

UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Thiago Fonseca Silva

Associação entre fatores ambientais, genéticos e epigenéticos na
leucoplasia bucal: ênfase na metilação do gene *p16_{CDKN2A}*, infecção pelo
HPV e polimorfismo do gene DNMT3B (C46359T)

Montes Claros
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metilação do gene *p16_{CDKN2A}*, infecção pelo HPV e polimorfismo
do gene DNMT3B (C46359T)

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Orientador: Prof. Dr. André Luiz Sena Guimarães
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Meus pais Antônio Lúcio da Silva e Santa Helena Fonseca Silva e

meus irmãos Ângelo Fonseca Silva e Nayara Fonseca Silva.

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“...Temos o destino que merecemos. O nosso destino está de acordo com os nossos méritos...”

“...A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original...”

Albert Einstein

RESUMO

Alterações genéticas e epigenéticas estão associadas ao desenvolvimento e comportamento do câncer. A metilação do DNA foi associada a diversos tipos de neoplasias malignas a partir da alteração da expressão de diversos genes, entre eles o gene *p16_{CDKN2A}*. Modificações no padrão da expressão desse gene podem gerar alterações na checagem do ciclo celular, determinando ou potencializando os eventos relacionados ao câncer. Outro fator atualmente relacionado a modificações no padrão de expressão do gene *p16_{CDKN2A}* é a infecção pelos vírus HPV. Esse presente estudo correlacionou eventos genéticos, epigenéticos e ambientais em amostras de pacientes portadores de Leucoplasia Bucal (LB). Foram analisadas possíveis diferenças relacionadas ao status de metilação do gene *p16_{CDKN2A}*, o polimorfismo genético do DNMT3B (C46359T), à infecção pelos HPV 16/18 e a expressão tecidual das proteínas DNMT1, DNMT3B e P16 nas lesões de LB ($n = 48$) e em amostras de mucosa bucal normal (MB) ($n = 24$), utilizadas como grupo de comparação. Espécimes parafinados foram submetidos a cortes histológicos e processos de extração do DNA para avaliações morfológicas, reações de imuno-histoquímica e reações de PCR-FRLP e PCR-MSP. Os resultados deste trabalho mostraram que a presença do HPV 16/18 e a metilação do gene *p16_{CDKN2A}* foram associados à LB ($p = 0.001$). Também foi percebido um aumento significativo da expressão da proteína DNMT1 no grupo LB ($p = 0.001$) comparado ao grupo controle. Entretanto, nem a infecção pelo HPV 16/18, nem o polimorfismo DNMT3B (C46359T) associaram-se ao status de metilação do gene *p16_{CDKN2A}*. Da mesma forma, não foi observada associação entre infecção pelo HPV 16/18 e o polimorfismo DNMT3B (C46359T) com a expressão tecidual das proteínas DNMT1, DNMT3B e P16. Os nossos resultados sugerem que a infecção pelo HPV 16/18, a metilação do gene *p16_{CDKN2A}* e a expressão da proteína DNMT1 podem estar associadas à gênese e malignização das lesões de LB.

Palavras-chave: Leucoplasia bucal. Carcinogenese bucal. Polimorfismo genético. Metilação. Imunoistoquímica. Gene *p16*. DNMT3B. HPV. Lesão cancerizáveis

ABSTRACT

Genetic and epigenetic alterations are associated with the development and behavior of cancer. DNA methylation was associated with several types of malignancies by altering the expression of several genes, including gene *p16_{CDKN2A}*. Changes in the expression pattern of this gene may cause changes in cell cycle check and establishing or empowering the events related to cancer. Another factor currently linked to changes in the pattern of gene expression *p16_{CDKN2A}* is infection by the HPV virus. The present study correlated genetic events, epigenetic and environmental samples from patients with oral leukoplakia (OL). We analyzed possible differences related to the gene *p16_{CDKN2A}* methylation status of, to DNMT3B (C46359T) polymorphism, to infection with HPV 16/18 and tissue expression of proteins DNMT1, DNMT3B and P16 in the lesions of OL (n = 48) and samples of normal oral mucosa (OM) (n = 24), used as a comparison group. Paraffin specimens were subjected to histological and DNA extraction procedures for morphological, immunohistochemical reactions and reactions FRLP-PCR and MSP-PCR. These results showed that the presence of HPV 16/18 and *p16_{CDKN2A}* methylation of the gene were associated with OL (p = 0.001). Was also realized a significant increase in DNMT1 protein expression in the OL group (p = 0.001) compared to OM. However, neither HPV 16/18, or DNMT3B (C46359T) polymorphism were associated with gene *p16_{CDKN2A}* methylation status of. Likewise, no association was observed between HPV 16/18 and DNMT3B (C46359T) polymorphism with the tissue expression of proteins DNMT1, DNMT3B and P16. Our study suggests that infection with HPV 16/18, methylation of the gene *p16_{CDKN2A}* and DNMT1 protein expression may be associated with the genesis and malignant lesions of OL.

Key-words: Oral leukoplakia. Oral carcinogenesis. Genetic polymorphism. Methylation. Immunohistochemistry. *p16* gene. DNMT3B. HPV. Premalignant lesions.

LISTA DE ABREVIATURAS

DNA	Ácido desoxirribonucléico
CCECP	Carcinoma de células escamosas de cabeça e pescoço
CDK	Quinase dependente de ciclina
CpG	Dinucleotídeo citosina-guanina
DNMT	DNA-metiltransferase
DNMT1	DNA-metiltransferase-1
DNMT3B	DNA-metiltransferase-3B
HPV	<i>Papiloma Virus Humano</i>
LB	Leucoplasia bucal
MB	Mucosa Bucal
MSP	PCR de metilação específica
Pb	Pares de bases
PCR	Reação em cadeia da polimerase
pRB	Proteína do retinoblastoma
RFLP	Polimorfismo de comprimento de fragmentos de restrição
SAH	S-adenosil-metionina
SAM	S-adenosil-hemocisteína
Taq DNA polimerase	<i>Thermus aquaticus</i> DNA polimerase

LISTA DE SIGLAS

μL	Microlitro
μm	Micrômetro
Ng	Nanograma
Pmol	Picomol

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1 INTRODUÇÃO

1.1 Leucoplasia bucal

As primeiras descrições para o termo leucoplasia revelaram uma lesão cuja definição ou descrição clínica era de uma área esbranquiçada sobre as mucosas da cavidade bucal e que não poderia ser removida por raspagem (1,2). Atualmente, a Organização Mundial da Saúde (OMS) define a leucoplasia bucal (LB) como sendo a principal lesão cancerizável da boca, apresentando-se como uma placa ou mancha branca, não destacável e que não poderia ser diagnosticada como nenhuma outra lesão específica (2-5).

As leucoplasias podem se apresentar sob vários aspectos clínicos de colorações diferenciadas com componentes leucoplásicos (esbranquiçadas), eritoplásicos (avermelhadas) e leucoeritoplásicos (esbraquiçadas e avermelhadas) (1,4,6). Também podem ser classificadas em homogêneas ou não-homogêneas(7). As leucoplasias homogêneas são aquelas que exibem uma superfície totalmente branca, de textura lisa ou verrucosa (7,8). As leucoplasias não homogêneas apresentam componentes brancos e eritematosos, podendo apresentar também superfície nodulares ou verrucosas (7,9).

Acredita-se que a LB se origina de reações inespecíficas do epitélio em resposta a vários estímulos endógenos e exógenos (10-12), podendo ser associada a uma ampla gama de agentes físicos, químicos e biológicos, tais como radiação ultravioleta, álcool, fumo, má higiene bucal e infecções viróticas, além de alterações genéticas individuais (4,13,14).

As alterações genéticas associadas ao surgimento e progressão das leucoplasias são de extrema importância e atualmente têm sido muito estudadas. Já são conhecidos alguns genes que, quando alterados, atuam de forma efetiva no processo de carcinogênese. Dentre eles encontram-se genes supressores de tumor e protooncogenes que podem atuar nas diversas fases da carcinogênese como a iniciação, promoção e progressão (15). Geralmente as primeiras alterações genéticas presentes na carcinogênese são mutações e deleções, sendo também encontradas alterações epigenéticas como metilações (16,17).

Histologicamente as leucoplasias são denominadas hiperceratoses e classificadas de acordo com o grau de acometimento celular. São classificadas como hiperceratose na ausência de atipia celular ou como atipia discreta, moderada ou grave na presença de alterações celulares (18). Dentre as alterações celulares observadas na LB, estão o hipercromatismo nuclear, inversão e perda de polarização das células da camada basal, pleomorfismos, alteração de estratificação e alteração na proporção núcleo/citoplasma (19). Estes variados graus de displasia epitelial juntamente com estudos clínicos-epidemiológicos foram capazes de indicar a natureza cancerizável das leucoplasias (4,13,20).

Diversos estudos têm enfatizado o potencial de malignização da leucoplasia bucal (4,19). Tal fato foi inicialmente sugerido com base em sua relação direta com carcinomas de cavidade bucal, nas características clínicas das lesões e na alta frequência de epitélios com graus de atipia celulares variados. Entretanto, uma dificuldade estaria em se identificar quais lesões teriam um risco potencial de se transformar em câncer, já que a avaliação dos parâmetros citados e sua reprodução não puderam predizer com absoluta confiabilidade a evolução das lesões (5,20-22). Um exemplo importante é retrado em um estudo o qual 7% das biópsias de LB apresentavam carcinomas em alguma parte da lesão que não seria selecionada para a biópsia (23).

Marcadores biológicos têm sido utilizados para determinar quais leucoplasias bucais apresentam uma maior probabilidade de transformação maligna. Atualmente são conhecidas várias alterações genéticas associadas às etapas iniciais da progressão do câncer, dentre estas incluem a expressão aumentada ou diminuída de oncogenes e genes supressores tumorais, tais como *EGFR*, *TP53*, *Rb*, *p65*, *COX-2*, *p16*, *CCND1*, e *PTEN* (24,25).

Estas alterações foram associadas com etapas distintas da progressão a carcinoma oral (26-28). Particularmente, alterações no gene supressor tumoral *TP53* representam um evento importante em estágios iniciais da progressão, ao passo que mutações no gene *p16*, um inibidor da quinase ciclina-dependente (proteína reguladora do ciclo celular), são consideradas alterações tardias durante a progressão tumoral (27,29-33).

Pesquisas realizadas no campo da biologia molecular refletem a grande importância de se estabelecer de forma precoce o diagnóstico de lesões neoplásicas, consequentemente, promovendo melhorias para o tratamento e prognóstico dos pacientes (30,34).

1.2 Gene *p16_{CDKN2A}*

O gene supressor de tumor *p16_{CDKN2A}*, também conhecido como *INK4A*, *CDK41* ou *MTS1*, localizado na região 9p21, é composto por três exons, os quais codificam uma proteína de 15,6 kDa com 158 aminoácidos (33). O gene codifica a proteína conhecida como P16, pertencente a uma família de inibidores de cinases dependentes de ciclinas (CDC) responsáveis pelo controle do ciclo celular (33,35). (Figura 1)

No ciclo celular normal, a P16 possui habilidade bioquímica de se ligar ao complexo ciclina D-CDK4 ou 6, inibindo a fosforilação da pRb (proteína do retinoblastoma). Com isso, tem-se a finalização dos processos do “ponto de checagem” G1-S do ciclo celular (36,37).

