PATRÍCIA CARLOS CALDEIRA

LEUCOPLASIAS BUCAIS: ESTUDO COMPARATIVO ENTRE O GRAU HISTOLÓGICO DE DISPLASIA, IMUNOEXPRESSÃO DE hMLH1 E p53 E ANÁLISE QUANTITATIVA DE AgNOR

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Dissertação apresentada ao Colegiado do Programa de Pós-graduação da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do grau de Mestre em Odontologia - área de concentração em Patologia Bucal.

Orientadora: Profª. Drª. Maria Auxiliadora Vieira do Carmo.

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Dizem que a pressa é inimiga da perfeição. Entretanto, aprendi que a perfeição deve ser almejada em qualquer trabalho, independente do tempo a ele conferido. Perseverar, com paciência e sem hesitação diante das dificuldades. Este trabalho é dedicado àqueles que assim me ensinaram, meus amados pais.

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RESUMO

hMLH1 é uma proteína do sistema de reparo de erros de pareamentos (MMR) em humanos, responsável pela manutenção da estabilidade genômica durante duplicações celulares repetidas. Relação entre a expressão alterada desta proteína e a ocorrência de instabilidade de microssatélite foi observada em leucoplasias e carcinomas de células escamosas de boca, indicando um possível papel desta proteína na carcinogênese bucal. Além disso, essas alterações parecem estar relacionadas com o grau histológico de severidade destas lesões. É também sugerida uma interação entre o sistema MMR e a p53, uma proteína supressora tumoral que está frequentemente alterada em cânceres humanos. A técnica de AgNOR é utilizada para avaliar o índice de proliferação celular. A fim de contribuir para melhor compreensão dos eventos precoces da carcinogênese bucal, objetivou-se avaliar e comparar a imunoexpressão de hMLH1 e p53 e o número de AgNOR em leucoplasias bucais com diferentes graus de displasia epitelial. Foram incluídas 62 amostras de leucoplasia, sendo 17 sem displasia, 19 com displasia discreta, 16 com displasia moderada e 10 com displasia severa, considerando-se os índices totais, bem como os valores obtidos em camadas distintas do epitélio. Para as proteínas hMLH1 e p53, os resultados foram expressos em porcentagem de células imunopositivas e, para a técnica de AgNOR, considerou-se o número médio de pontos marcados, sendo os dados submetidos à análise estatística. Observou-se uma diminuição nos índices de hMLH1 e aumento nos índices de p53 e AgNOR à medida que o grau de displasia se tornava mais severo, a despeito da presença ou não de significância estatística. Considerando os diversos graus de displasia, os índices de hMLH1 na camada suprabasal e os índices totais mostraram significância estatística em todas as comparações, exceto entre leucoplasia com displasia moderada e severa. Pôde-se observar, ainda, uma correlação inversa entre as marcações de hMLH1 e de ambos, p53 e AgNOR e uma correlação direta entre p53 e AgNOR. Nossos resultados sugerem que, durante o desenvolvimento progressivo de um fenótipo mais displásico da leucoplasia, os ceratinócitos parecem apresentar uma diminuição na capacidade de reparo de DNA e de supressão tumoral, sugerindo que esses fenômenos sejam alterações precoces no processo de carcinogênese, podendo essas vias estarem interrelacionadas, assim como ao aumento da proliferação celular.

Unitermos: hMLH1, p53, AgNOR, imunoistoquímica, leucoplasia bucal.

ABSTRACT

"ORAL LEUKOPLAKIAS: COMPARATIVE STUDY BETWEEN THE HISTOLOGICAL GRADE OF EPITHELIAL DYSPLASIA, IMMUNOEXPRESSION OF hMLH1, p53 AND QUANTITATIVE ANALYSIS OF AgNOR."

hMLH1 is a protein of the mammalian mismatch repair system (MMR), which maintains genomic stability during repeated duplication. Investigations point to a role of hMLH1 in oral carcinogenesis, and its expression pattern may be related to epigenetic events and microsatellite instability in oral leukoplakias and squamous cell carcinomas. It seems that those alterations are related to the histological severity of these lesions. It has been suggested that the MMR machinery could activate and be linked to regulation of p53, a tumor supressor protein which is commonly altered in human cancers. The AgNOR technique is used to assess the cell proliferation. This study aimed to assess and to compare the immunoexpression of hMLH1 and p53, and the AgNOR number, in oral leukoplakias with different degrees of epithelial dysplasia. Sixty-two specimens of oral leukoplakia were classified into four groups: 17 without dysplasia, 19 with mild dysplasia, 16 with moderate dysplasia, and 10 with severe dysplasia. Immunohistochemistry for hMLH1 and p53, and AgNOR technique were performed. Results are shown as percentage of immunopositive cells and mean AgNOR number and statistical analysis was made. Decreasement in hMLH1 and increasement in p53 and AgNOR indexes were observed throughout the development of more severe dysplasia, despite statistical significance. Considering oral leukoplakia groups, indexes of hMLH1 expression in suprabasal and all layers were statistically significant for all comparisons except for oral leukoplakia with moderate and severe dysplasia. An inverse correlation between hMLH1 and both p53 and AgNOR, and a direct correlation between p53 and AgNOR could be observed. Ou results suggest that during the development of more dysplastic phenotypes in oral leukoplakias, keratinocytes seem to present diminished capacity of DNA repair and tumor supression, indicating that these alterations could be early events in oral cacinogenesis. These pathways can be somehow interconected, as well as the increasement in cellular proliferation.

Keywords: hMLH1, p53, AgNOR, immunohistochemistry, oral leukoplakia.

ABREVIATURAS E SIGLAS

AgNOR – Regiões Argirofílicas Organizadoras Nucleolares

COEP – Comitê de Ética em Pesquisa

DAB – Diaminobenzidina

DNA – Ácido desoxirribonucléico

Exo1 – Exonuclease I

hMLH1 – Proteína MLH em humanos

hMSH2 – Proteína MSH em humanos

HNPCC – “Hereditary Non-polyposis Colorectal Cancer” - Câncer Colo-retal Hereditário sem Polipose

HPV – Papiloma Vírus Humano

LB – Leucoplasia Bucal

MMR – “Mismatch Repair System” - Sistema de reparo de erros de pareamentos

MSI – “Microsatellite instability” - Instabilidade de microsatélite

MutL – Proteína L do MMR de procariotas

MutS – Proteína S do MMR de procariotas

NOR – Regiões Organizadoras Nucleolares

OMS – Organização Mundial de Saúde

p53 – Proteína p53

rRNA – Ácido ribonucléico ribossomal

TP53 – Gene que codifica p53

SUMÁRIO

1 INTRODUÇÃO

A leucoplasia bucal é a principal lesão potencialmente maligna da boca, em que a frequência de alterações displásicas varia de 15,6 a 39,2%. Muitas leucoplasias regridem ou permanecem quiescentes, porém algumas progridem e 3-6% transformam-se em carcinoma de células escamosas. Embora a graduação de displasia epitelial seja feita baseada em critérios histológicos até certo ponto subjetivos, estando sujeita a variações intra e interexaminadores, este é ainda hoje o critério mais utilizado na avaliação do potencial de malignização destas lesões. Entretanto, leucoplasias com graus semelhantes de displasia podem ter cursos clínicos diferentes. Portanto, a busca por marcadores que reflitam o comportamento biológico dessas lesões é necessária, a fim de identificar características preditivas de transformação maligna que contribuam para uma abordagem menos empírica dessas lesões.

hMLH1 é uma das principais proteínas do sistema *mismatch repair* (MMR) humano, o qual participa no reparo de danos ao DNA. Considerando-se suas funções biológicas, estudos apontam para uma possível relação entre as proteínas do sistema MMR e a proteína p53. Recentemente, foram demonstradas correlação e associação entre a imunoexpressão de hMLH1 e a graduação histológica de malignidade em carcinomas de células escamosas de boca, assim como uma relação entre a imunoexpressão dessa proteína e a ocorrência de instabilidade de microssatélite.

A proteína p53 é produzida pelo gene supressor de tumor TP53, considerado o “guardião do genoma”. TP53 participa do processo de carcinogênese bucal, sendo inativado diretamente por mutações genéticas em aproximadamente 50% dos cânceres humanos. Vários estudos observaram que a imunomarcação de p53 aumenta de acordo com o grau de displasia epitelial de leucoplasias. Entretanto, a avaliação isolada da imunomarcação de p53 ainda não pode ser utilizada como único marcador para predizer o potencial de transformação maligna, uma vez que alguns estudos encontraram resultados conflitantes.

Existem fortes evidências que a técnica de AgNOR, empregada para mensurar proliferação celular, seja sensível à identificação precoce de alterações intranucleares, sendo que estudos relataram a utilidade desta técnica na distinção entre os diferentes graus de displasia de leucoplasia bucal.

Assim, o objetivo deste estudo foi a análise comparativa entre a imunoexpressão de hMLH1 e p53 e as médias de número de AgNOR, como indicadores de reparo de DNA, supressão tumoral e proliferação celular, respectivamente, em leucoplasias bucais com diferentes graus de displasia epitelial. Desta forma, busca-se contribuir para melhor compreensão dos eventos precoces da carcinogênese bucal. Apesar dos trabalhos existentes na literatura, o tema permanece controverso e novos estudos são claramente necessários.

2 REVISÃO DE LITERATURA

2.1 LEUCOPLASIA BUCAL

Estima-se que 14.120 novos casos de câncer de boca afetarão a população brasileira em 2010, sendo o carcinoma de células escamosas o mais comum deles (BRASIL, 2009). Apesar dos avanços alcançados em relação ao tratamento da doença, a taxa de sobrevida de 5 anos é de aproximadamente 50 a 55%, sendo que a prevenção e o diagnóstico precoce contribuem para a redução da incidência e aumento da sobrevida dos pacientes (NEVILLE e DAY, 2002).

O carcinoma de células escamosas de boca pode ser precedido por alterações potencialmente malignas, e a leucoplasia bucal (LB) é a mais comum delas, com prevalência populacional estimada em 2,6% (PETTI, 2003; HAYA-FERNÁNDEZ *et al*., 2004). LB é definida como uma "placa branca que não pode ser caracterizada clinicamente ou microscopicamente como nenhuma outra entidade" e o principal fator relacionado ao desenvolvimento da doença é o uso do tabaco (OMS, 2005; van der WAAL*,* 2009). O efeito do álcool, uso do betel, papiloma vírus humano (HPV) e dieta também são associados, porém o papel exato desses fatores etiológicos ainda não está bem estabelecido (CAMPISI *et al.,* 2007; NAPIER e SPEIGHT, 2008; van der WAAL*,* 2009). As LB acometem principalmente mucosa jugal e mucosa alveolar de homens de meia idade ou idosos (NEVILLE e DAY, 2002; NAPIER e SPEIGHT, 2008).

Clinicamente, as LB homogêneas aparecem como uma placa levemente elevada, fina, de coloração branco-acinzentada uniforme, podendo apresentar bordas bem definidas ou fundirem-se gradualmente à mucosa normal adjacente. Já as lesões não homogêneas podem ser nodulares, verrucosas ou salpicadas (eritroplásicas) (WARNAKULASURIYA *et al.,* 2007; van der WAAL*,* 2009). Existe ainda a leucoplasia verrucosa proliferativa, caracterizada por envolvimento multifocal, preferencialmente em pacientes do gênero feminino e que não apresentam fatores de risco conhecidos (WARNAKULASURIYA *et al.,* 2007; van der WAAL*,* 2009).

As LB podem permanecer estáveis, progredir, regredir, ou até mesmo desaparecer (RAY *et al.,* 2003; NAPIER e SPEIGHT, 2008). O risco de transformação maligna varia de 3,6 a 36%, sendo que fatores como presença e grau de displasia, gênero feminino, tempo de duração, paciente não tabagista, localização em língua e soalho bucal, tamanho maior que 200mm², apresentação clínica não homogênea parecem estar associados a pior prognóstico (CRUZ *et al.,* 2002; HOLMSTRUP *et al.,* 2006; HSUE *et al.,* 2007; SMITH *et al.,* 2009; van der WAAL, 2009).

A apresentação histológica de uma LB pode variar de lesões levemente hiperceratóticas a lesões com displasia severa (BRENNAN *et al.,* 2007; WARNAKULASURIYA *et al*., 2008). A frequência de alterações displásicas ou malignas em LB varia de 15,6 a 39,2%, sendo que uma taxa de 19,9% foi observada em um estudo retrospectivo de 3.300 lesões brancas de boca (NEVILLE e DAY, 2002).

A displasia epitelial é caracterizada pela alteração da arquitetura acompanhada por atipia citológica, podendo ser graduada em leve, moderada, severa e carcinoma *in situ* (van der WAAL*,* 2009). O grau de displasia epitelial da LB parece ser o indicador mais importante do potencial de transformação maligna da lesão, interferindo diretamente na conduta clínica do caso (CHATTOPADHYAY *et al*., 2002; KUROKAWA *et al.,* 2003; WARNAKULASURIYA *et al.,* 2007; PIMENTA *et al.,* 2008; SMITH *et al.,* 2009; van der WAAL*,* 2009). No entanto, há notável variação na interpretação e classificação das displasias, tanto inter-observador quanto intra-observador (KUJAN *et al.,* 2007; WARNAKULASURIYA *et al*., 2008).

