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Dissertação de Mestrado

Carcinoma espinocelular : características clínicas intra-orais e demográficas em uma população do Sul do Brasil e potenciais interações com as células endoteliais linfáticas.

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Pelotas, março de 2013.

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Epígrafe:

“E me fala de coisas bonitas
Que eu acredito
Que não deixarão de existir
Amizade, palavra, respeito
Caráter, bondade, alegria e amor
Pois não posso
Não devo
Não quero
Viver como toda essa gente
Insiste em viver
E não posso aceitar sossegado
Qualquer sacanagem ser coisa normal”

(Milton Nascimento, *Bola de meia, bola de gude)*

RESUMO

ALVES, Alessandro Menna. **Carcinoma espinocelular: características clínicas e demográficas intraorais em uma população do Sul do Brasil e potenciais de interação com as células endoteliais linfáticas.** 2013. 62f. Dissertação de Mestrado. Programa de Pós-Graduação em Odontologia, Universidade Federal de Pelotas, Pelotas

Esta dissertação foi dividida em dois trabalhos distintos, os quais podem ser resumidos da seguinte maneira:

Artigo 1: O carcinoma espinocelular oral (CEO) é o tumor maligno mais prevalente na cavidade oral, sendo um importante problema de saúde pública. O objetivo deste estudo foi avaliar o perfil clínico e epidemiológico dos casos registrados de CEO em um centro de diagnóstico clínico e histopatológico, localizado no Sul do Brasil. Oitocentos e seis indivíduos com CEO e suas variantes histológicas foram incluídos neste estudo, num período entre 1959 e 2012. As variáveis anotadas dos arquivos foram: idade, sexo, cor da pele, sítio, tamanho, tempo de evolução (relatado pelo paciente), assim como a presença de dor, linfonodos palpáveis, hábitos de tabagismo e etilismo, e a profissão. CEO foi mais prevalente em homens (76,6%), com a maioria dos casos distribuídos entre os 51 e 70 anos de idade (53,9%). Os sítios mais prevalentes foram vermelhão do lábio inferior [23,3% (20,4; 26,4)], seguido por borda lateral/ventre de língua [20,2% (17,5; 23,2)], gengiva/rebordo alveolar [18,1% (15,5; 21,0)], e assoalho bucal [14,9% (12,5; 17,5)]. Foi encontrada uma forte associação entre ocupações ao ar livre e CEO de vermelhão de lábio inferior. As lesões localizadas na língua, gengiva/rebordo alveolar e assoalho bucal foram comumente mais dolorosas, maiores que 2 cm, a frequentemente apresentavam envolvimento de linfonodos. A maioria dos nossos resultados confirmam os dados da literatura. O autoexame bucal deveria ser recomendado e campanhas de prevenção e detecção precoce do CEO deveriam ser realizadas periodicamente na tentativa de aumentar o sentimento pessoal em relação ao CEO.

Artigo 2: Dentro do microambiente tumoral (MT), as células neoplásicas estão numa constante *crosstalk* com células endoteliais linfáticas (CELs) e sanguíneas a fim de permitir o crescimento tumoral e metástase. Supondo que haja um *crosstalk* entre as CELs e as células do carcinoma espinocelular (CE) que exerce um importante papel na metástase, nosso objetivo foi identificar potenciais interações entre as CELs e linhagens celulares de CE, através de alguns ensaios *in vitro*. Células endoteliais linfáticas primárias adultas humanas da microvasculatura dérmica (HMVECs) e as linhagens de CE A431, UM-SCC-1, UM-SCC-22A e UM-SCC-22B foram cultivadas nos seus meios específicos. UM-SCC foram tratadas com rhIL-6, sendo a expressão de VEGF-C verificada por Elisa. Produção natural de IL-6 pelas HMVECs foi avaliada da mesma maneira. A presença de receptor de IL-6 (IL-6R) foi analisada por Western Blot nas linhagens UM-SCC. Meios condicionados(MC) das HMVECs foram preparados com diferentes tratamentos e incubados com a linhagem A431, a fim de verificar a atividade genatinolítica das MMPs por zimografia. Nossos resultados demonstraram que há interações entre as células tumorais e as CELs, uma vez que MC-CELS foram capazes de aumentar a atividade genatinolítica da MMP-2. Além disso, nós mostramos que as CELs secretam IL-6, e diferentes linhagens de CE possuem receptores para esta citocina. Sendo assim, nossos resultados indicam potenciais interações entre as LECs e as células tumorais, sendo necessário outros estudos para elucidar as vias de sinalização envolvidas.

Palavras-chave: Carcinoma espinocelular. Estudo epidemiológico. Célula endotelial linfática. Célula tumoral. Metaloproteinase da matriz. IL-6. VEGF-C.

ABSTRACT

ALVES, Alessandro Menna. **Squamous cell carcinoma: clinical intraoral and demographics characteristics in a Southern Brazil population and potential interactions with lymphatic endothelial cells.** 2013. 62f. Dissertação de Mestrado. Programa de Pós-Graduação em Odontologia, Universidade Federal de Pelotas, Pelotas.

This dissertation was divided into two distinct works, which can be summarized as follows:

Article 1: The oral squamous cell carcinoma (OSCC) is the most prevalent malignance in mouth, being an important public health problem. The aim of this study was to evaluate the clinical and epidemiological profile of the OSCC cases registered in a center of clinical and histopathological diagnosis, located in Southern Brazil. Eight hundred and six individuals with OSCC and its variants were included in this study, over 1959-2012 period. The variables recorded from the files were: age, gender, skin color, tumor location, size and evolution time of the lesions (referred by the patients), as well as, the presence of pain lymph nodes, habits of tobacco and alcohol, and also the profession. OSSC was more frequent in males (76.6%), with the majority of cases distributed between 51 and 70 years old (53.9%). The most prevalent sites were lower lip vermilion [23.3% (20.4; 26.4)], followed by lateral border/ventral surface of the tongue [20.2% (17.5; 23.2)], gingiva/alveolar ridge [18.1% (15.5; 21.0)], and floor of the mouth [14.9% (12.5; 17.5)]. A strong association between outdoor occupation and OSCC in lower lip vermilion was found. The OSCC lesions located in tongue, gingiva/alveolar ridge and floor of the mouth were commonly more painful, bigger than 2 cm, and frequently presenting lymph nodes involvement. Most of the results confirm the data from literature. Mouth self-examination should be recommended and campaigns of prevention and early detection of OSCC should be periodically performed in order to increase people's feelings of personal risk.

Article 2: Inside the tumor microenvironment (TM) the neoplastic cells are in dynamic crosstalk with the vascular and lymphatic endothelial cells in order to allow the tumor to growth and metastasize. Hypothesizing that there is a crosstalk between lymphatic endothelial cells and tumor cells from squamous cell carcinoma (SCC) that plays an

important role in metastasis, we aimed to identify potential interactions between lymphatic endothelial cells and tumor cells lines from SCCs, through some in vitro assays. Primary adult human dermal lymphatic microvascular endothelial cells (HMVECs) and the human head and neck SCC cell lines like A431, UM-SCC-1, UM-SCC-22A and UM-SCC-22B were cultured in their specific media. UM-SCC cells lines were treated with rhIL-6, being VEGF-C expression checked by Elisa. Baseline IL-6 was evaluated in HMEVCs using the same assay. Also the IL-6 receptor (IL-6R) was analyzed by Western blot in UM-SCC cells. Conditioned media from HMVECs were prepared with different treatments and incubated with SCC A431 cells, in order to verify the MMPs enzymatic activities by gelatin zymography. Our results demonstrated that there are interactions between tumor cells and LECs, since the LECs-CM were able to enhance MMP-2 gelatinolytic activity. Moreover, we showed that LECs secrete IL-6, and different SCC lines have receptors for this cytokine. Therefore, our results indicate some potential interactions between LECs and TCs, being necessary other studies to elucidate the involved signaling pathways.

Keywords: Squamos cell carcinoma. Epidemiological study. Lymphatic endotelial cell. Tumor cell. Matrix metalloproteinases. IL-6. VEGF-C.

SUMÁRIO

1 Introdução

O câncer de boca corresponde à aproximadamente 5% de todos os tumores malignos do corpo (GILLISON, 2007), embora haja uma ampla variação geográfica na incidência deste câncer (WARNAKULASURIYA, 2010). O câncer de boca apresenta alta incidência em diferentes partes do globo, como Sri Lanka, Paquistão, Taiwan, França, Leste Europeu, Rússia, EUA, Uruguai, Porto Rico, Brasil, Cuba, sendo que na Índia e em algumas ilhas da Melanésia é o tipo de malignidade mais comum. (WARNAKULASURIYA, 2010), (RASTOGI et al., 2008), (JOHNSON; JAYASEKARA; AMARASINGHE, 2011). No Brasil, o câncer oral é a sexta neoplasia mais comum (INCA, 2010). O carcinoma espinocelular, também chamado de carcinoma de células escamosas ou epidermóide, é o tipo mais prevalente (94% dos tumores orais malignos), e ocorre, normalmente, em indivíduos acima dos 45 anos de idade. Embora várias pesquisas tenham sido realizadas sobre o carcinoma espinocelular, em torno de 50% dos pacientes morrem em 5 anos.

Pesquisas sobre a história natural dessa doença, particularmente os quais avaliam a progressão das lesões potencialmente malignas ao longo do tempo, ajudariam a desenvolver programas de rastreamento mais adequados às desordens individuais. Desenvolvimento de marcadores tumorais com alta sensibilidade e especificidade poderiam auxiliar a detecção de pacientes e lesões de risco (WARNAKULASURIYA, 2009).

Vários processos biológicos estão envolvidos no desenvolvimento do câncer, os quais levam à proliferação das células neoplásicas, invasão de tecidos vizinhos e vasos sanguíneos e linfáticos, e, consequentemente, metástases loco regionais e à distância (TSANTOULIS et al., 2007). Recentemente, houve descobertas de que estes eventos envolvem mecanismos de *crosstalk* entre as populações celulares (células tumorais, endoteliais, entre outras) do microambiente tumoral e entre estas células e a matriz extracelular (GOMES et al., 2013; ISSA et al., 2009; KANEKO et al., 2007; NEIVA et al., 2009). Anteriormente, se pensava que a metástase linfática envolvia apenas a passagem das células malignas para o interior de vasos linfáticos

pré-existentes. Entretanto, atualmente é sabido que a metástase tumoral é um mecanismo complexo constituído por múltiplas etapas (YADAV et al., 2011) e estudos recentes sugerem que a linfangiogênese pode ser induzida pelas células tumorais e promover disseminação tumoral (BENKE et al., 2010; SIRIWARDENA et al., 2008). Neste contexto, citocinas, quimiocinas e fatores de crescimento presentes no microambiente tumoral, podem regular a angiogênese/linfangiogênese e também a produção de metaloproteinases da matriz, os quais são eventos chaves para o crescimento tumoral e disseminação metastática (HANAHAN; WEINBERG, 2011).

