UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

DOUTORADO EM PATOLOGIA BUCAL

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O MICROAMBIENTE TUMORAL COMO FATOR MODIFICADOR NO PROCESSO DE INVASÃO E PROGRESSÃO TUMORAL NO CARCINOMA ESPINOCELULAR DE ORIGEM BUCAL

Linha de Pesquisa: Câncer Bucal

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Rio Grande do Sul, como pré-requisito final à obtenção do título de doutora em Odontologia.

Área de Concentração: Patologia Bucal

Orientador: Prof. Dr. Marcelo Lazzaron Lamers

PORTO ALEGRE

2016

CIP - Catalogação na Publicação

Ramos, Grasieli de Oliveira
O MICROAMBIENTE TUMORAL COMO FATOR MODIFICADOR NO PROCESSO DE INVASÃO E PROGRESSÃO TUMORAL NO CARCINOMA ESPINOCELULAR DE ORIGEM BUCAL / Grasieli de Oliveira Ramos. -- 2016.
123 f.

Orientador: Marcelo Lazzaron Lamers.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Programa de Pós-Graduação em Odontologia, Porto Alegre, BR-RS, 2016.

1. Câncer bucal. 2. Migração celular. 3. Microambiente tumoral. 4. Metaloproteinase de matriz. 5. Contratilidade celular. I. Lamers, Marcelo Lazzaron, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

AGRADECIMENTOS

Agradeço,

À Universidade Federal do Rio Grande do Sul, à Faculdade de Odontologia da UFRGS, e a CAPES por possibilitarem a realização do meu curso de pós-graduação.

Ao CNPQ, por meio do programa Ciências sem Fronteiras, e a Universidade da Virginia (UVA), VA, EUA, pela possibilidade de realização de estágio de doutorado sanduiche, na universidade citada.

A equipe de cirurgia de cabeça e pescoço do HCPA, que sempre me recebeu super bem e possibilitou a realização da coleta das amostras para a pesquisa. Aos pacientes que aceitaram em participar desse trabalho, meu muito obrigada, espero que um dia esse trabalho possa contribuir para que outras pessoas tenham um tratamento mais eficiente que o atual.

Aos meus pais, que não mediram esforços para que eu sempre buscasse meus objetivos e sonhos, esse título com certeza é um pouco de vocês, essa vitória é nossa!

Ao Thiago, que sempre me apoiou em todos os momentos, fazendo o possível e o impossível para que essa caminhada fosse o mais agradável possível, e levou a sério aquela máxima contigo onde você for. Agradeço por todo o carinho, companheirismo, compreensão, além de todas as ajudas nas análises ao longo da tese, sempre buscando uma maneira de colaborar no processo e facilitar minha vida.

Ao meu orientador, Marcelo muito obrigada por todos os ensinamentos nesse caminho, tenho certeza que hoje sou uma professora/pesquisadora muito melhor graças a você. Quero que saibas que além de um excelente orientador, você foi um grande amigo, e isso com certeza vai ficar para sempre. Trabalhar com você foi sempre muito bom, sempre com novos desafios e conhecimentos. Sei que posso contar contigo sempre e que essa nossa parceria acadêmica não termina aqui, vou pedir muita ajuda ainda. Isso é só o inicio de uma parceria de trabalho e de vida.

Aos professores da patologia e histologia, Manoel, Pantelis, Marcia, Fernanda, Laura, Anna, Manoela, Marco, Vinicius, o meu muito obrigada por todos os momentos e conhecimentos compartilhados durante esse período.

Ao professor Rick Horwitz, responsável pelo laboratório na Universidade da Virginia, EUA, muito obrigada pela oportunidade oferecida e todo o conhecimento adquirido.

Aos membros do laboratório na UVA: Samuel (Sam), Karen, Hana, Alexia, Jessica, Leanna, por todos os ensinamentos e experiências passados juntos durante os 4 meses que estive no laboratório, mas em especial a Karen e Lucy, Jessica e Kees-Jan, Leanna e família, Sam e Leire que tornaram esse momento de aprendizado científico muito mais agradável e uma experiência cultural inexplicável.

Aos membros do laboratório de patologia, Chris, Alessandra, Pedro, Isabel, obrigada pelos ensinamentos nas mais diversas técnicas desenvolvidas ao longo do curso.

Aos meus colegas de pós – graduação em patologia bucal, Thaise, Alessandro, Alessandra, Natalia K, Natalia D, Liana, Vivian, Viviane, Artur, Carolina, Luciano, Rubia, Marina, Anaclaudia, Kelly, Francine, Bruna, Felipe, Carlos, Francisca, e todos os que passaram pela patologia nesses 4 anos, vocês foram a minha família em Porto Alegre, obrigada por todos os momentos que passamos juntos, momentos de estudos, que foram muitos, mas também de alegria e descontração, vocês estarão para sempre no meu coração e lembranças, Santa Catarina espera a visita de vocês.

Aos meus queridos amigos do LAMOC (orientados do Marcelo) Alessandro, Alessandra, Carlos, Sérgio, Silvia, Bibiana, Paloma, Lisiane, Leonardo, Daniele, Francisca, vocês tornaram meus dias mais alegres e a minha caminhada mais tranquila, obrigada por tudo, vocês estarão para sempre no meu coração e lembrança. Desejo a todos um futuro brilhante, independente da escolha de cada um. Esse grupo é formado por pessoas extremamente heterogêneas, mas que agregam valor e contribuem para que esse grupo seja especial. Não poderia deixar de agradecer minha dupla dinâmica, que sempre me ajudou e sempre nos entendemos na nossa dislexia, Lisi, foi muito bom trabalhar contigo, saibas que és uma pessoa brilhante, e super competente e terás um futuro de ouro pela frente.

RESUMO

INTRODUÇÃO: O carcinoma espinocelular de origem bucal (CEC) apresenta uma alta taxa de mortalidade devido à invasividade das células tumorais. A migração celular, principal evento da invasão e metástase, pode ser regulada tanto por fatores intrínsecos, como adesão e contratilidade celular, quanto extrínsecos, como composição, densidade e remodelagem da matriz extracelular (MEC).

OBJETIVO: Avaliar o papel de elementos intrínsecos e extrínsecos sobre o processo invasivo do carcinoma espinocelular de origem bucal.

MÉTODOS: Foi realizada imuno-histoquímica para as proteínas: Miosina II (isoformas A, B e C), metaloproteinases de matriz (1, 2, 9 e 14); imunofluorescência as proteínas: e-caderina, n-caderina, FAK, paxilina, vinculina e fibronectina em amostras de CEC oral. Foi realizado ensaio de migração nas seguintes condições: 1 – matriz 2D com o substrato de fibronectina, ou laminina ou matrigel; 2 – matriz 3D com colágeno na presença ou não de fibronectina ou laminina; 3 – matriz 3D com diferentes concentrações de colágeno (0,6; 1,2 e 1,8 mg/ml) + fibronectina na presença ou não de um inibidor de MMP. Foi realizado análise de adesão celular utilizando-se o microscópio TIRF e o microscópio confocal, tanto em matrizes 2D quanto 3D. Foram realizados esferoides celulares para avaliar a contratilidade celular, através do plaqueamento das células em gel de agarose e a utilização de drogas que inibem ou que induzem a contratilidade, bem como a partir de células transfectadas com versões fosfomiméticas para a cadeia leve de miosina. Foi realizado ainda western blotting para proteínas: e-caderina, FAK, vinculina, paxilina, N-caderina, integrinas e as isoformas de miosina II, bem como foi avaliado os níveis de ativação das proteínas da família RhoGTPase, as quais estão envolvidas no controle da migração celular.

RESULTADOS: A expressão das MMPs analisadas e das isoformas de miosinas foi maior nas zonas de invasão tumoral, sendo que o CEC oral também apresenta uma maior expressão de proteínas associadas à adesão

com a MEC. A migração celular foi afetada pela densidade e a composição da MEC, bem como pela atividade das MMPs. Adicionalmente, a modulação das proteínas de adesão célula-matriz altera a velocidade de migração, a direcionalidade dessa migração e também a forma de migração, mudando de uma migração coletiva para uma migração individual. O aumento na contratilidade células resulta numa dispersão celular enquanto que a diminuição da contratilidade resulta numa melhor adesão célula – célula.

CONCLUSÕES: O comportamento das células tumorais pode ser modulado através de fatores extrínsecos como, por exemplo, a alteração no microambiente tumoral, seja ela por mudança no substrato ou na densidade da matriz, e também dos fatores intrínsecos como a alteração nos níveis de miosina.

PALAVRAS CHAVES: Câncer Bucal; Migração Celular; Microambiente Tumoral; Metaloproteinase de Matriz; Contratilidade Celular; Adesão celular.