Considerando-se que na maioria dos laboratórios de diagnóstico a avaliação de tecidos biopsiados se faz através de microscopia de luz, a classificação de displasia epitelial pode ser subjetiva, tornando o valor preditivo de transformação maligna muito aquém do ideal (CHATTOPADHYAY *et al*., 2002; CRUZ *et al*., 2002; RAY *et al*., 2003; FAN *et al*., 2006; CHATTOPADHYAY e RAY, 2008; WARNAKULASURIYA *et al*., 2008). Desta forma, diversos biomarcadores relevantes são investigados como indicadores prognósticos precoces, uma vez que a expressão e função aberrantes de moléculas são resultados dos múltiplos eventos genéticos e epigenéticos que ocorrem na oncogênese (CHATTOPADHYAY *et al*., 2002; RAY *et al*., 2003; FAN *et al*., 2006; KONDOH *et al.,* 2007; CHATTOPADHYAY e RAY, 2008; KURIBAYASHI *et al.,* 2009; SCULLY e BAGAN, 2009). Contudo, ainda não há um biomarcador ou uma gama de biomarcadores que possam ser utilizados para se estabelecer prognóstico mais preciso de lesões displásicas bucais, levando à necessidade de novas pesquisas (SMITH *et al.,* 2009; van der WAAL*,* 2009).

2.2 PROTEÍNA hMLH1

O sistema MMR (*mismatch repair*) de reparo de DNA participa na manutenção da

estabilidade genômica em duplicações repetidas, durante as quais nucleotídeos mal pareados podem ser incorporados ao DNA (FISHEL, 1998; JASCUR e BOLAND, 2006; JUN *et al*., 2006; FERNANDES *et al*., 2008). Este sistema pode ser entendido como uma maquinaria de excisão e ressíntese de porções mal pareadas de DNA, cujas principais proteínas são MutS e MutL (KOLODNER e MARSISCHKY, 1999; JASCUR e BOLAND, 2006; JIRICNY, 2006; JUN *et al*., 2006).

Os maus pareamentos são reconhecidos por homólogos de MutS em humanos (hMSH). Os heterodímeros MutSα, composto por hMSH2 e hMSH6, e MutSβ, a combinação entre hMSH2 e hMSH3, em funcionamento, formam uma braçadeira deslizante que se liga à fita dupla de DNA nas regiões mal pareadas (CHUNG e RUSTGI, 2003; JASCUR e BOLAND, 2006; JIRICNY, 2006; JUN *et al*., 2006). Esta ligação recruta o complexo MutLα, formado por hMLH1 e PMS2, homólogos de MutL. (JASCUR e BOLAND, 2006; JIRICNY, 2006; JUN *et al*., 2006; O'BRIEN e BROWN, 2006). A interação entre os complexos de MutL e MutS ativa exonuclease I (Exo1), que faz a excisão da sequência incorreta de DNA (JASCUR e BOLAND, 2006; JIRICNY, 2006). Uma vez removido o erro, Exo1 não é mais estimulada e passa a ser inibida (JIRICNY, 2006). A DNA polimerase δ sintetiza o novo DNA, utilizando a fita-mãe como molde (JASCUR e BOLAND, 2006; JIRICNY, 2006). Por fim, a DNA ligase I sela a ruptura remanescente e finaliza o processo de reparo (JIRICNY, 2006).

Microssatélites são múltiplas seqüências repetidas de nucleotídeos e estão presentes em grande número no genoma humano, em porções codificadoras e não codificadoras (CHUNG e RUSTGI, 2003; JASCUR e BOLAND, 2006; JIRICNY, 2006). Essas regiões são particularmente propensas à ocorrência de *loops* de DNA mal pareado (JASCUR e BOLAND, 2006; JIRICNY, 2006). Além disso, a função de conferência da DNA polimerase, que por si só corrige 99% dos erros de replicação, tende a ser menos eficiente em regiões de microssatélite (JASCUR e BOLAND, 2006). Na presença de um erro de replicação, caso o sistema MMR esteja inativo, um fenótipo mutado acompanhado por instabilidade de microssatélite (MSI) e, eventualmente, câncer podem ser consequentes (CHUNG e RUSTGI, 2003; JASCUR e BOLAND, 2006; JIRICNY, 2006; FERNANDES *et al*., 2007).

Defeitos em genes MMR foram associados com diversos tumores, sendo que os principais alvos são hMLH1 e hMSH2 (JASCUR e BOLAND, 2006; O'BRIEN e BROWN, 2006;

FERNANDES *et al.,* 2007). Estudos acerca do sistema MMR foram amplamente desenvolvidos em pacientes portadores da Síndrome do Câncer Coloretal Hereditário sem Polipose (HNPCC), uma doença autossômica dominante com alta penetrância, caracterizada por risco aumentado de desenvolvimento de câncer coloretal. Em aproximadamente 90% dos pacientes com HNPCC são observadas mutações *germline* principalmente em hMLH1 e hMSH2 (PARAF *et al.,* 2001; CHUNG e RUSTGI, 2003; SENGUPTA *et al*., 2007). De maneira semelhante, 15% dos casos de tumores coloretais esporádicos são associados a alterações somáticas de genes MMR, sendo a mais comum delas a hipermetilação da região promotora de hMLH1, bloqueando a síntese protéica (PARAF *et al.,* 2001; CHUNG e RUSTGI, 2003; SENGUPTA *et al*., 2007). Pacientes sindrômicos e não sindrômicos apresentam prognóstico estritamente relacionado ao seu padrão de MSI (CHUNG e RUSTGI, 2003).

Tendo em vista a principal função do sistema MMR, células proliferativas apresentam maiores níveis de proteínas MMR do que células quiescentes (JASCUR e BOLAND, 2006). A investigação de hMLH1 através de imunoistoquímica, uma técnica simples e barata, foi sugerida e empregada em diversos estudos e apresentou alta sensibilidade (67-79%) e especificidade (> 99%) na detecção de MSI (CASTRILLI *et al.,* 2001; PARAF *et al*., 2001; STONE *et al*., 2001; CASTRILLI *et al.,* 2002; LINDOR *et al*., 2002; SHIA *et al*., 2004; FERNANDES *et al*., 2007; FERNANDES *et al*., 2008).

FERNANDES e colaboradores (2007) demonstraram que hMLH1 está expressa em epitélio bucal normal e que a mesma não foi relacionada à idade, gênero e localização da amostra. O uso de tabaco aumentou a expressão de hMLH1 na camada suprabasal, enquanto a presença de inflamação diminuiu a expressão da proteína na camada basal.

Estudos sobre hMLH1 em carcinoma de células escamosas de cabeça e pescoço foram realizados e foi sugerido que o fenótipo de MSI seja um evento precoce no desenvolvimento desta lesão (SENGUPTA *et al.,* 2007). LIU e colaboradores (2002) encontraram que 26% das lesões apresentaram quase ausência de imunoexpressão de hMLH1. Dentre os 13 casos negativos, 12 apresentaram hipermetilação da região promotora enquanto dos 22 casos imunopositivos, 17 não apresentaram esta alteração epigenética. De maneira semelhante, PURI e colaboradores (2005) encontraram hipermetilação de hMLH1 em 23% dos casos. ZUO e

colaboradores (2009) encontraram que 32,5% das amostras apresentaram hipermetilação e 18,3% apresentaram imunoexpressão reduzida de hMLH1. Em outro estudo, FERNANDES e colaboradores (2008) observaram significativa correlação e associação entre a imunoexpressão de hMLH1 e a graduação histológica de malignidade dos tumores, sendo que tumores bem diferenciados apresentavam superexpressão de hMLH1, enquanto expressão reduzida foi observada em tumores pouco diferenciados.

Em relação às LB, poucos estudos foram desenvolvidos. SENGUPTA e colaboradores (2007) encontraram que 15% das lesões com displasia apresentaram hipermetilação da região promotora de hMLH1 e 30% em hMLH1 e hMSH2 simultaneamente. Além disso, os autores relataram que houve uma correlação entre o nível de MSI e a freqüência de hipermetilação. HA e colaboradores (2002) encontraram que 14% das lesões potencialmente malignas avaliadas apresentaram MSI e que houve tendência de maior prevalência nas lesões com maior grau de displasia epitelial.

2.3 PROTEÍNA p53

O gene TP53 codifica uma proteína supressora tumoral, a proteína p53, considerada a "guardiã do genoma" (VOGELSTEIN *et al.,* 2000; WHYTE *et al.,* 2002; KUROKAWA *et al.,* 2003). Esta proteína interage com várias outras e participa de diversas vias de sinalização, direcionando a resposta ao dano de DNA, para manutenção da integridade genômica (WHYTE *et al.,* 2002; RODIER *et al.,* 2007). Em resposta a algum fator oncogênico ou outros desencadeantes de estresse celular, a cascata de resposta ao dano de DNA preferencialmente converge para p53, que é ativada e, dependendo da severidade dos sinais de estresse, induz super ou sub regulação de vários genes envolvidos na parada do ciclo celular, reparo de DNA, envelhecimento celular ou apoptose (VOGELSTEIN *et al.,* 2000; WHYTE *et al.,* 2002; KUROKAWA *et al.,* 2003; SANTOS-GARCÍA *et al*., 2005; RODIER *et al.,* 2007; JOERGER e FERSHT, 2008).

Tais características conferem à p53 uma habilidade eficiente de suprimir a carcinogênese, uma vez que a mesma é capaz de reduzir a ocorrência de mutações somáticas e impedir a sobrevivência e proliferação de células mutadas (VOGELSTEIN *et al.,* 2000; RODIER

et al., 2007). Portanto, caso a proteína p53 esteja com função alterada, defeitos nos mecanismos a ela relacionados podem levar à manutenção de células com danos genotóxicos severos, levando ao acúmulo excessivo de mutações e transformação neoplásica (WHYTE *et al.,* 2002; RODIER *et al.,* 2007).

Em aproximadamente 50% dos cânceres humanos, TP53 é inativado diretamente por uma mutação, enquanto nos casos remanescentes a atividade de p53 pode estar suprimida devido a alterações em mecanismos associados a esta proteína (VOGELSTEIN *et al.,* 2000; JOERGER e FERSHT, 2008). Essas mutações do gene TP53 comumente resultam na superexpressão e/ou estabilização da proteína (MURTI *et al.,* 1998; SANTOS-GARCÍA *et al*., 2005; JOERGER e FERSHT, 2008). Portanto, considerando que a proteína "selvagem" apresenta um período de meia-vida curto e usualmente não é detectada por imunoistoquímica, a presença de proteína superexpressa detectável é interpretada como indicador de proteína inativa, que pode ser observada em diversos tipos de câncer, inclusive em carcinoma de células escamosas de boca (KERDPON *et al.,* 1997; MURTI *et al.,* 1998; WHYTE *et al.,* 2002; SANTOS-GARCÍA *et al.,* 2005; SHAH *et al.,* 2009). Estudos revelam que a mucosa bucal normal é imunonegativa ou apresenta baixos índices de células p53 positivas, sendo estas restritas às camadas basal e parabasal (KERDPON *et al.,* 1997; CRUZ *et al.,* 1998; KUROKAWA *et al.,* 2003; SANTOS-GARCÍA *et al.,* 2005; FAN *et al.,* 2006).

Alguns autores sugerem que alteração de p53 e sua consequente superexpressão seja um evento precoce na carcinogênese da cavidade bucal, uma vez que 15-19% das LB apresentam alguma mutação em TP53, inclusive lesões com displasia leve (CRUZ *et al.,* 1998; MURTI *et al.,* 1998; SANTOS-GARCÍA *et al*., 2005). De acordo com a literatura, o índice de imunomarcação de p53 aumenta com o aumento da severidade da displasia em LB, sendo que a marcação suprabasal foi associada com displasias moderada e severa e desenvolvimento de carcinoma de células escamosas (KERDPON *et al.,* 1997; CRUZ *et al.,* 1998; LEE *et al.,* 2000; NYLANDER *et al.,* 2000; CRUZ *et al.,* 2002; KUROKAWA *et al.,* 2003; SANTOS-GARCÍA *et al.,* 2005; FAN *et al.,* 2006). Entretanto, em alguns estudos o índice de expressão de p53 ou mutações em TP53 não puderam ser relacionados com o potencial de malignidade ou grau de displasia da LB (KERDPON *et al.,* 1997; CRUZ *et al.,* 1998; MURTI *et al.,* 1998; OGMUNDSDÓTTIR *et al.,* 2009). Foi sugerido que a avaliação conjunta de achados histológicos

e alguns marcadores, como a expressão de p53, poderia identificar indivíduos com LB de alto risco (CRUZ *et al.,* 1998; LEE *et al.,* 2000; FAN *et al.,* 2006).

Em algumas situações, a fosforilação de p53 é dependente de hMutS□ e hMutL□ funcionantes (LI, 1999; BERNSTEIN *et al.,* 2002). Além disso, p53 é capaz de paralisar o ciclo celular para que o reparo de DNA seja realizado (BERNSTEIN *et al.,* 2002). Portanto, parece que estas proteínas e as vias em que elas estão envolvidas podem estar relacionadas (Figura 1) (ZABKIEWICZ e CLARKE*,* 2004; SENGUPTA e HARRIS, 2005; JUN *et al*., 2006; O'BRIEN e BROWN, 2006). Entretanto, a comparação entre a imunoexpressão de p53 e hMLH1 em LB não foi investigada até o momento.

