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EXPRESSÃO IMUNOISTOQUÍMICA DAS ENZIMAS DNA METILTRANSFERASES 1, 3a E 3b EM LEUCOPLASIAS E CARCINOMAS DE CÉLULAS ESCAMOSAS BUCAIS

FILIPE IVAN DANIEL

PORTO ALEGRE
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Tese apresentada à Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul, como parte dos requisitos para a obtenção do título de Doutor em Odontologia, área de concentração em Estomatologia Clínica.

Orientadora: Prof[a]. Dra. Fernanda Gonçalves Salum

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Aprovada em 13 de novembro de 2009.

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Dedico esta Tese à Inah, minha esposa, que através de seu

amor incondicional, uniu seus sonhos aos meus

e me apoiou nesta longa caminhada.

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"Senhor, dê-me serenidade para aceitar as coisas que não posso mudar,

coragem para mudar as coisas que posso e sabedoria para distinguir

umas das outras."

(Oração da Serenidade)

RESUMO

Objetivos: Neste estudo foi investigada a expressão das enzimas DNA metiltransferases (DNMTs) 1, 3a e 3b em carcinomas escamocelulares (CEC) e leucoplasias bucais, relacionando-a com a graduação histopatológica e presença de displasia epitelial, respectivamente, bem como com os parâmetros clínicos dos pacientes.

Metodologia: A técnica de imunoistoquímica utilizando anticorpos anti-DNMT1, anti-DNMT3a e anti-DNMT3b (diluição de 1:700) foi realizada para detectar a expressão das três DNMTs em 21 amostras de leucoplasias com diagnóstico histopatológico de acantose e/ou hiperceratose (leucoplasia sem displasia), 16 leucoplasias com diagnóstico histopatológico de displasia epitelial, 20 CEC bem diferenciados (grau I), 20 CEC moderadamente diferenciados (grau II) e 20 CEC pobremente diferenciados (grau III). Vinte amostras de tecidos orais não tumorais foram utilizadas como controles. O material, incluído em parafina, foi obtido do arquivo do Laboratório de Patologia do Serviço de Estomatologia e Prevenção do Câncer Bucomaxilofacial do Hospital São Lucas da PUCRS. A análise estatística foi realizada por meio da Análise de Variância (ANOVA), teste Student-Newman-Keuls, correlação de Pearson e teste t.

Resultados: A incidência da imunorreatividade nuclear para DNMT3a nos grupos de CEC (39,9%) foi significativamente superior a do grupo-controle (22,6%) ($p<0,05$), mas não diferiu dos grupos de leucoplasias (28,2%). Para a DNMT1 e DNMT3b não houve diferença estatisticamente significativa entre os grupos de CEC (65% e 74,7%), de leucoplasias (68,3% e 70,9%) e controle (65,4% e 76,5%). A expressão imunoistoquímica das enzimas DNMTs não exibiu correlação com a idade dos pacientes nem associação com gênero e consumo de chimarrão. Houve uma significativa associação entre DNMT3a e uso de álcool ($p = 0,01$) e uma associação inversa entre DNMT1 e tabagismo ($p = 0,048$).

Conclusões: Apesar do aumento do nível de imunorreatividade à enzima DNMT3a nos grupos de CEC, as três enzimas estudadas não possuem capacidade preditora de susceptibilidade ao CEC em pacientes com leucoplasia bucal, nem exibem associação com as características histopatológicas ou com os parâmetros clínicos idade, gênero e uso de chimarrão. O consumo de bebidas alcoólicas foi associado com uma maior expressão de DNMT3a e o uso de cigarro com uma menor expressão de DNMT1.

DESCRITORES[1]:

Metilação de DNA, Metiltransfease, Câncer bucal, Carcinoma de células escamosas, leucoplasia, imunoistoquímica.

[1] DeCS – Descritores em Ciências da Saúde, disponível em http://decs.bvs.br

ABSTRACT

Objectives: This study investigated the expression of DNA methyltransferase (DNMT) 1, 3a, and 3b enzymes in oral squamous cell carcinoma (SCC) and leukoplakia, their relationship with histopathologic graduation and dysplasia, respectively, and with clinical parameters.

Study design: Immunohistochemistry using antibodies anti-DNMT1, anti-DNMT3a, and anti-DNMT3b (dilution of 1:700, Imgenex, San Diego, USA) was carried out to detect the expression of the 3 DNMTs proteins in 21 oral leukoplakias with histopathologic diagnosis of acanthosis and/or hyperkeratosis (leukoplakia without dysplasia), 16 leukoplakias with histopathologic diagnosis of epithelial dysplasia, 20 oral well differentiated SCC (grade I), 20 oral moderately differentiated SCC (grade II), and 20 oral poorly differentiated SCC (grade III). Twenty samples of non-tumor oral tissues were obtained as control. The tissues were embedded in paraffin and obtained from the pathologic archive of Division of Stomatology and Prevention of Bucomaxillofacial Cancer, São Lucas Hospital, PUCRS. Statistical analysis was done using Analysis of Variance (ANOVA), Student-Newmann-Keuls test, Pearson correlation and t test.

Results: The incidence of nuclear DNMT3a immunoreactivity in oral SCC groups (39.9%) was significantly higher than in control (22.6%) ($P<0.05$), but not when compared to oral leukoplakias groups (28.2%). For DNMT1 and DNMT3b, there were no statistically significant difference between oral SCC groups (65% and 74.7%), oral leukoplakia groups (68.3% and 70.9%) and control (65.4% and 76.5%). DNMT protein expression exhibited no correlation with age and no association with gender and mate. There were a significant association between the DNMT3a and alcohol use ($P = 0.01$), and an inverse association between DNMT1 and smoking ($P = 0.048$).

Conclusions: Although there was a higher level of DNMT3a immunopositivity in SCC groups, the three studied enzymes had no predictive capacity for SCC development in patients with oral leukoplakia, and exhibited no association with their histopathological features or the clinical parameters age, gender and mate consumption. Alcohol intake was associated with a higher DNMT3a expression and smoking with a lower DNMT1 expression.

DESCRIPTORS[2]

DNA methyltransferase, DNMT, oral cancer, squamous cell carcinoma, oral leukoplakia, immunohistochemistry.

[2] MeSH – Medical Subject Headings, available at: www.nlm.nih.gov/mesh

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LISTA DE ABREVIATURAS

AdoMet	*S*-adenosyl-L-methionine
ANOVA	Analise de variância / Analysis of variance
APC	Adenomatosis polyposis coli gene
C	Citosina / Cytosine
CEC	Carcinoma de células escamosas
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CpG	Dinucleotídeo Citosina-Guanina / Cytosine-Guanine dinucleotide
DAP-K	Death-associated protein kinase
DNA	Ácido desoxirribonucléico / deoxyribonucleic acid
DNMT	DNA metiltransferase / DNA methyltransferase
EDTA	Ethylenediamine tetraacetic acid
FHIT	Fragile histidine triad gene
G	Guanina / Guanine
H_2O_2	Hydrogen peroxide
HAC	Histone acetylase
HDAC	Histone deacetylase
HE	Hematoxilina e eosina
IHC	Immunohistochemistry
K-ras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of heterozygosity
MBD	Methyl-CpG-binding domain
MeCP2	Methyl-CpG-binding protein 2
MGMT	Methylguanine-DNA methyltransferase
MLH1	DNA mismatch repair protein Mlh1
mRNA	Ácido ribonucleic mensageiro / Messenger ribonucleic acid
NA	Not available
NBH	Northem blot hybridization
^oC	Graus Celsius
p	Probabilidade / probability
p15	Cyclin-dependent kinase inhibitor 2B gene
p16	Cyclin-dependent kinase inhibitor 2A gene
p21	cyclin-dependent kinase inhibitor 1A gene

p53	Tumor protein p53
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Reação em cadeia da polymerase
pH	Potential of hydrogen
PUCRS	Pontifícia Universidade Católica do Rio Grande do Sul
qRT-PCR	Real-time polymerase chain reaction
r	Coeficiente de Correlação de Pearson
RAR	Retinoic acid receptor
RASSF1	Ras association domain family member 1 gene
Rb	Retinoblastoma gene
RB1	Retinoblastoma 1 gene
SCC	Squamous cell carcinoma
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
TpG	Timine-guanine dinucleotide
TRD	Transcriptional repression domain
W	Watts
µm	Micrometer

SUMÁRIO

Introdução

1 INTRODUÇÃO

O carcinoma de células escamosas ou carcinoma escamocelular (CEC) é a neoplasia maligna mais comum da cavidade bucal, representando 90% a 95% de todos os cânceres dessa região[1,2], com cerca de 14 mil novos casos no Brasil em 2008.[3] Como toda neoplasia maligna, o CEC bucal é uma doença genética com modificações moleculares complexas, mais especificamente nos genes que regulam a proliferação celular, impedindo que os controles normais de crescimento sejam eficientes.[4,5]

Os fatores de risco mais comumente associados a essa neoplasia são o consumo crônico de cigarro e álcool, sendo este risco proporcional à quantidade e ao tempo de uso desses agentes.[4,6,7] A interação sinérgica entre eles está comprovadamente envolvida na etiologia do CEC bucal e na evolução das lesões cancerizáveis[6,8].

As lesões precursoras de câncer ou potencialmente malignas possuem algumas das características moleculares e fenotípicas similares às células neoplásicas, apresentando risco aumentado de transformação em câncer.[9] A leucoplasia é o exemplo mais comum e, ainda que não necessariamente evolua para o carcinoma, pode apresentar uma ou mais modificações genéticas que se traduzem em diferentes graus de displasia epitelial. Entretanto, mesmo na ausência de displasia, elas podem já possuir características genéticas em comum às do CEC bucal[4,7,10] e nestes casos a habilidade de identificar o risco de transformação maligna é limitada.[4] Para Reibel (2003)[11], as características clínicas e histopatológicas (presença ou ausência de displasia epitelial) ainda são os parâmetros mais importantes para a predição do desenvolvimento do CEC em tais lesões.

As estratégias terapêuticas do câncer bucal visam a sua cura, com a preservação e restauração das funções dos órgãos envolvidos, minimizando as seqüelas da doença e/ou tratamento. As modalidades atualmente disponíveis envolvem cirurgia, radioterapia e quimioterapia.[12] A morbidade resultante de tratamentos agressivos, necessários para aumentar os índices de cura, afeta a qualidade de vida da maioria dos indivíduos que sobrevivem. A ineficácia de muitos

tratamentos e a alta taxa de mortalidade do câncer de boca são atribuídos ao fato de grande parte das lesões serem diagnosticadas em estágio avançado.[7]

Os principais fatores prognósticos do câncer bucal ainda são o estadiamento clínico do tumor, a sua localização e a graduação histopatológica, embora, muitas vezes, estes fatores falhem na sua função preditiva. Diversas moléculas relacionadas à carcinogênese do CEC bucal, como aquelas envolvidas na regulação do ciclo celular (p53, p21, ciclina D1), apoptose (Bcl-2, Bax), angiogênese (Fator de crescimento endotelial vascular) e metástase (E-caderina, laminina, colágeno tipo IV) têm sido estudadas com o intuito encontrar um marcador biológico que possa auxiliar no diagnóstico precoce, no seguimento clínico dos pacientes e na escolha das medidas terapêuticas.[13-15]

Um melhor entendimento das alterações moleculares que ocorrem desde os estágios iniciais da carcinogênese bucal, da displasia epitelial, carcinoma *in situ* até carcinoma invasivo, pode aumentar as possibilidades de se detectar o potencial de transformação maligna das lesões cancerizáveis, bem como diagnosticar precocemente o câncer. A descoberta de marcadores moleculares para diferenciar as leucoplasias que apresentem maior propensão a sofrer transformação maligna poderá auxiliar o profissional na conduta terapêutica frente a lesões precursoras.[11] Entretanto, as evidências científicas disponíveis sobre esses marcadores permanecem inconclusivas.[8]

O desenvolvimento do câncer envolve, basicamente, o acúmulo de mutações gênicas com perda do controle do crescimento celular.[16,17] Duas classes de genes que sofrem mutações estão envolvidas no câncer: os proto-oncogenes e os genes supressores tumorais.[5] Os proto-oncogenes estão diretamente relacionados com a regulação do crescimento celular e, quando alterados por mutações, translocações e amplificações, são chamados de oncogenes, pois promovem a proliferação das células independente de qualquer controle.[16,18] Já os genes supressores tumorais codificam proteínas que de alguma forma impedem ou reduzem o crescimento celular. A sua inativação contribui para o desenvolvimento do câncer, por resultar na diminuição desses inibidores do crescimento.[5,6]

A oncogênese não é resultado unicamente de mutações gênicas. As alterações conhecidas como epigenéticas são aquelas que modificam os padrões de expressão gênica sem alterar a sequência de nucleotídeos do DNA.[19] Enquanto a

informação genética fornece a sequência de aminoácidos para a síntese protéica, a epigenética informa quando, como e onde estas informações genéticas devem ser utilizadas.[20] A alteração epigenética mais encontrada nas células de mamíferos é a metilação de DNA, ou seja, a adição covalente de um radical metil para o carbono 5 do nucleotídeo citosina[20,21], além de modificações das histonas[22], que são proteínas nucleares responsáveis pela condensação da cromatina.[23] Essas alterações podem ser transmitidas por meio da divisão celular às células filhas e contribuir para a inativação de genes supressores tumorais no câncer.[24,25]

A metilação de genes resulta no seu silenciamento, enquanto a não metilação permite a sua transcrição. Após a metilação, o produto do gene não é produzido, embora a seqüência do DNA seja mantida.[19,22,26] Quando isto ocorre em um gene supressor tumoral a célula perde a propriedade de transcrição de substâncias importantes para o controle do crescimento celular.[20]

A metilação ocorre nas ilhas CpG, que são sítios do DNA ricos em nucleotídeos Citosina (C) e Guanina (G), localizados dentro de regiões promotoras de quase metade de todos os genes dos mamíferos, que geralmente não estão metiladas. A metilação dessas regiões está associada com a perda de função do gene, por meio do bloqueio da transcrição, o que pode levar a uma proliferação celular descontrolada quando esta inativação ocorre em genes supressores tumorais.[22,27] Essas modificações têm sido encontradas durante os estágios iniciais da oncogênese.[22,28]

Neoplasias podem exibir dois tipos de defeitos no padrão de metilação: hipometilação global, que ocorre em regiões não reguladoras de genes, capaz de causar instabilidade/alterações estruturais nos cromossomos[27,29] e hipermetilação de regiões promotoras ou reguladoras da expressão gênica, bloqueando a transcrição de genes supressores tumorais.[28,30]

A metilação é um processo realizado por três enzimas denominadas DNA metiltransferases (DNMTs): DNMT1, DNMT3a e DNMT3b.[27,31,32] A primeira delas, DNMT1, é responsável por manter o padrão de metilação após cada ciclo de replicação do DNA, utilizando o padrão presente na fita-mãe. Após cada divisão celular, apenas uma das fitas de DNA possui radicais metil, que é a fita proveniente da célula-mãe. A enzima DNMT1, por sua vez, realiza a metilação da segunda fita, exatamente nas regiões em que o radical metil encontra-se na fita-mãe. As DNMT3a

e DNMT3b, por outro lado, como não são capazes de diferenciar entre nucleotídeos hemimetilados e não metilados, são consideradas responsáveis pela adição de grupos metil ao DNA não previamente metilado, processo denominado metilação *de novo.*[28,33,34] Estas últimas são altamente expressas em células embrionárias e neoplásicas, e pouco expressas após a diferenciação celular e em células somáticas adultas.[24,35-37] Embora as DNMT1 e DNMT3a/DNMT3b sejam consideradas responsáveis pelas metilações de manutenção e *de novo*, respectivamente, é provável que todas as três possuam ambas as funções durante a carcinogênese.[20] A expressão aumentada das enzimas DNMTs leva a um padrão de metilação descontrolado no genoma e em muitos genes supressores de tumor.[28]

O padrão de metilação das células tumorais vem sendo estudado como marcador tanto para o diagnóstico precoce e determinação do prognóstico, quanto para avaliação da resposta à terapia antineoplásica.[19] Por se tratar de uma alteração reversível, certas drogas inibidoras de DNA metiltransferase como a decitabina (5-aza-2'-deoxicitidina) têm sido testadas em linhagens celulares neoplásicas, mostrando efeito antiproliferativo[38] e radiossensibilizador.[39] Esta droga é capaz de reverter o estado de metilação, permitindo a re-expressão dos genes silenciados.[40]

O papel da metilação do DNA e a expressão das enzimas DNMTs são estudados tanto em tecidos tumorais quanto nas lesões precursoras de câncer de bexiga, colorretal, rins, fígado, pâncreas e colo de útero.[28,32,36,41,42]

Robertson et al. (1999)[36] investigaram o padrão de expressão de mRNA de DNMT1, DNMT3a e DNMT3b em tecidos tumorais de bexiga, cólon, rim e pâncreas, bem como em tecidos normais adjacentes a essas lesões. Foi observado significativo aumento da expressão de DNMT3b nos tumores, enquanto DNMT1 e DNMT3a demonstraram uma moderada elevação da expressão e com uma freqüência menor.

A expressão imunoistoquímica das enzimas DNMT1 e DNMT3a foi investigada por Choi et al. (2003)[41] em tecido hepático normal, nódulos cirróticos, nódulos hepáticos displásicos e em hepatocarcinomas iniciais e avançados de 59 pacientes. Os autores verificaram que 71,2% dos tecidos normais não apresentavam imunorreatividade para DNMT1. Em relação à DNMT3a, nenhuma imunorreatividade nuclear foi detectada nos casos de tecido normal ou com displasia leve. A positividade para ambas as enzimas foi progressivamente aumentando quando os

tecidos pré-neoplásicos e neoplásicos foram avaliados. Os dados sugerem que a expressão da DNMT1 e DNMT3a tem importante papel nos estágios precoces da hepatocarcinogênese.

Em 48 pacientes com carcinoma colorretal, Zhu et al. (2007)[28] verificaram que a porcentagem de células mostrando imunorreatividade para DNMT1 nos tumores (75,3%) foi significativamente maior do que no tecido normal (39%).

