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SUELEN TEIXEIRA LUIZ

ANÁLISE DE ASSOCIAÇÃO GENÉTICA E IMUNOISTOQUÍMICA DA SOX-2 EM LEUCOPLASIA BUCAL

Curitiba
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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do título de Doutor em Odontologia, Área de Concentração em Clínica Odontológica Integrada (Ênfase em Estomatologia).

Orientadora: Profa. Dra. Aline Cristina Batista Rodrigues Johann
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SUELEN TEIXEIRA LUIZ

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“Ora, àquele que é poderoso para fazer infinitamente mais do que tudo quanto pedimos ou pensamos, segundo o poder que opera em nós.”

Efésios 3:20

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ARTIGO EM PORTUGUÊS

Página título

TÍTULO:

Análise de associação genética e imunoistoquímica da SOX-2 em leucoplasia bucal

TÍTULO CURTO:

Associação do polimorfismo no gene *SOX2* com a leucoplasia bucal

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Resumo

Objetivo: investigar a associação do polimorfismo do gene *SOX2* com a leucoplasia bucal (LB) e comparar com a expressão imunoistoquímica da SOX-2. **Material e Métodos:** Estudo de caso-controle. A amostra foi composta por 64 pacientes com LB e 20 com mucosa bucal normal (grupo controle), que foram submetidos à genotipagem dos polimorfismos do gene *SOX2* rs77677339 (G/A) por PCR em tempo real e à imunoistoquímica para SOX-2 (expressão epitelial basal, suprabasal e total; área nuclear e intensidade). Os testes estatísticos incluíram Qui–quadrado e Exato de Fisher, significância de 5%. **Resultados:** Não houve associação significativa (*p*=0,578) na distribuição dos genótipos para o marcador rs77677339 (G/A) entre LB e controle. Foi observado o genótipo GG (96,9%) no grupo LB e 100% nos controles. O genótipo GA não foi observado no grupo controle. Os cruzamentos estatísticos entre os resultados imunoistoquímicos e a genética não foram significantes. **Conclusão:** Houve ausência da associação do polimorfismo rs77677339 (G/A) no gene SOX2 e da imunoistoquímica no grupo com LB, porém a presença do alelo A nos indivíduos heterozigotos com LB sugere um importante papel desse alelo como marcador de risco.

Palavras-chave: Leucoplasia, oral; Polimorfismo Genético; Fatores de transcrição SOX; Gene; Células-tronco, tumorais.

Introdução

O carcinoma de células escamosas (CCEB) é o tipo mais comum de câncer de boca (Tandon *et al*., 2017), cuja estimativa global para o ano de 2018 foi de 354.9 mil casos incidentes e 177.4 mil casos de mortalidade (Ferlay *et al*., 2019). Sendo assim, com o intuito de reduzir esses índices é essencial o diagnóstico precoce e o entendimento da patogênese das desordens potencialmente malignas, como a leucoplasia bucal (LB) (Awadallah *et al*., 2018; Khan *et al*., 2019). A LB é classificada como a desordem bucal potencialmente maligna mais frequente em boca (Awadallah *et al*., 2018), e descrita pela OMS – 2017 como "um termo clínico usado para descrever placas brancas de risco questionável, uma vez que outras condições específicas e outras desordens bucais potencialmente malignas tenham sido descartadas" (Neville *et al*., 2017). Embora haja variações entre as populações e áreas geográficas, a prevalência é de 4,47% e a taxa de transformação maligna é de 3% a 14,5% (Anderson *et al*., 2015; Warnakulasuriya & Ariyawardana, 2016; Mello *et al*., 2018).

A LB é mais comum no gênero masculino, de meia idade e idosos. Clinicamente é descrita em dois tipos: homogênea (plana e fina, tem superfície lisa e pode exibir fissuras rasas) e não homogênea (salpicada, nodular e exofítica) (Warnakulasuriya, 2018; Ganesh *et al*., 2018). O uso de tabaco, consumo de álcool e noz de betel são fatores etiológicos para o desenvolvimento das LB, e portanto dependendo do hábito pode ser encontrada em diferentes sítios anatômicos. As LB que são idiopáticas, podem estar relacionadas com maior risco em progredir para o câncer (Warnakulasuriya & Ariyawardana, 2016; Speight *et al*., 2018). As características histopatológicas são descritas como aumento da camada de ceratina (hiperortoceratinizada ou hiperparaceratinizada) e acantose do epitélio. Podem ocorrer displasias no epitélio (leves, moderadas ou severas) dependendo das alterações citológicas e arquitetônicas (Neville *et al*., 2017). Entretanto, a classificação histológica não é suficiente para a determinação do prognóstico, pois as alterações genéticas apresentam o risco de progressão que não pode ser definido pelo diagnóstico histopatológico atual (Kil *et al*., 2016).

Tem-se buscado identificar polimorfismos genéticos em lesões orais potencialmente malignas (Shridhar *et al*.,2016), por meio dos genes que estão

envolvidos: no metabolismo carcinogênico (*GSTM, GSTT1, GSTP1, CYP1A1, CYP2E1, CYP2E1 - Li et al., 2013)*, reparo do DNA (*MRE11A, PRKDC* - Mondal *et al.,* 2013), controle do ciclo celular (*Tp53, Tp21/27, CDK4, CDK6, CCND1, STK15* - Ye *et al*., 2008), alteração da matriz extracelular (*MMP2, MMP9* - Chaudhary *et al., 2011)* e na imunoinflamação (*TNF-α, TGF-β1, IL-10/6, IFN-ɤ* - Hsu *et al.*, 2014). A associação dos polimorfismos de nucleotídeos únicos (SNPs) nos marcadores de células-tronco tumorais com a LB ainda não foi investigada. As células-tronco tumorais são subpopulações de células cancerígenas com propriedade de auto-renovação e diferenciação de múltiplas linhagens para estimular o crescimento e a heterogeneidade do tumor (Ayob & Ramasamy, 2018).

Dentre os marcadores de células-tronco tumorais, destaca-se o fator de transcrição SOX-2 (Arnold *et al*., 2011), que possui 317 aminoácidos e três domínios principais (domínio N-terminal, HMG e C-terminal). Na região denominada transativação ocorre a ligação do promotor, que desencadeia a expressão ou repressão dos genes alvo (Weina & Utikal, 2104). A superexpressão pode desencadear mecanismos que possibilitam que as células cancerígenas adquiram fenótipo correspondente às células-tronco, independente da linhagem celular (Novak *et al*., 2019). No CCEB a proteína SOX-2 está relacionada com a progressão do tumor, metástase e pior prognóstico (Yoshihama *et al*., 2016).

Na avaliação imunoistoquímica, por meio de expressão proteica, SOX-2 encontrou-se superexpressa quando avaliada em LB (baixo e alto risco), na qual apresentou maior número médio de células positivas e maior média de área nuclear positiva nas lesões em comparação com tecido bucal normal (Luiz *et al*., 2018). Essa superexpressão levou o presente grupo de pesquisa a investigar se haveria alguma interação do gene *SOX2* com a suscetibilidade ou proteção na LB. Diante disso, no presente estudo buscou-se investigar a associação do polimorfismo no gene *SOX2* com a LB.

O gene *SOX2*, localizado no cromossomo 3q26.3, codifica fatores de transcrição [membro da HMG-box relacionados com o SRY (SOX)] e desempenha papel importante fisiologicamente na diferenciação celular e na organogênese precoce (Avilion *et al*., 2003, Boiani *et al*., 2005; Sarkar *et al*., 2013). Os efeitos *do SOX2* parecem ser altamente dependentes do tipo de tumor,

podendo atuar como supressores ou na oncogênese (Wuebben & Rizzino, 2017). As desregulações genéticas e epigenéticas de *SOX2* podem contribuir para a heterogeneidade do tipo celular intratumoral, favorecendo a formação das células-tronco tumorais e resistentes a quimioterápicos (Mamun *et al*., 2018). *SOX2* é importante regulador de processos celulares relacionados ao câncer, incluindo a sinalização WNT / β-catenina, EMT e JAK / STAT3 (Weina & Utikal, 2104) sendo que no CCEB parecem estar envolvidos com a aquisição de fenótipos malignos, com a progressão da transição epitelial-mesenquimal (EMT) e com a β-catenina como articulador nessa progressão (Liu *et al*., 2018). No CCEB, a superexpressão do gene *SOX2* demonstrou estar envolvida na oncogenicidade, exibindo maior capacidade de invasão tumoral, já o silenciamento de *SOX2* reduziu as características de transição epitélio-mesênquima e suprimiu a expressão de genes de resistência a drogas e antiapoptóticos (Chou *et al*., 2015).