Figura 1 – Interação entre as proteínas MMR e p53 na sinalização de apoptose e parada no ciclo celular.

2.4 REGIÕES ORGANIZADORAS NUCLEOLARES (NOR)

O nucléolo é um domínio bem definido no núcleo da célula em intérfase, no qual genes ribossomais estão alocados e a biogênese ribossomal ocorre (TRERÈ, 2000; DERENZINI *et al.,* 2006). Portanto, no nucléolo encontram-se cromatina ribossomal, substâncias envolvidas no

controle da transcrição ribossomal e estruturas contendo transcritos ribossomais (DERENZINI *et al.,* 1990).

As regiões organizadoras de nucléolo (NOR) contêm genes ribossomais e proteínas não-histonas e podem ser identificadas através de coloração pela prata (AgNOR) (DERENZINI *et al.,* 2006). Assim, as AgNOR são visualizadas em microscopia de luz como pequenos pontos negros bem definidos no nucléolo e refletem a atividade transcricional de genes ribossomais (ZACZEK *et al.,* 1994; TRERÈ, 2000). Existe uma relação entre a atividade funcional nucleolar e a proliferação celular, sendo que quanto maior a síntese de rRNA, mais rápida é a duplicação celular (DERENZINI *et al.,* 1998). Através de análises quantitativa e morfométrica, a técnica de AgNOR é empregada para avaliar a atividade metabólica da célula, como proliferação, diferenciação e síntese protéica (AHMED *et al.,* 2009). Em células quiescentes ou inativas, as AgNOR formam um ponto central e homogêneo, enquanto em células proliferativas esta distribuição é desorganizada, com múltiplos pontos dispersos (LÓPEZ-BLANC *et al.,* 2009).

Células malignas freqüentemente exibem maior número de AgNOR quando comparadas a suas equivalentes normais ou benignas (XIE *et al.,* 1997; TRERÈ, 2000). Além disso, foi demonstrado que lesões displásicas da mucosa bucal apresentam maior número de AgNOR que epitélio normal, assim como carcinoma de células escamosas em relação às displasias, tendo sido esta análise capaz de diferenciar estas lesões (WARNAKULASURIYA e JOHNSON, 1993; CHATTOPADHYAY *et al.,* 1994; KOBAYASHI *et al.,* 1995; XIE *et al.,* 1997; CHATTOPADHYAY *et al.,* 2002). Portanto, a quantidade de proteínas AgNOR na intérfase é utilizada para avaliar a atividade nucleolar e proliferação celular, sendo que altos níveis de AgNOR indicam pior prognóstico e maior taxa de proliferação (METZE e LORAND-METZE, 1999; SIRRI *et al.,* 2000; CHATTOPADHYAY e RAY, 2008; ELANGOVAN *et al.,* 2008).

Na busca por um critério mais objetivo para o diagnóstico e graduação de displasia em leucoplasias bucais, recentemente foi sugerido um ponto de corte (2,3) na contagem de AgNOR, o qual distinguiria entre lesões com displasia leve e moderada (CHATTOPADHYAY e RAY, 2008), embora outros estudos sejam necessários para validação dessas observações.

3 JUSTIFICATIVA

A leucoplasia bucal é a principal lesão potencialmente maligna da boca, cuja apresentação histológica varia de lesões hiperceratóticas a lesões com displasia severa. Muitas leucoplasias regridem ou permanecem quiescentes, porém algumas progridem e podem evoluir para carcinoma de células escamosas, uma doença multifatorial que corresponde à maioria dos cânceres bucais, com altas taxas de mortalidade. Apesar da subjetividade da avaliação histológica para o estabelecimento do grau de displasia, este é ainda o método mais utilizado na identificação de lesões de comportamento mais agressivo. No entanto, lesões fenotipicamente semelhantes podem apresentar comportamentos clínicos distintos, o que justifica a realização de novos estudos em busca de marcadores moleculares que possam elucidar eventos precoces envolvidos na carcinogênese bucal. A abordagem clínica das leucoplasias bucais poderá, desta forma, se tornar um procedimento menos empírico.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar, comparativamente, os índices de marcação de proteínas envolvidas no reparo de DNA e na supressão tumoral e a proliferação celular em leucoplasias bucais com diversos graus histológicos de displasia.

4.2 OBJETIVOS ESPECÍFICOS

1. Identificar a relação entre a imunoexpressão de hMLH1 e o grau de displasia epitelial.
2. Avaliar a relação entre a imunomarcação de p53 e o grau de displasia epitelial.
3. Analisar a média de número de AgNOR de acordo com o grau de displasia epitelial.
4. Correlacionar a imunoexpressão de hMLH1 e p53 e o número médio de AgNOR nas lesões.

5 METODOLOGIA

5.1 SELEÇÃO DA AMOSTRA

Os arquivos do Serviço de Patologia Bucomaxilofacial da Faculdade de Odontologia da Universidade Federal de Minas Gerais e da Universidade Federal de Goiás foram revisados e lesões clinicamente diagnosticadas como LB foram avaliadas. Lâminas coradas em hematoxilina e eosina foram analisadas por dois examinadores independentes (*Kappa* = 0,85) e o grau de displasia foi estabelecido de acordo com os critérios da Organização Mundial de Saúde (OMS, 2005), como descrito nos quadros 1 e 2. Sessenta e duas amostras foram selecionadas, sendo divididas em quatro grupos: 1) LB sem displasia (n=17); 2) LB com displasia discreta (n=19); 3) LB com displasia moderada (n=16); e 4) LB com displasia severa (n=10). Lesões em lábio foram excluídas. Sete amostras de mucosa bucal normal obtidas de extração cirúrgica de terceiros molares totalmente inclusos foram utilizadas para comparação. Cortes histológicos seriados de 3 µm foram utilizados para a realização das reações.

Quadro 1 – Critérios morfocitológicos para diagnóstico de displasia epitelial (OMS, 2005)

Alterações da arquitetura	*Alterações citológicas*
Estratificação epitelial irregular	Alteração do tamanho nuclear e celular
Perda de polaridade da camada basal	Pleomorfismo nuclear e celular
Projeções epiteliais em forma de gota	Aumento da proporção núcleo / citoplasma
Aumento do número de mitoses	Núcleos aumentados de tamanho
Mitoses superficiais anormais	Figuras de mitose atípicas
Ceratinização celular prematura (disceratose)	Nucléolos numerosos e aumentados
Presença de pérolas de ceratina	Hipercromasia

Quadro 2 – Critérios de graduação histológica de displasia epitelial para leucoplasias de boca (OMS, 2005)

Graduação histológica	*Critério para classificação*
Ausência de atipia	Ausência de alterações estruturais e atipia citológica.
Atipia epitelial discreta	Presença de alterações estruturais e atipia citológica confinadas ao terço inferior do epitélio.
Atipia epitelial moderada	Presença de alterações estruturais e atipia citológica estendendo até o terço médio do epitélio.
Atipia epitelial severa	Presença de alterações estruturais e atipia citológica estendendo além do terço médio do epitélio.

5.2 REAÇÃO IMUNOISTOQUÍMICA PARA PROTEÍNAS hMLH1 E p53

Cortes histológicos foram desparafinizados em xilol e hidratados em etanol. Especificações sobre a reação estão descritas no quadro 3. Após recuperação antigênica, realizou-se bloqueio da peroxidase endógena com solução de metanol e H_2O_2 3%. As lâminas foram incubadas com os anticorpos primários e, posteriormente, com o sistema de detecção. A reação foi revelada com solução cromógena de 3,3' diaminobenzidina (DAB) e a contracoloração foi feita com hematoxilina de Mayer. Fragmentos de lesões sabidamente positivas para cada anticorpo foram incluídos como controle positivo. Para o controle negativo, o anticorpo primário foi omitido.

Quadro 3 – Anticorpos primários: fabricantes, recuperação antigênica, diluição, incubação e sistema de detecção.

Anticorpo monoclonal	*Clone / fabricante*	*Recuperação antigênica*	*Diluição*	*Incubação*	*Sistema de detecção*
Anti-hMLH1	Clone G168-15; BD Pharmingen™, código 550838	TRIS-EDTA, pH 9.0, banho maria 96°C 30'.	1:100	15h / 4°C	Advance HRP (Dako, código K4068)
Anti-p53	Clone D07 – Dako, código M7001	TRIS-EDTA, pH 9.0, panela de pressão 20'.	1:100	18h / 25°C	LSAB (Dako, K0690)

5.2.1 ANÁLISE DA IMUNOMARCAÇÃO DAS PROTEÍNAS hMLH1 E p53

Células com núcleo acastanhado foram consideradas positivas, independente da intensidade da coloração. A contagem foi realizada por um observador em microscópio ótico com retículo acoplado. As camadas basal e suprabasal foram avaliadas, sendo a basal correspondente à primeira camada de células, adjacentes ao tecido conjuntivo, e as demais agrupadas como suprabasais. De acordo com cálculo realizado por Fernandes e colaboradores (2007), foram avaliados 16 campos em aumento de 400x em cada lâmina, contando-se as células positivas e negativas. Nos casos em que toda a extensão do corte não perfazia os 16 campos, o tecido foi contado em sua totalidade. O número de células positivas em cada camada separadamente e em todas as camadas agrupadas foi dividido pelo total de células contadas

(positivas e negativas) na camada e o resultado foi multiplicado por 100. Logo, os resultados são demonstrados como porcentagem de células positivas.

5.3 TÉCNICA DA AgNOR

Cortes histológicos foram desparafinizados em xilol e hidratados em etanol. Realizou-se recuperação antigênica, com solução de ácido cítrico pH 6.0 em panela de pressão por 20 minutos. Após resfriarem, as lâminas foram imersas em solução de etanol e ácido acético. Depois de lavadas, procedeu-se à incubação com solução de prata e gelatina, misturadas na proporção de 2:1, respectivamente [solução de prata (5 gramas de nitrato de prata em 10 mL de água destilada); gelatina (1 grama de gelatina em pó e 0,5 mL de ácido fórmico em 50 mL água destilada)]. Esta incubação foi realizada em ambiente escuro, à temperatura ambiente, por 25 minutos. Procedeu-se lavagem com água destilada aquecida. As lâminas foram desidratadas em etanol, diafanizadas em xilol e montadas com lamínulas.

5.3.1 CONTAGEM DE AgNOR

Foram avaliadas 100 células em cada lâmina, por um único observador, em microscópio ótico com retículo acoplado, em aumento de 1000x. Pontos negros bem definidos ou agregados (pontos sobrepostos) no núcleo celular foram contados como um AgNOR. As camadas do epitélio foram avaliadas conforme descrito no item 5.2.1. O total de número de AgNOR contado foi dividido pelo número de células contadas em cada camada separadamente e para ambas as camadas agrupadas. Portanto, foram obtidos os números médios de AgNOR na camada basal, suprabasal e total.

5.4 ANÁLISE ESTATÍSTICA

A análise estatística foi realizada utilizando-se o programa SPSS® para Windows versão 12.0. Análise de normalidade foi feita através do teste de Kolmogorov-Smirnov. Para as variáveis normais, os testes ANOVA e Tukey foram empregados e valores de $p<0,05$ foram considerados significantes. Para as variáveis não-normais, os testes de Kruskal-Wallis e Mann-Whitney foram utilizados e, após correção de Bonferroni, consideraram-se estatiscamente significantes valores

de $p<0,005$. Análise de correlação foi feita através dos testes de Pearson e Spearman.

5.5 ASPECTOS ÉTICOS E LEGAIS

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais (COEP-UFMG), parecer número ETIC 030/09, obedecendo ao exigido pela legislação brasileira, conforme as resoluções CNS nº196/96 e 304/00 do Conselho Nacional de Saúde, sobre Diretrizes e Normas Regulamentadoras de Pesquisas Envolvendo Seres Humanos.

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7 RESULTADOS E DISCUSSÃO

7.1 Artigo 1

"hMLH1 immunoexpression is related to the degree of epithelial dysplasia in oral leukoplakia".

(submetido à *Journal of Oral Pathology and Medicine*).

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hMLH1 immunoexpression is related to the degree of epithelial dysplasia in oral leukoplakia.

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Methods:	Immunohistochemistry
Keywords:	Leukoplakia, Oral precancer, Oral cancer
Abstract:	Background: hMLH1 is a protein of the mammalian mismatch repair system, responsible for genomic stability during repeated duplication. Relation between its altered expression linked to microsatellite instability has also been observed in oral leukoplakias and squamous cell carcinomas pointing to a possibly role of hMLH1 in oral carcinogenesis. To our knowledge, this is the first study evaluating the immunoexpression of hMLH1 in oral leukoplakias regarding their different degrees of epithelial dysplasia. Methods: Sixty-two specimens of oral leukoplakia were classified in four groups: 17 without dysplasia, 19 with mild dysplasia, 16 with moderate dysplasia, and 10 with severe dysplasia. Immunohistochemistry for hMLH1 was performed and percentage of positive cells was assessed. In the statistical analysis, p values <0.005 were considered significant. Results: hMLH1 indexes decreased from a lower degree of dysplasia to a higher one, despite statistical significance. Statistical difference was found mainly between suprabasal layers and total indexes. Conclusions: hMLH1 immunoexpression was inversely related to the

	OL degree of dysplasia. The total epithelial hMLH1 index seems to be of more clinical relevance than the evaluation stratified by layers. Our findings also suggest a role of such alterations in this pathway of DNA repair as an early event in oral carcinogenesis.