Sendo assim, a presente dissertação foi dividida em dois artigos, sendo que o primeiro aborda as características clínicas e demográficas de pacientes com carcinoma espinocelular oral, e o segundo artigo aborda, em alguns ensaios *in vitro*, possíveis interações entre dois tipos celulares presentes no microambiente tumoral: as células tumorais e as células endoteliais linfáticas.

2 Projeto de Pesquisa

O câncer, em âmbito mundial, é uma das maiores causas de morte contabilizando um total aproximado de 7,6 milhões (13%) de óbitos no ano de 2008. (GLOBOCAN, 2008). Para o Brasil, foi estimado um total de 489.270 novos casos de câncer em 2010, sendo 236.240 (aproximadamente 48%) e 253.030 (aproximadamente 52%) para homens e mulheres, respectivamente. Com relação ao câncer oral, 10.330 (4,37%) de novos casos dessa forma de neoplasia foram estimados para homens e 3.790 (1,5%) para mulheres (Estimativas INCA, 2010). O câncer é uma doença com importante componente genético, em que, a partir de alterações, oriundas de heranças familiais ou de fatores externos, no genoma de um indivíduo pode levá-lo a desenvolver uma condição cancerosa (TSANTOULIS et al., 2007).

A disseminação metastática de células tumorais é a causa básica de muitas mortes relacionadas ao câncer (SU et al., 2007) e, apesar dos inúmeros estudos nesta área, o índice de sobrevida 5 anos não tem aumentado muito nas últimas décadas, permanecendo em torno de 53% (SMITH et al., 2000). A disseminação de células cancerígenas em sítios distantes ocorre através de vasos sanguíneos e linfáticos (HE et al., 2005), onde estes últimos seriam uma das principais rotas da metástase para os nódulos linfáticos (TOBLER & DETMAR, 2006), a qual é a via de eleição para os diversos tipos de carcinomas, incluindo o Carcinoma Espinocelular (CEC) de boca.

Neste aspecto, a angiogênese exerce papel fundamental, não apenas no crescimento e aporte sanguíneo neoplásico, mas também na metástase tumoral, e, mesmo sendo um processo natural do corpo humano servindo a diversas finalidades, acaba sendo utilizada também pelo tumor no seu desenvolvimento (FOLKMAN et al., 1971; HANAHAN & FOLKMAN, 1996).

O tipo de vaso que terá maior atividade proliferativa estimulada pelo tumor pode ditar, então, a forma de migração, hematogênica ou linfática (MAREEL & LEROY, 2003). Vários estudos já demonstraram que uma variedade de tumores humanos tem capacidade de expressar membros da família do VEGF. A descoberta dos

fatores linfangiogênicos (VEGF-C e VEGF-D) permitiu expandir os conhecimentos a respeito da biologia linfática, tanto fisiológica como patológica. Tem sido observada uma correlação estatística positiva entre a expressão de fatores linfangiogênicos e a habilidade metastática do tumor sólido primário (STACKER et al., 2002). Para a angiogênese, é necessária uma maior quantidade de VEGF-A, principalmente, secretada pelas células tumorais, sendo que o VEGF-A também é secretado por células da linhagem macrofágica e células mesenquimais (DONG et al., 2004; LIANG et al., 2006). Na migração linfática associada à disseminação tumoral, o sistema molecular mais extensivamente estudado é a via de sinalização que envolve o eixo VEGF-C/VEGF-D/ receptor VEGFR3 (ACHEN & STACKER, 2008). Dados mostram que o receptor VEGFR-2 também está presente em vasos linfáticos, sugerindo sua possível atividade regulatória na linfangiogênese (ACHEN et al., 2002; HIRAKAWA et al., 2005; WHITEHURST et al., 2006; ACHEN & STACKER, 2008), através da ligação com VEGF-C, o que, até certo ponto, implica também em uma certa atividade angiogênica do mesmo sob determinadas condições (WHITEHURST et al., 2006).

Grande ênfase tem sido dada a premissa de que os sinais iniciais provenientes das células tumorais são o evento predominante na linfangiogênese e na metástase. Entretanto, pouco se sabe a respeito da influência das células constituintes dos vasos linfáticos e do microambiente neoplásico sobre as células tumorais. O chamado *crosstalk* entre esses tipos celulares é um assunto que vem sendo abordado com mais freqüência atualmente (KANEKO et al., 2007; ISSA et al., 2009; NEIVA et al., 2009). Kaneko et al. (2007) verificaram uma sinalização orquestrada por Bcl-2, através do fator ativador de transcrição STAT3, onde a célula endotelial vascular secreta VEGF-A para a célula tumoral. Através desse estímulo inicial, a célula neoplásica, após uma reação em cascata, aumenta a expressão de Bcl-2 para si própria, bem como a expressão de CXCL8 e CXCL1, citocinas pró-angiogênicas, encontra-se elevada.

Recentemente um estudo conduzido por Neiva et al. (2009) revelou uma via de sinalização iniciada por células endoteliais sanguíneas que resultava na ativação de STAT3, Akt, e ERK em carcinomas espinocelulares de cabeça e pescoço (HNSCCs). O bloqueio desta sinalização inicial proveniente das células endoteliais

causou um impacto direto sobre a sobrevivência e migração das células tumorais (NEIVA et al., 2009).

As células endoteliais linfáticas, no entanto, são distintas biológica, funcional e estruturalmente das suas correspondentes sanguíneas, bem como no que diz respeito ao seu repertório protéico. Estas células endoteliais linfáticas podem desempenhar um importante papel na patogênese do câncer, modificando o microambiente tumoral (ISSA et al. 2009).

Assim, acredita-se que ocorra um preparo do microambiente linfático e linfonodal para que as células tumorais epiteliais sejam atraídas no movimento metastático, através da ação de quimiocinas diversas (PSAILA et al., 2007; ACHEN & STACKER, 2008; ISSA et al., 2009). As quimiocinas (citocinas quimiotáticas) compõem uma família de mais de 40 membros que se ligam a um receptor associado à proteína G nas células-alvo (ACHEN & STACKER, 2008). As quimiocinas são subdivididas em quatro grupos, denominados CC, CXC, C e CX3C, baseando-se na forma com que as suas primeiras duas cisteínas conservadas são arranjadas, embora a maioria das quimiocinas conhecidas pertença às famílias CC e CXC (COSCIA & BIRAGYN, 2004).

No que diz respeito às Metaloproteinases da Matriz Extracelular, as MMPs, elas possuem papel fundamental no processo de invasão dos tecidos vizinhos e metástase dos tumores epiteliais (VERSTAPPEN et al, 2006; WERNER, RATHCKE & MANDIC, 2002). Diversos trabalhos na literatura tem mostrado que há uma interação entre as células tumorais e as células presentes na matriz extracelular e entre o tumor e a própria matriz extracelular. Yang et al. (2010) demonstraram que as células tumorais ovarianas, induzidas por fatores secretados pelo endotélio linfático (provavelmente por ação de algumas citocinas) poderiam ter seus níveis de MMP-9 aumentados e do inibidor da MMP-2 (TIMP-2) reduzidos. Além disso, Zhou et al. (2010) encontraram uma expressão mais elevada das MMPs -2 e -9 em carcinomas de língua com metástase linfonodal, sugerindo que essas duas MMPs, especificamente, possuem um importante papel na progressão e metástase linfonodal nos carcinomas espinocelulares da região de cabeça e pescoço.

Seguindo a linha de investigação que procura identificar a existência de um mecanismo de *crosstalk* entre as células endoteliais linfáticas e as células tumorais

no processo de metástase, Issa et al. (2009) demonstraram que as mesmas interagem entre si, e que, após serem estimuladas por VEGF-C, as células endoteliais linfáticas secretam uma quimiocina denominada CCL21. Esta liga-se ao receptor CCR7, o qual está presente na célula maligna. Com isso, ocorre uma quimioatração das células neoplásicas em direção aos vasos linfáticos, aumentando o potencial de migração e a atividade proteolítica por parte das células tumorais, devido ao próprio VEGF-C de forma autócrina, em uma matriz de três dimensões. É interessante notar que CCL21 possui também uma atividade antitumoral, observada por Sharma et al. (2003), devido à sua capacidade de atrair linfócitos T e células dendríticas (AKNIN et al., 2009). Segundo Sharma et al. (2003) os linfócitos T recrutados começam a produzir lNFɣ, o qual induz a produção de outras duas citocinas angiostáticas chamadas CXCL10 e CXCL9, sendo que possivelmente o efeito antitumoral dessas três citocinas justifique a atividade terapêutica de CCL21.

A citocina IL-6, com importante papel no processo inflamatório agudo, tem também expressão aumentada em muitos tipos de tumores malignos (TANG et al., 2005; YAN et al., 2006). Tang et al. (2005), estudando tumores pancreáticos, observaram que as interleucinas IL-1α e lL-6 secretadas pelas células inflamatórias e tumorais estimulavam a produção de VEGF-A e VEGF-C.

Assim, a metástase tumoral ocorre em uma sequência de eventos que consiste da proliferação neoplásica no tumor primário, da invasão de suas células tumorais aos tecidos vizinhos e vasos, da migração destas células nos vasos e, subseqüentemente, da adesão neoplásica aos órgãos alvo. É possível que algumas dessas etapas da metástase, incluindo proliferação, invasão e migração celulares, possam ser reguladas por quimiocinas e seus receptores. Acredita-se que a partir de uma melhor compreensão da ação destas quimiocinas nas células tumorais do CEC e no seu microambiente será possível o desenvolvimento de terapias antitumorais baseadas em estratégias que visem bloquear a interação entre tais citocinas e seus respectivos receptores.

2.1. Objetivos:

Objetivo Geral:

O objetivo geral deste projeto é avaliar em um estudo *in vitro* se algumas citocinas presentes no microambiente tumoral são expressas pelas células endoteliais linfáticas e participam do crosstalk entre estas células e as células tumorais de CECs.

Objetivos específicos:

- Avaliar se o VEGF-C produzido e secretado *in vitro* pelas células tumorais de CECs induz a expressão das citocinas CCL21 e IL-6 nas células endoteliais linfáticas.