ABSTRACT

INTRODUCTION: Oral squamous cell carcinoma (OSCC) presents high mortality index due to the invasive phenotype of tumor cells. Cell migration is the main event in cell invasion and metastasis and it can be regulated by intrinsic factor, such as adhesion and cell contractility, and extrinsic factors, such as density and extracellular matrix (EMC) remodeling.

OBJECTIVE: Analyze the role of intrinsic and extrinsic factor during the invasive process of oral squamous cell carcinoma.

METHODS: We performed immunostaining in OSCC samples for the following proteins: myosin II (isoforms A, B and C), matrix metalloproteinase (1, 2, 9 and 14) e-cadherin, n-cadherin, FAK, paxillin, vinculin and fibronectin. We also performed migration assays with OSCC cell line in the following conditions 1 – 2D matrix with fibronectin or laminin or matrigel; 2 – 3D matrix with collagen in the presence or not of fibronectin or laminin; 3 – 3D matrix with different collagen concentration (0,6; 1,2 e 1,8 mg/ml) with fibronectin in the presence or not of the MMP inhibitor. In order to analyze cell adhesion, it was performed Total Internal Reflectance Fluorescence and Confocal microscopy, in 2D and 3D matrix. To analyze cell contractility, cells were plated in agarose gel in order to produce spheroids, which were treated with drugs that inhibit or induce cell contractility or cells were previously transfected with Myosin Light Chain phosphomimetics mutants. It was also performed western blotting to: e-cadherin, n-cadherin, FAK, paxillin, vinculin and myosin II isoforms, as well as it was analyze the levels in RhoGTPase family, which are involved in cell migration control.

RESULTS: The expression to MMPs and myosin II isoforms were higher at invasion zone of the tumor, and the OSCC presented higher expression of proteins associated to adhesion to ECM. Cell migration was affected by the EMC composition and density and by MMP activity. Also, the modulation of cell-matrix adhesion proteins altered migration speed, cell directionality as well as influenced the switch between collective and single cell migration. The increase

in cell contractility resulted in cell dispersion while the decrease in cell contractility resulted in a better cell-cell adhesion.

CONCLUSIONS: The behavior of cell tumor can be modulate by extrinsic factors, for example, the change in tumor microenvironment, by the change in the EMC substrate or density and by intrinsic factors such as the alteration in myosin levels.

KEY WORDS: Oral Cancer; cell migration; tumor microenvironment; matrix metaloproteinase; cell contractility; cell adhesion.

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LISTA DE ABREVIATURAS E SIGLAS

MMP – Metaloproteinase de matriz

CEC – Carcinoma espinocelular/OSCC (inglês)

HCPA – Hospital de Clínicas de Porto Alegre

TIMP – inibidor de metaloproteinase de matriz

INCA – Instituto Nacional do Câncer

UV – ultravioleta

HPV – vírus do papiloma humano

TNM – sistema de estadiamento (T – tumor; N – nodo; M – metástase)

TEM – Trânsição epitélio – mesênquima/EMT (inglês)

MEC – Matriz extracelular/EMC (inglês)

GTP - guanosina trifosfato

GDP - guanosina difosfato

MNMII – miosina não muscular do tipo II

MIIA – miosina II isoform A

MIIB – miosina II isoforma B

MIIC – miosina II isoforma C

ATP – adenosina trifosfato

MLCK – miosina de cadeia leve

ELC – cadeia leve essencial de miosina II

RLC – cadeia leve regulatória de miosina II

Ser19 – Serina 19

Tre18 – treonina 18

EMMPRIM – indutor extracelular de metaloproteinase de matriz

TNF – fator de necrose tumoral

H ^{INV}/L $^{E\text{-}CAD}$ – SCC 25 – alta invasividade/ baixa expressão de e-caderina (*high invasive/low e-cadherin expression*)

L ^{INV}/H $^{E\text{-}CAD}$ – CAL 27 – baixa invasividade/alta expressão de e-caderina (*low invasive/ high e-cadherin expression*)

TAE – epitélio adjacente ao tumor (*tumor adjacent epithelia*)

CT – centro de tumor

TIRF microscopy – *Total internal reflection fluorescence microscope*

GFP – proteína fluorescente verde (*green fluorescence protein*)

SPSS – *Statistical Package for the Social Sciences*

BSA – Soro de albumina bovina

DMEM – meio de eagle modificado por Dulbecco

FBS – soro fetal bonivo

FAK – cinase de adesão focal

PBS – tampão fosfato salino.

LISTA DE SÍMBOLOS

% - Por cento

ºC – Graus Celsius

H_2O_2 – Peróxido de hidrogênio

µ - micro

SUMÁRIO

* Artigo Científico 1 aceito para a publicação na revista PlosOne.

*Artigo Científico 2 formatado de acordo com as instruções da revista Matrix Biology.

* Artigo Científico 3 formatado de acordo com as instruções da revista Tumor Biology.

1. INTRODUÇÃO

Segundo Willis (1952) “neoplasia é uma massa anormal de tecido, cujo crescimento é excessivo e não coordenado com os tecidos normais, e esse crescimento persiste mesmo após a interrupção do estímulo que o originou”. As neoplasias se formam a partir de mutações no genoma celular; essas mutações, comuns no genoma humano, normalmente são barradas no ciclo celular, onde as células entrarão em apoptose, impedindo que essa mutação seja passada adiante. Entretanto quando ocorre um descontrole neste mecanismo, a célula mutada prosseguirá seu ciclo celular ganhando autonomia e iniciando uma proliferação excessiva que resulta em crescimento desordenado e consequentemente acúmulo de novas mutações nas células neoplásicas, permitindo assim a permanência do tumor (1).

Clinicamente, os tumores podem ser divididos em benignos e malignos. Os tumores benignos são aqueles onde a proliferação das células neoplásicas fica confinada ao tecido onde elas surgem, sem a capacidade de invadir os tecidos adjacentes nem de disseminar para tecidos distantes (metástase), além de apresentarem um crescimento lento. Já os tumores malignos possuem a capacidade de invadir e destruir os tecidos adjacentes e metastatizar para locais distantes formando novos tumores semelhantes ao primeiro tumor (1).

O câncer é um problema de saúde pública mundial. Nos Estados Unidos (EUA), no ano de 2013, uma a cada quatro pessoas morreram em decorrência da doença. No ano de 2012 ocorreram cerca de 14.1 milhões de novos casos no mundo e 8.2 milhões de pessoas morreram devido ao câncer. Para o ano de 2030 espera-se que ocorram 21.4 milhões de novos casos de câncer causando 13.2 milhões mortes (2). Nos EUA, estima-se que no ano de 2013 ocorreram 41.380 casos de câncer de boca e faringe e desses 7.890 pessoas morreram em decorrência desse câncer.

No Brasil, o Instituto Nacional do Câncer (INCA) estima para 2016 que ocorram 596 mil novos casos de câncer, e desses 15.49 mil são em cavidade bucal, sendo o quinto mais comum em homens representando 5.2% dos casos

(3). No ano de 2010, o câncer foi a segunda maior causa de mortes no Brasil (cerca de 16%), sendo que 4.891 dos óbitos foram decorrentes de câncer de boca. A estimativa de sobrevida dos pacientes com câncer de boca em 5 anos foi de 50% para homens e 60% para mulheres, e essa taxa não tem modificado muito ao longo do tempo (3, 4). Dentre os tumores malignos que acometem a cavidade oral o carcinoma espinocelular (CEC), é o mais frequente (95%), se origina da camada espinhosa do epitélio oral e apresenta diferentes graus de diferenciação escamosa. O CEC apresenta alta taxa de mortalidade e morbidade, principalmente devido a propensão de realizar metástases extensas e precoces para linfonodos regionais (5).

Além das mutações, as células tumorais passam por diversas alterações bioquímicas e estruturais que permitirão que ela se adapte a novos ambientes com maior facilidade. Dentre essas modificações destacam-se: desregulação energética celular, manutenção para sinalização de proliferação, fuga dos fatores de supressão, fuga do sistema imune, ativação de replicação e imortalidade, ativação de invasão e metástase, indução da angiogênese e resistência à morte celular (6). Destas modificações, a ocorrência de metástase é um dos principais eventos relacionados ao insucesso clínico das terapias atuais, sendo influenciada tanto por características intrínsecas das células tumorais, como a ativação de vias de sinalização relacionadas à migração celular e a aquisição de um fenótipo característico de células mesenquimais, quanto por elementos extrínsecos presentes no microambiente tumoral, o qual é composto por fibroblastos, células endoteliais, células do sistema imune, além da matriz extracelular.