A avaliação do SNP no gene *SOX2* foi realizada somente no câncer de mama, evidenciando que *OCT4* (rs3130932), *NANOG* (rs11055786), e *SOX2* (rs11915160) podem ter grande influência na suscetibilidade a este câncer e na redução da resposta à quimioterapia neoadjuvante (Tulsyan *et al*., 2014). Com relação à outras doenças, os SNPs no gene *SOX2* estão associados ao desenvolvimento de nefropatia no Diabetes Mellitus tipo 1 (Zhang *et al*., 2010), malformação ocular grave (Zhou *et al*., 2008) e anoftalmia (Osborne *et al*., 2011).

Apesar das associações dos SNPs do gene *SOX2* em outras doenças/lesões já estarem descritas na literatura, nas LB ainda são desconhecidas. Portanto, de acordo com o que se conhece até o momento, este é o primeiro estudo com o objetivo de avaliar a associação de polimorfismo genético do *SOX2* com a LB e com a expressão imunoistoquímica da SOX-2. Para isso, a hipótese nula formulada é a de que não há associação no polimorfismo no gene *SOX2* com LB, e com a expressão imunoistoquímica de SOX-2.

Material e Métodos

Trata-se de um estudo transversal de caso-controle, e inclui pacientes com leucoplasia bucal (LB) submetidos a análise genética e imunoistoquímica. O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa da Pontifícia Universidade Católica do Paraná (# 2.971.307).

Foram obtidas amostras (fichas, blocos de parafina e lâminas) dos arquivos da Pontifícia Universidade Católica do Paraná (PUCPR), Universidade Federal de Minas Gerais e Universidade Federal de Santa Catarina, de pacientes com diagnóstico de LB (clínico de LB associado ao histológico de hiperceratose e atipias) e de mucosa bucal normal (grupo controle). A partir das lâminas, as imagens foram digitalizadas no programa ZEN 2.3 lite (ZEISS Microscope Software ZEN Lite) e a verificação histológica foi realizada por dois patologistas. Os fragmentos de mucosa bucal normal (grupo controle), foram obtidos partir do rebordo alveolar por meio de cirurgia para a exodontia de terceiros molares. As informações sobre o gênero dos indivíduos foram coletadas dos prontuários.

Os critérios de exclusão foram: blocos de parafina ausentes, com pouca quantidade de tecido epitelial para análise e com inflamação.

Foram incluídos um total de 89 pacientes, sendo 68 do grupo LB e 21 pacientes do grupo controle. A distribuição conforme a localização anatômica de acordo com o grupo LB foi a seguinte: 10 casos em língua, 40 casos em rebordo alveolar, 2 casos em mucosa alveolar, 4 casos em gengiva, 4 casos em assoalho de boca, 7 casos no palato, 14 casos em mucosa jugal, 3 casos em mucosa labial, 1 caso em arco amigdaliano, e em 4 casos não foram informados a localização nos prontuários. O grupo controle constituiu em 100% e o grupo LB em 54,4% de mucosa bucal oriunda de região ceratinizada, porém toda a amostra do grupo LB revelou-se com hiperceratose e displasia epitelial.

Análise Genética

a) Preparo dos cortes e extração do DNA

Três cortes de 10μm de tecido de cada paciente foram desparafinizados por meio de xilol e etanol. O protocolo para extração de DNA, a partir de material parafinado com o sistema comercial QIAamp DNA minikit® foi realizado de acordo com as normas do fabricante. As amostras foram diluídas à concentração final de 20 ng/ul, para solução de trabalho, e armazenadas em freezer a –20ºC.

b) Seleção dos marcadores

O marcador do tipo tag SNP rs77677339 (G/A) do gene *SOX2*, foi selecionado com base no *International HapMap Project* (http://www.hapmap.org), segundo os parâmetros de frequência alélica mínima de 5% e desequilíbrio de ligação de 80% na população europeia (CEU). Esse marcador indicado pelo *International HapMap Project* captura toda informação do gene em termos de variabilidade, assim reduzindo custos e tempo.

c) Genotipagem por PCR em tempo real

O DNA purificado dos 89 pacientes foi amplificado pela técnica de PCR em tempo real utilizando a tecnologia TaqMan® Genotyping Master Mix (Applied Biosystems 7500 Real Time PCR System) para a genotipagem e análise da discriminação alélica do tag SNP rs77677339 (G/A), utilizando sondas fluorescentes. Dependendo do anelamento da sonda tem-se a descriminação do genótipo. Duas fluorescências iguais determinam o genótipo homozigoto (GG ou AA) e duas fluorescências distintas indicam genótipo heterozigoto (GA). Foi utilizado o controle negativo em toda a genotipagem realizada. Quantificaram-se, assim, amostras de 84 pacientes e em 5 o genótipo não foi determinado.

Reação imunoistoquímica e análise de imunomarcação para SOX-2

Utilizou-se o protocolo de reação imunoistoquímica e análise de imunomarcação descrito na literatura (Luiz *et al*., 2018), constando de forma resumida em: *imunno Retriever* (Dako, Carpinteria, CA, USA), anticorpo primário - anti SOX-2 monoclonal de coelho na diluição de 1:50 (clone: EPR3131, ABCAM Cambridge, MA), *Advance link* e *enzyme* (Dako, código K40689), DAB (Spring Bioscience Corp, Pleasanton, CA, código DAB-999) e hematoxilina Harris (Biotec, Curitiba, Brasil). A omissão do anticorpo primário foi usada para o controle negativo e o seminoma foi usado como controle positivo.

As lâminas foram digitalizadas no programa ZEN 2.3 lite (ZEISS Microscope Software ZEN Lite) e analisou-se: a) contagem das células positivas e negativas no epitélio na camada basal (<64,1 ou ≥64,1), suprabasal (<68,6 ou ≥68,6) e total (<67,2 ou ≥67,2); b) segmentação semiautomatizada para quantificar a área nuclear imunopositiva em micrômetros quadrados (< 0 ou ≥ 0);

assim como descrito por Luiz, *et al*. (2018) e c) intensidade de marcação (pontuação: 1= quando não foram encontradas as células positivas e coloração fraca; 2= coloração moderada e forte: ≤1 ou >1) (Figura 1).