Sawada et al. (2007)[42] investigaram, por imunoistoquímica, a expressão de DNMT1 em 30 amostras de CEC cervical, 97 de lesões precursoras e 34 de epitélio normal, provenientes de 49 pacientes. A imunorreatividade foi significativamente mais elevada nos tumores, quando comparada com o tecido histologicamente normal. A expressividade da enzima foi superior nas lesões precursoras classificadas como de alto grau e nos carcinomas micro-invasivos, reduzindo nos carcinomas invasivos, sugerindo que o aumento da expressão da DNMT1 foi um evento precoce da oncogênese.

A literatura científica a respeito de pesquisas envolvendo a expressão dessas três enzimas no câncer de boca e em suas lesões precursoras é ainda escassa. O objetivo desta tese foi estudar e comparar a expressão imunoistoquímica das três enzimas DNA metiltransferases (DNMT1, DNMT3a e DNMT3b) em leucoplasias e CEC bucais, relacionando-a com a presença de displasia epitelial e diferenciação histopatológica, respectivamente, bem como com os parâmetros clínicos idade, gênero e hábitos de tabagismo, alcoolismo e consumo de chimarrão dos pacientes envolvidos.

Artigo de Revisão

2 ARTIGO DE REVISÃO

THE ROLE OF EPIGENETIC TRANSCRIPTION REPRESSION AND DNA METHYLTRANSFERASES IN CANCER

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ABSTRACT

Epigenetic alterations such as DNA methylation have been implicated in the development and progression of various cancers. DNA methylation consists of the reversible addition of a methyl group to the carbon 5 position of cytosine in CpG dinucleotides, and is considered essential for normal embryonic development. However, global genomic hypomethylation and aberrant hypermethylation of regulatory regions of tumor suppressor genes have been associated with chromosomal instability and transcription repression, respectively, providing neoplastic cells with a selective advantage. DNA methyltransferases are the enzymes responsible for the addition of methyl groups to CpG dinucleotides, which together with histone modifiers, initiate the events necessary for transcription repression to occur. It has been demonstrated that increased expression of DNA methyltransferases may contribute to tumor progression through methylation-mediated gene inactivation in various human cancers. Given their importance, this article reviews the main epigenetic mechanisms for regulating transcription and its implications in cancer development.

KEYWORDS

Epigenesis, DNA methylation, Methyltransferases, Histone code.

INTRODUCTION

Heritable and reversible mechanisms known as epigenetic alterations do not require direct alterations of DNA sequences, but they can be responsible for modifying gene expression and are related to cancer development. [1-5] While the genetic information provides the sequence for protein synthesis, the epigenetic information provides instructions on how, where, and when the genetic information will be used.[6]

Several epigenetic mechanisms regulate gene expression: DNA methylation, modifications of histone proteins and functional noncoding RNAs.[1] The major form present in mammalian cells is DNA methylation, which is the covalent addition of a methyl group to the carbon 5 position of cytosine predominantly in the CpG dinucleotide.[6,7] This cytosine modification pattern can be transmitted through cell division and may contribute to gene inactivation in cancer.[3,4]

DNA methylation is essential for normal embryonic development and has a variety of important functions, such as the regulation of gene expression, control of cell differentiation and development, chromatin modification, mutation accumulation, silencing of endogenous retroviruses, preservation of chromosomal integrity, genomic imprinting control, and X chromosome inactivation.[6,8-12] Initially discovered as a mechanism for control of development, it plays an important role in many tumor types.[13] Genomic methylation patterns are frequently altered in tumor cells with global hypomethylation accompanying region-specific hypermethylation sites. When hypermethylation occurs within the promoter of a tumor suppressor gene, it can silence expression of the associated gene and provide the cell with a growth advantage in a manner akin to deletions or mutations.[6,14,15]

This review summarizes the main epigenetic mechanisms involved in cancer development, with special focus on DNA methyltransferase enzymes and hypermethylation of tumor suppressor genes.

METHYLATION PROCESS AND CANCER

In mammalian cells, the DNA targets for modification through methylation are cytosine bases adjacent to guanine bases (CpG dinucleotides).[16,17] Sequences of CpG, when found at a high frequency in the genome, are referred to as CpG islands.[18] Most of the 29,000 CpG islands found in the human genome are in the promoter regions of almost half of the genes and are generally unmethylated in normal cells.[3,15,19]

The modification of cytosine is catalyzed by the enzymes DNA methyltransferases (DNMTs) using *S*-adenosyl-L-methionine (AdoMet) as the methyl donor (figure 1).[8,12,18] The methyl group of AdoMet is bound to a sulfonium ion which thermodynamically destabilizes the molecule and makes the relatively inert methylthiol of the methionine moiety very reactive toward activated carbon atoms[8]. The reaction involves DNMT-DNA binding, flipping the target cytosine out of the double helix, and formation of a transient covalent complex with the cytosine residue.[17] DNMT adds a cysteine thiolate to the 6-carbon of the substrate cytosine followed by transfer of the methyl group to the 5-carbon.[20]

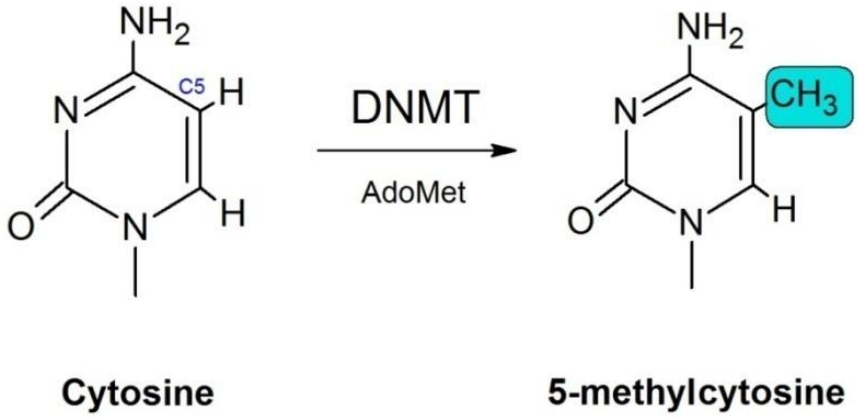

Figure 1: Structures of cytosine before and after the transfer of a methyl group from the cofactor AdoMet, catalyzed by DNA methyltransferases.

The distribution of methylated and non-methylated CpG dinucleotides is not random, but rather conforms to a pattern.[16] Certain genomic sites, such as pericentromeric regions, imprinted regions, and genes on the inactive X chromosome in females, are hypermethylated while other sites, such as CpG islands which are often associated with gene promoter regions, are hypomethylated.[6,12,21]

Cancers exhibit at least two types of methylation defects: hypomethylation characterized by a global loss of methylation and hypermethylation of CpG islands of regulatory regions of tumor suppressor genes.[22,23]

Hypomethylation of non-promoter regions of DNA (known as global hypomethylation) may cause genomic instability and structural changes in chromosomes in cancer, although the relationship between the two processes is not clear.[15,24] Two resulting effects of losses of methylation in tumorigenesis have been proposed. First, weakening of transcriptional repression in normally silent regions of the genome could cause the potentially harmful expression of inserted viral genes and of normally silenced genes, such as imprinted genes and genes on the inactive X chromosome. Second, losses of methylation of nuclear structures other than genes could affect the functional stability of chromosomes, such as pericentromeric regions.[25]

Methylation of CpG islands in gene promoter regions may be involved in carcinogenesis as a result of three possible mechanisms: cytosine methylation facilitates gene mutation as 5-methylcytosine is deaminated to thymine,[26,27] aberrant DNA methylation may be associated with allelic loss,[6,28] and tumor suppressor genes may be inactivated by DNA hypermethylation.[3,29]

According to the Knudson two-hit hypothesis, promoter methylation may occur on a gene acting on the wild-type allele, while the other is mutated, contributing to the biallelic inactivation of tumor suppressor genes, either as a primary or second hit in both familial and sporadic forms of cancer.[25,30] In this case, genetic and epigenetic changes can collaborate to prevent the expression of a functional gene product in cancer cells.[31] Otherwise, hypermethylation of both alleles may also be present in some cases (figure 2).[25]

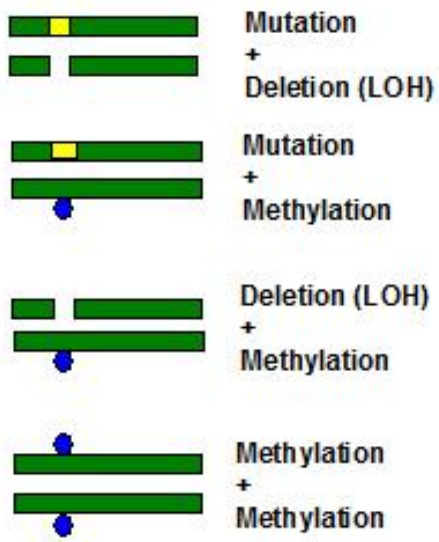

Fig. 2: Genetic and epigenetic events that can block the expression of tumor suppressor genes in cancer. When only one allele is hit either by genetic or epigenetic alteration, the other can still express the protein that controls cell growth (first hit). However, if the other allele is inactivated (second hit), gene expression will be blocked, contributing to the development of cancer. LOH: Loss of heterozygosity.

It is important to note that only methylation within or around the promoter region is associated with gene silencing. Dense methylation within the body of a gene, even within CpG islands, does not hinder transcription.[25,32] Many tumor suppressor genes associated with mutations in cancer have been found with promoter hypermethylation (table 1).

It has been recognized that aberrant hypermethylation events can occur early in tumorigenesis, predisposing cells to malignant transformation.[3,6,13] Renal tumors were demonstrated to have their average number of methylated CpG islands increase significantly and progressively from precancerous conditions to invasive tumors,[11] and thus, precursor lesions of oral,[33] liver,[34] and uterine cervix[35] cancers have been the focus of studies. Moreover, methylation of genes such as APC in the formation of intestinal polyps, H19 in preneoplastic kidney parenchyma of Wilms tumor patients and RB1 in familial cases of unilateral retinoblastoma is almost certainly implicated in the earliest stages of tumorigenesis.[3]

Table 1: Most studied hypermethylated promoters of genes implicated in carcinogenesis

Gene	Tissue	Reference
p15 (cell cycle regulator)	Oral cancer Hepatic cancer	Ogi et al.[36] Oh et al.[37]
p16 (cell proliferation inhibitor)	Oral cancer Saliva of oral cancer patients Saliva of leukoplakia patients Head and neck cancer Hepatic cancer Colorectal cancer Renal cancer Lung cancer	Kulkarni, Saranath.[38]; Shaw et al.[39]; Ogi et al.[36] Rosas et al.[40] López et al.[33] Sanchez-Cespedes et al.[41]; Maruya et al.[42] Oh et al.[37] Eads et al.[43]; Arai et al.[11] Lin et al.[44]
RASSF1 (cell cycle regulator)	Nasopharyngeal cancer Hepatic cancer Bladder cancer	Fendri et al.[45] Oh et al.[37] Friedrich et al.[46]; Abbosh et al.[47]
MLH1 (cell cycle regulator)	Colorectal cancer Renal cancer	Eads et al.[43]; Herman et al.[48] Arai et al.[11]
MGMT (DNA repair)	Oral cancer Saliva of oral cancer patients Saliva of leukoplakia patients Head and neck cancer Bladder cancer Lung cancer	Kulkarni; Saranath.[38] Rosas et al.[40] López et al.[33] Sanchéz-Cespedes et al.[41]; Maruya et al.[42] Abbosh et al.[47] Vallböhmer et al.[49]
FHIT (DNA replication regulator)	Lung cancer	Lin et al.[44]; Kim et al.[50]
DAP-K (proapoptotic protein)	Oral cancer Saliva of oral cancer patients Nasopharyngeal cancer Head and neck cancer Pancreatic cancer Renal cancer Lung cancer	Kulkarni; Saranath.[38]; Ogi et al.[36] Rosas et al.[40] Fendri et al.[45] Sanchez-Cespedes et al.[41]; Maruya et al.[42] Dansranjavin et al.[51] Christoph et al.[52] Vallböhmer et al.[49]
APC (cell adhesion)	Colorectal cancer Lung cancer	Eads et al.[43]; Esteller et al.[53] Vallböhmer et al.[49]
Cadherin (cell adhesion)	Hepatic cancer Pancreatic cancer Lung cancer	Kanai et al.[28]; Oh et al.[37] Dansranjavin et al.[51] Kim et al.[50]
RAR (retinoic acid receptor)	Nasopharyngeal cancer Head and neck cancer Lung cancer	Fendri et al.[45] Maruya et al.[42] Kim et al.[50]; Lin et al.[44]

MECHANISMS OF SILENCING

In higher eukaryotes, DNA methylation and histone modifications appear to be the main events responsible for the formation of transcriptionally active or inactive chromatin.[54]

DNA methylation inhibits transcription by interfering with its initiation.[3] Because 5-methylcytosine is located in the major groove of the DNA helix,[8] it is possible that this modified cytosine interferes directly with the binding of transcription

factors.[21,55] Many factors are known to bind CpG-containing sequences, and some of these fail to bind when the CpG is methylated.[21] However, it is unlikely to be a widespread mechanism for transcriptional silencing, since most transcription factors do not have CpG dinucleotides within their DNA binding sites.[6] Another possible mechanism is that specific transcriptional repressors may recognize methyl-CpG and turn off transcription.[55]

Four proteins with a methyl-CpG-binding domain (MeCP2, MBD1, MBD2, and MBD3) recognize methylated DNA and are implicated in transcriptional repression. These proteins also have an affinity for histone modifying enzymes which cause chromatin condensation and gene silencing.[56] MeCP2 contains both a methyl-CpG-binding domain and a transcriptional repression domain (TRD), which can be tethered to another protein called Sin3A which interacts with histone deacetylase, a member of another transcriptional repression system.[57]

Histones, nuclear proteins that interact with DNA to form nucleosomes, besides being responsible for packing DNA within chromosomes, are also essential for transcription regulation.[10,58] Histone modifications such as acetylation and methylation may be read by the DNA methylation machinery, leading to either methylation of or failure to methylate a particular CpG dinucleotide.[21] Histone acetylation occurs at sites where transcription takes place, resulting in chromatin decondensation (euchromatin) to permit binding of transcription factors to DNA.[18,58] Acetylation is controlled by histone acetylases (HACs) and histone deacetylases (HDACs).[59] Deacetylation of these proteins (in particular H3 and H4) by HDAC leads to a tighter nucleosomal packing and the formation of a compacted chromatin environment (heterochromatin) that inhibits transcription.[3,10] Moreover, histone methylation is linked to euchromatic and heterochromatic states.[59,60] Methylation of lysine 9 in the core histone H3 is associated with silenced genes,[61] whereas methylation of lysine 4 in histone H3 is a feature of active genes.[21]

DNA methylation and histone modifications are intricately connected with each other.[7,24] Thus, the methylation level is connected to the broad organization of chromatin, with unmethylated DNA usually being part of euchromatin, whereas heavily methylated DNA is part of heterochromatin.[8] HDAC may play an important role, in cooperation with DNA methyltransferases, in maintaining tumor suppressor gene silencing.[29] DNA methylation recruits methyl-CpG binding proteins and their

associated co-repressors and HDACs, resulting in tighter packaging of DNA and reduced access of transcription factors to their binding sites (figure 3).[10,62,63]

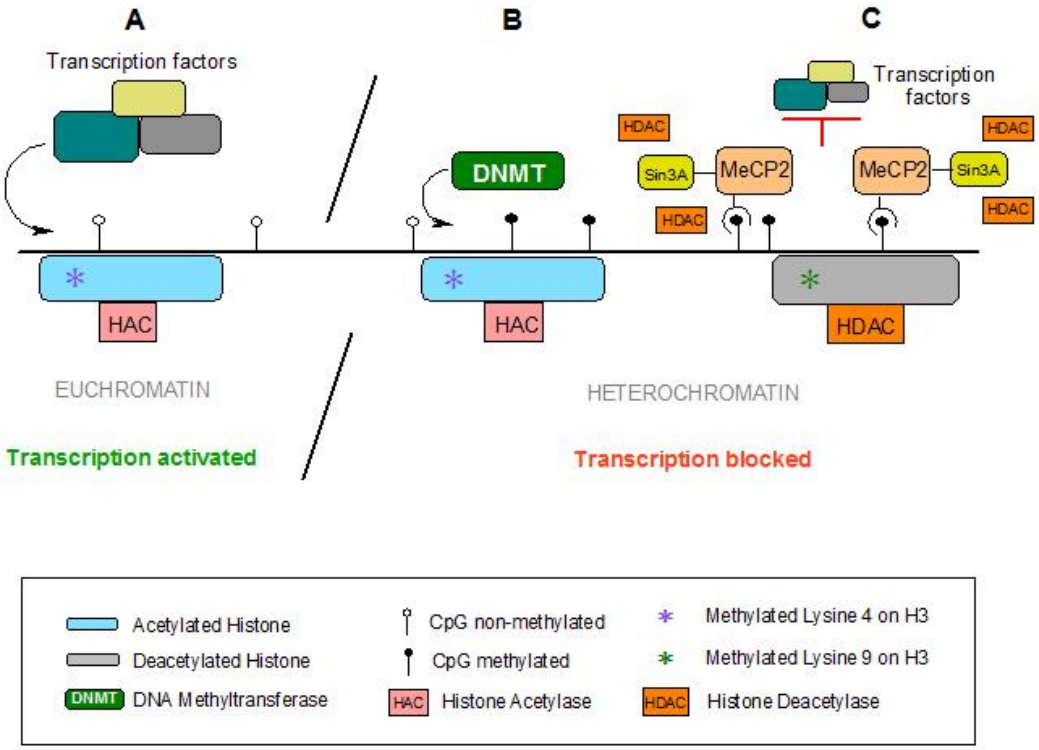

Fig. 3: Mechanism of transcriptional silencing by DNA methylation. (A) Promoter of gene in a transcriptionally active state. Chromatin in this phase is occupied by spaced nucleosomes composed of acetylated histone complexes and with tails of histone H3 methylated at lysine 4, which configures the euchromatin, making the region accessible to components of the transcription machinery. (B) DNA methyltransferase adds the methyl group to the cytosine of CpG islands. (C) The methylated CpG sites attract methyl-binding proteins such as MeCP2 which, in turn, attract Sin3A and HDAC to the region. Chromatin structure is modified, with deacetylated histone and methylated lysine 9 of histone H3, configuring the heterochromatin. Once these changes have occurred, the transcription factors are repelled and transcription is blocked.

