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BRUNO AUGUSTO LINHARES ALMEIDA MARIZ

EXPRESSÃO IMUNO-HISTOQUÍMICA DE FGF-2 E FGFR-1 EM CARCINOMA ESPINOCELULAR ORAL DE LÍNGUA

FGF-2 AND FGFR-1 IMMUNOEXPRESSION IN ORAL TONGUE SQUAMOUS CELL CARCINOMA

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Estomatopatologia, na Área de Patologia.

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Orientador: Prof. Dr. Jacks Jorge Júnior

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“Today we still yearn to know why we are here and where we came from. Humanity's deepest desire for knowledge is justification enough for our continuing quest”

Stephen Hawking

RESUMO

O fator de crescimento fibroblástico 2 (FGF-2) e o receptor do fator de crescimento fibroblástico 1 (FGFR-1) estão associados à maior capacidade de invasão tumoral, proliferação celular, angiogênese e ao potencial de metástase. Este estudo teve como objetivo investigar a expressão de FGF-2 e FGFR-1 na displasia epitelial oral (DEO) e carcinoma espinocelular de língua (CECL). Foram selecionados retrospectivamente cento e sessenta e sete casos, incluindo 85 espécimes cirúrgicos de pacientes com CECL, provenientes do Hospital Onofre Lopes, Natal, Brasil, além de 46 biópsias incisionais de CECL e 36 de DEO provenientes do arquivo do Laboratório de Patologia Oral da Faculdade de Odontologia de Piracicaba (UNICAMP). Cortes de tecido parafinado foram submetidas à reação imunoistoquímica para FGF-2 e FGFR-1. As lâminas foram escaneadas e a marcação imunoistoquímica foi quantificada digitalmente pelo software Aperio Positive Pixel Count v9. Cinco áreas iguais foram selecionadas no estroma e no epitélio tumoral. Os resultados foram exportados e o score final de cada lesão foi obtido através da soma da porcentagem de pixels fracos, moderados e fortes, gerando um valor que variou de 100 a 300. Os casos foram divididos como tendo “baixa expressão” ou “alta expressão” das proteínas de acordo com a mediana dos grupos. FGF-2 e FGFR-1 foram mais expressos em DEO de alto grau do que em DEO de baixo grau, tanto no epitélio, como nas células do estroma ($p<0.05$). A sobrevivência doença específica (SDE) em 5 anos foi de 47,3% dos pacientes com CECL. A alta expressão de FGF-2 nas células inflamatórias e mesenquimatosas do estroma foi associada à invasão vascular e pior prognóstico. Pacientes com alta expressão de FGF-2 no estroma apresentaram taxa de SDE em 5 anos de 36,7%, contra 59,3% dos pacientes com baixa expressão (HR: 2,272; IC95%: 1.213-4.254; p=0,008). A alta expressão de FGFR-1 no estroma foi correlacionada com metástases linfonodais e metástases à distância. Pacientes com alta expressão de FGFR-1 no epitélio apresentaram taxa de SDE em 5 anos de 22,9%, contra 75,6% dos pacientes com baixa expressão (HR: 2,594; IC95%: 1,390-4,841; p=0,003). O mesmo aconteceu com FGFR-1 no estroma, com taxa de SDE em 5 anos de 32,9%, contra 64,0% (HR: 3,378; IC95%: 1,816-6,286; p=0,001). A análise multivariada de Cox confirmou que a expressão de FGF-2 no estroma (HR: 2,197; IC95%: 1,128-4,282; p=0,02), FGFR-1 no epitélio (HR: 3,178; IC95%: 1,505-6,709; p=0,002) e

FGFR-1 no estroma (HR: 3,041; IC95%: 1,454-6,356; p=0,003) estão fortemente associadas a um maior risco de morte relacionada ao CECL. Em conjunto, nossos achados demonstram que FGF-2 e FGFR-1 desempenham um papel importante na DEO e no CECL, estando associados à presença de metástase e à sobrevivência dos pacientes.

Palavras-chave: fator de crescimento fibroblástico, câncer oral, FGF-2, FGFR-1, displasia epitelial oral

ABSTRACT

Fibroblast growth factor 2 (FGF-2) and fibroblast growth factor receptor 1 (FGFR-1) expression is associated with tumour invasiveness, cell proliferation, angiogenesis and metastasis potential. This study aimed to investigate FGF-2 and FGFR-1 expression in oral epithelial dysplasia (OED) and tongue squamous cell carcinoma (TSCC). One hundred and sixty-seven cases were retrospectively selected, including 85 surgical specimens of patients with TSCC, from Onofre Lopes Hospital, Natal, Brazil, besides 46 TSCC and 36 OED incisional biopsies from the Laboratory of Oral Pathology, Piracicaba Dental School, University of Campinas (UNICAMP). Tissue sections were submitted to immunohistochemical reaction for FGF-2 and FGFR-1. Slides were scanned and the immunostaining was digitally quantified by the Aperio Positive Pixel Count v9 software. Five areas were selected from the stroma and the epithelium. Results were exported and the final score of each lesion was obtained by the sum of the percentage of weak, moderate and strong pixels, resulting in a value ranging from 100 to 300. Cases were classified as “weak expression” or “high expression” of the proteins, accordingly to the group median. FGF-2 and FGFR-1 were more expressed in high-grade OED than in low-grade OED, either in the stroma or in the epithelium ($p<0.05$). The 5-year disease-specific survival (DSS) rate was 47.3% of the patients with TSCC. FGF-2 high expression in the inflammatory and mesenchymal cells of the stroma was associated with vascular invasion and worse prognosis. Patients with high expression of FGF-2 in the stroma had a 5-year DSS of 36.7%, against 59.3% of patients with low expression (HR: 2.272; CI(95%): 1.213-4.254; $p=0.008$). FGFR-1 high expression in the stroma was correlated with lymph node metastasis and distant metastasis. Patients with high expression of FGFR-1 in the tumour had a 5-year DSS of 22.9%, against 75.6% of patients with low expression (HR: 2.594; CI(95%): 1.390-4.841; $p=0.003$). The same was observed with FGFR-1 in the stroma, with a 5-year DSS of 32.9%, against 64.0% (HR: 3.378; CI(95%): 1.816-6.286; $p=0.001$). The Cox multivariate analysis confirmed that the expression of FGF-2 in the stroma (HR: 2.197; CI(95%): 1.128-4.282; $p=0.02$), FGFR-1 in the tumour (HR: 3.178; CI(95%): 1.505-6.709; $p=0.002$) and FGFR-1 in the stroma (HR: 3.041; CI(95%): 1.454-6.356; $p=0.003$) are strongly associated with a higher risk of death related to TSCC. Taken together, our findings demonstrate that FGF-2 and FGFR-1

play an important role in OED and TSCC, and are associated with the presence of metastasis and patients disease-specific survival.

