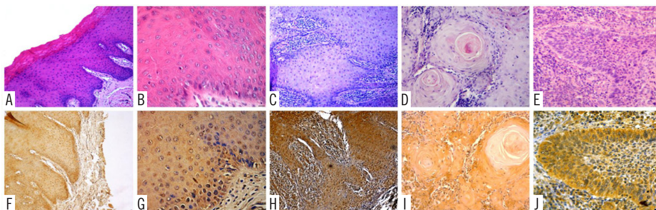

FIGURE 4 – *Photomicrographs of leukoplakia and oral SCCs in HE staining (A-E) e and immunolabeleing for TIMP-1*

Mild leukoplakia (A and F); moderate leukoplakia (B and G); severe leukoplakia (C and H); stage I SCC (D and I); stage II SCC (E and J); scale bar = 20 µm.
SCC: squamous cell carcinoma; HE: hematoxylin and eosin; TIMP-1: tissue inhibitor of metalloproteinase-1.

DISCUSSION

The present study aimed to describe the TIMP-1 expression and distribution in leukoplakia and SCC from incisional and excisional biopsies from SAPB-UFES. Immunohistochemistry was used, as it allows detecting the location and correlation with morphology, especially with cellular events at the stroma/tumor interface.

It is already known that the proteolysis process is important at all stages of cancer development and progression. Within this context, Chambers and Matrisian, in review (1997)[20] showed evidence that suggests a complex role for MMP in the previous stages and after the ECM degradation. Both enzymes and their inhibitors, such as TIMP, proved to be important regulators of tumor growth, acting on the primary and metastatic sites of neoplasms.

The imbalance between MMP and TIMP is a recognized feature that favors the ECM degradation, facilitating tumor invasion and metastasis. Among the various MMP involved in tumorigenesis, studies have shown that MMP-2 and MMP-9 play an important role in tumor progression. Their increased expressions have already been observed in several neoplasms, and this increase is almost always accompanied by greater invasive and metastatic activities, which lead to a decrease in patient survival[21-23].

Although all members of the TIMP family have the ability to inhibit MMP activity, it has been described that this inhibition is selective. Regarding TIMP-1, the target of this study, Baker *et al*. (2002)[24] and Lambert *et al*. (2004)[18] describe it as an inhibitor of all MMP, except for membrane-type MMP and MMP-19. Also, its preferred targets are MMP -7, -9, -1, and -3[25].

The literature has also shown, however, that TIMP are involved in several biological activities, including cell differentiation, growth, migration, invasion, angiogenesis, and apoptosis, and all of these cellular effects are mediated regardless of their ability to inhibit MMP activity[26].

Another important aspect to be highlighted is the diversity of activities of this family of molecules. Although they are secreted proteins with extracellular functioning, they have already had surface receptors identified, suggesting, therefore, control of cellular behavior and signaling known as "from outside to inside the cell"[25].

When we demonstrated that lesions of leukoplakia and oral SCC express the TIMP-1 molecule, it becomes possible to search for correlations between the expression profile with histopathological features which are relevant in determining the severity of each lesion, whether the carcinoma itself or its precursor lesion.

The immunolabeling profile varied between lesions (leukoplakia and SCCs), and also within each lesion. In leukoplakia cases, regardless of the degree of dysplasia, all of them expressed TIMP-1 in the epithelial, connective, and skeletal muscle tissue. For leukoplakia classified as mild dysplasia, TIMP-1 expression was more notable in the basal layer and in areas of basal hyperplasia. In a different way, the cells of the basal layer that presented loss of polarity had less expression of the molecule when compared to the other cells. Since TIMP-1 is a synthesized and secreted molecule, it is suggested that the disorganization of the cytoplasm and the organelles involved in the synthesis, due to the loss of polarity, may have resulted in decreased production and accumulation of the protein.

For moderate leukoplakia, no correlation was established between dysplastic factors and TIMP-1 expression, whereas for severe leukoplakia, all layers of epithelial tissue showed TIMP-1 expression, however in a heterogeneous manner. The cells of the spinous layer showed more intense immunolabeling and the keratinocytes of that layer also had variation in the expression of the protein, counting between strong and weakly labeled, independent of the cellular and nuclear pleomorphism.