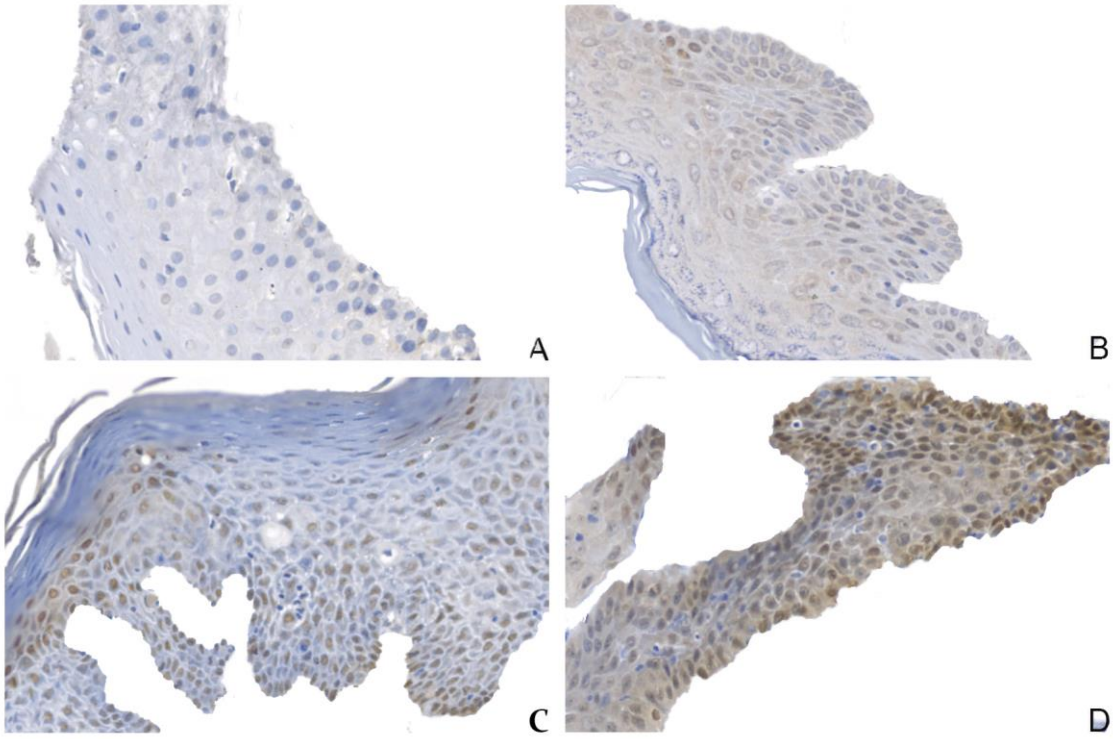

Figura 1. Fotomicrografia revelando níveis de intensidade da coloração de SOX-2, de acordo com os critérios de pontuação ≤1: A) Epitélio demonstrando ausência de células positivas. **B)** Coloração fraca. **Pontuação >1: C)** Coloração moderada. **D)** Coloração forte. (Imunoistoquímica SOX-2 100x).

Análise Estatística

Os dados foram analisados por meio do programa SPSS 25.0 (SPSS Inc, Chicago, Illinois, USA).

Os modelos dominante (GG+GA *vs* AA) e recessivo (AA+GA *vs* GG) não foram reprodutíveis nessa amostra devido à ausência do genótipo AA. Portanto, apenas o modelo aditivo (GG *vs* GA e AA) foi utilizado para análise genotípica do *SOX2*. O teste Qui-quadrado de Pearson e Teste exato de Fisher foram utilizados para as variáveis categóricas. O nível de significância adotado em todos os testes foi de 5% (p<0,05).

Resultados

O total de 84 pacientes foram incluídos, sendo 64 do grupo LB e 20 do grupo controle. No grupo LB, 54,4% pertenciam ao gênero feminino e no controle 47,6%.

Não houve associação significativa (*p*=0,578) na distribuição dos genótipos para o marcador rs77677339 (G/A) entre os grupos LB e controle. Foi observado o genótipo GG em 96,9% das LB e 100% nos controles. O genótipo GA estava ausente no grupo controle (tabela 1).

Tabela 1- Análise genotípica do tag SNP rs77677339 (G/A) no gene *SOX2*, no modelo aditivo – PUCPR, 2019.

Gene	Tag SNPs DbSNP ID[a]	Variação	Grupo	GG n (%)	GA n (%)	AA n (%)	Valor de *p**
SOX2	**rs77677339**	**[G/A]**	Leucoplasia	62 (96,9)	2 (3,1)	-	0,578
			Controle	20 (100,0)	0 (0,00)	-	

NOTA: a: SNP identificados na base de dados do NCBI; * Teste exato de Fisher

Na análise genotípica do tag SNAP rs77677339 (G/A) no gene *SOX2* com a análise imunoistoquímica (porcentagem de células positivas na camada basal, suprabasal e total do epitélio, área nuclear e intensidade) do modelo aditivo, também não houve associação com a LB (Tabela 2).

Tabela 2 - Análise genotípica do tag SNP rs77677339 (G/A) no gene *SOX2*, com análise imunoistoquímica – PUCPR, 2019.

Imunopositividade para SOX-2	Grupo	GG n (%)	GA n (%)	AA n (%)	Valor de *p**
Camada basal					
<64,1	Leucoplasia	22 (95,7)	1 (4,3)	-	0,535
	Controle	20 (100,0)	0A (0,00)	-	
≥64,1	Leucoplasia	40 (97,6)	1 (2,4)	-	-
	Controle	0 (0,0)	0 (0,0)	-	-
Camada suprabasal					
<68,6	Leucoplasia	21 (95,5)	1 (4,5)	-	0,537
	Controle	19 (100,0)	0 (0,0)	-	
≥68,6	Leucoplasia	41 (97,6)	1 (2,4)		0,977
	Controle	1 (100,0)	0 (0,0)		
Total					
<67,2	Leucoplasia	22 (95,7)	1 (4,3)	-	0,535
	Controle	20 (100,00)	0 (0,0)	-	
≥67,2	Leucoplasia	40 (97,6)	1 (2,4)	-	-
	Controle	0 (0,0)	0 (0,0)	-	
Área nuclear (área total)					
<0	Leucoplasia	7 (87,5)	1 (12,5)	-	0,471
	Controle	9(100)	0 (0,0)	-	
≥0,1	Leucoplasia	55 (98,2)	1 (1,8)	-	0,836
	Controle	11 (100,0)	0 (0,0)	-	
Intensidade					
≤1	Leucoplasia	29 (96,9)	1 (3,1)	-	0,640
	Controle	20 (100,0)	0 (0,0)	-	
>1	Leucoplasia	33 (96,7)	1 (3,3)		0,882
	Controle	0 (0,0)	0 (0,0)		

NOTA: a: SNP identificados na base de dados do NCBI; * Teste exato de Fisher

Discussão

No presente estudo a hipótese nula de que não haveria associação do polimorfismo do gene *SOX2* com leucoplasia bucal e com a expressão imunoistoquímica (porcentagem de células positivas na camada basal, suprabasal e total do epitélio, porcentagem da área nuclear e intensidade da imunomarcação) em LB foi aceita. Porém, há resultados genéticos que podem ser inferidos contendo significância biológica.

Em estudo preliminar realizou-se a avaliação quantitativa da imunoexpressão de SOX-2 em LB isolada (não agrupadas com líquen plano), e observou-se uma superexpressão dessa proteína em LB de baixo e alto risco de transformação maligna comparado à mucosa bucal normal, o que demonstrou o possível envolvimento desse fator de transcrição na patogênese da LB (Luiz *et al*., 2018). Do mesmo modo, a imunoexpressão de SOX-2 em LB foi avaliada por meio da coloração nuclear e observou-se que a expressão proteica aumentou significativamente com o grau de displasia no epitélio. No entanto, epitélios adjacentes normais apresentaram expressão negativa de SOX-2 (Vicente *et al*., 2019). Tendo em vista as atribuições proteicas, até o presente momento, as associações do gene *SOX2* ainda não foram elucidadas.

O tag SNP rs77677339 é um marcador, segundo o *International HapMap Project,* suficiente para capturar toda informação do gene em termos de variabilidade, o que torna desnecessária a genotipagem dos demais marcadores para o *SOX2* na população estudada. No modelo aditivo investigado, não houve diferença entre o grupo LB e controle. No grupo LB 3,1% dos pacientes apresentaram genótipo heterozigoto GA e no grupo controle foi ausente. Embora não se tenha evidenciado diferença estatística dos genótipos entre os grupos estudados, ressalta-se que o alelo A é raro na população, sendo aproximadamente 4% usando a base *1000Genomes* (*National Center for Biotechnology* - db SNP rs77677339). A sua presença em heterozigose já poderia influenciar em uma possível associação de risco desse polimorfismo do gene *SOX2* na LB. No entanto, mais estudos são necessários com amostra maior para analisar a possível associação.

