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EM SAÚDE

ALDINI BEUTING PEREIRA KITAHARA

EXPRESSÃO IMUNOHISTOQUÍMICA DE NANOG EM LEUCOPLASIA BUCAL

Curitiba
2019

ALDINI BEUTING PEREIRA KITAHARA

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do título de Mestre em Odontologia, Área de Concentração em Multidisciplinaridades em Saúde (Ênfase em Biociências).

Orientador: Profª. Drª. Aline Cristina Batista Rodrigues Johann

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Título: EXPRESSÃO IMUNOHISTOQUÍMICA DE NANOG EM LEUCOPLASIA BUCAL

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Resumo

Objetivo: Leucoplasia bucal é a lesão potencialmente maligna mais frequente. A gradação de displasia da Organização Mundial da Saúde é pobremente reproduzível, já o sistema binário apresenta maior concordância entre observadores, mas requer validação. O NANOG é um potencial marcador na avaliação da leucoplasia bucal. Comparou-se a imunoexpressão do NANOG em leucoplasia bucal com a mucosa normal. **Métodos:** Sessenta e cinco casos de leucoplasia bucal (pela classificação binária 32 de baixo risco e 33 de alto risco ou Organização Mundial da Saúde 27 displasias discreta, 27 moderada e 11 severa) e 12 controle foram submetidas a imunohistoquímica para NANOG realizando a contagem das células positivas e negativas do epitélio. Os testes incluíram Qui-quadrado, Kruskal-Wallis e Dunn. **Resultados:** A classificação binária não apresentou diferenças na expressão de NANOG entre os grupos. Pela Organização Mundial de Saúde, observou-se uma maior porcentagem de células positivas para NANOG na leucoplasia bucal moderada comparada com a severa e o controle. **Conclusão:** A expressão de NANOG foi maior em leucoplasia bucal moderada comparada com a severa e o controle, sugere-se que o NANOG pode ter um papel na transformação maligna em uma etapa de transição entre a leucoplasia bucal com displasia discreta e severa.

Palavra-chave: Proteína Homeobox Nanog; leucoplasia bucal, células tronco

Introdução

O câncer de cabeça e pescoço é o sexto tipo de câncer mais comum no mundo e o câncer de boca, um dos tipos de câncer mais prevalentes e emergentes (Liu et al., 2010). No Brasil, segundo dados do Instituto Nacional de Câncer (INCA), em 2013 foram registradas 5.401 mortes por câncer de boca sendo 4.223 homens e 1.178 mulheres. Em 2018, estimam-se 14.700 novos casos de câncer de boca sendo 11.200 homens e 3.500 mulheres (INCA, 2018). Mais de 90% dos cânceres de boca são carcinomas de células escamosas bucais (CCEB) (El-Naggar, Chan, Grandis, Takata & Slootweg, 2017), que é uma neoplasia maligna do epitélio de revestimento que pode acometer qualquer estrutura das mucosas da cavidade bucal ou lábios, sendo um dos dez cânceres mais comuns no mundo (Rivera, 2015), com taxa de sobrevida de aproximadamente 50% após 5 anos (Liu et al., 2010).

Lesões epiteliais bucais potencialmente malignas podem dar origem ao CCEB. Dentre essas lesões, a mais frequente é a leucoplasia bucal (LB) (Awadallah, Idle, Patel & Kademani, 2018). LB é um termo clínico usado para descrever placas brancas de risco questionável, sempre que outras condições específicas e outros distúrbios orais potencialmente malignos foram descartados (El-Naggar et al., 2017). A literatura reporta que 16 a 48% dos CCEB estavam associados a uma LB quando diagnosticados. Estimando pobremente a taxa anual de transformação maligna da LB de 1,36% em várias populações e áreas geográficas (Liu et al., 2010). O diagnóstico precoce, tratamento efetivo e compreensão da possível progressão para CCEB, de lesões epiteliais bucais potencialmente malignas, como a LB, podem minimizar a morbimortalidade e ter um efeito direto na sobrevida do paciente com a doença (Awadallah et al., 2018).

É importante a compreensão de que a progressão das lesões epiteliais bucais potencialmente malignas para o CCEB é um evento complexo, que integra um processo gradual de alterações genéticas e histológicas, que levam a um acúmulo e avanço de alterações moleculares, evoluindo para a transformação maligna (Awadallah et al, 2018).

A gradação da displasia epitelial da Organização Mundial de Saúde (OMS) por meio de análise histopatológica é o método de escolha para determinar o risco de transformação maligna da LB (El-Naggar et al., 2017), entretanto, ela é

subjetiva, apresentando diferentes interpretações pelos observadores (Warnakulasuriya**,** Reibel, Bouquot, & Dabelsteen, 2008). O sistema binário de classificação (Kujan et al., 2006) apresenta maior concordância global inter e intraobservador (Krishnan et al., 2016). Diante disto faz-se necessária a busca de marcadores que auxiliem nesta avaliação. Dentre esses marcadores destacam-se os marcadores de células tronco tumorais. As células-tronco são dotadas de uma capacidade de se auto renovar e transformar-se em células especializadas. Somente uma subpopulação específica de células tumorais é capaz de iniciar e perpetuar o crescimento tumoral, especialmente sob tratamento (Reers, Pfannerstill, Maushagen, Pries & Wollenberg, 2014). Os fatores de transcrição OCT-4, NANOG e SOX-2, conhecidos por serem críticos na manutenção da pluripotência das células tronco, compõem um circuito essencial para regulação da pluripotência das células tronco (Okumura-Nakanishi et al., 2005; Li, 2010; Wang et al., 2010). Esses fatores estão relacionados com proliferação, crescimento, tumorigenicidade, quimio-resistência e metástase (Liu et al., 2013).

O fator de transcrição NANOG possui atributos protumorigênicos. Além de promover a autorrenovação e o potencial proliferativo a longo prazo de células tronco tumorais, a reprogramação oncogênica mediada por NANOG pode estar correlacionada com o comportamento clínico nas neoplasias malignas. A hipótese é que o NANOG potencializaria o circuito molecular da tumorigênese e, portanto, poderia representar um novo alvo terapêutico ou biomarcador para o diagnóstico, prognóstico, auxiliando o tratamento do câncer (Jeter, Yang, Wang, Chao & Tang, 2015). A alta e moderada expressão do NANOG mostrou-se associada com CCEB de alto grau e a expressão fraca ou negativa mostrou-se relacionada com carcinoma bem diferenciado (Lee et al., 2015). A alta expressão de NANOG tem sido correlacionada com carcinomas de alto grau histológico e prognóstico ruim (Zhang et al.,2012; Watanabe et al.,2014; Qiao, He, Cai, & Yang, 2015). Apesar dos estudos que investigam o papel do NANOG no CCEB, de acordo com nosso conhecimento, não existem estudos que avaliem a imunoexpressão do NANOG em LB comparado com a mucosa normal, o que poderá contribuir para o entendimento da patogênese da LB.

O objetivo do nosso estudo é verificar a imunoexpressão do NANOG em LB pelo sistema de classificação OMS (displasia discreta, moderada e severa) e

Binário (baixo risco e alto risco), comparando com a mucosa bucal normal, sendo a hipótese nula a ausência de diferença na expressão de NANOG.

Materiais e métodos

A metodologia foi desenvolvida no Laboratório de Patologia Experimental da Pontifícia Universidade Católica do Paraná (PUCPR). O estudo dos casos de lesões de LB realizado foi retrospectivo documental, observacional e transversal aprovado pelo Comitê de Ética em Pesquisa local sob o número 1.110.687.

Amostras

As amostras utilizadas foram dos arquivos do laboratório de patologia da PUCPR, Universidade Federal de Minas Gerais e Universidade Federal de Santa Catarina que apresentavam a) casos diagnosticados clinicamente como LB associados ao histológico de hiperceratose com displasia leve, moderada ou severa, b) mucosa bucal normal (grupo controle). As amostras eram oriundas da mucosa do rebordo alveolar e obtidas a partir da cunha distal realizada para permitir acesso na exodontia de terceiro molar incluso. Idade e gênero dos pacientes foram coletados dos prontuários e os pacientes foram classificados de acordo com as faixas etárias (<30, 30-49, ≥50 anos) (Gopinath, Thannikunnath, & Neermunda, 2016).

As lâminas foram coradas com hematoxilina e eosina e escaneadas pelo programa ZEN 2.3 lite (ZEISS Microscope Software ZEN Lite, Oberkochen, Alemanha). Foram utilizados dois sistemas de classificação: OMS e Binário. No sistema de gradação proposto pela OMS (El-Naggar *et al*., 2017), o grau é de acordo com o terço do epitélio afetado: atipia discreta no terço basal, atipia moderada se estende ao terço médio e severa no terço superior do epitélio. Pelo sistema binário as lesões de LB foram classificadas da seguinte maneira (Kujan *et al*., 2006), quando há menos de quatro alterações arquitetônicas ou menos de cinco alterações citológicas a lesão é classificada como “baixo risco” e quando há pelo menos quatro mudanças arquitetônicas e cinco alterações citológicas é classificada como “alto risco” de transformação maligna. Foram consideradas alterações arquitetônicas: Estratificação epitelial irregular; Inversão da polaridade

das células basais; Projeções epiteliais na forma de uma gota; Aumento do número de mitoses; Mitoses superficiais anormais; Disqueratose; Presença de pérolas de queratina. Foram consideradas citológicas: Aumento no tamanho do núcleo; Anisonucleose; Anisocitose; Pleomorfismo Nuclear; Pleomorfismo Celular; Aumento na proporção de núcleo/citoplasma; Figuras mitóticas atípicas, Hipercromatismo nuclear e Aumento do número e tamanho do nucléolo (Kujan *et al*., 2006). Todos os casos foram classificados por dois patologistas e quando houve desacordo um terceiro participou do processo de classificação. O teste de Wilcoxon inter-examinador revelou não haver diferenças entre as avaliações (p = 0,16) para todos os casos.