Indeed, DNA methylation appears to be the dominant mechanism of silencing genes.[25] Drugs that inhibit HDAC can increase the expression of genes with unmethylated promoters, but they cannot induce the re-expression of hypermethylated genes in cancer cells. However, if some demethylation is first effected by low doses of demethylating drugs, histone deacetylase inhibitors act synergistically in re-expressing the silent gene.[64,65] Thus, the use of a combination of inhibitors of DNMTs and of histone deacetylases is an attractive therapeutic strategy.[25]

METHYLATION AND MUTATION

Cytosine methylation can increase mutation rates because of the spontaneous hydrolytic deamination of methylated cytosine, which causes C → T transition mutation.[15,18,27] This phenomenon was used to explain the high incidence of CpG to TpG transition mutations observed in the p53 tumor suppressor gene.[66]

The epigenetic silencing of the DNA repair enzyme O^6-MGMT (O^6-methylguanine-DNA methyltransferase) is another example of how abnormal methylation may lead to increased rates of mutation.[67] The O^6-MGMT protein removes carcinogen-induced O^6-methylguanine adducts from DNA, which produce G → A transition mutations if left unrepaired.[15] Tumors with silenced O^6-MGMT alleles seem to be predisposed to mutation in key genes, such as p53[67] and K-ras.[68]

DNA METHYLTRANSFERASES (DNMTs)

Three DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) are responsible for adding methyl groups to CpG dinucleotides.[6,13]

DNMT1

DNM T1 is often referred as the maintenance methyltransferase since it is believed to be the primary enzyme responsible for copying methylation patterns after DNA replication.[6,22,34,69] Its predominant splicing isoform in somatic cells in humans comprises 1616 amino acids, with a large N-terminal regulatory domain and smaller C-terminal catalytic domain.[8] It is the most abundant DNMT targeted to replication

foci and, under experimental conditions, has up to 50-fold preference for hemimethylated DNA substrate.[14,70]

This enzyme can maintain CpG methylation after DNA replication by methylating the daughter DNA strand, using the methylation pattern of the parental strand as a template (figure 4A).[8,16,71] Its inactivation produces global demethylation, which is consistent with the fact that DNMT1 is required for maintenance methylation.[71] The structural and mechanistic basis for the specificity of the enzyme for CpG sites as well as its preference for hemimethylated DNA is still unknown.[8]

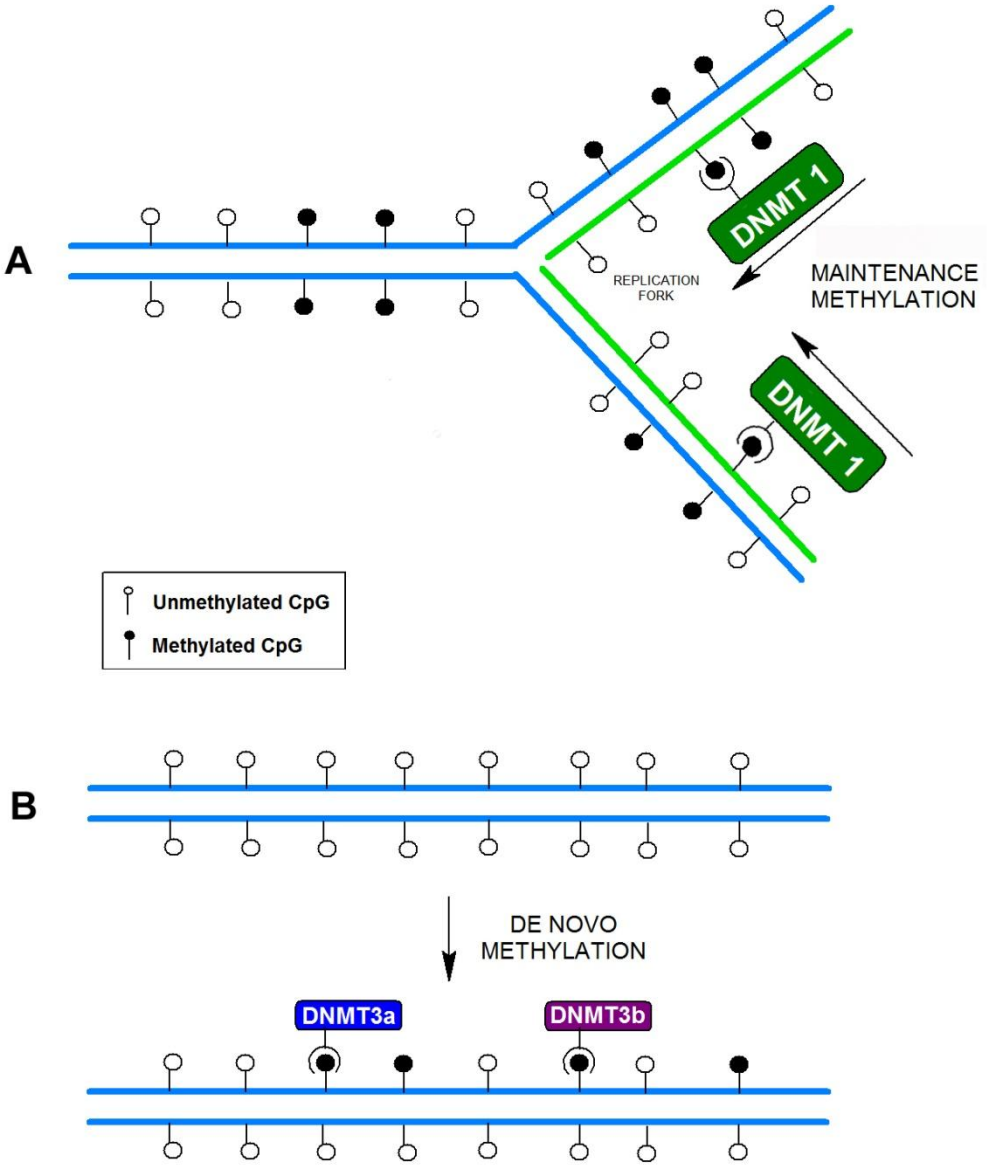

Fig. 4: Maintenance versus *de novo* methylation. (A) After semiconservative DNA replication, the daughter strand is base-paired with one of the methylated parental strand. The enzyme DNMT1 is responsible for maintaining the methylation pattern by completing half-methylated sites. (B) *De novo* methylation of unmethylated sequences, catalyzed by DNMT3 family enzymes.

Three sequences located in the N-terminal increase the precision of maintenance methylation and give the enzyme direct access to the nuclear replication site: proliferating cell nuclear antigen (PCNA) binding domain,[72] replication foci targeting sequence[73] and polybromo homology domain.[17,74] PCNA is required for DNA replication, and the DNMT1-PCNA interaction may allow the newly synthesized daughter strands to be rapidly remethylated before being packaged into chromatin.[6,72] This tight association of the DNMT1 with the replication machinery allows DNMT1 to bind newly replicated and still naked DNA.[17]

Otherwise, some genes may make this interaction difficult with replicating foci. p21, a cell cycle regulator, can disrupt the DNMT-PCNA interaction, suggesting that p21 may negatively regulate methylation by blocking access of DNMT to PCNA,[72] particularly during DNA damage, when p21 protein is induced.[75] It was also demonstrated that p21 may inhibit DNMT1 gene expression.[75] The retinoblastoma gene product, Rb, another cell cycle regulator protein, can bind to DNMT1 and inhibit its methyltransferase activity during DNA replication in the cell cycle.[76] Loss of functional Rb may grant DNMT1 free access to the genome which could allow for aberrant *de novo* methylation of CpG.[6] These observations point to a complicated network of connections between DNMT1 and several cellular proteins involved in gene regulation and epigenetic signaling during cell replication.[54]

DNMT3 FAMILY

Although DNMT1 is the major DNMT in humans, two other enzymes, DNMT3a and DNMT3b, have also been shown to possess DNMT activity.[11] They catalyze DNA methylation at CpG dinucleotides in unmethylated genomic sequences.[29]

Since DNMT3a and DNMT3b cannot differentiate between unmethylated and hemimethylated CpG sites, they obviously cannot copy a specific pattern of methylation or contribute to the maintenance of methylation pattern.[8] Because they show no preference for hemimethylated DNA, both enzymes appear to function as *de novo* methyltransferases,[77] and show a disperse distribution throughout the nucleus,

not associated with replication sites even during S-phase (figure 4B).[17] This fact suggests that these DNMTs utilize a different mechanism for accessing the densely packed chromatin and for interacting with their target sites which may involve auxiliary factors such as chromatin remodeling complexes.[17]

DNMT3a and DNMT3b are highly expressed in early embryonic cells, the stage in which most programmed *de novo* methylation events occur, are downregulated after differentiation and in adult somatic tissues, and are overexpressed in tumor cells.[3,21,77-79] DNMT3b has been shown to play a crucial role in incorporating *de novo* hypermethylation of promoter CpG islands, a possible mechanism for tumor suppressor gene inactivation within human cancer cells.[7,18]

Another member of the DNMT3 family is DNMT3L, a regulatory factor for the *de novo* methylation without methylation capacities. Its amino acid sequence is very similar to that of DNMT3a and DNMT3b, but lacks the residues required for DNA methyltransferase activity in the C-terminal domain.[7]

DNMT AND CANCER

Although the DNMT1 and DNMT3 families have been considered, respectively, maintenance and *de novo* methyltransferases, it is likely that all three DNMTs possess both functions in vivo, particularly during carcinogenesis.[6]

Excessive amounts of DNMT1, which cannot target replication foci, may participate in the *de novo* methylation of CpG islands that are not methylated in normal cells,[80] supporting the idea that DNMT can contribute to tumor progression through CpG island methylation-mediated gene inactivation.[81] Thus, its increased expression may play an important role in the malignant progression of cancer, leading to aberrant methylation in many important tumor suppressor genes.[22]

Increased expression of DNMT protein may be an early and significant event in urothelial,[80] hepatic,[34,37,82-84] gastric,[85] pancreatic,[86] lung,[44,49,50] breast,[87] and uterine cervix[35] carcinogenesis (table 2).

Table 2: Summary of DNA methyltransferase studies in human cancer tissues and cell lines

DNMT	Tissue	Sample	Method	Expression	Reference
DNMT1	Hepatic cancer	59 patients	IHC	↑	Choi et al.[82]
		42 patients	IHC/qRT-PCR	↑	Fan et al.[83]
		53 patients	IHC	↑	Saito et al.[34]
		27 patients	qRT-PCR	↑	Sun et al.[84]
		25 patients	IHC/qRT-PCR	↑	Oh et al.[37]
	Gastric cancer	38 patients	IHC	↑	Ding et al.[85]
	Pancreatic cancer	100 patients	IHC	↑	Peng et al.[86]
		NA	qRT-PCR	↑	Robertson et al.[79]
	Colorectal cancer	25 patients	qRT-PCR	↑	Eads et al.[43]
		36 patients	IHC	Heterogeneous expression	De Marzo et al.[88]
		48 patients	IHC/qRT-PCR	↑	Zhu et al.[22]
	Renal cancer	110 patients	IHC	↑	Arai et al.[11]
		NA	qRT-PCR	↑	Robertson et al.[79]
	Bladder cancer	102 patients	IHC	↑	Nakagawa et al.[80]
		NA	qRT-PCR	↑	Robertson et al.[79]
	Lung cancer	153 patients	qRT-PCR	↑	Kwon et al.[89]
		102 patients	qRT-PCR	↑	Kim et al.[50]
		91 patients	qRT-PCR	↑	Vallböhmer et al.[49]
		100 patients	IHC	↑	Lin et al.[44]
	Cervix cancer	127 patients	IHC	↑	Sawada et al.[35]
DNMT3a	Hepatic cancer	59 patients	IHC	↑ (Nuclear staining) ↓ (cytoplasmic staining)	Choi et al.[82]
		25 patients	IHC/qRT-PCR	↑	Oh et al.[37]
	Gastric cancer	38 patients	IHC	↑	Ding et al.[85]
	Pancreatic cancer	NA	qRT-PCR	↑	Robertson et al.[79]
	Colorectal cancer	25 patients	qRT-PCR	↑	Eads et al.[43]
	Renal cancer	NA	qRT-PCR	↑	Robertson et al.[79]
	Bladder cancer	NA	qRT-PCR	↑	Robertson et al.[79]
	Lung cancer	91 patients	qRT-PCR	↑	Vallbömer et al.[49]
		100 patients	IHC	↑	Lin et al.[44]
	Tumor cell lines (not specified)	NA	NBH	↑	Xie et al.[77]
DNMT3b	Hepatic cancer	25 patients	IHC/qRT-PCR	↑	Oh et al.[37]
	Gastric cancer	38 patients	IHC	↑	Ding et al.[85]
	Colorectal cancer	25 patients	qRT-PCR	↑	Eads et al.[43]
	Renal cancer	NA	qRT-PCR	↑	Robertson et al.[79]
	Bladder cancer	NA	qRT-PCR	↑	Robertson et al.[79]
	Lung cancer	100 patients	IHC	↑	Lin et al.[44]
		102 patients	qRT-PCR	↑	Kim et al.[50]
		91 patients	qRT-PCR	↑	Vallböhmer et al.[49]
	Breast cancer	12 cell lines	qRT-PCR	Aberrant expression	Roll et al.[87]
	Tumor cell lines (not specified)	NA	NBH	↑	Xie et al.[77]

NA: not available; IHC: immunohistochemistry; qRT-PCR: real-time PCR; NBH: Northern blot hybridization; ↑: increased expression; ↓: decreased expression.

DNMT INHIBITORS

In contrast to genetic alterations, epigenetic changes in cancer are potentially reversible, which has spurred the development of pharmacologic inhibitors of DNA methylation and histone deacetylation.[90] Indeed, the reactivation of epigenetically silenced genes in cancer may have a profound antitumor effect, thereby being a rational target for therapy and prevention.[25]

DNA methylation inhibitors such as 5-aza-2'-deoxycytidine (Decitabine) can be utilized to reverse the effects of methylation, including the reduction of mutations at methylated CpG sites, reactivation of genes suppressed by hypermethylation and restoration of cell growth control.[91] Treatment of cultured cells with this drug has been shown to cause cell growth inhibition, G2/M arrest, and cell apoptosis.[78] The disadvantage of this demethylating agent is its myelosuppressive effect, particularly when used at high doses.[15]

The combination of DNMTs and HDAC inhibitors may have an advantage in the treatment of cancer.[25] The use of a histone deacetylase inhibitor such as trichostatin A and phenylbutyrate in combination with 5-aza-2'-deoxycytidine has resulted in a strong synergistic growth inhibition in both cell lines and tumor.[92,93]

CONCLUSION

Here, we described the main epigenetic mechanisms for regulating gene transcription, mainly of tumor suppressor genes. Among them are DNA methylation and histone modification such as deacetylation, intricately related to chromatin configuration. This transcriptional control already exists in normal cells and is essential for adequate development. However, the aberrant epigenetic regulation of gene expression plays an important role in cancer development, as do the genetic alterations, making it an important topic for molecular oncology research.

Since it is a potentially reversible change, the epigenetic event represents new opportunities for the clinical management of cancer through the development of strategies to reverse gene silencing. Further, the associated molecular changes (such as DNMT/HDAC overexpression and gene promoter hypermethylation) may serve as markers for risk assessment, diagnosis and prognosis of cancer.

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Artigo de Pesquisa

3 ARTIGO DE PESQUISA

IMMUNOHISTOCHEMICAL EXPRESION OF DNA METHYLTRANSFERASES 1, 3a AND 3b IN ORAL LEUKOPLAKIAS AND SQUAMOUS CELL CARCINOMAS

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ABSTRACT

OBJECTIVES: Overexpression of DNA methyltransferases DNMT1, DNMT3a and DNMT3b has been reported in various cancers and precancerous lesions. This study investigated the expression of DNMT1, DNMT3a and DNMT3b proteins in oral squamous cell carcinoma (SCC) and leukoplakia, and their relationship with histopathologic and clinical parameters, in order to evaluate their significance in multistage oral carcinogenesis.

MATERIALS AND METHODS: Immunohistochemistry was carried out to detect the expression of the 3 DNMTs in 60 samples of oral SCC and 37 samples of oral leukoplakia (21 with histopathologic diagnosis of acanthosis and/or hyperkeratosis and 16 with epithelial dysplasia), using antibodies anti-DNMT1, anti-DNMT3a, and anti-DNMT3b (dilution of 1:700). For comparison, 20 samples of non-tumor oral mucosa were used.

RESULTS: The incidence of nuclear DNMT3a immunoreactivity in the three groups of oral SCC (39.8%) was significantly higher than in control (22.6%) ($P<0.05$), but not when compared to oral leukoplakias groups (28.2%). For DNMT1 and DNMT3b, there were no statistically significant differences between oral SCC groups (65% and 74.7%), oral leukoplakia groups (68.3% and 70.9%) and control (65.4% and 76.5%). DNMT protein expression exhibited no correlation with age and no association with gender or use of *mate*. There was a significant statistically association between the DNMT3a and habitual alcohol use ($P=0.01$), and an inverse association between DNMT1 and smoking ($P=0.048$).

CONCLUSION: The three DNMTs analyzed had no predictive capacity for SCC development in patients with oral leukoplakia, and exhibited no association with their histopathological features or the clinical parameters age, gender and mate consumption. Alcohol intake was associated with a higher DNMT3a expression, and smoking with a lower DNMT1 expression. DNMT3a immunopositivity increase may be used as oral SCC tumor presence marker.

KEYWORDS

Methyltransferase, oral cancer, squamous cell carcinoma, leukoplakia, immunohistochemistry.