A pesquisa atual não evidenciou associação do polimorfismo no gene *SOX2* e imunoexpressão da SOX-2 com LB. Estudo publicado previamente avalia

a expressão imuno-histoquímica da SOX-2 em uma amostra composta por 94 displasias de laringe. A expressão nuclear foi detectada em 40% das displasias, enquanto as células estromais e epitélios adjacentes normais apresentaram expressão negativa. Por meio do DNA extraído dos mesmos blocos de tecido em parafina, os autores avaliaram a amplificação do gene *SOX2* utilizando a PCR em tempo real em 55 pacientes de displasias laríngeas. A amplificação do gene *SOX2* foi detectada em 33% desses pacientes e não correlacionou com a gravidade das lesões. Quando realizaram a correlação da amplificação do gene e a expressão proteica, observaram que a amplificação genética conduz apenas parcialmente à expressão da proteína SOX-2 (Granda-Díaz *et al*., 2019). O presente estudo está em consonância com esses autores, embora tenhamos avaliado em localização anatômica diferente, e com análise de polimorfismo e não de amplificação gênica (Granda-Díaz *et al*. 2019). Portanto, como foi postulado pelos autores supracitados, outros eventos moleculares e epigenéticos podem estar associados aos eventos transcricionais do gene *SOX2.*

A regulação da expressão de *SOX2* pode ocorrer em níveis transcricional, por microRNAs, por RNAs longos não-codificantes, e modificações pós-traducionais de *SOX2* (Wuebben & Rizzino, 2017). Em carcinoma de células escamosas de boca, a expressão da proteína SOX-2 foi detectada em porcentagens muito mais altas do que a expressão do mRNA *SOX2*, sugerindo o possível envolvimento de mecanismos pós-transcricionais (Vicente *et al*., 2019).

Estudos posteriores deverão ser conduzidos com outras ferramentas de análise como as alterações no número de cópias (amplificação), através de ensaio de Hibridização In-situ por Fluorescência (FISH); estudos epigenéticos e de sinalizações intracelulares (Alonso *et al*., 2011; Gut *et al*., 2018) com o propósito de elucidar as funções do gene *SOX2* na patogênese da LB.

Embora a amostra tenha sido coletada de três centros, a limitação no presente estudo encontra-se na dificuldade de se obter participantes com o diagnóstico de LB, e da busca de associação dos dados com outros possíveis fatores etiológicos, devido ao uso de bancos secundários com informações ausentes. As lesões de LB podem ocorrer em vários locais anatômicos, porém os fragmentos do grupo controle foram coletados apenas de um sítio, que era decorrente da própria técnica cirúrgica para exodontia dos terceiros molares, evitando danos aos tecidos de mucosa bucal normal de outras localizações.

Portanto, estudos podem ser necessários para a análise da função do polimorfismo do gene *SOX2* como fator de risco na LB em mucosas originalmente ceratinizadas, no desenvolvimento CCB; e correlacionar com dados imunoistoquímicos.

Conclusão

A ausência da associação do polimorfismo rs77677339 (G/A) e da imunoistoquímica SOX-2 não exclui o papel da proteína SOX-2 no grupo de pacientes com LB. Além disso, a presença do alelo A apenas em indivíduos com LB heterozigotos, sugere o papel de marcador de risco desse alelo no rs77677339 G/A do gene *SOX2*.

Declaração de conflito de interesse

Os autores declaram que não há conflito de interesse.

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ARTIGO EM INGLÊS

Title page

TITLE:

Analysis of genetic association and immunohistochemistry of SOX-2 in oral leukoplakia

SHORT TITLE:

Association of SOX2 gene polymorphism with oral leukoplakia

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Abstract

Objective: to investigate the association of SOX2 polymorphism with oral leukoplakia (OL) and to compare with immunohistochemistry expression of SOX-2. **Material and Methods:** Case control study. The sample was composed by 64 patients with OL and 20 presenting normal oral mucosa (control group) that were submitted to genotyping of SOX2 gene polymorphisms rs77677339 (G/A) by PCRP in real time and to immunohistochemistry for SOX-2 (basal epithelium expression, suprabasal and total; nuclear area and intensity). The statistical tests included Chi-square and Fisher's Exact Test, 5% of significance. **Results:** There was no significance (p=0,578) in genotypes distribution for rs77677339 (G/A) marker between OL and control. The genotype GG (96,9%) was observed in OL and 100% in controls. The genotype GA was not observed in the controls. The statistical crossings between immunohistochemistry and genetics results were not significant. **Conclusion:** there was no association of rs77677339 (G/A) polymorphism and immunohistochemistry on OL, but in the presence of allele A on heterozygotes with OL suggests an important role of this allele as a risk marker.

Key words: Leukoplakia, oral; Polymorphism, Genetic; SOX Transcription Factors; Gene; Neoplastic Stem Cells.

Introduction

Squamous cell carcinoma (OSCC) is the most common type of mouth cancer (Tandon *et al*., 2017), whose global estimate for 2018 was 354.9 thousand incident cases and 177.4 thousand mortality cases (Ferlay *et al*., 2019). Thus, in order to reduce these rates, it is essential the early diagnosis and understanding of the pathogenesis of potentially malignant disorders, as oral leukoplakia (OL) (Awadallah *et al*., 2018; Khan *et al*., 2019). OL is classified as the most frequent oral potentially malignant disorder (Awadallah *et al.*, 2018), and described by WHO – 2017 as “white plaques of questionable risk, having excluded other known diseases or disorders that carry no increased risk for cancer” (Neville *et al*., 2017). Although there are variations between populations and geographical areas, the prevalence is 4.47% and the rate of malignant transformation is 3% to 14.5% (Anderson *et al*., 2015; Warnakulasuriya & Ariyawardana, 2016; Mello *et al*., 2018).

Leukoplakia is more common in males, middle aged and elderly. Clinically it is described in two types: homogeneous (flat and thin, present a smooth surface and may exhibit shallow cracks) and not homogeneous (speckled, nodular and exophytic) (Warnakulasuriya, 2018; Ganesh *et al*., 2018). Tobacco use, alcohol consumption and betel nut are etiological factors for the development of OL, and therefore depending on the habit can be found in different anatomical sites. Idiopathic OL may be related to higher risk of cancer progression (Warnakulasuriya & Ariyawardana, 2016; Speight *et al*., 2018). Histopathological features are described as increased keratin layer (hyperortoceratinized or hyperparaceratinized) and acanthosis of the epithelium. Epithelial dysplasia may occur (mild, moderate or severe) depending on cytological and architectural changes (Neville *et al*., 2017). However, histological classification is not enough to determine prognosis, since genetic alterations present the risk of progression that cannot be defined by the current histopathological diagnosis (Kil *et al*., 2016).

It has been sought to identify genetic polymorphisms in potentially malignant oral lesions (Shridhar *et al*.,2016), through the genes involved in: carcinogenic metabolism (*GSTM, GSTT1, GSTP1, CYP1A1, CYP2E1, CYP2E1 - Li et al., 2013)*, DNA repair (*MRE11A, PRKDC* - Mondal *et al.,* 2013), cell cycle control (*Tp53, Tp21/27, CDK4, CDK6, CCND1, STK15* - Ye *et al*., 2008), extra

cell matrix (*MMP2, MMP9* - Chaudhary *et al., 2011)* and immunoinflammation (*TNF-α, TGF-β1, IL-10/6, IFN-ɤ* - Hsu *et al.*, 2014). The association of single nucleotide polymorphisms (SNPs) in tumor stem cell markers with OL has not yet been investigated. Tumor stem cells are subpopulation of cancerous cells that are able of self-renewing and multiple lineage differentiation to stimulated tumor growth and heterogeneity (Ayob & Ramasamy, 2018).

Among tumor stem cell markers, the transcription factor SOX-2 stand out (Arnold *et al*., 2011), which has 317 amino acids and three main domain (N-terminal domain, HMG and C-terminal), in the called transaction region is where a promoter binding occurs, which triggers an expression or repression of target genes (Weina & Utikal, 2014). Over expression may trigger mechanisms that enable cancerous cells to acquire phenotype stem cells-related, independent of the cell linage (Novak *et al*., 2019). In SCC the protein SOX-2 is related with tumor progression, metastasis and worse prognoses (Yoshihama *et al*., 2016).