A amostra foi constituída de casos com diagnóstico de leucoplasias (32 casos de baixo risco e 33 casos de alto risco classificados pelo sistema binário ou 27 casos de displasia discreta, 27 casos de displasia moderada e 11 casos de displasia severa classificados pela OMS) e 12 de grupo controle, totalizando 77 casos.

O grupo de LB de acordo com sua localização teve a seguinte distribuição: 11 casos na língua (baixo risco/alto risco = 6/5), 20 em rebordo alveolar (15/5), 2 em mucosa alveolar (0/2), 3 na gengiva (0/3), 6 no assoalho bucal (1/5), 5 no palato (1/4), 11 casos na mucosa jugal (5/6), 2 na mucosa labial (0/2), e 5 não informados (4/1). Como grupo controle foi composto de mucosa bucal normal do rebordo alveolar todos os casos eram queratinizados, enquanto 59,4% de baixo risco e 42,4% de LB de alto risco originaram-se sítios queratinizados, entretanto todas as lesões apresentaram hiperceratose.

Reação imunohistoquímica para NANOG

A partir dos blocos de parafina foram obtidos cortes histológicos de 4µm de espessura. Os cortes foram desparafinizados em xilol, hidratados em soluções decrescentes de álcool, incubados em peróxido de hidrogênio e metanol 5%, imersos em Immuno Retriever (Dako, Carpinteria, CA, EUA) e incubados em câmara úmida a 4ºC overnight com anticorpo primário monoclonal de coelho anti NANOG (1:50 clone: EPR3131, Abcam, Cambridge, UK). Na sequência foram incubados em Advance link, followed by Advance enzyme (Dako, code K406889), por 30 min a 25-28 ° C em uma câmara úmida e revelados por 3,3’-diaminobenzina

(Sigma Chemical, St Louis, EUA, código D7679), seguindo de contra-coloração com hematoxilina de Harris (Biotec), desidratação em etanol e diafanização em xileno. O controle negativo não foi incubado com o anticorpo primário. O seminoma foi utilizado como controle positivo.

Análise de imunomarcação

Um examinador foi cegado para os grupos de amostra. A lesão foi digitalizada usando o programa ZEN 2.3 lite. Para a padronização e delimitação da área de avaliação, um retângulo de mesma dimensão foi desenhado em todas as imagens usando a ferramenta "retângulo" em uma ampliação de 100X. A região de escolha foi a de “hot spot” de NANOG no epitélio (Barakat & Siar, 2015). Na qual foi analisada uma imagem, todas as células epiteliais positivas e negativas foram contadas (usando a “ferramenta de eventos”), estratificada por camadas (basal e suprabasal). A camada basal foi considerada a primeira camada de células epiteliais adjacentes ao tecido conjuntivo, enquanto outras camadas, excluindo o estrato córneo, foram consideradas como suprabasal (Shimizu, 2006).

Os núcleos marrons correspondiam as células positivas e para a análise da porcentagem foram utilizadas as seguintes fórmulas:

1. Porcentagem total de células positivas para NANOG: número total de células positivas × 100) / (número total de células positivas + número total de células negativas);

2. Porcentagem de células positivas para NANOG na camada basal (ou suprabasal): células positivas na camada basal (ou suprabasal) × 100 / células positivas da camada basal (ou suprabasal) + células negativas na camada basal (ou suprabasal).

Para análise quantitativa de coloração imunohistoquímica em cada caso foram contadas uma média de 533 células epiteliais que se encontravam contidas dentro do retângulo.

Análise Estatística

Os dados foram analisados utilizando o software SPSS 23.0 (SPSS, Inc., Chicago, IL, EUA). Para fins analíticos os casos sem marcação (negativos) para NANOG foram eliminados n=31, sendo 24 casos de LB (14 casos eram de baixo

risco, 10 de alto risco, 10 displasias discreta, 13 moderada e 1 severa) e 7 de controle, restando 46 positivos que foram analisados. Para a análise do gênero e faixa etária conforme o tipo da lesão foi utilizado o teste qui-quadrado. Os testes de normalidade e grupo controle Kolmogorov-Smirnov e de Shapiro-Wilk para ambas as classificações, evidenciaram a ausência de normalidade ($p<0,05$) entre os grupos, portanto o teste de escolha foi o KrusKal-Wallis seguido do teste de comparações múltiplas não paramétricas de Dunn. O nível de significância adotado em todos os testes foi de 5% ($p <0,05$). Para verificar a reprodutibilidade do examinador, foi realizada a contagem do número de células positivas pelo total de células epiteliais em uma imagem e esta contagem foi repetida após 21 dias. O teste de reprodutibilidade intra-examinador de Dalberg, mostrou um erro de 0,49% na porcentagem de células positivas na camada basal, 0,27% na camada suprabasal e 0,20% no total de células positivas indicando que o examinador reproduziu as medidas de forma aceitável. O teste t de Student para amostras emparelhadas indicou não haver erro sistemático na medida efetuada com $p= 0,11$ na porcentagem de células positivas na camada basal, $p= 0,14$ na camada suprabasal e $p= 0,06$ no total de células.

Resultados

Os grupos com lesões de LB de baixo risco apresentou distribuição semelhante em todas as faixas, o de alto risco concentrou-se nas faixas 30 a 49 e maiores que 50 anos e o grupo controle apresentou maior frequência na faixa menor que 30 anos. (Tabela 1).

Tabela 1 – Frequência da faixa etária, gênero na lesão de LB de baixo e alto risco, e no grupo controle.

Grupo / Variável	LB Baixo risco n (%)	LB Alto risco n (%)	Grupo controle n (%)
Faixa etária			
< 30 anos	2 (40,0) A	0 (0,0) A	3 (60,0) A
30 - 49 anos	3 (27,3) A	7 (63,6) B	1 (9,1) B
≥ 50 anos	13 (43,3) A	16 (53,3) B	1 (3,3) B
Gênero			
Feminino	6 (30,0) A	12 (60,0) A	2 (10,0) A
Masculino	12 (46,2) A	11 (42,3) A	3 (11,5) A

Teste de Qui-quadrado. Letras distintas indicam diferenças estatisticamente significantes em coluna. N= número de casos.

Os grupos com lesões de LB discreta e severa apresentaram distribuição semelhante em todas as faixas, a displasia moderada concentrou-se na faixa maior que 30 anos e o grupo controle apresentou maior frequência na faixa menor que 30 anos. (Tabela 2).

Tabela 2 – Frequência da faixa etária, gênero na lesão de LB de displasia discreta, moderada, severa e no grupo controle.

Grupo/Variável	LB Discreta n (%)	LB Moderada n (%)	LB Severa n (%)	Grupo Controle n (%)
Faixa etária				
< 30 anos	2 (40,0) A	0 (0,0) A	0 (00,0) A	3 (60,0) A
30 - 49 anos	3 (27,3) A	6 (54,5) B	1 (9,1) A	1 (9,1) B
≥ 50 anos	12 (40,0) A	8 (26,7) AB	9 (30,0) A	1 (3,3) B
Gênero				
Feminino	5 (25,0) A	7 (35,0) A	6 (30,0) A	2 (10,0) A
Masculino	12 (46,2) A	7 (26,0) A	4 (15,4) A	3 (11,5) A

Teste de Qui-quadrado. Letras distintas indicam diferenças estatisticamente significantes em coluna. N= número de casos.

Quando a LB foi classificada de acordo com o sistema binário, não houve diferença estatística na porcentagem de células positivas para NANOG entre as LB de baixo e alto risco e grupo controle (Tabela 3 e Figura 1).

Tabela 3 – Mediana da porcentagem de células positivas na camada basal, suprabasal e total para NANOG em lesões de LB de baixo e alto risco (sistema binário) e grupo controle

Grupo / Variável	LB Baixo risco n (%)	LB Alto risco n (%)	Grupo controle n (%)	Kruskal Wallis Valor de p
Células positivas na camada basal	8,41	0,85	1,17	0,15
Células positivas na camada suprabasal	10,70	3,87	0,87	0,30
Total de células positivas	14,58	4,34	0,65	0,15

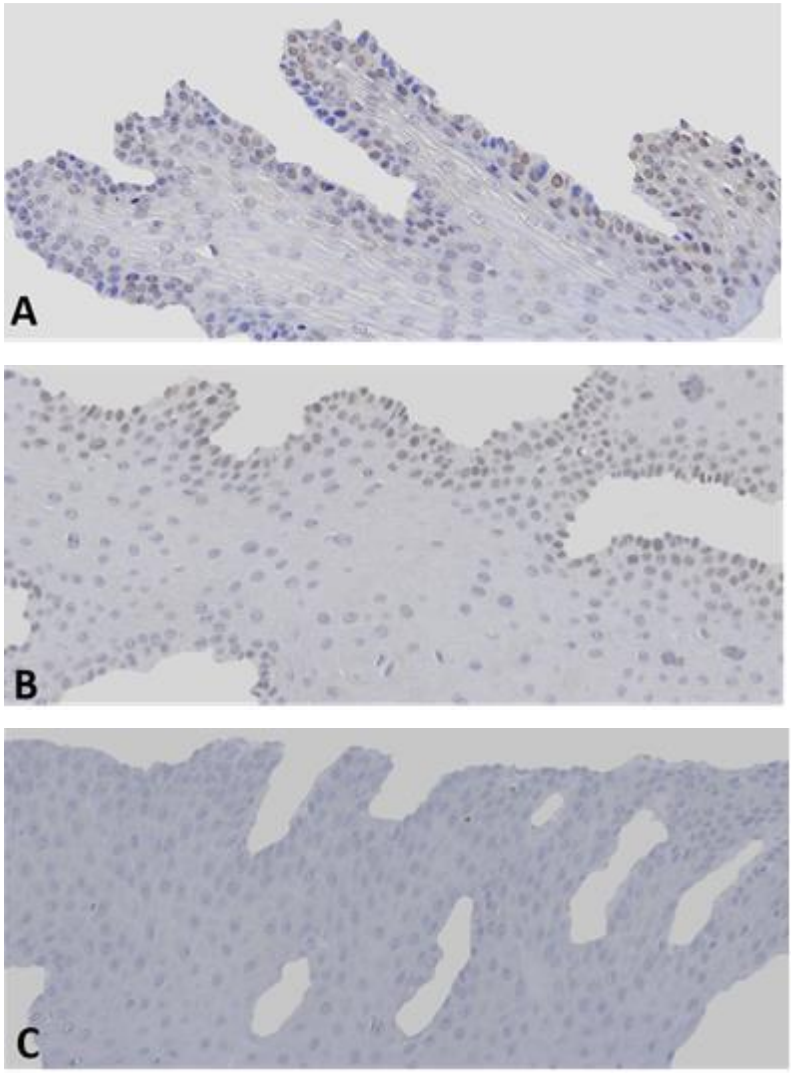

Figura 1: Fotomicrografia revelando a expressão imunohistoquímica para NANOG para os casos de (a) lesão de leucoplasia bucal de alto risco de transformação maligna, (b) baixo risco e (c) mucosa normal (100X).