INTRODUCTION

Epigenetic events are reversible and mitotically heritable chemical/structural alterations that regulate gene activity in the absence of underlying changes in DNA sequence.[1-3] The major form of epigenetic modification in mammalian cells is DNA methylation, or the covalent addition of a methyl group to the 5-carbon position of cytosine predominantly in the CpG dinucleotide.[4,5] First discovered as a mechanism of developmental control, it plays an important role in many cancers, beside the widely known genetic alterations.[6] Global hypomethylation and aberrant methylation of CpG islands of tumor suppressor gene promoters are frequently found in human cancers.[7,8] It is not exactly known how hypomethylation of non-promoter regions of DNA play a role in carcinogenesis, but genomic instability and structural changes in chromosomes may be potential effects.[9,10] However, aberrant methylation of promoter regions is a notable process in cancer. It can silence gene expression[3,11] and also predispose cytosine to mutation, through its deamination to thymine.[12,13] Promoter methylation may affect a specific gene, acting on the wild-type allele, while the other is mutated, contributing to the biallelic inactivation of tumor suppressor genes,[14] or the methylation of both alleles may also occur in some cases.[15] Therefore, genetic and epigenetic changes can collaborate to repress the transcription of important gene products in cancer.[16]

Three DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) are responsible for adding methyl groups to CpG dinucleotides.[5,6] DNMT1 is often referred to as a maintenance methyltransferase, since immediately after DNA replication it can copy the methylation pattern to the daughter DNA strand using the parental strand as a template.[5,8,17-21] It is the most abundant DNMT targeted to replication foci and has up to 50-fold preference for hemimethylated DNA substrate.[22,23] On the other hand, since DNMT3a and DNMT3b do not differentiate between unmethylated and hemimethylated CpG sites, they cannot copy a specific pattern of methylation or contribute to the maintenance of the methylation pattern,[18] where they are believed to function as *de novo* methyltransferases.[24] However, it is probable that all three possess both maintenance and *de novo* functions in vivo, particularly during carcinogenesis.[5] Increased expression of DNMTs may play an

important role in the progression of cancer, leading to aberrant methylation in many important tumor suppressor genes, particularly in urothelial,[25] hepatic,[21,26-29] gastric[30], pancreatic,[31] lung,[32-34] breast,[35] and uterine cervix[36] cancers.

There are currently few reports in the literature that have investigated the expression of DNMTs in oral cancer. In this study, we carried out an immunohistochemical analysis of DNMT1, DNMT3a and DNMT3b in a series of tissue samples of oral squamous cell carcinoma (SCC) and leukoplakia to evaluate their expression in multistage oral carcinogenesis and their relationship with histopathologic and clinical parameters.

MATERIALS AND METHODS

A total of 117 specimens were included in this study: 21 leukoplakias with histopathologic diagnosis of acanthosis and/or hyperkeratosis (leukoplakia without epithelial dysplasia), 16 leukoplakias with histopathologic diagnosis of epithelial dysplasia, 20 well-differentiated oral squamous cell carcinomas (grade I), 20 moderately differentiated oral squamous cell carcinomas (grade II), and 20 poorly differentiated oral squamous cell carcinomas (grade III). It has not been made distinction of epithelial dysplasia graduation in the groups of leukoplakia because of its subjectiveness and the low number of cases. As control group, 20 specimens of oral fibroepithelial hyperplasia were also obtained, since ethical principles prevented obtaining healthy mucosa samples. All the tissues were obtained from the pathology archive of the Division of Stomatology, São Lucas Hospital, Brazil. Histopathologic diagnosis was performed by two previously calibrated raters (kappa=0.794) according to the World Health Organization classification.[37,38] There were no patients from whom multiple tissue samples were obtained metachronously, and none of them received preoperative treatments such as radiotherapy or chemotherapy. Lip (vermilion), pharyngeal, and proliferative verrucous leukoplakia lesions were excluded. Information on age, sex, smoking, and alcohol and *mate*[3] consumption

[3] Traditional south brazilian beverage with carcinogenic potential when consumed as very hot tea.

history were obtained from hospital records and are shown in table 1. This study was approved by the Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul.

Immunohistochemistry

We performed immunohistochemical assays using the streptavidin-peroxidase method. Proper formalin-fixed and paraffin-embedded tissues as 3-µm thick sections were obtained and mounted on slides coated with Histogrip™ (Zymed Laboratory, Carlsbad, CA), and the slides baked at 60°C for 15 min. Slides were deparaffinized in xylene, rehydrated in a graded alcohol series, and washed in tap water. Endogenous peroxidase activity was blocked with 6% H_2O_2 in methanol for 15 min. For antigen-retrieval, the sections were placed in a pressure cooker filled with EDTA solution, pH 8.0, for 20 min in a microwave oven at 1050 W. Non-specific reactions were blocked with 5% skim milk in phosphate-buffered saline (PBS). The sections were incubated with mouse monoclonal antibodies against DNMT1 (IMG-261A, 1:700 dilution, Imgenex, San Diego, USA), DNMT3a (IMG-268A, 1:700 dilution, Imgenex, San Diego, USA) and DNMT3b (IMG-184A, 1:700 dilution, Imgenex, San Diego, USA) at 4°C overnight, followed by incubation with PicTure™-MAX (Zymed Laboratory, Carlsbad, USA) polymer conjugate and Dako Liquid DAB+ Substrate Chromogen System™ (3,3'-diaminobenzidine) (DAKO North America Inc., Carpinteria, USA) for the visualization of antigen-antibody complexes. All sections were counterstained with Harris Hematoxylin. As a negative control, the primary antibodies were omitted from the reaction sequence. Tissue specimens of placenta, in which we had detected positive immunoreactivity for DNMT1, DNMT3a and DNMT3b were used as a positive control in each slide. The surrounding non-neoplastic stroma served as an internal negative control for each slide.

We assessed DNMT1, DNMT3a and DNMT3b immunoreactivity only in the nucleus[8] of epithelial cells and nuclear staining above the levels of any cytoplasmic background was considered evidence of expression.[39] For each sample, at least 500 cells were counted using the public domain program ImageJ version

1.41o (National Institute of Health, Bethesda, USA) in at least five fields equidistantly captured at a magnification of 200X under a light microscope (Axiostar Plus, Carl Zeiss, Oberkochen, Germany). If the lesion was small with less than 500 cells, all the cells were counted.[31,36] Every 15 cases the rater repeated the count of one case to guarantee the method reproductibility. DNMT immunopositivity in each sample was expressed as a percentage of all the cells counted. Evaluation was conducted with the rater blinded to knowledge of the clinical and pathologic characteristics of the cases.

Statistical analysis

The SPSS™ program version 15 (SPSS Inc., Headquarters, USA) was used for statistical analysis. The incidence of immunopositivity for each DNMT was compared between each group using analysis of variance (ANOVA), followed by Student-Newman-Keuls test, when a difference was encountered. Comparison of DNMT immunopositivity with each category of clinical parameters was analyzed by ANOVA for smoking and alcohol history, and t test for gender and habitual mate consumption. Pearson's correlation test was used to correlate age and DNMT, and to correlate the immunopositivity of the three enzymes. For all tests, differences were considered significant when P was less than 0.05.

RESULTS

The samples were obtained from 79 males and 38 females with an overall mean age of 55.6 ± 13.0 (mean ± standard deviation) years at diagnosis. Seventy-six (65%) patients were smokers and 23 (19.6%) ex-smokers, and 18 (15.4%) had never smoked. Forty-six (39.3%) patients were alcohol users and 57 (48.7%) ex-users, and 14 (12%) never drank alcohol. With regards to *mate* consumption, this habit was

present in 38 (32.5%) patients. A summary of the relevant characteristics of all study-enrolled individuals is presented in table 1.

Table 1. Clinical characteristics of study subjects

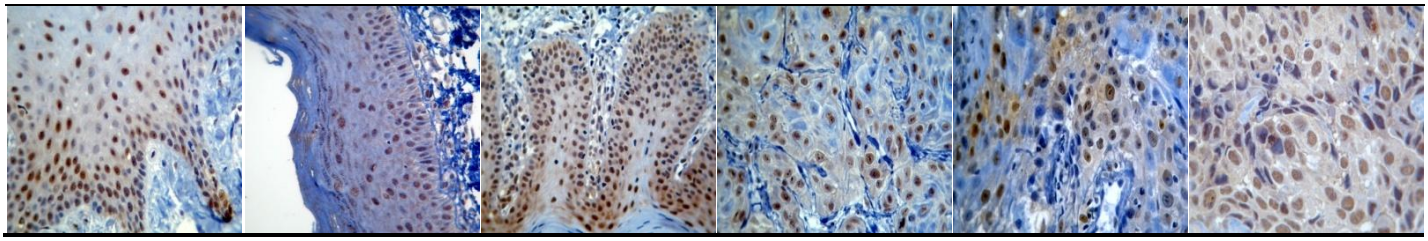
Characteristic	Control	Leukoplakia without dysplasia	Leukoplakia with dysplasia	SCC grade I	SCC grade II	SCC grade III	Total
Sample	20	21	16	20	20	20	117
Sex							
Male	12 (60%)	9 (42.9%)	11 (68.8%)	17 (85%)	16 (80%)	14 (70%)	79 (67.5%)
Female	8 (40%)	12 (57.1%)	5 (31.2%)	3 (15%)	4 (20%)	6 (30%)	38 (32.5%)
Age (years)							
Range	33–82	28–72	24–76	39–83	44–86	44–81	24–86
Mean ± SD	58.4 ± 14.5	48.1 ± 11.7	53.2 ± 14.3	57.9 ± 14.1	60.2 ± 11.3	55.2 ± 9.2	55.6 ± 13.0
Smoking habit							
Yes	8 (40%)	13 (61.9%)	9 (56.2%)	16 (80%)	15 (75%)	15 (75%)	76 (65%)
No	6 (30%)	5 (23.8%)	5 (31.3%)	1 (5%)	4 (20%)	2 (10%)	23 (19.6%)
Ex-smoker	6 (30%)	3 (14.3%)	2 (12.5%)	3 (15%)	1 (5%)	3 (15%)	18 (15.4%)
Alcohol habit							
Yes	3 (15%)	2 (9.5%)	6 (37.5%)	11 (55%)	11 (55%)	13 (65%)	46 (39.3%)
No	15 (75%)	18 (85.7%)	9 (56.3%)	5 (25%)	7 (35%)	3 (15%)	57 (48.7%)
Ex-user	2 (10%)	1 (4.8%)	1 (6.2%)	4 (20%)	2 (10%)	4 (20%)	14 (12%)
Mate habit							
Yes	7 (35%)	10 (47.6%)	6 (37.5%)	7 (35%)	7 (35%)	1 (5%)	38 (32.5%)
No	13 (65%)	11(52.4%)	10 (62.5%)	13 (65%)	13 (65%)	19 (95%)	79 (67.5%)
Site							
Tongue	3 (15%)	6 (28.6%)	5 (31.3%)	8 (40%)	9 (45%)	6 (30%)	37 (31.6%)
Floor	0 (0%)	3 (14.3%)	3 (18.7%)	6 (30%)	5 (25%)	9 (45%)	26 (22.2%)
Buccal mucosa	7 (35%)	5 (23.8%)	2 (12.5%)	1 (5%)	2 (10%)	0 (0%)	17 (14.5%)
Alveolar ridge/gum	2 (10%)	4 (19%)	1 (6.3%)	2 (10%)	2 (10%)	2 (10%)	13 (11.1%)
Palate	2 (10%)	1 (4.8%)	2 (12.5%)	2 (10%)	2 (10%)	2 (10%)	11 (9.4%)
Others	6 (30%)	2 (9.5%)	3 (18.7%)	1 (5%)	0 (0%)	1 (5%)	13 (11.2%)

SCC: Squamous cell carcinoma
SD: Standard deviation

In all specimens, the immunohistochemical staining of all three DNMTs was localized in the nuclei of epithelial cells. The percentage of positive cells for DNMT1, DNMT3a, and DNMT3b are shown in tables 2, 3, and 4, respectively. For DNMT1 and DNMT3b there was no significant difference between any group (ANOVA, $P = 0.327$ and $P = 0.27$, respectively). The majority of cells of noninvasive lesions and all carcinoma grades stained positive for DNMT1 and DNMT3b (tables 2 and 4).

Table 2: Incidence of nuclear immunoreactivity for DNMT1 (%, mean ± SD)

Control	Leukoplakia without dysplasia	Leukoplakia with dysplasia	SCC grade I	SCC grade II	SCC grade III
65.4 ± 32.0	70.8 ± 27.3	65.8 ± 22.4	75.3 ± 12.2	58.3 ± 29.2	61.4 ± 24.2

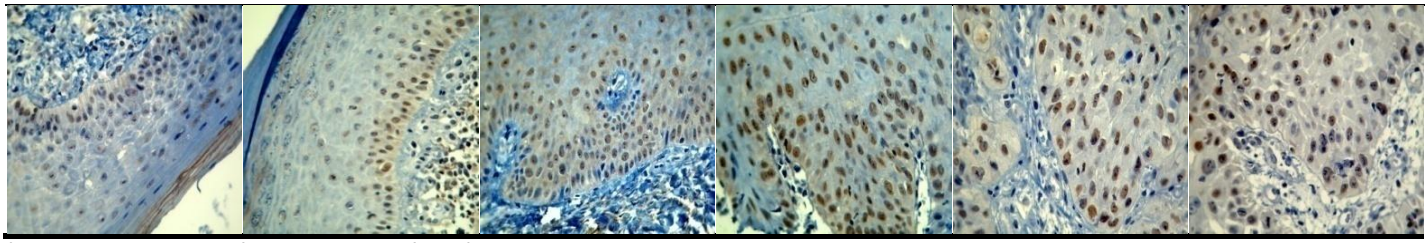


(P = 0.327, ANOVA)

Table 3: Incidence of nuclear immunoreactivity for DNMT3a (%, mean ± SD)

Control	Leukoplakia without dysplasia	Leukoplakia with dysplasia	SCC grade I	SCC grade II	SCC grade III
22.6 ± 12.3	30.1 ± 18.0	26.3 ± 13.1	40.5 ± 22.8	38.6 ± 19.7	40.5 ± 21.6

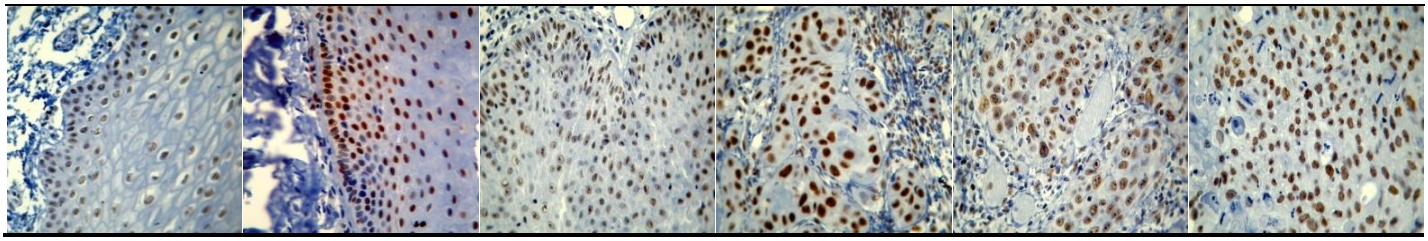
P<0.05



(P = 0.006, ANOVA, Student-Newman-Keuls test)

Table 4: Incidence of nuclear immunoreactivity for DNMT3b (%, mean ± SD)

Control	Leukoplakia without dysplasia	Leukoplakia with dysplasia	SCC grade I	SCC grade II	SCC grade III
76.5 ± 13.7	67.5 ± 15.7	74.3 ± 15.0	73.3 ± 10.8	75.5 ± 11.4	75.4 ± 10.8



(P = 0.27, ANOVA)

There was a progressive increase in the percentage of DNMT3a positive cells from control lesion through leukoplakias and SCC. Although, statistically significant differences in the percentage of DNMT3a were only found when control was compared to squamous cell carcinoma grades I, II, and III (Student-Newman-Keuls multiple comparison test, P<0.05) (table 3). Figures inside tables 2, 3 and 4

show examples of immunohistochemical staining in each group for DNMT1, DNMT3a, and DNMT3b, respectively.

The degree of nuclear immunopositivity for DNMT3a was lower than for DNMT1 and DNMT3b (figure 1). However, it was not possible to make a correlation between the levels of any DNMT immunoreactivity (Pearson's correlation, r = 0.057, P = 0.543 for DNMT1 x DNMT3a; r = - 0.04, P = 0.966 for DNMT1 x DNMT3b; r = - 0.027, P = 0.769 for DNMT3a x DNMT3b).

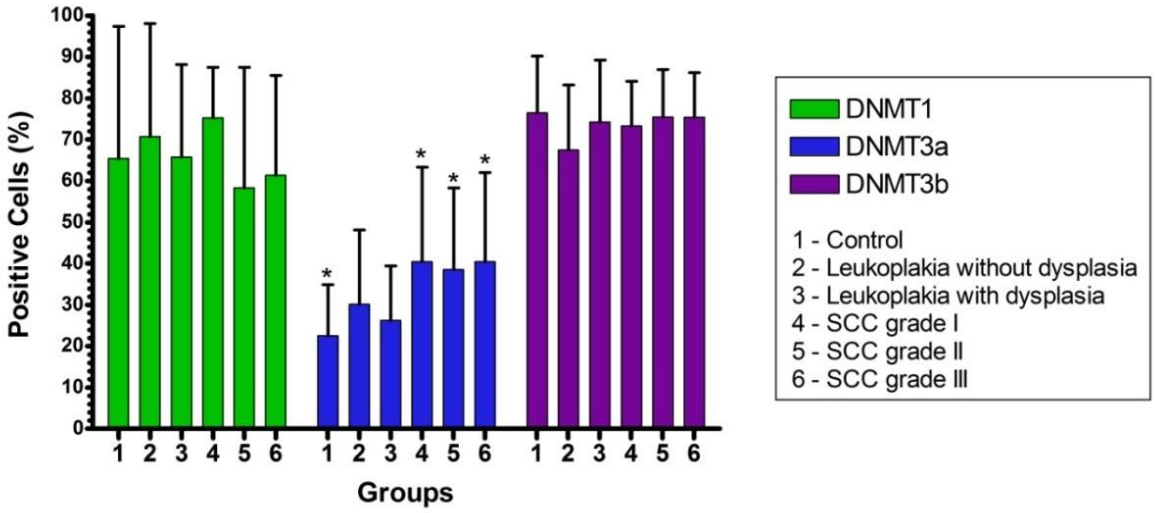


Figure 1: DNMT immunohistochemical expression in control, leukoplakia and squamous cell carcinoma groups. * Statistically significant difference between group 1 and groups 4, 5, and 6 (P<0.05, ANOVA, Student-Newman-Keuls post hoc test).

We analyzed the immunohistochemical expression of DNMTs and the clinical data obtained from these patients. There was no correlation between any DNMT enzyme immunoreactivity and age (Pearson correlation, r = 0.005, 0.046, and 0.130, for DNMT1, DNMT3a, and DNMT3b, respectively, P>0.05), or association with gender (t test, P>0.05) and *mate* consumption (t test, P>0.05). However, there was a significantly higher mean percentage of DNMT1 immunoreactivity in non-smokers (ANOVA, P = 0.048), and a higher DNMT3a immunoreactivity in alcohol users (ANOVA, P = 0.010).