A compreensão de como ocorre à interação dos fatores intrínsecos e extrínsecos relacionadas à metástase das células tumorais é crucial para o entendimento da progressão tumoral, bem como para o desenvolvimento de novas terapias que auxiliem no controle do câncer. Esta tese está dividida em capítulos onde serão abordados os temas migração celular, contratilidade celular, microambiente tumoral, bem como as repercussões que alterações nesses fatores causam no comportamento agressivo do CEC bucal.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Carcinoma espinocelular bucal

O Carcinoma Espinocelular Bucal (CEC) é uma neoplasia de origem epitelial invasiva com vários graus de diferenciação escamosa. Ele apresenta propensão a metástases precoces e extensas para linfonodos. O CEC ocorre principalmente em homens na 5ª ou 6ª década de vida (5).

O CEC é responsável por aproximadamente 90% de todas as neoplasias malignas da cavidade bucal, ocorrendo principalmente nas regiões de lábio, língua e assoalho de boca (7). Para o ano de 2014, a Organização Mundial da Saúde (OMS) considerou esse tumor como o oitavo tipo de tumor mais frequente em todo o mundo (8). Anualmente mais de 500.000 casos de câncer de cabeça e pescoço são diagnosticados, sendo 50.000 apenas nos Estados Unidos, o que faz do CEC o sexto mais comum nesse país em 2013 (9). No Brasil, o Instituto Nacional do Câncer (3), estimou para o ano de 2016 que ocorram 15.490 novos casos de CEC bucal, sendo 11.140 em homens. Para o estado do Rio Grande do Sul, o risco estimado é de 15.49 novos casos entre os homens e 3.4 entre as mulheres para cada 100 mil habitantes.

A etiologia do CEC é multifatorial, sendo composta por fatores endógenos, como a predisposição genética, e fatores exógenos ambientais e comportamentais, como uso do tabaco, ingestão de bebidas alcoólicas, exposição à radiação solar e produtos químicos carcinogênicos (10-12). Os CECs podem se originar a partir de lesões prévias presentes na mucosa bucal (desordens potencialmente malignas) ou devido a uma alteração direta das células epiteliais normais em células tumorais (13).

Durante a carcinogênese, inicialmente ocorrem alterações proliferativas e posteriormente alterações no processo invasivo. A maioria dos casos está associada ao etilismo e tabagismo, sendo que esses fatores possuem um efeito sinérgico, onde o álcool atua como um agente permeabilizante na mucosa oral permitindo a penetração de carcinógenos contidos no cigarro,

aumentando em até 7 vezes o risco de desenvolvimento de CEC bucal (14). Os agentes carcinogênicos presentes no tabaco podem causar alterações no DNA resultando em alterações na estrutura do mesmo e com isso causando mutações (15). Essas mutações podem causar, por exemplo, perda de função do gene p53, que codifica uma proteína supressora de tumor (proteína p53) que interrompe o ciclo celular. Falhas na atividade de p53 resultam em falhas na regulação do ciclo celular e apoptose, causando uma proliferação celular descontrolada e a perpetuação de novas mutações (16).

O CEC no lábio é causado principalmente pela exposição à radiação ultravioleta (UV). A luz UV causa um aumento no número de mutações no DNA durante o ciclo celular (17) as quais, quando não corrigidas podem realizar a ativação de proto-oncogenes como o RAS, resultando na proliferação celular desordenada (17, 18).

Entre os agentes biológicos relacionados ao CEC, o papiloma vírus (HPV) possui posição de destaque (19, 20). Durante a infecção viral, o vírus utiliza o material genético do hospedeiro para controlar seu ciclo celular. Em casos de uma infecção persistente, o vírus perde o controle sobre sua expressão gênica causando a produção de proteínas que irão degradar a proteína p53 e inativar outras proteínas da célula hospedeira que levarão a desregulação do ciclo celular e consequente aumento da proliferação celular e das características malignas da lesão (14, 18, 21).

2.2 Características clínicas, microscópicas e moleculares

Clinicamente, o CEC pode se apresentar como uma placa branca ou vermelha não removível a raspagem, um nódulo ou aumento de volume ou ainda como uma úlcera que não cicatriza, com mais de duas semanas de evolução, endurecimento dos tecidos adjacentes, perda dental, dor ou sangramento bucal (11, 22).

Microscopicamente, o CEC é caracterizado pela invasão do tecido conjuntivo pelas células epiteliais tumorais na forma de ilhas, lençóis ou cordões. Estas ilhas apresentam diferentes graus de variação escamosa

podendo apresentar disceratose e pérolas de ceratina. Adicionalmente, podem apresentar atipia nuclear e celular e alterações arquiteturais como estratificação epitelial irregular, projeções epiteliais em forma de gota, alteração na polaridade das células da camada basal, aumento no número de mitoses e perda de adesão celular (5). Histologicamente eles podem ser graduados em 4 níveis, baseado no grau de diferenciação do tumor no fronte de invasão. De acordo com os critérios de Bryne, estabelecidos em 1992 (23) o carcinoma de grau I ou bem diferenciado contém menos de 20% de células indiferenciadas, as quais são células que não passaram pelo processo de diferenciação celular tornando suas características muito diferentes da célula que deu origem, neste caso a célula epitelial; grau II ou moderadamente diferenciado com menos de 50% das células indiferenciadas; grau III ou pouco diferenciado com menos de 75% das células indiferenciadas e; grau IV ou pleomórfico com mais de 75% de células indiferenciadas (24).

O prognóstico desses pacientes é baseado no sistema TNM onde são considerados o tamanho do tumor (T), a presença ou não de metástase nos linfonodos (N) e metástase a distância (M). A partir da associação destes parâmetros, esses tumores são classificados em estadios, onde o primeiro estadio é o que apresenta melhor prognóstico, pois são tumores menores e sem a presença de metástases, já o estadio IV possui o pior prognóstico, pois geralmente os tumores são maiores e/ou com a presença de metástases (25).

O tratamento desses tumores está baseado no estadio clínico. Pacientes com estadios mais iniciais (I e II) normalmente são tratados apenas com uma modalidade de tratamento, nesses casos normalmente opta-se por cirurgia ou radioterapia, porém pacientes em estadios mais avançados (III e IV) esses tratamentos não são suficientes, necessitando de tratamentos complementares como a quimioterapia ou ainda associação de mais de um tratamento (26, 27).

Além das características clínicas e microscópicas, nas últimas décadas têm sido estudados extensivamente os mecanismos moleculares relacionados à carcinogênese, com o potencial uso destas informações no processo de decisão de tratamento. Os tumores de origem epitelial passam pelo processo

denominado Transição Epitélio Mesenquima (TEM), onde eles perdem suas características epiteliais e passam a apresentar características mesenquimais, o que auxilia durante o processo de invasão e metástase. Dentre as alterações fenotípicas que ocorrem durante esse processo, as células tumorais perdem ou diminuem a expressão de proteínas como a E-caderina e a queratina e passam a expressar proteínas tipicamente mesenquimais como a N-caderina e a Vimentina. Diversos estudos já demostraram que essa alteração apresenta uma forte relação com a agressividade tumoral, onde células com maior expressão de vimentina e N-caderina apresentam um comportamento mais agressivo e consequentemente um pior prognóstico (6, 21, 28, 29). Porém dentro de um mesmo tumor aparecem populações celulares com fenótipos heterogêneos, onde células localizadas na região de fronte de invasão apresentam características mesenquimais o que facilita o deslocamento dessas células através da matriz extracelular (MEC) e a implantação das mesmas no novo sítio dando origem a uma metástase (29-31). Apesar de todos os estudos e avanços realizados na área com o intuito de auxiliar no tratamento ainda se faz necessário entender como essas alterações moleculares influenciam o comportamento das células tumorais.

2.3 Fatores envolvidos na progressão tumoral

Os tumores malignos podem apresentar aspectos comportamentais distintos de acordo com a evolução da doença. A progressão tumoral depende da adaptação que a célula sofre durante o processo de carcinogênese, para que adquira capacidade de sobreviver aos diferentes desafios a que esta será submetida durante a progressão tumoral. Essas adaptações resultam em uma maior capacidade de manter ativado o processo de proliferação celular, indução de angiogênese (formação de novos vasos sanguíneos), resistir aos mecanismos de morte celular, fugir dos sistemas de supressão tumoral e imune, além de potencializar os processos de ativação de invasão e metástase. Outros meios de adaptação também são utilizados como a ativação de replicação e imortalidade, além de causar uma desregulação energética

celular, possibilitando que a mesma possa sobreviver aos diferentes ambientes e condições que a célula tumoral será submetida (6).