Quando as LB foram classificadas pela OMS, observou-se maior porcentagem de total de células positivas para NANOG na LB moderada comparada com a severa e o controle (Tabela 4).

Tabela 4 – Mediana da porcentagem de células positivas na camada basal, suprabasal e total para NANOG; em lesão de LB discreta, moderada e severa (classificadas pela OMS) e grupo controle

Grupo variável	LB discreta n (%)	LB moderada n (%)	LB severa n (%)	Grupo controle n (%)	Kruskal Wallis Valor de p
Células positivas na camada basal	8,13	2,56	0	1,17	0,33
Células positivas na camada suprabasal	6,69	12,40	0,99	0,87	0,10
Total de células positivas	6,44 AB	9,71A	0,76B	0,65B	0,03

Comparações múltiplas não paramétricas de Dunn: Letras diferentes indicam diferenças estatisticamente significativas. Controle x moderada p= 0,02; severa x moderada p= 0,02.

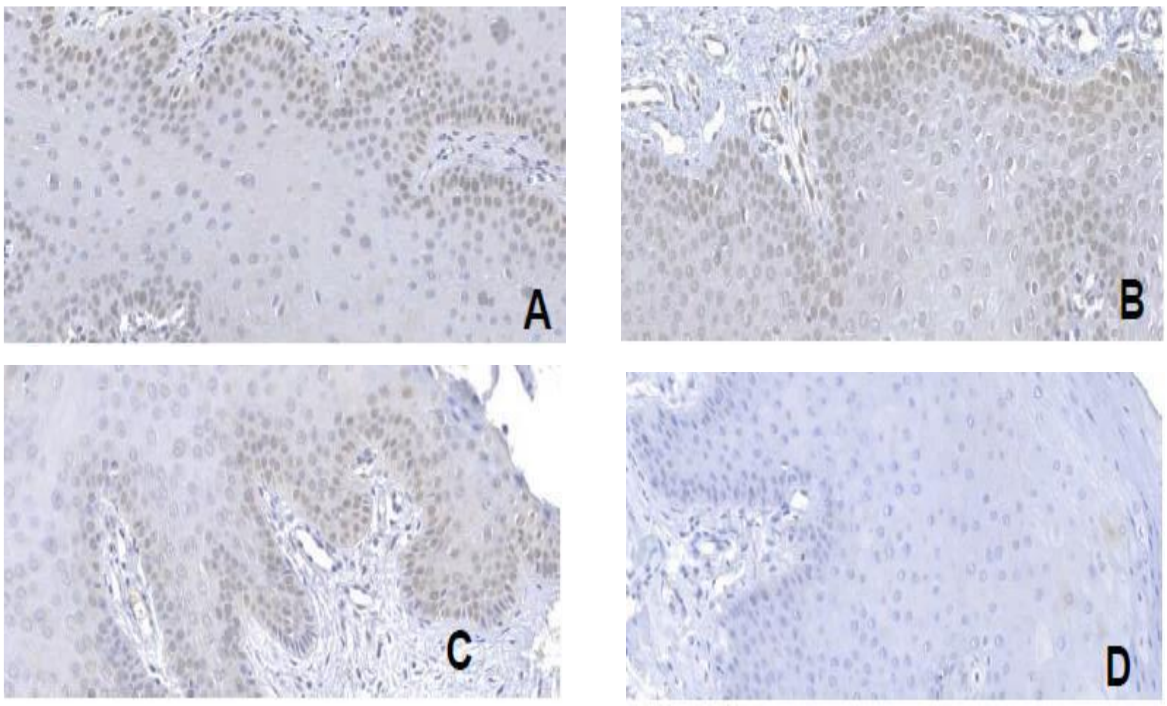

Figura 2. Fotomicrografias do epitélio e conjuntivo, revelando a expressão imunohistoquímica para NANOG nos casos de lesões LB de displasia discreta (A), moderada e (B) severa e (C) grupo controle (D) (100x).

Discussão

Este foi o primeiro estudo que avaliou a imunoexpressão do NANOG em lesões de LB comparado com a mucosa bucal normal, pelo sistema de classificação binária e OMS. A hipótese nula foi aceita quando se utilizou a classificação binária e rejeitada quando se utilizou a OMS, já foi observada uma maior expressão de NANOG em displasia moderada quando comparada com a mucosa bucal normal e com a displasia severa.

Estudos mostram que a maior expressão de NANOG foi observada em: CCEB com menor diferenciação histológica (Watanabe *et al*., 2014; Ravindran *et al*., 2015; Lee *et al*., 2015; Kim *et al*., 2017); tecidos normais adjacentes ao CCEB e no CCEB do que tecidos bucais normais (Fu *et al*., 2016); metástase linfonodal (Ravindran *et al*., 2015; Kim *et al*., 2017), (Lee *et al*., 2015); focos metastáticos (Watanabe *et al*., 2014); menores taxas de sobrevida global e pior prognóstico (Kim *et al*., 2017), (Lee *et al*., 2015). Considerando que tecidos normais adjacentes ao CCEB e células tumorais do CCEB apresentam maior expressão de NANOG que em tecidos bucais normais (Fu *et al*., 2016), hipotetizou-se no presente estudo que a expressão também seria maior em LB comparado com a mucosa normal, já que esta é uma lesão potencialmente maligna. Isto foi verificado na LB com displasia moderada, entretanto isto não ocorreu para a discreta e severa. O NANOG poderia ter um papel na transformação maligna em uma etapa de transição entre a LB com displasia discreta e severa, promovendo a autorrenovação e o potencial proliferativo de células com o fenótipo de células tronco tumorais por meio da reprogramação oncogênica mediada por NANOG. Desta forma, uma vez adquirida essas propriedades, a expressão de NANOG diminui na displasia severa.

A expressão do NANOG em queilite actinica, outra lesão potencialmente maligna de boca, revelou-se semelhante quando comparada as displasias discreta, moderada e severa (Scotti et al., 2018). Diferentemente deste estudo, a presente pesquisa revelou uma maior expressão na lesão de LB com displasia moderada comparada com displasia severa, essa possível diferença pode ser devida as diferenças dos fatores etiológicos destas lesões já que a queilite está relacionada com a exposição aos raios ultravioleta (Dos Santos, de Sousa, Nunes, Sotto, & Araújo, 2003) e os principais fatores etiológicos associados a LB intrabucais são o hábito de fumar e o uso crônico de álcool (Porter, Gueiros, Leão & Fedele, 2018).

Na ausência de estudos que avaliem o NANOG na LB, um outro marcador, a metalotioneína, apresentou maior expressão na displasia moderada comparada com o controle achado semelhante ao encontrado no presente estudo, entretanto não encontraram diferenças entre a severa e a moderada diferentemente do presente estudo. Esses autores sugerem que a metalotioneína pode ser um marcador para displasia moderada desempenhando um papel na carcinogênese bucal (Johann et al., 2008).

A gradação de displasia da Organização Mundial de Saúde é o método de escolha para determinar o risco de transformação maligna da LB (El-Naggar et al., 2017) entretanto é pobremente reproduzível (Warnakulasuriya et al, 2008). O sistema binário apresenta maior concordância entre observadores (Krishnan *et al*., 2016), mas requer validação antes de ser aplicado rotineiramente em LB (El-Naggar et al., 2017). Shubhasini et al., 2017 avaliaram classificação e concordância entre dois patologistas em lesões potencialmente malignas utilizando OMS e sistema binário evidenciando que os sistemas existentes para classificação de displasia não são competentes para descartar subjetividade. Diante disto, o presente estudo realizou a análise da imunoexpressão de NANOG e mostrou achados semelhantes em ambas as classificações para porcentagem de células positivas para as camadas basal e suprabasal. Entretanto, a classificação da OMS permitiu, diferentemente do Sistema binário, identificar, com relação a porcentagem total de células positivas, maior expressão do NANOG em lesão de LB com displasia moderada comparada com o controle, o que possivelmente foi subestimado pelo agrupamento das lesões na classificação binária. O presente estudo sugere que a classificação da OMS seja a de escolha para a avaliação do NANOG.

Diferentemente do presente estudo, outros autores analisando a imunoexpressão de hMLH1, p53 e AgNOR primeiramente por meio da classificação da OMS (Caldeira, Aguiar, Mesquita & Do Carmo, 2011; Caldeira, Abreu, Batista & Do Carmo, 2011) e posteriormente pelo sistema binário (Caldeira, Abreu & do Carmo, 2012) postularam que o uso do sistema binário daria um suporte mais confiável na abordagem clínica de remoção de LB de alto risco. Os autores sugerem que são necessários maiores estudos sobre o sistema binário de classificação para o fortalecimento do mesmo reduzindo possivelmente a

variabilidade e melhorar a reprodutibilidade da avaliação da displasia epitelial (Caldeira et al., 2012).