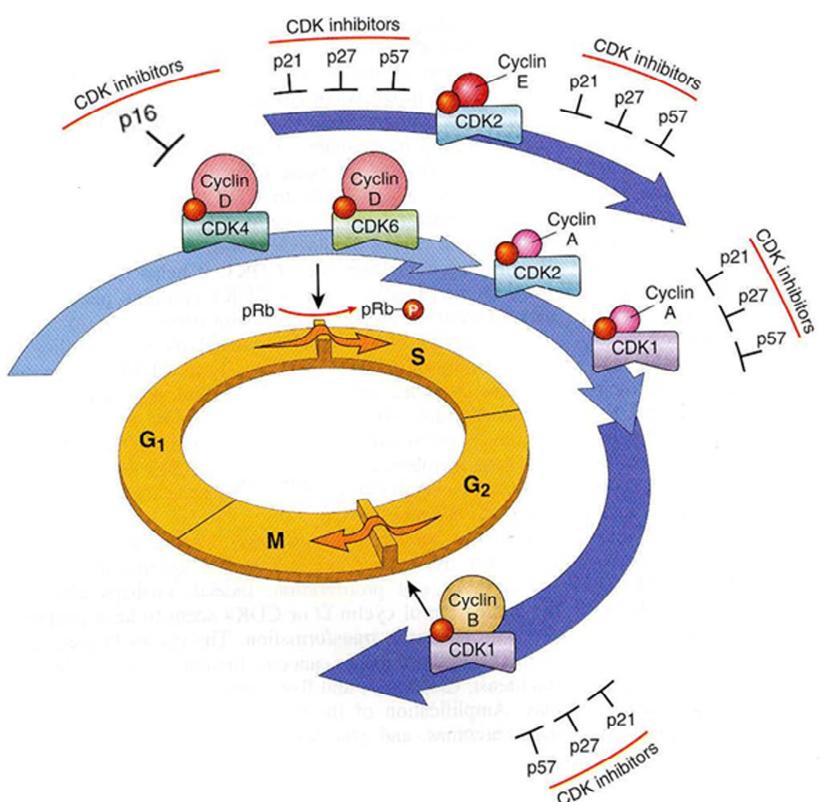


Figura 1: Proteína P16 e o ciclo celular.
Fonte: Adaptado de Robbins, 1994 (38).

No ciclo de divisão celular, a super-expressão da P16 inibe a progressão pela fase G1 do ciclo celular, através da sua ligação ao complexo CDK4 ou 6/ciclina D. Desse modo, ocorre o bloqueio da atividade quinase da enzima. (33,39,40).

A perda funcional do P16 resulta na proliferação celular anormal pela perda da função de checagem do ciclo celular, o que permite que as células progridam, irrestritamente, para a fase S (26,35,41,42). A inativação ou hipoexpressão desse gene pode gerar uma proliferação aberrante e potencializa o processo tumoral, resultando em rompimento do controle do ciclo celular, com consequente desenvolvimento de tumor, o que está associado aos fenômenos carcinogênicos (26,41).

A perda da expressão do P16 está associada a varios mecanismos, incluindo mutações e deleções homozigóticas dentro da região codificante do gene e metilações (40). A hipermetilação da região promotora do gene resulta em um silenciamento do gene mediado epigeneticamente, com consequente repressão ou inativação gênica (43,44).

A inativação do gene *p16CDKN2A* além de estar associada a diversos tipos de cânceres, este achado também tem sido frequentemente reportada em lesões cancerizáveis, sugerindo um papel precoce importante desse gene no desenvolvimento do câncer (26,27,32).

1.3 Metilação

As alterações epigenéticas são modificações caracterizadas pela alteração na estrutura da cromatina, porém não afetam a sequência dos nucleotídeos e são herdáveis no genoma durante a divisão celular (45,46). Como não alteram a sequência do DNA, estas alterações são reversíveis (28).

A adição do radical metil (CH₃) em regiões específicas do DNA contendo, predominantemente, citosinas denomina-se metilação e essa modificação epigenética é responsável pelo controle da atividade gênica (47,48).

Em 1948, foi descoberta a “quinta base” do DNA, a 5-metilcitosina (49). A 5-metilcitosina é uma citosina metilada, na qual ocorreu a adição de um grupamento metil à estrutura da base. Porém, nem toda citosina presente no genoma pode ser metilada. Para que ocorra o processo de metilação é necessário que a citosina esteja na seqüência 5' - CG - 3', conhecido como dinucleotídeo CpG (28,43,45,50). Os dinucleotídeos CpG estão distribuídos heterogeneamente no genoma, sendo encontrados principalmente em regiões de DNA altamente repetitivo e heterocromatina (28,51).

O processo de metilação é catalisado por uma família de enzimas denominadas DNA-metiltransferases (DNMT). Para as DNMT transferirem um grupamento metil para o carbono 5 do anel da citosina, é preciso um doador de metil, o S-adenosilmetionina (AdoMet) (51-53). A DNMT catalisa a transferência do metil do AdoMet para o DNA resultando em um DNA metilado e em uma S-adenosilhomocisteína (AdoHcy), produto do AdoMet sem o metil (47,54). (Figura 2)

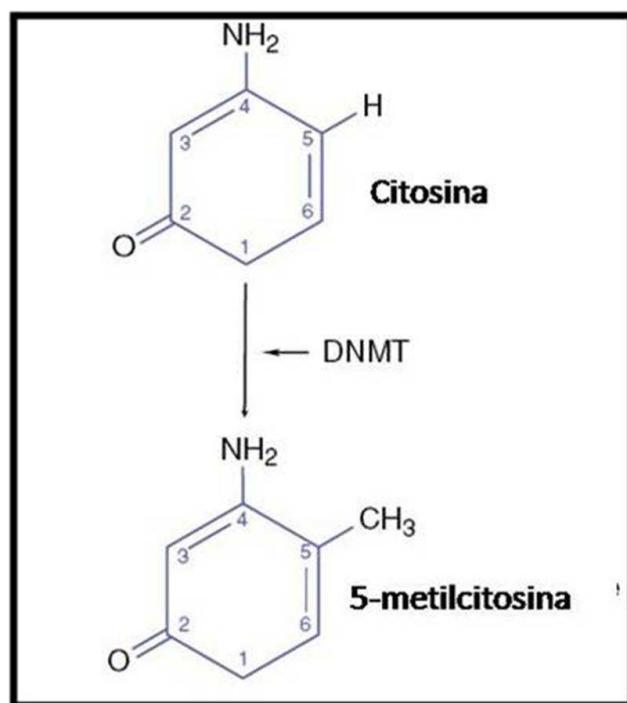


Figura 2: Esquema demonstrando o mecanismo de metilação do DNA.
Fonte: Adaptado de FAZZARI, 2004 (55).

1.4 DNA-Metiltransferases

As DNA metiltransferases (DNMTs) são um grupo de enzimas responsáveis pelos eventos de metilação do DNA. Atualmente são descritos três DNA-Metiltrasferases identificados em mamíferos, DNMT1, DNMT2, DNMT3, com suas respectivas isoformas. (56,57).

Os estudos sobre as funções das DNMTs apontam que a DNMT1 seria uma metiltransferase de manutenção, responsável pela metilação do DNA a partir de uma fita molde em células somáticas (58,59). A metilação de manutenção é responsável pela cópia dos padrões de metilação do DNA durante a divisão celular, tendo preferência por DNA hemimetilado. Assim, quando uma nova seqüência CpG é gerada pela replicação do DNA, um sítio hemimetilado é formado se o CpG da fita parental estiver metilado, servindo como substrato para a DNMT1 (57,60-62). (Figura 3)

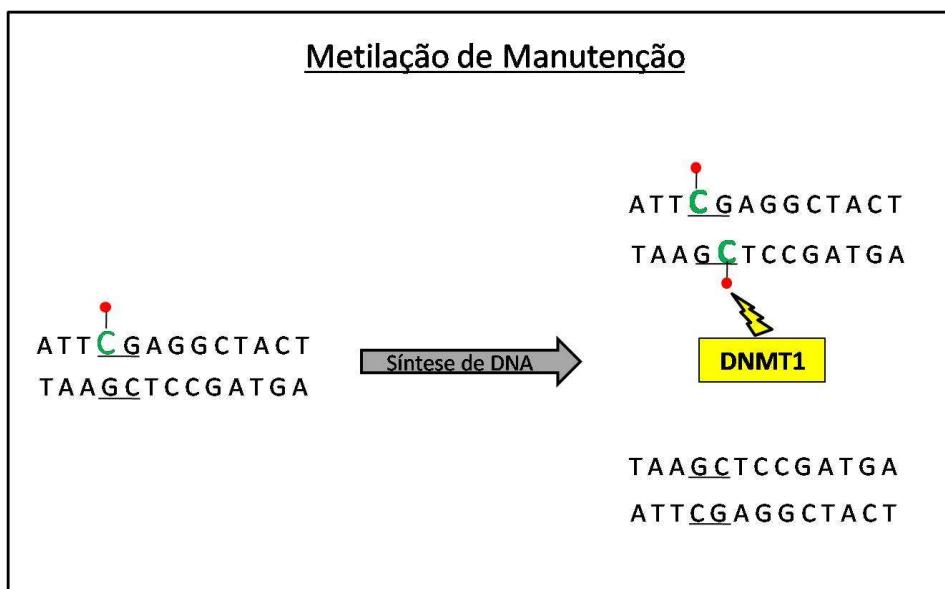


Figura 3: Representação esquemática demonstrando o mecanismo da metilação de manutenção do DNA.
Fonte: Construção pessoal.

A DNMT2 possui um domínio catalítico sem atividade em humano e, consequentemente, não apresenta capacidade de metilação (63).

Os fenômenos de metilação de regiões gênicas não metiladas é denominado metilação *de novo*. A metilação *de novo* é o processo pelo qual ocorre a metilação de citosinas

anteriormente não metiladas. Este processo é catalisado pelas DNMT3A e DNMT3B, as quais são responsáveis pela adição do radical CH₃ nas citosinas do DNA não-metilado (64-68). (Figura 4)

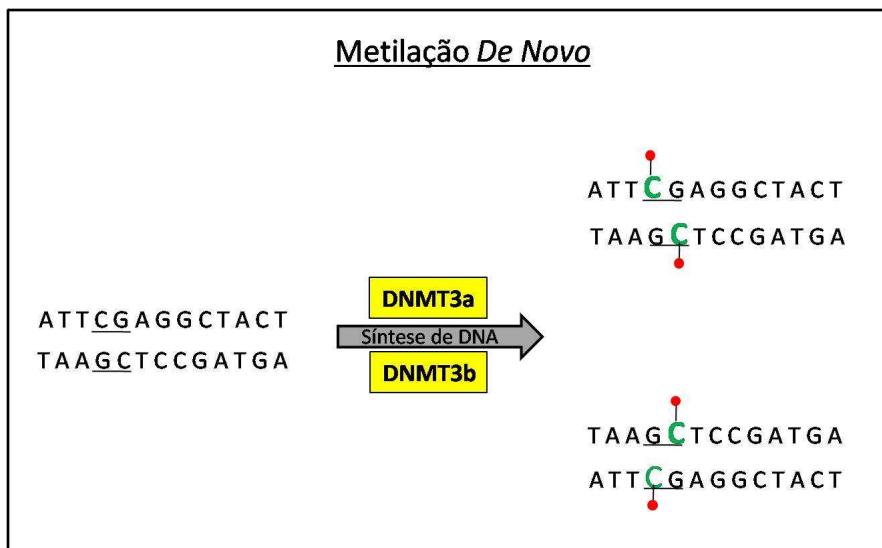


Figura 4: Representação esquemática demonstrando o mecanismo da metilação *de novo* do DNA.
Fonte: Construção pessoal.