Dentre as adaptações que a célula tumoral passa durante o processo de progressão tumoral, a invasão tecidual e o desenvolvimento do potencial metastático dos tumores malignos podem ser considerados a maior causa de insucessos clínicos em termos de terapias e prognósticos. A invasão pode ocorrer individualmente, onde células migram isoladamente para formar uma interface tumoral difusa; ou coletivamente, em que grupos de células invadem os tecidos e mantêm contatos celulares firmes. Estas formas de invasão são caracterizadas por alterações moleculares importantes, tais como modificação da adesão célula-célula e na adesão célula-matriz extracelular além de outros fatores como a regulação da contratilidade celular, que podem influenciar o processo de invasão (32-35).

2. 4 Migração celular

A migração celular é uma característica crítica de numerosos fenômenos fisiológicos e patológicos, incluindo desenvolvimento, reparo de feridas, angiogênese e metástases. Nos processos fisiológicos para que ocorra homeostasia nos tecidos a migração celular precisa manter uma direcionalidade, para que cada célula consiga desempenhar adequadamente suas funções. As células migratórias de um tumor não são coordenadas, estão randomicamente orientadas e se dividem em grupos de confusa organização ou se isolam, levando à alteração da estrutura tecidual (36-39). Por exemplo, nos tumores de origem epitelial ocorre a ruptura da lâmina basal que separa o epitélio do tecido conjuntivo subjacente e consequente invasão do tumor. Este processo invasivo é influenciado pela TEM onde as células perdem marcadores de adesão célula-célula facilitando a transição de uma migração coletiva para uma migração individual (36, 40, 41). Assim sendo, a invasão tumoral facilita a emergência das metástases, espalhando as células do câncer para outras partes do corpo e contribuindo para a formação de tumores secundários (36-38).

A migração celular é um fenômeno que depende tanto de fatores intrínsecos, como a ativação de vias de sinalização e contratilidade celular, quanto de fatores extrínsecos como os componentes da matriz extracelular (MEC). Para que a migração ocorra é necessária a ativação de diversas moléculas estruturais e de sinalização que vão determinar uma assimetria espacial, tornando a célula com uma morfologia polarizada (figura 1). Inicialmente, ocorre a polimerização de actina na porção frontal da célula (*leading edge*), promovendo a formação de projeções de membranas, chamadas de lamelipódio. Após, formam-se novas adesões ao substrato , que podem ser do tipo adesões nascentes, pontos e complexos focais. Este é um processo extremamente dinâmico e ocorre através da interação entre proteínas transmembranas como as integrinas com proteínas da MEC. A adesão formada irá desencadear uma cascata de sinalização intracelular e também o recrutamento de proteínas a qual é dependente do tipo e do número de moléculas de integrinas recrutadas na ligação com a MEC. Algumas dessas adesões nascentes formadas inicialmente irão amadurecer através da troca definida de moléculas de adesão (*turnover*), as quais permitirão a ancoragem de feixes de actina-miosina II que resultam em fibras de estresse e com isso gerando forças contráteis que irão auxiliar na movimentação do corpo celular na direção determinada. Finalizando o ciclo acontecerá a liberação das adesões na porção posterior da célula e com isso o deslocamento celular no sentido determinado pelo lamelipódio (42-49).

Cada etapa da migração celular é regulada por GTPases de baixo peso molecular pertencentes à família Rho (de Ras-homology), que desempenham um papel fundamental nesse processo. Estas proteínas ciclam entre um estado inativo (ligadas a GDP) e um estado ativo (ligadas a GTP). Quando ativadas, exercem seus efeitos por meio de uma vasta quantidade de proteínas efetoras. Entre as GTPases mais expressas encontra-se a proteína Rac1, que está principalmente envolvida na formação de lamelipódios e complexos focais, enquanto RhoA está envolvida principalmente na formação das fibras de estresse, na maturação de adesões e na contratilidade celular através da

ativação de miosinas (50, 51). O balanço recíproco entre estas duas GTPases determinara a morfologia celular e o comportamento migratório (52, 53).

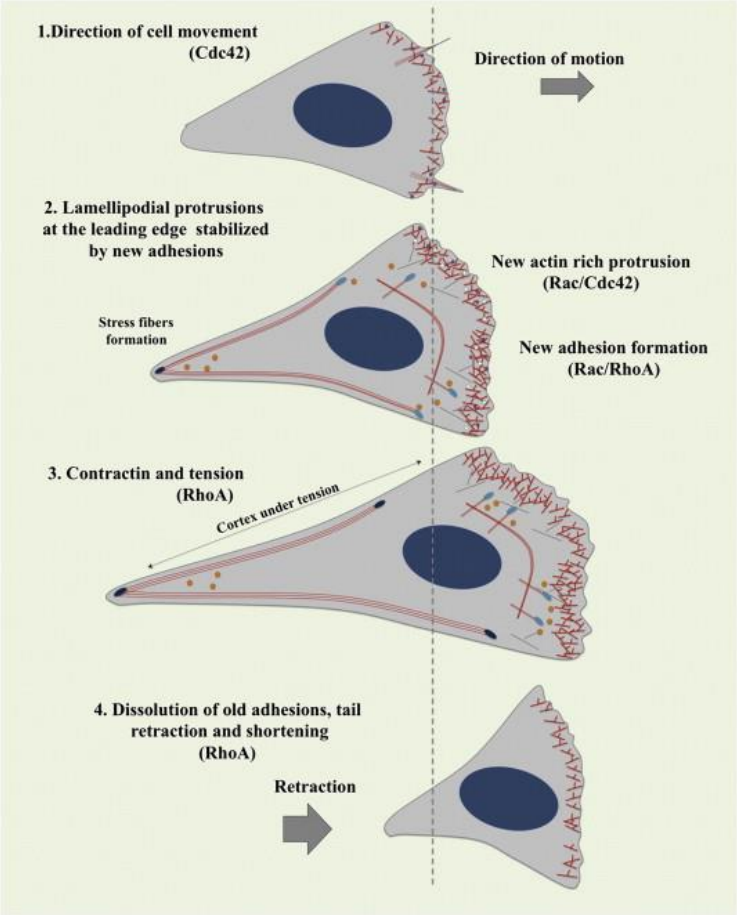

Figura 1: Etapas de migração celular e sua regulação através da ação de GTPases. 1 – Direção da movimentação é definida pelo Cdc42; 2 – a estabilização do lamelipódio no fronte celular é realizado através da estabilização das novas adesões, a projeção é regulada pela Rac e Cdc42, já a formação de novas adesões é regulada pela Rac e RhoA, ocorre ainda a formação de fibras de estresse; 3 – a contração e tensão do córtex celular é regulada pela ação da RhoA; 4 – dissolução das adesões maduras, retração da parte posterior da célula e encurtamento celular, essas etapas são reguladas pela RhoA. Fonte: Hanna S. e El-Sibai, 2013 (54).

2.5 Contratilidade celular

A indução da contratilidade celular é um dos principais fenômenos necessários para que ocorra a migração e é realizada através de proteínas motoras como a miosina não muscular do tipo II (MNMII) (Figura 2).

Fisiologicamente ela está envolvida na organização do citoesqueleto de actina, polarização e regulação celular, porém ela também está envolvida durante a tumorigênese, divisão celular e invasão (55). A MNMII apresenta três isoformas: miosina IIA (MIIA), miosina IIB (MIIB) e miosina IIC (MIIC). Essas três isoformas são codificadas por três diferentes genes. Elas exibem diferenças em suas propriedades enzimáticas, localização celular e modelos de expressão tecidual. A distinção entre as três moléculas de miosina II é baseada em suas cadeias pesadas, uma vez que cada isoforma realiza a mesma função molecular básica, que é a ligação e a contração de F-actina de uma forma ATP-dependente, e a atividade das três isoformas é regulada de uma maneira semelhante, através da fosforilação da cadeia leve de miosina principalmente via atividade da enzima myosin light chain kinase (MLCK) (56). As isoformas colaboram em diversas etapas durante o processo de migração celular, a MIIA participa do processo de maturação das adesões na parte anterior da célula, formação do lamelipódio, além de estar associada com funções Rho-quinase dependente. Já a MIIB auxilia na contração do corpo celular, estabilização da polaridade celular, deslocamento do núcleo e descolamento da célula do substrato (57, 58). O papel da Miosina IIC ainda é controverso, sendo que a sua expressão encontra-se aumentada em diferentes tipos de tumores (59).