Introduction

Oral squamous cell carcinoma is the most common cancer of the oral cavity, and despite the advances concerning treatment of patients, its overall prognosis remains poor, mainly due to late diagnosis (1,2). This malignancy can be preceded by oral leukoplakia (OL), the main potentially malignant oral disorder, which is often related to tobacco use, although some cases are idiopathic (3,4). Oral lesions may persist unchanged, enlarge, reduce or even disappear (4). It seems that there are some predictors of malignant transformation, as lesion duration, patient's age, gender, the affected site, clinical appearance, smoking habit, and presence of epithelial dysplasia (5).

Despite its subjectivity and intra and inter-examinators disagreement, light microscopic assessment of dysplasia is yet the most useful method for grading epithelial dysplasia in OL, as it is considered a predictive marker for cancer and, though, for the management of the potentially malignant lesions (6). Nevertheless, lesions with similar histological phenotypes may show different biological behavior (7,8). Studies looking toward biological markers for early events involved in OL cellular transformation are clearly warranted, in order to identify lesions with greatest potential for malignant transformation (8,9).

Among other functions, the mammalian mismatch repair system (MMR) is responsible for maintaining genomic stability during repeated duplication (10,11). It was demonstrated that MMR-defective cells are resistant to cell death caused by alkylating and methylation agents, cisplatin and UV radiation (10,12). hMLH1 is a human MutL homolog of the MMR system, which plays a major role in mutation avoidance, taking part in the repair of specific mismatches (10). Most patients of Hereditary Non-Polyposis Colorectal Cancer Syndrome (HNPCC), a dominant autosomal condition, carry on a mutation at MMR genes, mainly hMLH1 and hMSH2, and present high risk for the development of colorectal carcinomas (13). As repeated sequences of nucleotides within the DNA, i.e. microsatellites, are hypersensitive to MMR dysfunction, the malignancies that affect those patients usually develop the microsatellite instability (MSI) phenotype, resulting from accumulating errors during DNA replication (14).

It was suggested that the genomic instability would emerge as a crucial early event in carcinogenesis (8). Studies concerning the MMR proteins and genes were performed on diverse entities, including oral lesions. Alterations such as MSI and hypermethylation of the promoter regions of hMLH1 and hMSH2 were detected in 14-70% and 63% of OL, respectively (15,16). Moreover, a trend toward a higher prevalence of MSI in OL with more severe grades of dysplasia was reported (15).

Apart of the PCR-based molecular assay, immunohistochemical staining for hMLH1 showed a great sensitivity (67 - 79%) and specificity (> 99%) in detecting MSI phenotype (17). Likewise, the histological grade of malignancy of oral squamous cell carcinoma interfered on hMLH1, but not on hMSH2 immunoexpression, with poorly differentiated lesions presenting reduced or negative hMLH1 immunoexpression (18).

Despite investigations carried out on hMLH1 immunoexpression in oral lesions and its possible role in carcinogenesis, its relation to OL with different degrees of dysplasia has never been studied before (16,18,19). In order to add information whether the altered expression pattern of hMLH1 protein, reflecting MSI, would be an early event in carcinogenesis, this study aimed to assess the relation between the immunoexpression of hMLH1 and different degrees of epithelial dysplasia in OL.

Material and methods

Tissue samples

The study protocol was approved by the Ethics Committee of Universidade Federal de Minas Gerais (ETIC 030/09). Files of Oral Pathology Service were revised, and lesions clinically diagnosed as OL were evaluated. Slides stained with hematoxylin and eosin were analyzed by two independent investigators and the degree of epithelial dysplasia was established in accordance with the criteria of WHO (20). Sixty-two samples of OL were selected, being 17 histologically classified as OL without dysplasia, 19 with mild dysplasia, 16 with moderate dysplasia, and 10 with

severe dysplasia. Lip lesions were not included. Seven samples of normal oral mucosa obtained from surgical extraction of totally included third molars were used for a comparative purpose.

Immunohistochemical staining

For immunohistochemical staining, 3μm sections were dewaxed in xylene and hydrated with graded ethanol. TRIS-EDTA (ethykene-diamine tetraacetic acid) solution pH 9.0 was used for antigen retrieval, heated to 96°C in water bath for 30 minutes. Endogenous peroxidase was blocked with a 1:1 solution of methanol and 3% H_2O_2. Primary antibody anti-hMLH1 (clone G168-15; BD Pharmingen™, code number 550838) was diluted 1:100 in 1% bovine serum albumin (BSA) and slides were incubated for 15 hours at 4°C. Detection was undertaken with a two step, highly sensitive, ready-to-use, peroxidase-based system (Advance™; Dako, code number K4068). Reactions were revealed with DAB chromogen solution (Dako, code number K3468). Mayer's hematoxylin was used for counterstaining. Negative controls were obtained by omission of primary antibody and samples of normal oral mucosa with known positive reactivity were included as positive controls.

Cell Counting and statistical analysis

Cells were considered positive if they presented brown nuclear staining, regardless of intensity. Counting was performed by one investigator using an eyepiece grid in light microscope (Standard 20 ZEISS, Carl Zeiss, Gottingen, Germany). Firstly, epithelial layers were evaluated separately. Basal layer was considered as the first layer of cells, adjacent to connective tissue, whereas cells above this layer were considered as suprabasal. A total index was obtained adding the results of all layers (basal and suprabasal). Considering the statistical analysis performed by Fernandes *et al.* (21), in order to obtain a representative index, sixteen high-power fields (400x) were analyzed for each slide, and both positive and negative cells were counted. Samples in which there were less than sixteen fields, all fields were evaluated. The number of positive cells in each layer separately and in all layers grouped was divided by the total of counted cells in the layer (positive and negative) and the result was then multiplied by 100, so the indexes are demonstrated as a percentage of positive cells.

Statistical analysis was performed using SPSS software version 12.0. Kolmogorov-Smirnov was applied as a normality test and Kruskal Wallis and Mann-Whitney tests were used for comparisons (p values <0.05). After performing Bonferroni correction, statistical significance was achieved when p values were <0.005.

Results

Out of the 62 patients with OL, 50 (80.6%) were Caucasian and 12 (19.4%) were Black, being 40 males (64.5%) and 22 females (35.5%), with age ranging from 31 to 82 years (mean = 52 years). Forty-two were smokers, and only two were not tobacco users. Seventeen patients reported alcohol consumption, and they were smokers as well. Lesions ranged from 1 to 50 mm (mean = 15.8 mm), and the most affected site was the buccal mucosa (n=17), followed by alveolar mucosa (n=16), retromolar region (n=7), and lateral border of tongue (n=7). Other sites included floor of mouth (n=3), comissure (n=3), palate (n=2), and tongue dorse (n=1). Five patients had multifocal involvement. This information is summarized in Table 1.

All samples of normal mucosa and OL presented basal and suprabasal immunostained cells. As a non-normal distribution was found for all hMLH1 indexes, median values were assumed. Comparing OL with different degrees of dysplasia, all hMLH1 indexes decreased from a lower degree to a higher one, despite statistical significance. Differences were more evident in the suprabasal layer indexes than in the basal ones (Table 2, Figure 1). Normal mucosa and OL without dysplasia showed similar indexes in hMLH1 staining when layers were analyzed either separately or together.

Table 3 shows p values for comparisons between groups and according to epithelial layers. It is noteworthy that analyzing the total indexes of hMLH1 staining, as well as the suprabasal indexes, all comparisons between groups showed statistical significance ($p<0.005$), except between normal mucosa and OL without dysplasia, normal mucosa and OL with mild dysplasia, and OL with moderate dysplasia and OL with severe dysplasia. Moreover, for these indexes, the

highest difference was noticed between OL with mild dysplasia and OL with moderate dysplasia. Basal layer did not reach statistical significance for any comparison.

Analyzing the affected site, lesions in floor of the mouth and lateral border of tongue showed the lowest suprabasal and total indexes. It is interesting to observe that the two non-smoker patients were elderly women, who presented OL at lateral border of the tongue, with moderate and severe dysplasia, and had lower hMLH1 indexes than median values of their OL groups. In this study, neither gender nor smoking habit were related to the hMLH1 indexes. The association of tobacco use could not be assessed neither for OL without dysplasia, nor for OL with mild dysplasia, as all those patients were smokers.

Discussion

During consecutive replications, mismatched nucleotides can be incorporated into DNA which can lead to activation of MMR system in order to correct the mismatch and to maintain the copy fidelity and genomic stability, otherwise the cell could develop a mutator phenotype and MSI, due to accumulating errors in microsatellite regions of DNA (10,11,14,22,23). Though, replicative cells have higher levels of MMR proteins than resting ones (22). The oral epithelium is known to be under constant renewal and it has been demonstrated that hMLH1 and hMSH2, the two major MMR proteins, are highly expressed in this tissue (21). This was also observed in the present study, as normal oral epithelium showed high levels of hMLH1 immunostaining, reaching a maximum index of 89.2%. It seems, therefore, that this protein is present not only in the replicative stratum, but that it is conserved throughout the maturation process of the keratinocytes. Nevertheless, the exact role of hMLH1 in these cells needs to be clarified. Overall, we can infer that the immunoexpression pattern of this protein in normal oral mucosa is compatible with the maintenance of genomic stability.

Cells with deficient MMR are prone to maintain mismatches, leading to MSI and, eventually, developing cancer (14,22,23). Indeed, in approximately 90% of patients with HNPCC syndrome, known to have a higher risk to colorectal cancer, a germline mutation can be detected, mainly at

hMLH1 and hMSH2 (13,14). In addition, defects on MMR genes have been associated with many malignancies, being hMLH1 and hMSH2 the main targets (12,22).

Squamous cell carcinoma is the most common oral malignancy and OL is the main potentially malignant lesion preceding this cancer, being both associated with smoking habit in most of the cases (1). It was hypothesized that the tobacco use would lead to epigenetic alterations of MMR genes, even in normal oral mucosa, which would then become more susceptible to malignant transformation, possibly with MSI phenotype (16). Tanic *et al.* (8) could identify genomic instability in OL samples, and the authors proposed that lesions with the highest degrees of genomic instability had less chance to develop squamous cell carcinoma, probably due to accumulation of deleterious or lethal mutations which would prevent cell replication. On the other hand, cells carrying a reasonable number of mutations would survive and give rise to tumors (8). Investigations point to the occurrence of hMLH1 hypermethylation, leading to a reduced immunexpression of this protein in squamous cell carcinoma of the head and neck, as well as the presence of MSI and epigentic alterations in OL (15,16,19,24,25). However, the role of MMR proteins in these lesions is still not fully understood (1,3,16).

Our results show similar hMLH1 immunoexpression in normal oral mucosa and OL without dysplasia. It was noticed a progressive decrease in hMLH1 indexes throughout the development of a higher dysplasic phenotype of keratinocytes in OL, assessed by the degree of dysplasia. Similarly, a trend toward a higher prevalence of MSI in OL with more severe grades of dysplasia was reported (15). Moreover, well-differentiated oral squamous cell carcinoma showed higher immunoexpression of hMLH1 than poorly differentiated lesions (18). These findings suggest that this MMR protein may be involved in the progressive transformation of these cells. As they present a defective MMR system, the keratinocytes may gradually acquire diminished mutation avoidance capacity, but their cellular viability is still kept (8). These observations are in consonance with previously reported data which detected hypermethylation of the promoter region of hMLH1 gene in squamous cell carcinoma of the head and neck and in OL, which could be related to the decreased immunostaining of the protein (16,17,19,24,25).

We observed that differences in hMLH1 indexes between OL groups were higher for the suprabasal layers than the basal ones. It seems that this finding corroborates with the current criteria used to grade dysplasia, which considers the acquirement of cellular mutator phenotype throughout the epithelial layers. Another interesting finding was that, comparing hMLH1 suprabasal and total indexes between OL groups, the highest difference in median values was found between OL with mild dysplasia and OL with moderate dysplasia, suggesting that the progression of a lesion from a mild to a moderate dysplasia involves the most notable alteration in hMLH1 immunoexpression. This seems to be in accordance with the suggested binary system for classification of OL dysplasia, which would group lesions of "no/questionable/mild" dysplasia into low risk OL, and "moderate/severe" into high risk (26). Though, as proposed by some authors, grading OL dysplasia would become easier and more reliable, and mild dysplasia would be the cut-off point to decide about removing or not the lesion (6). Nevertheless, those cases in which histological grading is not compatible with clinical outcome would still be misdiagnosed (8,9).

Considering that the carcinogenic process involves many interrelated genetic and epigenetic events, efforts have been made in order to identify possible early molecular markers, which would precede phenotypic alterations, improving the prediction of cancer development (2,9). In the present study, considering all indexes found in OL groups, a somehow ample range can be observed within the same group, as revealed by minimum and maximum values. Though, we infer that the isolated assessment and interpretation of hMLH1 index from an individual case may be performed with caution, as overlapping values may occur among OL with different degrees of dysplasia. Thus, we consider that the evaluation of immunohistochemical indexes of hMLH1 alone is not sufficient to grade epithelial dysplasia in OL, in spite of its possible role in the development and progress of OL dysplastic phenotype. Despite its subjectivity and variability, the currently used method of grading OL dysplasia still seems to be the main predictive indicator of malignant transformation (5-7,9).