Key Words: fibroblast growth factor, oral cancer, FGF-2, FGFR-1, oral epithelial dysplasia

LISTA DE ABREVIATURAS E SIGLAS

AP – Adenoma Pleomorfo

CEC – Carcinoma Espinocelular

CI – Confidence interval

CxAP – Carcinoma ex-Adenoma Pleomorfo

DEO – Displasia epitelial oral

DSS – Disease-specific survival

DOPM – Desordens orais potencialmente malignas

EMT – Epithelial-mesenchymal transition

FGF-2 – Fibroblast growth factor 2

FGFR-1 – Fibroblast growth factor receptor 1

HPV – Papiloma vírus humano

HNSCC – Head and Neck Squamous cells carcinoma

HR – Hazard ratio

IC – Intervalo de confiança

LLLT – Low level laser therapy

LPO – Líquen plano oral

OED – Oral epithelial dysplasia

OSCC – Oral squamous cells carcinoma

PMD – Potentially malignant disorder

RNA – Ácido ribonucleico

SCC - Squamous cells carcinoma

TEM – Transição Epitélio Mesênquima

TSCC – Tongue squamous cells carcinoma

SUMÁRIO

1 INTRODUÇÃO:

O câncer oral é uma doença multifatorial considerada um problema de saúde pública, principalmente em países em desenvolvimento. Dados do projeto GLOBOCAN apontam que em 2012 surgiram 300.373 casos de câncer na cavidade oral e que mais de 145.000 pessoas morreram em decorrência da doença em todo o mundo. No Brasil, a estimativa para o ano de 2018 é de 11.200 novos casos de câncer na cavidade oral em homens e 3.500 casos em mulheres, tornando-o a quinta neoplasia maligna mais comum em homens no Brasil, quando excluímos o câncer de pele não-melanoma (INCA, 2018).

Os Carcinomas Espinocelulares (CEC) orais representam mais de 90% dos casos de câncer oral em todo o mundo, sendo mais comum entre a sexta e sétima décadas de vida e comumente associado a fatores de risco, como o tabagismo (Sloan et al., 2017). O consumo de bebidas alcoólicas age juntamente com o hábito de fumar como um fator de risco sinérgico. O sachê de bethel, associado ou não ao tabaco é responsável por casos de câncer em regiões específicas onde esses hábitos são culturais. No câncer de orofaringe, o HPV (principalmente o tipo 16) também é considerado fator de risco, apesar de presente em uma pequena quantidade de casos (Saraiya et al., 2015). Já no câncer de lábio, a radiação ultravioleta é o principal fator de risco (Sloan et al., 2017). O CEC oral de língua é o tipo de câncer oral mais agressivo e muitas vezes seu tratamento deve ser multimodal mesmo nos estágios iniciais da doença, mostrando uma maior taxa de mortalidade que em outros sítios da cavidade oral (Almangush et al., 2015; Kauppila et al., 2015).

O Fator de Crescimento Fibroblástico 2 (FGF-2, Fibroblast Growth Factor 2), também conhecido como Fator de Crescimento Fibroblástico básico (b-FGF, *basic Fibroblast Growth Factor*), é um dos principais constituintes da família FGF (Nayak et al., 2015), que compreende 22 membros de proteínas secretadas agindo como fatores parácrinos, autócrinos ou endócrinos (Giacomini et al., 2016) que sinalizam por meio de dos receptores FGF (Turner & Grose, 2010).

A família dos receptores dos Fatores de Crescimento Fibroblásticos (FGFR) é composta por quatro (FGFR-1-4) receptores transmembrana de tirosina quinase expressos em diferentes tipos celulares (Ipenburg et al., 2016). A sinalização FGF-FGFR é responsável por diferentes mecanismos celulares, incluindo comportamentos

malignos, como proliferação e invasão tumoral, aumento da angiogênese e consequente impacto nas taxas de sobrevida (Turner e Grose, 2010).

FGF-2 é uma citocina multifuncional expressa em diversos tecidos e atua numa grande variedade de atividades biológicas (Delrieu, 2000; Nayak et al., 2015), como proliferação e diferenciação em vários tipos celulares (Delrieu, 2000). Além do seu papel na embriogênese, ela regula funções homeostáticas na vida adulta (Giacomini et al., 2016), participando de diferentes processos, incluindo angiogênese (Brizeno et al., 2016) e reparo tecidual (Harada et al., 2017).

FGF-2 tem cinco isoformas criadas de um único RNA mensageiro por iniciação de tradução alternativa (Delrieu, 2000). A proteína de baixo peso molecular tem 18kDa e está presente no citoplasma, de onde é secretada, agindo por meio de seus receptores (FGFR) (Martinez et al., 2010). Por outro lado, as quatro isoformas de alto peso molecular estão localizadas no núcleo, onde seus sinais agem por meio de um mecanismo intácrino, ou seja, independentemente dos FGFR (Delrieu, 2000).

FGFR-1 tem sido indicado como um potencial alvo molecular em diferentes tipos de câncer, incluindo o CEC oral (Clauditz et al., 2017), e inibidores do FGFR-1 têm sido usados na tentativa de impedir os processos de carcinogênese e transição epitélio-mesênquima (TEM) (Nguyen et al., 2013).

1.1 Expressão de FGF-2/FGFR-1 na mucosa oral normal e durante o processo de reparo:

FGF-2 é normalmente expresso nas camadas basal e parabasal do epitélio da mucosa oral (Forootan et al., 2000; Wakulich et al., 2002), enquanto FGFR-1 é expresso nas camadas sobrejacentes (Forootan et al., 2000). Brizeno et al. (2016) estudou os efeitos da diabetes no processo de reparo da mucosa oral, encontrando uma menor expressão de FGF-2 em ratos diabéticos que em controles normoglicêmicos, e portanto, um atraso significativo nos processos de angiogênese e produção de colágeno, prejudicando o mecanismo de reparo de feridas. Fujisawa et al. (2003) aplicou FGF-2 para tratar úlceras em coelhos, e foi capaz de promover a cicatrização das lesões induzindo a proliferação de fibroblastos e ceratinócitos.

Usumez et al. (2014) usou terapia com laser de baixa intensidade (LLLT) para tratar mucosite oral em ratos, mostrando que o tratamento acelerava o processo de cicatrização da mucosite pelo aumento dos níveis de FGF-2. Harada et al. (2017) também estudou mucosite oral em hamsters, induzida por 5-fluorouracil, e notou que a maior expressão de FGF-2 estava relacionada a uma recuperação acelerada da mucosa.

Morelli et al. (2011) notou a importância do FGF-2 durante os eventos iniciais do reparo em pacientes tratados com um enxerto celular vivo como um procedimento de aumento gengival, e uma pasta contendo FGF-2 foi usada clinicamente por Jiang et al. (2013) para tratar estomatite aftosa menor recidivante, aliviando substancialmente a intensidade da dor e acelerando a cicatrização das lesões nesses pacientes.

Jansen et al. (2009) implantou enxertos de colágeno com FGF-2 no palato de ratos, o que levou a um aumento na celularidade e vascularização. Além disso, houve um número diminuído de miofibroblastos, diminuindo a contração das feridas, um dos maiores problemas na cicatrização de feridas no palato. Estes resultados são similares aos de Hata et al. (2008), que administrou FGF-2 em lesões no palato de ratos, melhorando o suprimento vascular durante o reparo. Matsumoto et al. (2012) usou uma esponja de colágeno contendo FGF-2 para regenerar defeitos ósseos em cachorros, promovendo um reparo mais eficiente. Kanda et al. (2003) estudou a imunoexpressão de FGFRs em feridas na mucosa palatina de ratos, e achou uma alta expressão de FGFR-1 em miofibroblastos durante o processo de reparo.

Mullane et al. (2008) tratou polpas dentais com FGF-2, melhorando a neovascularização, podendo ser usada como um tratamento tópico antes do reimplante de dentes avulsionados. Além disso, FGF-2 é naturalmente secretado pelas células da polpa dental, principalmente após algum trauma (Tran-Hung et al., 2008). Além disso, a injeção de FGF-2 na câmara pulpar e canal radicular de dentes humanos tratados endodonticamente por Kim et al. (2010) resultou na regeneração de tecido semelhante à polpa dental após implantação ectópica in vivo, o que representa uma possível alternativa futura durante o tratamento endodôntico.