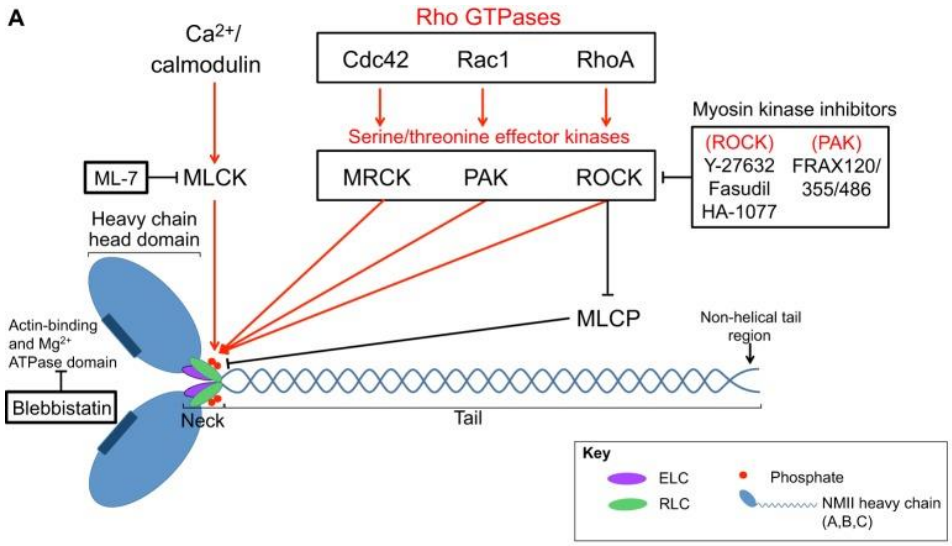

Figura 2: Estrutura da MNMII e sua regulação através de serina/treonina cinases. A MNMII consiste em uma cadeia pesada que é formada por uma cabeça globular onde se ligam a actina e o ATP; a região do pescoço, onde se ligam as cadeias leves regulatórias (ELC

e RLC); e uma cauda que está formada por homodímeros organizados em formato helicoidal, bem como uma região de cauda não helicoidal onde está localizado a isoforma da MNMII. As serina/treonina cinases regulam a atividade da MNMII através da fosforilação da miosina RLC no resíduo Tre18 e Ser19. Essas cinases são reguladas pelas Rho GTPases, bem como pelas via de sinalização Ca^{2+}/calmodulina. Essa imagem ainda indica como inibidores farmacológicos podem modular a atividade da MNMII. Fonte: Newell – Litwa K et al., 2015 (60)

Durante a transição epitélio mesênquima, a contratilidade mediada pela ação das MNMII pode influenciar o comportamento migratório através da mudança de uma migração coletiva para uma migração individual (61). A expressão anormal dessas proteínas pode interferir nos mecanismos moleculares envolvidos na carcinogênese, podendo estar relacionado com o grau de malignidade dos tumores. Essas alterações levariam ao aumento da contratilidade celular resultando em alterações no processo de divisão celular (59, 62), bem como auxiliando na invasão das células tumorais em matrizes rígidas (63). Drogas que inibem a contratilidade, como a blebistatina e a ML-7 influenciam o comportamento invasivo das células tumorais, reduzindo a migração celular (64-69), demonstrando a influência da contratilidade no comportamento das células tumorais. Adicionalmente, evidências mostram que fatores extrínsecos ao tumor podem influenciar a atividade das miosinas como, por exemplo, a ação de quimioatraentes, fatores de crescimento e a organização da matriz extracelular (60). Além disso, elementos do microambiente também podem interferir na atividade das miosinas e consequentemente no comportamento invasivo dos tumores, e a compreensão de como esses fatores extrínsecos modulam o comportamento invasivo das células tumorais é de extrema importância na busca por terapias para o tratamento dessa doença.

2.6 Microambiente tumoral

Durante muito tempo o câncer foi considerado como um processo em que células autônomas, com uma série de alterações genéticas e epigenéticas, se desenvolvem e progridem independentes do meio em que estão inseridas (70). Contudo evidências mostram que o tumor se utiliza de todo o

microambiente em que está inserido, onde além das células tumorais outros grupos celulares interagem formando o denominado microambiente tumoral (71).

O conceito de microambiente tumoral é antigo. Em 1889 Stephen Paget publicou um artigo onde apresentou o conceito do "*seed and soil*" que afirma que quando uma planta vai semear, suas sementes são lançadas para todas as direções, mas ela somente vai viver e crescer se cair em solo adequado e esse autor acredita que as células tumorais possuem esse mesmo princípio (72). Neste contexto, as células que serão semeadas podem ser chamadas de células progenitoras, iniciadoras, células tronco ou ainda metastáticas e o solo adequado será o microambiente tumoral, estroma e ainda os fatores do hospedeiro.

O microambiente tumoral é um fator crucial na tumorigênese, sendo que a progressão tumoral e a própria patogênese do câncer são em grande parte dependentes de suas interações com os componentes do microambiente. O microambiente do tumor é uma dinâmica rede que inclui as células tumorais, células endoteliais, pericitos, fibroblastos, fibroblastos associados ao tumor, miofibroblastos, células imunes e pericitos, bem como a matriz extracelular que a rodeia (6, 20, 73). Neste trabalho, será focado o papel da organização da MEC sobre as células tumorais.

2.7 Matriz extracelular

A MEC é formada por um complexo de proteínas e glicoproteínas que são secretados e formam uma rede organizada que envolve as células. É constituída por proteínas estruturais como colágeno, laminina, fibronectina, vitronectina, elastina, glicoproteínas e proteoglicanos, porém essa composição pode variar de tecido para tecido (74, 75). Além de propiciar a sustentação dos tecidos, a MEC desempenha um papel essencial no desenvolvimento de processos biológicos de reparo tecidual e metástase através da regulação da proliferação, diferenciação, adesão, migração e expressão gênica celular (76-81).

2.8 Remodelação da matriz extracelular

A organização da MEC pode ser dinâmica através de elementos que atuam na sua remodelação, sendo este processo capaz de alterar o comportamento celular (81). A remodelação da MEC ocorre tanto durante processos fisiológicos como na remodelação óssea ou nas glândulas mamárias, quanto em processos patológicos como o câncer (81). A remodelação da matriz extracelular é resultado de um processo múltiplo que varia de acordo com um estímulo inicial (82). O processo de remodelamento depende de duas etapas principais: a síntese de novos componentes da MEC pelas células e a degradação através de enzimas proteolíticas como as metaloproteinases de matriz (MMPs) (82). Adicionalmente, modificações físicas e químicas também podem ocorrer, sugerindo que este processo é altamente regulado de maneira a garantir a homeostase tecidual (83).

2.9 Metaloproteinase de matriz

As metaloproteinases de matriz (MMPs) são enzimas proteolíticas que estão envolvidas na degradação da MEC. Em condições fisiológicas, desempenham funções importantes na regulação da MEC durante a embriogênese ou remodelação de tecidos. Em condições patológicas, as MMPs participam da destruição da MEC em processos inflamatórios, além do crescimento de tumores e cistos (77, 84, 85). As MMPs são secretadas por células como fibroblastos, macrófagos, neutrófilos, linfócitos, ceratinócitos e células tumorais (86).

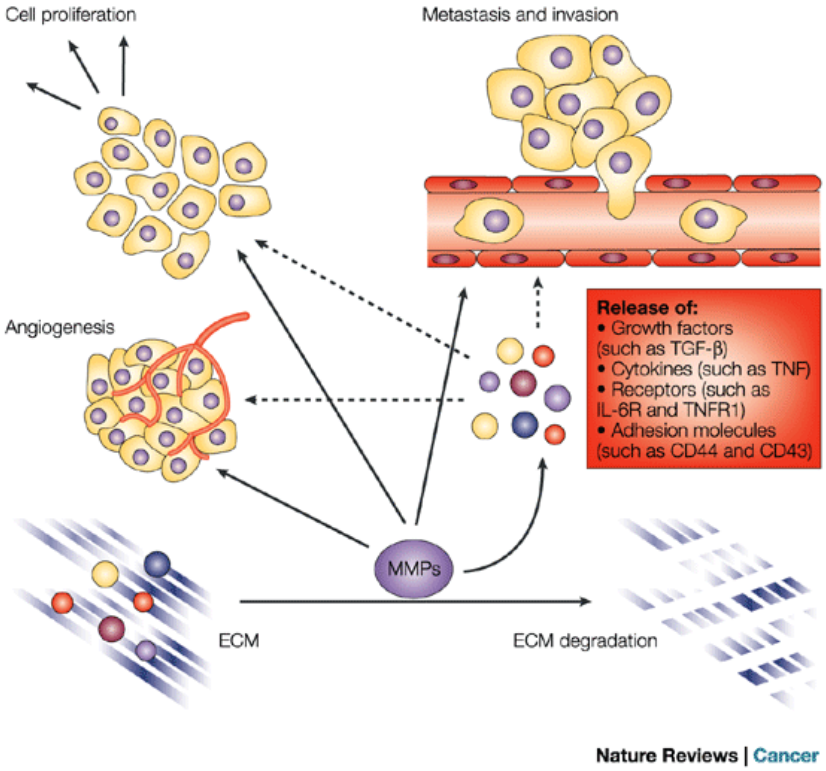

Figura 3: Potenciais ações das MMPs – MMPs estão envolvidas em diferentes eventos celulares como proliferação celular; angiogênese; formação e degradação da MEC, liberação de fatores de crescimento, citocinas, receptores e moléculas de adesão (CD44 e CD43); invasão e metástase. Fonte: Rao JS et. al. 2003 (87)

Muitos tumores produzem as MMPs como parte do mecanismo de invasão; no entanto, o processo ainda não é muito conhecido (77, 78, 84, 85, 88-90). A degradação dos componentes da MEC pelas MMPs favorece a migração, diferenciação e proliferação celular que ocorrem nos processos de reparo, angiogênese, invasão de células neoplásicas e metástase. A ação das MMPs disponibiliza fatores de crescimento no meio, modificando a MEC e expondo sítios ocultos que alteram o ambiente tecidual, favorecendo assim o desenvolvimento e crescimento de tumores primários e metastáticos (76, 77, 84, 85, 90-93).