In the immunohistochemistry evaluation, through protein expression, SOX-2 was over expressed when it was evaluated in OL (low and high risk), where it presents higher mean number of positive cells and highes mean positive nuclear area in the lesions, compared to the normal oral tissue (Luiz *et al*, 2018). This over expression led this research group to investigate if there would be any interaction of *SOX2* gene with susceptibility or protection on OL. Therefore, the present study investigates the association of *SOX2* gene in polymorphism with OL.

SOX2 gene, located in the chromosome 3q26.3, encodes transcription factors [member of HMG-box related with SRY (SOX)] and plays a physiological important roe in cell differentiation and early organogenesis (Avilion *et al*., 2003, Boiani *et al*., 2005; Sarkar *et al*., 2013). The effects of *SOX2* seem to be highly dependent on tumor type and may act as suppressor or on the oncogenesis (Wuebben & Rizzino et al., 2017). Genetic an epigenetic deregulations of *SOX2* may contribute to intratumoral cell type heterogeneity, favoring tumor stem cells and chemotherapy resistant cells (Mamun *et al*., 2018). *SOX2* is an important regulator of cells process related to cancer, including WNT / β-catenina, EMT e JAK / STAT3 signaling (Weina & Utikal, 2104). In SCC seem to be involved in the acquisition of malignant phenotypes, with the progression of epithelial-mesenchymal transition (EMT) and with β-catenina with articulator in this

progression (Liu *et al*., 2018). In SCC, over expression of SOX2 gene was involved in oncogenicity , showing higher capacity of tumor invasion; in SOX2 silencing the epithelial-mesenchymal transition was reduced and the expression of drug-resistant genes and apoptotic genes was suppressed (Chou *et al*., 2015).

SNP evaluation in *SOX2* gene was only performed in breast cancer, highlighting that *OCT4* (rs3130932), *NANOG* (rs11055786), e *SOX2* (rs11915160) may influence in the susceptibility of this cancer and in response reduction in neoadjuvant chemotherapy (Tulsyan *et al*., 2014). Regarding other diseases, SNPs in *SOX2* gene are associated with nephropathy in type I Diabetes Mellitus (Zhang *et al*., 2010), severe eye malformation (Zhou *et al*., 2008) and anophthalmia (Osborne *et al*., 2011).

SNPs of *SOX2* gene association in others diseases/lesions, on OL it remains unknown. Therefore, according to what is known so far, this is the first study that aims to evaluate the association of *SOX2* genetic polymorphism with OL and with immunohistochemistry expression of SOX-2. So, the null hypothesis is that there is no association on polymorphism on *SOX2* gene with OL, and with the immunohistochemistry expression of SOX-2.

Material and Methods

This is a cross-sectional case-control study and includes patients with oral leukoplakia (OL) submitted to genetic and immunohistochemical analysis. The present study was approved by Research Ethics Comitee of Pontifical Catholic University of Paraná (# 2.971.307).

Samples (files, paraffin blocks and slides) were obtained for the Pontifical Catholic University, Federal University of Minas Gerais and Federal University of Santa Catarina archives of patients diagnosed with OL (clinical findings associated with histological hyperkeratosis with atypia) and normal oral mucosa (control group). The slides were scanned on ZEN 2.3 Lite program (ZEISS Microscope Software ZEN Lite) and the histological verification was performed by two pathologists. The normal oral mucosa (group control) were obtained from the alveolar ridge during third molar extraction. Information on the gender of individuals was collected from medical records.

The exclusion criteria were: paraffin blocks absent, showing not enough epithelial tissue for analysis and inflammation.

A total of 89 patients were included, being 68 from OL group and 21 from the control group. Patients aged less than 48 corresponded to 30,8% on OL e 100% on the control group. The distribution according to the anatomical location according to the LB group was as follows: 10 cases on the tongue, 40 cases on the alveolar ridge, 2 cases on the alveolar mucosa, 4 cases on the gingiva, 4 cases on the mouth floor, 7 cases on the palate, 14 cases on the buccal mucosa, 3 cases on the lip mucosa, 1 case on the tonsil arch and 4 cases were not informed in the location in the medical records. The control group comprised 100% and the OL group 54.4% of the oral mucosa from the keratinized region, however the all sample of the OL group revealed hyperkeratosis and epithelial dysplasia.

Genetic Analysis

a) Sections preparation and DNA extraction

Three tissue sections of each patient of 10 μm were deparaffinized using xylol and ethanol. DNA extraction protocol, from paraffin material, was performed with QIAamp DNA minikit® commercial system, according to manufacturer recommendations. The samples were diluted to a final concentration of 20 ng/ul, and stored in a freezer on –20ºC.

b) Markers selection

The *SOX2* gene tag marker SNP rs77677339 (G/A) was selected based on *International HapMap Project* (http://www.hapmap.org), according to minimum of 5% allelic frequency parameters and 80% linkage disequilibrium in European population (CEU). This marker indicated by *International HapMap Project* captures all variability gene information, reducing expense and time.

c) Genotyping by CRP in real time

Patients' purified DNA was amplified by CRP in real time technique using TaqMan® Genotyping Master Mix (Applied Biosystems 7500 Real Time PCR System) technology for genotyping and discrimination allelic analysis of tag

SNP rs77677339 (G/A), using fluorescents probes. Depending on hybridization probe there will be genotype discrimination. Two equal fluorescence determine homozygous genotype (GG or AA) and two distinct fluorescence indicate heterozygous genotype (GA). A negative control was used in every genotyping. Thus, samples from 84 patients were quantified and in 5 the genotype was not determined.

Immunohistochemistry reaction SOX-2 immunostaining analysis

The protocol of immunohistochemistry reaction was performed and immunostaining analysis described on literature (Luiz *et al*., 2018), in brief: *imunno Retriever* (Dako, Carpinteria, CA, USA), primary antibody - anti SOX-2 rabbit monoclonal in 1:50 dilution (clone: EPR3131, ABCAM Cambridge, MA), *Advance link* e *enzyme* (Dako, code K40689), DAB (Spring Bioscience Corp, Pleasanton, CA, code DAB-999) e hematoxylin Harris (Biotec, Curitiba, Brazil). Primary antibody omission was used for negative control and seminoma was used as positive control.

The slides were scanned in ZEN 2.3 lite program (ZEISS Microscope Software ZEN Lite) and it was analyzed: a) counting of positive and negative cells on basal layer of epithelium (<64,1 or ≥64,1), suprabasal (<68,6 or ≥68,6) e total (<67,2 or ≥67,2); b) semiauthomatized segmentation for quantification of immunopositive nuclear area in square micrometers (< 0 or ≥ 0); as described by Luiz, *et al.* (2018) e c) staining intensity (1 = no positive cells and poor staining; 2 = moderate staining and strong - ≤1 or >1) (Figure 1).