- Avaliar se as citocinas com ação quimiotática CCL21 e IL-6 são expressas pelas células endoteliais linfáticas e tem a capacidade de estimular a expressão de VEGF-C, MMP-2 e MMP-9 *in vitro* nas células tumorais de CECs, aumentando o seu potencial metastático.

2.2 Materiais e métodos:

Cultivo Celular. Células endoteliais linfáticas adultas da microvasculatura dérmica humana (*Adult human dermal lymphatic microvascular endothelial cells – HMVECs, Lonza*) serão cultivadas a 37°C e 5% de CO2, em meio EGM-2 MV (Lonza), suplementado de acordo com as instruções do fabricante, sendo semeadas em placas de cultivo de 60mm ($4.5x10^5$). Será cultivada uma linhagem de célula tumoral proveniente de CEC de assoalho bucal com metástase linfonodal (linhagem H376, Banco de Células Europeu/ European Collection of Cell Culture - ECACC). Essa linhagem de células neoplásicas será cultivada em Meio de Eagle modificado por Dulbecco (DMEM, Gibco, Invitrogen, Carlsbad, CA), suplementado com 10% de soro fetal bovino (FBS), 100U/ml de penicilina e 100 mg/ml de estreptomicina, sendo as células mantidas a 37°C e 5% de CO2. As células tumorais serão plaqueadas em placas de cultivo de 60 mm ($1x10^6$).

Expressão protéica e Western blotting. Inicialmente, os níveis basais de VEGF-C e VEGFR3 serão obtidos de lisados protéicos da linhagem de célula tumoral e das HMVECs, utilizando, para ambos, anticorpos policlonais anti-humanos (Santa Cruz Biotechnology Inc., Holly Ditch Farm, UK). Também os níveis basais de IL-6 e CCL21 serão analisados nas HMVECs e H376, utilizando os anticorpos anti-

humanos anti-IL-6 (Santa Cruz Biotechnology Inc., Holly Ditch Farm, UK) e anti-CCL21 (R & D Systems, Minneapolis, USA).

Para avaliar o papel de VEGF-C na indução da expressão protéica de CCL21 e IL-6 nas HMVECs, serão avaliados os níveis protéicos dessas citocinas nestas células endoteliais através da técnica de Western Blot. As mesmas serão deixadas em meio sem soro *overnight* e tratadas com rhVEGF-C (R & D Systems, Minneapolis, USA) por 24 horas em um experimento dose-resposta, utilizando as concentrações de 1, 10, 50 e 100ng/ml. As HMVECs serão também expostas ao meio condicionado e coletado das linhagens celulares tumorais em situação de subconfluência, cultivadas por 24h em DMEM sem soro.

Para analisar o efeito das citocinas produzidas pela HMEVCs nas células tumorais e sua possível relação com o potencial metastático neoplásico, será verificada a expressão de VEGF-C, MMP-2 e MMP-9 nestas últimas, através da análise de seus respectivos *immunoblots*. Para tal, as linhagens de células tumorais, após serem deixadas em condições de privação de nutrição *overnight*, serão tratadas com rhIL-6 (1, 10, 50 e 100ng/ml) e rhCCL21 (50, 100, 200 e 350ng/ml), sendo todas as proteínas recombinantes humanas obtidas da R & D Systems (Minneapolis, USA). Além dos experimentos dose-resposta, serão também realizados experimentos tempo-dependente, avaliando a expressão protéica após tratamentos de 12, 24 e 36 horas de duração. A linhagem H376 será ainda tratada com meio condicionado e coletado das HMVECs em EGM sem suplementação, em situação de subconfluência e cultivo por 24h, sendo também avaliadas as expressões de VEGF-C, MMP-2 e MMP-9 nessas células tumorais.

Zimografia. Para avaliar o efeito das citocinas de CCL21 e IL-6 na atividade da MMP-2 e MMP-9 da linhagem celular tumoral de CEC o processamento será feito de forma semelhante à relatada para o ensaio de Western blotting. Inicialmente, as células tumorais serão plaqueadas em uma concentração de 1×10^6 em placas de cultivo de 60mm. As células serão incubadas em estufa de CO_2 por 24h com DMEM e 10% de soro fetal bovino. No dia seguinte, as referidas citocinas (rhCCL21 e rhIL-6) serão incubadas em contato com as células por 12, 24 e 36 h. Após estes períodos, o meio de incubação será trocado por DMEM sem soro. Então, as células ficarão com DMEM sem soro por mais 24h. Finalmente, o meio de cultivo

condicionado pelas células será separado, adicionado de 1% de inibidor de protease e congelado em *freezer* – 80ºC, até a realização da zimografia, para conservação das metaloproteinases 2 e 9. A atividade proteolítica será examinada em gel de poliacrilamida a 10% contendo 0,05% de gelatina. O meio condicionado pelas MMPs será misturado ao tampão de amostra (2% SDS; 125 mM Tris-HCl (pH 6,8); 10% de glicerol e 0,001% de azul de bromofenol) e então a eletroforese será realizada. Após a eletroforese, o gel será lavado duas vezes em Triton X-100 (2%) por 60 min a temperatura ambiente e então incubado a 37ºC por 24 h em tampão 50 mM Tris-HCl (pH 7,4) contendo 5 mM $CaCl_2$ (Tris-$CaCl_2$). Após a incubação, os géis serão corados com Azul de Coomassie G-250 a 0.05% (Bio Rad, Richmond, CA). A atividade gelatinolítica será detectada como bandas não coradas no gel que ficará corado em azul. Para quantificar a inibição relativa as bandas serão digitalizadas e as suas transmitâncias, serão analisadas com o programa Image J (NIH, Bethesda, MD, USA). Para cada grupo tratado, será usado como controle negativo amostras não tratadas, já o controle positivo será feito através de saliva. A intensidade de transmitância das bandas serão quantificadas em pixels, na escala de cinza, com o auxílio do programa Image J (NIH, Bethesda, MD, USA). O programa que será usado permite igualar o *background*, e deste modo, somente as bandas são analisadas. Os dados obtidos serão transformados em valores numéricos. Cada valor encontrado será dividido pelo valor do respectivo controle, obtendo-se assim, percentuais.

3 Relatório do trabalho de campo

Após a submissão à qualificação do projeto de dissertação de mestrado, levando-se em consideração o parecer exarado pela Comissão de Avaliação, o projeto intitulado “Crosstalk entre células endoteliais linfáticas e células tumorais do carcinoma espinocelular bucal e o papel das citocinas CCL21 e IL-6 na metástase”, foi submetido às seguintes alterações:

- contextualização do carcinoma espinocelular de boca enfocando os seus aspectos epidemiológicos e clínicos;

- em função de intercorrências no processo de importação de alguns reagentes e linhagens celulares, assim como algumas dificuldades no estabelecimento de metodologias e cultivo das células HMVECs, a verificação dos níveis basais de VEGFR3 e CCL21 das HMVECs e CCL21 e IL-6 nas linhagens tumorais, até o fechamento desta dissertação, ainda não haviam sido avaliados;

- A estimulação das HMVECs com o rhVEGF-C para o experimento dose resposta foi realizado, no entanto, devido à problemas na otimização da técnica laboratorial, os dados não foram incluídos na dissertação;

- Foi realizada a estimulação da linhagem tumoral por meio condicionado provenientes das HMVECs, tratados ou não com VEGF-C;

- Nos testes relacionados à zimografia optou-se por incluir a avaliação das células tumorais com rhVEGF-C, com a justificativa de que a literatura aponta que possa ocorrer um efeito autócrino do VEGF-C;

- Além disso, foi incorporado ao documento final, o levantamento dos casos de carcinoma espinocelular oral do Centro de Diagnóstico das Doenças da Boca.

4 Artigo1

Este trabalho será submetido à *Journal of Oral Pathology and Medicine*, já estando formatado conforme as normas da revista citada.

Article title: Demographic and clinical profile of oral squamous cell carcinoma lesions in a service-based population from Southern Brazil: A retrospective study of 53 years.

Running title: Oral cancer in Southern Brazil population

Keywords: oral squamous cell carcinoma; oral lesions; epidemiological study, demography.

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ABSTRACT

The oral squamous cell carcinoma (OSCC) is the most prevalent malignance in mouth, being an important public health problem. The aim of this study was to evaluate the clinical and epidemiological profile of the OSCC cases registered in a center of clinical and histopathological diagnosis, located in Southern Brazil. Eight hundred and six individuals with OSCC and its variants were included in this study, over 1959-2012 period. The variables recorded from the files were: age, gender, skin color, tumor location, size and evolution time of the lesions (referred by the patients), as well as, the presence of pain lymph nodes, habits of tobacco and alcohol, and also the profession. OSSC was more frequent in males (76.6%), with the majority of cases distributed between 51 and 70 years old (53.9%). The most prevalent sites were lower lip vermilion [23.3% (20.4; 26.4)], followed by lateral border/ventral surface of the tongue [20.2% (17.5; 23.2)], gingiva/alveolar ridge [18.1% (15.5; 21.0)], and floor of the mouth [14.9% (12.5; 17.5)]. A strong association between outdoor occupation and OSCC in lower lip vermilion was found. The OSCC lesions located in tongue, gingiva/alveolar ridge and floor of the mouth were commonly more painful, bigger than 2 cm, and frequently presenting lymph nodes involvement. Most of the results confirm the data from literature. Mouth self-examination should be recommended and campaigns of prevention and early detection of OSCC should be periodically performed in order to increase people's feelings of personal risk.

INTRODUCTION

The oral cancer represents around 5% of all the malignances of the body(1), although there is a wide geographical variation in the incidence of this cancer(2). In some parts of the globe it is the most frequent type of cancer(2), like in South Asia, including India and some islands in Melanesia, mainly due to the smoking and chewing tobacco habits(3). Besides, in others areas oral cancer shows great incidence, like Sri Lanka, Pakistan and Taiwan; in some European countries; in some Latin America countries as Brazil, Uruguay, Puerto Rico and Cuba; (4). In Brazil oral cancer represents the sixth neoplasm(5). The oral squamous cell carcinoma (OSCC) is the most prevalent neoplasm (94% of all the oral tumors), which occurs in individuals over 45 years old. Although several researches about OSCC have been performed, more than 50% of the patients die within 5 years from the diagnosis.

