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AVALIAÇÃO IMUNOISTOQUÍMICA DE MARCADORES DA TRANSIÇÃO EPITÉLIO-MESÊNQUIMA NO FRONTE DE INVASÃO DO CARCINOMA DE CÉLULAS ESCAMOSAS DE BOCA

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Dissertação apresentada ao Programa de Pós-graduação em Odontologia da Pontifícia Universidade Católica de Minas Gerais, como requisito parcial para a obtenção do título de Mestre em Odontologia, Área de Concentração em Clínicas Odontológicas - Ênfase: Estomatologia.

Orientador: Prof. Dr. Martinho Campolina Rebello Horta

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A Deus...

... Que sempre está comigo em todos os momentos.

Dando-me sabedoria e discernimento.

Que sabe exatamente o que se passa em meu coração.

A Ele seja dada toda a honra e louvor.

“Feliz o homem que acha sabedoria,

e o homem que adquire conhecimento.”

Provérbios 3:13

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RESUMO

O carcinoma de células escamosas de boca (CCEB) é uma das neoplasias malignas que mais acometem a população mundial. As áreas mais profundas e invasivas desta neoplasia, denominadas fronte de invasão (FI), têm sido identificadas como responsável por seu comportamento biológico. Transição epitélio-mesênquima (EMT) é um evento biológico no qual células epiteliais perdem suas características fenotípicas e ganham propriedades de células mesenquimais, sendo importante durante o processo de invasão local e metástase das neoplasias epiteliais malignas. O objetivo deste estudo foi avaliar a expressão dos marcadores de EMT caderina-E e vimentina em CCEB. Detecção imunoistoquímica e análise semi-quantitativa de caderina-E e vimentina foi realizada em 20 amostras. Diferenças na expressão de caderina-E e vimentina entre o FI e as áreas centrais e superficiais (ACS) do tumor foram avaliadas através do teste de McNemar. O teste exato de Fischer foi utilizado para avaliar a existência de diferenças na expressão de caderina-E e vimentina no FI entre tumores com alto e baixo grau de invasividade histológica. O teste exato de Fischer foi também utilizado para avaliar a existência de associação entre expressão de cadeina-E e de vimentina no FI. Redução na expressão de caderina-E foi observada em 15 amostras (75%). A expressão de caderina-E no FI mostrou-se reduzida quando comparada às ACS ($p<0,05$). Adicionalmente, a expressão de caderina-E no FI mostrou-se reduzida nos tumores de alto grau de invasividade histológica quando comparada aos tumores de baixo grau ($p<0,05$). Expressão positiva de vimentina foi observada em 6 amostras (30%). Não foi observada diferença na expressão de vimentina entre o FI e as ACS ($p>0,05$). Não foi observada diferença na expressão de vimentina no FI entre tumores de alto e baixo grau de invasividade histológica ($p>0,05$). Não foi observada associação entre expressão de caderina-E e de vimentina no FI ($p>0,05$). A expressão heterogênea de caderina-E e vimentina no CCEB sugere que essas proteínas são importantes marcadores de EMT nessa neoplasia. A expressão reduzida de caderina-E no FI e sua associação com a invasividade histológica identifica esta proteína como um importante marcador de EMT no FI. As alterações na expressão de vimentina não foram limitadas ao FI e não estavam relacionadas à invasividade histológica.

Palavras-chave: Câncer bucal. Carcinoma de células escamosas. Transição epitélio-mesênquima. Caderina-E. Vimentina.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is one of the most common malignances worldwide. The histologically most deep and invasive areas of this tumor, named as the invasive front (IF), have been identified as chiefly responsible for its clinical behavior. Epithelial-mesenchymal transition (EMT) is a biological event in which epithelial cells lose many of its phenotypical features and gain typical properties of mesenchymal cells. EMT is a significant event during tumor invasion and metastasis of malignant epithelial tumors. The aim of this study was to evaluate the expression of the EMT markers E-cadherin and vimentin in OSCC. Immunohistochemical detection and semiquantitative analysis of E-cadherin and vimentin was performed in 20 OSCC samples. Differences in the expression of E-cadherin and vimentin between the IF and the central/superficial areas (CSA) of the tumor were analyzed using the McNemar test. The Fischer exact test was used to assess differences in the expression of each protein at the IF between high- and low-invasive OSCC. The Fischer exact test was also used to evaluate association between E-cadherin and vimentin expression at the IF. Reduced expression of E-cadherin was detected in 15 OSCC samples (75%). The E-cadherin expression was reduced at the IF when compared to the CSA ($p<0.05$). Moreover, the E-cadherin expression was reduced at the IF of high-invasive OSCC when compared to low-invasive OSCC ($p<0.05$). Positive expression of vimentin was observed in 6 OSCC samples (30%). There was no significant difference in vimentin expression between the IF and the CSA ($p>0.05$). In addition, no difference in vimentin expression at the IF was observed between low- and high-invasive OSCC ($p>0.05$). Finally, no association was observed between E-cadherin and vimentin expression at the IF of OSCC ($p>0.05$). A heterogeneous E-cadherin and vimentin expression was observed in OSCC, suggesting that these proteins are significant EMT markers in this neoplasia. The reduced E-cadherin expression at the IF and its association with histological invasiveness identify this protein as a significant marker of EMT at the IF. Changes in vimentin expression are neither limited to the IF nor related to the IF histological invasiveness.

Key words: Oral cancer. Squamous cell carcinoma. Epithelial-mesenchymal transition. E-cadherin. Vimentin.

SUMÁRIO

1 INTRODUÇÃO

1.1 O carcinoma de células escamosas de boca

O carcinoma de células escamosas, também denominado carcinoma epidermóide ou carcinoma espinocelular, representa mais de 90% das neoplasias malignas da boca (BARNES et al., 2005; PETERSEN 2009).