1.5 Polimorfismo DNMT3B (C46359T)

Polimorfismos genéticos são alterações na sequencia do DNA que ocorrem em mais de 1% da população, que pode causar ou não alterações na expressão do gene acometido (69). Neste contexto vários estudos têm demonstrado que o alelo T do polimorfismo do gene DNMT3B (C46359T) está associado aos processos do câncer (70-72).

O gene DNMT3B está localizado no cromossomo humano 20q11.2. É composto por 23 exons e 22 íntrons, abrangendo cerca de 47 kb do DNA genômico. Esse gene exerce um importante papel no mecanismo da metilação *de novo* e, também, pode estar envolvido nos eventos relacionados com a carcinogênese (63,73,74). Um aumento na expressão do gene pode levar à uma modificação no padrão de metilação gerando fenômenos metilatórios aberrantes, favorecendo o desenvolvimento de diversos tipos de câncer (63,73,75,76).

O gene DNMT3B possui 13 polimorfismos descritos na sua região promotora e aproximadamente 345 polimorfismos em toda sequência (77). O polimorfismo de nucleotídeo único de transição C para T (C46359T) na região promotora -149bp- em relação ao sítio inicial de transcrição, conferindo um aumento de 30% na atividade promotora (70,72). Foi postulado que a variante T regula o gene DNMT3B, podendo levar a um aumento de sua expressão e, com isso, resultar em uma predisposição para metilações *de novo* aberrantes em ilhas CpG de genes supressores de tumor e genes de reparo (71,78,79). A relação entre as variantes polimórficas do gene DNMT3B (C46359T) ainda não foi estudada em lesões cancerizáveis.

1.6 Metilação e expressão gênica

Estudos moleculares demonstram que o maior número de citosinas na região CpG intensifica a metilação, podendo gerar um estado de hipo-expressão ou silenciamento gênico (80). A CpG metilada pode reprimir a transcrição diretamente pela inibição de fatores de transcrição ligados ao DNA, e também, indiretamente, recrutando proteínas que ativam a enzima histona deacetilase, responsável por possuir complexos repressores do DNA metilado (48,50,52,58,81). Embora as modificações derivadas da metilação da citosina sejam pouco freqüentes, elas podem ocasionar mutações (52,68).

O mecanismo preciso pelo qual a metilação reprime a transcrição não é bem estabelecido. Algumas teorias sugerem que a metilação interfere na ligação de fatores de transcrição em sequências regulatórias do DNA; outra teoria relacionada ao silenciamento gênico através da metilação é a modificação da estrutura da cromatina que bloqueia o acesso dos fatores de transcrição; outra hipótese seria através da afinidade de ligação do DNA metilado a proteínas que suprimem a expressão gênica (45,46,51,53,54,80,82).

O mecanismo de metilação pode estar envolvido tanto na causa como na consequência do processo carcinogênico (43). Além disso, a freqüência de metilações ou mutações genéticas variam entre os genes. Essas variações podem resultar em diferentes tipos tumorais e consequentemente no diagnóstico e prognóstico (47,68,83). Além da

hipermetilação, a hipometilação também pode estar relacionada aos processos de carcinogênese, através de alterações nos mecanismos de expressão gênica (26).

Metilações vêm sendo associadas ao desenvolvimento de cânceres através da inativação transcricional de genes supressores de tumor principalmente aqueles relacionados com o controle do ciclo celular (81,82,84). O padrão de metilação já foi associado a uma grande variedade de carcinomas incluindo o carcinoma de células escamosas de cabeça e pescoço (46,82,83).

1.7 Papiloma vírus humano

Os papilomavírus humanos constituem um grupo de vírus-DNA da família *Papillomaviridae* epiteliotrópicos capazes de induzir lesões de pele e/ou mucosa, associado à etiologia de diversas neoplasias benignas e malignas. (85,86).

Atualmente são conhecidos mais de 200 subtipos diferentes de HPV sendo que mais de 80 já foram identificados e classificados em HPV de alto e baixo risco, de acordo com sua associação a lesões malignas ou benignas e com sua homologia na sequência de DNA (85,87,88).

Em boca, pelo menos 25 tipos de HPV já foram identificados e classificados em baixo risco (HPV: 1, 2, 3, 4, 7, 10, 11, 13, 32, 40, 55, 57, 69, 72) e alto risco (HPV: 16, 18, 31, 33, 35, 45, 52, 58, 59, 73) (89-91).

Vários trabalhos na literatura relataram associação da infecção pelos HPV com neoplasias malignas. Os vírus HPV de alto risco (principalmente o subtipo 16 e o 18) têm sido identificados como fatores etiológicos dos cânceres de cérvix uterino, anogenitais e mais recentemente associados ao CCECP (87-90,92).

Os vírus HPV infectam os ceratinócitos na camada basal do epitélio estratificado escamoso e se replicam exclusivamente no núcleo das células do hospedeiro. Apesar do mecanismo

da expressão gênica e replicação viral não serem completamente elucidados, sabe-se que a expressão inicial de proteínas do vírus ocorre em estágios iniciais de diferenciação dos ceratinócitos. Seis proteínas não estruturais (E1, E2, E4, E5, E6 e E7) e duas proteínas do capsídeo (L1 e L2) são inicialmente transcritas na infecção e a interação entre algumas destas proteínas e as células do hospedeiro são responsáveis pela proliferação dos ceratinócitos (93,94). (Figura 5)

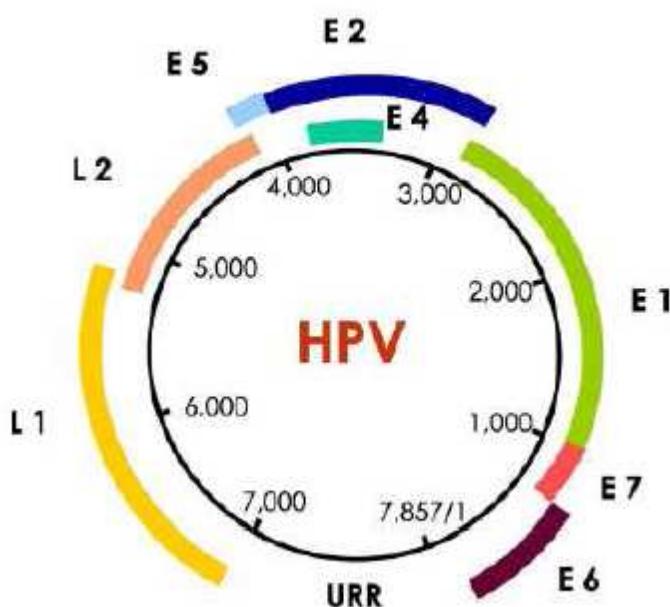


Figura 5: Representação esquemática do genoma do HPV.

Fonte: Muñoz, et al, 2006 (95).

Os genes virais E6 e E7 codificam proteínas com o mesmo nome responsáveis pelo potencial oncogênico do vírus. Tais proteínas fortemente associadas aos HPVs de alto risco formam complexos com algumas proteínas celulares, principalmente as responsáveis pela supressão tumoral, o que pode gerar uma inativação dos componentes controladores do ciclo celular, com consequentes transtornos no crescimento celular e nos processos de reparação do DNA, promovendo instabilidade genética, acúmulo de mutações e desenvolvimento de neoplasias (35,86,96,97). Estudos apontam que a presença das oncoproteínas E6 e E7 podem ser capazes de desregular a expressão de vários genes relacionados com o controle do ciclo-celular, como, *p53* e *p16_{CDKN2A}*. Além disso, foi demonstrado que estas proteínas podem contribuir de forma relevante para progressão

maligna através de indução de anormalidades de centrossomo levando à instabilidade genômica que pode gerar aneuploidia (10,86,87,89,91,93).

Atualmente, tem-se relacionado à presença dos HPV 16 e 18 com alterações epigenéticas associadas à carcinogênese bucal. Diversos estudos apontam um possível associação entre a presença do HPV e transtornos funcionais e genéticos do gene *p16_{CDKN2A}*, o que estabelece uma outra via potencial para a ocorrência dos fenômenos neoplásicos (10,35,86,91,96).

1.8 Carcinogênese

A transformação de um tecido normal em maligno é um processo multifatorial caracterizado por alterações genéticas, epigenéticas e fenotípicas que culminam na indiferenciação e imortalização celular (10,11). O início (gênese) do processo de cancerização envolve a ativação de vias de sinalização metabólicas que favorecem o crescimento celular e as características de sobrevida da célula (50,98,99).

A literatura retrata a carcinogênese como sendo um processo de múltiplas etapas que culminam na diferenciação celular anormal em um tecido neoplásico autônomo. Estas etapas podem ser divididas em iniciação, promoção, progressão e manifestação (10,17).

Classicamente a cancerização era dividida em três etapas: iniciação era caracterizada por lesões irreversíveis no DNA (mutação) induzida pela exposição a agentes químicos, físicos ou biológicos; a promoção era entendida por um processo lento, reversível, e associada à proliferação focal de células iniciadas (100,101). Os agentes promotores eram capazes de alterar os sinais moleculares envolvidos no controle da proliferação celular (16,17,101). Consecutivamente, a progressão era caracterizada, morfológicamente e biologicamente, por crescimento autônomo. A etapa de manifestação se estabelece quando as células adquirem capacidade de invadir e se disseminar para outros tecidos (16). Assim, todos estes fatores atuam para o favorecimento do desenvolvimento neoplásico e devem ser considerados para a estimativa de risco para a carcinogênese (10,45,98).

Atualmente, estudos moleculares para rastreamento de possíveis genes que podem estar envolvidos no processo de carcinogênese vêm sendo alvo de várias investigações (12,27,28,100). Neste contexto, as alterações epigenéticas têm ganhado destaque no campo da etiopatogênese das doenças neoplásicas (45). Fenômenos epigenéticos estão associados a alterações de expressão gênica sem, no entanto, modificar a seqüência dos nucleotídeos do DNA. Por não alterar a seqüência do DNA, essas modificações podem ser reversíveis (28,45,47,50,82). Essas características fazem destes mecanismos, alvos atrativos para a melhor compreensão dos eventos neoplásicos.

Agentes ambientais, tais como exposição à radiação ultravioleta e a poluentes industriais (50,102), além de fatores relacionados ao estilo de vida, como dieta, hábito tabagista e etilista representam fatores de risco importantes que podem influenciar nos mecanismos da carcinogênese (28). Infecções virais, enfatizando o *Papiloma Virus Humano* (HPV), têm sido associadas à etiopatogênese e risco para alguns tipos de câncer (43,81). O HPV, vírus associado ao câncer cérvix uterino, foi associado também ao câncer pulmonar e ao câncer de cabeça e pescoço (84,86).

Classicamente, a etiopatogênese do câncer está relacionada a alterações genéticas progressivas, associadas à ruptura dos mecanismos de sinalização celular relacionados à carcinogênese (82,103,104).

Vários estudos têm demonstrado a importância do gene *p16_{CDKN2A}* para os eventos relacionados à carcinogênese. Modificações no padrão da expressão protéica desse gene podem gerar alterações na checagem do ciclo celular, com consequente instabilidade cromossômica e mitoses aberrante, determinando ou potencializando os eventos da gênese do cancer (43;101;105). Entretanto, pouco se sabe a respeito dos mecanismos moleculares envolvidos no processo de modulação da expressão gênica do *p16_{CDKN2A}*. Atualmente, esta lacuna na literatura é alvo de vários estudos relacionados com a gênese e progressão do câncer.