A ausência de imunomarcação para NANOG foi verificada em 33,84% dos casos de LB e 58,33% de mucosa bucal normal, esses casos foram retirados da análise estatística, portanto as diferenças encontradas no presente estudo devem ser consideradas apenas para casos imunopositivos para NANOG. Na ausência de estudos prévios que avaliem a expressão de NANOG na LB, a ausência de marcação para o NANOG também foi descrita para CCEB em 13,3 % (Watanabe et al., 2014) 28,33% (Ravidran et al., 2014), 31,58% (Lee et al., 2015), dados esses semelhantes ao encontrado para LB. Watanabe et al., (2014) verificaram que apesar de 13.3% dos casos apresentarem o foco primário negativo para NANOG, todos os focos metastáticos correspondentes a estes casos apresentaram alta expressão de NANOG. Os tumores NANOG negativos podem conter um número limitado de células CCEB indiferenciadas, incluindo célula tronco tumorais. Portanto em pacientes de NANOG negativos, as células tronco tumorais expressam NANOG em focos primários em estágio inicial, metastatizam e formam o tumor secundário. Posteriormente as células tumorais indiferenciadas positivas para NANOG podem ser mantidas em focos metastáticos e desaparecer dos focos primários.

Uma limitação do estudo, foi que o grupo controle eram oriundas do rebordo alveolar e não pareados de acordo com a localização. Isso também impossibilitou o pareamento da idade, pois os indivíduos submetidos a extração do terceiro molar que possibilitou a obtenção da amostra normal apresentavam faixa etária abaixo dos 30 anos e os portadores de LB maiores que 30. Entretanto, por motivos éticos, não foi possível obter amostra normal de outra região.

Conclusão

A expressão de NANOG foi maior em leucoplasia bucal moderada comparada com a severa e o controle, sugere-se que o NANOG pode ter um papel na transformação maligna em uma etapa de transição entre a leucoplasia bucal com displasia discreta e severa.

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ARTIGO EM INGLÊS

Title: IMMUNOHISTOCHEMICAL EXPRESSION OF NANOG IN ORAL LEUKOPLAKIA.

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Abstract

Objective: Oral leukoplakia (OL) is globally recognized as the most frequent potentially malignant lesion. The World Health Organization (WHO) classification of dysplasia is poorly reproducible, but the binary grading system, which requires validation, presents a higher concordance between observers. NANOG is a potential biomarker for OL, and its immunoexpression in OL was compared to that in normal mucosa. **Methods:** Sixty-five cases of OL (32 low-risk and 33 high-risk according to the binary grading system or 27 mild dysplasia, 27 moderate dysplasia and 11 severe dysplasia according to the WHO classification) and 12 controls were analyzed by immunohistochemistry for NANOG with counts of positive and negative epithelial cells. Chi-square, Kruskal-Wallis, and Dunn's tests were used. **Results:** The binary grading system showed no differences in the NANOG expression between groups. The WHO classification showed a higher percentage of NANOG-positive cells in moderate OL compared to moderate to severe OL and the control groups. **Conclusion:** NANOG expression was higher in the moderate OL group than in the severe OL and control groups. Thus, NANOG may have a role in malignant transformation in a transitional stage between OL and mild and severe dysplasia.

Keywords: Homeobox protein Nanog; oral leukoplakia; stem cells

Introduction

Head and neck cancer is the sixth most common type of cancer in the world and oral cancer is one of the most prevalent and emerging types of cancer (Liu et al., 2010). In Brazil, according to data from the National Cancer Institute (INCA), 5,401 deaths due to oral cancer were recorded in 2013, consisting of 4,223 men and 1,178 women. In 2018, 14,700 (11,200 men and 3,500 women) new cases of oral cancer are estimated (INCA, 2018). More than 90% of oral cancers are oral squamous cell carcinomas (OSCCs) (El-Naggar, Chan, Grandis, Takata & Slootweg, 2017), a malignant neoplasm of the epithelial lining which can affect any mucosal structure of the oral cavity or lips. It is one of the ten most common cancers worldwide (Rivera, 2015), with a 5-year survival rate of approximately 50% (Liu et al., 2010).

Potentially malignant oral epithelial lesions may give rise to OSCC. Among these lesions, oral leukoplakia (OL) is the most frequent (Awadallah, Idle, Patel & Kademani, 2018). OL is the clinical term used to describe white plaques of questionable risk when other specific conditions and potentially malignant oral disorders are discarded (El-Naggar et al., 2017). It is reported that 16–48% of OSCCs are associated with OL when diagnosed. The annual rate of malignant transformation of OL is 1.36% in various populations and geographical areas (Liu et al., 2010). An early diagnosis, effective treatment and understanding of the possible progression of potentially malignant oral epithelial lesions, such as OL, to OSCC can minimize morbidity and mortality and have a direct effect on patient survival (Awadallah et al., 2018).

It is important to understand that the progression of potentially malignant oral epithelial lesions to OSCC is a complex event, which includes gradual genetic and histological changes, leading to a build-up and progression of molecular changes, evolving to malignant transformation (Awadallah et al., 2018).

The World Health Organization (WHO) grading of epithelial dysplasia by histopathological analysis is the method of choice to determine the risk of malignant transformation of OL (El-Naggar et al., 2017), but it is subjective, presenting different interpretations by observers (Warnakulasuriya**,** Reibel, Bouquot, & Dabelsteen, 2008). The binary classification system (Kujan et al., 2006) presents greater global inter- and intra-observer agreements (Krishnan et al., 2016).

Therefore, markers that assist in this assessment, including tumor stem cell markers, are required. Stem cells are endowed with an ability to self-renew and differentiate into specialized cells. Only a specific subpopulation of tumor cells are able to initiate and perpetuate tumor growth, especially under treatment (Reers, Pfannerstill, Maushagen, Pries & Wollenberg, 2014). The OCT-4, NANOG and SOX-2 transcription factors, known to be critical for the maintenance of stem cell pluripotency, constitute an essential circuit for regulating stem cell pluripotency (Okumura-Nakanishi et al., 2005; Li, 2010; Wang et al., 2010). These factors are related to proliferation, growth, tumorigenicity, chemo-resistance and metastasis (Liu et al., 2013).

The NANOG transcription factor has pro-tumorigenic attributes. Besides promoting self-renewal and the long-term proliferative potential of tumor stem cells, NANOG-mediated oncogenic reprogramming may be correlated with clinical behavior in malignant neoplasms. The hypothesis is that NANOG would potentiate the molecular circuit of tumorigenesis and thus, could represent a new therapeutic target or biomarker for diagnosis and prognosis, aiding cancer treatment (Jeter, Yang, Wang, Chao & Tang, 2015). High and moderate NANOG expression was associated with high-grade OSCC and low or negative expression was related to well-differentiated carcinoma (Lee et al., 2015). High NANOG expression has been correlated with carcinomas of high histological grade and poor prognosis (Zhang et al., 2012; Watanabe et al., 2014; Qiao, He, Cai, & Yang, 2015). Although several studies have assessed the role of NANOG in OSCC, to our knowledge, there are no studies evaluating the immunoexpression of NANOG in OL compared to normal mucosa, which may contribute to the understanding of OL pathogenesis.

Thus, the objective of our study is to assess the immunoexpression of NANOG in OL according to the WHO classification (mild, moderate, and severe dysplasia) and the binary grading system (low-risk and high-risk), compared to

normal oral mucosa. The null hypothesis is the absence of differences in NANOG expression.

Material and Methods

The methodology was developed in the Laboratory of Experimental Pathology of the Pontifical Catholic University of Paraná (PUCPR). The study of the cases of OL lesions was retrospective, documentary, observational and transversal, approved by the Local Research Ethics Committee under number 1.110.687.

Samples

Samples from the archives of the Laboratory of Pathology of the PUCPR, the Federal University of Minas Gerais, and the Federal University of Santa Catarina that presented a) cases clinically diagnosed as OL associated with histological hyperkeratosis with mild, moderate, or severe dysplasia, and b) normal oral mucosa (control group), were used. The samples were derived from the mucosa of the alveolar ridge and obtained during a distal wedge procedure to allow for the extraction of the third molar. The age and gender of patients were collected from medical records and patients were classified according to the age ranges (<30, 30-49, ≥50 years) (Gopinath, Thannikunnath, & Neermunda, 2016).

The samples on the slides were stained with hematoxylin and eosin and scanned using the ZEN 2.3 lite software (ZEISS Microscope Software ZEN Lite, Oberkochen, Germany). Two classification systems, WHO and binary, were used. In the grading system proposed by the WHO (El-Naggar et al., 2017), the classification is based on the third section of the affected epithelium: mild atypia in the basal third, moderate atypia extending to the middle and severe in the upper third of the epithelium. According to the binary system, OL lesions were classified as follows (Kujan et al., 2006): the lesion is classified as "low-risk" regarding malignant transformation when there are less than four architectural or five cytological changes, and as "high-risk" when there are at least four architectural and five cytological changes. The following architectural changes were considered: Irregular epithelial stratification, inversion of polarity of basal cells, drop-shaped epithelial projections, increased number of mitoses, abnormal surface mitoses,

dyskeratosis, and presence of keratin pearls. Cytological changes were: increase in the size of the nucleus, anisonucleosis, anisocytosis, nuclear pleomorphism, cellular pleomorphism, increase in the nucleus/cytoplasm ratio, atypical mitotic figures, nuclear hyperchromatism, and increased number and size of the nucleolus (Kujan *et al*., 2006). All cases were classified by two pathologists and, when there were disagreements, a third party participated in the classification process. The Wilcoxon inter-examiner test revealed no differences between evaluations ($p = 0.16$) for all cases.

The 77 samples were composed of 65 cases from individuals diagnosed with leukoplakia (32 low-risk and 33 high-risk cases according to the binary system, or 27 cases of mild dysplasia, 27 cases of moderate dysplasia and 11 cases of severe dysplasia according to the WHO classification) and 12 cases in the control group.

The locations of the lesions in the OL group were distributed as follows: 11 cases in the tongue (low risk/high risk = 6/5), 20 in the alveolar ridge (15/5), 2 in the alveolar mucosa (0/2), 3 in the gingiva (0/3), 6 in the floor of the mouth (1/5), 5 in the palate (1/4), 11 in the buccal mucosa (5/6), 2 in the labial mucosa (0/2), and 5 unknown (4/1). As the control group was composed of normal oral mucosa of the alveolar ridge, all cases were keratinized, 59.4% of low risk and 42.4% of high-risk OL originated from keratinized sites; however, all lesions presented hyperkeratosis.