DISCUSSION

Evidence suggests that DNA methylation plays an important role in tumor suppressor gene silencing of several cancer types and their precursor lesions,[3,5,6,25,30,31] including oral squamous cell carcinoma.[40] DNA methyltransferases, which may be of importance in deregulating gene expression and leading to cancer formation, have been found to be overexpressed in tumor cells of some types of human cancers.[8,21,25-27,29-32,36,41] Currently, there are no data about their expression in oral tumorigenesis. In the present paper, we describe for the first time the immunohistochemical expression of the three known catalytically active DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) in a series of oral SCC and its precursor lesion leukoplakia. The other two known DNA methyltransferase family enzymes DNMT2 and DNMT3L have not been shown to possess methylation ability[4,18] and for this reason were not included in this study.

Leukoplakia is the most representative precursor lesion of oral cancer, possessing many of the genetic modifications present in oral SCC, whether or not showing dysplasia.[42,43] In this study, there was no significant difference in DNMTs protein levels between leukoplakias either with or without dysplasia and SCC of all grades or the non-tumor control group. It was not possible to suggest that the DNMTs enzymes could exert a role in early events during multistage oral carcinogenesis. Since this study included only 16 cases of leukoplakias with epithelial dysplasia, we combined the mild, moderate, and severe cases, which may have introduced certain errors. Thus, until this moment, there is no practical diagnostic value in determining percentages of immunopositivity in potentially precancerous lesions, for differentiating them according to the presence of epithelial dysplasia or from oral SCC and non-tumor tissues. Clinical and histopathologic characteristics are still important factors for predicting the development of SCC in these lesions.[44]

Our study showed that DNMT3a immunoreactivity was significantly higher in the three oral SCC grades than in non-tumor tissue, suggesting that deregulation of DNMT3a expression was present in tumor cells of oral SCC. It was possible to detect a progressively increasing percentage of immunopositivity, although without statistical significance, from non-tumor tissues (22.6%) through leukoplakias (28.2%)

and SCC (39.8%), suggesting that aberrant DNMT3a expression may play a role in oral carcinogenesis. Other studies have discovered similar trends of higher nuclear expression of DNMT3a in hepatic and lung cancer tissues, compared to normal tissue. Choi et al. (2003)[26] found a progressively increasing frequency of nuclear DNMT3a immunoreactivity from non-neoplastic liver (0%), through high-grade dysplastic nodules (29.2%) and advanced hepatic carcinoma (39.3%). Lin et al. (2007)[32] detected a strong immunostaining in 32% of lung cancer cases, whereas the expression levels in surrounding non-neoplastic tissues showed a pale staining. This higher incidence of *de novo* DNMT3a could be responsible, at least in part, for the silencing of tumor suppressor genes in oral carcinogenesis, leading the proliferation of tumor cells without adequate control.

For DNMT1 and DNMT3b, there was no progressive increase or decrease in immunopositivity among the six groups studied. Other studies showed a higher immunohistochemical expression of DNMT1 in hepatic,[26,29] gastric,[30] colorectal,[8] bladder,[25] and cervix cancer,[36] and of DNMT3b in hepatic[29] and gastric cancer.[30] These studies analyzed the intensity of positive cells, which may vary because of the tissue fixation and the immunohistochemistry technique. In the present research we assessed the percentage of positive cells, which is more adequate for the immunohistochemical evaluation. The oral hyperplasic tissues used as control-group may have contributed to absence of difference between the groups for DNMT1 and DNMT3b, since these cells have a higher proliferative rate than normal cells, although without maturation/differentiation changes. Based on our results, increase in DNMT1 and DNMT3b protein expression may not be an early and significant event during oral carcinogenesis. However, other methods that could detect small elevations in DNMT mRNA levels in precancerous conditions may help in determining a more significant difference between these groups, since immunohistochemistry cannot detect differences until protein expression reaches a certain level in malignant lesions.

Similarly to other studies,[8,30,36] there was no statistically significant correlation/association between the levels of immunopositivity for all DNMTs and the clinical parameters age and gender. With respect to *mate* consumption, this habit has been compared to the DNMTs immunoreactivity since there may be an association with esophagus, larynx, and oral cavity cancer as reviewed by Loria et al.[45] The

possible mechanism of *mate* in oral carcinogenesis involves the high temperature at drinking and the carcinogenic contaminants introduced during the processing of the leaves.[45] This is the unique study that collected this information and an absence of association with DNMTs has been shown.

A correlation between increased expression of DNMT1 and smoking has been reported, but no relation for DNMT3a and DNMT3b has been found.[46,47] The mechanisms for the effects of tobacco carcinogens on epigenetic mechanisms are not known.[46] In this study, there was an inverse relation between smoking and DNMT1 expression, with a higher incidence of immunostaining in those patients without a history of smoking. The tobacco carcinogens may be responsible for a lower expression of the DNMT1 gene, which in turn, may provide an inferior capacity to maintain the epigenetic pattern during cell division, leading to a global hypomethylation. Genes that should be silenced in normal cells may lose its inactivation during mitosis, and lower methylation of pericentromeric regions may result in an abnormal separation of the chromosomes during cell division. On the other hand, this lower DNMT1 level may influence the oncogenes inactivation, allowing its expression and also contributing to tumor development. Although, since the rate of smokers (65%) were higher than ex-smokers (15.4%) and non-smokers (19.6%), it is desirable to enlarge the sample size in order to confirm this inverse association.

With respect to alcohol consumption, it is the first time that this habit is associated with DNMT immunopositivity. The higher expression of DNMT3a, in a theoretical manner caused by alcohol intake, may lead to a new pattern of methylation (as result of *de novo* methylation) in normally non-methylated genes, including tumor suppressor genes, contributing to the carcinogenesis. It is important to note that the clinical data were collected from hospital records, which may introduce some bias to the results discussed here. Moreover, it is difficult to isolate confounding risk factors such as alcohol and tobacco use, which are the most important risk factors for cancer of the upper aerodigestive tract. Further studies with larger samples are needed, since the finding of a relation with smoking is not consistent with previous reports and because the role of alcohol in DNMT expression needs to be confirmed.

CONCLUSION

Although there was a higher level of DNMT3a immunopositivity in SCC groups, the three studied enzymes had no predictive capacity for SCC development in patients with oral leukoplakia, and exhibited no association with their histopathological features or the clinical parameters age, gender and mate consumption. Alcohol intake was associated with a higher DNMT3a expression and smoking with a lower DNMT1 expression.

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Discussão Geral

4 DISCUSSÃO GERAL

Embora muitos avanços tenham ocorrido no campo da oncologia, o câncer de boca permanece com elevada taxa de diagnóstico tardio, sendo este um dos principais fatores responsáveis por reduzir as chances de cura e aumentar as seqüelas, contribuindo para uma menor qualidade de vida dos pacientes afetados. Diversas pesquisas visam identificar uma molécula que possa auxiliar não só o diagnóstico precoce, mas também a predição da progressão de lesões cancerizáveis e a escolha de uma terapia antineoplásica mais adequada.

A epigenética tem sido extensivamente estudada por envolver modificações reversíveis que podem ser alvo de agentes com potencial antineoplásico. As alterações epigenéticas são importantes não apenas para a manutenção, mas também para a iniciação de muitos tumores.[19] As enzimas responsáveis por catalisar a reação de metilação, evento epigenético mais conhecido e estudado atualmente, constituem uma peça-chave neste processo, podendo não somente ser o alvo de drogas inibidoras, mas também constituir um biomarcador para o diagnóstico precoce. Para validar a hipótese de que as três enzimas DNA metiltransferases (DNMT1, DNMT3a e DNMT3b) pudessem ser marcadores biológicos para lesões epiteliais potencialmente malignas e tumorais da cavidade bucal, foi realizada uma avaliação imunoistoquímica em fragmentos de tecidos bucais do arquivo do Serviço de Estomatologia e Prevenção do Câncer Bucomaxilofacial do Hospital São Lucas da PUCRS.

Os grupos de lesões potencialmente malignas foram constituídos por leucoplasias, cujo diagnóstico foi definido por meio de avaliação clínica e histopatológica. A presença ou ausência de displasia epitelial, determinada pela análise microscópica, foi a graduação adotada nesta pesquisa, para separar os casos em dois grupos. As lesões foram estratificadas com o objetivo de avaliar a expressão das três enzimas em função da gravidade histopatológica das alterações, visto que a presença de displasia aumenta o potencial para o desenvolvimento do CEC bucal.[11,43,44] As amostras de carcinoma de células escamosas bucais foram divididas em três grupos, de acordo com sua graduação histopatológica (CEC grau I,

CEC grau II e CEC grau III)[43,44], também com o intuito de avaliar o comportamento das DNMTs em função da diferenciação celular dos tumores.[44]

Para comparar os resultados obtidos nos grupos de lesões tumorais e potencialmente malignas com um grupo-controle, foram utilizados fragmentos de tecidos com diagnóstico clínico e histopatológico de hiperplasias fibroepiteliais, por se tratarem de lesões benignas com características e comportamento não tumorais.[7] As células de tecido hiperplásico embora não apresentem alterações no seu grau de maturação e diferenciação histopatológica, possuem uma taxa de proliferação maior do que as células de epitélio normal, o que pode ter contribuído para a ausência de diferença com os demais grupos. A utilização de tecidos saudáveis foi impossibilitada pelo aspecto ético. Submeter pacientes aos riscos inerentes a um procedimento cirúrgico sem indicação, com o único objetivo de captar tecido não patológico para a pesquisa, sem que isto trouxesse benefícios diretos ao mesmo, constituiu uma forte barreira para a obtenção de amostras de tecido normal para compor o grupo-controle.

A expressão das três enzimas DNMT ocorreu somente no núcleo das células presentes nas lesões avaliadas. Nos grupos de leucoplasia e controle ocorreu uma marcação positiva nas camadas basal e suprabasal, sem que fosse possível realizar distinção entre qualquer grupo com base apenas neste aspecto. Em todos os casos, a imunorreatividade ocorreu predominantemente nas células epiteliais e em algumas células inflamatórias presentes no tecido conjuntivo, demonstrando uma grande afinidade e especificidade dos anticorpos anti-DNMT1, anti-DNMT3a e anti-DNMT3b para o tecido epitelial.

A DNMT1, considerada uma enzima de manutenção do padrão normal de metilação, deve-se mostrar presente em certa quantidade no núcleo das células somáticas, já que é uma enzima essencial para que a metilação seja copiada corretamente após cada divisão celular.[20,28,45] Além disso, segundo Nakagawa et al. (2003)[46], excessivas quantidades dessa enzima, que não se ligam aos focos de replicação, poderiam participar do processo de metilação *de novo* em ilhas CpG que não estão metiladas nas células normais. No presente estudo, a maioria das células epiteliais, tanto de lesões não tumorais (controle e leucoplasias), quanto de carcinomas exibiram imunorreatividade para a DNMT1. Entretanto, não foi observado aumento significativo da sua expressão nos grupos de leucoplasia com

displasia e de carcinoma. Desta forma, não se pôde comprovar, baseado nestes resultados, que uma maior expressão desta enzima seja responsável pelo silenciamento de genes supressores tumorais implicados na progressão do câncer de boca.

As enzimas DNMT3a e DNMT3b, responsáveis pela adição de novos radicais metil a nucleotídeos não metilados, devem estar subexpressas nas células somáticas normais.[36,37] Os resultados obtidos para DNMT3a mostraram uma maior expressão nos grupos de carcinoma (39,5%), quando comparado com os grupos de lesões não tumorais (22,6% e 28,2%, controle e leucoplasias, respectivamente), com significância estatística na comparação entre carcinomas e grupo-controle. Este aumento nas lesões tumorais pode indicar maior atividade de metilação *de novo* presente nestes tecidos e contribuir para o silenciamento de genes supressores tumorais. Além disso, como proposto por Robertson et al. (2001)[20], a DNMT3a é também capaz de desempenhar um papel de manutenção do padrão de metilação durante os ciclos de divisão celular, contribuído para que os genes silenciados permaneçam neste estado durante o crescimento do tumor. Porém, o mesmo não pôde ser afirmado para DNMT3b, visto que não houve um aumento na sua expressão imunoistoquímica no CEC bucal nas amostras envolvidas nesta pesquisa.

É importante ressaltar que a atividade catalítica das enzimas não foi avaliada neste trabalho. A carcinogênese pode afetar a sua função, aumentando a sua capacidade de metilação, mesmo que não haja uma maior produção destas enzimas pela célula. Ainda assim elas seriam capazes de influenciar o silenciamento de genes supressores tumorais e contribuir para a proliferação celular descontrolada. Os resultados aqui apresentados, embora não tenham demonstrado uma maior incidência de DNMT1 e DNMT3b no câncer bucal, requerem confirmação por meio de técnicas que avaliem também a expressão dos genes DNMT1 e DNMT3b como, por exemplo, o PCR quantitativo. Esta técnica requer a coleta de material a fresco ou congelado a -80ºC, o que implica na realização de uma pesquisa prospectiva, inviabilizando a utilização dos casos utilizados nesta pesquisa retrospectiva.

Os efeitos do tabagismo nos mecanismos epigenéticos são desconhecidos. Neste estudo, houve uma relação inversa entre este hábito e a expressão de DNMT1, com uma maior prevalência da imunopositividade naqueles

pacientes sem história de cigarro. Os carcinógenos do tabaco podem ser responsáveis por uma menor expressão do gene DNMT1, o que leva a uma capacidade menor de manter o padrão epigenético durante a divisão celular, resultando em uma hipometilação global. Por outro lado, foi verificada uma relação direta entre o consumo de bebidas alcoólicas e a expressão imunoistoquímica de DNMT3a, podendo esse hábito influenciar na expressão do gene dessa enzima e estar relacionada com a hipermetilação regional de genes supressores tumorais no câncer.

Conclusões

5 CONCLUSÕES

Com base nos resultados deste estudo:

- Somente para a enzima DNMT3a detectou-se um aumento dos níveis de imunorreatividade nos grupos de carcinoma de células escamosas bucais.

- As enzimas DNMT1, 3a e 3b não podem ser utilizadas como preditoras de susceptibilidade ao carcinoma escamocelular em pacientes com leucoplasia bucal;

- As três enzimas não podem ser empregadas como marcadores para a graduação histopatológica do carcinoma escamocelular bucal.

- O hábito de tabagismo e o consumo de bebidas alcoólicas foram estatisticamente associados com uma menor expressão imunoistoquímica de DNMT1 e uma maior expressão de DNMT3a, respectivamente.

- Gênero, idade e consumo de chimarrão não foram associados a uma maior ou menor expressão imunoistoquímica das enzimas DNMT1, 3a e 3b.

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Apêndices

APÊNDICE A – Ficha para coleta de dados

Ficha: _____
AP: _____

DADOS DE IDENTIFICAÇÃO

Nome: _____

Gênero: ( ) M ( ) F - D.N.:___/___/_____

HÁBITOS

( ) Fumante ( ) Ex-fumante ( ) Não fumante

( ) Etilista ( ) Ex-etilista ( ) Não etilista ( ) Chimarrão

DADOS DA LESÃO

Localização: ( ) Assoalho ( ) Palato Duro ( ) Palato Mole ( ) Pilar amigdaliano
( ) Mucosa labial ( ) Rebordo alveolar ( ) Gengiva

( ) Língua
( ) Dorso
( ) Ventre
( ) Borda lateral
( ) Mucosa jugal
( ) Fundo de sulco

EXAME ANÁTOMO-PATOLÓGICO

Data da Biópsia: __/__/____

( ) Hiperplasia fibroepitelial
( ) Acantose e/ou hiperceratose (Leucoplasia sem displasia)
( ) Displasia epitelial (Leucoplasia com displasia)
( ) Carcinoma escamocelular grau I
( ) Carcinoma escamocelular grau II
( ) Carcinoma escamocelular grau III

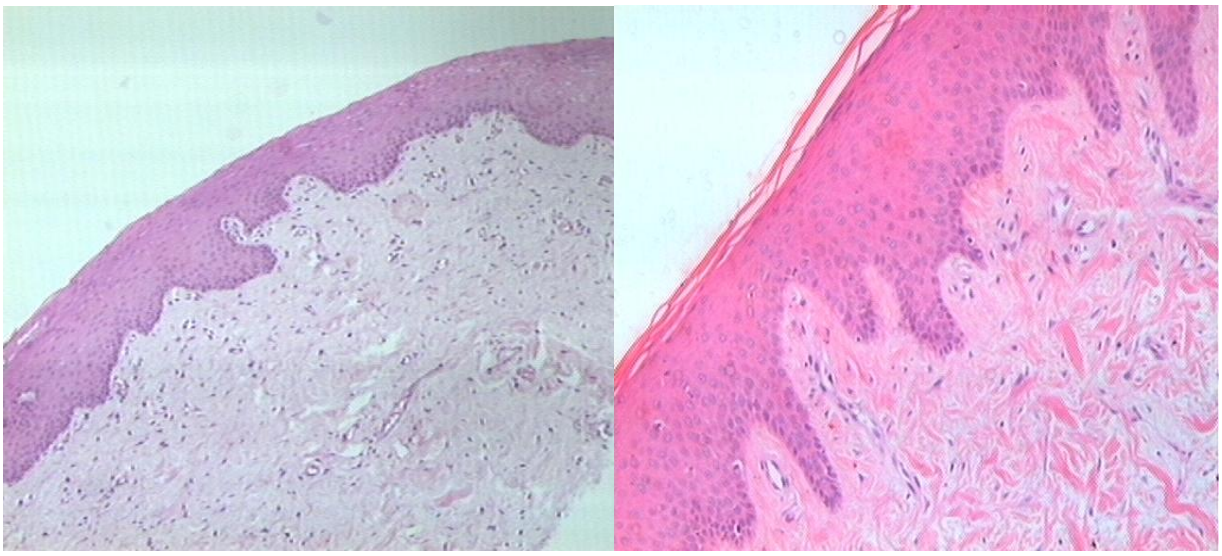
EXAME IMUNOISTOQUÍMICA

DNMT1	Campos					Total	%
	1	2	3	4	5		
No. Núcleos Positivos							
No. Núcleos Negativos							