2 OBJETIVOS

- Investigar possíveis associações entre a metilação do gene *p16_{CDKN2A}*, o polimorfismo do gene DNMT3B (C46359T) e a infecção pelo HPV 16 e/ou 18 em lesões de LB.

3 PRODUTOS

3.1 *Increased p16_{CDKN2A} methylation, presence of HPV 16/18 and DNMT1 protein imunolocalization in Bucal Leukoplakia*, será submetido para publicação no periódico Histopathology.

O presente produto revela a possível associação entre a metilação do gene *p16_{CDKN2A}* e a infecção pelo HPV 16 e/ou 18 na gênese e malignização da leucoplasia bucal.

3.1 ARTIGO

Increased *p16_{CDKN2A}* methylation, presence of HPV 16/18 and DNMT1 protein imunolocalization are associated with Oral Leukoplakia

Running title: HPV 16/18, DNMT1 and *p16_{CDKN2A}* methylation in oral leukoplakia

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Abstract:

Since evidence suggest a possible role of methylation in development of neoplasias, an increasing number of studies using methylation analyses have been published. However epigenetics unbalance seems to be important in oral carcinogenesis, little is known about the interaction among epigenetics and the others cofactors involved in oral carcinogenesis.

The purpose of the present study was initially to investigate the association between *p16_{CDKN2A}* methylation and OL. In addition, we tested if HPV16/18 or *dnmt3b* (C46359T) polymorphism could alter *p16_{CDKN2A}* methylation status. Finally, we also evaluated DNMT1, DNMT3B and P16 proteins expression. In order to test these hypotheses, a case-control study with 72 lesions was performed.

OL was associated with the presence of HPV 16 and/or 18 and *p16_{CDKN2A}* methylation. However, neither HPV16/18 nor *dnmt3b* (C46359T) polymorphism influenced *p16_{CDKN2A}* methylation. Additionally, HPV16/18 and *dnmt3b* (C46359T) had no impact on the immunohistochemical expression of DNMT1, DNMT3B, and P16CDKN2A. In addition, we found increased expression of DNMT1 in OL.

In conclusion, OL is associated with p16CDKN2A gene methylation and DNMT1 expression. However *p16_{CDKN2A}* methylation status is not associated with HPV 16/18 infection or *dnmt3b* (C46359T) polymorphism.

Key words: DNMT3B; polymorphism; *p16_{CDKN2A}*; methylation; head and neck cancer, oral premalignant lesions.

Introduction

Oral squamous cell carcinoma (OSCC) is a disease associated with major morbidity and mortality and represents a major worldwide public health problem in many countries ¹⁻⁴. OSCC can be preceded by a group of lesions denominated potentially malignant disorders ^{5, 6}. From these, Oral Leukoplakia (OL) is the classical oral lesion associated with malignant transformation ^{5, 7-9}.

It was demonstrated that high risk HPV infection is strongly associated with OSCC ¹⁰⁻¹². On the contrary of cervical cancer, where HPV has been established as a primary cause, in oral cavity tobacco is the primary cause and high risk HPV seems to be an important cofactor for oral carcinogenesis ^{13, 14}.

The development of OSCC in humans has been viewed as a disease involving progressive genetic alterations ¹⁵⁻²⁰. Recently, evidence make epigenetic an important piece for this puzzle ^{13, 21-24}. The DNA methylation is the addition of methyl radical to specific regions of DNA containing, predominantly, cytosine nucleotides. It is catalyzed by a family of enzymes denominated DNA methyltransferase (DNMTs) ²⁵. DNMT1 is a maintenance enzyme, and both DNMT3A and DNMT3B are responsible for de novo methylation, which is the establishment of a new methylation pattern ^{26, 27}. It was postulated that the variant T of *dhmt3b* (C46359T) genetic polymorphism might promote increased protein expression, resulting in a predisposition to aberrant de novo methylation ²⁸⁻³⁰. Moreover, it was demonstrated that DNMT3B expression is relevant to *p16CDKN2A* inactivation in oesophageal cancer ^{31, 32}. Additionally, recent studies suggest that high-risk HPV infection has an important role in epigenetic regulation and could induce the methylation of *p16CDKN2A* gene ^{33, 34 21, 35}.

Taken these facts, the purpose of the present study was initially to investigate the association between *p16_{CDKN2A}* methylation and OL. In addition, we tested if HPV16/18 or dmt3b (C46359T) polymorphism could alter *p16_{CDKN2A}* methylation status. Finally, we also evaluated DNMT1, DNMT3B and P16 proteins expression.

Samples and Methods

Tissue Specimens and Patients

The current study was based on a case-control design. Data and tissue samples of 72 patients were obtained from the Department of Dentistry at the Universidade Estadual of Montes Claros, Minas Gerais, Brazil. The samples were divided in 2 groups: control group (C) was composed from normal oral mucosa tissues obtained during surgery of oral mucocele (n= 24 samples; group male-to-female ratio 1:1.4; group mean age= 32.83 years; SD 15.78 years; range, 15- 74 years). The study group was composed OL lesions (n= 48 samples; group male-to-female ratio = 1.5:1; group mean age=53.90; SD 12.45 years; range, 33- 93 years). The study was approved by the local Ethics Committee (process number 1133/08).

Morphological Classification

For the purposes of morphological analysis, samples were fixed in formalin, embedded in paraffin, serially sectioned at 5 µm, and were stained with hematoxylin and eosin and evaluated under a conventional light microscope. The samples were classified according to International Classification of Diseases for Oncology (ICD-10th revision) and

World Health Organization (WHO) criteria ³⁶, and the morphological analysis was carried out by the same oral pathologist without prior knowledge of the demographic or clinical characteristics related to the samples.

DNA isolation and Bisulfite Conversion of DNA for Methylation-specific PCR (MSP)

DNA was isolated from ten 10- μm -thick tissue sections from each tissue block of the specimens using the DNeasy Tissue Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. The *p16_{CDKN2A}* gene methylation profile was evaluated by methylation-specific (MSP) PCR. DNA samples were bisulphite-treated for 3 hours, and MSP-PCR was performed as previously described ³⁷ and posteriorly modified ³⁸. The *p16_{CDKN2A}* promoter was defined by two distinct PCR reactions as methylated or unmethylated based on fragment sizes of 150 and 151 bps, respectively. All samples that presented even one of allele methylated were considered as methylated (Figure1).

Dnmt3b Genotypes

Dnmt3b (C46359T) polymorphism was assessed by RFLP (Table 1). Polymerase chain reaction for DNMT3B was performed in a total volume of 25 μL containing approximately 100ng genomic DNA as template, 0.5 μL of each primer (20 pmol/ μL), 2.5 μL dNTP-mix (25 mM of each, AMRESCO, Ohio, CA, USA), 2.5 μL 10X PCR buffer, 1.25 μL magnesium chloride (50 mM), and 2.5 units of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA).

The 230-bp PCR product from the DNMT3B gene was digested with Bln1 restriction endonuclease (Sigma-Aldrich, St. Louis, MO, USA), that recognize a restriction

site (C/CTAGG) in T allele; wild-type C allele lacks the Bln1 restriction site. Thus, the wild-type C allele has only one band (230-bp), while the polymorphic T allele has two bands (172 and 58-bp). For that, 10 µL amplified DNA was digested with 2.5 U of Bln1 for 16 h at 37°C. PCR and restriction reactions were performed into a termocycler (Eppendorf AG, Hamburg, Germany) (Figure 1). DNA sequencing was realized to confirm the DNMT3B genotyping by PCR-RFLP.

HPV identification

HPV-DNA sequences were first PCR amplified by L1 and then by HPV-16 and HPV-18. Beta globin gene primers were used as an internal control. All primers sequences were described by Katiyar et al³⁹ (Table 1). Polymerase chain reaction was performed in a total volume of 25 µL containing approximately 100 ng genomic DNA as a template, 0.5 µL of each primer (20 pmol/µL), 2.5 µL dNTP-mix (25 mM of each, AMRESCO, Ohio, CA, USA), 2.5 µL 10X PCR buffer, 1.25 µL magnesium chloride (50 mM), and 2.5 units of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) (Figure 1).

Electrophoresis

The PCR products were verified on a 6.5% polyacrylamide gel that was electrophoresed at a constant voltage of 120V for 1.5 hours and stained with silver nitrate. Electrophoresis results were estimated against a 100-bp ladder.

Immunohistochemical analyses

Paraffin sections (3 µm) were mounted on glass and dried overnight at 37°C. All sections were then deparaffinised in xylene, rehydrated through a series of alcohol washes and washed in phosphate-buffered saline. The sections were incubated with anti-DNMT1, anti-DNMT3B (diluted 1:250, IMGENEX, CA, USA) or anti-P16 clone JC8 (1:500, SANTA CRUZ, USA) monoclonal antibodies at 4°C for 18 h. Endogenous peroxidase was blocked by incubating the sections with 0.03% H₂O₂ in ethanol for 30 minutes. For antigen retrieval, sections were heated in a steam cooker for 5 min at 125°C in Tris-EDTA buffer (1mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0). Signals were developed with 3'3-diaminobenzidine-tetrahydrochloride for 5 min and counter-stained with Harris hematoxylin for 30 sec. Normal mucosa was used as positive control, and the primary antibody was replaced with phosphate buffered saline for a negative control. After staining, tissue sections were scored according to the percentage of positive cells among the total cells.

Statistical analysis

The statistical significance of differences between case and control group distributions for clinicopathological features, methylation and genotype status was determined using Fisher or chi-squared tests. The association between categorical variables (HPV infection, methylation and genotype status) and immunostaining was evaluated by the mann whitney test. The Pearson correlation test was used to evaluate correlations among DNMT3B polymorphism or HPV positivity, *p16_{CDKN2A}* methylation, and expression of P16, DNMT1 and DNMT3B. In order to test the association between categorical

variables (*p16_{CDKN2A}* methylation status, HPV-16 or 18 infection and *dmt3b* (C46359T) polymorphism) chi square or Fisher's exact tests were used. Binary logistic regression models were fitted to assess the association between the variables and the risk of advanced histological staging. The associations were expressed by odd ratios (ORs) with the corresponding 95% CIs.

All analyses were assessed using SPSS 17.0 (SPSS Inc., Chicago, USA) and statistical significance was set at p<0.05.

Results

The immunoexpression of P16, DNMT1, DNMT3B, together with an illustrative sample positive for HPV16 or 18, or a MSP reaction for methylated *p16_{CDKN2A}* is shown in the figure 1.

OL lesions presented more *p16_{CDKN2A}* methylation and HPV16 and/or HPV 18 than control. However, no association was found between *dmt3b* (C46359T) polymorphism and OL (Table 2). The *p16_{CDKN2A}* methylation was not influenced by the presence of HPV16 and/or HPV 18 (p=0.380) or *dmt3b* (C46359T) polymorphism (p=0.15) (data not shown).

No correlation was found regarding DNMT3B, DNMT1 and P16 expression (data not shown). Neither HPV infection nor *dmt3b* (C46359T) polymorphism influenced the expression of P16, DNMT1 and DNMT3B (data not shown). The expression of DNMT1 was higher in OL than control (figure 2).

Discussion

A large number of studies tried to identify a biological marker associated with oral carcinogenesis^{6, 11, 12, 18, 40-42}. In this field, molecular biology has emerged as an important toll to identify susceptible groups for diseases^{6, 37, 41, 43-45}. For example, it was demonstrated that loss of heterozygosity could be responsible for a 33 - fold increased risk of leukoplakia malignant transformation¹⁸. Recently, epigenetics unbalance has been emerged as an important cause for carcinogenesis⁴⁶. In view of the reversible nature of epigenetic modifications^{47, 48} they are attractive targets for therapeutic intervention, especially for premalignant lesions^{49, 50}.