Although TIMP molecules have a regulatory function for ECM remodeling, it is important to note that TIMP-1 binding sites have already been found on the cell surface of keratinocytes, fibroblasts, and tumor cells, as in breast cancer. Although this group of receptors still needs further studies, it is believed that they can participate in the transduction of intracellular signals[27, 28].

Although the distribution pattern of TIMP-1 varied among leukoplakias, as mentioned, and it was possible to correlate some cellular aspects of dysplasia with a greater or lesser presence of the molecule, there are still gaps that need to be filled to define the relationship between expression of the enzyme, as well as other TIMP, and dysplasia grading in this important precursor lesion of the oral SCC.

Some of these gaps have been filled with studies on potentially malignant disorders, such as leukoplakia, and the different dysplastic degrees of lesions and conditions. Chen *et al.* (2008)[29], evaluated MMPs-2 and -9 and TIMP-2 in the spectrum of non-atrophic, atrophic lichen planus and carcinomas originating from lichen planus, in an attempt to elucidate the balance between these proteins in the progression to SCC. In lesions without the presence of dysplastic factors, TIMP-2 expression was moderate to strong, mainly in the cytoplasm of the basal and spinous cells, unlike MMP immunolabeling. Despite the increase in TIMP-2 during the cancerization of lichen planus, its expression was weaker than that of MMP.

Few studies have linked the TIMP-1 expression with leukoplakia and its degrees of dysplasia. This fact became a challenge during the analysis, but at the same time it was motivating, in the understanding of its role in carcinogenesis, widely described in other types of cancer. Sutinen *et al.* (1998)[30] compared TIMP-1 mRNA expression in lesions with oral epithelial dysplasia (mild, moderate, and severe), oral lichen planus, SCC, and lymph nodes with metastasis. The results were not remarkable for epithelial dysplasia and lichen planus, however, positive and weak immunolabeling was observed in the stroma of oral SCCs and lymph nodes, suggesting less activation in the areas of the properly established carcinoma.

Our analysis showed that, in SCC lesions, it was possible to identify the protein both in tumor stroma and parenchyma, and stage II SCC fibroblasts showing more evident labeling. Vicente *et al.* (2005)[31], in a TIMP-1 immunoreactivity study of 45 SCC cases, reported 19 cases (28%) in which the molecule was also expressed in the stroma and around tumor cells. It is worth mentioning that the microenvironment, previously considered only as a passive support structure, was recognized as an effective dynamic factor in tumor progression. In addition to stromal cells, this environment contains ECM, proteins and their inhibitors, vessels, nerves and immune cells, which are important participants in the process.

However, it was in the tumor parenchyma that the labeling variations had some important aspects that should be considered and scored, especially regarding the presence or absence of TIMP-1 in invasive areas, such as tumor islands and less differentiated cells.

For stage I SCC, TIMP-1 was detected in all layers of the epithelium, the same did not occur in stage II SCC. In the latter, basal cells with hyperplasia showed intense labeling, as well as cells in the spinous layer. However, between these two regions, a strip of unlabeled keratinocytes, corresponding to the parabasal layer, was detected.

Vicente *et al.* (2005) also detected the presence of the protein in SCC with heterogeneous expression. The authors described that 45 (66.2%) from 68 SCC cases were labeled, of which 11 (16.2%) strongly expressed, 16 (23.5%) with immunolabeling between 10% and 50% of the tumor cells, 18 (26.5%) expressed less than 10% positive cells, and 23 cases were negative.

The analysis carried out in this work also allowed characterizing the deeper layers of SCC as those with intense labeling in cells with pleomorphism and in areas with mitosis, whether they are typical or atypical. The cells with loss of polarity were weakly expressed. This last aspect was similar to that described for mild and severe leukoplakia.