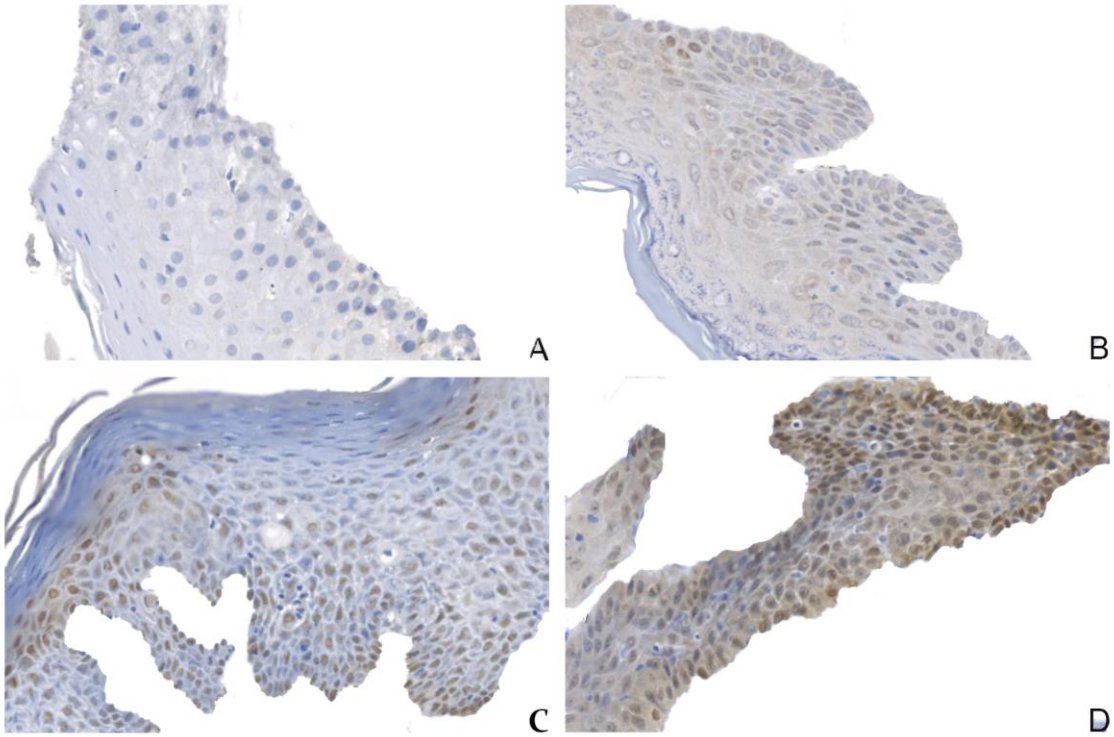

Figure 1. Photomicrograph revealing levels of intensity of SOX-2 coloration, according to the punctuation ≤1: A) Epithelium demonstrating absence of positive cells. **B)** Weak staining. **Punctuation >1: C)** Moderate staining. **D)** Strong coloring. (Immunohistochemistry SOX-2 100x).

Statistical Analysis

The data was analyzed using SPSS 25.0 program SPSS Inc, Chicago, Illinois, USA).

Dominant model (GG+GA *vs* AA) and recessive (AA+GA *vs* GG) were not reproducible in this sample because of the absent of AA genotype. Therefore, only the additive model (GG *vs* GA and AA) was used for genotyping analysis of SOX-2. Pearson qui-square test and Fischer's Exact Test were used for categorical variable. The significance level for all the tests were 5% (p<0,05).

Results

A total of 84 patients were included, being 64 from OL group and 20 from the control group. Patients aged less than 48 corresponded to 30,8% on OL e 100% on the control group. On OL group, 54,4% were female and on control group 47,6%.

There was no significance association (p=0,578) on genotypes distribution for the marker rs77677339 (G/A) for the group (OL) and control. It was observed thar genotype GG (96,9%) on OL and 100% on controls. Genotype GA was absent in controls (Table 1).

Table 1 - Genotype analysis of *SOX2* gene tag marker SNP rs77677339 (G/A), on additive model.

Gene	Tag SNPs DbSNP ID[a]	Variation	Group	GG n (%)	GA n (%)	AA n (%)	*p value**
SOX2	**rs77677339**	**[G/A]**	Leukoplakia	62 (96,9)	2 (3,1)	-	0,578
			Control	20 (100,0)	0 (0,00)	-	

NOTE: [a] SNP identified on NCBI database; * Fisher's Exact Test.

On genotype analysis of *SOX2* gene tag marker SNP rs77677339 (G/A) with the immunohistochemistry analysis (percentage of positive cells in basal, suprabasal and total epithelium layer, nuclear area and intensity) of additive model showed no association with OL either (Table 2).

Table 2 - Genotype analysis of *SOX2* gene tag marker SNP rs77677339 (G/A), with immunohistochemistry analysis – PUCPR, 2019.

Immunopositivity for SOX-2	Group	GG	GA	AA	*p* value *
		n (%)	n (%)	n (%)	
Basal layer					
<64,1	Leukoplakia	22 (95,7)	1 (4,3)	-	0,535
	Control	20 (100,0)	0A (0,00)	-	
≥64,1	Leukoplakia	40 (97,6)	1 (2,4)	-	-
	Control	0 (0,0)	0 (0,0)	-	-
Suprabasal layer					
<68,6	Leukoplakia	21 (95,5)	1 (4,5)	-	0,537
	Control	19 (100,0)	0 (0,0)	-	
≥68,6	Leukoplakia	41 (97,6)	1 (2,4)		0,977
	Control	1 (100,0)	0 (0,0)		
Total					
<67,2	Leukoplakia	22 (95,7)	1 (4,3)	-	0,535
	Control	20 (100,00)	0 (0,0)	-	
≥67,2	Leukoplakia	40 (97,6)	1 (2,4)	-	-
	Control	0 (0,0)	0 (0,0)	-	
Nuclear area (total area)					
<0	Leukoplakia	7 (87,5)	1 (12,5)	-	0,471
	Control	9(100)	0 (0,0)	-	
≥0,1	Leukoplakia	55 (98,2)	1 (1,8)	-	0,836
	Control	11 (100,0)	0 (0,0)	-	
Intensity					
≤1	Leukoplakia	29 (96,9)	1 (3,1)	-	0,640
	Control	20 (100,0)	0 (0,0)	-	
>1	Leukoplakia	33 (96,7)	1 (3,3)		0,882
	Control	0 (0,0)	0 (0,0)		

NOTE: [a] SNP identified on NCBI database; b: first allel is the larger on and the second is the smaller allel; * Fisher's Exact Test.

Discussion

The null hypothesis, in the present study, that there would be no association of SOX-2 gene polymorphism with oral leukoplakia and immunohistochemistry expression (percentage of positive cells in basal, suprabasal and total epithelium layer, percentage of nuclear area and immunostaining intensity) in OL was accepted. However, there are genetic results that can be inferred containing biological significance.

In a preliminary study, quantitative immunoexpression of SOX-2 isolated was performed (not grouped with lichen planus), and an over expression of this protein was observed in OL lesion low and high risk for malignant transformation when compared with normal oral mucosa, which demonstrated the possible involvement of this transcription factor in the pathogenesis of OL (Luiz *et al*., 2018). In the same way, SOX-2 immunoexpression on OL was evaluated by nuclear coloration and it was observed that the protein expression increased, in a significant way, with epithelium dysplasia degree. However, normal adjacent epithelium showed negative expression of SOX-2 (Vicente *et al*., 2019).Till present moment the protein assignments of SOX-2 gene have not been elucidated.

SNP rs77677339 (G/A) tag is a marker, according to *International HapMap Project,* that is able to capture all variability gene information, which makes unnecessary the genotyping of others markers for *SOX2* in the studied population. In the investigated additive model, there was no difference between OL group and control. On OL group 3,1% of the individuals showed heterozygous genotype GA and it was absent in the control group. Although there was no statistic difference of genotypes between the studied groups, it is important to highlight that allel A is rare in population, being approximately 4% using the database 1000Genomes (NCBI - db SNP rs77677339), its presence in heterozygous could influence in a possible risk association of *SOX2* gene polymorphism on OL. However, further studies are necessary with bigger sample to analyze this possible association.

This research did not point association of *SOX2* gene polymorphism and SOX-2 immunoexpression with OL. Previously published article evaluates SOX-2 immunohistochemistry expression in a sample of 94 laringeal dysplasia. The nuclear expression was detected in 40% of dysplasia, while stromal cell and

normal adjacent epithelia showed no expression. Through DNA extracted from the same paraffin tissue blocks, the authors evaluated the amplification of *SOX2* gene using CRP in real time in 55 patients of laryngeal dysplasia. The amplification of *SOX2* gene was detected in 33% of these patients and was not correlated with lesion severity. When correlation between gene amplification and protein expression it was observed the genetic amplification leads only partially to SOX-2 protein expression (Granda-Díaz *et al*., 2019). In the present study, although the anatomical area evaluated was different from the study previously mentioned, and with polymorphism analyze, not genetic amplification; our study is in line with those authors (Granda-Díaz *et al*., 2019). Therefore, as it was stated by the mentioned authors, other molecular and epigenetic events can be associated with transcriptional events of *SOX2* gene.