In the current study, we demonstrated that OL lesions are associated with *p16_{CDKN2A}* methylation. A recent study suggests that *p16_{CDKN2A}* methylation might be associated with oral carcinogenesis⁴⁰. Moreover, evidence suggest that *p16_{CDKN2A}* methylation may serve as a useful molecular marker for predicting local recurrence in carcinoma tongue⁵¹. So taken these facts, we might speculate that *p16_{CDKN2A}* methylation is an important for oral cancerization.

The presence of HPV16 and/or HPV 18 was associated with OL development, but not with *p16_{CDKN2A}* methylation. However epigenetics unbalance seems to be important in oral carcinogenesis, little is known about the interaction among epigenetics and the others cofactors involved in oral carcinogenesis. In theory there would be no advantage for of high-risk HPV to downregulate P16⁵²⁻⁵⁶. Additionally, the process of *p16_{CDKN2A}* aberrant gene methylation had not begun in E6/E7 immortalized cells⁵⁷. On the other hand, studies using lung SCC samples suggested a possible role of high-risk HPV infection in epigenetic regulation, specifically in *p16_{CDKN2A}* methylation^{21, 33}. Taken our previously published data⁴¹ as well as the lack of association among the expression of DNMT1, DNMT3B and

HPV positivity observed in the current study, we could speculate that ^{p16}CDKN2A methylation and HPV16 and/or HPV 18 infection are important factors related to the oral cancerization but, both are independent each other.

The importance of functional genetic polymorphisms has been demonstrated in a vast majority of oral diseases^{58, 59, 59-61}. However, the allele T of *dnmt3b* (C46359T) polymorphism was not associated with OL or *p16*_{CDKN2A} methylation status. No association was observed among *dnmt3b* (C46359T) polymorphism and the expression of DNMT1, DMNT3B and P16 proteins. Our study does not support that the allele T of *dnmt3b* (C46359T) polymorphism regulates protein expression, at least in the context of OL lesions.

An interesting finding observed in the current study was the increased expression of DNMT1 protein in OL lesions independently of HPV16 or 18 infection or *dnmt3b* (C46359T) polymorphism. It was demonstrated that reduction of DNMT1 in a transgenic mice reduce the tongue cancer progression⁶². These data suggest that the increased DNMT1 protein expression is associated with OL.

Conclusion

OL is associated with HPV 16/18 infection, DNMT1 expression and *p16*_{CDKN2A} methylation. However, *p16*_{CDKN2A} methylation status in OL is not influenced by HPV 16/18 infection or *dnmt3b* (C46359T) polymorphism.

Competing interests

None declared

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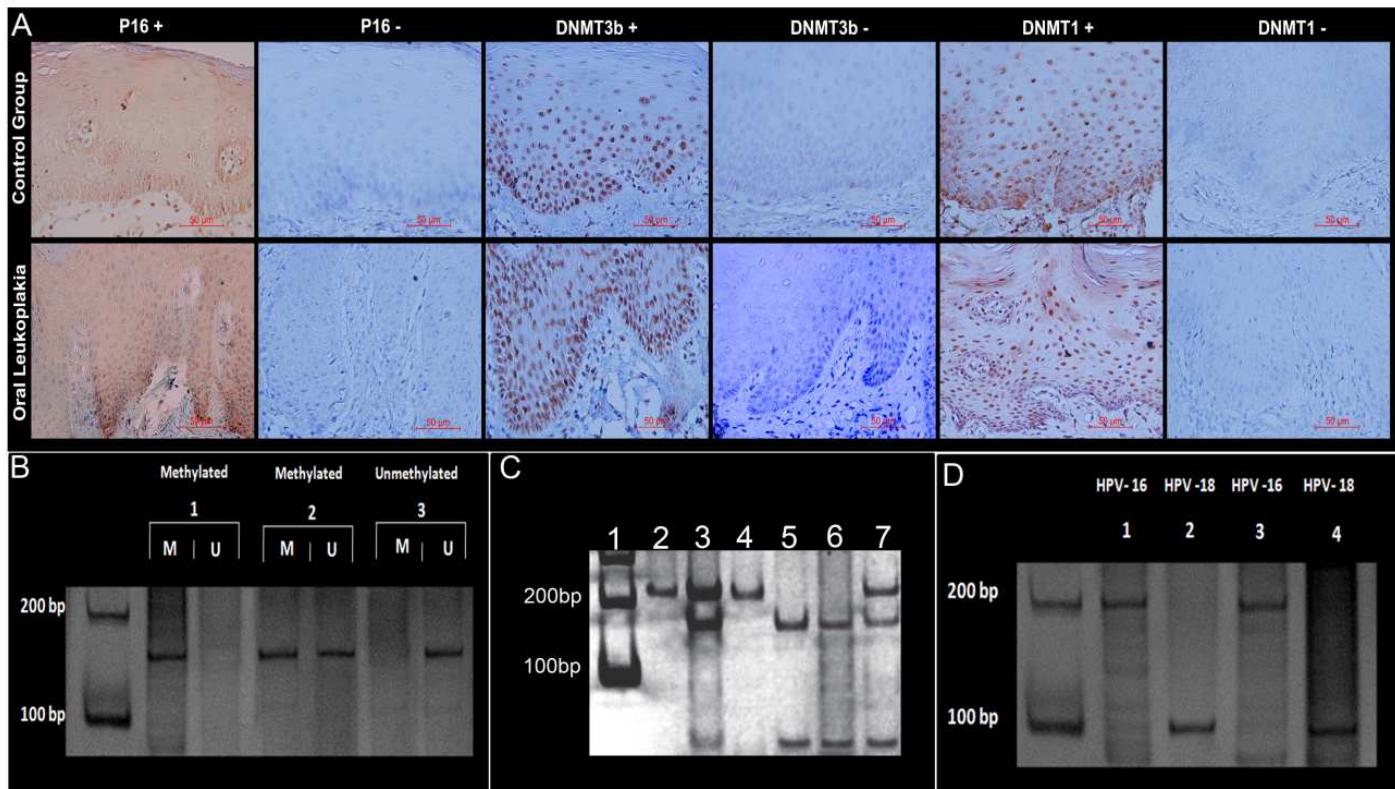
Figure1: Molecular diagnosis

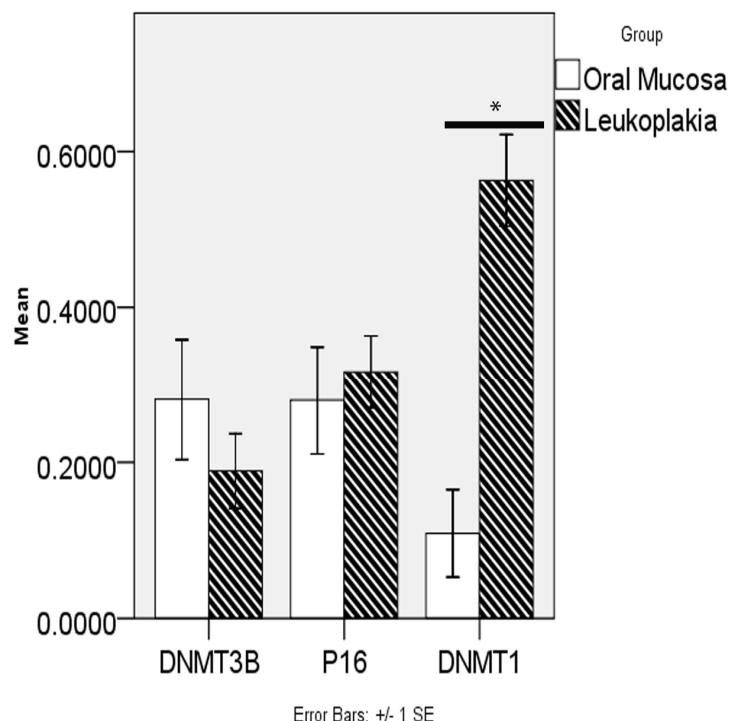
Figure 1A- Positive and negative immunostaining samples of the different groups.

Figure 1B- Methylation-specific PCR of *p16_{CDKN2A}* gene. ‘M’ (150bp) and ‘U’ (151bp) represent primer sets specific to methylated and unmethylated DNA, respectively. Samples 1 and 2 contain methylated DNA (M) indicative of the presence *p16_{CDKN2A}* methylation. Contrariwise, sample 3 showed only positive amplification for the unmethylated.

Figure 1C- PCR-RFLP for genotyping of DNMT3B (C46359T) polymorphism. Lane 1: 100-bp molecular marker, lanes 2 and 4: CC genotype, lane 3 and 7: CT genotype, lane 5 and 6: TT genotype.

Figure-1D: 100-bp molecular marker lane 1 and 3 HPV-16 positive, lane 2 and 4: HPV-18 positive (100-bp).

Figure 2- Expression of DNMT3B, P16 and DNMT1 in OL and normal bucal mucosa.



* ($p=0.001$)

Tables

Table I- Primer sequences, PCR product and polymerase chain reaction thermal conditions

	Primer sequences	PCR product (bp)	References	PCR thermal conditions
DNMT3B C46358T	F 5'-TGGCTACCAAGGTCTCCTGGCC-3' R 5'-GGTAGCCGGAACTCCACGG-3'	230	Own design*	1 x 95°C-5' 35 x 95°C-1' 68.4°C-1' 72°C-1' 1 x 72°C-10'
Methylated P16	F 5'- TTATTAGAGGGTGGGGCGGATCGC -3' R 5'- GACCCCGAACCGCGACCCTGA -3'	150	Moreira et al., 2009	1 x 95°C-5' 35 x 95°C-1' 64°C-1' 72°C-1' 1 x 72°C-10'
Unmethylated P16	F 5'-TTATTAGAGGGTGGGGTGATTGT-3' R 5'-CAACCCCAAACCAACCATAA-3'	151	Moreira et al., 2009	1 x 95°C-5' 35 x 95°C-1' 64°C-1' 72°C-1' 1 x 72°C-10'
L1 consensus primer	F:5'-GCMCAGGGWCATAAYAATGG-3' R 5'-CGTCCMAARGGAWACTGATC-3' M=A or C, R=A or G, W=A or T, Y=C or T		Katiyar et al 2005(31)	1 x 95°C-5' 35 x 95°C-1' 58°C-1' 72°C-1' 1 x 72°C-
HPV-16	F5'-AAGGCCAACTAAATGTCA-C-3' R 5'-CTGCTTTTATACTAACCGG-3'		Katiyar et al 2005(31)	1 x 95°C-5' 35 x 95°C-1' 60°C-1' 72°C-1' 1 x 72°C-
HPV-18	F 5'-ACCTTAATGAAAAACCAACGA-3 R 5'-CGTCGTTAGAGTCGTT-3'		Katiyar et al 2005(31)	1 x 95°C-5' 35 x 95°C-1' 61°C-1' 72°C-1' 1 x 72°C-
Bglobin	F 5'-GAAGAGCCAAGGCACAGGTAC-3' R 5'-CCAATTCCATCCACGTTACACC-3'		Katiyar et al 2005(31)	1 x 95°C-5' 35 x 95°C-1' 61°C-1' 72°C-1' 1 x 72°C

* It was designed based on the GenBank reference sequence (accession n°.NG_007290), using the software "Annhyb" (<http://annhyb.free.fr>) and Blast (www.ncbi.nlm.nih.gov/blast).