Cerca de vinte e quatro tipos de MMPs (tabela 1) já foram identificados em humanos, sendo classificadas de acordo com sua estrutura molecular e o substrato que degradam. Elas se dividem em MMPs associadas à membrana e em MMPs solúveis. As MMPs associadas à membrana são representadas por

seis tipos, divididas de acordo com o mecanismo de ancoragem à membrana. As MMPs solúveis são divididas principalmente em: 1- colagenases, as quais degradam o colágeno intersticial tipo I, II, III e V; 2- gelatinases, responsáveis por degradar colágenos previamente desnaturados; e 3- estromelisinas, as quais destroem especificamente fibronectina, elastina, laminina e a proteína central das proteoglicanas (76, 92).

A metaloproteinase de matriz–1 (MMP-1), ou colagenase A, faz parte do grupo das colagenases e participa da clivagem de colágeno tipo I, II, III, VII, VIII e X, além de outras moléculas como a tenascina C. Ela participa de processos como migração de ceratinócitos e reepitelização, aumento da proliferação celular e ação pró e anti–inflamatória (76, 92, 93). A MMP-1 é sintetizada por células tumorais, fibroblastos, macrófagos, plasmócitos, linfócitos e neutrófilos. Durante o processo de invasão, sua produção ocorre em resposta a fatores produzidos pelas células neoplásicas, e sua expressão é observada no estroma junto ao local de invasão (76, 85, 94).

A metaloproteinase de matriz-2 (MMP-2), ou gelatinase A, tem como principal função degradar colágeno tipo IV e outros componentes da MEC como colágeno tipo V, VII, X, gelatina tipo I, fibronectina e elastina (76, 92, 93). Sua expressão está associada a cistos odontogênicos (95), ameloblastomas (88) e na invasão do CEC (96, 97). Segundo Zhang et al. (2009), a MMP-2 é uma das mais importantes enzimas proteolíticas que degradam a membrana basal e a MEC, estando relacionada à invasão tumoral e metástase.

A metaloproteinase de matriz–9 (MMP-9), ou gelatinase B, assim como a MMP-2, possui a capacidade principal de degradar colágeno do tipo IV e V, além das gelatinas I e V. É secretada por células tumorais e células inflamatórias, sendo responsável pelo aumento da afinidade ao colágeno, ação pró e anti–inflamatória e resistência das células tumorais aos mecanismos de defesa do hospedeiro (76, 84, 92, 93).

A metaloproteinase de matriz -14 (MMP-14) ou metaloproteinase de matriz tipo membrana - 1 (MT-MMP-1) é uma MMP associada à membrana

responsável principalmente pela degradação do colágeno tipo I, II e III, gelatina, caseína, fibronectina, laminina, vibronectina, entactina, nidogena, proteoglicanos e fibrina (84). Na clivagem do colágeno hidrolisado tipo I e gelatina, a MMP-14 é mais efetiva que a MMP-1 (84, 90, 92). A MMP-14 está associada à angiogênese, migração e adesão celular, apoptose e morte celular por fagocitose. Além disso, está presente na artrite, tumores de pulmão, isquemia – reperfusão e adenocarcinomas de mama (98, 99).

As MMPs são reguladas pelos inibidores teciduais de metaloproteinases (TIMPs), que são uma pequena família de proteínas que inibem a atividade das MMPs (98), e foram identificados quatro TIMPs (TIMPs 1 – 4) (98). As TIMPs participam de processos como inibição da invasão celular, tumorigênese, apoptose, metástases e angiogênese (98, 99). A homeostase tecidual é atingida através do balanço entre a expressão de MMPs e TIMPs (84). Na tabela 1 podemos observar a atividade de cada TIMP especificamente (90, 100).

Muitas proteinases encontradas no fronte de invasão, recebem a contribuição das células do estroma (fibroblastos, células inflamatórias e células endoteliais). As células tumorais usam essas enzimas para romper a membrana basal invadir os tecidos adjacentes e metastatizar para os tecidos à distância. A produção de MMPs pelas células do estroma é regulada pelas células tumorais através da produção de quimiocinas, citocinas e EMMPRIN (indutor extracelular de metaloproteinase de matriz). A influência dos TIMPs no comportamento invasivo as células tumorais é autônomo, depende apenas do genótipo do TIMP do tumor e não de fatores relacionados ao paciente (101). Diversos estudos já descreveram a expressão das MMPs no CEC oral (102-105), e eles mostraram que as MMPs estão correlacionadas com a pobre diferenciação do tumor (103-105), com a graduação histológica do tumor e metástases para linfonodos (102), além disso, a MMP-1 está mais expressa quando comparada com a mucosa normal (103).

Tabela 1: Classificação das MMPs, seus substratos e inibidores.

MMP	TIPO	SUBSTRATO	INIBIDOR
MMP-1	Colagenase	Colágeno (I, II, III, VII, VIII, X), Caseína, Laminina, Pró-MMP-1, 2 e 9; MMP-2 e 9, Agrecana, Gelatina, IL-1β.	TIMP- 1, 2, 3 e 4
MMP-2	Gelatinase	Gelatina, Colágeno (I, IV, V, VI, X, XI, XIV), Elastina, Fibronectina, Laminina, Agrecana, Osteonectina, MMP-1; MMP-9; MMP-13.	TIMP- 1, 2, 3 e 4
MMP-3	Estromelisinas	Colágeno (III, IV, V e IX), Agrecana, Elastina, Caseína, Plasminogênio, Laminina, Gelatina, Fibronectina, IL-1β; MMP-2/ TIMP-2; MMP-7; MMP-8; MMP-9; MMP-13.	TIMP- 1, 2, 3 e 4
MMP-7	Matrilisina	Colágeno (IV, V, VI, VII, VIII, IX, X), Fibronectina, Laminina, Gelatina, Pró-MMP-9, Agrecana, Decorina, Entactina, Elastina, Caseína, Plasminogênio, β4-integrina; MMP-1; MMP-2; MMP-9; MMP-9/TIMP-1	TIMP- 1, 2 e 4
MMP-8	Colagenase	Colágeno (I, II, III, V, VII, VIII, X), Gelatina, Fibronectina, Agrecana.	TIMP- 1, 2
MMP-9	Gelatinase	Gelatina, Colágeno (IV, V, VII, X, XIV), Elastina, Fibrilina e Osteonectina, Plasminogênio, Agrecana, Fibronectina.	TIMP- 1, 2, 3 e 4
MMP-10	Estromelisinas	Colágeno (III, IV, V), Gelatina, Caseína, Elastina, Agrecana, MMP-1, 8	TIMP- 1, 2
MMP-11	Estromelisinas tipo MMP	Fibronectina, Laminina, Gelatina.	TIMP- 1, 2
MMP-12	Estromelisinas tipo MMP	Elastina, Gelatina, Colágeno (I e IV), Fibronectina, Laminina, Vitronectina, Proteoglicanas, Caseína, Fibrina, Fibrinogênio, Plasminogênio.	TIMP- 1, 2
MMP-13	Colagenase	Colágeno (II, V, IX, X, XIV), Gelatina, Plasminogênio e Fibronectina, Agrecana; Perlecana, Onteonectina, MMP-9	TIMP- 1, 2 e 3
MMP-14	Associada a membrana	Colágeno (I, II, III), Gelatina, Fibronectina, Laminina, Tenacina, Caseína,	