NANOG immunostaining

Paraffin blocks were cut into 4 µm thick sections. The sections were deparaffinized in xylol, hydrated in alcohol solutions of decreasing concentrations, incubated in 5% hydrogen peroxide and 5% methanol, immersed in Immuno Retriever (Dako, Carpinteria, CA, USA), and incubated in a humid chamber at 4 °C overnight with rabbit anti NANOG primary monoclonal antibody (1:50 clone: EPR3131, Abcam, Cambridge, UK). They were then incubated in Advance link, followed by Advance enzyme (Dako, K406889), for 30 min at 25-28 ºC in a humid chamber, revealed with 3,3'-diaminobenzine (Sigma Chemical, St. Louis, USA, code D7679), counterstained with Harris hematoxylin (Biotec), dehydrated in

ethanol, and diaphanizated in xylene. The negative control was not incubated with the primary antibody. A seminoma was used as a positive control.

Immunostaining analysis

The examiner was blinded to the sample groups. The lesion was scanned using the ZEN 2.3 lite software. In order to standardize and delimit the assessment area, a rectangle of the same size was drawn on all images using the "rectangle" tool at 100× amplification. The region of interest was the NANOG "hot spot" in the epithelium (Barakat & Siar, 2015). In the analyzed image, all positive and negative epithelial cells were counted (by using the "events" tool) and stratified by layers (basal and suprabasal layers). The basal layer was considered the first layer of epithelial cells adjacent to the connective tissue, while other layers, excluding the stratum corneum, were considered as suprabasal layers (Shimizu, 2006).

Brown nuclei corresponded to positive cells and the following formulae were used for the analysis:

1. Total percentage of NANOG-positive cells: total number of positive cells × 100) / (total number of positive cells + total number of negative cells);

2. The percentage of NANOG-positive cells in the basal layer (or suprabasal layer): positive cells in the basal layer (or suprabasal layer) × 100/positive cells of the basal layer (or suprabasal layer) + negative cells in the basal layer (or suprabasal layer).

In each case, an average of 533 epithelial cells that were contained within the rectangle were counted for the quantitative analysis.

Statistical analysis

The data were analyzed using the SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). For analytical purposes, the NANOG unlabeled (negative) cases were eliminated (n = 31), with 24 cases of OL (14 low risk, 10 high risk, 10 mild dysplasia, 13 moderate dysplasia, and 1 severe dysplasia), and 7 controls, leaving 46 positive cases that were analyzed. The Chi-square test was used for gender and age group analysis, according to the type of lesion. The Kolmogorov-Smirnov and Shapiro-Wilk control and normality tests for both classifications revealed the absence of

normality ($p < 0.05$) between the groups, therefore the test of choice was Kruskal-Wallis followed by Dunn's multiple nonparametric comparison test. The level of significance adopted in all tests was 5% ($p < 0.05$). To verify the reproducibility of the examination, the number of positive cells among the total number of epithelial cells in one image were counted and this count was repeated after 21 days. Dalberg's intra-examiner reproducibility test showed an error of 0.49% in the percentage of positive cells in the basal layer, 0.27% in the suprabasal layer and 0.20% in the total of positive cells, indicating that the counts were acceptable. The Student's t-test for paired samples indicated no systematic error in the measurement performed with $p = 0.11$ in the percentage of positive cells in the basal layer, $p = 0.14$ in the suprabasal layer and $p = 0.06$ in all cells.

Results

The group with low risk OL lesions showed a similar distribution across all age groups. High risk lesions were concentrated in the 30 to 49 and >50 years age groups, and the highest frequency of cases in the control group belonged to individuals <30 years old (Table 1).

Table 1: Frequency of occurrence of the selected age groups and genders among low and high risk OL lesion cases, and the control group.

Group / Variable	Low risk OL N (%)	High Risk OL N (%)	Control Group N (%)
Age Range			
< 30 years	2 (40.0) A	0 (0.0) A	3 (60.0) A
30 - 49 years	3 (27.3) A	7 (63.6) B	1 (9.1) B
≥ 50 years	13 (43.3) A	16 (53.3) B	1 (3.3) B
Gender			
Female	6 (30.0) A	12 (60.0) A	2 (10.0) A
Male	12 (46.2) A	11 (42.3) A	3 (11.5) A

OL= Oral leukoplakia; Chi-square test. Distinct letters indicate statistically significant differences in the column. N= number of cases.

The group with mild and severe OL lesions displayed a similar distribution across all age groups, moderate dysplasia was concentrated in the >30 years age

group, and the highest frequency of cases in the control group belonged to individuals <30 years old (Table 2).

Table 2: Frequency of occurrence of the selected age groups and genders among OL lesion cases with mild, moderate, and severe dysplasia, and the control group.

Group/Variable	Mild OL N (%)	Moderate OL N (%)	Severe OL N (%)	Control Group N (%)
Age Range				
< 30 years	2 (40.0) A	0 (0.0) A	0 (00.0) A	3 (60.0) A
30 - 49 years	3 (27.3) A	6 (54.5) B	1 (9.1) A	1 (9.1) B
≥ 50 years	12 (40.0) A	8 (26.7) AB	9 (30.0) A	1 (3.3) B
Gender				
Female	5 (25.0) A	7 (35.0) A	6 (30.0) A	2 (10.0) A
Male	12 (46.2) A	7 (26.0) A	4 (15.4) A	3 (11.5) A

OL= Oral leukoplakia; Chi-square test. Distinct letters indicate statistically significant differences in the column. N= number of cases.

When OL was classified according to the binary system, there was no statistical difference in the percentage of NANOG-positive cells between the low and high risk OL and the control group (Table 3 and Figure 1).

Table 3: Median percentage of NANOG-positive cells in the basal layer, suprabasal layer and total cells in low and high risk OL lesions (binary system) and the control group

Group / Variable	Low risk OL %	High Risk OL %	Control Group %	Kruskal Wallis p Value
Positive cells in the basal layer	8.41	0.85	1.17	0.15
Positive cells in the suprabasal layer	10.70	3.87	0.87	0.30
Total number of positive cells	14.58	4.34	0.65	0.15

OL= oral leukoplakia.

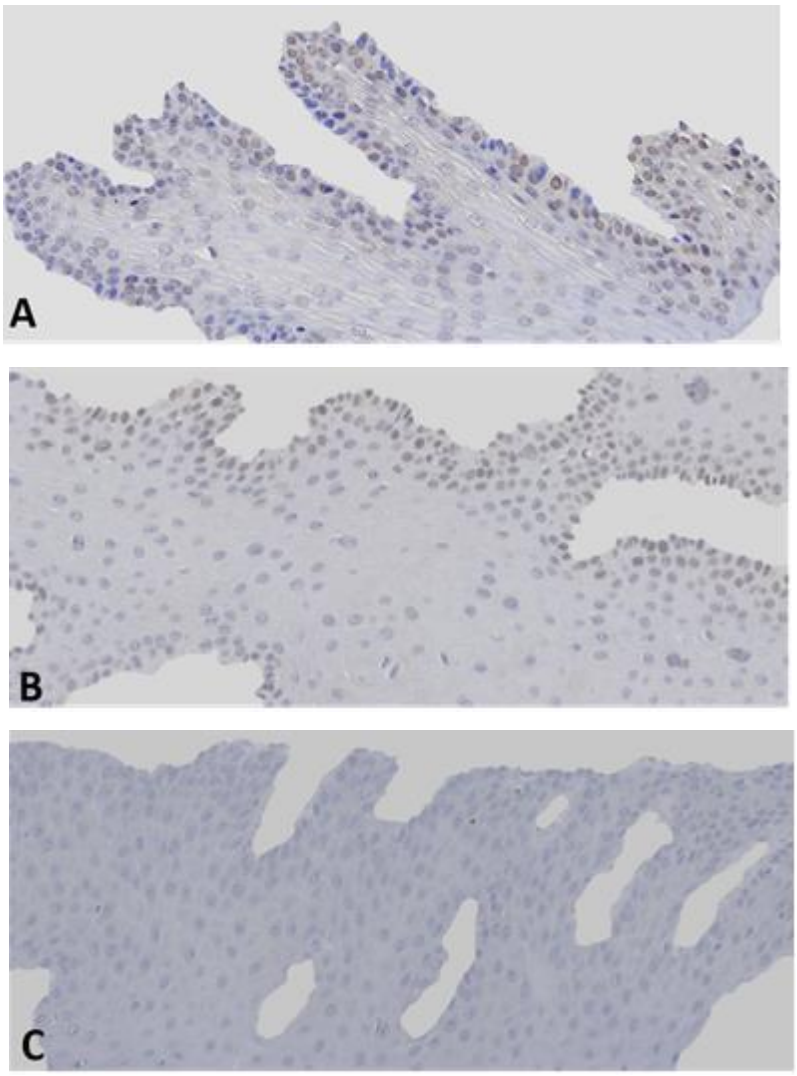

Figure 1: Photomicrograph revealing NANOG expression in cases of (a) oral leukoplakia lesion with high risk of malignant transformation, (b) low-risk and (c) normal mucosa (100×).

When OL were classified according to the WHO classification, a higher percentage of the total number of NANOG-positive cells was observed in the moderate OL compared to the severe OL and control groups (Table 4).

Table 4: Median percentage of NANOG-positive cells in the basal layer, suprabasal layer and total cells in mild, moderate, and severe cases of OL lesions (according to the WHO) and the control group

Group / Variable	Mild OL %	Moderate OL %	Severe OL %	Control Group %	Kruskal Wallis p Value
Positive cells in the basal layer*	8.13	2.56	0	1.17	0.33
Positive cells in the suprabasal layer*	6.69	12.40	0.99	0.87	0.10
Total number of positive cells**	6.44 AB	9.71A	0.76B	0.65B	0.03

OL= oral leukoplakia; WHO = World Health Organization; non-parametric multiple comparisons of Dunn: different letters indicate statistically significant differences. Control x Moderate p= 0.02; severe x moderate p= 0.02.