Table II - Molecular features in Buccal Leukoplakia and normal buccal mucosa

	Leukoplakia		Buccal Mucosa		<i>p</i> value	
	Negative	12	25.0%	19	79.2%	
HPV 16 or 18	positive	36	75.0%	5	20.8%	0.001
	methylated	42	87.5%	2	8.3%	
<i>p16CDKN2A</i> status	unmethylated	6	12.5%	22	91.7%	0.001
	CC	15	31.3%	13	54.2%	
<i>DNMT3B</i> (C46359T)	CT/TT	33	68.8%	11	45.8%	0.053

In bold: significant *p*-value<**0.05**, * Analyzed by X² test.

4 CONSIDERAÇÕES FINAIS

Estudos apontam que a carcinogênese bucal é um processo multifatorial, caracterizado por alterações genéticas, epigenéticas e ambientais, atuando de forma sinérgica, culminando no estado celular maligno. Entretanto, as vias pelas quais estes fatores atuam, ainda não são bem compreendidas.

Desta forma, o presente estudo buscou investigar a influência dos fatores genéticos, epigenéticos e ambientais em de pacientes portadores de CCECP e LB analisados separadamente.

Assim o presente trabalho sugere que o polimorfismo DNMT3B (C46359T) ou metilação do gene *p16_{CDKN2A}*, analisados separadamente, são fatores relevantes associados a diferentes parâmetros clínicos em pacientes portadores de CCECP, quando categorizados pela idade, e sugere também, que a metilação do gene *p16_{CDKN2A}* e a expressão da proteína DNMT1 podem estar associadas ao desenvolvimento da LB e serem possíveis fatores relacionados à gênese e malignização das LB.

Tendo em vista a complexidade dos processos carcinogênicos e a necessidade de um maior entendimento dos mecanismos envolvidos na carcinogênese e na progressão neoplásica maligna de cabeça e pescoço, mais estudos são necessários para melhor compreensão da etiopatogênese dos eventos neoplásicos malignos.

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6 ANEXO

Effect of age on the association between p16CDKN2A methylation and DNMT3B polymorphism in head and neck carcinoma and patient survival. Artigo científico publicado no periódico INTERNATIONAL JOURNAL OF ONCOLOGY (2010).

Effect of age on the association between p16CDKN2A methylation and DNMT3B polymorphism in head and neck carcinoma and patient survival

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Abstract. *De novo* DNA methylation is a relevant epigenetic mechanism, which represses gene transcription and commonly inactivates tumor suppressor genes in carcinogenesis. A single nucleotide polymorphism of DNMT3B, C46359T (-149C-T) was reported to modulate individual's susceptibility to cancer. We investigated the role of this polymorphic variant regarding the methylation status of the p16CDKN2A gene in young and older patients with head and neck squamous cell carcinoma (HNCC) matched by the TNM staging system, together with its impact on patients survival. The results showed that the presence of the allele T of the polymorphism DNMT3B (-149C-T) was associated with advanced TNM staging and smoking habit, but no association was found between this polymorphisms and DNMT3B immunostaining. While p16CDKN2A methylation was significantly associated with smoking habit in older patients, this parameter was associated with family history of cancer in young patients. Moreover, in older patients the absence of p16CDKN2A promoter methylation had a negative impact on survival. In conclusion, nucleotide polymorphism of DNMT3B is not associated with methylation of p16CDKN2A gene in HNSCC. The association of p16CDKN2A gene methylation with smoking, family history of cancer and survival is dependent on age.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer and represents 350,000 cancer deaths worldwide every year (1,2). It includes malign epithelial neoplasms that arise in the paranasal sinuses, nasal cavity, oral cavity, pharynx, and larynx (3). In Brazil, considering only oral cavity, the estimate for 2009 is 10,300 new cases of squamous cell carcinoma according to the National Institute of Cancer (INCA) (4). HNSCC has been regarded as a disease that generally affects men between the sixth through the eighth decades of life following long-term exposure to smoking and alcohol intake (5). However, an increase in the incidence of head and neck cancer among younger patients under the age of 45 years has been reported worldwide (6-12). Evidence suggests that carcinogenesis in young adults have a distinct mechanism of disease and often is not associated with classic risk factors for HNSCC (6-10,12). Differences in prognosis were observed in HNSCC patients according to age (13). Classically, the development of cancer in human has been viewed as a disease related to progressive genetic alterations (14-16). Recently, evidence indicates that not only genetic factors but also epigenetic modifications are similarly relevant in carcinogenesis (17,18). In contrast to genetic alterations, epigenetic modifications are reversible (19,20). This feature makes them attractive targets for therapeutic intervention (21,22).

The DNA methylation is the addition of methyl radicals to specific regions of DNA containing, predominantly, cytosine nucleotides. It is catalyzed by a family of enzymes denominated DNA methyltransferase (DNMTs), including three catalytically active enzymes - DNMT1, DNMT3a and DNMT3b. Although these enzymes act cooperatively to establish a pattern of genomic methylation, specific functions are performed by DNMTs. DNMT1 is an enzyme of maintenance, while DNMT3a and DNMT3b are responsible for the establishment of a new methylation pattern, known as *de novo* methylation (23,24). In addition to an important role in controlling gene activity, embryonic development, genomic

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imprinting (25), methylation has been associated with the development of cancer by transcriptional inactivation of tumor suppressor genes (17,25). The pattern of methylation has been linked to several cancer types, such as lung (17), oral (26) and head and neck cancer (27). Hypermethylation as well as hypomethylation can promote the development of the carcinogenesis (28). Genetic polymorphisms of the DNMT3b gene were described and it is associated with susceptibility of a variety of cancers (29-34), including head and neck squamous cell carcinoma (35-37). Genetic polymorphism of DNMT3b was described in the -149 position (C46359T). It was postulated that the variant T might regulate this gene, promoting an increase in its expression, and resulting in a predisposition to aberrant *de novo* methylation of tumor suppressor genes and repair genes (38-40).

The *p16CDKN2A* is a tumor suppressor gene that encodes a cyclin-dependent kinase inhibitor which plays an important role in the regulation of the G1/S phase cell cycle checkpoint. The inactivation of this gene was observed in many tumor types (41-45). P16 protein can be inactivated by point mutation, homozygous deletion and methylation of the promoter region (46,47). Although increased expression of DNMT3B gene is associated with P16 inactivation in esophageal and lung cancer (48,49), their role in HNSCC has not been established. We hypothesized that the polymorphism of DNMT3B (C46359T) could promote high levels of DNMT3B expression and induce consequently *p16CDKN2A* methylation. Furthermore, we attempted to verify whether this possible association is dependent on age and has impact on patient survival.

Patients and methods

Patients. The present analysis was based on a case-control study design. The patients were recruited from databases of the head and neck surgery services in Montes Claros, Brazil from 1996 through 2007 (6). The study group included 75 patients with HNSCC consisting of a case group of 25 patients aged ≤45 years (young) and a control group of 50 patients aged >45 years (older patients), that were matched for TNM staging, smoking and alcohol intake. Young and older patients were from the same geographical area.

Clinical data. The mean age was 42.1 years (SD 3.17 years; range, 33-45 years) for young and 62.2 years (SD 8.0 years; range, 49-82 years) for older patients with HNSCC. Physical description of skin color was not used because, in Brazil, it is a poor predictor of genomic ancestry (50,51). The study was approved by the local Ethics Committee (process no. 1085). Information on age, sex, tobacco smoking, alcohol drinking, medical history, family cancer history, tumor site, TNM clinical staging, and survival were obtained from medical charts.

All patients were staged according to the UICC TNM Classification of Malignant Tumors (1997) (52). Lesions of HNSCC were classified according to the primary site as described in the International Classification of Diseases (ICD-10) for Oncology. The anatomical sites reviewed in this study included: i) 28 (37.3%) mouth and perioral region (C00, C01, C02, C04, C05, C06.0, C06.2); ii) 22 (29.3%) oropharynx (C09-C10) of the patients; and iii) hypopharynx-

larynx 25 (33.4%) (C12, C13, C32). Lesions located in the oral cavity were considered as the anterior group and those located in the oropharynx-hypopharynx-larynx as the posterior group. Patients with diagnosis of carcinoma *in situ* or multiple head and neck carcinomas were excluded. All patients were asked about the occurrence of cancer in a first degree relative. The term cancer was defined using the WHO definition of 'an uncontrolled growth and spread of cells that may affect almost any tissue of the body'.

Histological gradation. Histological sections of tissues were stained with hematoxylin-eosin and evaluated under conventional light microscopy. All patients had histologically confirmed squamous cell carcinoma of head and neck. Histopathological classification of the tumors as moderate, or poorly differentiated was based on the World Health Organization criteria (WHO, 1997) (53) and invasive front area was also evaluated as described elsewhere (54).

DNA isolation and bisulfite conversion of DNA for methylation-specific PCR (MSP). DNA was isolated from ten 10-μm-thick tissue sections from each tissue block of HNSCC specimens, using the DNeasy Tissue Kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. The *p16CDKN2A* gene methylation profile was evaluated through methylation-specific PCR (MSP). DNA samples were bisulfite-treated for 3 h and MSP-PCR was performed as described (55) and posteriorly modified (56). Primer sequences, PCR product and polymerase chain reaction thermal conditions for defining methylation status are presented in Table I. The *p16CDKN2A* promoter methylation status for methylated or unmethylated reactions was identified by a fragment of 150 and 151 bp respectively (Fig. 1).

DNMT3B genotyping. DNMT3B (C46359T) polymorphism was assessed by RFLP (Table I). Polymerase chain reaction for DNMT3B was performed in a total volume of 25 μl containing ~100 ng genomic DNA as template, 0.5 μl of each primer (20 pmol/μl), 2.5 μl dNTP-mix (25 mM of each, Amresco, Ohio, CA, USA), 2.5 μl 10X PCR buffer, 1.25 μl magnesium chloride (50 mM), and 2.5 U of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA).

The 230-bp PCR product from the DNMT3B gene was digested with Bln1 restriction endonuclease (Sigma-Aldrich, St. Louis, MO, USA), that recognizes a restriction site (C/CTAGG) in T allele; wild-type C allele lacks the Bln1 restriction site. The wild-type C allele has only one band (230-bp), while the polymorphic T allele has two bands (172 and 58-bp). Thus, 10 μl amplified DNA was digested with 2.5 U of Bln1 for 16 h at 37°C. PCR and restriction reactions were performed into a thermocycler (Eppendorf AG, Hamburg, Germany) (Fig. 1). DNA sequencing was realized to confirm the DNMT3B genotyping by PCR-RFLP.

Electrophoresis. The PCR products for methylation and digested fragments were verified on 6.5% polyacrylamide gel electrophoresis at 120 V of constant voltage for 1.5 h and stained with silver nitrate. Electrophoresis results were estimated regarding a 100-bp ladder.

Table I. Primer sequences, PCR product and polymerase chain reaction thermal conditions.

	Primer sequences	PCR product (bp)	Refs.	PCR thermal conditions
DNMT3B C46358T	F 5'-TGGCTTACCAAGGTCTCCTTGGCC-3' R 5'-GGTAGCCGGAACTCCACGG-3'	230	Own design*	1x95°C-5' 35x95°C-1' 68.4°C-1' 72°C-1' 1x72°C-10
Methylated <i>p16</i>	F 5'-TTATTAGAGGGTGGGGCGGATCGC-3' R 5'-GACCCCGAACCGCGACCGTA-3'	150	(56)	1x95°C-5' 35x95°C-1' 64°C-1' 72°C-1' 1x72°C-10
Unmethylated <i>p16</i>	F 5'-TTATTAGAGGGTGGGGTGATTGT-3' R 5'-CAACCCCAAACCACAACCATAA-3'	151	(56)	1x95°C-5' 35x95°C-1' 64°C-1' 72°C-1' 1x72°C-10

*Designed based on the GenBank reference sequence (accession no. NG_007290), using the software 'Anhyb' (<http://anhyb.free.fr>) and Blast (www.ncbi.nlm.nih.gov/blast).