1.2 Expressão de FGF-2/FGFR-1 nas Glândulas Salivares:

Nas glândulas salivares normais, FGF-2 é expresso na membrana basal dos ductos intercalados, ácinos e células basais dos ductos excretores (Kusafuka et al., 1998; Myoken et al., 1996), e como as glândulas salivares apresentam propriedades regenerativas fracas, FGF-2 tem sido usado na regeneração tecidual de glândulas submandibulares de ratos cirurgicamente danificadas, melhorando sua capacidade de regeneração (Kobayashi et al., 2016).

FGF-2 também tem sido administrado para tentar reparar células das glândulas salivares danificadas pela radioterapia (Guo et al., 2014; Kojima et al., 2011; Thula et al., 2005). Thula et al. (2005) administrou FGF-2 antes e imediatamente após irradiar células da glândula parótida de ratos, e encontrou um efeito radioprotetor associado com aumento de parada do ciclo celular em G2, permitindo maior tempo para corrigir danos ao DNA causados pela radioterapia. Kojima et al. (2011) administrou FGF-2 nas glândulas submandibulares irradiadas de camundongos, que mostraram um aumento do número de células acinares no grupo tratado. Guo et al. (2014) testou um adenovírus codificante de FGF-2 nas glândulas parótidas irradiadas de porcos, limitando o declínio do fluxo salivar, causado pela proteção das células endoteliais da microvasculatura da parótida pelo tratamento com FGF-2.

Em Adenomas Pleomórficos (AP), o tumor de glândula salivar mais comum (Sloan et al., 2017), FGF-2 e FGFR-1 estão presentes na membrana basal dos ninhos de células mioepiteliais, ao redor de células mioepiteliais nas áreas mixoides e nas células das lacunas das áreas condroides (Kusafuka et al., 1998). Miguita et al. (2010) encontrou expressão citoplasmática de FGF-2 e expressão nuclear de FGFR-1 em células mioepiteliais de AP, enquanto Persson et al. (2008) encontrou fusão e amplificação do gene FGFR-1 nessas lesões. Além disso, Soares et al. (2012) encontrou alta expressão de FGF-2 em AP que recorreram. Em sua contraparte maligna, o Carcinoma ex-Adenoma Pleomórfico (CxAP), as células mioepiteliais benignas apresentaram alta expressão de FGF-2 (tanto a isoforma de baixo peso molecular como de alto peso molecular) e ausência de expressão de FGFR-1. Já as células epiteliais malignas mostraram menor expressão de FGF-2, mas apresentaram expressão de FGFR-1, tanto citoplasmática como nuclear (Furuse et al., 2010; Martinez et al., 2010). A presença do receptor de FGF-2 no epitélio maligno pode indicar que existe uma resposta parácrina nessa lesão.

Myoken et al. (1996) encontrou maior expressão de FGF-2 e FGFR-1 em tumores malignos de glândula salivar, e sugeriram que esses fatores podem contribuir para a progressão do tumor de forma autócrina. Resultados similares foram encontrados por Sumitomo et al. (1999), onde essas proteínas foram co-expressas nas células que sofreram transformação maligna em CEC de glândulas submandibulares de ratos. Além disso, níveis de FGF-2 e FGFR-1 estão aumentados no soro e na saliva de pacientes com tumores de glândulas salivares benignos e malignos (Huang et al., 2012). Adicionalmente, Ach et al. (2016) encontrou aberrações no gene FGFR-1 em carcinomas de glândula salivar.

1.3 Expressão de FGF-2/FGFR-1 em Desordens Orais Potencialmente Malignas:

FGF-2 parece estar superexpresso no epitélio displásico de desordens orais potencialmente malignas (DOPM) (Wakulich et al., 2002; Raimondi et al., 2006; Bishen et al., 2008; Nayak et al., 2015). Wakulich et al. (2002) estudou a expressão de FGF-2 em diferentes graus de displasia epitelial, carcinoma *in situ* e CEC oral, e encontrou aumento da expressão de FGF-2 de acordo com a progressão das displasias para CEC, principalmente nas camadas superiores do epitélio. Na fibrose submucosa oral (FSO), uma DOPM caracterizada pela fibrose da mucosa que dificulta a abertura bucal, FGF-2 é superexpresso nos fibroblastos e células endoteliais nos estágios iniciais da doença, enquanto sua expressão na matriz do estroma aumenta nos estágios finais da doença (Bishen et al., 2008).

Nayak et al. (2015) estudou 72 casos de DOPM, incluindo leucoplasia e FSO, onde a expressão de FGF-2, tanto pela técnica de imunoistoquímica, como a nível molecular (expressão gênica), estava associada com a transformação maligna de DOPM em CEC oral. Gorugantula et al. (2012) encontrou altos níveis de FGF-2 na saliva de pacientes com Líquen Plano Oral (LPO), em comparação com controles saudáveis. Não existem estudos correlacionando a expressão de FGFR-1 com DOPM.

1.4 Expressão de FGF-2/FGFR-1 em Carcinoma Espinocelular Oral:

A expressão de FGF-2 e FGFR-1 tem sido correlacionada com menor diferenciação, maior potencial de invasão e pior prognóstico em pacientes com CEC oral (Forootan et al., 2000; Hase et al., 2006; Freier et al., 2007; Harada et al., 2007; Young et al., 2013; Nayak et al., 2015; Peng et al., 2015; Ozretić et al., 2016; Clauditz et al., 2017), além de influenciar a TEM em linhagens celulares de CEC oral (Jiao et al., 2015; Nguyen et al., 2013). Além disso, a expressão de FGF-2 e FGFR-1 em fibroblastos no fronte de invasão de CEC oral foi correlacionado com maior potencial invasivo, metástases linfonodais e pior prognóstico (Hase et al., 2006).

Myoken et al. (1994) inicialmente descreveu a expressão de FGF-2 em tecidos e linhagens celulares de CEC oral. Forootan et al. (2000) mostrou que FGF-2 está superexpresso em células mais atípicas de CEC oral e em linfócitos que infiltram o tumor, estando significativamente associado ao grau de diferenciação do tumor (Nayak et al., 2015) e à invasão linfovascular (Lassig et al., 2017). Interessantemente, a supressão de FGF-2 por agentes anticancerígenos está correlacionada à diminuição do crescimento e da neoangiogênese em linhagens celulares de CEC oral (Harada et al., 2007).

Além disso, os níveis de FGF-2 na saliva de pacientes recentemente diagnosticados com CEC oral encontram-se elevados em relação a pacientes em remissão ou indivíduos saudáveis (Gorugantula et al., 2012).

A amplificação do gene FGFR-1 está associada à presença de metástases distantes e pior prognóstico em pacientes com CEC oral (Peng et al., 2015). A amplificação de FGFR-1 foi encontrada em 10% dos casos de CEC oral (Clauditz et al., 2017), e em 5.6% dos CECs de orofaringe (Ozretić et al., 2016). Já Freier et al. (2007) encontrou amplificação de FGFR-1 em 17.4% dos casos de CEC oral. Young et al. (2013) correlacionou a amplificação de FGFR-1 com o hábito de fumar de pacientes com CEC de língua.