We must highlight the profile of immunolabeling invasive regions, such as tumor islands. In these locations, both for stage I and stage II SCC, there was variation in immunolabeling; the less differentiated cells presented weaker labeling, in turn, keratinocytes with increased nucleoli and changes in the nucleus-cytoplasm ratio showed stronger expression. The cells that preserved microscopic features that characterize the epithelium, such as juxtaposition and some polarity, were those that still expressed TIMP-1. However, stage II SCC presented islands with a higher proportion of undifferentiated cells and, therefore, weakly labeled when compared to stage I. These data corroborate with Vicente *et al.* (2005)[32], who observed the presence of TIMP-1 in nests and epithelial cords in three distinct patterns, but homogeneous only in the central part of the tumor cell nest and dispersed or irregular.

The literature is still controversial regarding the TIMP-1 expression and its role in tumorigenesis. TIMP-1 overexpression has been shown to inhibit melanoma growth and metastasis[33], and suppress the metastatic capacity of human gastric cancer cells[34] and oral squamous cells carcinoma[35].

On the other hand, there are also reports associating TIMP-1 with the potential for invasion and metastasis. Palikhe *et al.* (2010)[19], in a study of patients with head and neck cancer, evaluated the TIMP level in the plasma and found an association between TIMP concentration and increased survival; as TIMP inhibits MMP and also functions as a growth factor, it can directly influence cancer progression.

Our findings suggest that in invasive areas, such as tumor islands, where it is possible to detect cells that are losing their epithelial characteristics and, therefore, have more aggressive behavior, the TIMP-1 expression is reduced. However, for better evaluation, a study with semi-quantitative or quantitative analysis, in addition to the descriptive one, would be necessary.

In areas with well-arranged keratin pearls, intense expression was observed in peripheral keratinocytes and weak or absent labeling in central cells. Pearls are areas of keratinization that refer to the regular event of the corneal layer formation, but in SCC they occur intraepithelially and not on tissue surface[36]. The negative immunolabeling in these structures, a SCC feature, suggests that flattened, eosinophilic and translucent cells, whose cytoplasmic nuclei and organelles were digested by lysosomal enzymes, and whose cytoplasm full of numerous keratin tonofilaments, prevented TIMP-1 labeling[37].

We must emphasize that, in the study of carcinogenesis and the established disease, invasion and metastasis are aspects that can be evaluated microscopically and clinically, since TIMP-1 expression may be a valuable tool to aid in diagnosis, and its analyze is enabeled through immunohistochemistry. Although labeling with monoclonal antibodies are rather specific and show the biological behavior of cells, visual quantification is not always accurate. Therefore, it is important to emphasize that a significant challenge of this technique is the determination of labeling intensity, as it may present great variability.

Therefore, the study of TIMP-1 expression pattern has mostly presented subjective or semi-quantitative analyzes, assigning scores to different immunolabeling levels. In this study, the analysis of the expression of the protein in question was restricted to the comparison of intensity between different tissues in the same section, ruling out the comparison with other studies that assign scores.

However, the conduction of studies that seek precisely to identify biomarkers that may inhibit the MMP activity, and thereby a possible reduction in the progression of the disease or even in the malignancy of precursor lesions, has its value as an auxiliary tool not only in the prognosis, as well as for the establishment of future candidates for therapeutic agents. Among these drugs are peptidomimetics, non-peptidomimetics, tetracycline derivatives, and bisphosphonates, all of which interfere with MMPs expression and activation, reducing the function of these enzymes and consequent degradation of MEC, thereby inhibiting the MMP function as an alternative way of anticancer therapy[38].

CONCLUSION

Based on the experiments and analyzes carried out, we conclude that:

• the TIMP-1 expression pattern was associated with dysplastic cellular aspects, such as hyperplasia of the basal layer and loss of polarity in leukoplakia and SCCs, regardless of the degrees of dysplasia and differentiation, respectively;

• in SCCs, in areas of the tumor parenchyma populated by cells that underwent the de-differentiation process, presented weak or even absence of TIMP-1;

• in stage II SCC, areas of tumor islands with loss of microscopic features of epithelial tissue and weak TIMP-1 expression, were more frequent than in stage I SCC.

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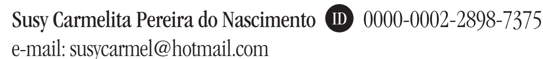

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