The Center of Diagnosis of Oral Diseases (CDOD) is considered a reference Center for the clinical and histopathological diagnosis of oral diseases, having a collection of approximately 20,000 biopsy samples. In the last years, CDOD attends around 1,500 patients per year, mainly the population who lives in the in the extreme south of Rio Grande do Sul, the state located in Southern Brazil. The CDOD started the activities in 1959 and in the last 15 years, campaigns of prevention and detection of oral cancer have been performed, in order to aware the population about the importance of the self-examination and early diagnosis of oral cancer and how to avoid its occurrence. Some studies have evaluated the impact of oral cancer campaigns in prevention and early diagnosis of this disease(6, 7).

The consumption of tobacco and alcohol is strongly associated to the development of tumors like OSCC and upper aerodigestive tract neoplasms(4, 8). However, in the last decades, there is an increasing trend for OSCC development in females and young adults, being speculated the existence of other etiological factors like genetic heritage, dietary habits, and HPV(9-12).

Frequently, OSCC represents a heterogeneous disease whose prognosis is difficult to establish, because it is conditioned by multiples variables such as the extension and location of primary tumor, degree of invasion of neighboring structures, presence of regional and distant metastasis, histological types, the chosen therapy, and the status of general health of the patient (4).

The aim of this study was to evaluate the clinical and epidemiological profile of the OSCC cases registered in a center of clinical and histopathological diagnosis, located in Southern Brazil.

MATERIALS AND METHODS

Eight hundred and six individuals with oral squamous cell carcinoma (OSCC) and its variants (basaloid squamous carcinoma, verrucous carcinoma, and spindle cell carcinoma), who attended the Center of Diagnosis of Oral Diseases (CDOD), School of Dentistry, University of Pelotas, Brazil were included in this study, over 1959-2012 period. This study had the approval of the Ethic Committee of School of Dentistry, Pelotas, Brazil.

From a total of 20,206 biopsies, 806 cases were OSCC. The following clinical parameters were recorded from the files: age, gender, skin color, tumor location, size and evolution time of the lesions (referred by the patients), as well as their symptomatology, the presence of lymph nodes, habits of tobacco and alcohol, and profession. The data were categorized as followed: age of individual in decades; gender (male and female); skin color in white and non-white (referred by professional); historic of tobacco and alcohol habits (dichotomic variable); workplace (indoor or outdoor); histological variant (squamous cell carcinoma, verrucous carcinoma, basaloid squamous carcinoma, and spindle cell carcinoma), clinical appearance (ulcer, leukoplakia, erithroplakia, leukoerithroplakia, and association), location of the lesion (vermilion of lower and upper lip, labial mucosa, lateral border/ventral surface of the tongue, dorsum of the tongue, floor of the mouth, gingiva/alveolar ridge, buccal mucosa, palate and oropharynx); size of the lesions (up to 2cm, 2.1-4cm, and over to 4cm); referred evolution time (up to 6 months, 6.1-12 months, and over to 12 months); lymph node involvement (dichotomic variable); and presence of pain (dichotomic variable).

The data were entered in duplicate using EPIDATA 3.1 version (EpiData Association, Odense, Denmark) and analyzed using STATA 11.0 (Stata Corporation, College Station, TX, USA). Descriptive analyses were performed and associations between variables were assessed using the chi-square and Fisher's tests, with confidence level of $p<0.05$.

RESULTS

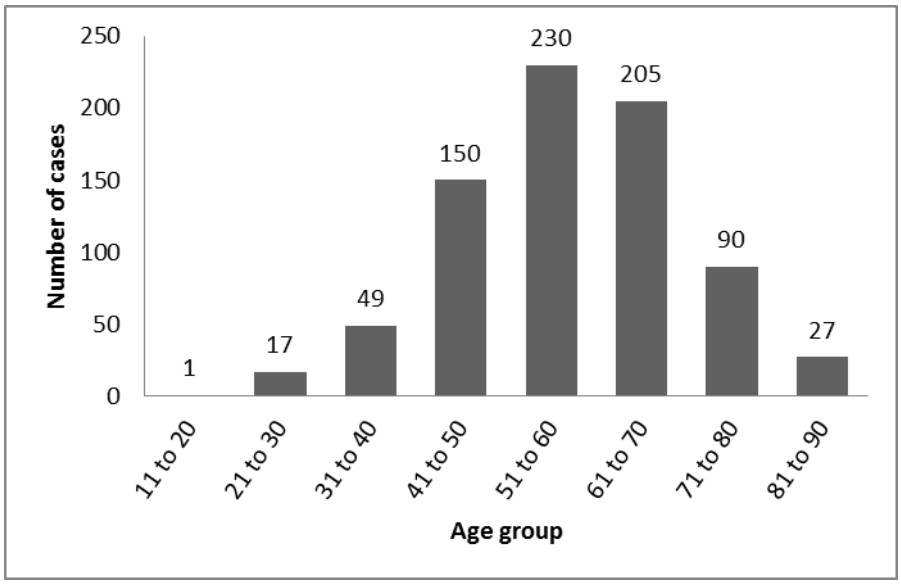
From January 1959 to December 2012, a total of 20,206 samples were processed in our service, with 806 (3.9%) cases being diagnosed as squamous cell carcinoma or its histological variants, with a high prevalence in males [76.6%(73.5;79.4)] and white individuals [92.2%(90.0;94.0)] (Table 1). From the total of squamous cell carcinoma, 186 (23.1%) were diagnosed during 1959-1996 period (total number of biopsies = 8,148), being the other 620 (76.9%) cases diagnosed from 1996 to 2012 (total number of biopsies = 12,058). The mean age was 57.7 years (19-90), being the majority of cases distributed between 51 and 70 years old (53.9%) (Figure 1).

Table 1: Descriptive analysis of individual variables (demographic and behavioral).(N=806). Pelotas, Brazil.

Variable	Absolute frequency (nº)	Relative frequency [% (95%CI)]
Gender		
Female	189	23.4 (20.6; 26.5)
Male	617	76.6 (73.5; 79.4)
Skin color[1]		
White	661	92.2 (90.0; 94.0)
Black	56	7.8 (6.0; 10.0)
Smoking[2]		
Yes	427	89.5 (86.4; 92.1)
No	50	10.5 (7.9; 13.6)
Alcoholism[3]		
Yes	211	84.4 (79.3; 88.7)
No	39	15.6 (11.3; 20.7)
Profession[4]		
Indoor	272	40.6 (36.9; 44.4)
Outdoor	236	35.2 (31.6; 39.0)
Retired	162	24.2 (21.0; 27.6)

[1] 89 data were not informed; [2]329 data were not informed; [3]556 data were not informed; [4]136 data were not informed.

Figure 1 - Distribution of cases according to age



The most prevalent sites were lower lip vermilion [23.3% (20.4; 26.4)], followed by lateral border/ventral surface of the tongue [20.2% (17.5; 23.2)], gingiva/alveolar ridge [18.1% (15.5; 21.0)], and floor of the mouth [14.9% (12.5; 17.5)]. Ulcer was the commonest clinical apresentation [69.9% (66.6; 73.0)] (Table 2). From the total of the cases, the most commom histological diagnosis was squamous cell carcinoma [96.8% (95.3; 97.9)] (Table 2). The most frequent evoltion time reffered by the patients was up to 6 months (Table 2).

Table 2 – Descriptive analysis of lesions characteristics(histological variant, referred time of evolution, clinical appearance, size, site, pain, and presence of lymph nodes). Pelotas, Brazil. N=806

Variable	Absolute frequency (nº)	Relative frequency [% (95%CI)]
Histological variant		
Squamous cell carcinoma	780	96.8 (95.3; 97.9)
Verrucous carcinoma	24	3.0 (1.9; 4.4)
Basaloid squamous carcinoma	1	0.1 (0.0; 0.6)
Spindle cell carcinoma	1	0.1 (0.0; 0.6)
Referred evolution time[1]		
Up to 6 months	294	55.8 (52.3; 59.3)
6-12 months	114	21.6 (18.8; 24.6)
Over 12 months	119	22.6 (18.7; 25.6)
Clinical appearance[2]		
Ulcer	446	69.9 (66.6; 73.0)
Leukoplakia	81	12.7 (10.4; 15.1)
Erithroplakia	14	2.2 (1.3; 3.5)
Leukoerithroplakia	12	1.9 (1.0; 3.1)
Association	85	13.3 (11.0; 15.8)
Size[3]		
Up to 2 cm	272	52.3 (48.8; 55.9)
2.1 – 4 cm	180	34.7 (31.5; 38.1)
Over 4 cm	88	17.0 (14.5; 19.8)
Site		
Lower lip vermilion	241	23.3 (20.4; 26.4)

Upper lip vermilion	8	0.8 (0.2; 1.6)
Labial mucosa	18	1.7 (0.9; 2.9)
Lateral border/ventral surface of the tongue	209	20.2 (17.5; 23.2)
Dorsum of the tongue	9	0.9 (0.3; 1.8)
Buccal mucosa	62	6.0 (4.4; 7.8)
Floor of the mouth	154	14.9 (12.5; 17.5)
Gingiva/alveolar ridge	187	18.1 (15.5; 21.0)
Palate	66	6.4 (4.9; 8.4)
Oropharynx	81	7.8 (6.0; 9.9)
Pain[4]		
Yes	285	66.1 (62.7; 69.4)
No	146	33.9 (30.6; 37.3)
Lymph node involvement[5]		
Yes	155	58.5 (55.1; 62.0)
No	110	41.5 (38.0; 44.9)

[1] 279 data were not reported; [2] 168 data were not reported; [3] 286 data were not reported; [4] 375 data were not reported; [5] 541 data were not reported.

A strong association between outdoor occupation, represented mainly by agricultors, and OSCC in lower lip vermilion was found. While in individuals who works indoor 22.5% of the lesions were found in this site, in those who work outdoor the percentage increases to 47.4% (p<0.001). Skin color was also associated with this site. Lower lip vermilion lesions were more commonly found in white individuals in comparison with non-white. (p<0.001) (Table 3).

Table 3: Association between lower lip vermilion lesions and individual characteristics.

	Lower lip vermilion, n (%)		
Variable/Category	**Yes**	**No**	**P-value**
Profession			<0.001
Indoor	53 (22.5)	183 (77.5)	
Outdoor	129 (47.4)	143 (52.6)	
Retired	38 (23.5)	124 (76.5)	
Skin color			<0.001
White	226 (34.2)	435 (65.8)	
Black	1 (1.8)	55 (98.2)	

The OSCC lesions located in lateral border/ventral surface of the tongue were commonly bigger than 2 cm (Table 4) and frequently presented lymph nodes involvement (Table 5). Similar positive and statistically significant association were also observed for gingiva/alveolar ridge and floor of the mouth (Table 4 and 5).