A expressão de FGFR-1 foi relacionada ao processo da TEM em CEC oral (Nguyen et al., 2013; Jiao et al., 2015). Nguyen et al. (2013) encontrou maior expressão de FGFR-1 em CEC de cabeça e pescoço e linhagens celulares de TEM. Sua maior expressão foi correlacionada com pleomorfismo nuclear, tumores mais invasivos e menor diferenciação histológica. Quando as linhagens celulares de TEM

foram tratadas com um inibidor de FGFR-1, houve menor proliferação e invasão, além de mudança do seu formato original fusiforme para uma morfologia poliédrica, indicando que FGFR-1 tem um papel na TEM. Do mesmo modo, Jiao et al. (2015) mostraram superexpressão de FGFR-1 em CEC de língua associada à menor diferenciação e maior potencial de metástases. Quando a expressão de FGFR-1 foi silenciada nas linhagens celulares, suas propriedades motoras e de invasão foram altamente reduzidas.

Portanto, o objetivo deste estudo foi avaliar a expressão de FGF-2 e FGFR-2 em casos de Displasia Epitelial Oral e Carcinoma Espinocelular de língua, além de correlacionar essa expressão com os dados clínico-patológicos dos pacientes.

2 ARTIGO:

Title: FGF-2 and FGFR-1 might be considered as prognostic factors in Tongue Squamous Cell Carcinoma

Short Running Title: FGF-2 and FGFR-1 expression in oral cancer

Keywords: fibroblast growth factor, oral cancer, FGF-2, FGFR-1

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Abstract:

Fibroblast growth factor-2 (FGF-2) and Fibroblast growth factor receptor-1 (FGFR-1) are associated with tumour invasiveness, cell proliferation, angiogenesis and metastasis potential. **Aims:** investigate FGF-2 and FGFR-1 expression in oral epithelial dysplasia (OED) and tongue squamous cell carcinoma (TSCC). **Methods and results:** one hundred and sixty-seven cases were retrospectively selected, including 85 surgical specimens of patients with TSCC, 46 incisional biopsies of TSCC and 36 OED. Tissue sections were submitted to immunohistochemical staining for FGF-2 and FGFR-1. FGF-2 and FGFR-1 were more expressed in high-grade OED than in low-grade OED, either in the stroma or in the epithelium ($p<0.05$). The 5-year disease-specific survival (DSS) rate was 47.3% of the patients with TSCC. FGF-2 high expression in the inflammatory and mesenchymal cells of the stroma was associated with vascular invasion and worse prognosis. Patients with high expression of FGF-2 in the stroma had a 5-year DSS of 36.7%, against 59.3% of patients with low expression (HR: 2.272; CI(95%): 1.213-4.254; p=0.008). FGFR-1 high expression in the stroma was correlated with lymph node metastasis and distant metastasis. Patients with high expression of FGFR-1 in the tumour had a 5-year DSS of 22.9%, against 75.6% of patients with low expression (HR: 2.594; CI(95%): 1.390-4.841; p=0.003). The same was observed with FGFR-1 in the stroma, with a 5-year DSS of 32.9%, against 64.0% (HR: 3.378; CI(95%): 1.816-6.286; p=0.001). The Cox multivariate analysis confirmed that the expression of FGF-2 in the stroma (HR: 2.197; CI(95%): 1.128-4.282; p=0.02), FGFR-1 in the tumour (HR: 3.178; CI(95%): 1.505-6.709; p=0.002) and FGFR-1 in the stroma (HR: 3.041; CI(95%): 1.454-6.356; p=0.003) are strongly associated with a higher risk of death related to TSCC. **Conclusions:** taken together, our findings

demonstrate that FGF-2 and FGFR-1 play an important role in OED and TSCC, and are associated with the presence of metastasis and patients disease-specific survival.

Keywords: fibroblast growth factor, oral cancer, FGF-2, FGFR-1

Introduction:

Tongue squamous cell carcinoma (TSCC) represents the type of oral SCC with the worst prognosis[1–3]. Most of patients underwent multimodality therapy even in early stages of the disease[4]. Oral epithelial dysplasia (OED) is a potentially malignant disorder (PMD) caused by accumulation of genetic changes in the oral mucosa[5], associated with an increased risk of progression to oral cancer. However, few studies have focused in stablishing useful key biological molecules or markers involved in OED progression and with prognostic value in TSCC.

Fibroblast growth factor-2 (FGF-2) is a multifunctional cytokine involved in several biologic activities, such as proliferation, differentiation, angiogenesis and tissue repair. Under homeostatic situations, fibroblast growth factor receptor-1 (FGFR-1) is signalled through growth factors, such as FGF-2[6]. Changes in the FGF-2/FGFR-1 signalling has been identified in several cancers, including non-small cell lung cancer[7], breast cancer[8,9], oesophageal cancer[10,11], lymphoma[12], hepatocellular carcinoma[13,14] and glioblastoma[15]. Although the role of FGF2 and FGFR1 has been previously investigated in oral SCC tumorigenicity and metastasis progression[4,16,17], mainly with experimental models, few studies evaluated the impact of these markers in the outcome of patients with oral cancer[18–20].

Thus, in this study, we evaluated FGF-2 and FGFR-1 immunoexpression in TSCC and OED. The expression of these biological markers was further correlated with clinicopathological parameters and survival data.

Materials and Methods:

Patients' Cohorts:

A total of 85 cases of TSCC (surgical specimens) were retrieved from Onofre Lopes Hospital, Natal, Brazil. Clinical data regarding patient's age, sex, tumour location, tumour stage, lymph node involvement, distant metastasis, treatment, clinical status and follow-up were retrieved from patient's files. The time between the diagnosis and the death, or the last contact with the patient (when still alive) was used to perform the survival analyses. Furthermore, 82 incisional biopsies from the Pathology Department of the Piracicaba Dental School, University of Campinas (FOP/UNICAMP) of TSCC (n=46) and Oral Epithelial dysplasia (n=36) were used to compare the immunoexpression of FGF-2 and FGFR-1. The cases were reviewed by two experienced pathologists and classified according to the World Health Organization criteria[21]. Cases with insufficient material or incomplete clinical data were excluded. The Ethics Committee of the Piracicaba Dental School, University of Campinas (protocol 69181417.9.0000.5418), approved the ethical aspects of this study.

Immunohistochemistry:

Immunohistochemical reactions were performed on 3µm-thick sections of paraffin-embedded tissues. Antigen retrieval was performed using EDTA/Tris solution (pH 9.0) for 15 minutes in an electric pressure cooker (for FGF-2) and, using citrate buffer solution (pH 6.0) in a microwave for 30 minutes (for FGFR-1). The endogenous

peroxidase activity was suppressed with 10% H_2O_2, in five cycles of 5 minutes each, before the sections were incubated with the primary antibodies for 2 hours. Primary antibodies utilized were mouse monoclonal anti-FGF-2 (clone G-2, 1:50 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit polyclonal anti-FGFR-1 (1:50 dilution; Santa Cruz Biotechnology). Immunohistochemical staining was performed with Envision (for FGF-2) and Advance (for FGFR-1), both of which were purchased from Dako and used following the manufacturer's instructions. Sections were then exposed to diaminobenzidine tetra- hydrochloride (DAB; Sigma-Aldrich, St Louis, Missouri, USA) and counterstained with Carazzi's haematoxylin. Breast carcinoma cases were used as positive controls. For negative controls, the protocol was followed without adding the primary antibody.