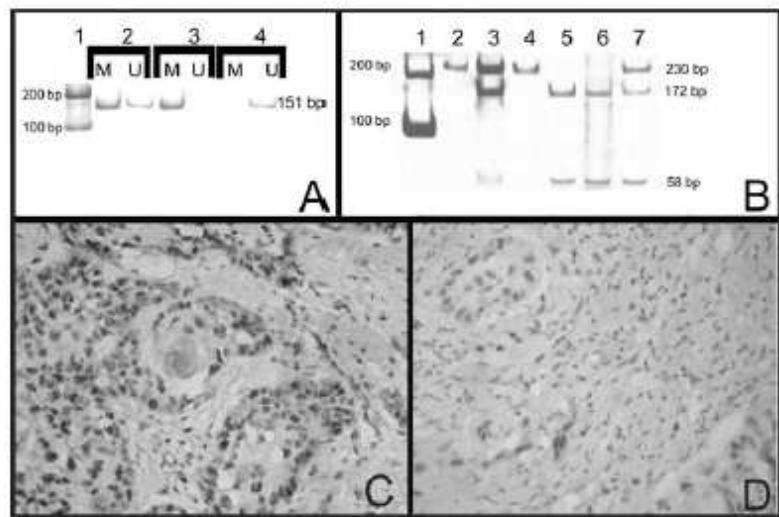


Figure 1. (A) Methylation-specific PCR of *p16* gene. 'M' (150 bp) and 'U' (151 bp) represent primer sets specific to methylated and unmethylated DNA, respectively. Samples 2 and 3 contain methylated DNA (M) indicative of the presence *p16* methylation. Sample 4 shows the unmethylated status of *p16* gene because of absence of methylated reaction (M). Lane 1, 100-bp molecular marker. (B) PCR-RFLP for genotyping of DNMT3B (C46358T) polymorphism. Lane 1, 100-bp molecular marker; lanes 2 and 4, CC genotype; lanes 3 and 7, CT genotype; lanes 5 and 6, TT genotype. (C) Positive immunostaining of DNMT3B, magnification x400. (D) Negative immunostaining of DNMT3B, magnification x400.

Immunohistochemical analyses. Paraffin sections (3-μm) were mounted on glass, and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated through a series of alcohol, and washed in phosphate-buffered saline. Anti-DNMT3b monoclonal antibody (diluted 1:250, IMGENEX, CA, USA) was used as the primary antibody and the incubation time was 18 h at 4°C. Endogenous peroxidase was blocked by incubation with 0.03% H₂O₂ in ethanol for 30 min. For antigen retrieval, sections were heated in a steam cooker filled for

5 min at 125°C in Tris-EDTA buffer (1 mM Tris base, 1 mM EDTA solution, 0.05% Tween-20, pH 9.0). Signals were developed with 3,3'-diaminobenzidine-tetrahydrochloride for 5 min and counter-stained with Harris hematoxylin for 30 sec. Normal mucosa was used as positive control and, for negative control, the primary antibody was replaced with phosphate-buffered saline. After staining, tissue sections were scored according to the percentage of positive cells among the neoplastic cells.

Table II. *p16* methylation and their association with molecular and clinicopathological features in case and control HNSCC.

Variables	All patients (%) <i>p16</i> methylation status		Younger patients (%) <i>p16</i> methylation status		Older patients (%) <i>p16</i> methylation status	
	Positive	Negative	Positive	Negative	Positive	Negative
Age						
Younger	17 (68)	8 (32)				
Older	42 (84)	8 (16)				
p-value		0.099				
Gender						
Male	53 (89.8)	11 (68.7)	16 (94.1)	05 (62.5)	37 (88.1)	06 (75.0)
Female	06 (10.2)	05 (31.3)	01 (5.9)	03 (37.5)	05 (11.9)	02 (25.0)
p-value		0.035		0.044		0.328
Family history of any cancer						
Absent	29 (49.2)	10 (62.5)	05 (29.4)	06 (75.0)	24 (57.1)	04 (50.0)
Present	30 (50.8)	06 (37.5)	12 (70.6)	02 (25.0)	18 (42.9)	05 (50.0)
p-value		0.343		0.032		0.709
Smoking status						
Smokers	47 (79.7)	13 (81.3)	14 (82.4)	07 (87.5)	33 (78.6)	06 (75.0)
Non-smokers	0 (0.0)	03 (18.7)	0 (0.0)	01 (12.5)	0 (0.0)	02 (25.0)
Ex-smokers	12 (20.3)	0 (0.0)	03 (17.6)	0 (0.0)	09 (21.4)	0 (0.0)
p-value		0.001		0.169		0.002
Alcohol consumption						
Drinkers	37 (62.7)	07 (43.8)	12 (70.6)	03 (37.5)	25 (59.5)	04 (50.0)
Non-drinkers	03 (5.1)	02 (12.4)	01 (5.9)	01 (12.5)	02 (4.8)	01 (12.5)
Ex-drinkers	19 (32.2)	07 (43.8)	04 (23.5)	04 (50.0)	15 (35.7)	03 (37.5)
p-value		0.318		0.289		0.676
Anatomic sites						
Anterior	19 (32.2)	09 (56.2)	04 (23.5)	05 (62.5)	15 (35.7)	04 (50.0)
Posterior	19 (32.2)	03 (18.8)	13 (81.3)	03 (18.8)	27 (87.1)	04 (12.9)
p-value		0.176		0.075		0.351
TNM clinical stage						
I/II	06 (10.2)	03 (18.8)	02 (11.8)	01 (12.5)	04 (9.5)	02 (25.0)
III/IV	53 (89.8)	13 (81.2)	15 (88.2)	07 (87.5)	38 (90.5)	06 (75.0)
p-value		0.349		0.958		0.217
Tumor size						
T1/T2	13 (22.0)	07 (43.8)	04 (23.5)	04 (50.0)	09 (21.4)	03 (37.5)
T3/T4	46 (78.0)	09 (56.2)	13 (76.5)	04 (50.0)	33 (78.6)	05 (62.5)
p-value		0.081		0.186		0.329
Locoregional metastasis						
Absent	21 (35.6)	06 (37.5)	05 (29.4)	03 (37.5)	16 (38.1)	03 (37.5)
Present	38 (60.4)	10 (62.5)	12 (70.6)	05 (62.5)	26 (61.9)	05 (62.5)
p-value		0.888		0.686		0.975
WHO grade						
I	17 (28.8)	02 (12.5)	02 (11.8)	02 (25.0)	15 (35.7)	0 (0.0)
II	18 (30.5)	06 (37.5)	09 (52.9)	03 (37.5)	09 (21.4)	03 (37.5)
III	24 (40.7)	08 (50.0)	06 (35.3)	03 (37.5)	18 (42.9)	05 (62.5)

Table II. Continued.

Variables	All patients (%) <i>p16</i> methylation status		Younger patients (%) <i>p16</i> methylation status		Older patients (%) <i>p16</i> methylation status	
	Positive	Negative	Positive	Negative	Positive	Negative
Invasive front grade						
Score 4-8	05 (8.5)	0 (0.0)	01 (5.9)	0 (0.0)	04 (9.5)	0 (0.0)
Score >8	54 (91.5)	16 (100.0)	16 (94.1)	08 (100.0)	38 (90.5)	08 (100.0)
p-value		0.288		0.489		0.363
DNMT3B genotype						
CC	10 (16.9)	03 (18.8)	03 (17.6)	02 (25.0)	07 (16.7)	01 (12.5)
CT	41 (69.5)	11 (68.8)	11 (64.8)	06 (75.0)	30 (71.4)	05 (62.5)
TT	08 (13.6)	02 (12.5)	03 (17.6)	0 (0.0)	05 (11.9)	02 (25.0)
p-value		0.983		0.440		0.615
Allele frequency						
C allele	51 (86.4)	14 (87.5)	14 (82.4)	08 (100.0)	37 (88.1)	06 (75.0)
T allele	08 (13.6)	02 (12.5)	03 (17.6)	0 (0.0)	05 (11.9)	02 (25.0)
p-value		0.912		0.296		0.310
DMNT3B immunohistochemistry*						
Mean rank of positivity	37.90	28.9	12.81	10.14	25.31	20.44
p-value		0.133		0.384		0.368

In bold, significant p-value <0.05. *Analyzed by Kruskal-Wallis test, the other analyses were done using the χ^2 test.

Statistical analysis. Statistical significance of differences between case and control group distributions for alleles, genotypes, methylation status, immunohistochemical analysis and clinicopathological features was determined using Fisher or χ^2 tests. The possible association between genotypes and DNMT3B immunostaining was evaluated by Kruskal-Wallis test.

Time to survival was calculated from date of diagnosis to time of last follow-up visit or to time of death. The records of each patient were reviewed, considering the same parameters, for 0-2500 days. All deaths were caused by locoregional and/or metastatic disease. For the purposes of analysis, patients who died without evidence of recurrence were excluded. Time survival was displayed by means of the Kaplan-Meier method for the variables. The results of Kaplan-Meier were compared by the log-rank test. Variables with $p \leq 0.25$ additional to age, *p16CDKN2A* methylation status, DNMT3B genotypes and immunostaining were included in the Cox proportional hazards multivariate model. Categorical variables considered as referents were those associated with less risk of death in accordance with the literature. All analyses were assessed using SPSS 17.0 (SPSS Inc., Chicago) and statistical significance was set at $p < 0.05$.

Results

Association of *p16CDKN2A* promoter methylation, DNMT3b polymorphism and clinicopathological parameters of HNSC

patients. The frequency distributions of *p16CDKN2A* promoter methylation according to age, molecular features and clinicopathological parameters are summarized in Table II. No association between *p16CDKN2A* methylation and age was observed. In young patients we identified a significant association between *p16CDKN2A* methylation with the presence of family history of cancer and male gender. On the other hand, in the older patients, *p16* methylation was significantly increased regarding the presence of smoking habit. Considering all patients, *p16* methylation was increased in male and smoker subjects (Table II). Differences in gender habits were observed according to age. In young patients, no differences of tobacco addiction and gender were observed ($p=0.072$). However, considering all patients together or only older patients, the male gender was associated with the presence of tobacco habit when compared with women ($p=0.001$) (data not shown). No relation between DNMT3B immunostaining and *p16CDKN2A* methylation was observed.

Table III presents the distribution of molecular and clinicopathological parameters grouped by age and DNMT3B variants. No association between clinicopathological parameters and the polymorphic variables were observed in young patients. The T allele of DNMT3B genotype was significantly associated with advanced TNM staging and tumor size in the older patients. Considering all samples, the distribution of allele T was increased in the T3/T4 tumors. No relation between polymorphic variants of DNMT3B gene and *p16CDKN2A* promoter methylation was observed.

Table III. DNMT3B genotype and their association with p16 methylation and clinicopathological features in case and control HNSCCs.