O Instituto Nacional do Câncer estimou, para o ano de 2010, a ocorrência de 489.270 novos casos de câncer no Brasil. Entre estes casos, estavam previstos 14.120 novos casos de câncer bucal, sendo 10.330 em homens e 3.790 em mulheres. Portanto, essa neoplasia representa o sexto tipo de câncer mais comum em homens e o oitavo mais comum em mulheres em nosso país (INSTITUTO NACIONAL DO CÂNCER, 2011).

A maioria dos casos de carcinoma de células escamosas de boca está associada à exposição da mucosa bucal a carcinógenos, podendo também haver predisposição individual vinculada a características genéticas específicas. Portanto, a sua etiologia é multifatorial e os principais fatores de risco para o seu desenvolvimento são tabaco, álcool, radiação solar e infecção pelo Papilomavírus Humano (HPV, subtipos 16 e 18) (BARNES et al., 2005; SCULLY, 2011).

Esta neoplasia ocorre principalmente em indivíduos do sexo masculino, entre a quinta e sexta décadas de vida. As regiões da mucosa bucal mais acometidas pelo tumor são lábio inferior, língua, assoalho bucal, gengiva, palato duro e mucosa jugal (GERVÁSIO et al., 2001; BARNES et al., 2005; XAVIER et al., 2007).

O carcinoma de células escamosas origina-se a partir de queratinócitos do epitélio de revestimento da mucosa bucal. Sua anatomia patológica microscópica caracteriza-se pela presença de invasão da lâmina própria e tecidos adjacentes por lençóis, ninhos, cordões ou pequenos grupos de células epiteliais de revestimento neoplásicas (BARNES et al., 2005).

Os tratamentos de escolha para esta neoplasia são cirurgia, radioterapia ou ambos, dependendo da localização e do estadiamento clínico do tumor, obtido através do sistema TNM (MISRA; CHATURVEDI; MISRA, 2008).

1.2 O fronte de invasão e sua importância no comportamento biológico do carcinoma de células escamosas de boca

O prognóstico do carcinoma de células escamosas de boca está diretamente relacionado ao estadiamento clínico do tumor através do sistema TNM. Entretanto, observa-se frequentemente que tumores de mesmo estadio clínico apresentam diferenças em seu comportamento, tornando-se necessário o uso de métodos complementares para o estabelecimento de um prognóstico mais preciso. A graduação da diferenciação histológica do tumor é um destes métodos complementares. Esta graduação histológica está baseada em evidências de que variações nas características morfológicas microscópicas do tumor podem refletir diferentes graus de comportamento, sendo de valor auxiliar no estabelecimento do seu prognóstico (BRYNE, 1991; LINDENBLATT et al., 2011).

Entre as diversas propostas de métodos a serem utilizados para a graduação histológica do carcinoma de células escamosas de boca, destaca-se o sistema de Bryne et al. (1989, 1992), no qual são avaliadas apenas as áreas mais profundas e invasivas do tumor, local denominado fronte de invasão. Os parâmetros morfológicos analisados por este sistema são grau de queratinização, pleomorfismo nuclear, padrão de invasão (modo de invasão histológica) e infiltrado inflamatório. Cada parâmetro morfológico é graduado de 1 a 4, em ordem crescente, na medida em que denota maior grau histológico de malignidade. Os valores estabelecidos para os quatro parâmetros são então somados perfazendo um produto final, denominado grau histológico de malignidade, que pode variar de quatro a dezesseis. Quanto maior o valor do grau histológico de malignidade, mais agressivo é o tumor e pior é o seu prognóstico (BRYNE et al., 1992).

O valor prognóstico do sistema de graduação histológica do fronte de invasão para o carcinoma de células escamosas de boca têm sido constantemente confirmado (ODELL et al., 1994; PIFFKÒ et al., 1996; PIFFKÒ et al., 1997; SAWAIR et al., 2003; KUROKAWA et al., 2005). Segundo Bànkfalvi e Piffkò (2000), o reconhecimento do fronte de invasão como região responsável pela determinação do comportamento biológico do carcinoma de células escamosas de boca é uma das descobertas mais promissoras no estudo do prognóstico desta neoplasia. Cabe destacar que, entre os quatro parâmetros morfológicos avaliados pelo sistema de

graduação histológica do fronte de invasão, o padrão de invasão (modo de invasão histológica, que expressa as características infiltrativas do tumor) é um dos mais importantes fatores relacionados ao prognóstico (ODELL et al., 1994; BRYNE, JENSSEN; BOYSEN, 1995; SAWAIR et al., 2003).

1.3 Marcadores da transição epitélio-mesênquima (EMT) e sua importância no processo de invasão local e metástase das neoplasias epiteliais

O termo “transição epitélio-mesênquima” (*Epithelial-mesenchymal transition* - EMT) descreve uma série de eventos durante os quais células epiteliais perdem muitas de suas características e ganham propriedades típicas de células mesenquimais, o que implica em mudanças complexas na arquitetura e no comportamento celular (THIERY; SLEEMAN, 2006; ZEISBERG; NEILSON, 2009). A EMT pode ser classificada em três categorias: EMT tipo 1, 2 e 3. EMT tipo 1 envolve células epiteliais primitivas e ocorre durante a gastrulação e a migração de células da crista neural. EMT tipo 2 envolve células epiteliais secundárias (maduras) que migram para o estroma resultando na formação de fibroblastos residentes ou induzidos por processo inflamatório. A EMT tipo 3, objeto de estudo desta dissertação, envolve as células epiteliais neoplásicas que deixam o nódulo tumoral primário dos carcinomas e migram para um novo sítio, em um processo caracterizado por alterações fenotípicas que as capacitam a realizar o processo de invasão local e metástase (ZEISBERG; NEILSON, 2009; VERED et al., 2010).