We could not demonstrate statistical differences between basal layer indexes for any group. Nevertheless, the suprabasal and the total indexes were different between almost all comparisons. In fact, a precise determination of epithelial layers may be difficult, specially in the

presence of architectural changes, as seen in OL with severe dysplasia. These data suggest that, concerning the dysplastic transformation of epithelium, the total hMLH1 indexes may be more reliable than stratified ones for comparisons between OL with different degrees of dysplasia.

Comparing OL grouped according to the affected site, lesions in the floor of mouth and lateral border of the tongue presented both lowest suprabasal and total indexes. Besides, we could observe that the two non-smokers patients with OL were elderly women who presented lesions at lateral border of the tongue with moderate and severe dysplasia, respectively. Those features are pointed out as some of the high risk factors for OL malignant transformation, i.e. female gender, non-smokers, location in tongue or floor of the mouth, presence of epithelial dysplasia, long duration of OL, size greater than 200 mm^2, and non-homogeneous type (5). It is important to emphasize that those non-smokers patients presented lower hMLH1 indexes than median values of their OL groups. These observations, although punctual, point to a possible role of this protein and the MMR system in OL pathogenesis and severity, and in oral carcinogenesis overall. In this study, the gender was not related to the hMLH1 index, as reported before (21). We could not evaluate the reported association between the smoking habit and hMLH1 immunoexpression (16,21), as there were only two non-smoker patients in OL group.

To our knowledge, this is the first study assessing the immunohistochemical expression of one of the major MMR proteins, hMLH1, in OL with different degrees of dysplasia. Likewise, we intended to improve the current understanding of the complex multistep process of oral carcinogenesis, in the light of MMR system analysis, through a simple technical procedure, i.e. immunohistochemistry, which was reported to present a close association with MSI detection. As it is a cross-sectional study, it is not possible to ensure cause-effect relation among the analyzed data as well as monitoring hMLH1 indexes over time. The immunohistochemical method identifies the cytological presence of the protein, but its functional status is not assessed. Moreover, investigation of genetic and epigenet events was not a purpose of this study. So, further studies are encouraged.

In conclusion, this study demonstrated that hMLH1 in OL showed decreasing indexes from a lower degree of dysplasia to a higher one. For comparisons between OL with different degrees of

dysplasia, the assessment of the total hMLH1 index may be more relevant than considering layers separately. Our results suggest that the altered expression of hMLH1, and maybe the MSI, could be early events in the carcinogenic process.

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Conflict of interest statement

None declared.

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Tables

Table 1 – Demographic and clinical data.

Case number	Gender	Age (years)	Ethnic group	Smoking habit	Alcohol use	Site	OL size (mm)
OL without dysplasia (n=17)							
1	Female	48	Caucasian	Yes	NA	Buccal mucosa	NA
2	Male	65	Caucasian	NA	NA	Alveolar mucosa	NA
3	Female	31	Black	Yes	NA	NA	20
4	Male	59	Caucasian	NA	NA	Retromolar	30
5	Female	41	Black	NA	NA	Tongue (dorse)	50
6	Male	52	Black	Yes	Yes	Alveolar mucosa	10
7	Male	38	Caucasian	Yes	NA	Retromolar	11
8	Male	76	Caucasian	NA	NA	Alveolar mucosa	20
9	Male	49	Caucasian	Yes	Yes	Alveolar mucosa	50
10	Male	60	Caucasian	Yes	Yes	Alveolar mucosa	5
11	Female	48	Caucasian	Yes	No	Retromolar	10
12	Male	51	Caucasian	Yes	Yes	Palate	15
13	Female	56	Caucasian	Yes	NA	Buccal mucosa	1
14	Male	NA	Caucasian	Yes	NA	Buccal mucosa	NA
15	Male	65	Caucasian	NA	NA	Alveolar mucosa	NA
16	Male	32	Caucasian	Yes	No	Comissure	20
17	Male	72	Black	Yes	NA	Buccal mucosa and comissure	20
OL with mild dysplasia (n=19)							
18	Male	55	Black	Yes	Yes	Palate	5
19	Male	57	Caucasian	Yes	Yes	Alveolar mucosa	10
20	Male	36	Caucasian	Yes	Yes	Tongue (venter) and buccal mucosa	50
21	Male	73	Black	Yes	Yes	Buccal mucosa	20
22	Female	45	Caucasian	Yes	No	Alveolar mucosa	30
23	Male	68	Black	NA	NA	Buccal mucosa	NA
24	Male	34	Caucasian	Yes	Yes	Retromolar, palate and buccal mucosa	10
25	Male	34	Caucasian	Yes	Yes	Retromolar, palate and buccal mucosa	10
26	Male	43	Caucasian	Yes	Yes	Retromolar	20
27	Male	52	Caucasian	Yes	No	Retromolar	NA
28	Female	48	Caucasian	Yes	Yes	Alveolar mucosa	NA
29	Male	48	Caucasian	Yes	No	Buccal mucosa	10
30	Male	58	Caucasian	Yes	Yes	Buccal mucosa	30
31	Female	56	Caucasian	Yes	No	Buccal mucosa	10
32	Female	55	Caucasian	Yes	NA	Alveolar mucosa	6
33	Male	36	Caucasian	Yes	Yes	Retromolar	NA
34	Male	40	Caucasian	Yes	NA	Retromolar	NA
35	Male	40	Caucasian	Yes	NA	Buccal mucosa	15
36	Male	35	Caucasian	Yes	No	Buccal mucosa	7
OL with moderate dysplasia (n=16)							
37	Male	55	Caucasian	Yes	No	Buccal mucosa	NA
38	Male	56	Caucasian	NA	NA	Buccal mucosa	NA
39	Female	55	Caucasian	Yes	NA	Tongue (lateral border)	2
40	Female	70	Caucasian	No	No	Tongue (lateral border)	40
41	Female	45	Caucasian	Yes	No	Buccal mucosa	20
42	Male	56	Caucasian	Yes	NA	Tongue (lateral border)	NA
43	Male	44	Caucasian	Yes	Yes	Buccal mucosa	5
44	Male	69	Caucasian	NA	NA	Alveolar mucosa	10
45	Female	55	Caucasian	NA	NA	Buccal mucosa	5
46	Male	47	Caucasian	Yes	Yes	Tongue (lateral border)	3
47	Male	65	Caucasian	NA	NA	Tongue (lateral border)	15
48	Male	46	Caucasian	Yes	NA	Alveolar mucosa	15
49	Female	43	Black	Yes	No	Alveolar mucosa	15
50	Male	53	Caucasian	NA	NA	Alveolar mucosa	10
51	Female	55	Caucasian	NA	NA	Buccal mucosa	2
52	Female	63	Caucasian	NA	NA	Floor of mouth	10
OL with severe dysplasia (n=10)							
53	Female	81	Caucasian	NA	NA	Alveolar mucosa	6
54	Female	43	Black	NA	NA	Alveolar mucosa	30
55	Female	38	Black	Yes	Yes	Buccal mucosa	NA
56	Female	57	Caucasian	Yes	NA	Comissure	10
57	Female	82	Black	No	No	Tongue (lateral border)	10
58	Male	55	Caucasian	NA	NA	Comissure	NA
59	Male	43	Caucasian	Yes	No	Buccal mucosa and comissure	NA
60	Female	74	Caucasian	NA	NA	Floor of mouth	10
61	Male	46	Black	NA	NA	Floor of mouth	NA
62	Male	60	Caucasian	Yes	NA	Tongue (lateral border)	NA
Normal mucosa (n=7)							
63	Female	22	Caucasian	No	No	Retromolar	NM
64	Male	18	Caucasian	No	No	Retromolar	NM
65	Female	23	Caucasian	No	No	Retromolar	NM
66	Male	32	Caucasian	No	No	Retromolar	NM
67	Female	16	Caucasian	No	No	Retromolar	NM
68	Male	29	Caucasian	No	No	Retromolar	NM
69	Female	22	Caucasian	No	No	Retromolar	NM

OL = oral leukoplakia; *n* = number of cases; NA = data not avaiable; NM = normal mucosa.

Table 2 – Percentage of immunopositive cells for hMLH1 in oral leukoplakias according to the degree of epithelial dysplasia.

	Basal layer*	Suprabasal layer*	All layers*
Normal mucosa (n=7)	97.3 (94.2-98.8)	79.3 (74.1-82.1)	85.4 (85.2-89.2)
OL without dysplasia (n=17)	97.3 (94.0-99.7)	83.7 (62.9-92.5)	89.2 (77.4-94.4)
OL with mild dysplasia (n=19)	97.2 (87.2-99.4)	71.3 (58,7-90.4)	78.7 (67.1-92.9)
OL with moderate dysplasia (n=16)	96.6 (86.9-98.7)	57.0 (33.1-70.9)	68.1 (55.2-78.8)
OL with severe dysplasia (n=10)	91.9 (82.1-97.8)	43.8 (28.5-68.2)	59.9 (43.1-75.7)

* median values (minimum-maximum); OL = oral leukoplakia; *n* = number of cases.

Table 3 – Statistical analysis (*p* values) for comparisons of percentage of immunopositive cells for hMLH1 between groups.

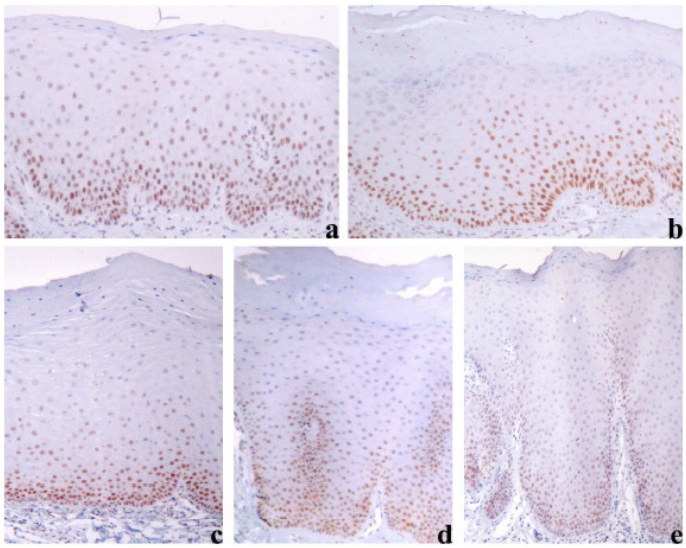
	Normal mucosa	OL without dysplasia	OL with mild dysplasia	OL with moderate dysplasia
OL without dysplasia	b – 0.852 sb – 0.099 t – 0.234			
OL with mild dysplasia	b – 0.735 sb – 0.055 t – 0.035	b – 0.471 sb – 0.003* t – 0.001*		
OL with moderate dysplasia	b – 0.175 sb – 0.000* t – 0.000*	b – 0.146 sb – 0.000* t – 0.000*	b – 0.441 sb – 0.000* t – 0.000*	
OL with severe dysplasia	b – 0.019 sb – 0.000* t – 0.000*	b – 0.006 sb – 0.000* t – 0.000*	b – 0.012 sb – 0.000* t – 0.000*	b – 0.041 sb – 0.036 t – 0.010

p values for Mann-Whitney test; *statistically significant ($p<0.005$); OL = oral leukoplakia.

b – comparison of percentage of hMLH1 positive cells in basal layer only;

sb - comparison of percentage of hMLH1 positive cells in suprabasal layer only;

t - comparison of percentage of hMLH1 positive cells in all layers (total).



hMLH1 immunohistochemical staining (streptoavidin-biotin, 200X) gradually decreases from (a) normal oral epithelium and (b) oral leukoplakia without dysplasia to; (c) oral leukoplakia with mild dysplasia; (d) oral leukoplakia with moderate dysplasia; and (e) oral leukoplakia with severe dysplasia.

7.2 Artigo 2

"Oral leukoplakias presenting different degrees of dysplasia: a comparative study of hMLH1, p53 and AgNOR".

(Artigo a ser submetido à publicação).

Abstract

OBJECTIVES: To assess and to compare the immunoexpression of hMLH1 and p53, and the AgNOR number in oral leukoplakias with different degrees of epithelial dysplasia.

METHODS: Sixty-two samples of oral leukoplakia were evaluated by immunohistochemistry for hMLH1 and p53, and AgNOR technique, being 17 without dysplasia, 19 with mild dysplasia, 16 with moderate dysplasia, and 10 with severe dysplasia. Results are shown as percentage of immunopositive cells and mean AgNOR numbers.

RESULTS: Decrease in hMLH1 and increase in p53 and AgNOR indexes were observed throughout the development of more severe dysplasia, despite statistical significance. Considering oral leukoplakia groups, indexes of hMLH1 expression in suprabasal and all layers were statistically significant for all comparisons except for oral leukoplakia with moderate and severe dysplasia. An inverse correlation between hMLH1 and both p53 and AgNOR, and a direct correlation between p53 and AgNOR could be observed.

CONCLUSIONS: Alterations in the immunoexpression pattern of p53 and hMLH1 seemed to be early events in oral carcinogenesis. During the progressive acquirement of a more dysplastic phenotype, keratinocytes may show diminished capacity of DNA repair and tumor supression, as well as higher cellular proliferation, and these pathways can be somehow interconected.