Immunohistochemical analysis:

After the immunohistochemical reactions, the slides were scanned into high-resolution images using the Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc., Vista, California, USA). FGF-2 and FGFR-1 immunoexpression was digitally assessed on a quantitative scale, by the Aperio Positive Pixel Count v9 software with specific input parameters as follows: hue value, 0.1; hue width, 0.5; colour saturation threshold, 0.04; and intensity threshold ranging from 100 to 175. Five areas were selected from the epithelial cells and inflammatory cells in the stroma, and a separate analysis was performed. These data were exported, and the final score of each tumour was calculated as the sum of the percentage of each category multiplied by their intensity scores using the following formula: [tumour score = (percentage weak × 1) + (percentage moderate × 2) + (percentage strong × 3)]. The results ranged from 100 to 300, and cases were classified as having "weak expression" or "high expression" of

the proteins, accordingly to the group median, in agreement with previous studies from our group[22,23].

Statistical Analysis:

The scores obtained by digital analysis were submitted to normal distribution tests (D'Agostino–Pearson and Shapiro–Wilk). Kruskal-Wallis test was used to compare the differences between score means in the respective groups. For frequency analysis in contingency tables, chi-square and Fisher's exact test were performed. Survival rate curves for disease-specific survival (DSS), based on clinicopathological parameters and immunohistochemical staining, were constructed using the Kaplan–Meier method and compared using the log-rank test. The multivariate Cox regression model was used to estimate the hazard ratio and respective 95% confidence interval. The level of significance considered was 5% ($p \leq 0.05$). All the tests were performed using statistical software SPSS 22.0 (IBM, Armonk, New York, EUA).

Results:

Clinicopathological characteristics of the patients:

A total of 85 cases of TSCC with complete clinical information was used in this study (Table 1). The five-year disease-specific survival rate for the patients with TSCC was 47.3%. A slight male predominance was found (M:F ratio of 1:0.8) with a mean age of 55.5 years (range of 19-89 years). Most of the patients consumed tobacco (63.5%) or alcohol (58.8%). Stage I/II tumours accounted for 42.4%, and stages III/IV 57.6% of the malignancies, with 51.8% of the patients presenting lymph node metastasis and 24.7% presenting distant metastasis. Most of the patients (52/85, 61.2%) were solely treated with surgery. Radiotherapy as the single-modality treatment

was delivered to only 8/85 (9.4%) patients. Combined-modality treatment was delivered to 21 patients (surgery plus radiotherapy and/or chemotherapy), and 4 patients received only palliative measures. Regarding the microscopic aspects of the tumours, 29.4% were well differentiated, 37.7% moderate and 32.9% poorly differentiated. Furthermore, vascular and neural invasion were present in 43.5% and 49.4%, respectively. Mean follow-up time was 40 months. The clinical data of the OED and TSCC incisional biopsies are shown in table 2.

FGF-2 expression:

FGF-2 immunoexpression was assessed in the epithelial cells and in the inflammatory cells within the stroma of the OED and TSCC. FGF-2 was expressed in the basal and suprabasal layers of the epithelium in low-grade OED (Fig. 1A), while in high-grade OED, this expression was more prominent and extended to the upper layers of the epithelium (Fig. 1B). FGF-2 was expressed in the cytoplasm of malignant cells in more well differentiated TSCC (Fig. 1C) and could be expressed in the nucleus of these cells in poorly differentiated tumours (Fig. 1D). The immunoexpression digital score was significantly higher in high-grade OED ($p=0.001$) when compared to low-grade OED (Fig. 1E). This was also true when comparing well differentiated and poorly differentiated TSCC ($p=0.027$) as shown in tables 2 and 3. FGF-2 high expression was correlated with tumour size ($p=0.024$) and the presence of distant metastasis ($p=0.007$) as shown in Table 3. However, epithelial expression of FGF-2 did not significantly correlate with the DSS probabilities in the Kaplan-Meier analysis (Fig. 1G and Table 4).

Comparing the FGF-2 expression in the inflammatory cells, it was significantly lower in the low-grade OED, when compared with the high-grade OED and the TSCC lesions ($p=0.001$), but there was no difference between the other groups (Fig. 1F).

Cases with high expression of FGF-2 in the inflammatory and mesenchymal cells of the stroma were associated with vascular invasion (Table 3). Patients with high expression of FGF-2 in the stroma had a 5-year DSS of 36.7%, against 59.3% of patients with low expression (HR: 2.272; CI(95%): 1.213-4.254; p=0.008). The Cox multivariate analysis confirmed that the high expression of FGF-2 in the stroma (HR: 2.197; CI(95%): 1.128-4.282; p=0.02), is strongly associated with a higher risk of death related to TSCC, as shown in Figure 1H and Table 4.

FGFR-1 expression:

FGFR-1 immunoexpression was also examined in the epithelial cells and in the inflammatory cells within the stroma of OED and TSCC. FGFR-1 was expressed in the upper layers of the epithelium of both low-grade and high-grade OED, but with much more intensity in the high-grade lesions (Fig. 2A and 2B). This difference was statistically significant (p=0.001), as in Fig. 2E. In the TSCC epithelial cells, the same pattern of the FGF-2 analysis was observed, with cytoplasmic expression in the more differentiated cases and nuclear expression in the poorly differentiated lesions (Fig. 2C and 2D, respectively).

The FGFR-1 expression in the stroma was also significantly lower in the low-grade OED when compared with the other groups ($p<0.05$), but no other differences were seen (Fig. 2F). FGFR-1 high expression in the stroma were also correlated with lymph node metastasis and distant metastasis (Table 3).

The survival curve rates using log-rank test showed that FGFR-1 high expression either in the malignant cells or in the stroma cells were strongly correlated with lower DSS (Fig. 2G and 2H). Patients with high expression of FGFR-1 in the tumour had a 5-year DSS of 22.9%, against 75.6% of patients with low expression

(HR: 2.594; CI(95%): 1.390-4.841; p=0.003). The same was observed with FGFR-1 in the stroma, with a 5-year DSS of 32.9%, against 64.0% (HR: 3.378; CI(95%): 1.816-6.286; p=0.001). The Cox multivariate analysis confirmed that the high expression of FGFR-1 in the tumour (HR: 3.178; CI(95%): 1.505-6.709; p=0.002) and FGFR-1 in the stroma (HR: 3.041; CI(95%): 1.454-6.356; p=0.003) are strongly associated with a higher risk of death related to TSCC, as shown in Table 4.

Discussion:

FGF-2 and FGFR-1 aberrant expression has been associated with different types of malignancies[8,10–12,24] and has been suggested as interesting molecular therapeutic targets. FGF-2 is a potent angiogenic factor and cells transfected with the FGF-2 gene underwent malignant transformation[25]. Moreover, FGF-2 and FGFR-1 inhibitors have been suggested as therapeutic strategies in oral cancer[26,27]. Here, we evaluated the expression of FGF-2 and FGFR-1 in TSCC and OED biopsies.

In patients with oral squamous cell carcinoma (OSCC), FGF-2 and FGFR-1 expression has been correlated with poorer differentiation, higher invasion potential and worse prognosis[3,16–18,28–32]. Indeed, these proteins might influence the epithelial-mesenchymal transition (EMT) in TSCC and OSCC cell lines[4,33]. Furthermore, FGF-2 is overexpressed in the dysplastic epithelium of oral potentially malignant disorders (PMD)[17,34,35] but there are no studies investigating the FGFR-1 expression in OED or in any other oral PMD.