Vários marcadores têm sido utilizados para demonstrar a ocorrência de EMT. Entre as proteínas da superfície celular, destacam-se as caderinas (N, E, OB) e as integrinas. Entre as proteínas do citoesqueleto, destacam-se vimentina, α-SMA (α actina de músculo liso) e β-catenina. A EMT tipo 3, observada no processo de invasão local e metástase das neoplasias, é caracterizada principalmente por variações na expressão de marcadores como caderina-E, caderina-N, β-catenina e vimentina (THIERY; SLEEMAN, 2006; ZEISBERG; NEILSON, 2009).

Células com fenótipo epitelial são firmemente coesivas, sem mobilidade, apresentando polarização apical-basolateral e organizada distribuição das moléculas de adesão no citoesqueleto. Na EMT ocorre transformação dessas células epiteliais

polarizadas em células com características mesenquimais: sem polarização e com grande mobilidade. Esta aquisição de um fenótipo migratório é importante para o processo de invasão local e metástase das neoplasias epiteliais (THIERY; SLEEMAN, 2006; CHAFFER; THOMPSON; WILLIANS, 2007; GUARINO; RUBINO; BALLABIO, 2007; LOGULLO et al., 2009).

A adesão célula-célula determina a polaridade celular e as principais moléculas responsáveis por este fenômeno pertencem à superfamília das caderinas, glicoproteínas transmembrana dependentes de cálcio, que podem ser divididas em clássicas e não clássicas. As clássicas são denominadas de acordo com os principais tecidos onde são encontradas: caderina-E (localizada principalmente no epitélio), caderina-N (localizada no tecido endotelial, nos neurônios, músculo estriado, cristalino e fibroblastos), caderina-P (localizada em placenta, epiderme e epitélio glandular da mama), caderina-VE (localizada em células endoteliais) (STEMMLER, 2008; ALBERTS et al., 2010). As caderinas são moléculas transmembrana que apresentam um domínio extracelular e um domínio intracelular. Para a adesão célula-célula, é necessário que o domínio intracelular esteja ligado ao citoesqueleto através de proteínas de ancoragem denominadas cateninas (α, β, gama, p120). O braço citoplasmático das caderinas une-se ao complexo multiprotêico β-catenina, gama-catenina e p120-catenina. Este complexo liga-se diretamente à α-catenina, sendo esta responsável por unir o complexo ao citoesqueleto (CAVALLARO; SCHAFFHAUSER; CHRISTOFORI, 2002; GUARINO; RUBINO; BALLABIO, 2007; ALBERTS et al., 2010).

A caderina-E, objeto de estudo desta dissertação, é a principal molécula de adesão do tecido epitelial, fundamental para o estabelecimento e manutenção da polaridade celular ápico-basal e da integridade estrutural do epitélio (ALBERTS et al., 2010). Esta glicoproteína transmembrana é codificada pelo gene CDH1, localizado no cromossomo 16q21-22 (BERX; ROY, 2009). A expressão membranosa da E-caderina é observada principalmente na camada suprabasal e espinhosa do epitélio de revestimento da mucosa bucal normal (MANDAL et al., 2008; WANG et al., 2009). A expressão ou a perda de expressão de algumas moléculas responsáveis pela adesão celular estão relacionadas ao processo de invasão local e metástase de várias neoplasias epiteliais malignas, incluindo o carcinoma de células escamosas de boca. A redução na expressão de caderina-E é considerada um dos mais importantes indicadores e reguladores da EMT, representando um passo

determinante neste processo. Diferentes mecanismos para inativação da caderina-E têm sido identificados: mutação hereditária ou somática; hipermetilação gênica; indução de repressores de transcrição; fosforilação de resíduos de tirosina no domínio citoplasmático da caderina-E; perda ou deslocamento da β-catenina ou da p120; rompimento do domínio extracelular por metaloproteinases (BERX; VAN ROY, 2009; GUARINO; RUBINO; BALLABIO, 2007).

A redução na expressão de caderina-E é um fenômeno frequente em neoplasias malignas de origem epitelial e tem sido associada a menor diferenciação histológica tumoral e maior potencial invasivo e metastático, inclusive em carcinoma de células escamosas de boca (BÁNKFALVI et al., 2002; YOKOYAMA et al., 2001; MAHOMED; ALTINI; MEER, 2007; WANG et al.. 2009; LIU et al., 2010).

A vimentina é uma proteína da família dos filamentos intermediários expressa em células mesenquimais como fibroblastos, células endoteliais, células hematopoiéticas e células da glia (ALBERTS et al., 2010; SATELLI; LI, 2011). Os filamentos intermediários, os microtúbulos e os microfilamentos constituem os três maiores grupos de proteínas não musculares do citoesqueleto (ALBERTS et al., 2010). São descritas seis classes de filamentos intermediários, cada uma restrita a determinados tipos celulares: tipos I e II (queratinas ácidas e básicas, presentes em células epiteliais); tipo III (vimentina, presente em células mesenquimais; desmina, presente em células musculares); tipo IV (neurofilamentos, presentes em neurônios); tipo V (laminas, constituintes da membrana nuclear); tipo VI (nestina, presentes em neurônios embrionários) (SATELLI; LI, 2011). O monômero de vimentina apresenta um domínio alfa-hélice central altamente conservado flanqueado por domínios N terminal (cabeça) e C terminal (cauda). A unidade básica para a formação dos filamentos de vimentina são os dímeros formados pela associação desses monômeros. Os filamentos de vimentina funcionam como organizadores do espaço citoplasmático, suportando e ancorando a posição de organelas no citosol e contribuindo para a manutenção da integridade celular e tecidual (MININ; MOLDAVER, 2008; ALBERTS et al., 2010; SATELLI; LI, 2011).