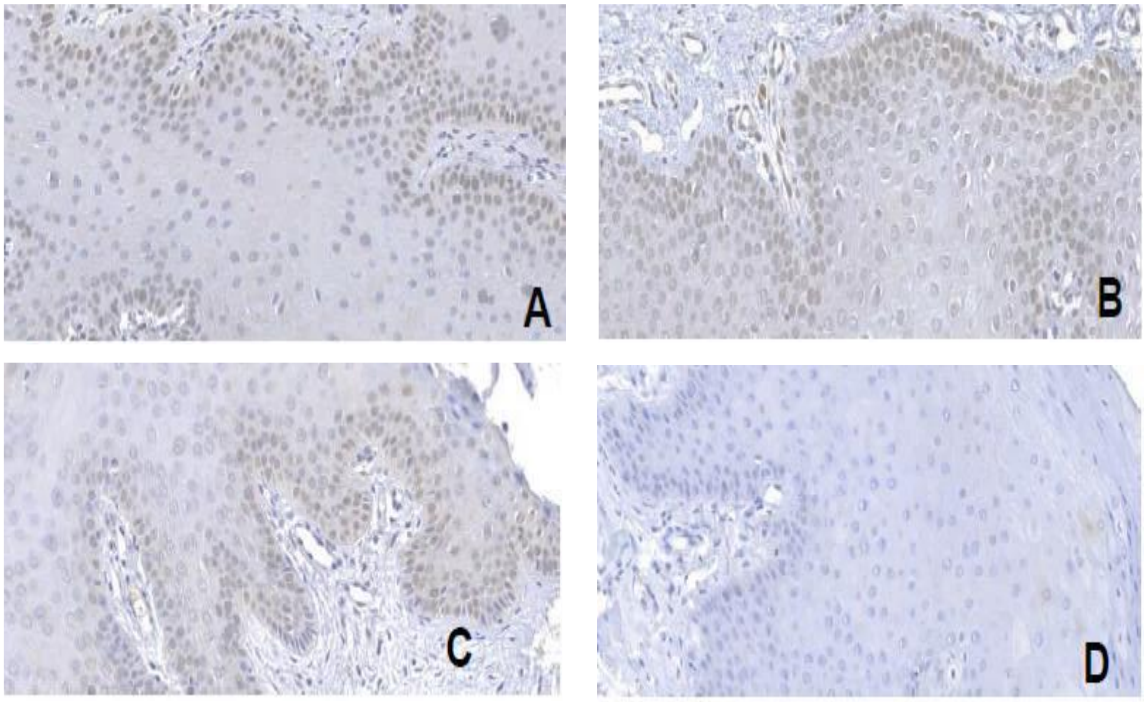

Figure 2: Photomicrographs of the epithelium and connective tissue, revealing the immunohistochemical expression of NANOG in cases of OL lesions with mild (A), moderate (B) and severe (C) dysplasia, and the control group (D) (100×).

Discussion

To our knowledge, this is the first study to assess the immunoexpression of NANOG in OL lesions compared to the normal buccal mucosa, using the binary and WHO classification systems. The null hypothesis was accepted when the binary classification was used and rejected when the WHO system was used, as a higher NANOG expression was observed in cases with moderate dysplasia when compared with those with normal buccal mucosa and severe dysplasia.

Several studies have reported a higher NANOG expression in OSCC with lower histological differentiation (Watanabe *et al*., 2014; Ravindran *et al*., 2015; Lee *et al*., 2015; Kim *et al*., 2017), normal tissues adjacent to the OSCC and the OSCC tumor cells compared to normal oral tissues (Fu *et al*., 2016), lymph node metastasis (Ravindran *et al*., 2015; Kim *et al*., 2017), (Lee *et al*., 2015), metastatic foci (Watanabe *et al*., 2014), OSCC with lower rates of overall survival and worse prognoses (Kim *et al.*, 2017), (Lee *et al*., 2015). Given that normal tissues adjacent to the OSCC and OSCC tumor cells have higher expression of NANOG compared to normal oral tissues (Fu *et al*., 2016), it was hypothesized in this study that the expression would also be higher in OL compared to normal mucosa, since this is a potentially malignant lesion. This was verified in OL with moderate dysplasia, but not in mild and severe OL. NANOG could play a role in malignant transformation during a transitional stage between OL and mild and severe dysplasia, promoting self-renewal and the proliferative potential of cells with a tumor stem cell phenotype through NANOG-mediated oncogene reprogramming. Consequently, once these properties are acquired, NANOG expression decreases in severe dysplasia.

NANOG expression in actinic cheilitis, another potentially malignant lesion of the mouth, was similar when compared to mild, moderate, and severe dysplasia (Scotti et al., 2018). In contrast, the present study revealed a higher expression in OL lesions with moderate dysplasia compared to those with severe dysplasia and this difference may be due to differences in the etiological factors associated with these lesions since cheilitis is related to exposure to ultraviolet radiation (Dos Santos, de Sousa, Nunes, Sotto, & Araújo, 2003) and the main etiological factors associated with intrabuccal OL are smoking and chronic alcohol intake (Porter, Gueiros, Leão & Fedele, 2018).

In the absence of studies evaluating NANOG in OL, another biomarker, metallothionein, displayed higher expression in cases with moderate dysplasia compared to the control. This is similar to the results obtained in this study, but no differences were found between severe and moderate dysplasia, unlike our observations. The authors suggest that metallothionein may be a marker for moderate dysplasia, playing a role in oral carcinogenesis (Johann et al., 2008).

The WHO grading of dysplasia is the method of choice to determine the risk of malignant transformation of OL (El-Naggar et al., 2017), but it is poorly reproducible (Warnakulasuriya et al, 2008). The binary system presents a higher concordance among observers (Krishnan *et al*., 2016), but requires validation before being applied routinely in OL (El-Naggar et al., 2017). Shubhasini et al., (2017) evaluated the classification and concordance of two pathologists in examining potentially malignant lesions using the WHO and binary systems and reported that the existing dysplasia classification systems are not competent enough to rule out subjectivity. As such, the immunoexpression of NANOG was performed in this study and revealed similar findings for both classifications regarding the percentage of positive cells in the basal and suprabasal layers. However, unlike the binary system, the WHO classification identified higher NANOG expression in OL lesions with moderate dysplasia compared to the control, in relation to the total percentage of positive cells, which was possibly underestimated by the grouping of the lesions in the binary classification. Hence, the results from this study suggest that the WHO classification is the choice system for evaluating NANOG expression.

Unlike the present study, other authors analyzed the immunoexpression of hMLH1, p53 and AgNOR, first, using the WHO classification (Caldeira, Aguiar, Mesquita & Do Carmo, 2011; Caldeira, Abreu, Batista & Do Carmo, 2011) and subsequently, the binary system (Boiler, Abreu & do Carmo, 2012), and postulated that the use of the binary system would more reliably support the clinical approach to reducing high-risk OL. The authors suggested that further studies on the binary classification system are needed to strengthen it, possibly reducing variability and improving the reproducibility of epithelial dysplasia assessment (Caldeira et al., 2012).

The absence of NANOG immunostaining was observed in 33.84% of OL and 58.33% of normal buccal mucosa, and these cases were removed from the statistical analysis, so the differences found in this study should be considered only for NANOG-positive cases. In the absence of other studies evaluating the expression of NANOG in OL, the absence of NANOG staining was also described in OSCC in 13.3 % (Watanabe et al., 2014), 28.33% (Ravidran et al., 2014), and 31.58% (Lee et al., 2015), with similar findings to those found for OL. Watanabe et al., (2014) found that although 13.3% of the cases showed that the primary focus was negative for NANOG, all metastatic foci corresponding to these cases showed high NANOG expression. NANOG-negative tumors may contain a limited number of undifferentiated OSCC cells, including tumor stem cells. Therefore, in NANOG negative patients, the tumor stem cells express NANOG in primary foci at an early stage, metastasize and form the secondary tumor. Subsequently, NANOG positive undifferentiated tumor cells may be retained in metastatic foci and disappear from the primary foci.

One limitation of this study was that the control group was derived from the alveolar ridge and not paired according to the location. This also prevented age pairing, as the subjects submitted to third molar extraction, provided for the normal sample they were <30 years, and the OL patients were >30 years. However, for ethical reasons, it was not possible to obtain normal samples from another region.

Conclusion

NANOG expression was higher in moderate OL compared to severe OL cases and the control. NANOG may have a role in malignant transformation at a transitional stage between OL and mild and severe dysplasia.