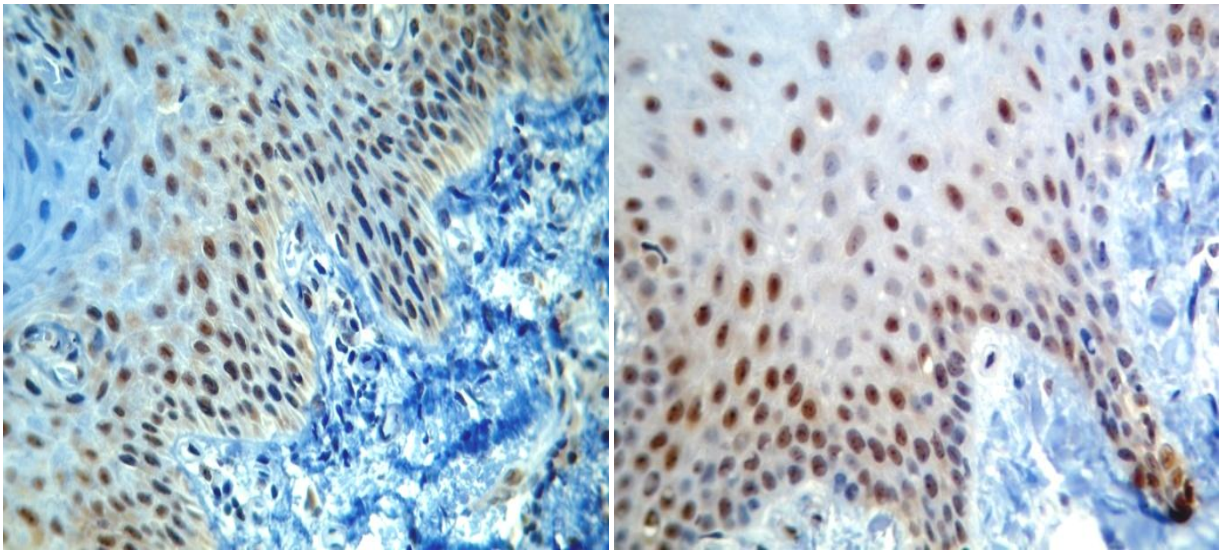
DNMT3a	Campos					Total	%
	1	2	3	4	5		
No. Núcleos Positivos							
No. Núcleos Negativos							

DNMT3b	Campos					Total	%
	1	2	3	4	5		
No. Núcleos Positivos							
No. Núcleos Negativos							

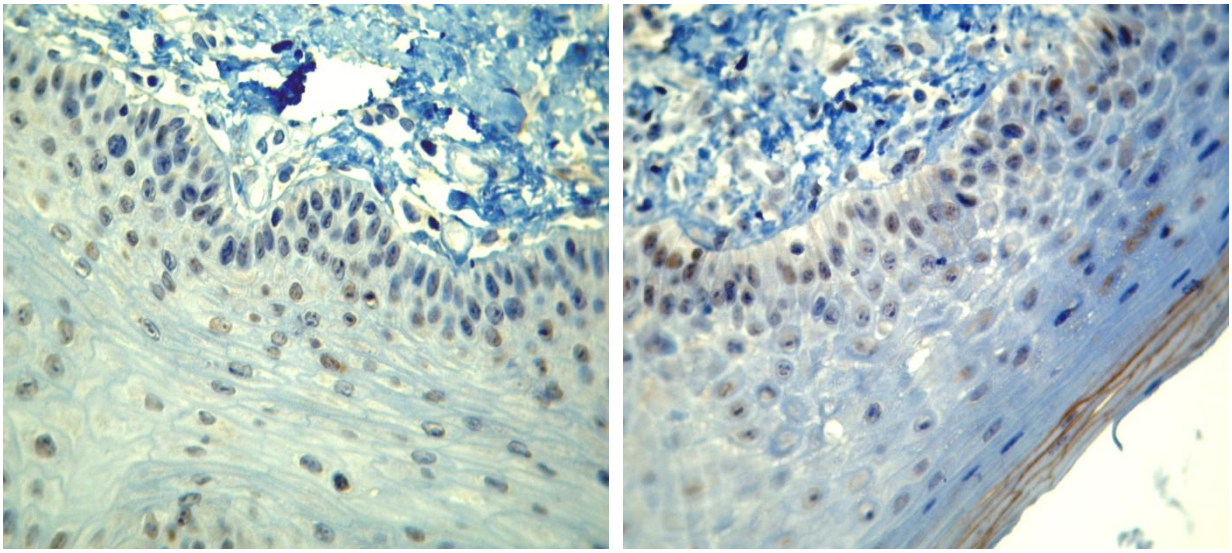
APÊNDICE B – Fotomicrografias (grupo-controle)



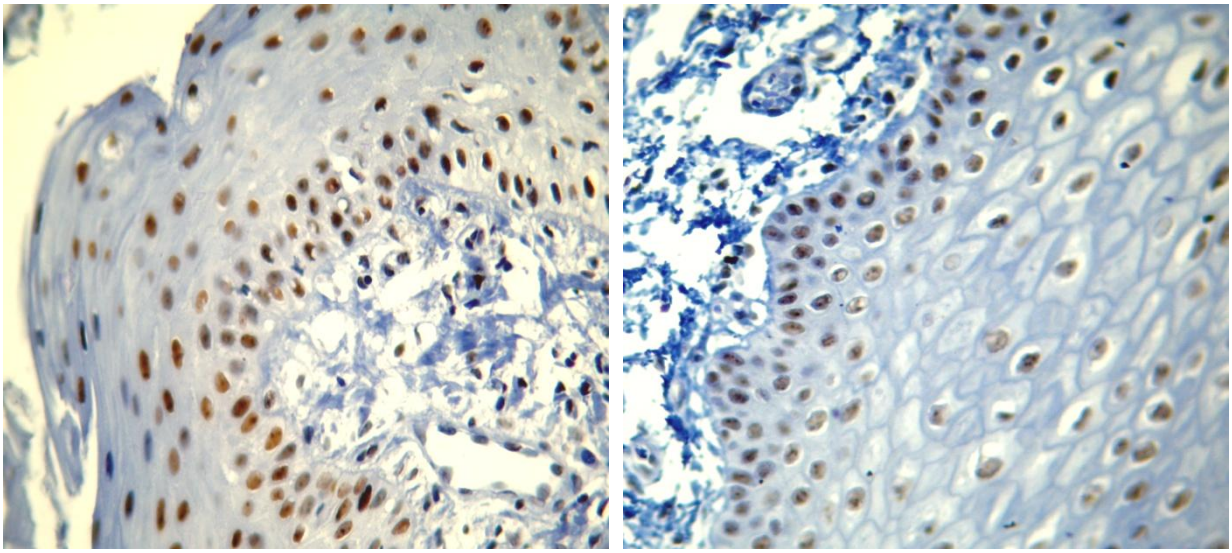
Figuras 1 e 2: Hiperplasia fibroepitelial evidenciando epitélio com áreas de hiperceratose e hiperplasia irregular das projeções epiteliais (HE, ~40X e ~100X).



Figuras 3 e 4: Hiperplasia fibroepitelial. Marcação do anticorpo anti-DNMT1 no epitélio (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

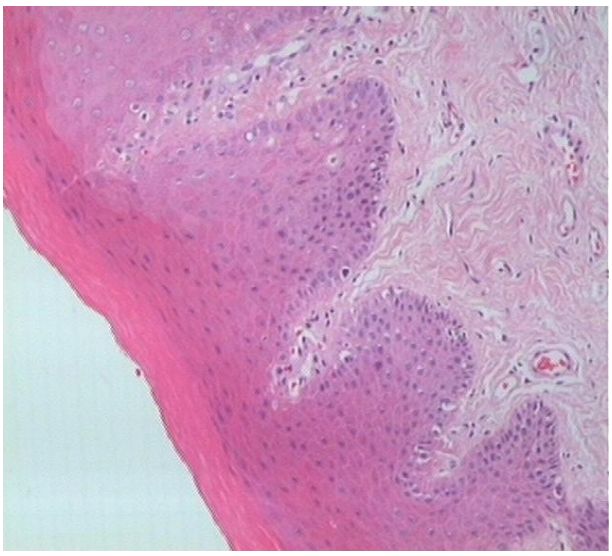
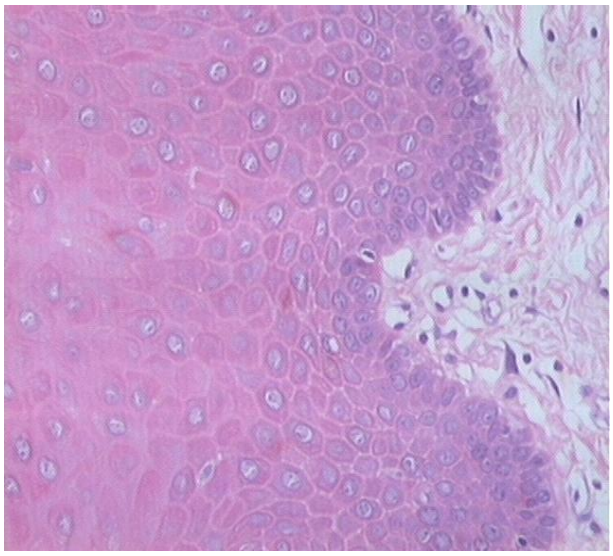


Figuras 5 e 6: Hiperplasia fibroepitelial. Marcação do anticorpo anti-DNMT3a no epitélio (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

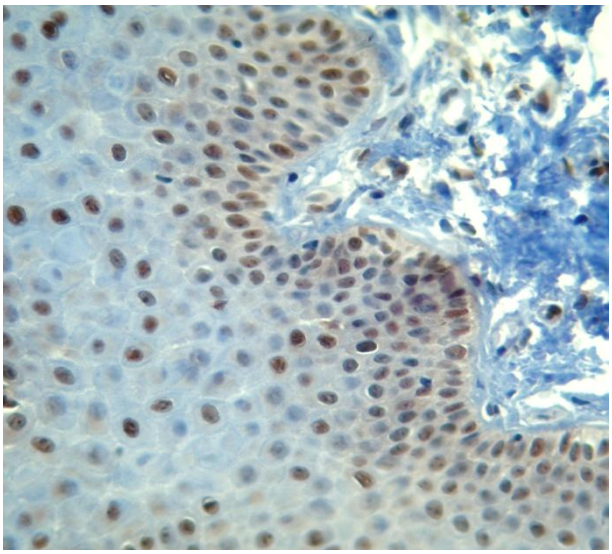
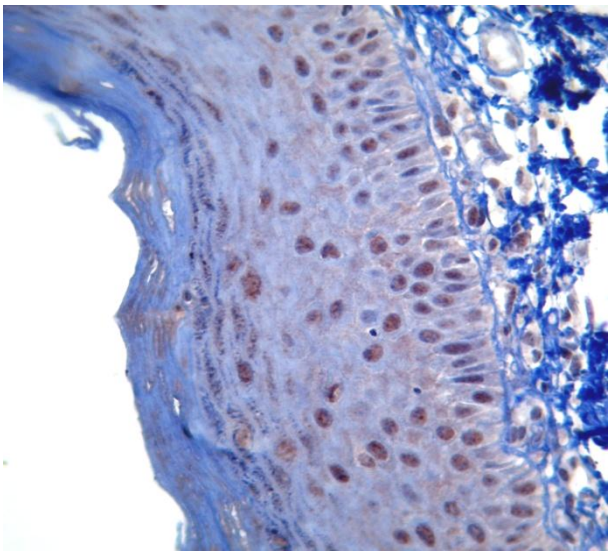


Figuras 7 e 8: Hiperplasia fibroepitelial. Marcação do anticorpo anti-DNMT3b (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

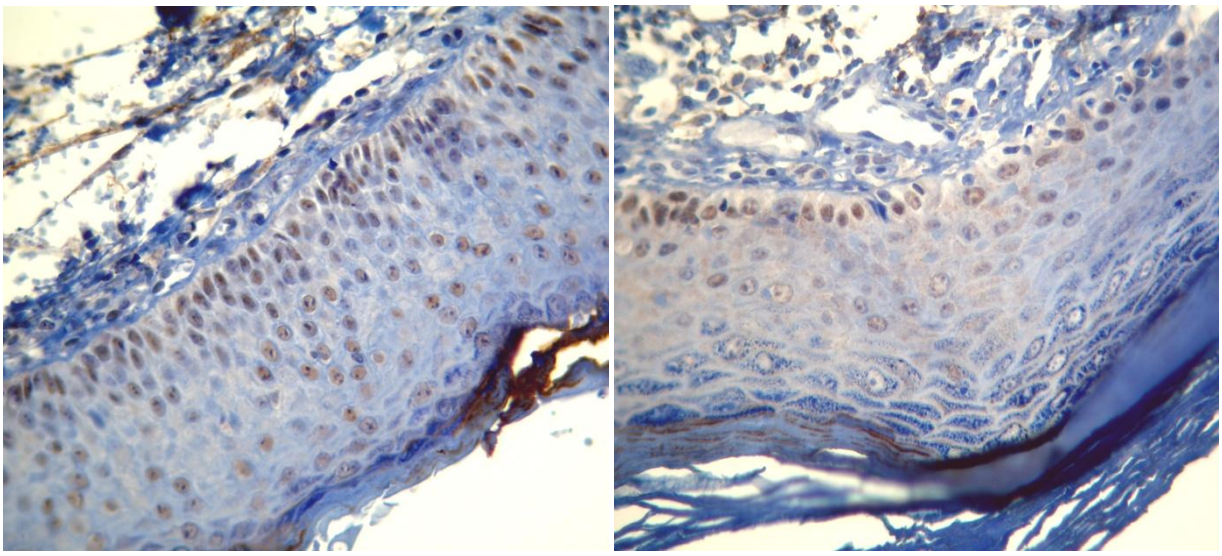
APÊNDICE C – Fotomicrografias (leucoplasias sem displasia)

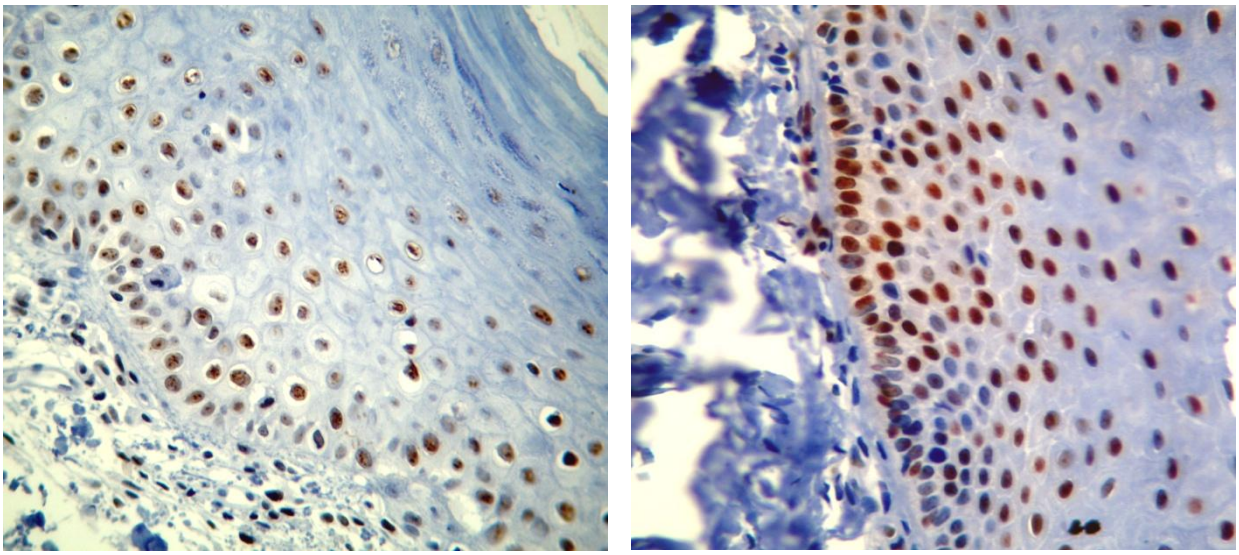
Figuras 9 e 10: Leucoplasia sem displasia. Observa-se tecido epitelial apresentando hiperceratose e acantose (HE, ~100X e ~200X).