SOX2 expression regulation may occur in transcriptional levels, by microRNAs, long non-coding RNAs and *SOX2* post-translations modifications (Wuebben & Rizzino, 2017). In oral squamous cells carcinoma, SOX-2 protein expression was detected in higher percentage than *SOX2* mRNA expression, suggesting possible involvement of post-transcriptional mechanism (Vicente *et al*., 2019).

Further studies should be conducted using another analysis tools, such as alterations in the number of copies (amplification), through Fluorescence In-situ Hybridization assay (FISH); epigenetic studies and intracellular singling (Alonso *et al*., 2011; Gut *et al*., 2018) in order to elucidate the role of *SOX2* gene in OL pathogenesis.

Although the sample was collected from three centers, the limitation in the present study is found in the difficulty of obtaining participants with the diagnosis of OL, and the search for an association of the data with other possible etiological factors, due to the use of secondary banks with missing information. LB lesions can occur in several anatomical sites, but the fragments of the control group were collected only from one site, which was due to the surgical technique for extraction of third molars, avoiding damage to the normal oral mucosa tissues from other locations. Thus, studies may be needed for further evaluation of the role of SOX2 gene polymorphism as a risk factor in originally keratinized mucosa in the LB, and for the development of CCEB and to correlate with immunohistochemical data.

Conclusion

Absence association of rs77677339 (G/A) polymorphism and SOX-2 immunohistochemistry do not exclude the role of SOX-2 protein in the group of patients with OL. Besides the presence of allel A only in heterozygous individuals suggests that the role of risk marker of this allel on rs77677339 (G/A) of *SOX2* gene.

Conflict of interest

The authors declare no conflict of interest.

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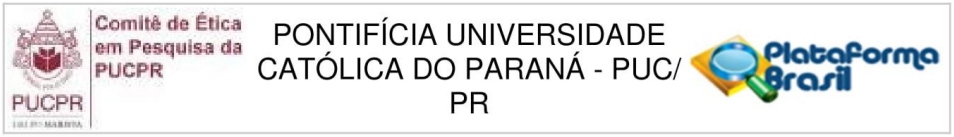
ANEXOS

Parecer do comitê de ética



PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: ASSOCIAÇÃO DOS POLIMORFISMOS E IMUNOEXPRESSÃO DE OCT4, SOX2 E NANOG COM CARCINOMA DE CÉLULAS ESCAMOSAS DE BOCA E LEUCOPLASIA BUCAL

Pesquisador: Aline Cristina Batista Rodrigues Johann

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 5

CAAE: 37645714.0.0000.0020

Instituição Proponente: Pontifícia Universidade Católica do Parana - PUCPR

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.971.307

Apresentação do Projeto:

A presente emenda tem o objetivo de alterar a descrição do projeto que consta no parecer de aprovação. Na metodologia foi descrito que se avaliariam 360 lâminas que fazem parte do acervo do Centro de Simulação Clínica e na realidade e de acordo com o que consta no projeto enviado para o CEP, serão utilizados lâminas e blocos de parafina dos arquivos do Laboratório de Patologia Experimental. A metodologia do projeto descrita segundo a autora é a seguinte:

Será realizado um estudo retrospectivo por meio de uma pesquisa documental e observacional de casos de CCEB (CCEB) e LB Bucal. O presente estudo já obteve aprovação correlata a imunohistoquímica dos Comitês de Ética em Pesquisa da Pontifícia Universidade Católica do Paraná (PUCPR) (Parecer número:1.110.687) e do Hospital Erasto Gaertner (parecer número 2.371.598) e uma emenda ao projeto será feita para a análise genética. Toda a metodologia de processamento e análise imuno-histoquímica foi desenvolvida no Laboratório de Patologia Experimental e para a análise genética será realizada no Laboratório Multiusuário. Os equipamentos que serão usados na execução do projeto já estão disponíveis.A partir da amostra selecionada (PUCPR, UFMG, UFSC, HEG, UP) a análise genética será realizada.Preparo dos cortes:Inicialmente serão realizadas a limpeza e a remoção da parafina externa dos blocos. Posterior a esse passo serão coletados 10 cortes de 5mm de tecido de cada bloco, utilizando-se micrótomo limpo, com navalhas

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descartáveis. Após o corte, as fatias serão acondicionadas em tubos plásticos de 1,5ml identificados previamente e mantidas à temperatura ambiente até o momento da extração. Os procedimentos laboratoriais serão realizados por um único técnico de laboratório.Antes da extração do DNA, os cortes passarão pelo processo de desparafinização, sendo que cada método requereu procedimentos distintos.Para purificação com o kit da Qiagen (QIAamp DNA minikit®) será seguido os seguintes procedimentos: será adicionado 1.200l de xilol as amostras, agitando-se por 15 segundos (1). Em seguida, os tubos serão centrifugados a 14.000rpm (rotações por minuto) durante cinco minutos (2). O sobrenadante será desprezado (3) e 1.200l de etanol será adicionado ao sedimento formado (4). Os tubos serão agitados por 15 segundos (5) e centrifugados a 14.000rpm durante cinco minutos (6). Esse procedimento será repetido e, ao final, os tubos com as tampas abertas serão colocados em centrífuga à vácuo a 37°C durante 15 minutos, objetivando a evaporação do etanol remanescente (7). Extração manualO protocolo para extração de DNA a partir de material parafinado com o sistema comercial QIAamp DNA minikit® será realizado de acordo com as normas do fabricante. Em cada tubo será adicionado 180l de Buffer ATL (Tissue Lysis Buffer), além de 20l de proteinase K. Após homogeneização, as amostras serão incubadas em banho-maria a 55°C, durante três horas, e agitadas gentilmente a cada hora. Após esse período, deverá ser adicionado a cada tubo 200l de Buffer AL (fornecido pelo fabricante), sendo os mesmos previamente aquecidos a 70°C durante 10 minutos para a inativação da proteinase residual. Em seguida, será adicionado 200l de etanol seguido de agitação durante 15 segundos e centrifugando brevemente. A solução resultante será transferida para o dispositivo da coluna QIAamp DNA minikit® e que deverá ser centrifugada a 8.000 rpm por um minuto. O dispositivo com a coluna será removido do tubo e recolocado em um tubo limpo. Posteriormente serão adicionados 500l de Buffer AW1 (Wash Buffer 1) ao dispositivo com a coluna, que será centrifugado a 8.000 rpm por um minuto. O procedimento de lavagem será repetido com 500l de Buffer AW2 (Wash Buffer 2), seguido de centrifugação a 14.000 rpm durante três minutos. O DNA extraído da coluna será eluído pela adição de 100l de Buffer AE (fornecido pelo fabricante). Adicionando primeiramente 50l Buffer AE, aguardando-se um minuto e centrifugando-se a 8.000 rpm durante um minuto. Em seguida, será adicionado o restante (50l) de Buffer AE. Aguardar por cinco minutos e centrifugar a 8.000 rpm por um minuto. Após as amostras serem transferidas do dispositivo para tubo de recuperação, as mesmas serão armazenadas a -20°C. Análises de polimorfismos genéticos - Genotipagem por PCR em tempo real: Os polimorfismos alvo (tag SNPs) nos genes dos biomarcadores de lesão e resposta tecidual propostos foram escol.

Continuação do Parecer: 2.971.307

Objetivo da Pesquisa:

A presente emenda tem o objetivo de alterar a descrição do projeto que consta no parecer de aprovação. Na metodologia foi descrito que se avaliariam 360 lâminas que fazem parte do acervo do Centro de Simulação Clínica e na realidade e de acordo com o que consta no projeto enviado para o CEP, serão utilizados lâminas e blocos de parafina dos arquivos do Laboratório de Patologia Experimental.