Introduction

Through the carcinogenic process, the keratinocyte tend to acquire progressively altered phenotype and genotype, leading to the development of a potentialy malignant cell, which can evolve to a malignant entity (Scully and Bagan, 2009). Oral leukoplakia (OL) is the most common potentially malignant disorder of the oral cavity, which can preced oral squamous cell carcinoma. It is usually related to tobacco use, nevertheless some cases are idiopathic (Napier and Speight, 2008). OL may present different clinical behaviors, and some predictors of malignant transformation were pointed out, as the duration of the lesion, patient's age, gender, the affected site, clinical appearance, smoking habit, and presence of epithelial dysplasia (Napier and Speight, 2008; van der Waal, 2009). The histological examination is yet the most useful method for grading epithelial dysplasia in OL, as it is considered a predictive marker for cancer risk and, though, for the management of the patients (Warnakulasuriya et al., 2008). Nevertheless, lesions disclosing similar histological presentation may evolve differently (Tanic et al., 2009).

hMLH1 is one of the major proteins of the mammalian mismatch repair system (MMR), which is responsible for the maintenance of genomic stability during repeated duplication (Kolodner and Marsischky, 1999; Jun et al., 2006). Mutation in MMR genes, mainly hMLH1 and hMSH2, are frequently dected in patients of Hereditary Non-Polyposis Colorectal Cancer Syndrome (HNPCC), who shows high risk for the development of colorectal carcinomas (Paraf et al., 2001). As microsatellites regions of DNA are hypersensitive to MMR dysfunction, malignancies affecting those patients usually develop the microsatellite instability (MSI) phenotype, resulting from acumulating errors during DNA replication (Chung and Rustgi, 2003).

It was suggested that the genomic instability, would emerge as a crucial early event in carcinogenesis (Tanic et al., 2009). Studies concerning the MMR proteins and genes were performed on diverse diseases, including oral lesions. Alterations such as MSI and hypermethylation of the promoter regions of hMLH1 and hMSH2 were detected in 14-70% and 63% of OL, respectively (Ha et al., 2002; Sengupta et al., 2007). Moreover, a trend toward a higher prevalence of MSI in OL with more severe degrees of dysplasia was reported (Ha et al., 2002). Apart of the PCR-based molecular assay, immunohistochemical staining for hMLH1 showed a great sensitivity (67 – 79%) and specificity (> 99%) in detecting MSI phenotype (Shia et al., 2004). Likewise, the histological degree of malignancy of oral squamous cell carcinoma

interfered on hMLH1, but not on hMSH2 immunoexpression, with poorly differentiated lesions presenting reduction or negative hMLH1 immunoexpression (Fernandes et al., 2008).

The p53 protein shows a pivotal role in tumor avoidance, as it can induce DNA repair, cell cycle arrest, cell death or senescense (Joerger and Fersht, 2008). Its alteration is a common finding in human cancers, including those of oral mucosa (Kurokawa et al., 2003). Most but not all studies reveal that p53 immunoexpression, mainly at the suprabasal epithelial layer of OL, may be related with high risk lesions (Cruz et al., 2002; Ogmundsdóttir et al., 2009).

Loss of apoptosis competence coupled with loss of capability to repair DNA damage increases genomic instability, which in turn accelerates progession to cancer (Bernstein et al., 2002). Interactions among many pathways and genes may participate in the evolution from premalignant to malignant oral lesions, with both, MMR and tumor supressor genes having a role in this process (Liu et al., 2002). There is evidence for mediation of apoptosis through p53 and MMR interaction, for example with the MMR machinery proposed to activate and to regulate p53, nevertheless p53-independent pathways can also take place (Bernstein et al., 2002; Zabkiewicz and Clarke, 2004; Jun et al., 2006; O'Brien and Brown 2006).

The nucleolar organizer regions (NOR) are loops of ribosomal DNA associated with non-histone argyrophilic proteins, which can be identified by AgNOR coloration (Derenzini et al., 2006). The AgNOR technique is used to assess the nucleolar activity and cell proliferation, though malignant entities would present higher AgNOR number than benign ones (Chattopadhyay et al., 1994). Also, it has been suggested that AgNOR counting may be able to distinguish OL with different degrees of dysplasia (Chattopadhyay and Ray, 2008).

Considering that the carcinogenesis is a multistep process, in which many cellular mechanisms can be altered (Scully and Bagan, 2009), the present study aimed to assess and to compare the immunoexpression of hMLH1 and p53, as well as the AgNOR number, in OL with different degrees of epithelial dysplasia.

Material and methods

Tissue samples

The study protocol was approved by the Ethics Committee of Universidade Federal de Minas Gerais (ETIC 030/09). Hematoxylin and eosin stained slides of 62 lesions clinically diagnosed as

OL were evaluated and the degree of dysplasia was assessed by two independent examinators, according to the criteria of WHO (2005). Seventeen samples were histologically classified as OL without dysplasia, 19 with mild dysplasia, 16 with moderate dysplasia, and 10 with severe dysplasia. Lip lesions were not included. Seven samples of normal oral mucosa obtained from surgical extraction of totally included third molars were included for a comparative purpose.

Immunohistochemical staining

For immunohistochemical staining, 3µm sections were dewaxed in xylene and hydrated with graded ethanol. Specifications concerning antigen retrieval, primary antibodies and detection system are listed in Table 1. After antigen retrieval, endogenous peroxidase was blocked with a 1:1 solution of methanol and 3% H_2O_2. Slides were incubated with primary antibody, followed by the detection system. Reactions were revealed with DAB chromogen solution (Dako, code number K3468), and Mayer's hematoxylin was used for counterstaining. Negative controls were obtained by omission of primary antibody, and samples of normal oral mucosa and oral squamous cell carcinoma which were known to be reactive against hMLH1 and p53, respectively, were included as positive controls.

AgNOR technique

Sections of 3 µm were dewaxed in xylene and hydrated in ethanol. The slides were immersed in a citric acid solution, pH 6.0, and heated in a pressure cooker for 20 minutes. After cooling down, sections were immersed in a 3:1 ethanol: acetic acid solution. After washing, silver solution was applied in a dark room, for 25 minutes, at 25°C. Slides were washed with warm water, dehydrated in ethanol, cleared in xylene, and mounted.

Cell Counting and statistical analysis

For immunohistochemical stain, cells were considered positive if they presented brown nuclear coloring, regardless of intensity. Cell counting was performed by one examinator using an eyepiece grid in light microscopy (Standard 20 ZEISS, Carl Zeiss, Gottingen, Germany). Epithelial layers were evaluated separately. Basal layer was composed of the first layer of cells, adjacent to connective tissue, whereas cells above this layer were considered as suprabasal. A score for all

layers was also obtained adding the results of basal and suprabasal layers. According to the analysis performed by Fernandes et al. (2007), in order to obtain a representative index, sixteen high-power fields (400x) were analysed for each slide, and both positive and negative cells were counted. Samples in which there were less than sixteen fields, all fields were evaluated. The number of positive cells in each layer separately and in all layers grouped was divided for the total of counted cells in the layer (positive and negative) and the result was then multiplied by 100, so the indexes are demonstrated as percentage of positive cells.

For AgNOR, 100 cells were counted in each slide, at a 1000x magnification, with immersion oil and an ocular grid. Black dots or aggregated clusters within cellular nucleoli were counted as one AgNOR. Moreover, basal and suprabasal layers were analised. The total AgNOR number was divided by the number of cell in each layer saparately, as well as for all layers grouped. Likewise, we could assess the mean AgNOR values for basal, suprabasal and all layers.

Statistical analysis was performed using SPSS software version 12.0. Analysis of normality was performed with Kolmogorov-Smirnov test. Only the AgNOR indexes of basal and suprabasal layers showed a normal distribution, though ANOVA and Tukey tests were performed and p values <0.05 were considered significant. For all other indexes, Kruskal-Wallis and Mann-Whitney tests were used, with significance achieved when p values were <0.005, after Bonferroni correction. Appropriate correlation analyses were undertaken with Pearson and Spearman's tests for normal and not normal data, respectively.

Results

Fifty patiens (80.6%) were Caucasian and 12 (19.4%) were Black, been 40 males (64.5%) and 22 females (35.5%), with age raging from 31 to 82 years (mean = 52 years). Forty-two were smokers, and only two were not tobacco users. Seventeen patients reported alcohol consumption, and they were smokers as well. Lesions ranged from 1 to 50 mm (mean = 15.8 mm), and affected buccal mucosa (n=17), alveolar mucosa (n=16), retromolar region (n=7), lateral border of tongue (n=7), floor of mouth (n=3), comissure (n=3), palate (n=2), and tongue dorse (n=1). Five patients had multifocal involvement.

Table 2 shows hMLH1 and p53 indexes as well as AgNOR number in OL groups. It is

interesting to notice that the gradual acquirement of a more severe phenotype was accompained by a decrease in hMLH1 and an increase in p53 and AgNOR indexes, irrespective of statistical significance. When lesions were grouped according to the affected site, irrespective of the degree of dysplasia, the lowest hMLH1 indexes and the highest p53 and AgNOR values were observed for lesions in the floor of the mouth and in the lateral border of the tongue.

Table 3 shows *p* values for comparisons of hMLH1, p53 and AgNOR indexes among groups. hMLH1 and AgNOR values in suprabasal and all layers were different when comparing normal mucosa with OL with moderate and severe dysplasia. Besides, for all p53 indexes, normal mucosa showed statistically signicant differences among all OL groups, except for the suprabasal layer of OL without dysplasia. Considering the four OL groups, indexes of all layers of hMLH1 and in suprabasal layer were statistically significant for all comparisons, except between OL with moderate and severe dysplasia, while the basal layer did not reach significance for any comparison. p53 suprabasal and all layers indexes, as well as AgNOR suprabasal index, were significant between OL without dysplasia and OL with severe dysplasia and OL with mild dysplasia and OL with severe dysplasia. Besides, p53 basal layer indexes revealed borderline *p* values for comparisons between these groups.

Concerning correlation analysis, summarized in Table 4, evaluating only the basal layer, hMLH1 index was inversely and weakly related to p53 and directly and weakly related to AgNOR values. Also, p53 indexes were directly and weakly related to AgNOR values. Analysing suprabasal layer, we could find an inverse and moderate relation between hMLH1 and p53 indexes and an inverse weak relation between hMLH1 and AgNOR values. Besides, p53 indexes showed a direct but weak relation to AgNOR values. Finally, when all layers indexes were assessed, hMLH1 showed an inverse moderate relation to both p53 and AgNOR values. In addition, it was observed direct moderate relation between p53 immunoexpression and AgNOR indexes.

Discussion

For comparisons between normal oral mucosa and all OL groups, p53 indexes in basal, suprabasal and all layers reached statistical significance, except for suprabasal index compared with OL without dysplasia. It seems that the suprabasal immunoexpression of p53 is related to

the emergence of the dysplastic phenotype, and that this alteration is kept by the cells throughout the maturation process, from the basal to the suprabasal layers, as well as on their progressive acquirement of a more severe dyplastic phenotype, assessed by the degree of epithelial dysplasia.

In fact, investigations demonstrated that normal oral mucosa presents negative or low indexes of p53 immunostained cells (Kurokawa et al., 2003; Fan et al., 2006). Likewise, p53 immunopositive cells were identified in OL with mild dysplasia, with increasing indexes from hyperplasia, to dysplastic and to oral squamous cell carcinoma, with immunopositivity found in superficial layers of moderate and severe dysplasias (Kerdpon et al., 1997; Cruz et al., 2002; Kurokawa et al., 2003). The detection of p53 in oral dysplastic lesions prompted many investigators to suggest that its abnormalities may constitute an early event, contributing to genomic instability and thus enhacing the risk of malignant transformation, nevertheless some studies do not support this finding (Cruz et al., 1998; Murti et al., 1998; Tanic et al., 2009).

Differences in hMLH1 indexes were seen between normal mucosa and OL with moderate and severe dysplasia only. On the other hand, considering *p* values for comparisons of hMLH1 indexes between OL groups, mainly at suprabasal and all layers, it seems that this protein presents altered immunoexpression according to the degree of OL dysplasia, with more severe OL showing lower hMLH1 indexes. Similarly, well-differentiated oral squamous cell carcinoma showed higher immunoexpression of hMLH1 than poorly differentiated lesions (Fernandes et al., 2008). Considering that immunohistochemical staining for hMLH1 has shown a great sensitivity and specificity in detecting MSI phenotype (Shia et al., 2004), we can infer that these observations apparently corroborate to a previous study which found a trend toward an increased prevalence of MSI in more aggressive histologic OL lesions (Ha et al., 2002). Liu *et al.* (2002) concluded that the loss of hMLH1 expression occurs in tumor progression rather than in tumor initiation in head and neck squamous cell carcinoma, suggesting that this MMR protein may be involved in the progressive transformation of these cells.

The AgNOR staining has been largely used to assess cellular proliferation, and oral normal epithelium showed lower AgNOR number than dysplastic OL, which in turn presents lower indexes than oral squamous cell carcinoma (Chattopadhyay et al., 1994). Recently, it was suggested that mean AgNOR number would be useful in distinguishing OL with mild and

moderate dysplasia (Chattopadhyay and Ray, 2008). Our results show that the AgNOR counting of all layers was not statisticaly significant between any OL group, nevertheless increasing values were found with the development of more dysplastic lesions. Our findings support that, indeed, keratinocytes show higher proliferation capacity throughout the evolution to more dysplastic phenotype. However, the AgNOR counting was not useful as a diagnostic tool neither for differentiating OL from normal mucosa nor for grading dysplasia, as reported before (Elangovan et al., 2008). It is interesting to emphasize that according to Cabrini *et al.* (1992), the increase in the number of AgNOR is a reflection of the proliferation indexes rather than the degree of malignancy.