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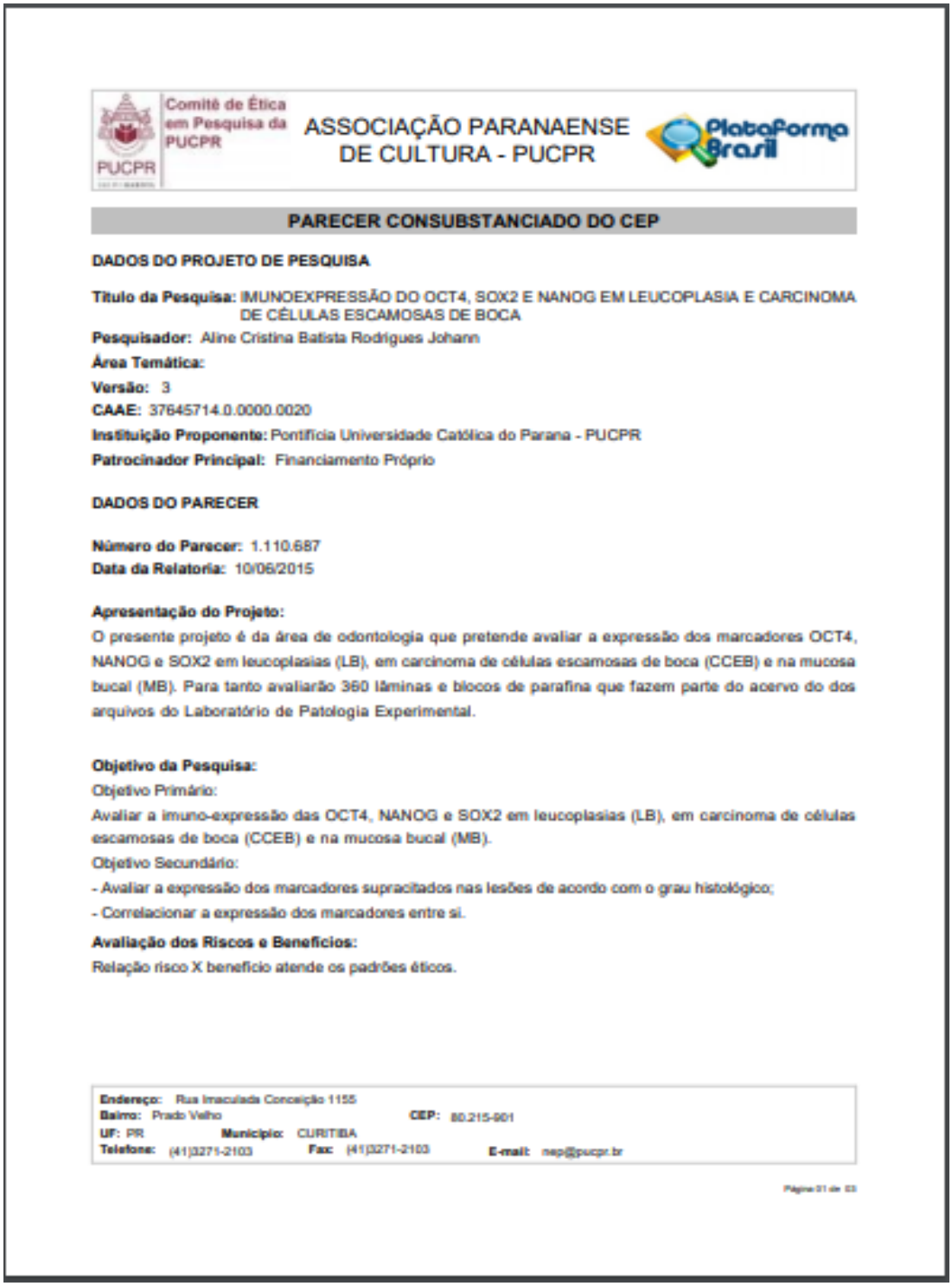
ANEXOS

Anexo A- Parecer de comitê de ética



Comitê de Ética em Pesquisa da PUCPR

ASSOCIAÇÃO PARANAENSE DE CULTURA - PUCPR

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: IMUNOEXPRESSÃO DO OCT4, SOX2 E NANOG EM LEUCOPLASIA E CARCINOMA DE CÉLULAS ESCAMOSAS DE BOCA

Pesquisador: Aline Cristina Batista Rodrigues Johann

Área Temática:

Versão: 3

CAAE: 37645714.0.0000.0020

Instituição Proponente: Pontifícia Universidade Católica do Parana - PUCPR

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.110.687

Data da Relatoria: 10/06/2015

Apresentação do Projeto:

O presente projeto é da área de odontologia que pretende avaliar a expressão dos marcadores OCT4, NANOG e SOX2 em leucoplasias (LB), em carcinoma de células escamosas de boca (CCEB) e na mucosa bucal (MB). Para tanto avaliarão 360 lâminas e blocos de parafina que fazem parte do acervo do dos arquivos do Laboratório de Patologia Experimental.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar a imuno-expressão das OCT4, NANOG e SOX2 em leucoplasias (LB), em carcinoma de células escamosas de boca (CCEB) e na mucosa bucal (MB).

Objetivo Secundário:

- Avaliar a expressão dos marcadores supracitados nas lesões de acordo com o grau histológico;
- Correlacionar a expressão dos marcadores entre si.

Avaliação dos Riscos e Benefícios:

Relação risco X benefício atende os padrões éticos.

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Página 01 de 03

Comentários e Considerações sobre a Pesquisa:

Trata-se de uma pesquisa que apresenta critérios de inclusão e exclusão, metodologia, cronograma e orçamento claros.

Considerações sobre os Termos de apresentação obrigatória:

Os termos de apresentação obrigatória estão adequados à Res. CNS 466/12.

Recomendações:

Solicita-se que seja encaminhado,apenas, a justificativa para a mudança ou retirada do centro pesquisador, se for o caso.

Conclusões ou Pendências e Lista de Inadequações:

A emenda do projeto se deu em virtude da necessidade adicional, para a composição do grupo mucosa normal, da remoção de um fragmento da mucosa do rebordo alveolar de indivíduos. Esta intervenção é um procedimento padrão de acesso para a exodontia de terceiro molar incluso, não acrescentando nenhum ônus ao paciente.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Lembramos aos senhores pesquisadores que, no cumprimento da Resolução 466/12, o Comitê de Ética em Pesquisa (CEP) deverá receber relatórios anuais sobre o andamento do estudo, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, para conhecimento deste Comitê. Salientamos ainda, a necessidade de relatório completo ao final do estudo.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEPPUCPR de forma clara e sucinta, identificando a parte do protocolo a ser modificado e as suas justificativas.

Se a pesquisa, ou parte dela for realizada em outras instituições, cabe ao pesquisador não iniciá-la antes de receber a autorização formal para a sua realização. O documento que autoriza o início da pesquisa deve ser carimbado e assinado pelo responsável da instituição e deve ser mantido em poder do pesquisador responsável, podendo ser requerido por este CEP em qualquer tempo.

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CURITIBA, 17 de Junho de 2015

Assinado por:
NAIM AKEL FILHO
(Coordenador)

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Anexo B- Normas para publicação na revista

ORAL DISEASES
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Author Guidelines

The median processing time from submission to first decision for manuscripts submitted to *Oral Diseases* in the prior 12 months is 22 days.

Content of Author Guidelines: 1. General, 2. Ethical Guidelines, 3. Manuscript Submission Procedure, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance.

Relevant Documents: Online Open Order Form, Standard Release Form for photographic consent

Useful Websites: Submission Site, Articles Published in *Oral Diseases*, Author Services, Wiley-Blackwell's Ethical Guidelines, Guidelines for Figures

1. GENERAL

The editors encourage submissions of original articles, review articles, reports of meetings, book reviews and correspondence in the form of letters to the editor. *Oral Diseases* does not accept case reports.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in *Oral Diseases*. Authors are encouraged to visit Wiley-Blackwell Author Services for further information on the preparation and submission of articles and figures.

Avoiding allegations of plagiarism

The journal to which you are submitting your manuscript employs text matching software (iThenticate) to ensure against plagiarism. By submitting your manuscript to this journal you accept that your manuscript may be screened for plagiarism against previously published work. Authors should consider whether their manuscript may raise concerns via iThenticate, which will signal whether a paper is likely in any way to be plagiarized in a formal sense. iThenticate will also, however, signal whether a paper may be plagiarized by repeating work of the submitting authors and thus be regarded as duplicate or redundant publication. Experience shows that, on occasion, large sections of submitted manuscripts can be close to verbatim in word choice from that seen in other papers from the authors' group. This has nothing to do with simple repetition of names/affiliations, but does involve common (not necessarily "standard") phrases that are more appropriately referenced instead of repeating. Alternatively, they can be rephrased differently. Previously published results, including numerical information and figures or images, should be labeled to make it clear where they were previously reported. Papers that present new analyses of results that have already been published (for example, subgroup analyses) should identify the primary data source, and include a full reference to the related primary publications. *Oral Diseases* will review and publish accepted mansucripts that report data included in conference proceedings in abstract form. In such cases, authors must be clear to readers that part of all of the manuscript's data have already been published in abstract form by so indicating using a footnote to the title that states the confrence proceedings in which the relevant abstract was published. For full guidance on text matching and plagiarism, please refer to Section 3 ('Research Integrity') of Wiley's Ethics Guidelines at https://authorservices.wiley.com/ethics-guidelines/index.html.

2. ETHICAL GUIDELINES

Oral Diseases adheres to the ethical guidelines given below for publication and research.

2.1. Authorship and Acknowledgements

Authorship: *Oral Diseases* adheres to the International Standards for Authors published by the Committee on Publication Ethics (COPE). All authors named on a paper should agree to be named on the paper, and all authors so named should agree to the submission of the paper to *Oral Diseases* and approve the submitted and accepted versions of the publication. Any change to the author list should

be approved by all authors, including any author who has been removed from the list.

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2.2. Ethical Approvals

Human Subjects: Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2002) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included.

Photographs of People: Oral Diseases follows current HIPAA guidelines for the protection of patient/subject privacy. If an individual pictured in a digital image or photograph can be identified, his or her permission is required to publish the image. The corresponding author must either submit a

letter signed by the patient authorizing Oral Diseases to publish the image/photo, or complete the 'Standard Release Form for photographic consent' available at the top of this page or by clicking the "instructions and Forms" link on the ScholarOne Manuscripts submission site. The approval must be received by the Editorial Office prior to final acceptance of the manuscript for publication. Otherwise, the image/photo must be altered such that the individual cannot be identified (black bars over eyes, tattoos, scars, etc.). Oral Diseases will not publish patient photographs that will in any way allow the patient to be identified, unless the patient has given their express consent.

Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

Animal Study: When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

2.3 Clinical Trials

Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flowchart should also be included in the submission material. Clinical trials can be registered in any free, public clinical trials registry such as http://www.clinicaltrials.gov or http://isrctn.org/. A list of further registries is available at http://www.who.int/ictrp/network/primary/en/. As stated in an editorial published in *Oral Diseases* (12:217-218), 2006), all manuscripts reporting results from a clinical trial must indicate that the trial was fully registered at a readily accessible website. The clinical trial registration number and name of the trial register will be published with the paper.

2.4 DNA Sequences and Crystallographic Structure Determinations

Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly.