Avaliação dos Riscos e Benefícios:

Os riscos e benefícios apresentados estão adequados e de acordo com a Resolução 466/2012.

Comentários e Considerações sobre a Pesquisa:

Os objetivos e a metodologia apresentados estão adequados e de acordo com a Resolução 466/2012.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos de apresentação obrigatória foram anexados e estão adequados e em acordo com a Resolução 466/2012.

Recomendações:

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Emenda aprovada.

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Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1231281_E4.pdf	02/10/2018 16:49:33		Aceito
Outros	ProjetoEmenda.docx	02/10/2018 16:39:53	Aline Cristina Batista Rodrigues Johann	Aceito
Declaração de Instituição e Infraestrutura	Autorizacao_Hospital_Erasto_Gaertner.pdf	04/04/2017 10:34:48	Aline Cristina Batista Rodrigues Johann	Aceito
Declaração de Instituição e Infraestrutura	Autorização UFSC.pdf	29/05/2015 11:21:57		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tcle.doc	28/05/2015 15:31:57		Aceito
Outros	Carta solicitação de correção do parecer -signed.pdf	24/03/2015 16:04:11		Aceito
Outros	TCUD 1.pdf	21/10/2014 09:24:50		Aceito
Folha de Rosto	Folha de rosto.pdf	21/10/2014 09:24:28		Aceito
Projeto Detalhado / Brochura Investigador	Projeto edital universal.doc	10/10/2014 18:21:33		Aceito
Outros	declaração custos-signed.pdf	10/10/2014 18:20:22		Aceito
Declaração de Instituição e Infraestrutura	Autorização UFMG.pdf	10/10/2014 17:50:56		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CURITIBA, 19 de Outubro de 2018

Assinado por:
NAIM AKEL FILHO
(Coordenador(a))

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Produção Científica

Artigos Publicados:

Luiz ST, Modolo F, Mozzer I, Dos Santos EC, Nagashima S, Camargo Martins AP, de Azevedo MLV, Azevedo Alanis LR, Hardy AMTG, de Moraes RS, Aguiar MCF, Ignácio SA, Jham BC, Noronha L, Johann ACBR. **Immunoexpression of SOX-2 in oral leukoplakia**. Oral Dis. 2018 Nov;24(8):1449-1457. doi: 10.1111/odi.12922.

Michels A, **Luiz ST**, Santos ECD, et al. **Erythroplasia: the oral epithelial lesion with the greatest potential for malignant transformation – a mini review**. J Dent Health Oral Disord Ther. 2018;9(6):522–524. doi: 10.15406/jdhodt.2018.09.00441

Pellizzari VA, Michels AC, **Luiz ST**, de Souza EM, Tabchoury C, Rached RN. **Fluoride Ion Release of Self-Adhesive Resin Cements and Their Potential to Inhibit In Situ Enamel and Dentin Demineralization.** Oper Dent. 2017 Sep/Oct;42(5):548-558. doi: 10.2341/16-115-L.

Artigos Submetidos:

Luiz ST, Michels AC, Modolo F, Mozzer I, Dos Santos EC, Nagashima S, Camargo Martins AP, de Azevedo MLV, Azevedo Alanis LR, Hardy AMTG, de Moraes RS, Aguiar MCF, Ignácio SA, Jham BC, Noronha L, Johann ACBR. **OCT-4 and SOX-2 in Oral Leukoplakia.** Journal of Oral Pathology and Medicine. (JOPM-11-19-OA-5786).

Luiz ST, Michels AC, Santos EC, Lima AAS, Caldeira PC, Johann ACBR. **Leukoplakia: a potentially malignant oral lesion – an update.** Stomatologija – Baltic Dental and Maxillofacial Journa**l** (SBDMJ-2019-33 - 390).

Kitahara A, **Luiz ST**, Michels AC, Modolo F, Mozzer I, Dos Santos EC, Nagashima S, Camargo Martins AP, de Azevedo MLV, Azevedo Alanis LR, Hardy

AMTG, de Moraes RS, Aguiar MCF, Ignácio SA, Jham BC, Noronha L, Johann ACBR. **Immunoexpression of NANOG in oral leukoplakia.** Oral Diseases (ODI-11-19-OM-7645).

Tschoeke A, Luiz ST, Couto PHC, Caldeira PC, Johann ACBR **Peripheral Odontogenic Fibroma: a systematic review.** Minerva Stomatologica (Minerva Stomatol-4155).

Alanis LRA, de Andrade VKH, **Luiz ST**, de Souza PTR, Vasconcelos IM, Donaduzzi LC, Souza PHC. **Accuracy of Ultrasonography vs CT vs Cone Beam CT for Sialolithiasis Diagnosis: a Systematic Review.** Dental MaxilloFacial Radiology (DMFR-D-19-00351).

Artigos em fase de redação/correção:

Luiz ST, Galina F, Hamadosh A, Junior SC, Johann ACBR, Alanis LRA, Souza PHC. **Morphometric evaluation of infraorbital foramen in 2D and 3D images by means of cone beam computed tomography.**

Luiz ST, Andrade VKH, Couto SAB, Gambus LCC, Souza PHC**. Palliative Care in Dentistry.**

Madruga MHM, **Luiz ST**, Werneck RI, Hardy AMTG, Alanis LRA, Tannous LA,Soares ICM, Johann ACBR. **Serum and salivary alterations in polytraumatized patients hospitalized in Intensive care units: a systematic review".**

Normas para publicação – Oral Diseases

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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

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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.

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It is a requirement that the corresponding author submit a short description of each individual's contribution to the research and its publication. Upon submission of a manuscript all co-authors should also be registered with a correct e-mail addresses. If any of the e-mail addresses supplied are incorrect, the corresponding author will be contacted by the Journal Administrator.

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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.

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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.

Preprint policy: This journal will consider for review articles previously available as preprints on non-commercial servers such as ArXiv, bioRxiv, psyArXiv, SocArXiv, engrXiv, etc. Authors may also post the submitted version of a manuscript to non-commercial servers at any time. Authors are requested to update any pre-publication versions with a link to the final published article.

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.

Letters to the Editors: Letters, if of broad interest, are encouraged. They may deal with material in papers published in *Oral Diseases* or they may raise new issues, but should have important implications. Only one letter may be submitted by any single author or group of authors on any one published paper. Letters to the Editors should not include an abstract and are limited to 500 words, with a maximum of 1 figure and 10 references.

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.

Meeting Reports: Will be considered by the editors for publication only if they are of wide and significant interest.

Short Communications: These are brief papers of any topic within the scope of *Oral Diseases*about significant and novel advances that are complete in research endeavor but not suitable for full publications. Short Communications should not include an abstract and are limited to 1000 words, with a maximum of 3 figures and 20 references.

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.

Invited Medical 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.

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.

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

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)
- (Tables)

Title Page: should be part of the manuscript uploaded for review and include:

- A title of no more than 100 characters including spaces
- A running title of no more than 50 characters
- 3-6 keywords
- Complete names and institutions for each author
- Corresponding author's name, address, email address and fax number
- Date of submission (and revision/resubmission)

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 cover 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).

Color figures

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.

Guidelines for Cover Submissions

If you would like to send suggestions for artwork related to your manuscript to be considered to appear on the cover of the journal, please follow these

general guidelines.

6. AFTER ACCEPTANCE

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.

Proof Corrections

The corresponding author will receive an e-mail alert containing a link to a website. A working e-mail address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site.

Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following website: www.adobe.com/products/acrobat/readstep2.html . This will enable the file to be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs.

Proofs must be returned to the Production Editor within **three days** of receipt.

As changes to proofs are costly, we ask that you only correct typesetting errors. Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately. Other than in exceptional circumstances, all illustrations are retained by the publisher. Please note that the author is responsible for all statements made in their work, including changes made by the copy editor.

Early View (Publication Prior to Print)

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