Figuras 11 e 12: Leucoplasia sem displasia. Marcação do anticorpo anti-DNMT1 (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

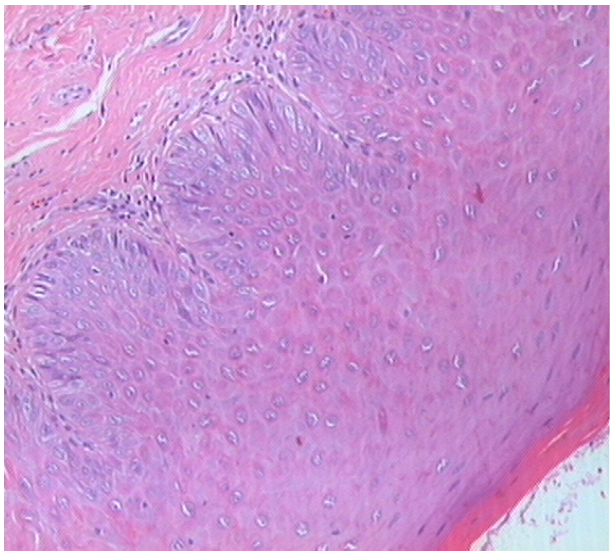
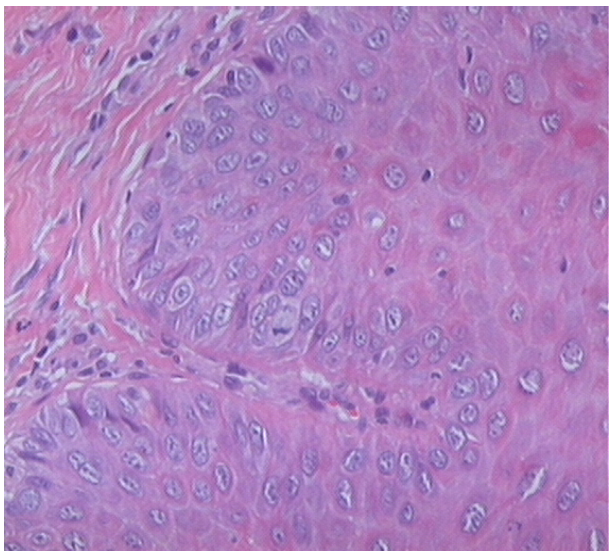


Figuras 13 e 14: Leucoplasia sem displasia. Marcação do anticorpo anti-DNMT3a (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

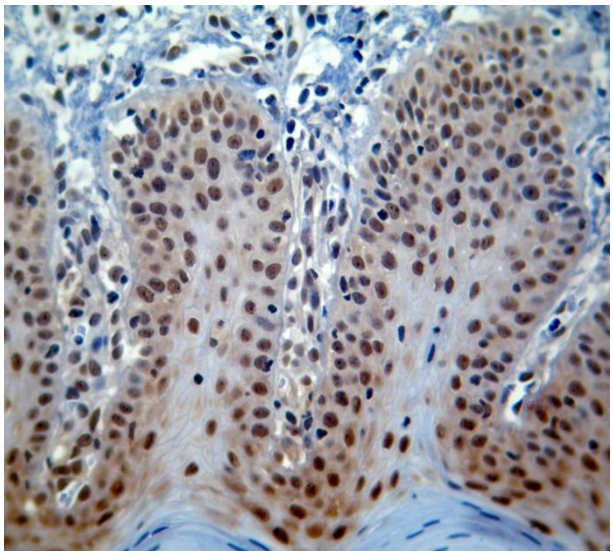
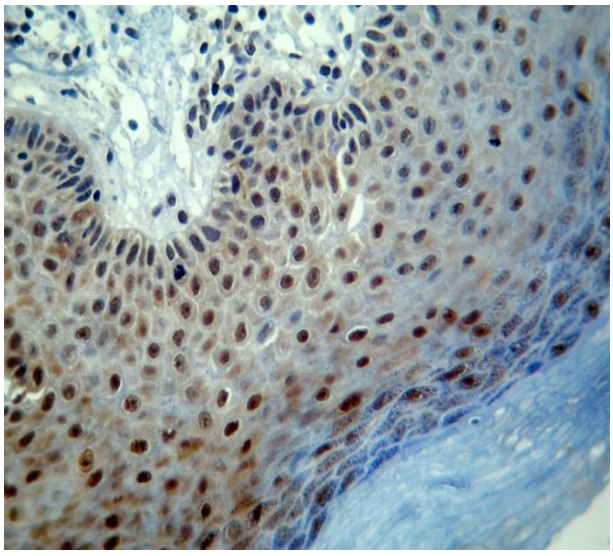


Figuras 15 e 16: Leucoplasia sem displasia. Marcação do anticorpo anti-DNMT3b ((núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

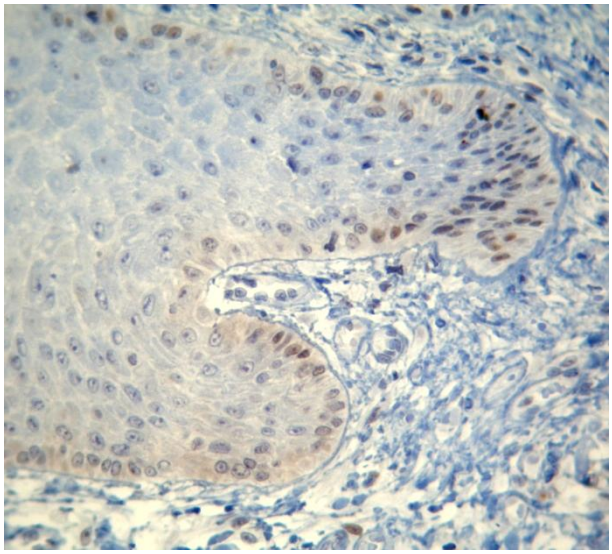
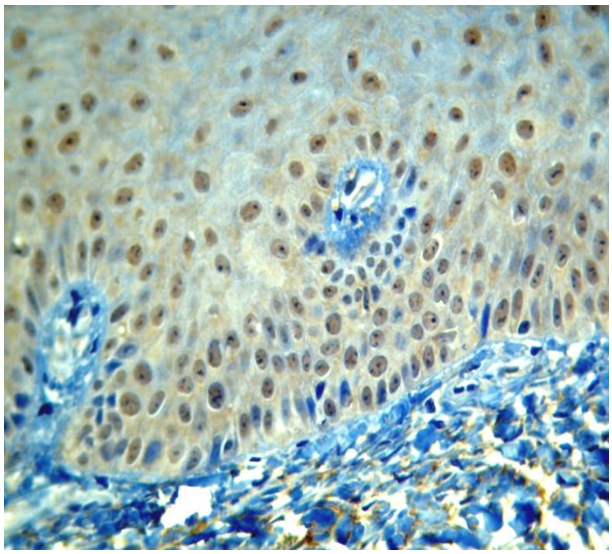
APÊNDICE D – Fotomicrografias (leucoplasias com displasia)

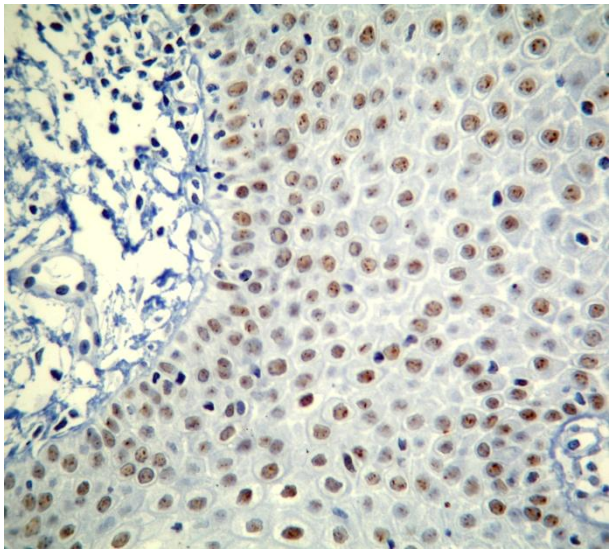
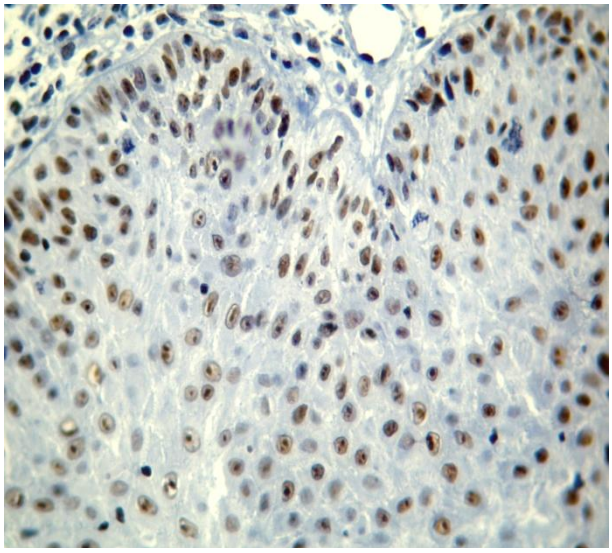
Figuras 17 e 18: Leucoplasia com displasia, demonstrando projeções epiteliais em forma de gota, aumento do número de figuras de mitose, pleomorfismo celular e nuclear e hipercromatismo nuclear (HE, ~100X e ~200X).

Figuras 19 e 20: Leucoplasia com displasia. Marcação do anticorpo anti-DNMT1 (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

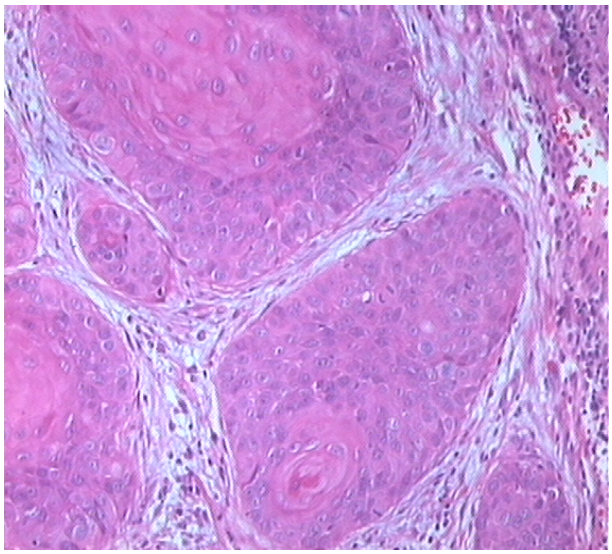
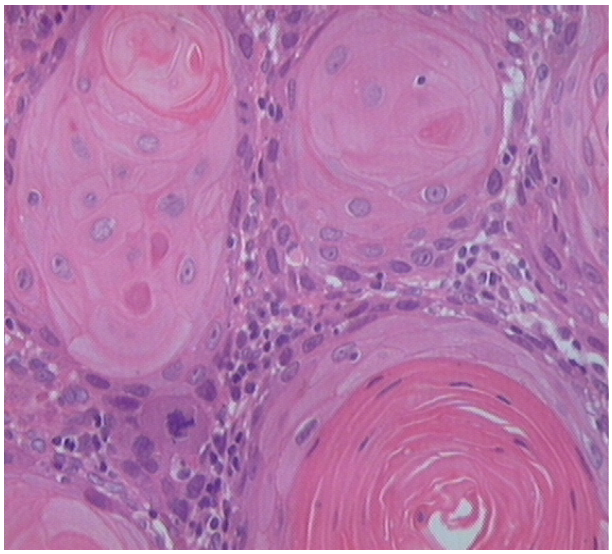



Figuras 21 e 22: Leucoplasia com displasia. Marcação do anticorpo anti-DNMT3a (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

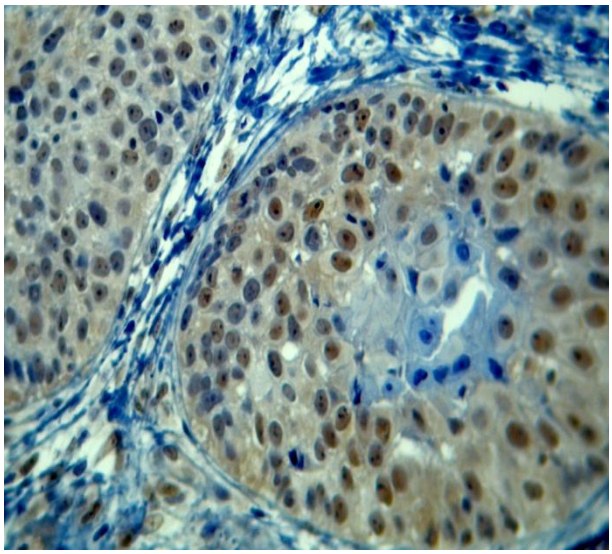
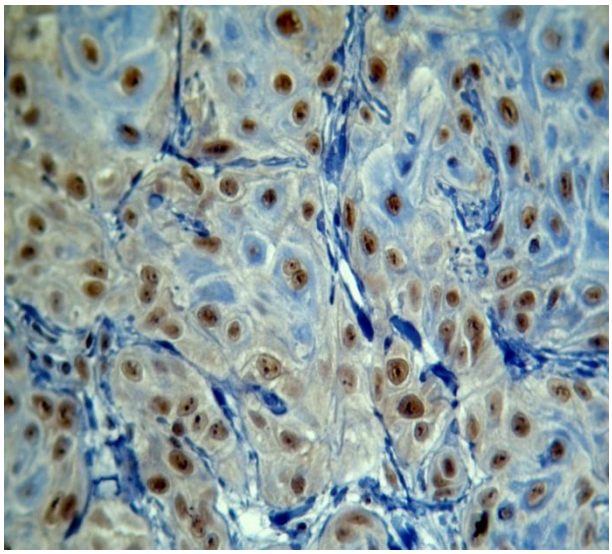



Figuras 23 e 24: Leucoplasia com displasia. Marcação do anticorpo anti-DNMT3b (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

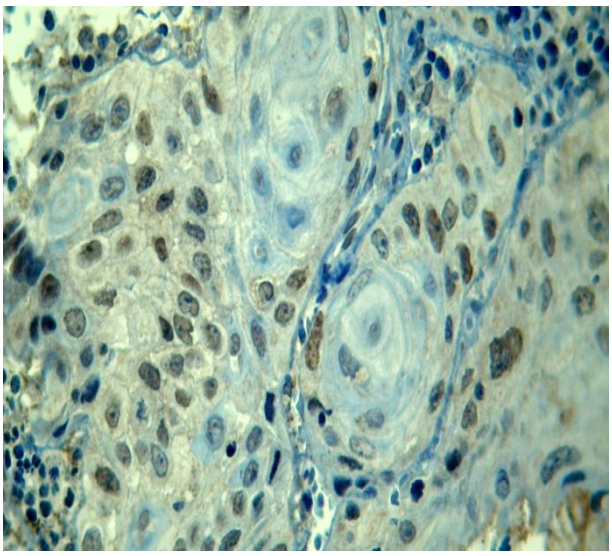
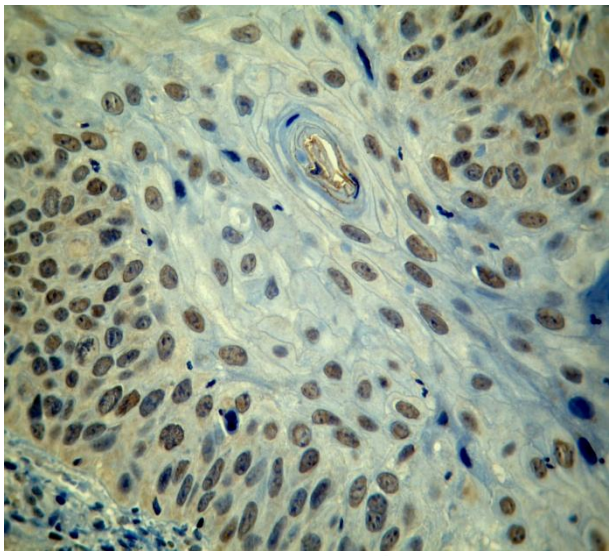
APÊNDICE E – Fotomicrografias (CEC grau I)

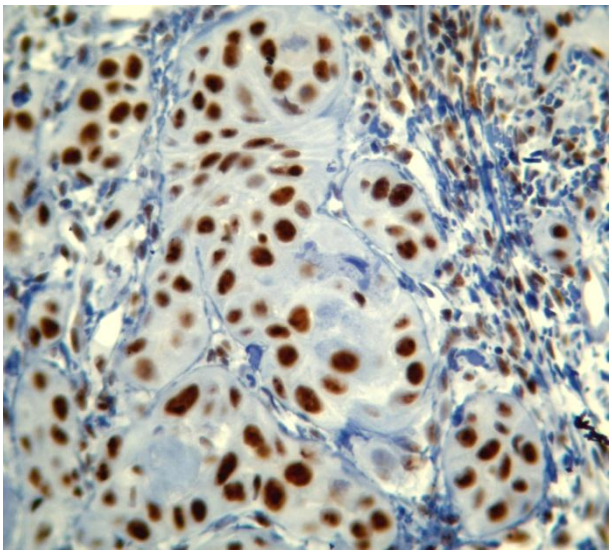
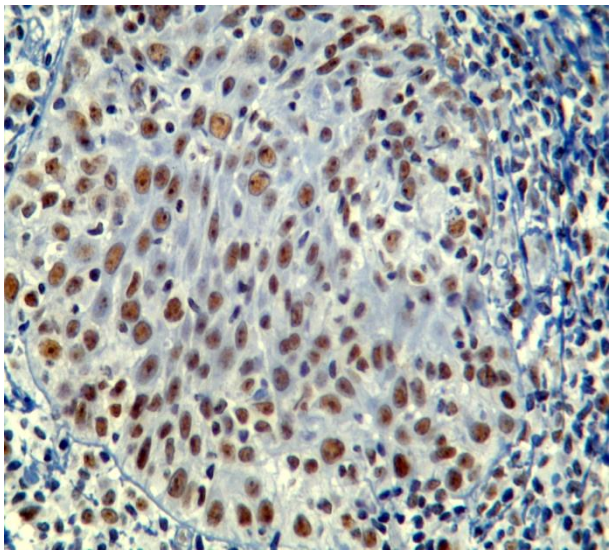
Figuras 25 e 26: CEC grau I. Observar proeminente ceratinização, poucas figuras mitóticas e mínimo pleomorfismo celular/nuclear (HE, ~100X e ~200X).