A vimentina não é expressa por células epiteliais normais e tem sido identificada como um importante marcador de EMT em neoplasias malignas epiteliais, nas quais está associada a uma maior capacidade migratória e invasiva das células tumorais (MCINROY; MÄÄTTÄ, 2007; ZEISBERG; NEILSON, 2009; SATELLI; LI, 2011). Enquanto filamentos intermediários de queratina estão

fortemente ligados a junções celulares promovendo adesão e integridade tecidual, filamentos de vimentina podem apresentar sítios de adesão transitórios que desempenham um papel importante durante a migração celular (MCINROY; MÄÄTTÄ, 2007).

Dandachi et al. (2001) observaram que células neoplásicas de adenocarcinoma de mama podem exibir expressão de vimentina associada à morfologia fusiforme, refletindo a aquisição de características mesenquimais e resultando em um fenótipo mais agressivo e invasivo. Paccione et al. (2008) demonstraram que células derivadas de nódulos metastáticos de carcinoma de células escamosas de boca expressam altos níveis de vimentina, não detectada em células tumorais derivadas da lesão primária. Eles também demonstraram que a reversão *in vitro* da expressão da vimentina pelas células neoplásicas resultou na reversão no fenótipo mesenquimal e redução das características de migração e invasividade. Jin et al. (2010) observaram que expressão de vimentina em carcinoma de células escamosas de esôfago está associada a metástase regionale à distância. Liu et al. (2010) demonstraram que a expressão de vimentina em carcinoma de células escamosas de boca está associada a um pior prognóstico.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a expressão dos seguintes marcadores da transição epitélio-mesênquima no carcinoma de células escamosas de boca: caderina-E e vimentina.

2.2 Objetivos específicos

a) avaliar a existência de diferença na expressão de caderina-E entre a região central/superficial e o fronte de invasão do carcinoma de células escamosas de boca;
b) avaliar a existência de diferença na expressão de caderina-E no fronte de invasão do carcinoma de células escamosas de boca entre tumores com alto grau e baixo grau de invasividade histológica;
c) avaliar a existência de diferença na expressão de vimentina entre a região central/superficial e o fronte de invasão do carcinoma de células escamosas de boca;
d) avaliar a existência de diferença na expressão de vimentina no fronte de invasão do carcinoma de células escamosas de boca entre tumores com alto grau e baixo grau de invasividade histológica;
e) avaliar a existência de associação entre a expressão de caderina-E e a expressão de vimentina no fronte de invasão do carcinoma de células escamosas de boca.

ARTIGO

O artigo intitulado “*Expression of epithelial-mesenchymal transition markers at the invasive front of oral squamous cell carcinoma*” será submetido ao periódico “*Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*”, tendo sido formatado de acordo com suas normas.

EXPRESSION OF EPITHELIAL-MESENCHYMAL TRANSITION MARKERS AT THE INVASIVE FRONT OF ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Objectives: To evaluate the expression of the Epithelial-mesenchymal transition (EMT) markers E-cadherin and vimentin in OSCC.

Study design: Immunohistochemical detection of E-cadherin and vimentin was performed in 20 OSCC samples. Differences in the expression of each protein between the IF and the central/superficial areas (CSA) of the tumor were accessed. Differences in the expression of each protein at the IF between histologically high- and low-invasive OSCC as well as association between E-cadherin and vimentin expression at the IF were also evaluated.

Results: E-cadherin expression was reduced at the IF when compared to the CSA. Moreover, E-cadherin expression was reduced at the IF of high-invasive when compared to low-invasive OSCC. There was no difference in vimentin expression between the IF and the CSA. Furthermore, no difference in vimentin expression at the IF was observed between low- and high-invasive OSCC. No association was observed between E-cadherin and vimentin expression at the IF.

Conclusions: A heterogeneous E-cadherin and vimentin expression was observed in OSCC, suggesting that these proteins are significant EMT markers in this tumor. The reduced E-cadherin expression at the IF and its association with histological invasiveness identify this protein as a significant marker of EMT at the IF. Nevertheless, changes in vimentin expression are neither limited to the IF nor related to the IF histological invasiveness.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the most common malignances worldwide[1,2] and its most significant risk factors are tobacco and alcohol.[3] This tumor mostly affects men over 40 years old and frequently occurs in lower lip, tongue, floor of mouth, soft palate, and gingival/alveolar ridge.[3] The histologically most deep and invasive areas of this tumor, named as the invasive front (IF), have been identified as chiefly responsible for its clinical behavior.[5,6]

Epithelial-mesenchymal transition (EMT) is a biological event in which epithelial cells lose many of its phenotypical features and gain typical properties of mesenchymal cells. One of the marks of EMT is the transformation of cohesive and polarized epithelial cells in mesenchymal-like cells showing no polarization and high mobility. This migratory phenotype is the hallmark of an important type of EMT that involves tumor cells of the epithelial malignant neoplasms during tumor invasion and metastasis.[7-11]

The transmembrane glycoprotein E-cadherin is the main molecule of cell-cell adhesion of epithelial tissue and plays and essential role in the establishment and maintenance of its polarity and structural integrity.[12,13] The loss of E-cadherin expression, that increases the mobility of epithelial cells and its ability for local invasion, has been used as a marker of EMT during tumor progression.[8,10,13,14]

Vimentin is a main component of intermediate filament family of proteins, expressed in mesenchymal cells such as fibroblasts, endothelial cells, hematopoietic cells and glial cells.[12,15] This protein is not expressed in normal epithelial cells and has been identified as a marker of EMT in epithelial malignant tumors, in which it is associated with an invasive tumor cell phenotype.[10,15]

The aim of this study was to evaluate the expression of the EMT markers E-cadherin and vimentin in OSCC. Differences in the expression of each protein between the IF and the central/superficial areas (CSA) of the tumor were accessed. Differences in the expression of each protein at the IF between histologically high- and low-invasive OSCC were also evaluated. Moreover, association between E-cadherin expression and vimentin expression at the IF was evaluated.