		Vitronectina, Proteoglicanas; MMP-2; MMP-13	
MMP-15	Associada a membrana	Fibronectina, Laminina, Perlecana, Agrecana, MMP-2	TIMP- 1, 2
MMP-16	Associada a membrana	Colágeno III, Caseína, Gelatina, Fibronectina, MMP-2	TIMP- 1, 2
MMP-17	Associada a membrana	Fibronectina, precursor de TNF.	TIMP- 1, 2
MMP-18	Colagenase	Colágeno (I, II, III, VIII, IX, X); gelatina; Agrecana.	TIMP-1, 2
MMP-19	Outras	Colágeno tipo I, Gelatina, Agrecana, Fibronectina.	TIMP- 1, 2
MMP-20	Outras	Amelogenina, Agrecana.	TIMP- 1, 2
MMP-21	Outras	Gelatina	TIMP- 1, 2
MMP-22	Desconhecido	Desconhecido	Desconhecido
MMP-23	Outras	Gelatina	TIMP- 1, 2
MMP-24	Associada a membrana	Proteoglicanas	TIMP- 1, 2
MMP-25	Associada a membrana	Colágeno IV, gelatina, Fibronectina, Fibrina, Pró-MMP-2.	TIMP- 1, 2
MMP-26	Matrilisina	Gelatina, Colágeno IV, Pró-MMP-9, Fibrinogênio, Fibronectina, Vitronectina.	TIMP- 1, 2
MMP-27	Desconhecido	Desconhecido	Desconhecido
MMP-28	Outras	Caseína	TIMP- 1, 2

Legenda: MMP – metaloproteinase de matriz; TIMP – inibidor tecidual de MMP; TNF – fator de necrose tumoral;

3. HIPÓTESE

A hipótese deste trabalho é que tanto fatores intrínsecos, como a contratilidade celular, quanto extrínsecos, como a composição do microambiente, são capazes de modular o comportamento invasivo do CEC de origem bucal.

4. OBJETIVOS

4.1 Objetivo geral

- Avaliar o papel de elementos intrínsecos e extrínsecos sobre o processo invasivo do carcinoma espinocelular de origem bucal.

4.2 Objetivos específicos

- Analisar o papel das miosinas não musculares do tipo II e da contratilidade celular na invasão tumoral.
- Avaliar os efeitos da composição da matriz extracelular sobre a migração celular de células tumorais.
- Caracterizar o papel da remodelação da matriz extracelular sobre a invasividade do carcinoma espinocelular oral.

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6. ARTIGO CIENTÍFICO 1

Artigo aceito para publicação na revista PLOS ONE

Fibronectin modulates cell adhesion and signaling to promote single cell migration of highly invasive Oral Squamous Cell Carcinoma

Running title: Extracellular matrix composition affects tumor invasion

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Abstract

Cell migration is regulated by adhesion to the extracellular matrix (ECM) through integrins and activation of small RhoGTPases, such as RhoA and Rac1, resulting in changes to actomyosin organization. During invasion, epithelial-derived tumor cells switch from laminin-enriched basal membrane to collagen and fibronectin-enriched connective tissue. How this switch affects the tumor migration is still unclear. We tested the hypothesis that ECM dictates the invasiveness of Oral Squamous Cell Carcinoma (OSCC). We analyzed the migratory properties of two OSCC lines, a low invasive cell line with high e-cadherin levels ($L^{inv}/H^{E\text{-}cad}$) or a highly invasive cell line with low e-cadherin levels ($H^{inv}/L^{E\text{-}cad}$), plated on different ECM components. Compared to laminin, fibronectin induced non-directional collective migration and decreased RhoA activity in $L^{inv}/H^{E\text{-}cad}$ OSCC. For $H^{inv}/L^{E\text{-}cad}$ OSCC, fibronectin increased Rac1 activity and induced smaller adhesions, resulting in a fast single cell migration in both 2D and 3D environments. Consistent with these observations, human OSCC biopsies exhibited similar changes in cell-ECM adhesion distribution at the invasive front of the tumor, where cells encounter fibronectin. Our results indicate that ECM composition might induce a switch from collective to single cell migration according to tumor invasiveness due to changes in cell-ECM adhesion and the resulting signaling pathways that alter actomyosin organization.

Keywords: Laminin, Paxillin, Vinculin, FAK, Rac1, RhoA

Introduction

Oral squamous cell carcinoma (OSCC) is an epithelial neoplasm found in 80 – 90% of head and neck cancer [1]. OSCC can occur at several sites of the oral mucosa and is originated from genetically altered keratinocytes arising from exposure to a wide range of mutagenic agents [2]. Histopathologically, OSCC lesions are characterized by the presence of different degrees of squamous differentiation, keratin production, nuclear pleomorphisms, mitotic activity, invasive growth and metastasis. Despite advances in treatment, the OSCC prognosis remains poor with a 5 year survival rate of around 50%. This prognosis has not improved over the past several years due to the development of distant metastasis, local recurrences and new tumors [1, 3, 4].

The ability of tumor cells to invade connective tissue is essential for them to access blood vessels and ultimately promote distant metastasis. Both events, tissue invasion and metastasis, are highly heterogeneous processes [5], requiring tumor cell adaptation to new environments that alter the migratory mode. Depending on the tumor origin, differentiation level, and tumor microenvironment, cancer cells migrate either as collective or single cells [6]. Amoeboid- and mesenchymal-like single cell migration involve the coordinated interaction of structural and signaling molecules that results in polymerization of actin at the leading edge, adhesion to the extracellular matrix (ECM) through integrins, contraction of the cell cortex and detachment of adhesions at the cell rear [7, 8], whereas cluster or strand like collective cell migration involves the single cell migration steps associated with the presence of cell-cell contacts, mainly mediated by cadherin family members [6, 9]. Rho family GTPases

orchestrates changes in actomyosin organization that drive these key events in cell migration. For example, Rac1 regulates actin filament nucleation associated with nascent adhesion formation, and RhoA controls cell contractility, actin elongation and adhesion maturation [7, 10]. Changes in RhoGTPase activation levels interfere with the balance between cell-cell and cell-ECM adhesions and likely influences collective vs single cell migration [10-13].

Tumor formation is sensitive to the microenvironment, which varies by the region of the tumor. The tumor microenvironment is characterized by intense angiogenesis, high concentrations of growth factors and inflammatory cytokines, and ECM remodeling [14, 15]. An abrupt adaptation occurs during invasion of epithelial-derived tumors when they move from the basal membrane, a laminin enriched environment, to the connective tissue region, which is rich in collagen and fibronectin [16, 17]. Oral squamous cell carcinoma biopsies exhibit decreased laminin content and increased fibronectin, depending on the aggressiveness and the location of the tumor [18, 19]. It is likely that the characteristics of the tumor microenvironment, such as the composition of the extracellular matrix, influence metastatic and invasive behavior due to biochemical or physical activation of migration-related proteins and signaling pathways.

In this study, we report that the change from a laminin- to a fibronectin-rich environment has a differential effect on the migration properties of OSCCs. In high invasive and low E-cadherin expressing OSCC cells ($H^{inv}/L^{E\text{-}cad}$), fibronectin induced a fast single cell migration phenotype that is associated with increased Rac1 activation levels and small cell-ECM adhesions; in low invasive

and high E-cadherin OSCC cells ($L^{inv}/H^{E\text{-}cad}$), fibronectin produces a collective, non-directional migration, with high RhoA activity and altered cell-ECM adhesion. Consistent with these results, human OSCC biopsies also demonstrated changes in cell-ECM and cell-cell adhesion according to the tumor region. Together, these data show that the composition of the extracellular matrix differentially affects cell-ECM adhesion, cell migration signaling pathways and the migratory output of OSCC cells and that these effects vary according to the differentiation level of the tumor.

Material and Methods

Human Biopsies and OSCC cell culture

The experimental design and the informed consent procedures were approved by the Ethical Committee of Federal University of Rio Grande do Sul - Brazil and of Hospital de Clínicas de Porto Alegre - Brazil (CAE#06397313.7.0000.5347) and all patients in this study provided written informed consent. Patients (n=10) with oral lesions were interviewed and submitted to surgery; OSCC diagnosis was confirmed histopathologically by a pathologist and fragments from regions corresponding to the center of the tumor and the carcinoma edge tissue, named as tumor adjacent epithelia (TAE) were collected. OSCC cell lines were obtained from the Tissue Culture Facility at School of Medicine of University of Virginia and checked for mycoplasma by this facility. Cal27 cells (ATCC® CRL-2095™) were cultivated in DMEM high glucose (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) while SCC25 cells (ATCC® CRL-1628™) in DMEM/F12 with 15mM HEPES and 0.5mM sodium pyruvate (Gibco) supplemented with FBS 10% and

hydrocortisone (400ng/ml, Sigma), and cells were maintained in incubator (37^oC, 5% CO_2). Cal27 cells are considered low invasive OSCC cells [20] with high E-cadherin levels ($L^{inv}/H^{E\text{-}cad}$), while SCC25 cells are highly invasive with low E-cadherin levels ($H^{inv}/L^{E\text{-}cad}$). Spheroids were performed plating $5x10^4$ cells in a 96 wells dish covered with 1.5% agarose and, after 3 days, spheroids were gently collected and used for experiments. For Total Internal Reflectance Fluorescence (TIRF) microscopy, cell lines ($1x10^6$) were nucleofected 24h before the experiment with 0.2µg Paxillin-GFP plasmid [21], using Amaxa Nucleofection System (Lonza).