We found higher expression of FGF-2 and FGFR-1 in high grade OED when comparing with the low-grade lesions, which is in agreement with previous studies[17,34], showing higher expression of FGF-2, as the severity of epithelial dysplasias increased,

mainly in the upper layers of the epithelium. FGF-2 expression has also been related with the malignant transformation of oral leucoplakia into OSCC[17]. Our results also suggest that the expression of FGF-2 and FGFR-1 in the stroma cells might be important to understand the behaviour of these lesions, and needs further investigation.

FGF-2 and FGFR-1 were associated with lower DSS both in univariate and multivariate analysis of TSCC cases. Only one previous study correlated FGF-2 expression with a worse prognosis in 61 OSCC patients[18], showing that FGF-2 high expression in the stroma was correlated with the presence of lymph node metastasis and a worse prognosis. However, their parameters to indicate the FGF-2 positivity were merely visual, based on the “presence or absence” of expression. They also found a correlation between FGF-2 expression and lymph node metastasis. Our results indicate that patients with lymph node metastasis present a higher FGF-2 expression, but without statistical significance. However, FGF-2 was significantly correlated with the presence of distant metastasis in our cases, which was not covered by Hase et al.[18].

In previous studies, FGFR-1 expression has been associated with TSCC poor differentiation and metastatic potential[4,16,33]. Our results show a correlation between the expression of FGFR-1 in the stroma cells and the presence of lymph node metastasis and distant metastasis, but there is no association, between its expression in neoplastic cells and other clinical features. However, FGFR-1 expression, either in the tumour or in the stroma, indicated higher risk of death in patients with TSCC. Hase et al.[18] also correlated FGFR-1 expression in fibroblasts of the invasion front with impaired prognosis in OSCC patients. FGFR-1 overexpression was also correlated with poor survival rates in HNSCC patients[19,20], using a semi quantitative analysis of

the immunostaining. Conversely, FGFR-1 amplification has been found in approximately 10-17% of OSCC cases[16,29–31], but no significant correlation with patient outcome has been found[3].

FGF-FGFR axis can promote tumour development and progression by downstream signal pathways, including MAPK/ERK and PI3K/AKT cascades, promoting cancer cells proliferation and survival, besides supporting tumour angiogenesis[4,14,36]. FGFR-1 expression has also been related with epithelial-mesenchymal transition (EMT) in TSCC[4,33], showing higher nuclear polymorphism, more invasiveness, poor histopathological grade and higher metastasis potential. Furthermore, treating the cells with FGFR-1 inhibitors or knocking down its expression made the OSCC proliferate and invade less.

FGF-2 inhibitors have also been used to treat OSCC cells *in vitro* and *in vivo*[27,37]. Furthermore, FGFR inhibitors reduced in vitro growth of head and neck SCC cell lines expressing FGF-2, which is consistent with its autocrine fashion[26]. Hence, FGF/FGFR inhibitors may represent a novel therapeutic modality for oral cancer, with potential as molecular-targeted anticancer drugs for FGF-2/FGFR-1 dependent lesions.

In conclusion, overexpression of FGF-2 and FGFR-1 were correlated with the presence of metastasis and worse outcome of patients with tongue squamous cell carcinoma and might be considered as potential biomarkers to predict the prognosis of TSCC patients.

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Author contributions:

BALAM performed the research and wrote the paper. CDS and JJ designed the research study, analysed the data and reviewed the content of the paper. MGFC provided cases and actively contributed with the revision of the paper.

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Tables:

Table 1: Clinicopathological features of the studied TSCC samples, and five-year disease-specific survival (DSS) rate calculated using Kaplan-Meier and log-rank test.

Parameter	***n***	***5-y DSS (%)***	**HR**	**CI (95%)**	**p value**
Age					
<55	35	49	1		
≥55	50	46	1.08	0.581-2.009	0.8
Gender					
Male	39	50.3	1		
Female	46	43.8	1.274	0.685-2.368	0.43
Tobacco use					
No	31	47.1	1		
Yes	54	47.4	0.896	0.462-1.737	0.73
Alcohol consumption					
No	35	55.1	1		
Yes	50	37.7	1.163	0.625-2.166	0.62
Tumor size (T)*					
T1/T2	44	52.1	1		
T3/T4	41	40.8	1.521	0.812-2.850	0.17
Lymph node metastasis (N)*					
N0	41	54.9	1		
N1/N2	44	37.2	1.664	0.892-3.103	0.1
Distant metastasis (M)*					
M0	64	50	1		
M1	21	32.5	1.174	0.556-2.479	0.65
Stage*					
I/II	36	56.3	1		
III/IV	49	39.1	1.7664	0.950-3.283	0.07
Grade of differentiation*					
Well	25	56.9	1		
Moderate/Poor	60	43.4	1.229	0.621-2.432	0.56
Vascular invasion					
No	48	52.5	1		
Yes	37	42.8	1.179	0.631-2.202	0.6
Neural Invasion					
No	43	55.5	1		
Yes	42	38.1	1.726	0.927- 3.213	0.08

***World Health Organization criteria (2017).

Table 2: Clinical data, FGF-2 and FGFR-1 expression in Oral epithelial dysplasias (OED) and Tongue Squamous cell carcinoma (TSCC) incisional biopsies.

	Incisional biopsies			
	Low-grade OED	High-grade OED	WD TSCC	MD/PD TSCC
Age				
<58 years	10	10	15	7
>58 years	8	8	13	11
Gender				
Female	7	**13**	5	2
Male	11	**5***	23	16
FGF-2 epithelium				
Low	9	7	14	**1**
High	9	11	14	**17***
FGF-2 stroma				
Low	2	3	1	1
High	16	15	27	17
FGFR-1 epithelium				
Low	6	8	10	5
High	12	10	18	13
FGFR-1 stroma				
Low	13	**5**	5	6
High	5	**13***	23	12

WD: Well differentiated; MD: moderately differentiated; PD: poorly differentiated. * $p<0.05$ (chi-square test)

Table 3: Number of TSCC cases with high expression of FGF-2 and FGFR-1 according to clinicopathological data.

	Number and percentage (%) of cases with high protein expression			
	FGF-2 epithelium	FGF-2 stroma	FGFR-1 epithelium	FGFR-1 stroma
Tumour size				
T1/T2	**15 (34.1)***	20 (44.5)	22 (50.0)	23 (52.3)
T3/T4	**24 (58.5)***	22 (53.7)	20 (48.8)	19 (46.3)
Lymph node metastasis				
No	17 (41.5)	21 (51.2)	22 (53.7)	**25 (61.0)***
Yes	22 (50.0)	21 (47.7)	20 (45.5)	**17 (38.6)***
Distant metastasis				
No	**24 (37.5)***	32 (50.0)	33 (51.6)	**36 (56.3)***
Yes	**15 (71.4)***	10 (47.6)	9 (42.9)	**6 (28.6)***
Stage				
I/II	14 (38.9)	18 (50.0)	20 (55.5)	21 (58.3)
III/IV	25 (51.0)	24 (49.0)	22 (44.9)	21 (43.9)
Vascular invasion				
No	21 (50.0)	**8 (19.0)***	19 (45.2)	20 (47.6)
Yes	18 (41.9)	**34 (79.0)***	23 (53.5)	22 (51.2)
Neural invasion				
No	18 (48.6)	14 (37.9)	15 (40.5)	16 (43.2)
Yes	21 (43.8)	28 (58.3)	27 (56.2)	26 (54.2)

* $p<0.05$ (chi-square test)

Table 4: Association between FGF-2 and FGFR-1 expression and Disease-specific survival (DSS) in the univariate analysis and Cox regression multivariate analysis.