MATERIAL AND METHODS

Tissue samples

This study was approved by the local ethics committee.

A total of 20 OSCC samples from archival formalin-fixed, paraffin-embedded specimens were evaluated.

The following inclusion criteria was applied: 1) OSCC samples obtained by total surgical resection; 2) OSCC samples in which the superficial areas as well as the invasive front could be clearly identified.

Evaluation of the pattern of invasion of OSCC

The pattern of invasion of OSSC samples was performed on hematoxylin and eosin stained sections, according to Bryne et al.[5] The pattern of invasion was classified as: grade 1 - pushing, well delineated infiltrating borders; grade 2 - infiltrating, solid cords, bands and/or strands; grade 3 - small groups or cords of infiltrating cells (n>15 cells); grade 4 - marked and widespread cellular dissociation in small groups and/or in single cells (n<15 cells). The samples were subsequently classified as low-invasive OSCC (pattern of invasion grade 1, 2 or 3) and high-invasive OSCC (pattern of invasion grade 4).

Immunohistochemistry

For immunohistochemical detection of E-cadherin and vimentin, four μm sections from the paraffin-embedded samples were used. Tissue sections were dewaxed with xylene, hydrated using graded alcohols, and treated with 0.6% H_2O_2 to eliminate endogenous peroxidase activity. Subsequently, antigen retrieval and primary antibodies incubation were performed. The applied primary antibodies, antigen retrieval protocols and stained conditions are described in Table I. The LSAB+ kit (Dako Corporation, Carpinteria, USA) was used for application of the biotinylated link antibody and peroxidase-labeled streptavidin, according to the manufacturer's instructions. The reactive products were visualized by immersing the sections for 3 min in 0.03% diaminobenzidine solution, containing 2 mM H_2O_2. The sections were then counterstained with Mayer's hematoxylin, dehydrated, and mounted. Samples of oral fibrous hyperplasia were used as positive control. In addition, immunostaining of morphologically normal oral mucosa epithelium adjacent

to tumor as well as tumor's connective tissue mesenchymal cells were respectively used as internal positive control for E-cadherin and vimentin. Negative control was determined by omission of the primary antibody.

Scoring of immunostaining results

A light microscopy was used to evaluate the immunohistochemical reactions. The expression of E-cadherin and vimentin was independently assessed at the invasive front and at the central/superficial areas of the tumor. The expression of E-cadherin was classified as following (Mahomed, Altini, Meer)[16] : 1) "preserved expression": membranous immunostaining in more than 50% of the epithelial tumor cells; 2) "reduced expression": membranous immunostaining in 50% or less of the epithelial tumor cells. The expression of vimentin was classified as following (Wang et al.)[17]: 1) "negative": cytoplasmatic immunostaining in less than 10% of the epithelial tumor cells; 2) "positive": cytoplasmatic immunostaining in 10% or more of the epithelial tumor cells. This analysis was performed independently by two authors (LCMCC and MCRH). Doubtful cases were reanalyzed and a consensus score agreed.

Statistical analysis

Differences in the expression of E-cadherin and vimentin between the IF and the CSA of the OSCC were analyzed using the McNemar test. The Fischer exact test was used to assess differences in the expression of each protein at the IF between high- and low-invasive OSCC. The Fischer exact test was also used to evaluate association between E-cadherin and vimentin expression at the IF. The level of significance was set at 5% and statistical analysis was performed by GraphPad Prism (GraphPad Software, San Diego, California, USA).

RESULTS

The results of E-cadherin expression are illustrated in Table II and Fig. 1. Preserved E-cadherin expression at both CSA and IF of OSCC was observed in 5 (25%) samples. Preserved E-cadherin expression at CSA and reduced expression at IF was observed in 11 (55%) samples. Reduced E-cadherin expression at both CSA

and IF of OSCC was observed in 4 (20%) samples. No samples showed reduced E-cadherin expression at CSA and preserved expression at IF. The E-cadherin expression was reduced at the IF when compared to the CSA ($p<0.05$).

The results of vimentin expression are demonstrated in Table II and Fig. 2. Negative vimentin expression at both CSA and IF of OSCC was observed in 14 (70%) samples. Negative vimentin expression at CSA and positive expression at IF was observed in 3 (15%) samples. Positive vimentin expression at both CSA and IF of OSCC was observed in 3 (15%) samples. No samples showed positive vimentin expression at CSA and negative expression at IF. There was no significant difference in vimentin expression between the IF and the CSA ($p>0.05$).

Comparisons of the expression of each protein at the IF between histologically high- and low-invasive OSCC are illustrated in Table III. The E-cadherin expression was reduced at the IF of high-invasive OSCC when compared to low-invasive OSCC ($p<0.05$). Nevertheless, no difference in vimentin expression at the IF was observed between low- and high-invasive OSCC ($p>0.05$).

The evaluation of association between E-cadherin expression and vimentin expression at the IF of OSCC was illustrated by Table IV. No association was observed between E-cadherin and vimentin expression at the IF of OSCC ($p>0.05$).

DISCUSSION

EMT is an important biological event in which cohesive and polarized epithelial cells switch to mesenchymal-like cells showing no polarization and high mobility. This migratory phenotype is significant during tumor invasion and metastasis of epithelial malignant neoplasms.[7-11] The altered expression of EMT markers such as E-cadherin, N-cadherin, β-catenin and vimentin have been recently studied in several malignant epithelial tumors.[8,10] Since the IF has been identified as chiefly responsible for the clinical behavior of OSCC[5,6] the evaluation of the EMT markers as E-cadherin and vimentin at this tumor area is noteworthy.