Variables	All patients (%)			Younger patients (%)			Older patients (%)		
	CC	CT	TT	CC	CT	TT	CC	CT	TT
Age									
Young	05 (20.0)	17 (68.0)	03 (12.0)						
Older	08 (16.0)	35 (70.0)	07 (14.0)						
p-value	0.899								
Gender									
Male	11 (84.6)	45 (86.5)	08 (80.0)	05 (100.0)	13 (76.5)	03 (100.0)	06 (75.0)	32 (91.4)	05 (71.4)
Female	02 (15.4)	07 (13.5)	02 (20.0)	0 (0.0)	04 (23.5)	0 (0.0)	02 (25.0)	03 (8.6)	02 (28.6)
p-value	0.864				0.326			0.235	
Family history of any cancer									
Absent	05 (38.5)	28 (53.8)	06 (60.0)	01 (20.0)	08 (47.1)	02 (66.7)	04 (50.0)	20 (57.1)	04 (57.1)
Present	08 (61.5)	24 (46.2)	04 (40.0)	04 (80.0)	09 (52.9)	01 (33.3)	04 (50.0)	15 (42.9)	03 (42.9)
p-value	0.527				0.395			0.933	
Smoking status									
Smokers	08 (61.5)	43 (82.7)	09 (90.0)	05 (100.0)	13 (76.5)	03 (100.0)	03 (37.5)	30 (85.7)	06 (85.7)
Non-smokers	01 (7.7)	02 (3.8)	0 (0.0)	0 (0.0)	01 (5.9)	0 (0.0)	01 (12.5)	01 (2.9)	0 (0.0)
Ex-smokers	04 (30.8)	07 (13.5)	01 (10.0)	0 (0.0)	03 (17.6)	0 (0.0)	04 (15.0)	04 (11.4)	01 (14.3)
p-value	0.437				0.692			0.054	
Alcohol consumption									
Drinkers	05 (38.5)	30 (57.7)	09 (90.0)	02 (40.0)	10 (58.8)	03 (100.0)	03 (37.5)	20 (57.1)	06 (85.7)
Non-drinkers	01 (7.7)	04 (7.7)	0 (0.0)	0 (0.0)	02 (11.8)	0 (0.0)	01 (12.5)	02 (5.7)	0 (0.0)
Ex-drinkers	07 (53.8)	18 (34.6)	01 (10.0)	03 (60.0)	05 (29.4)	0 (0.0)	04 (50.0)	13 (37.1)	01 (14.3)
p-value	0.165				0.367			0.425	
Anatomic sites									
Anterior	06 (21.4)	20 (71.4)	02 (7.1)	02 (40.0)	06 (35.2)	01 (33.3)	04 (50.0)	14 (40.0)	01 (14.3)
Posterior	07 (14.9)	32 (68.1)	08 (17.0)	03 (18.8)	11 (68.8)	02 (12.5)	04 (12.9)	21 (67.7)	06 (19.4)
p-value	0.418				0.976			0.330	
TNM clinical stage									
I/II	03 (23.1)	05 (9.6)	01 (10.0)	0 (0.0)	03 (17.6)	0 (0.0)	03 (37.5)	02 (5.7)	01 (14.3)
III/IV	10 (76.9)	47 (90.4)	09 (90.0)	05 (100.0)	14 (82.4)	03 (100.0)	05 (62.5)	33 (94.3)	06 (85.7)
p-value	0.401				0.448			0.043	
Tumor size									
T1/T2	07 (53.8)	12 (23.1)	01 (10.0)	01 (20.0)	07 (41.2)	0 (0.0)	06 (75.0)	05 (14.3)	01 (14.3)
T3/T4	06 (46.2)	40 (76.0)	09 (90.0)	04 (80.0)	10 (58.8)	03 (100.0)	02 (25.0)	30 (85.7)	06 (85.7)
p-value	0.036				0.301			0.001	
Locoregional metastasis									
Absent	06 (46.2)	18 (34.6)	03 (30.0)	01 (20.0)	07 (41.2)	0 (0.0)	05 (62.5)	11 (31.4)	03 (42.9)
Present	07 (53.8)	34 (65.4)	07 (70.0)	04 (80.0)	10 (58.8)	03 (100.0)	03 (37.5)	24 (68.6)	04 (57.1)
p-value	0.677				0.301			0.253	

Table III. Continued.

Variables	All patients (%)			Younger patients (%)			Older patients (%)		
	CC	CT	TT	CC	CT	TT	CC	CT	TT
WHO grade									
I	02 (15.4)	14 (26.9)	03 (30.0)	02 (40.0)	02 (11.8)	0 (0.0)	0 (0.0)	12 (34.3)	03 (42.9)
II	07 (53.8)	14 (26.9)	13 (30.0)	03 (60.0)	07 (41.1)	02 (66.7)	05 (40.0)	07 (20.0)	01 (14.2)
III	04 (30.8)	24 (46.2)	04 (40.0)	0 (0.0)	08 (47.1)	01 (33.3)	05 (40.0)	16 (45.7)	03 (42.9)
p-value	0.461		0.250		0.214				
Invasive front grade									
Score 4-8	01 (7.7)	04 (7.7)	0 (0.0)	0 (0.0)	01 (5.9)	0 (0.0)	01 (12.5)	03 (8.6)	0 (0.0)
Score >8	12 (92.3)	48 (92.3)	10 (100.0)	05 (100.0)	16 (94.1)	03 (100.0)	07 (87.5)	32 (91.4)	07 (100.0)
p-value		0.662			0.783			0.656	
p16 methylation status									
Positive	10 (76.9)	41 (78.8)	08 (80.0)	03 (60.0)	11 (64.7)	03 (100.0)	07 (87.5)	30 (85.7)	05 (71.4)
Negative	03 (23.1)	11 (21.2)	02 (20.0)	02 (40.0)	06 (35.3)	0 (0.0)	01 (12.5)	05 (14.3)	02 (28.6)
p-value		0.983			0.440			0.615	
DMNT3B immuno-histochemistry^a									
Mean rank of positivity	29.14	37.54	35.85	14.5	11.91	9.7	13.64	26.25	26.86
p-value		0.472			0.584			0.084	

In bold, significant p-value <0.05. ^aAnalyzed by Kruskal-Wallis test, the other analyzes were done using the χ^2 test.

Moreover, neither polymorphic variables nor clinicopathological parameters showed association with immunoexpression of DNMT3B.

Molecular results and survival of HNSCC patients. The mean overall survival of patients was 1021.5 days after the diagnosis. Factors which impacted in survival were differently distributed among the groups. In young patients no factor was associated with death. Considering both groups together, only TNM staging impacted on survival. However, in older patients, not only TNM staging but also the absence of p16CDKN2A promoter methylation showed a negative impact on survival (Table IV).

Discussion

The determinants of DNMT expression in human tissues have not been clearly defined. Recently, the role of ageing in DNA methylation was reported in liver cells (57). On the other hand, methylation of tumor suppressor genes, such as p16, has been associated with HNSCC development (48,58,59). In the present study we hypothesized that DNMT3B C46359T polymorphism may be associated with DNMT3B immuno-expression and p16CDKN2A methylation.

In addition, we tested if these parameters are related to the clinical stage, family history or the prognosis of HNSCC in young and older patients matched by the TNM staging, smoking and alcohol intake.

We observed that the frequency of p16 methylation in young patients was not different from older patients with HNSCC and we noted that this epigenetic alteration was present in most of the samples of both groups (76%). Taken together, the data indicate that p16 methylation is a common event in HNSCC development. Our data showed also that p16 methylation in young patients was associated with family history of cancer. However, there are some limitations that should be regarded. The high presence of subjects with family history of cancer in the young patients group could be a confounding factor. Therefore, a larger scale study is needed to confirm these data. Although we found association between p16 methylation in young patients and male gender, the limited number of patients of female gender indicates that the data must be analyzed cautiously.

We observed that p16CDKN2A methylation in older patients was significantly associated with smoking habit. Smoking is an important factor associated with the methylation of genes related to cancer (59,60). 7-methylguanine (m⁷Gua) is a biomarker of methylating agents present in tobacco (61).

Table IV. Cox regression analyses in the HNSCC patients with a follow-up of 0-2500 days.

Variables	p-value	All patients 95.0% CI				Young patients 95.0% CI				Older patients 95.0% CI			
		OR	Lower	Upper	p-value	OR	Lower	Upper	p-value	OR	Lower	Upper	
Age													
>45 years		Referent				-	-	-		-	-	-	
≤45 years	0.115	1.822	0.864	3.844	-	-	-	-	-	-	-	-	
P16 methylation													
Positive		Referent				Referent				Referent			
Negative	0.274	1.482	0.733	2.996	0.088	0.268	0.059	1.217	0.001	7.832	2.796	21.940	
DNMT3B genotype													
CC		Referent				Referent				Referent			
CT	0.822	1.110	0.449	2.744	0.438	1.991	0.350	11.336	0.973	0.980	0.297	3.233	
TT	0.871	1.096	0.362	3.318	0.327	0.286	0.023	3.496	0.824	1.168	0.297	4.595	
TNM													
Early		Referent				Referent				Referent			
Late	0.029	5.022	1.175	21.470	0.985	NA	0.011	8.449	0.011	8.449	1.622	44.015	
DNMT3B immuno-histochemistry													
	0.753	1.141	0.501	2.597	0.583	0.666	0.156	2.848	0.079	2.623	0.894	7.696	

In bold, significant p-value <0.05. The term referent is associated with categorical variables with a lower risk of developing the disease. NA, not applicable.

Recent reviews observed that age and cigarette smoking were positively correlated with the urinary m7Gua level (62). The urinary excretion of m7Gua was shown to be higher in smokers than in non-smokers (63). Furthermore, the m7Gua level in human urine decreased after smoking cessation (64). It is important to highlight that, in the present study, no p16CDKN2A methylation was observed in non-smokers. Therefore, our study gives additional support that both smoking and aging are important factors involved in DNA methylation.

In recent years, several studies have demonstrated that genetic polymorphisms are associated with susceptibility to, or protection from, cancer development (36,65,66). Until now, 13 polymorphisms were described in the coding region of gene DNMT3B according NCBI database, but the association between these genetic variations and cancer prognosis remains unclear. Polymorphisms may alter gene transcription and/or protein synthesis and function. Recently, an increased risk of HNSCC development was demonstrated with the T allele of DNMT3B -149 polymorphism in oral cavity, pharynx and larynx but not in nasopharynx (36). In the present study we attempted to verify the possible association between this polymorphism and early HNSCC development. We did not observe association between DNMT3B polymorphic variant and age, but in the older patients group we found association between the higher DNMT3B genotype (allele T) with an advanced TNM staging. Although this

finding suggests that polymorphisms might influence cancer progression, it will take a long time to prove this association. Here, the immunoexpression of DNMT3B protein was not related to the genetic polymorphisms in HNSCC samples studied. Moreover, this polymorphism did not show association with p16 methylation. This may be explained by the fact that other factors, such as HPV high-risk infection, may induce p16 methylation (47,67). Furthermore, other DNMT polymorphisms may be more relevant to this epigenetic alteration.

Evidence suggests that there are differences in molecular mechanisms between younger and older HNSCC patients (6,9). We found that absence of p16 methylation was associated with low survival in older patients. Therefore, it can be speculated that different epigenetic and genetic pathways may affect cancer evolution and prognosis. Although we are only just starting to understand the impact of molecular findings in patient prognosis, previous reports have already suggested that HPV infection has an impact on the prognosis of HNSCC (68,69).

In conclusion, our data suggest that while genetic factors are more importantly involved with p16 methylation in younger individuals with HNSCC, environmental factors are more relevant for this epigenetic change in older patients. Finally, the present study shows that p16 methylation status has impact on the prognosis of patients with HNSCC.

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