	5-y DSS (%)	**Univariate**			**Multivariate**		
		HR	CI (95%)	p value	HR	CI (95%)	p-value
Stage*							
I/II	56.3	1			1		
III/IV	39.1	1.7664	0.950-3.283	0.07	2.9989	1.475-6.097	**0.002**
FGF-2 expression (tumor)							
Low	51.2	1					
High	43.1	1.279	0.645-2.259	0.544	-	-	-
FGF-2 expression (stroma)							
Low	59.3	1			1		
High	36.7	2.272	1.213-4.254	**0.008**	2.197	1.128-4.282	**0.02**
FGFR-1 expression (tumor)							
Low	75.6	1			1		
High	22.9	2.594	1.390-4.841	**0.003**	3.178	1.505-6.709	**0.002**
FGFR-1 expression (stroma)							
Low	64	1			1		
High	32.9	3.378	1.816-6.286	**0.001**	3.041	1.454-6.356	**0.003**

*Tumour Stage was the only clinicopathological variable used in the multivariate analysis.

Figure legends:

Figure 1: Histopathological and graphical images of FGF-2 expression and its impact on DSS rate. (A) Low-grade oral epithelial dysplasia (200X); (B) high-grade epithelial dysplasia (200X); (C) TSCC, well differentiated (200X); (D) TSCC, poorly differentiated (200X); (E) FGF-2 expression in the epithelium; (F) FGF-2 expression in the stroma; DSS plots based on the expression of FGF-2 in the epithelium (G) and in the stroma (H).

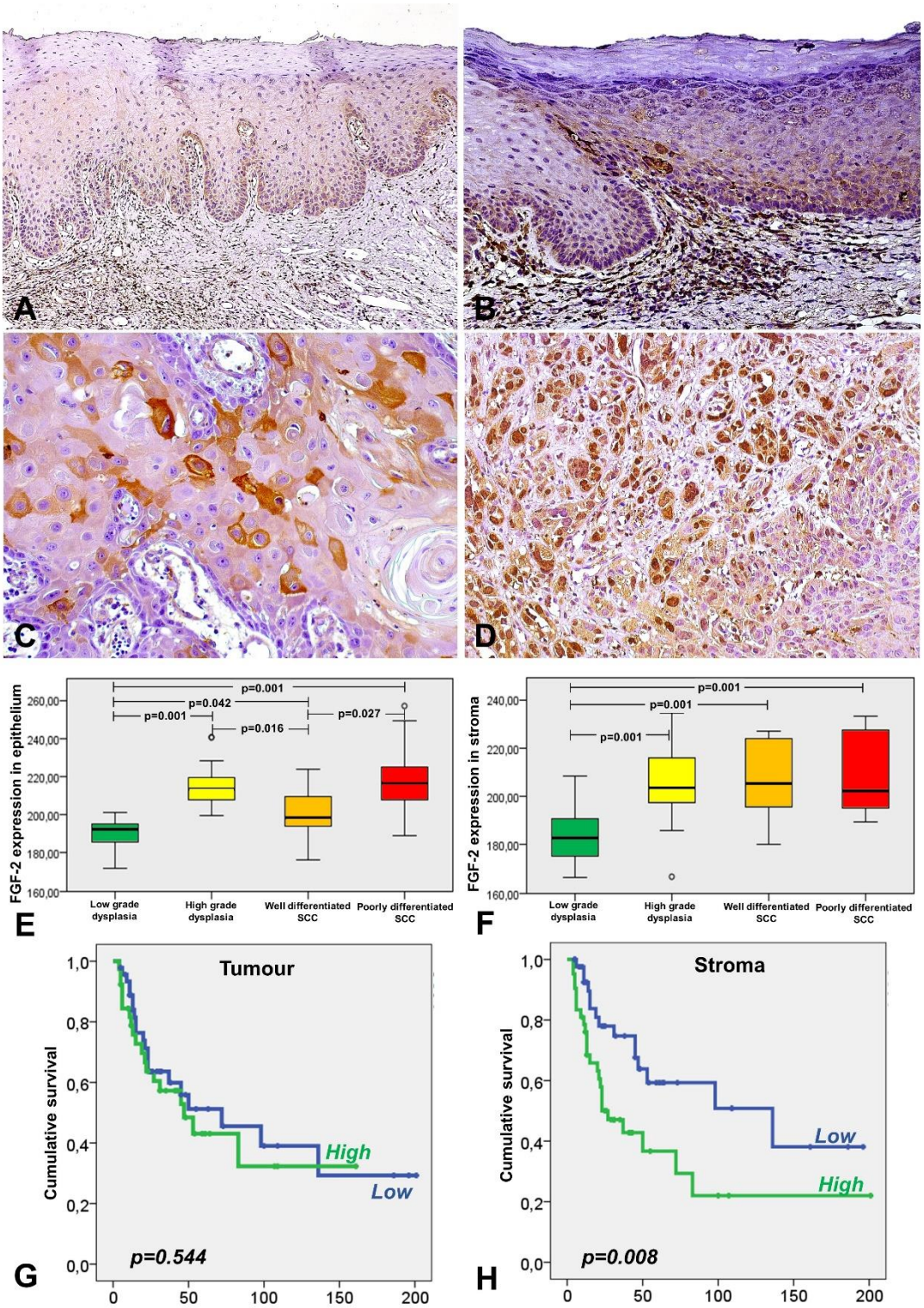
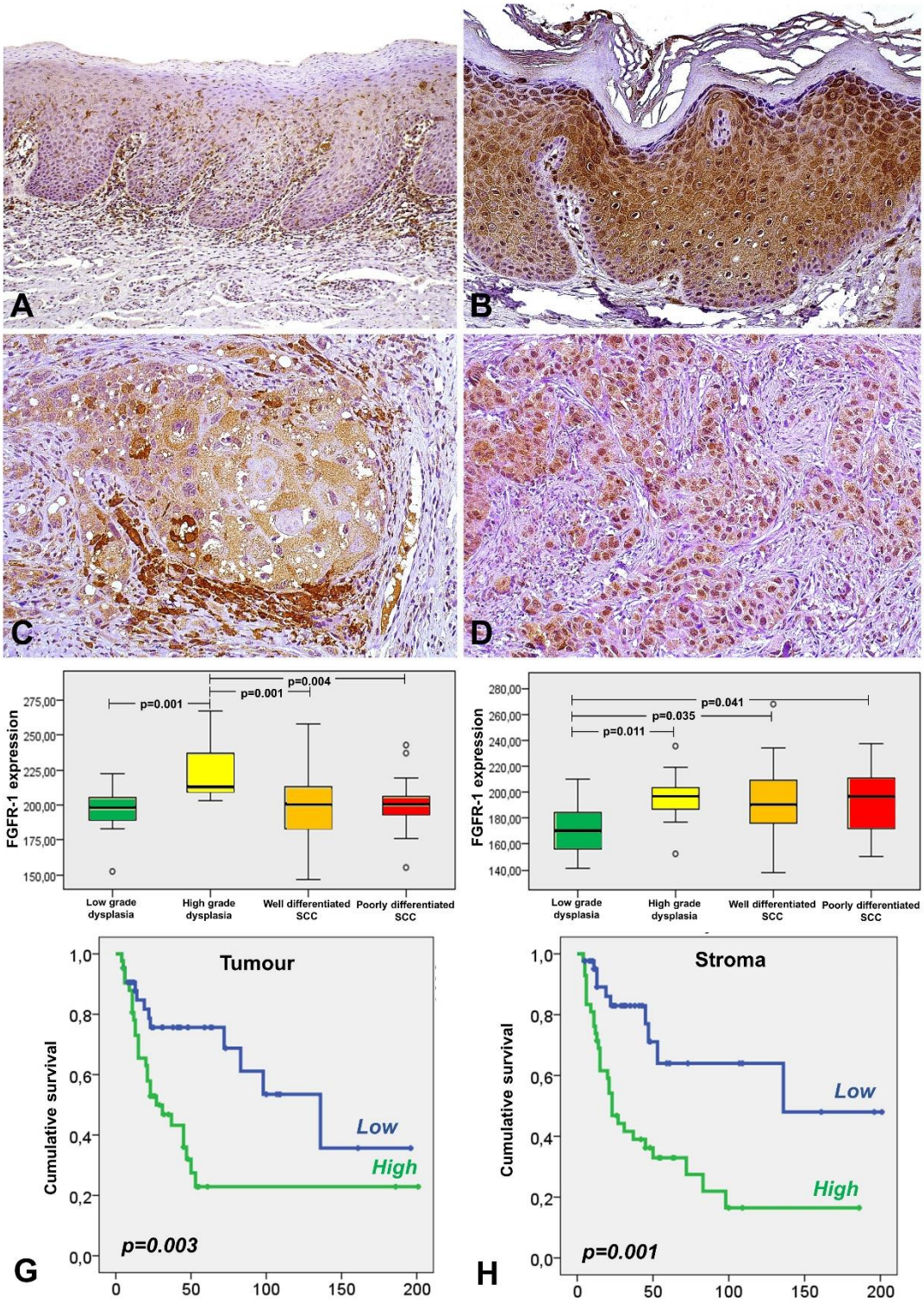