This study showed a heterogeneous loss of E-cadherin expression in OSCC, since reduced expression of this protein was observed in 15 samples (75%). Reduced expression of E-cadherin has been previously reported in OSCC.[16-24] E-cadherin is the main adhesion molecule of epithelial tissue and is essential in the

establishment and maintenance of the epithelial structural integrity.[12,13] Several mechanisms for inactivating E-cadherin protein or E-cadherin gene *CDH1* have been described in human malignant tumors: hereditary and somatic mutations, promoter hypermethylation, abnormal protein processing, and induction of transcriptional repressors.[14] Since the loss of E-cadherin expression increases the mobility of epithelial cells and its ability for local invasion, it has been used as a marker of EMT during tumor invasion and metastasis.[8,10,13,14] The loss of E-cadherin expression in OSCC has been associated with lesser disease-free time,[18,19] higher prevalence of lymph node metastasis[18,20,23] and lesser survival time,[17,18,19,21,22] even though no association with prognosis has also been reported.[16,24] Furthermore, the concomitant loss of E-cadherin and gain of N-cadherin (cadherin switching) has been recently reported in malignant epithelial tumors, including OSCC.[20] This phenomena, that increases the mobility of neoplastic epithelial cells and their capability to local invasion and mestastasis, has also been used as an indicator of the occurrence of EMT.[10,20]

The E-cadherin expression was reduced at the IF when compared to the CSA of the tumor ($p<0.05$). Similar results have been previously reported.[17,20,21] These findings advocate that E-cadherin loss occur mainly at the IF, suggesting that tumor cells at this important area could more easily detach and promote local invasion and metastasis.

The E-cadherin expression was reduced at the IF of histologically high-invasive OSCC when compared to low-invasive OSCC ($p<0.05$). This result suggests that the loss of E-cadherin expression at the IF of OSCC is associated with a higher histologically dissociation pattern of the tumor cells. It is important to highlight that the histological pattern of invasion, used to categorize the tumors as low- or high-invasive, is an important histological parameter that reflects the invasive features of the tumor[5,6] and has been pointed as a prognostic marker in OSCC.[6] Diniz-Freitas et al.[19] demonstrated that weak or absent E-cadherin expression in OSCC was associated with a more invasive histological pattern. Moreover, Wang et al.[21] reported that OSCC with loss of E-cadherin expression at the IF showed higher IF histological grading scores. In contrast, Mahomed et al.[16] observed no association between E-cadherin expression at the IF and IF grading score of OSCC.

A heterogeneous vimentin expression in OSCC was observed in the present study, since positive expression of this EMT marker was observed in 6 samples

(30%). Analogous results have been previously reported in OSCC.[17,22] Vimentin is an intermediate filament protein normally expressed by mesenchymal cells.[12,15] The expression of vimentin by epithelial malignant tumors cells increases its migratory and invasive capability and has been identified as a marker of EMT.[10,15] In vitro studies have shown that inhibition of vimentin expression results in reversal of the mesenchymal phenotype and reduction of migratory and invasive capability of OSCC cell lines.[25] In fact, vimentin expression have been associated with higher prevalence of lymph node metastasis in esophageal[26] as well as in head and neck squamous cell carcinoma.[27] Moreover, Liu et al.[22] reported a higher prevalence of recurrences and a lesser survival time in OSCC showing positive vimentin expression.

No significant difference in vimentin expression between the IF and the CSA was observed ($p>0.05$), even though negative vimentin expression at CSA and positive expression at IF was observed in 3 (15%) samples and no samples showed positive vimentin expression at CSA and negative expression at IF. This finding suggests that the expression of this EMT marker in OSCC is not limited to the IF cells. In contrast, Wang et al.[17] reported increased expression of vimentin at the IF of OSCC, when compared to CSA of the tumor.

No difference in vimentin expression at the IF was observed between low- and high-invasive OSCC ($p>0.05$). Liu et al.[22] also reported no association among vimentin expression at the IF and the histological pattern of invasion of OSCC. These findings suggest that vimentin expression is not related to the histological invasiveness of the OSCC. Nevertheless, association between higher histological invasiveness and positive vimentin expression was observed in head and neck squamous cell carcinoma.[27]

Finally, no association was observed between E-cadherin expression and vimentin expression at the IF of OSCC ($p>0.05$). This result suggests that the expression of different EMT markers in OSCC should be independent events, not necessarily occurring simultaneously. However, Liu et al.[22] reported correlation between loss of E-cadherin expression and gain of vimentin expression at the IF in OSCC.

In conclusion, a heterogeneous E-cadherin and vimentin expression was observed in OSCC, suggesting that these proteins are significant EMT markers in this tumor. E-cadherin expression was reduced at the IF when compared to the CSA as well as was also reduced at the IF of high-invasive when compared to low-

invasive OSCC. These findings reinforce the relevance of E-cadherin loss as a marker of EMT during the OSCC progression, since the IF has been identified as chiefly responsible for its behavior. No difference in vimentin expression was observed between the IF and the CSA. Moreover, no difference in vimentin expression at the IF was observed between low- and high-invasive OSCC. These findings suggest that, even though vimentin is a marker of EMT in OSCC, changes in vimentin expression are neither limited to the IF nor related to its histological invasiveness.

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Table I. Applied primary antibodies, antigen retrieval protocols and stained conditions

Specification	Clone	Source	Dilution	Incubation time	Antigen retrieval
E-cadherin	36B5	Novocastra	1:50	Overnight (18h)	Citrate pH 6.0, 98℃, 30 min
Vimentin	SRL33	Novocastra	1:200	Overnight (18h)	Tris/EDTA pH 9.0, 98℃, 30 min

Table II. E-cadherin and vimentin expression at the invasive front (IF) and at the central/superficial areas (CSA) of OSCC

E-cadherin expression	**n**	***p-value****
Preserved at CSA / Preserved at IF	5 (25%)	<0.05
Preserved at CSA / Reduced at IF	11 (55%)	
Reduced at CSA / Preserved at IF	0 (0%)	
Reduced at CSA / Reduced at IF	4 (20%)	
Total	20 (100%)	
Vimentin expression	**n**	***p-value****
Negative at CSA / Negative at IF	14 (70%)	n.s.
Negative at CSA / Positive at IF	3 (15%)	
Positive at CSA / Negative at IF	0 (0%)	
Positive at CSA / Positive at IF	3 (15%)	
Total	20 (100%)	

* *p*-values were obtained by the McNemar test.

n.s. - not significant.