Contrary, lesions located in lower lip demonstrated the smallest size at the time of the diagnosis (Table 4) and rare lymph node involvement (Table 5).

Table 4: Association between size and lesions characteristics.

	Size, n (%)			
Variable/Category	**Up to 2 cm**	**From 2.1 to 4 cm**	**Over 4 cm**	**P-value**
Site				
Lower lip vermilion	140 (51.5)	27 (16.9)	5 (5.7)	<0.001
Upper lip vermilion	4 (1.5)	0 (0.0)	1 (1.1)	0.313
Labial mucosa	4 (1.5)	2 (1.3)	2 (2.3)	0.815
Lateral border/ventral surface of the tongue	51 (18.8)	54 (33.8)	28 (31.8)	0.001
Dorsum of the tongue	3 (1.1)	3 (1.9)	0 (0.0)	0.414
Buccal mucosa	10 (3.7)	13 (8.3)	12 (12.8)	0.008
Floor of the mouth	34 (12.5)	34 (21.3)	30 (34.1)	<0.001
Gingiva/alveolar ridge	22 (8.1)	42 (26.3)	37 (42.1)	<0.001
Palate	10 (3.68)	16 (10.0)	16 (18.1)	<0.001
Oropharynx	11 (4.0)	17 (10.6)	13 (14.8)	0.002
Referred evolution time				0.214
Up to 6 months	102 (49.8)	72 (35.1)	31 (15.1)	
6 – 12 months	51 (64.6)	19 (24.1)	9 (11.4)	
Over 12 months	53 (59.6)	25 (28.1)	11 (12.4)	

Table 5: Lymph node involvement and associations.

	Lymph node involvement, n (%)		
Variable/Category	**Yes**	**No**	**P-value**
Site			
Lower lip vermilion	16 (26.2)	45 (73.8)	< 0.001
Upper lip vermilion	2 (66.7)	1 (33.3)	0.773

Labial mucosa	1 (100.0)	0 (0.0)	0.399
Lateral border/ventral surface of the tongue	59 (71.9)	23 (28.1)	0.003
Dorsum of the tongue	3 (60.0)	2 (40.0)	0.945
Buccal mucosa	9 (52.9)	8 (47.1)	0.647
Floor of the mouth	46 (73.0)	17 (27.0)	0.007
Gingiva/alveolar ridge	45 (70.3)	19 (29.7)	0.028
Palate	16 (69.6)	7 (30.4)	0.259
Oropharynx	28 (80.0)	7 (20.0)	0.006
Size			<0.001
Up to 2 cm	33 (38.4)	53 (61.6)	
From 2.1 to 4 cm	41 (63.1)	24 (36.9)	
Over 4 cm	37 (77.1)	11 (22.9)	
Referred evolution time			0.084
Up to 6 months	76 (65.5)	40 (34.5)	
From 6 to 12 months	22 (57.9)	16 (42.1)	
Over 12 months	14 (43.8)	18 (56.3)	
Number of sites			0.001
Single site	97 (51.9)	90 (48.1)	
Multiple sites	55 (75.3)	18 (24.7)	

The present study showed an association between pain and intraoral OSCC lesions, specially those located in the most frequent sites as tongue, floor of the mouth and gingiva/alveolar ridge (Table 6) and between pain and the size of the lesions (Table 6). The bigger were the lesions, more painful they were (Table 6).

Table 6: Pain and associations.

	Pain, n (%)		
Variable/Category	**Yes**	**No**	**P-value**
Site			
Lower lip vermilion	4 (34.4)	78 (65.6)	< 0.001
Upper lip vermilion	3 (75.0)	1 (25.0)	0.706
Labial mucosa	3 (37.5)	5 (62.5)	0.086
Lateral border/ventral surface of the tongue	103 (83.7)	20 (16.3)	<0.001

Dorsum of the tongue	4 (80.0)	1 (20.0)	0.510
Buccal mucosa	25 (71.4)	10 (28.6)	0.507
Floor of the mouth	70 (80.5)	17 (19.5)	0.002
Gingiva/alveolar ridge	79 (76.0)	25 (24.0)	0.015
Palate	31 (88.6)	4 (11.4)	0.003
Oropharynx	34 (89.5)	4 (10.5)	0.001
Size			<0.001
Up to 2 cm	84 (53.5)	73 (46.5)	
2.1 - 4 cm	80 (76.2)	25 (23.8)	
Over 4 cm	37 (78.7)	10 (21.3)	
Referred evolution time			0.037
Up to 6 months	145 (71.1)	59 (28.9)	
From 6 to 12 months	40 (57.1)	30 (42.9)	
Over 12 months	47 (58.8)	33 (41.2)	
Number of sites			<0.001
Single site	197 (60.4)	129 (39.6)	
Multiple sites	83 (86.5)	13 (13.5)	

DISCUSSION

Worldwide, oral cancer is a major problem in oral health, presenting high mortality and morbidity rates (2, 13) and being the sixth most common cancer (14). Oral squamous cell carcinoma (OSCC) is the most frequent type of oral cancer, accounting to approximately 94% of all the malignances in this site (2). Although several studies have been developed during the last decades with the objective of better understanding the etiopathogenesis of this disease, OSCC continue increasing in many populations around the world (2, 15). Analyzing a Mexican population data during a period of 20 years, Cepeda et al. (2011)(15) formulated many explanations for the increasing rates of incidence of OSCC, like the significant increase of the inhabitants in this country; the better ability of the general dentists in diagnosing this disease; and the increase in the consumption of tobacco and alcohol even in the younger Mexicans, creating a more permissive environment with this early exposure, specifically in females.

Population-based studies regarding to the prevalence of oral lesions are rare but very useful, since they provide a detailed description of the epidemiological profiles of these conditions, allowing the designing of the characteristics of oral health in specific population groups (16-19). The great majority of the epidemiological studies analyze hospital-based or oral health services-based cancer registries, which naturally gather biased information since just part of the population have access to these centers, mainly in developing countries (4). Therefore, the present study has the same limitation, in terms of the extrapolation of the data for the total population of the city, the state and the country in which it had performed. However, it is important to highlight that the data registered in the whole existence of the CDOD in Pelotas (Brazil) were evaluated, during a period of more than a half-century. Other Brazilian study performed in the University of Sao Paulo also analyzed data from the files of an oral pathology laboratory (20) for a similar period of time. In both, most of the biopsies received were incisional, since the services belong to Dental Schools. Thus, the majority of the cases represent a profile of OSCC patients when they first seek help.

In the last two decades the CDOD had performed campaigns in order to aware the population of the city about the importance of the self-oral examination and to promote the prevention and the early detection of oral cancer. For this reason, the prevalence data of the OSCC were categorized in two periods (1959-1996 and 1997-2012), since the first campaign occurred in November/1996). From the total of the OSCC biopsies, 76.9% were diagnosed after the 1996, reflecting the impact of the campaigns in the detection of the OSCC. Nevertheless, the minor number of samples in the first period of time limits the comparison between the two categories of time. Moreover, the total number of biopsies after November/1996 was 12,058, with OSCC cases corresponding to 5.2%. This percetual duplicate when we compare with the same diagnosis done before November/1996 (2.3% from 8,148).

Regarding the prevalence of sex, OSSC was more frequent in males (76.6%), corroborating the global literature for OSCC (4, 21, 22), that demonstrate twice level of oral cancer among males compared to females (9, 22-24). The still greater indulgence by men about the main risk factors of OSCC such as tobacco and alcohol consumption for intra-oral OSCC and sunlight exposure for lower lip OSCC, could explain it(4). However, some researches have reported a decrease of the male-

female ratio, attributing this changing to different facts as tobacco and alcohol alteration of habits, cultural and geographic peculiarities and other reasons (25, 26).

Even though we had found a high prevalence of smokers (59.2% - 427 out of 477) and alcohol drinkers (31% - 211 out of 250, we have not used these data in the analysis, due to a possible bias, once a high number of registers did not contain information regarding these habits and no specific questionnaire was used to get them.

Most diagnosed patients in this study were between 51 and 70 years old years old (53.9%), which are in accordance with the literature (4, 20-22, 27, 28), indicating age probably as a risk marker for OSCC (22). Different trends were observed by Marocchio et al (2010)(20) and Patel et al. (2011)(10). These last group analyzed incidence and survival data from the Surveillance, Epidemiology and End Results (SEER) Program of the National Cancer Institute in USA, from 1975 to 2007 for OCSCC and OSCC from tongue. They showed an increasing among young white individuals age 18 to 44 years, particularly among white women, postulating that they may be a new, emerging head and neck cancer patient population (10).

Concerning all the anatomical oral sites, lower lip represented the most frequent location (23.3%), corroborating data from the literature (21, 22, 29). The major prevalence of OSCC lesions in this site has great relevance when the studies are performed in tropical countries, besides the important influence by socio-demographical (like ethinic origin), main occupation, and cultural aspects (22, 29, 30) of the studied population.

It was showed a strong association between outdoor occupation, represented mainly by agricultors, and OSCC in lower lip vermilion and also between white skin colour and this site in our study, as demonstrated previously (21, 29). Although the biopsy registers do not provide the profession of the retired patients, probably most of then should be from rural areas, what would reinforce this correlation. Moreover, the lower lip, due to its anatomical position, is subjected to intense and chronic sun exposition to ultraviolet (UV) component of solar radiation and it is the most common site in males in populations charaterized by fair skinned pelople with high sun exposure (22, 29, 31). The potential protective factor of the women's lipstick should be considered (22). However, socio-demographic and lifestyle factors,

immunosuppression, and genetic susceptibility might produce a synergistic effect (29).

Lower lip were followed by lateral border/ventral surface of the tongue (20.2%), gingiva/alveolar ridge (18.1%), and floor of the mouth (14.9%). Considering the intraoral sites, tongue (specially the lateral borders) has been pointed as the most common site among European and US and other American populations (26, 28, 32). We grouped lateral border and ventral surface of the tongue because many times when one of this site is affected, the other also is, through the extension of the lesions.

Mostly confirming the literature data, the tongue OSCCs in this study were more painfull, frequently presenting lymph nodes involvement, and at least a T2 tumor or more (22, 30). Similar positive and estatisticaly significant association were also observed for gingiva/alveolar ridge and floor of the mouth.