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All sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential grant holders should be listed. Authors are also required to disclose any possible conflict of interest. These include financial (for example patent, ownership, stock ownership, consultancies, speaker's fee). Information on sources of funding and any potential conflict of interest should be disclosed at submission under the heading "Acknowledgements".

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3. MANUSCRIPT SUBMISSION PROCEDURE

Oral Diseases only accepts online submission of manuscripts. Manuscripts should be submitted at the online submission site: http://mc.manuscriptcentral.com/odi. Complete instructions for submitting a manuscript are available at the site upon creating an account. Assistance for submitting papers can be sought with the editorial assistant Lisa Walton at: odiedoffice@wiley.com

Upon successful submission, the journal administrator will check that all parts of the submission have been completed correctly. If any necessary part is missing or if the manuscript does not fulfil the requirements as specified below, the corresponding author will be asked either to adjust the submission according to specified instructions or to submit their paper to another journal.

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Launch your web browser (supported browsers include Internet Explorer 5.5 or higher, Safari 1.2.4, or Firefox 1.0.4 or higher) and go to the journal's online Submission Site:
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3.3. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc/.docx) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the entire manuscript including title page, abstract, text, references, acknowledgements, tables, and figure legends, but no embedded figures. In the text file, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.

3.4. Blinded Review

All manuscripts submitted to *Oral Diseases* will be reviewed by two experts in the field. *Oral Diseases*

uses single blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper.

3.5. Suggest a Reviewer

Oral Diseases attempts to keep the review process as short as possible to enable rapid publication of new scientific data. In order to facilitate this process, you must suggest the names and current e-mail addresses of from 2-4 potential reviewers whom you consider capable of reviewing your manuscript in an unbiased way.

3.6. Suspension of Submission Mid-way in the Submission Process

You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

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3.8. Manuscript Status

The average time from submission to first decision for manuscripts submitted to *Oral Diseases* is 20 days. You can access ScholarOne Manuscripts (formerly known as Manuscript Central) any time to check your 'Author Centre' for the status of your manuscript. The Journal will inform you by e-mail once a decision has been made.

3.9. Submission of Revised Manuscripts

To upload a revised manuscript, locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision'. Please remember to delete any old files uploaded when you upload your revised manuscript.

4. MANUSCRIPT TYPES ACCEPTED

Original Research Articles: Manuscripts reporting laboratory investigations, well-designed and controlled clinical research, and analytical epidemiology are invited. Studies related to aetiology, pathogenesis, diagnosis, prevention and treatment are all of interest, but all papers must be based on rigorous hypothesis-driven research. Areas of interest includediseases affectin any structures of the mouth; cancer and pre-cancerous conditions; saliva and salivary glands; bone and hard tissues; relationasip between oral, periodontal, and dental conditions and general health; pain; behaviotal dentistry; chemosensory, developmental, geriatric, and motor disorders.

Randomised trials must adhere to the CONSORT guidelines, and a CONSORT checklist and flowchart must be submitted with such papers. Please also refer to the notes under section 2.3 above.
Oral Diseases supports the ALLTRIALS initiative and encourages authors submitting manuscripts reporting a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/, http://isrctn.org/. The clinical trial registration number and name of the trial register will then be published with the paper.

Observational studies must adhere to the STROBE guidelines, and a STROBE checklist must be submitted with such papers. Diagnostic accuracy studies must adhere to the STARD guidelines, and a STARD checklist must be submitted with such papers.

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Review Papers: *Oral Diseases* commissions review papers and also welcomes uninvited reviews. Systematic reviews with or without meta-analyses must adhere to the PRISMA guidelines, and a PRISMA checklist and flowchart must be submitted with such papers. The word limit for Review Papers is 4,000 words, with a maximum of two tables or images and 50 references.

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Case Reports: *Oral Diseases* does not accept case reports and instead recommends that authors submit to *Clinical Case Reports* an open access journal published by Wiley.

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Invited Concise Reviews: These may be submitted by invitation of the Senior Editors only, and consist of around 2500-2750 words, with a maximum of one table or image and 25 references.

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Invited Commentaries: These may be submitted by invitation of the Senior Editors only.

Invited Editorials: These may be submitted by invitation of the Senior Editors only.

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5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Page Charge

Articles exceeding 6 published pages, including title page, abstract, references, table/figure legends and tables and figures, are subject to a charge of GBP70 per additional page. As a guide, one published page amounts approximately to 850 words, or two to four small tables/figures. Additional supplementary material (including text and figures), which does not fit within the page limits, can be published online only as supporting information.

5.2. Format

Language: Authors should write their manuscripts in British English using an easily readable style. Authors whose native language is not English should have a native English speaker read and correct their manuscript. Spelling and phraseology should conform to standard British usage and should be consistent throughout the paper. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Presentation: Authors should pay special attention to the presentation of their findings so that they may be communicated clearly. The background and hypotheses underlying the study as well as its main conclusions should be clearly explained. Titles and abstracts especially should be written in language that will be readily intelligible to any scientist.

Technical jargon: should be avoided as much as possible and clearly explained where its use is unavoidable.

Abbreviations: Oral Diseases adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. Non-standard abbreviations must be used three or more times and written out completely in the text when first used.

5.3. Structure: All papers submitted to *Oral Diseases* should include:

- Title Page
- Structured Abstract
- Main text
- References
- (Figures)
- (Figure Legends)
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Title Page: should be part of the manuscript uploaded for review and include:

- A title of no more than 100 characters including spaces
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Abstract: is limited to 200 words in length and should contain no abbreviations. The abstract should be included in the manuscript document uploaded for review as well as separately where specified in the submission process. The abstract should convey the essential purpose and message of the paper

in an abbreviated form set out under:

- Objective(s),
- Subject(s) (or Materials) and Methods,
- Results,
- Conclusions(s).

The Main Text of Original Research Articles should be organised as follows

Introduction: should be focused, outlining the historical or logical origins of the study and not summarize the results; exhaustive literature reviews are inappropriate. It should close with the explicit statement of the specific aims of the investigation.

Materials and Methods must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced. As a condition of publication, authors are required to make materials and methods used freely available to academic researchers for their own use. This includes antibodies and the constructs used to make transgenic animals, although not the animals themselves. Other supporting data sets must be made available on the publication date from the authors directly.

(i) Clinical trials: As noted above, these should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist should also be included in the submission material. Clinical trials can be registered in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/, http://isrctn.org/.As stated in an editorial published in *Oral Diseases* (12:217-218), 2006), all manuscripts reporting results from a clinical trial must indicate that the trial was fully registered at a readily accessible website. The clinical trial registration number and name of the trial register will be published with the paper.

(ii)Experimental subjects: As noted above, experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2002) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included.Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used. When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

***(iii) Suppliers*:** Suppliers of materials should be named and their location (town, state/county, country) included.

Results: should present the observations with minimal reference to earlier literature or to possible interpretations.

Discussion: may usually start with a brief summary of the major findings, but repetition of parts of the abstract or of the results sections should be avoided. The section should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

Acknowledgements: Should be used to provide information on sources of funding for the research, any potential conflict of interest and to acknowledge contributors to the study that do not qualify as authors. All sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential grant holders should be listed. Acknowledgements should be brief and should not include thanks to anonymous referees and editors. Where people are acknowledged, a covering letter demonstrating their consent must be provided.

5.4. References

References should be prepared according to the *Publication Manual of the American Psychological Association* (6th edition). This means in-text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text, for example, (Jones, 1998). For references with three to five authors, all authors should be listed only on the first occurrence of the in-text citation, and in subsequent in-text occurrences only the first author

should be listed followed by '*et al.*'. The complete reference list should appear alphabetically by name at the end of the paper.

A sample of the most common entries in reference lists appears below. Please note that a DOI should be provided for all references where available. For more information about APA referencing style, please refer to the APA website. Please note that for journal articles, issue numbers are not included unless each issue in the volume begins with page one.

Journal article

Example of reference with 2 to 7 authors

Beers, S. R., & De Bellis, M. D. (2002). Neuropsychological function in children with maltreatment-related posttraumatic stress disorder. *The American Journal of Psychiatry, 159*, 483–486. doi: 10.1176/appi.ajp.159.3.483

Ramus, F., Rosen, S., Dakin, S. C., Day, B. L., Castellote, J. M., White, S., & Frith, U. (2003). Theories of developmental dyslexia: Insights from a multiple case study of dyslexic adults. *Brain, 126*(4), 841–865. doi: 10.1093/brain/awg076

Example of reference with more than 7 authors

Rutter, M., Caspi, A., Fergusson, D., Horwood, L. J., Goodman, R., Maughan, B., ... Carroll, J. (2004). Sex differences in developmental reading disability: New findings from 4 epidemiological studies. *Journal of the American Medical Association, 291*(16), 2007–2012. doi: 10.1001/jama.291.16.2007

Book edition

Bradley-Johnson, S. (1994). *Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school* (2nd ed.). Austin, TX: Pro-ed.

5.5. Tables, Figures and Figure Legends

Figures: All figures and artwork must be provided in electronic format. Please save vector graphics (e.g. line artwork) in Encapsulated Postscript Format (EPS) and bitmap files (e.g. half-tones) or clinical or in vitro pictures in Tagged Image Format (TIFF).

Detailed information on our digital illustration standards can be found at http://authorservices.wiley.com/bauthor/illustration.asp.

Check your electronic artwork before submitting it: http://authorservices.wiley.com/bauthor/eachecklist.asp.

Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected.

Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same type size as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and unit, and follow SI nomenclature common to a particular field. Unusual units and abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc).

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Color figures may be published online free of charge; however, the journal charges for publishing figures in colour in print. If the author supplies colour figures at Early View publication, they will be invited to complete a colour charge agreement in RightsLink for Author Services. The author will have the option of paying immediately with a credit or debit card, or they can request an invoice. If the author chooses not to purchase color printing, the figures will be converted to black and white for the print issue of the journal.

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Upon acceptance of a paper for publication, the manuscript will be forwarded to the Production Editor who is responsible for the production of the journal.

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