Figuras 27 e 28: CEC grau I. Marcação do anticorpo anti-DNMT1 (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

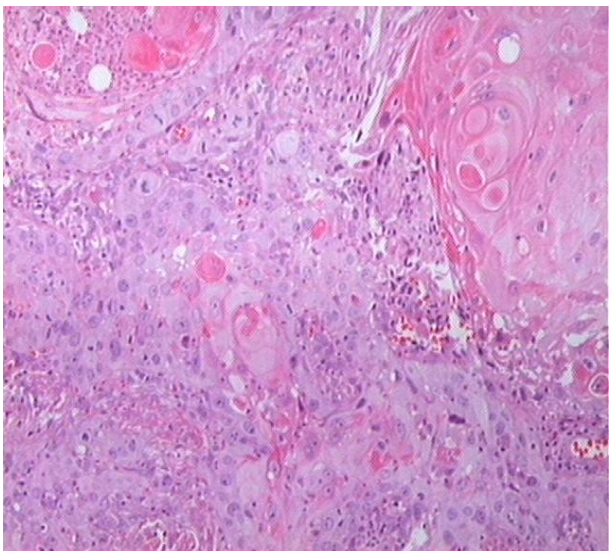
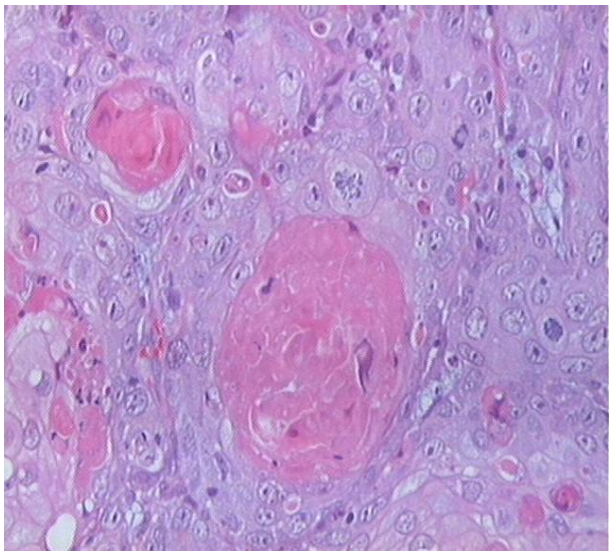



Figuras 29 e 30: CEC grau I. Marcação do anticorpo anti-DNMT3a (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

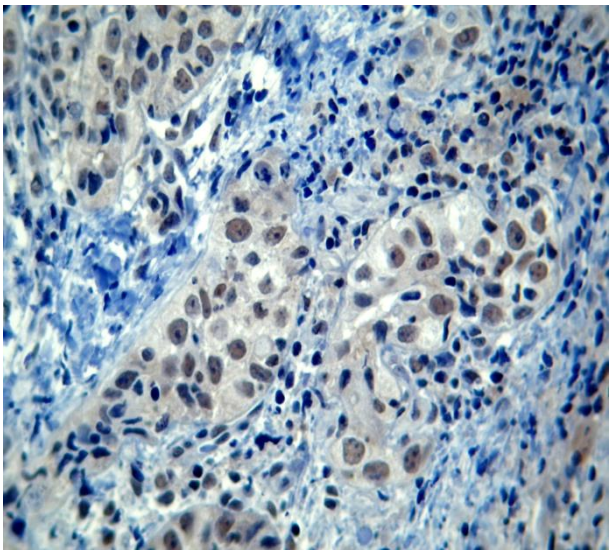
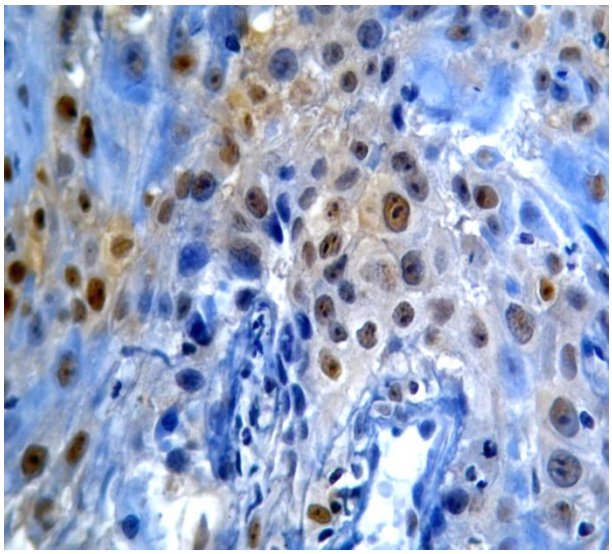



Figuras 31 e 32: CEC grau I. Marcação do anticorpo anti-DNMT3b (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

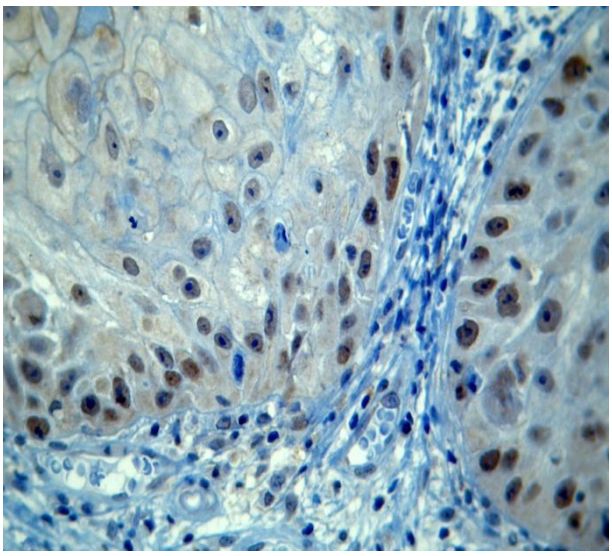
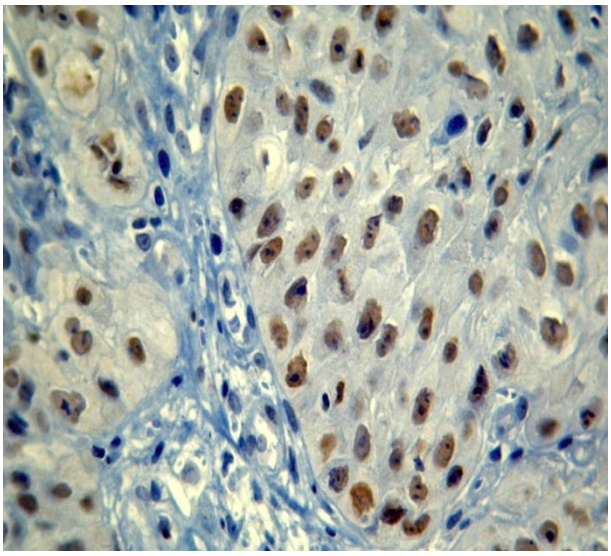
APÊNDICE F – Fotomicrografias (CEC grau II)

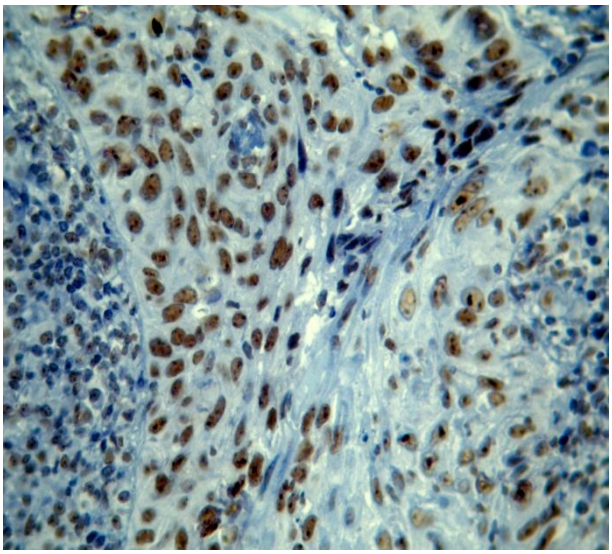
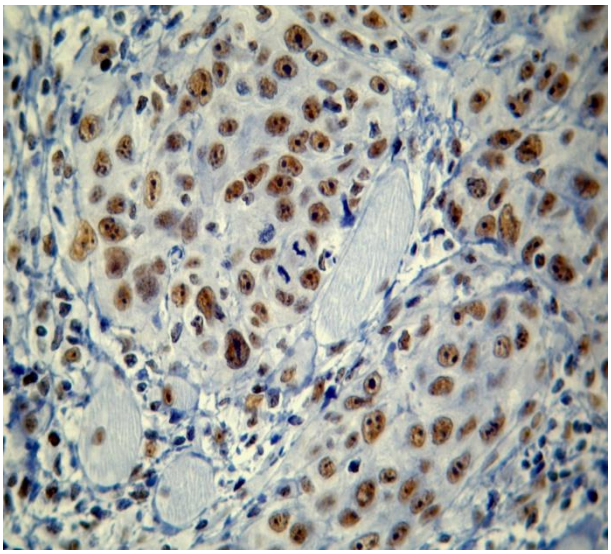
Figuras 33 e 34: CEC grau II, com menor grau de ceratinização, maior pleomorfismo celular/nuclear e com mitoses mais numerosas (HE, ~100X e ~200X).

Figuras 35 e 36: CEC grau II. Marcação do anticorpo anti-DNMT1 (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

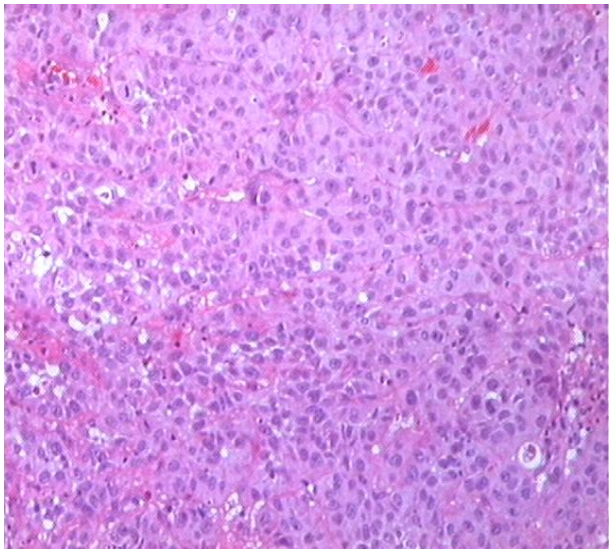
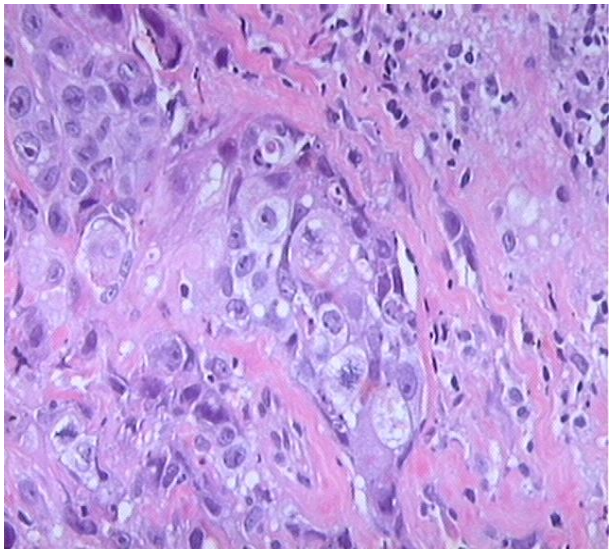



Figuras 37 e 38: CEC grau II. Marcação do anticorpo anti-DNMT3a (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

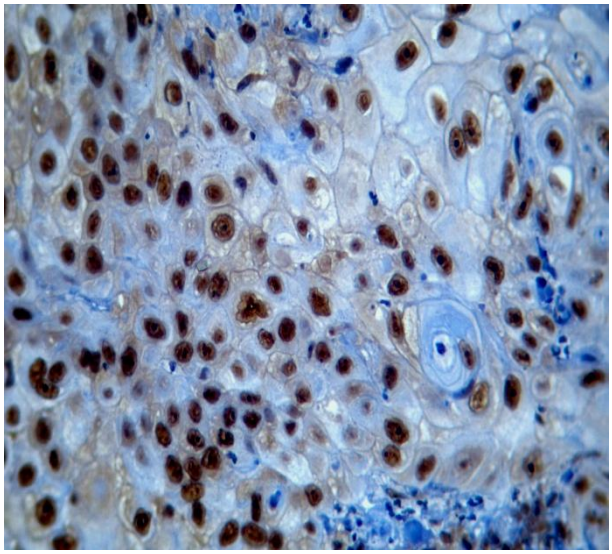
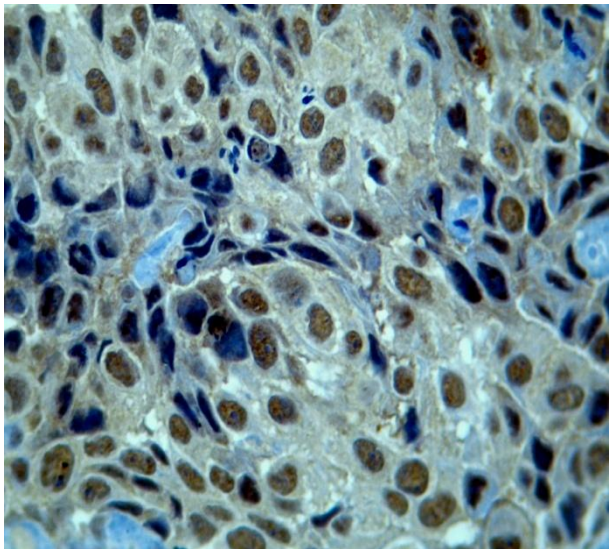



Figuras 39 e 40: CEC grau II. Marcação do anticorpo anti-DNMT3b (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

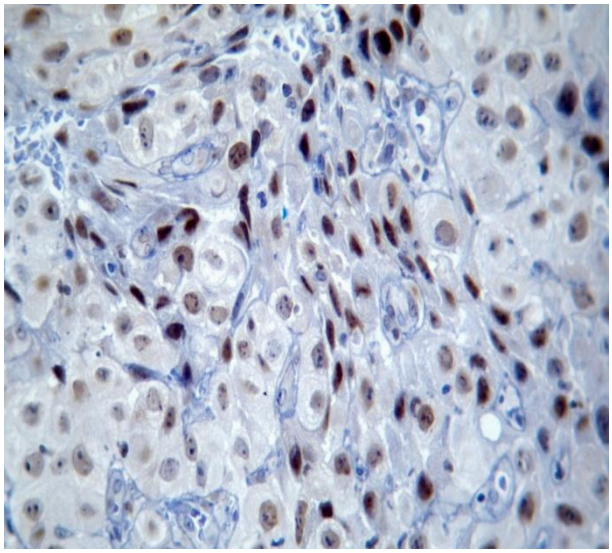
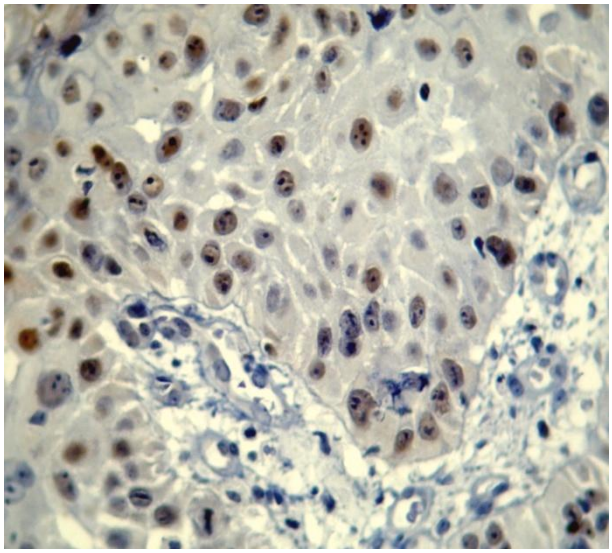
APÊNDICE G – Fotomicrografias (CEC grau III)

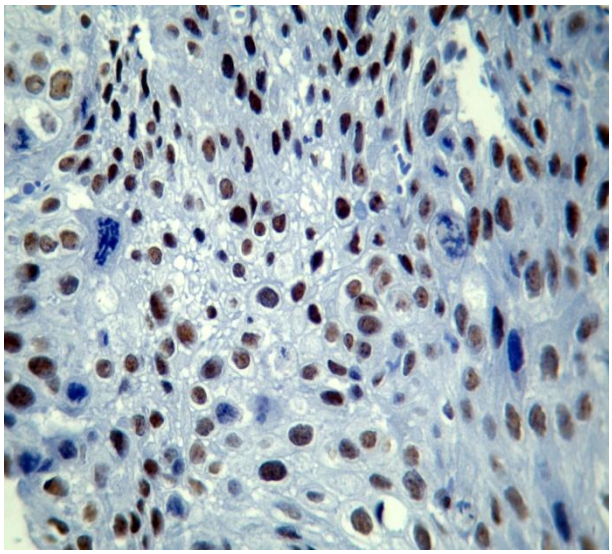
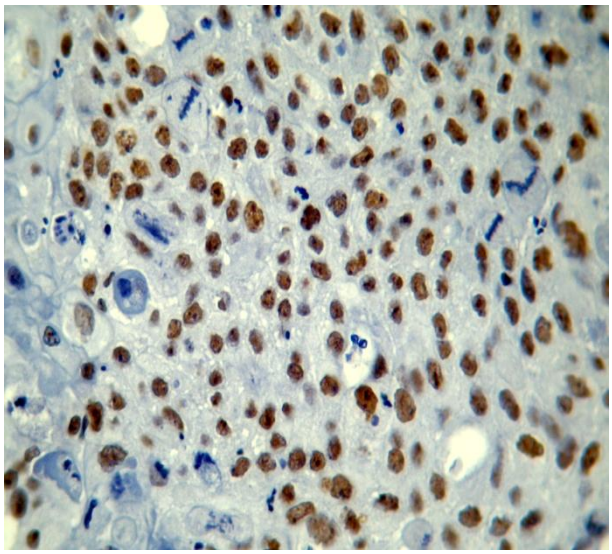
Figuras 41 e 42: CEC grau III evidenciando numerosas mitoses atípicas, ceratinização rara e pleomorfismo celular/nuclear evidente (HE, ~100X e ~200X).

Figuras 43 e 44: CEC grau III. Marcação do anticorpo anti-DNMT1 (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

Figuras 45 e 46: CEC grau III. Marcação do anticorpo anti-DNMT3a (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

Figuras 47 e 48: CEC grau III. Marcação do anticorpo anti-DNMT3b (núcleos corados em marrom), contra-corado com hematoxilina de Harris (núcleos corados em azul); ~200X.

Anexos

Anexo A – Carta de aprovação pela Comissão Científica e de Ética da Faculdade de Odontologia - PUCRS

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 03 de dezembro de 2007

O Projeto de: Tese

Protocolado sob nº:	0093/07
Intitulado:	Expressão imunoistoquímica das enzimas DNMT1, DNMT3A e DNMT3B em leucoplasiais e carcinomas de células escamosas bucais
Pesquisador Responsável:	Profa. Dra. Fernanda Gonçalves Salum
Pesquisadores Associados:	Filipe Ivan Daniel
Nível:	Doutorado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *30 de novembro de 2007.*

Este projeto deverá ser imediatamente encaminhado ao CEP/PUCRS

Profa. Dra. Marília Gerhardt de Oliveira
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

Av. Ipiranga, 6681, Prédio 06 sala 209
Porto Alegre /RS – Brasil – Cx. Postal:1429
90619-900

Fone/Fax: (51) 3320-3538
e-mail: odontologia-pg@pucrs.br

Anexo B – Carta de aprovação pelo Comitê de Ética em Pesquisa - PUCRS

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

Ofício 1537/07-CEP

Porto Alegre, 26 de dezembro de 2007.

Senhor(a) Pesquisador(a)

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 07/04045, intitulado: "**Expressão imunoistoquímica das enzimas DNMT1, DNMT3a e DNMT3b em leucoplasias e carcinomas de células escamosas bucais".**

Sua investigação está autorizada a partir da presente data.

Relatórios parciais e final da pesquisa devem ser entregues a este CEP.

Prof. Dr. José Roberto Goldim
COORDENADOR DO CEP-PUCRS

Ilmo(a) Sr(a)
Profa Fernanda Gonçalves Salum
N/Universidade

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