Table III. E-cadherin and vimentin expression at the invasive front (IF) of low- and high-invasive OSCC

	Low-invasive OSCC	High-invasive OSCC	*p*-value *
E-cadherin			
Preserved	4 (20%)	1 (5%)	<0.05
Reduced	3 (15%)	12 (60%)	
Vimentin			
Negative	6 (30%)	8 (40%)	n.s.
Positive	1 (5%)	5 (25%)	

* *p*-value was obtained by the Fischer Exact test.

n.s. - not significant.

Table IV. E-cadherin and vimentin expression at the invasive front (IF) of OSCC

	Vimentin (negative)	Vimentin (positive)	*p*-value*
E-cadherin (Preserved)	5 (25%)	0 (0%)	n.s.
E-cadherin (Reduced)	9 (45%)	6 (30%)	

* *p*-value was obtained by the Fischer Exact test.

n.s. - not significant.

Fig. 1. Immunohistochemical reactivity for E-cadherin in oral squamous cell carcinoma (OSCC). **A and B,** OSCC showing preserved E-cadherin expression at the morphologically normal oral mucosa epithelium adjacent to tumor (internal positive control), as well as at CSA and at the IF (A, X40; B, X200). **C,** OSCC displaying preserved E-cadherin expression at the CSA (X400). **D,** OSCC exhibiting reduced E-cadherin expression at the IF (X400). **E, F, G and H,** OSCC presenting preserved E-cadherin expression at the morphologically normal oral mucosa epithelium adjacent to tumor (E, X40), preserved E-cadherin expression at the CSA (E, X40; F, X200) and reduced E-cadherin expression at the IF (E, X40; G, X200; H, X400).