Figure 2: Histopathological and graphical images of FGFR-1 expression and its impact on DSS rate. (A) Low-grade oral epithelial dysplasia (200X); (B) high-grade epithelial dysplasia (200X). (C) TSCC, well differentiated (200X); (D) TSCC, poorly differentiated (200X); (E) FGFR-1 expression in the epithelium; (F) FGFR-1 expression in the stroma; DSS plots based on the expression of FGFR-1 in the epithelium (G) and in the stroma (H).



3 CONCLUSÃO:

A expressão de FGF-2 e FGFR-1 aumenta de acordo com o grau de displasia epitelial oral.

Carcinoma Espinocelular de língua com superexpressão de FGF-2 e FGFR-1 apresentam pior prognóstico, com maior risco de morte dos pacientes.

O uso dos marcadores FGF-2 e FGFR-1 parece ser útil como marcador de prognóstico de lesões de Carcinoma Espinocelular de língua, potencialmente determinando formas de tratamento individualizadas de acordo com as características clínicas e biológicas de cada tumor. Esse potencial ainda necessita confirmação por meio de estudos futuros.

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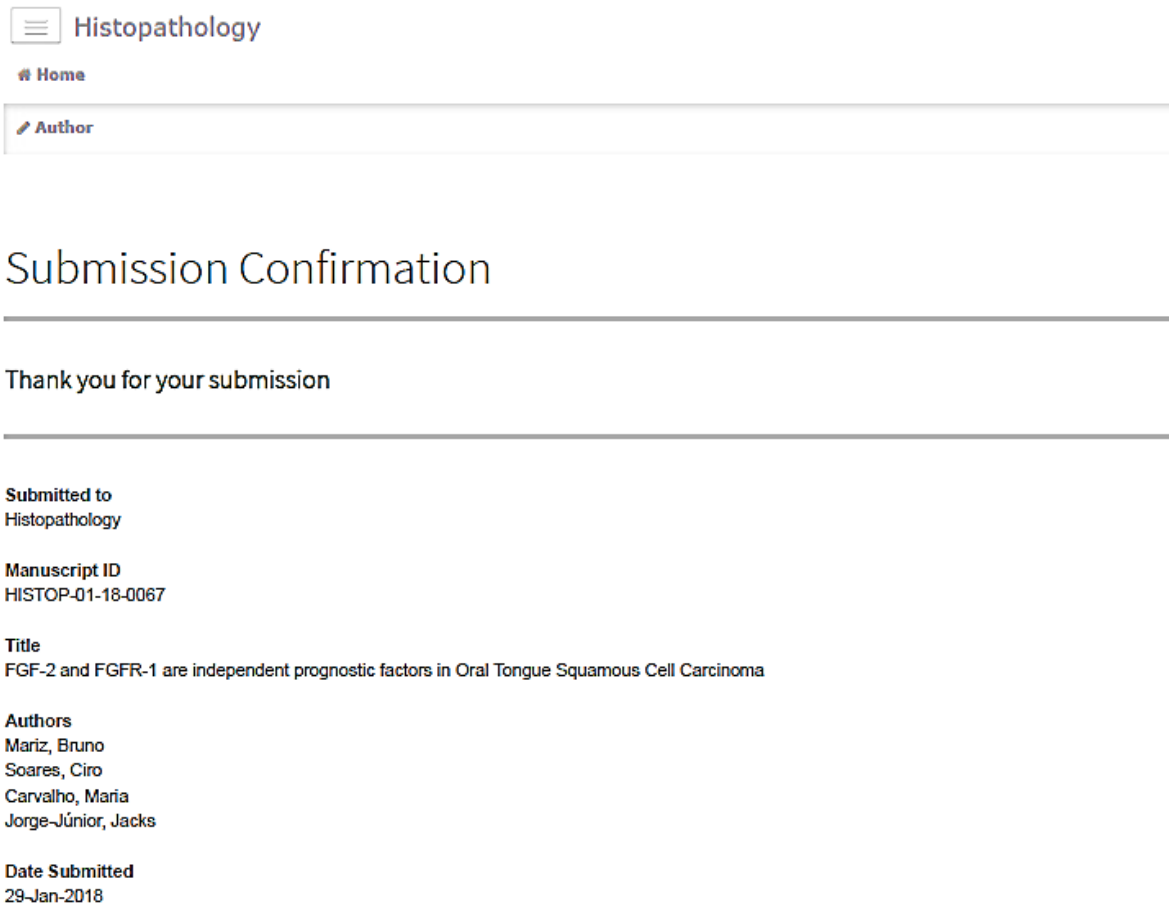
ANEXO 1:



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Date Submitted
29-Jan-2018

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Valor prognóstico da expressão do fator de crescimento fibroblástico 2 (FGF-2) e do receptor do fator de crescimento fibroblástico 1 (FGFR-1) em carcinomas espinocelulares orais**", CAAE **69181417.9.0000.5418**, dos pesquisadores **Bruno Augusto Linhares Almeida Mariz, Ciro Dantas Soares** e **Jacks Jorge Júnior**, satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado por este comitê em 05/07/2017.

The Research Ethics Committee of the School of Dentistry of Piracicaba of the University of Campinas (FOP-UNICAMP) certifies that research project "**Prognostic value of fibroblast growth factor 2 (FGF-2) and fibroblast growth factor receptor 1 (FGFR-1) expression in oral squamous cell carcinomas**", CAAE **69181417.9.0000.5418**, of the researcher's **Bruno Augusto Linhares Almeida Mariz, Ciro Dantas Soares** and **Jacks Jorge Júnior**, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health, and was approved by this committee on fifth of July of 2017.

Profa. Fernanda Miori Pascon

Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior

Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.