In the opposite manner, lesions located in lower lip demonstrated the smallest size at the time of the diagnosis, less pain and rare lymph node involvement. One point that shoud be emphazised is that the lesions located on lip are promptly detected by self-examination, compared with the intraoral lesions, which explain their minor size, and by consequence, their lowest symptomatology and the lowest incidence of cervical metastasis, and also allowing their early diagnosis and better prognosis (20-22, 26, 29, 30). Our results confirmed the reduced aggressiveness of lip OSCC, compared with intraoral cancer (30). The occurrence of the disease in this site is one of the reasons for early search of oral health care centers (29).

Gingiva is also the third intraoral most frequent site after tongue and floor of the mouth in another series (33), that sometimes can affect women, elderly patients who are non-smokers and non-drinkers, easly presenting bony invasion associated with significant increase of nodal metasyasis and worse survival (33). In another cross-sectional previous study performed in CDOD, gingiva and alveolar ridge were the most common intraoral sites, mainly in women (21).

Oropharynx deserves some special attention, since it was the forth intraoral OSSC localization in our study, and also because the biology of the disease in this site present some peculiarities (34). A distinct subset of these tumors presents

basaloid features, wild type p53 expression, being associated with the presence of human papilloma virus (especially type 16), having good prognosis and responding well to radiotherapy, with or without chemotherapy (4, 9, 34).

Cancer pain is also a public-health concern, once it creates a poor quality of life and limits normal function (35). The present study showed an association between pain and intraoral OSCC lesions and between pain and the size of the lesions. Ulcer was the more frequent OSSC clinical presentation. This findings are in accordance with the other authors (35-38) who demonstrated that pain in oral cancer may be associated with advanced, endophytic invasive tumors, determining a poor prognosis for the patients. Pain in turns leads to other symptoms, including anxiety, depression, and side-effects of high-dose opiate use (35). Nevertheless, some authors have described that early head and neck cancer can be painful at the time of initial diagnosis, being the orofacial pain an important predictor for the transition from precancer to cancer (39). Although this symptom is very useful in evaluating cancer progression, it has some subjectiviness in its evalutiion.

Many study endpoints comprise evolution time, time elapsed from referral to a health center and first appointment, time elapsed from first appointment and treatment, and total time elapsed from first noticing and starting treatment (13). We just have the refferred evolution time, which could be not the real time of duration for the lesions, once the patients frequently do not remember exactly when the alterations started and socio-cultural backgrounds can interfere in this data.

Our study was a demographic and clinical description of OSCC in a specific population in Southern Brazil. Most of the results confirm the data from literature about prevalence of sex, age, and tumor location. There was a significant correlation between lower lip vermilion OSCC lesions and occupation activities and between lip OSCC and skin color, highlighting the main socio-demographic and cultural characteristics of the studied population, who are mainly white, with European ascendency and working in agriculture. These lesions showed the smallest size, the lowest symptomatology and the lowest incidence of cervical metastasis, which can be easly perceived, explaining their early diagnosis and better prognosis. OSCC lesions in the other more prevalent sites as tongue, gingiva/alveolar ridge and floor

of the mouth were more painfull, frequently presenting lymph nodes involvement, and at least a T2 tumor or more, what also is confirmed by the literature data (22, 30).

Decrease in oral cancer incidence can be achieved and oral cancer awareness among the population and health care providers can be increased (22). Mouth self-examination should be estimulate as integral part of health policies, once it is a low-cost and non-invasive proccedure, as well as reliable and mass-applicable method to control oral cancer incidence (13, 19). In this context, the campaigns of prevention and early detection of OSCC performed in CDOD have been performed in order to increase people's feelings of personal risk and perceived susceptibility while at the same time avoiding unnecessary anxiety and encouraging inappropriate self-referrals. The clinician should be careful in examining high-risk patients (over the age of 45, light-skinned people exposed to sun, and people who are heavy smokers and alcohol drinkers), and also evaluating high-risk oral sites (lower lip, lateral sides of the tongue, floor of the mouth and gingiva/alveolar ridge. The complete filling of the biopsy files should be stimulated, once it provides consistent information for the pathologists in the histopathological diagnosis and also avoids the loss of important data in retrospective studies as the present one. In order to minimize the substantial inequalities in oral cancer both within and between countries, a multidisciplinary care is required, with more effective primary and secondary prevention (4), recognizing the problems of the target population and implementing effective politics to treat the disease. Research on the natural history of the disease, particularly which precancers will progress over time, would help to develop screening programs tailored more to the individual disorders. Development of tumor markers with high sensitivity and specificity could assist the detection of patients and lesions at risk (32).

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5 Artigo 2

Este trabalho será submetido à *European Journal of Oral Science*, já estando formatado conforme as normas da revista citada.

Article Title: Potential molecular interactions between lymphatic endothelial cells and SCC tumor cells lines.

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Running title: Interactions between LECs and SCC cells.

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Abstract:

Inside the tumor microenvironment (TM) the neoplastic cells are in dynamic crosstalk with the vascular and lymphatic endothelial cells in order to allow the tumor to growth and metastasize. Hypothesizing that there is a crosstalk between lymphatic endothelial cells (LECs) and tumor cells from squamous cell carcinoma (SCC) that plays an important role in metastasis, we aimed to identify potential interactions between LECs and tumor cells lines from SCCs, through in vitro assays. Primary adult human dermal lymphatic microvascular endothelial cells (HMVECs) and the human head and neck SCC cell lines like A431, UM-SCC-1, UM-SCC-22A and UM-SCC-22B were cultured in their specific media. UM-SCC cells lines were treated with rhIL-6, being VEGF-C expression checked by Elisa. Baseline IL-6 was evaluated in HMEVCs using the same assay. Also the IL-6 receptor (IL-6R) was analyzed by Western blot in UM-SCC cells. Conditioned media (CM) from HMVECs were prepared with different treatments and incubated with A431 cells, in order to verify the MMPs enzymatic activities by gelatin zymography. Our results demonstrated that there are interactions between tumor cells and LECs, since the LECs-CM were able to enhance MMP-2 gelatinolytic activity. Moreover, we showed that LECs secrete IL-6, and different SCC lines have receptors for this cytokine. Therefore, our results indicate some potential interactions between LECs and TCs, being necessary other studies to elucidate the involved signaling pathways.

Keywords: lymphatic endothelial cell, tumor cell, squamous cell carcinoma, matrix metalloproteinases, IL-6, VEGF-C.

Introduction:

Several biological processes are involved in the development of cancer which leads to the neoplastic cells to proliferate, invade neighboring tissues and blood/lymphatic vessels, and consequently, determine the locoregional and distant metastases(1). Recently, it was discovered that these events involve crosstalk mechanisms among cell population themselves (tumor cells, endothelial cells, and other cells in the tumor microenvironment) and among those cells and extracellular matrix (ECM)(2-5). It was previously thought that lymphatic metastasis involved only the passage of malignant cells along pre-existing lymph vessels near a tumor. However, nowadays it is known that tumor metastasis is a complex mechanism

consisting of multiple steps(6) and recent studies suggest that lymphangiogenesis can be induced by tumors and may promote tumor dissemination(7, 8) In this context, cytokines, chemokines and growth factors present in tumor microenvironment (TM), can regulate angiogenesis/lymphangiogenesis and also the matrix metalloproteinases (MMP) production, which are key events in cancer growth and metastatic spread(9).

The major factors related to angiogenesis/lymphangiogenesis belong to a family of vascular endothelial growth factors (VEGFs). VEGF-C, a member of VEGF family, is related to lymphangiogenesis and different studies have shown that the overexpression of this protein on cancer cells is related to increased lymphatic vessel density (LVD), invasion and metastasis(7, 8, 10). Furthermore, there are evidences that VEGF-C can promote an autocrine effect on tumor cells that have VEGF-C receptors (VEGFR-3), changing their phenotype, increasing motility and proliferation, and also acting as anti-apoptotic factor (8, 11, 12).

Such as VEGF-C, several studies have shown increased expression and serum levels of interleukin-6 (IL-6), a pleiotropic cytokine involved in acute inflammation, in different types of cancers(6, 13-15). Duffy et al. (2008)(15), in a study involving 444 patients shown that higher IL-6 serum levels were associated with increased recurrence and metastasis, and decreased survival. Moreover, other studies have demonstrated that IL-6 is able to induce increased expression and production of MMP-2 and -9, and VEGF, through JAK-STAT3 (janus-kinase – signal transducer and activator of transcription) signaling pathway(6, 16, 17). Neiva et al. (2009)(4) showed that blood endothelial cells are capable to produce this cytokine. IL-6 has been pointed as an inducer of epithelial-mesenchimal transition changes on tumor cells from head and neck tumor cells, via the activation of STAT3/Snail signaling pathway, increasing their metastatic potential(6).

MMPs are zinc-dependent enzymes capable to degrade basement membrane and other extracellular matrix components, such as collagen. Several reports have shown that different types of cancer cells can produce these proteolytic proteins, and related to squamous cell carcinoma (SCC), the MMPs more involved are -2 and -9(18-20). Roh et al. (2012)(19), through immunohistochemical and RT-PCR (real-time polymerase chain reaction) tests, showed that MMP-2 was stronger expressed in skin SCC than in normal epidermis, and Suarez-Roa et al. (2012)(20) reported that

MMP-2 expression was higher in deep areas and metastatic sites than of oral SCC, suggesting that MMP-2 play important role in invasion and metastasis. These enzymes can be regulated for several molecules from different cells in TM, such as IL-6 and CCL-21 (chemokine ligand 21) (2, 6, 16, 17).

Hypothesizing that there is a crosstalk between lymphatic endothelial cells (LECs) and tumor cells from squamous cell carcinoma (SCC) that plays an important role in metastasis, we aimed to identify potential interactions between lymphatic endothelial cells and tumor cells lines from SCCs, through in vitro assays.

Materials and methods

Cells and cell culture

Primary adult human dermal lymphatic microvascular endothelial cells (HMVECs) were obtained from Lonza (Walkersville, MD, USA) and cultured in endothelial cell growth medium 2 (EGM-2 MV; Lonza, Walkersville, MD, USA). The human epithelial carcinoma cell line A431 (Abcam, Cambridge, MA. USA); the human SCC cell line UM-SCC-1 from floor of the mouth, and the human hypopharynx cell lines (UM-SCC-22A and UM-SCC-22B, neck metastasis), gifts from Dr. Thomas Carey were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Auckland, NZ) and penicillin/streptomycin. All the cell lines were incubated at 37ºC in a humidified cabinet (95% air and 5% carbon dioxide). For the experiments, the UM-SCC 1 ($4x10^5$ cells per well), UM-SCC 22A and UM-SCC 22B ($6x10^5$ cells per well) cells were seeded in 60 mm dishes. The A431 cells were seeded in 6-well plates ($4x10^5$ cells per well). And HMVECs were seeded in 6-well plates ($3x10^5$ cells per well).