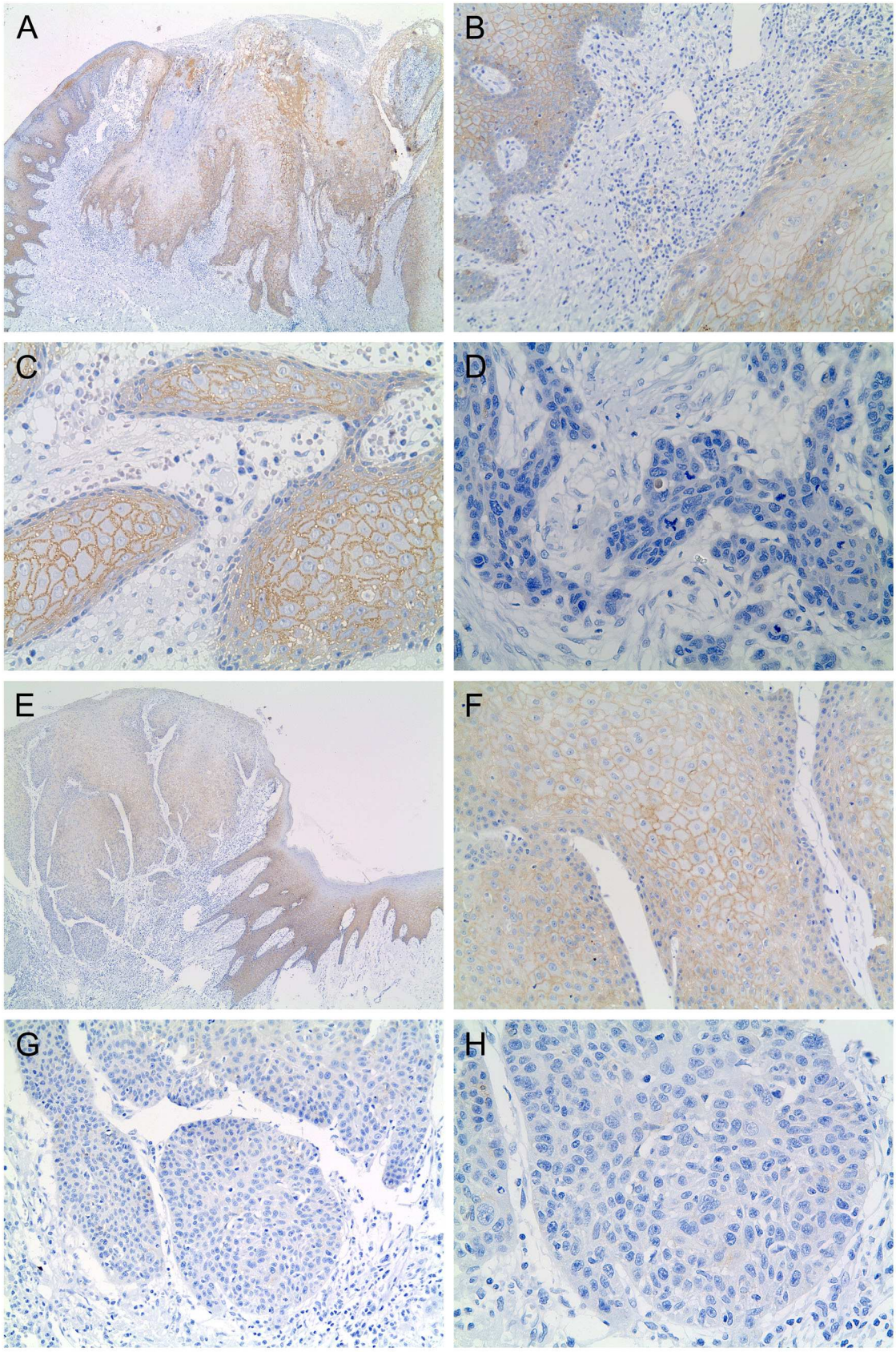
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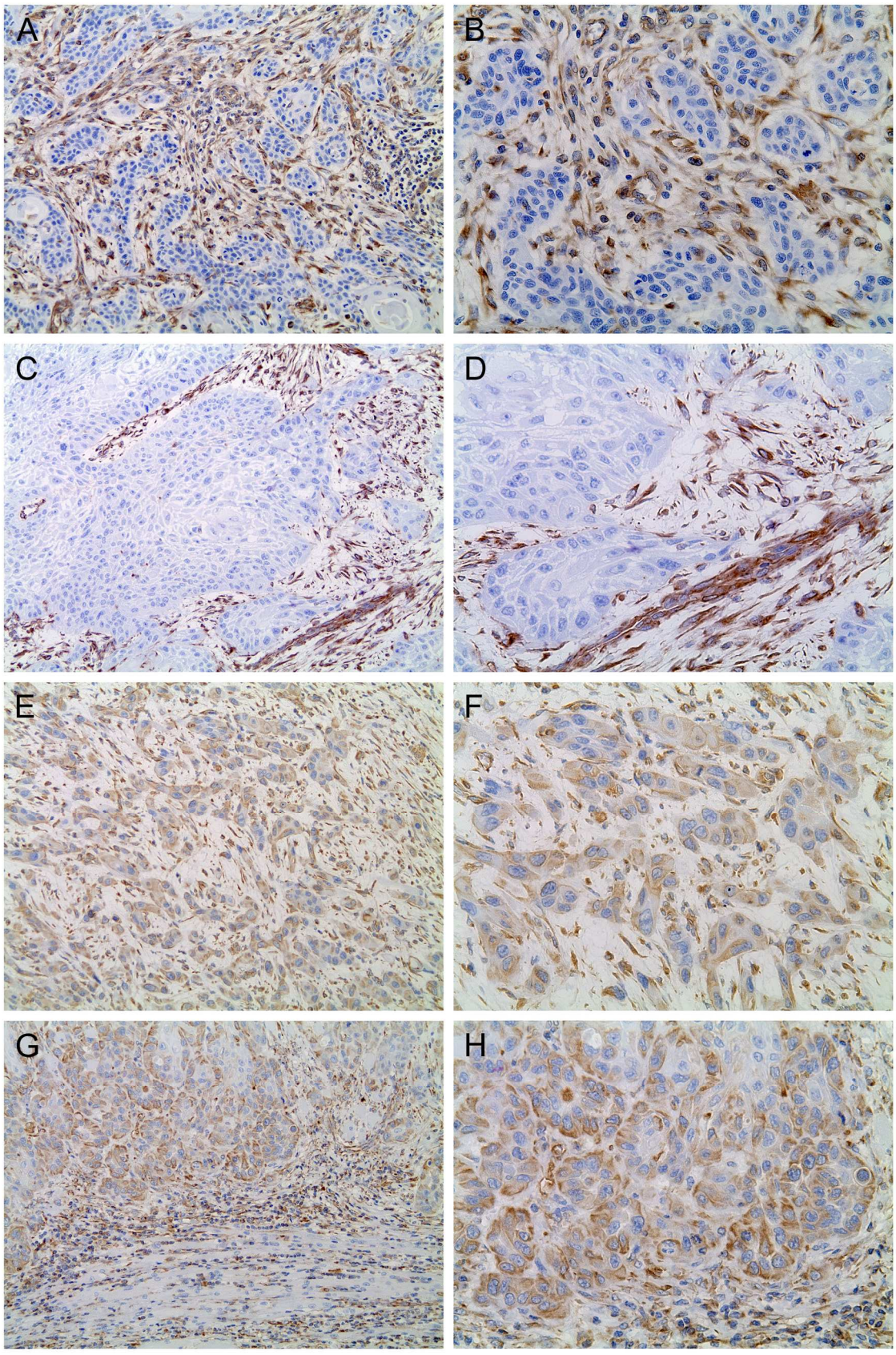
Fig. 2. Immunohistochemical reactivity for vimentin in oral squamous cell carcinoma (OSCC). Immunostaining of tumor's connective tissue mesenchymal cells were used as internal positive control. **A and B,** OSCC showing negative vimentin expression (A, X200; B, X400). **C and D,** OSCC exhibiting negative vimentin expression (C, X200; D, X400). **E and F,** OSCC displaying positive vimentin expression at the IF (E, X200; F, X400). **G and H,** OSCC presenting positive vimentin expression at the IF (G, X200; H, X400).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ANEXO - Carta de aprovação do Comitê de Ética em Pesquisa

Pontifícia Universidade Católica de Minas Gerais
Pró-Reitoria de Pesquisa e de Pós-Graduação
Comitê de Ética em Pesquisa

Belo Horizonte, 20 de outubro de 2010.

De: Profa. Maria Beatriz Rios Ricci
Coordenadora do Comitê de Ética em Pesquisa

Para: Martinho Campolina Rebello Horta
Departamento de Odontologia

Prezado (a) pesquisador (a),

O Projeto de Pesquisa CAAE – 0174.0.213.000-10 "*Avaliação imunoistoquímica de marcadores da transição epitélio-mesênquima no fronte de invasão do carcinoma de células escamosas de boca* " foi **aprovado** pelo Comitê de Ética em Pesquisa da PUC Minas.

Atenciosamente,

Profa. Maria Beatriz Rios Ricci
Coordenadora do Comitê de Ética em Pesquisa – PUC Minas