Morphological changes in suprabasal layers is regarded for grading epithelial dysplasia (WHO, 2005). Overall, we found that evaluation of the basal layer indexes isolately seemed to be less informative for distinguishing among different degrees of dysplasia. In addition, p53 basal layer indexes revealed borderline *p* values for some comparisons. It should be considered that a precise determination of epithelial layers may be difficult, mainly in the presence of great architectural changes, as seen in OL with more severe dysplasia. Though, we believe that the assessment of hMLH1, p53 and AgNOR indexes in all layers are more informative, giving support to the current grading system which analyzes the extent of morphological and achitectural changes within the epithelium, possibly reflecting the genetic and epigenetic alterations of those cells (Kerdpon et al., 1997; Cruz et al., 2002).

The gradual acquisition of a more severe phenotype was accompained by a decrease in hMLH1 and an increase in p53 and AgNOR indexes. Indeed, we found an inverse correlation between hMLH1 and both p53 and AgNOR indexes, and a direct correlation between p53 and AgNOR values, as expected. Taken together, our findings suggest that during the dysplastic transformation, the cells present a progressive altered protein expression, reflecting possible diminished capacities of DNA repair and tumor supression, as well as higher cellular proliferation, which are described as malignant cell features (Scully and Bagan, 2009). Moreover, our findings support that alterations in these pathways can be somehow interconected as proposed by some investigations, contributing to the gradual development of the altered phenotype (Bernstein et al., 2002; Liu et al., 2002; Zabkiewicz and Clarke, 2004, Jun et al., 2006, O'Brien and Brown, 2006).

The present study showed that the degree of epithelial dysplasia was related to the

immunoexpression of hMLH1 and p53 and the AgNOR number, with lesions with more severe dysplasia presenting patterns closer to those reported for malignant lesions. The analyses of indexes of all layers were more informative than evaluation of epithelial layers separately. Differences in the expression of hMLH1 and p53 semed to be earlier events in oral carcinogenesis. These pathways of DNA repair, tumor supression and cellular proliferation may be somehow conected and participating in the multistep carcinogenic process.

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Conflict of interest statement

None declared.

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Tables

Table 1 – Primary antibodies, antigen retrieval and detection system used.

Primary antibody	*Clone / manufacturer*	*Antigen retrieval*	*Dilution*	*Incubation*	*Detection system*
Anti-hMLH1	Clone G168-15; BD Pharmingen™, code 550838	TRIS-EDTA, pH 9.0, water bath, 96°C, 30'.	1:100	15h / 4°C	Advance HRP (Dako, code K4068)
Anti-p53	Clone D07 – Dako, code M7001	TRIS-EDTA, pH 9.0, pressure cooker, 20'.	1:100	18h / 25°C	LSAB (Dako, code K0690)

Table 2 – Percentage of hMLH1 and p53 immunostained cells and AgNOR number.

	hMLH1			***p53***			***AgNOR***		
	*Basal layer**	*Suprabasal layer**	*All layers**	*Basal layer**	*Suprabasal layer**	*All layers**	*Basal layer***	*Suprabasal layer***	*All layers**
Normal mucosa (n=7)	97.3 (94.2-98.8)	79.3 (74.1-82.1)	85.4 (85.2-89.2)	3.4 (1.2-7.7)	1.0 (0.5-2.9)	2.6 (1.1-3.8)	1.87 (0.21)	1.64 (0.20)	1.73 (1.6-1.9)
OL without dysplasia (n=17)	97.3 (94.0-99.7)	83.7 (62.9-92.5)	89.2 (77.4-94.4)	14.2 (1.6-68.0)	3.0 (0.2-12.0)	6.3 (0.8-28.0)	2.15 (0.26)	1.86 (0.23)	1.90 (1.5-2.4)
OL with mild dysplasia (n=19)	97.2 (87.2-99.4)	71.3 (58,7-90.4)	78.7 (67.1-92.9)	27.6 (3.2-45.0)	4.5 (2.3-10.0)	13.5 (3.1-20.0)	2.08 (0.50)	1.86 (0.20)	1.90 (1.5-2.4)
OL with moderate dysplasia (n=16)	96.6 (86.9-98.7)	57.0 (33.1-70.9)	68.1 (55.2-78.8)	27.8 (9.3-63.0)	4.5 (3.1-12.0)	10.9 (6.1-35.0)	2.27 (0.30)	2.08 (0.33)	2.11 (1.7-2.7)
OL with severe dysplasia (n=10)	91.9 (82.1-97.8)	43.8 (28.5-68.2)	59.9 (43.1-75.7)	49.9 (13.0-93.0)	12.5 (3.4-43.0)	23.8 (6.2-61.0)	2.22 (0.66)	2.29 (0.35)	2.16 (1.6-3.1)

* median values (minimum-maximum); ** mean values (standard deviation); OL= oral leukoplakia; n= number of cases.

Table 3 – Statistical significance (*p* values) for comparisons of hMLH1, p53 and AgNOR between groups.

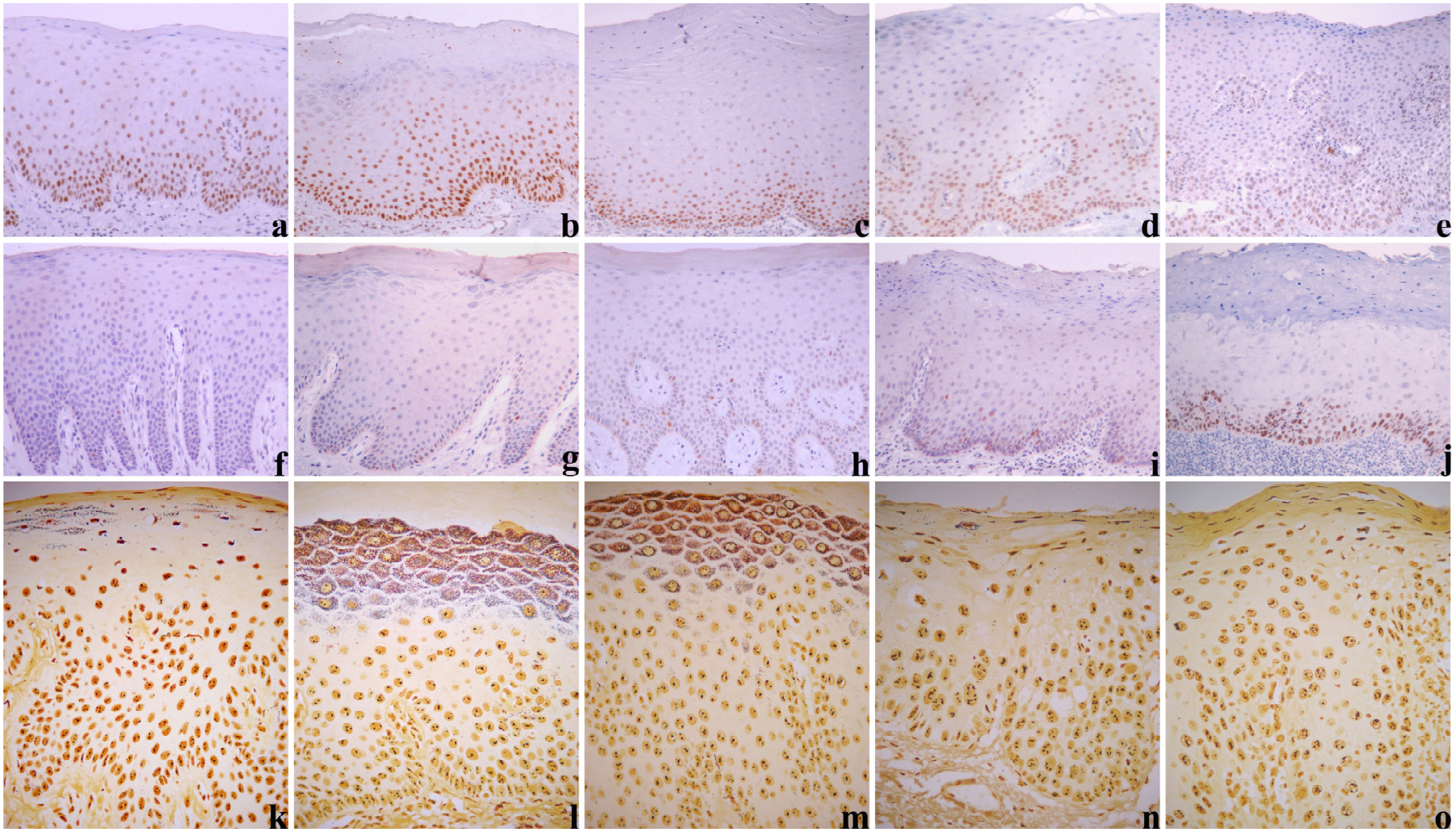
	hMLH1			***p53***			***AgNOR***		
	Basal layer[a]	*Suprabasal layer*[a]	*All layers*[a]	*Basal layer*[a]	*Suprabasal layer*[a]	*All layers*[a]	*Basal layer*[b]	*Suprabasal layer*[b]	*All layers*[a]
Normal mucosa x OL without dysplasia	0.852	0.099	0.234	0.000*	0.009	0.000*	0.563	0.379	0.007
Normal mucosa x OL with mild dysplasia	0.735	0.055	0.035	0.000*	0.000*	0.000*	0.798	0.372	0.035
Normal mucosa x OL with moderate dysplasia	0.175	0.000*	0.000*	0.000*	0.000*	0.000*	0.226	0.004**	0.001*
Normal mucosa x OL with severe dysplasia	0.019	0.000*	0.000*	0.000*	0.000*	0.000*	0.434	0.000**	0.002*
OL without dysplasia x OL with mild dysplasia	0.471	0.003*	0.001*	0.639	0.175	0.616	0.982	1.000	0.778
OL without dysplasia x OL with moderate dysplasia	0.146	0.000*	0.000*	0.068	0.058	0.041	0.926	0.118	0.045
OL without dysplasia x OL with severe dysplasia	0.006	0.000*	0.000*	0.006	0.001*	0.002*	0.993	0.001**	0.023
OL with mild dysplasia x OL with moderate dysplasia	0.441	0.000*	0.000*	0.230	0.567	0.333	0.647	0.098	0.044
OL with mild dysplasia x OL with severe dysplasia	0.012	0.000*	0.000*	0.004*	0.004*	0.002*	0.897	0.001**	0.021
OL with moderate dysplasia x OL with severe dysplasia	0.041	0.036	0.010	0.041	0.014	0.027	0.998	0.321	0.363

[a]Mann-Whitney test, *statistically significant ($p<0.005$). [b]Tukey test, **statistically significant ($p<0.05$). OL= oral leukoplakia.

Table 4 – Spearman's correlation coefficients for hMLH1, p53 and AgNOR comparisons, according to the epithelial layer.

	Basal layer	Suprabasal layer	All layers
hMLH1 x p53	-0.020	-0.439	-0.475
hMLH1 x AgNOR	0.018	-0.380	-0.410
p53 x AgNOR	0.274	0.329	0.418

Negative values = inverse correlation; positive values = direct correlation. Modules between: 0 and 0.400 = weak correlation; 0.401 and 0.700 = moderate correlation; > 0.700 = strong correlation.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8 CONCLUSÕES

1. Quanto mais severo o grau de displasia epitelial, menor foi índice de imunoexpressão de hMLH1, sugerindo que esta proteína e, possivelmente, a ocorrência de MSI, estão envolvidos na transformação displásica progressiva dessas lesões, além de serem eventos precoces na carcinogênese bucal.

2. Considerando-se a relação direta entre o grau aumentado de displasia e maior índice de imunoexpressão de p53, sugere-se que a imunoexpressão dessa proteína relaciona-se com a emergência do fenótipo displásico, que é mantido durante a maturação dos ceratinócitos, permitindo a aquisição de graus mais severos de displasia. Portanto, as alterações em p53 ocorreriam em estágios precoces da carcinogênese, possivelmente contribuindo para instabilidade genômica e risco de transformação maligna.

3. Embora o número médio de AgNOR tenha sido maior em lesões com graus de displasia mais severos, sugerindo maior capacidade proliferativa durante a evolução para fenótipos displásicos mais severos, a contagem de AgNOR não foi de auxílio, nesse estudo, na distinção entre os diversos graus de displasia, uma vez que as diferenças observadas não foram estatisticamente significantes entre os grupos de leucoplasia bucal.

4. A correlação inversa da imunoexpressão hMLH1 com imunoexpressão de p53 e o com número médio de AgNOR nas lesões pode ser reflexo de uma capacidade reduzida de reparo de DNA e de supressão tumoral, além de aumento no índice de proliferação celular. Além disso, essas vias podem estar interconectadas de alguma forma, contribuindo para o desenvolvimento gradual de fenótipos mais displásicos.

9 CONSIDERAÇÕES FINAIS

Considerando-se a importância clínica do carcinoma de células escamosas de boca, especialmente ligada à sua elevada incidência na população adulta e às altas taxas de mortalidade e morbidade, o diagnóstico precoce dessa entidade ainda é considerado o fator de prognóstico mais favorável ao paciente. Dessa forma, estudos visando o reconhecimento precoce de lesões com maior capacidade transformação maligna constituem um campo vasto de investigações, embora ainda mostrem resultados especulativos, conflitantes e não conclusivos.