Elisa - Production of IL-6 and VEGF-C

HMVEC, UM-SCC-1, UM-SCC-22A and UM-SCC-22B were cultured in 6 well plates or 60 mm dishes till they were 80% confluent. Cells were washed and plain medium was added. After 24 hours HMVEC culture supernatants were collected from 3 separate experiments. For the UM-SCC-1, UM-SCC-22A and UM-SCC-22B the plain medium was left overnight and after cells were treated with 0-20 ng/ml rhIL-6 (R & D Systems, Minneapolis, MN, USA) for 24 hours. Supernatants were collected from

3 independent assays. For both experiments, the cell number in each well was counted. The baseline level of IL-6 in culture supernatants from HMVEC and VEGF-C level in culture supernatants from the SCC tumor cell lines (baseline and from tumor cell lines treated with 20ng/ml of rh-IL-6) were measured using Quantitative Elisa kits (R&D Systems, USA) as manufacturer's instruction and normalized to $1x10^5$ cells.

Western Blot

The receptor for IL-6 (IL-6R) expression was evaluated by Western blots using polyclonal antibody rabbit anti-IL-6R (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Furthermore, UM-SCC-1, UM-SCC-22A and UM-SCC-22B were serum-starved overnight and treated with 0-20 ng/ml rhIL-6 (R & D Systems, Minneapolis, MN, USA) for 24 hours. The whole cell lysates were separated by 9% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels and transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubating the blots with 5% low fat milk for 1 hour in Tris buffered saline containing 0.1% Tween-20 (TBST). The blots were incubated with primary antibody described above in TBST (1:1,000) at 4°C overnight. After washing with TBST (3 times/15 min), the blots were incubated with horseradish peroxidades-conjugated goat anti-rabbit IgG (1:10,000) for 2 hours at room temperature (RT). A Super Signal West PICO chemiluminescent substrate detection system (Thermo Scientific, USA) was used to detect specific protein bands. Protein loading was normalized by incubating the membranes with anti-β-actin antibody, in all the experiments (1: 10,000). ImageJ software (NIH, Bethesda, MD, USA) was used to quantify Western blot bands.

Collection of conditioned media (CM)

HMVECs were seeded in 6-well plates ($3x10^5$ cells per well) and allowed to attach for 24 hours in EGM-2 MV. At that time period, cells were washed with PBS 1x and test medium were replaced (EBM + VEGF-C, EGM-2MV, and EGM-2MV + VEGF-C). Cells were also incubated for 24 hours at 37ºC, after which supernatant was collected and stored at -80ºC for the use with A431 cells.

Zymographic assays

Besides the three groups of CM described above, other three groups without CM were included: DMEM, DMEM + VEGF-C, and EGM-2MV. A431 cells were treated for 24 hours, with or without CM, and after this period, the supernatant was collected and stored at -80ºC for the zymography.

MMPs enzymatic activities were assayed using gelatin zymography. The samples media were mixed to the sample buffer (2% SDS; 125 mM Tris-HCl - pH 6.8; 10%; glycerol and 0.001% blue bromophenol). Samples were eletrophoresed on 0.05% gelatin-containing 10% SDS-polyacrylamide gel. The gels were washed twice in 2% Triton X-100 (2%) for 60 min in RT, followed by incubation with renaturing buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$ - Tris-$CaCl_2$) overnight at 37ºC. After that, the gels were stained by Coomassie Blue G-250 a 0.05% (Bio Rad, Richmond, CA), followed by destaining cycle, and clear zones of gelatin digestion were indicative of MMP activity. The average pixel intensities and areas of relevant bands were calculated using ImageJ software (NIH, Bethesda, MD, USA) and integrated densities were reported as pixel intensity-mm^2. In order to distinguish MMP activities from other assays, the calculated pixel values were normalized with the numbers in corresponding cells for each cell culture.

Results:

IL-6-induced SCC tumor cell lines expression of VEGF-C

The three differents SCC cell lines (UM-SCC-1, UM-SCC-22A and UM-SCC-22B) under stimulation of rhIL-6, expressed VEGF-C distinctly. UM-SCC-1 and UM-SCC-22A, both generated from primary tumor from head and neck sites, secreted more VEGF-C than UM-SCC-22B (from neck metastasis). However, rhIL-6 treatment (20 ng/ml) was not able to increase the releasing of this growth factor significantly when compared it with the untreated groups for all the studied cell lines (Figure 1).

HMVECs secrete IL-6 under normal cell culture conditions

The lymphatic endothelial cells (HMVECs) secreted a significant amount of IL-6 for the culture medium EGM2-MV (Figure 2), which was evaluated by Elisa.

Protein expression of IL-6R by SCC tumor cell lines:

The three SCC cell lines (UM-SCC-1, UM-SCC-22A and UM-SCC-22B) expressed IL-6R, when they were treated with different concentrations of rhIL-6 and evaluated by Western blot. The higher concentrated were rhIL-6 treatment, the more saturated were the receptors IL-6R in the SCC cell lines (Figure 3).

Metalloproteinases production

When the SCC A431 cell lines were treated with the CM obtained from LECs, regardless the additional stimulation with VEGF-C, gelatinolytic activity was always observed. For the three groups without CM, only the group treated with EGM-2MV showed band (Figure 4). When the pixels were measured, the groups treated with EGM-2MV - CM (with or without VEGF-C) showed bands more intense (Figure 5).

Discussion:

Inside the tumor microenvironment of OSCC, tumor cells are stimulated by several types of cells like fibroblasts, pericytes, inflammatory cells and vascular/lymphatic endothelial cells(5), and as a result they proliferate, invade and metastasize. This study emphasizes the potentialities of the molecular interactions between LECs and tumor cells from SCC and the role of this crosstalk, taking as a template, the analysis of in vitro experiments.

We have shown that some SCC cell lines can be stimulated by rhIL-6, showing distinct levels of VEGF-C expression by Elisa assay. VEGF-C is an important growth factor, in addition to acting as a lymphangiogenic or angiogenic factor(11). Tarquinio et al. (2012)(21) analyzing the baseline expression of VEGF-C, also showed variable levels of VEGF expression in different OSCC cell lines using Western blot. This fact may be the reflex of the distinct lymphatic metastatic potential and invasiveness of cancer cells(7, 21-24). IL-6, as well as a bunch of other cytokines and/or growth factors can increase VEGF expression in a hypoxic microenvironment, which is a key inducer of VEGF transcription(8). The VEGF isoforms have also been shown to participate in pathological processes, as angiogenesis and lympanhgiogenesis involved in cancer(8).

Many studies in experimental models of cancer have demonstrated that the VEGF-C and its receptor VEGFR-3 signaling system is a key regulator of tumor lymphangiogenesis(7, 11, 21). Sometimes VEGF-C produced by tumor cells also can

have an autocrine effect, as demonstrated by Kodama et al. (2008)(11) in the progressive growth of human gastric carcinoma. Therefore, the blockage of the VEGF-C/VEGFR-3 axis may be a good therapeutic approach in controlling cancer progression and dissemination.

We have also demonstrated that LECs are able to secrete IL-6 in normal cell culture conditions. IL-6 is one of the cytokines present in elevated levels in the serum of head and neck cancer patients, that can predict tumor recurrence, poor prognosis, and tumor metastasis(6, 15, 25). However, very little is known about the role of IL-6 in head and neck tumor biology. Neiva et al. (2009)(4) reported previously that blood endothelial cells can produce this cytokine. We have proved that also LECs can do that, hypothesizing that this cytokine plays an important role in the crosstalk between tumor and endothelial cells in SCC. These theories are based in the evidences reported in the literatures, which have shown that IL-6 can increase the expression and production of VEGF-, MMP-2 and -9(6, 16, 17). Moreover, IL-6 also has been the only cytokine related to the epithelial-mesenquimal transition changes on tumor cells in head and neck tumor invasion(6, 26).

In turn, some SCC cell lines in this study have shown that they can be induced by IL-6, once they have expressed IL-6R when treated with rhIL-6 in a dose-response experiment. In accordance with Yadav et al. (2011)(6), we suggest that IL-6 can be part of a chemokinetic network, in which cancer cell can modulate the host microinvironment for their own progression. In the same way, Lederle et al. (2011)(27) showed that IL-6 induces a complex reciprocally regulated cytokine network in human skin carcinoma model, that leads tumor cells to grow and invade. Using a model of glioblastoma multiforme si-RNA, Saidi et al. (2009)(28) inhibited VEGF-A and IL-6, getting complete abrogation of tumorigenesis, and reinforcing the potential benefits of combined therapies in cancer.

Studies have demonstrated that IL-6 is also able to increase MMPs secretion and gelatinolytic activity, mainly by JAK-STAT3 pathway signaling(16, 17). MMPs are important enzymes that degrade the extracellular matrix and several studies have shown that these proteins are overexpressed in malignant cells when compared to normal tissue, and this fact are related to metastasis and poor prognosis(18-20). Zhou et al. (2010)(18) evaluated through immunohistochemical that the MMP-2 and -9 were overexpressed in cases of metastatic oral SCC when compared with normal

tissue or non-metastatic oral SCC, suggesting that these two proteolytic proteins play key role in the lymph nodal spreading. Complementing these data, Suarez-Roa et al. (2012)(20) related that MMP-2 were strongly stained in invasive front of tumor, showing the participation of this enzyme on tissue invasion. Furthermore, the MMP-2 tumor-secreted seems to influence the production of these enzymes by stromal cells and guide proliferation of lymphatic vessel(20, 29).

In the present study, we showed that LECs-CM enhanced MMP-2 gelatinolytic activity by zymography. Other studies in the literature, using LECs or other cell types were also able to increase MMPs production by tumor cells(2, 30, 31). Koontongkaew et al. (2012)(31) using CM by fibroblasts and tumor cells, related that both MMP-2 and MMP-9 gelatinolytic activity were enhanced, and that this process is modulated through cell-cell interaction. In another study, Issa et al. (2009)(2) using co-culture test with LECs, also demonstrated MMP-2 and -9 increased gelatinolytic activity by CCL-21.

Despite the limitations of our study, we demonstrated that there are interactions between tumor cells and LECs, since the LECs-CM were able to enhance MMP-2 gelatinolytic activity. Moreover, we showed that LECs are able to produce IL-6 under normal conditions, and different carcinoma cell lines have receptor for this cytokine. Therefore, our results indicate some potential interactions between LECs and TCs, being necessary other studies to elucidate the involved signaling pathways.