As leucoplasias bucais são as principais lesões potencialmente malignas da boca, podendo evoluir para carcinoma de células escamosas, sendo que o grau histológico de displasia epitelial é, ainda hoje, o critério mais utilizado na avaliação do potencial de malignização dessas lesões. A carcinogênese é um processo multifatorial e envolve alterações em diversos genes e vias celulares, englobando eventos precoces e de evolução da doença. Dessa forma, buscou-se, através da análise de leucoplasias com diferentes graus de displasia, dados para o melhor entendimento das fases precoces da transformação maligna, através da avaliação de proteínas de reparo de DNA e de supressão tumoral, além do índice de proliferação celular, mecanismos estes que podem envolver vias interligadas no processo carcinogênico.

A proteína hMLH1 é uma das principais componentes do sistema MMR de reparo de DNA em humanos e evidências apontam para uma relação entre a imunoexpressão desta proteína e a graduação histológica de carcinoma de células escamosas de boca, assim como sua capacidade em identificar MSI, evento importante na aquisição de fenótipos celulares alterados. Além disso, a via do sistema MMR parece se relacionar com a via de p53, uma importante proteína supressora tumoral.

Os resultados do presente estudo sugerem que ceratinócitos apresentam, durante a aquisição de fenótipos mais displásicos, uma diminuição na capacidade de reparo de DNA via MMR e de supressão tumoral via p53, além de maior capacidade de proliferação celular, sendo que estas vias podem estar interelacionadas de alguma maneira, contribuindo para a evolução destas lesões. Nossos achados indicam, ainda, que alterações na expressão de hMLH1 e p53 parecem ser eventos precoces na carcinogênese bucal.

A falta de significância estatística entre o número médio de AgNOR e os diversos graus de displasia, nesse estudo, pode sugerir que o índice de proliferação celular não seja o evento mais relevante nesses estágios de transformação displásica, embora a utilização de outros

marcadores mais sensíveis de proliferação celular possa também ser estimulada.

É interessante observar que o grupo de lesões localizadas em borda lateral de língua e soalho bucal, além das lesões que acometeram mulheres não fumantes, dados considerados pela literatura como indicativos de maior potencial de transformação maligna, apresentaram, independente do grau de displasia, menor imunoexpressão de hMLH1 e maiores índices de p53 e número médio de AgNOR.

Este estudo propõe que a avaliação do epitélio em toda sua extensão seja mais informativa das alterações moleculares celulares que a análise estratificada por camadas, corroborando o atual critério histológico utilizado para graduar a displasia, uma vez que são consideradas as alterações morfológicas existentes em toda a extensão epitelial das leucoplasias.

Estudos longitudinais ajudarão a esclarecer se a análise conjunta destes marcadores pode ser útil para auxiliar na identificação de leucoplasias com maior potencial de malignização, tornando a conduta clínica dos casos menos empírica. Além disso, o padrão alterado de imunoexpressão de hMLH1, sugerindo a ocorrência de MSI precocemente nestas lesões, deverá ser melhor explorado através de novos trabalhos com outras metodologias.

10 ANEXOS

10.1 Anexo 1

Parecer do Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais.

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer nº. ETIC 030/09

Interessado(a): Profa. Maria Auxiliadora Vieira do Carmo
Depto. Clínica, Patologia e Cirurgia Odontológicas
Faculdade de Odontologia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 25 de março de 2009, o projeto de pesquisa intitulado **"Leucoplasias bucais: estudo comparativo entre o grau de displasia, análise quantitativa de AgNor e a imunoexpressão hMLH 1 e de p53"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2º andar – Sala 2005 – Cep:31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

10.2 Anexo 2 - Tabelas.

Table 1 – Indexes of hMLH1 and p53 immunostained cells and AgNOR count for each case.

	hMLH1 (%)			*p53 (%)*			*AgNOR (mean number)*		
Case number	*Basal layer*	*Suprabasal Layer*	*All layers*	*Basal layer*	*Suprabasal Layer*	*All layers*	*Basal layer*	*Suprabasal Layer*	*All layers*
OL without dysplasia (n=17)									
1	99.0	83.2	89.2	14.2	1.7	6.3	2.40	2.00	2.12
2	96.1	92.5	93.7	7.6	2.9	4.8	1.73	1.46	1.54
3	98.1	87.0	90.9	45.0	5.9	19.7	2.53	2.00	2.08
4	96.4	84.4	90.0	18.9	3.0	10.0	1.55	1.86	1.72
5	98.6	80.2	87.2	32.9	5.4	15.5	2.33	1.71	1.90
6	97.4	91.5	94.4	9.0	4.2	6.3	2.00	1.79	1.85
7	94.7	62.9	77.4	9.9	2.0	6.3	1.97	1.71	1.81
8	96.4	70.0	77.7	11.1	2.1	6.1	2.23	1.76	1.90
9	98.7	85.7	91.0	36.6	12.1	22.2	1.93	1.61	1.71
10	97.3	73.6	80.8	56.5	8.7	20.4	2.33	1.67	1.93
11	95.0	78.5	84.5	6.0	3.1	4.1	2.27	1.74	1.90
12	99.7	91.8	94.2	26.2	6.1	12.8	2.37	2.04	2.14
13	98.4	83.7	90.9	8.8	0.5	4.2	2.00	2.17	2.12
14	95.5	84.6	88.5	12.1	2.2	6.1	2.24	2.39	2.35
15	96.7	80.3	85.0	68.4	10.2	28.2	2.23	1.70	1.86
16	99.2	87.1	91.3	15.3	2.4	6.5	2.12	1.99	2.03
17	94.0	82.4	86.0	1.6	0.2	0.8	2.37	1.96	2.07
OL with mild dysplasia (n=19)									
18	87.2	68.9	76.2	3.7	2.8	3.2	1.73	1.67	1.70
19	98.9	81.4	88.4	9.7	2.8	5.8	1.87	1.91	1.90
20	90.3	60.5	69.5	27.6	5.0	12.1	1.77	1.68	1.71
21	95.2	71.2	78.7	45.4	10.0	20.3	1.85	1.71	1.76
22	97.6	66.5	78.2	27.1	6.4	14.2	2.68	1.72	2.10
23	92.0	58.7	67.1	13.0	3.7	6.9	2.43	1.94	2.35
24	98.7	90.4	92.9	5.3	4.5	4.9	2.03	1.80	1.87
25	92.2	81.9	85.9	3.2	3.0	3.1	1.73	1.68	1.70
26	98.2	72.8	81.1	9.4	3.5	5.8	1.44	1.49	1.47
27	97.4	65.8	76.0	43.1	6.8	19.8	1.60	2.15	2.04
28	93.4	84.1	87.7	8.6	2.3	4.9	1.88	1.92	1.91
29	99.4	74.5	83.2	39.9	5.1	18.0	3.42	2.11	2.36
30	95.2	71.3	77.7	32.6	7.0	16.5	2.32	1.78	1.90
31	98.0	78.8	84.7	36.0	4.5	17.6	2.05	2.00	2.01
32	97.7	87.4	90.4	27.8	6.2	13.7	1.30	1.79	1.69
33	99.1	69.4	77.5	30.2	4.4	13.5	2.00	1.64	1.72
34	97.2	73.9	82.1	40.5	5.4	19.1	2.27	2.23	2.24
35	96.6	60.7	72.0	34.0	4.7	16.7	2.68	1.94	2.10
36	97.2	62.1	74.0	15.3	2.9	8.6	2.40	2.08	2.14
OL with moderate dysplasia (n=16)									
37	94.2	69.2	77.7	20.6	4.6	10.3	2.30	2.67	2.55
38	93.5	57.2	70.6	17.7	3.4	9.2	2.10	1.87	1.94
39	98.6	69.1	78.8	43.8	9.1	24.8	1.90	2.01	1.98
40	95.9	53.5	65.9	9.3	4.6	6.1	2.57	2.39	2.43
41	98.2	64.0	73.4	54.6	5.5	22.4	2.14	2.13	2.13
42	97.5	55.3	67.5	44.8	11.1	24.2	2.87	1.93	2.21
43	96.5	61.3	73.8	21.8	3.5	10.5	2.28	2.07	2.15
44	98.7	60.9	68.5	42.6	3.7	13.0	1.75	1.64	1.66
45	96.7	58.0	67.6	30.8	4.0	10.6	2.50	2.00	2.09
46	89.6	49.7	60.1	34.5	11.5	19.9	2.33	2.13	2.16
47	98.6	70.9	78.4	24.7	4.4	11.1	2.20	2.01	2.04
48	93.9	47.5	61.3	14.0	3.5	8.3	2.07	1.67	1.78
49	97.2	36.2	55.2	21.6	3.5	9.6	2.09	1.99	1.93
50	92.0	33.1	60.8	62.6	10.0	34.8	2.02	1.64	1.81
51	86.9	56.5	64.8	12.7	3.1	6.8	2.45	2.45	2.45
52	96.9	56.8	70.2	39.7	5.9	15.7	2.77	2.73	2.74
OL with severe dysplasia (n=10)									
53	96.4	54.9	61.4	56.6	11.1	25.3	2.27	2.58	2.53
54	86.8	53.5	64.3	56.0	14.1	29.3	1.83	2.01	1.97
55	96.8	39.7	61.7	67.6	13.9	35.9	2.19	2.09	2.11
56	93.1	42.1	58.4	33.9	5.0	16.6	1.50	2.24	2.05
57	86.9	28.5	43.1	43.7	9.5	19.1	3.12	2.55	2.74
58	97.8	68.2	75.7	12.8	3.4	6.2	1.61	1.64	1.63
59	85.7	45.4	54.4	66.5	21.1	31.4	1.71	2.13	2.04
60	96.6	40.0	49.0	31.2	4.1	13.3	2.27	2.19	2.20
61	90.6	57.0	66.5	93.3	42.7	61.4	3.56	2.78	3.10
62	82.1	36.7	54.3	31.7	18.0	22.3	2.17	2.68	2.72
Normal mucosa (n=7)									
63	94.7	78.6	84.5	1.2	1.0	1.1	1.57	1.73	1.68
64	94.2	74.1	85.4	3.4	1.6	2.6	1.72	1.97	1.88
65	97.3	79.3	85.2	4.9	1.0	2.7	1.85	1.79	1.80
66	98.7	81.6	87.4	5.2	2.9	3.8	1.77	1.48	1.62
67	98.8	79.2	84.7	7.7	1.5	3.6	2.21	1.45	1.74
68	97.2	82.0	88.0	3.2	0.5	1.9	1.94	1.46	1.62
69	97.7	82.1	89.2	2.3	0.9	1.7	2.03	1.60	1.73

OL = oral leukoplakia.

Table 2 – Percentage of hMLH1 and p53 immunostained cells, and AgNOR number according to OL site.

	hMLH1			***p53***			***AgNOR***		
	*Basal layer**	*Suprabasal layer**	*All layers**	*Basal layer**	*Suprabasal layer**	*All layers**	*Basal layer***	*Suprabasal layer***	*All layers**
Buccal mucosa *(n=17)*	96.6	64.0	74.0	21.8	4.0	10.5	2.34	2.08	2.12
Alveolar mucosa *(n=16)*	97.0	71.8	79.5	27.5	5.2	13.4	2.01	1.80	1.88
Retromolar region *(n=7)*	97.2	72.8	81.1	18.9	3.5	10.0	1.87	1.83	1.81
Lateral border of tongue *(n=7)*	95.9	53.5	65.9	34.5	9.5	19.9	2.45	2.24	2.21
Floor of mouth *(n=3)*	96.6	56.8	66.5	39.7	5.9	15.7	2.87	2.57	2.74
Comissure *(n=3)*	97.8	68.2	75.7	15.3	3.4	6.5	1.74	1.96	2.03
Palate *(n=2)*	93.5	80.4	85.2	15.0	4.5	8.0	2.05	1.86	1.92
Tongue dorse *(n=1)*	98.6	80.2	87.2	32.9	5.4	15.5	2.33	1.71	1.90
Multifocal involvement *(n=5)*	92.2	81.9	85.9	5.3	4.5	4.9	1.92	1.85	1.87

OL = oral leukoplakia; *n* = number of cases; * median values; ** mean values.

Table 3 – Correlation coefficients for hMLH1, p53 and AgNOR comparisons.

		hMLH1		***p53***			***AgNOR***		
		Suprabasal layer	*All layers*	*Basal layer*	*Suprabasal layer*	*All layers*	*Basal layer*	*Suprabasal layer*	*All layers*
hMLH1	*Basal*	0.514*	0.548*	-0.020*	-0.133*	-0.074*	0.018*	-0.232*	-0.180*
	Suprabasal		0.989*	-0.463*	-0.439*	-0.478*	-0.223*	-0.380*	-0.414*
	Total			-0.465*	-0.440*	-0.475*	-0.218*	-0.382*	-0.410*
p53	*Basal*				0.872*	0.980*	0.274*	0.368*	0.403*
	Suprabasal					0.924*	0.236*	0.329*	0.361*
	Total						0.262*	0.383*	0.418*
AgNOR	*Basal*							0.502**	0.752*
	Suprabasal								0.897*

* Spearman test; ** Pearson test.