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Figures and Legends

Figure 1 - VEGF-C secreted by different UM-SCC cell lines under treatment with 20 ng/ml of rhIL-6. VEGF_C expression (pg/ml) was examined by ELISA assay. Data were normalized by cell counting.

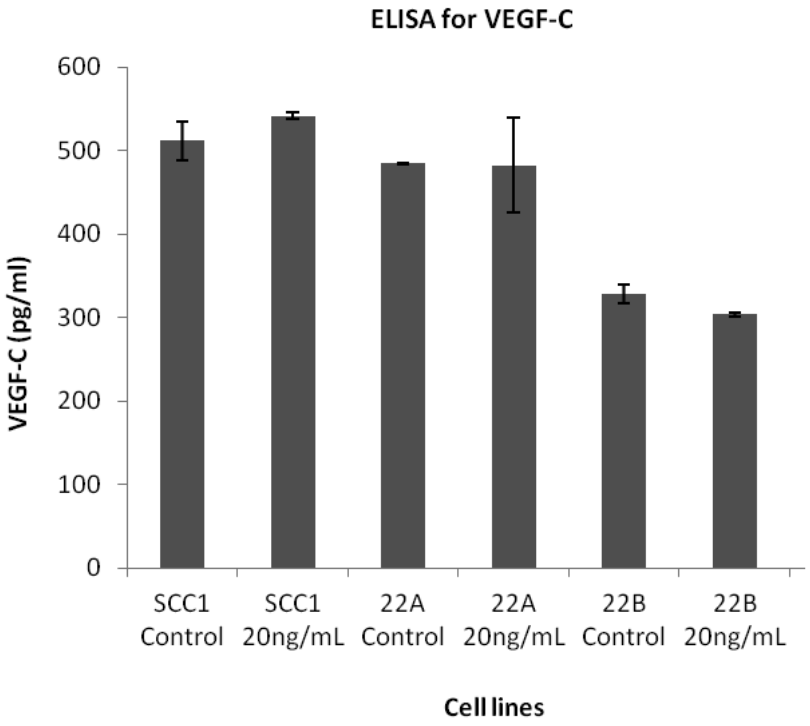


Figure 2 – IL-6 secreted by adult human dermal lymphatic microvascular endothelial cells (HMVECs) under usual cell culture condition (EGM2-MV). IL-6 expression (pg/ml) was examined by ELISA assay. Data were normalized by cell counting.

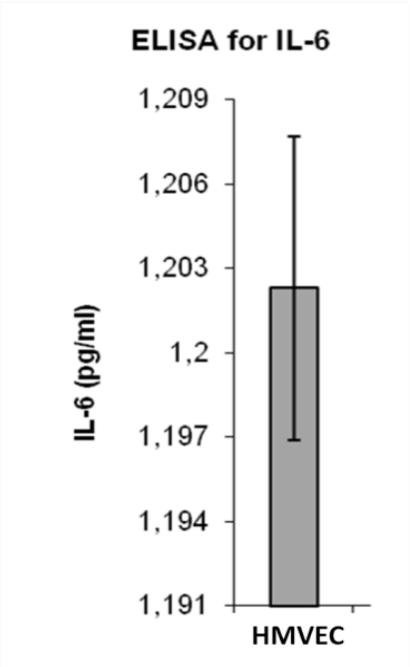


Figure 3 – Western blot for evaluation of IL-6R expression in different SCC cell lines. Dose-response test using rhIL-6 (0-20 ng/ml). β-actin expression was used as control.

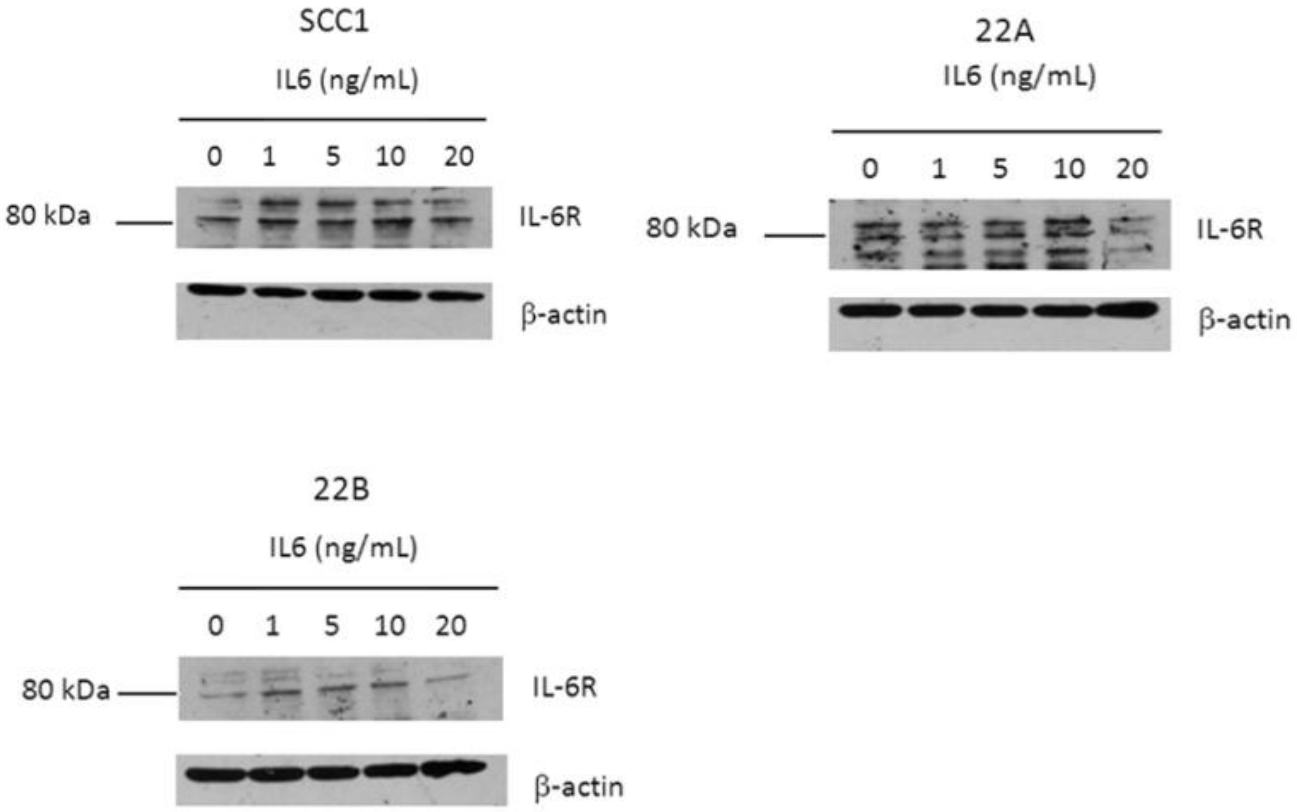


Figure 4 – Gelatin zymography of SCC A431 cell line with and without CMs. *CMs test.

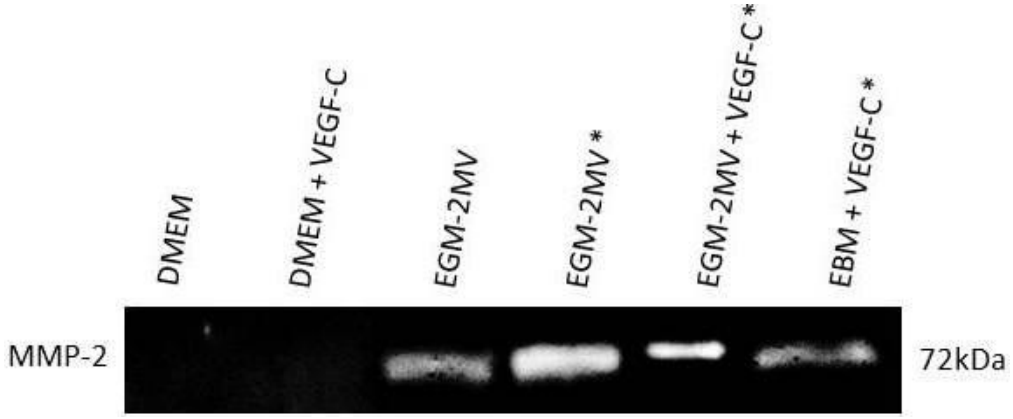
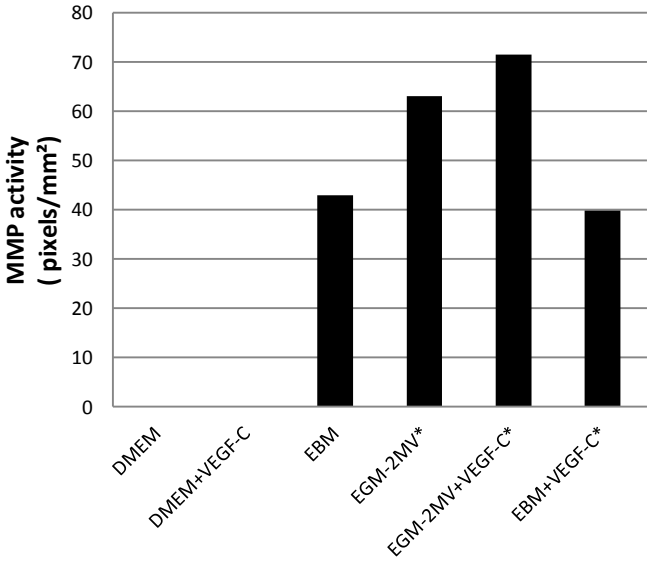


Figure 5 - MMP-2 gelatinolytic activity of SCC A431 cell line with and without CMs measured by ImageJ software. * CMs test.



6 Considerações finais

Em relação ao primeiro artigo, nosso resultados foram semelhantes ao da literatura atual. Além disso, sugerimos que o auto-exame bucal seja recomendado e, campanhas de prevenção e detecção precoce do CEO devam ser realizadas rotineiramente.

E em relação ao segundo artigo, nossos resultados apontam que as células tumorais e as células endoteliais linfáticas interagem entre si, uma vez que os meios condicionados aumentaram a atividade gelatinolítica da MMP-2. Além disso, demonstramos que a célula endotelial linfática é capaz de produzir IL-6, e que as células tumorais possuem receptores para tal proteína. Entretanto, outros estudos são necessários para elucidar as vias de sinalização envolvidas neste processo.

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