Experimental conditions

Unless stated otherwise, all reagents were purchased from Sigma Aldrich. For 2D imaging experiments, cells were trypsinized, washed and plated in glass-bottomed dishes covered with fibronectin (2µg/ml), laminin (poly-l-lysine (1mg/ml) + laminin (2µg/ml)) or Matrigel® (50µl/cm^2, BD Bioscience) in the presence of CCM1 media (Hyclone, Thermo Scientific). For 3D imaging experiments, it was used collagen (1.2mg/ml, rat tail collagen) matrices assembled according to the manufacturer (Gibco) in the presence/absence of fibronectin (10µg/ml) or laminin (10µg/ml). For each condition, a thin layer of the respective collagen matrix was initially plated at the surface of the glass-bottomed dishes. After polymerization, $3x10^4$ cells or spheroids were embedded in a new collagen matrix and, after 3h, imaged using CCM1 media. To ensure that cells were in the 3D matrix, it was verified the lower and the upper focus with detectable cells and it was always selected cells for imaging at an intermediate focus position.

Immunoblots

Antibodies were purchased from Cell Signaling (E-cadherin, N-cadherin, Integrins α4, α5, αv, β1, β3), BD-Transduction (Paxillin, FAK) and Sigma (□-Tubulin, Vinculin). Cells ($1x10^6$) were trypsinized, washed and lysed in RIPA Buffer (25mM Tris-HCL pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors cocktails. Cell lysates (20µg) were separated in 4-20% SDS Gels (Biorad) and proteins transferred to PVDF membranes, blocked (4% BSA) and immunoassayed for E-cadherin, FAK, Paxilllin, β-Tubulin, Vinculin or integrins (α4, α5, αv, β1, β3) using Pierce ECL Western Blotting Substrate (Thermo Scientific). Densitometry of the bands was performed using ImageJ software (http://rsb.info.nih.gov/ij), and values for each protein were normalized to the loading control.

Immunofluorescence

For tumor staining, human biopsies were fixed immediately after collection (4% formaldehyde, 4h, 4°C), cryoprotected with increasing sucrose concentrations (10-30%, 4°C), embedded in OCT compound, frozen (-20°C), cut using cryostat and seven µm-thick slices were collected in gelatin-covered slides. For cell lines staining, $L^{inv}/H^{E\text{-}cad}$ and $H^{inv}/L^{E\text{-}cad}$ were plated in coverslips covered with fibronectin (2µg/ml) or poly-l-lysine (1mg/ml) + laminin (2µg/ml) in the presence of CCM1 media. After 3h, cells were washed (PBS) and fixed (formaldehyde, 4%, 10min, RT). Fixed cells or human biopsies were permeabilized (Triton X-100 0.3%, RT 10 min), blocked (10% normal goat serum, RT, 1h), incubated with antibodies for E-cadherin, FAK, Paxillin, Vinculin

or Fibronectin (ON, 4^{o}C), washed (PBS) and incubated (2h, RT) with the corresponding secondary antibodies containing Alexa488 dye (Molecular Probes, Oregon, USA). Actin filaments were stained with phalloidin toxin conjugated to rhodamine (Molecular Probes, Oregon, USA) for 2h (RT). Samples were washed (PBS) and mounted with antifade medium (Vectashield, VectorLab, Burlingame, CA). Images were obtained in confocal microscope (Olympus Fluoview 1000, Tokyo, Japan) with a 63x objective (UPlanSApo x63, 1.20 NA, oil immersion objective) using FV-1000 ASW Fluoview software (Olympus, Tokyo, Japan). Alexa488 was excited with the 488nm laser line of an Argon ion laser (Melles Griot, Albuquerque, NM), while rhodamine with the 543nm laser line of a Helium-Neon laser (Melles Griot, Albuquerque, NM). Z-stacks were obtained from cells (0.1□m step size) and biopsy slices (0.5μm step size) with or without digital zoom (3x for cell lines; 5x for biopsies). In order to analyze the whole adhesion and avoid image background, 3 confocal-obtained slices were merged using the “Z-stack/maximum projection” tool from the ImageJ software. This new merged image corresponds to an equivalent 0.3μm or 1.5μm thick slice of the cell lines or the biopsy samples, respectively. Besides brightness/contrast corrections, no further image editing was performed and figures were prepared using Adobe Photoshop® 7 software.

RhoGTPase activity

For analysis of RhoGTPase activation, pull down assays [22] were performed. $L^{inv}/H^{E\text{-}cad}$ and $H^{inv}/L^{E\text{-}cad}$ cells were plated in plastic dishes covered with fibronectin (2μg/ml) or poly-l-lysine (1mg/ml) + laminin (2μg/ml) in the presence of CCM1 media. After 3h, cells were washed (PBS), harvested, lysed

with CRIBs buffer in the presence of protease and phosphatase inhibitors and incubated in the presence of GST-PAK-CRIB (Rac1) or GST-RBD-CRIB (RhoA) beads. After washing, samples were prepared for SDS-PAGE and submitted to immunoblotting for Rac1 (BD Bioscience) or RhoA (Santa Cruz Biotechnologies). Densitometry of the bands was performed using ImageJ software.

FRET imaging and analysis

$L^{inv}/H^{E\text{-}cad}$ and $H^{inv}/L^{E\text{-}cad}$ OSCC cells were nucleofected with Raichu-Rac1-WT or Raichu-Rac1-V12 plasmids for Rac1 activity and Raichu –RhoA-WT or Raichu-RhoA-Q63L plasmids [23] for RhoA activity (0.5µg/10^6 cells) and 24h later were trypsinized and plated in plastic dishes covered with fibronectin (2µg/ml) or poly-l-lysine (1mg/ml) + laminin (2µg/ml) in the presence of CCM1 media. Cells were washed (PBS), fixed in formaldehyde 4% and sacarose 4% (10 min, RT), washed (PBS) and analyzed by confocal microscopy with 2x digital zoom. Donor probe was excited with the 458nm laser line of an Argon ion laser (Melles Griot, Albuquerque, NM). Images were analyzed by Matlab® software (MathWorks, Natick, MA) using the Biosensor Processing software 2.1 [24]. The mean intensity values from FRET-ratio TIFF images were obtained on ImageJ software. Using ImageJ software, selected images were adjusted for the same levels of brightness/contrast and a 0.5 pixel-wide Gausian filter was applied.

Migration and adhesion dynamics assays

Imaging acquisition and analysis for migration assays were performed as previously described [25]. For phase microscopy movies, images were captured at 10min intervals using a Nikon TE300 microscope (10x 0.25 NA CFI Achro DL106 Nikon objective) with a charge coupled device camera (Orca II, Hamamatsu Photonics) using Metamorph software (Molecular Devices). For TIRF microscopy, images were taken at 3s intervals using an Olympus IX70 inverted microscope (63x 1.45 NA oil Olympus PlanAplo 660 TIRFM objective) fitted with a Ludl modular automation controller (Ludl Electronic Products) with a charge-couple device camera (Retiga Exi, Qimaging) and controlled by Metamorph software. GFP was excited with the 488nm laser line of an Argon laser (Melles Griot) and a dichroic mirror (HQ485/30) and an emission filter (HQ525/50) were used. All images and movies were analyzed using ImageJ software and panels mounted using Adobe Photoshop® 7 software. For analysis of migration parameters, it was performed at least 4 independent experiments (phase contrast microscopy movies) and the nucleus of each migratory cell was tracked using the “manual tracking” plug-in on ImageJ. It was considered as migratory cell only cells that migrated for at least 6h. In case of migratory cells that underwent mitosis, the tracking process was ended 1h before cytokinesis. To determine migration speed, it was performed the ratio between the total distance traveled (distance) and the number of slices (time) that cell migrated. To analyze the cell trajectory and persistence of migration, the X and Y coordinates obtained during the tracking of the nucleus of the migratory cell in each slice were normalized to start at a virtual X=0 and Y=0 position and the

variation on the position was plotted in a polar plot graph [25]. For analysis of adhesion properties, it was used $H^{inv}/L^{E\text{-}cad}$ cells expressing paxillin-GFP from at least 4 independent experiments (TIRF microscopy) for each experimental group. Adhesion length and area was determined by measuring, respectively, the long axis or the area of each adhesion that assembled during the movie. The percentage of total adhesion area in each newly formed protrusion was measured by the ratio of the sum of the area of all adhesions that assembled in the protrusion by the total area of the protrusion. The adhesion assembly speed was measured using the “kymograph” plug-in on ImageJ. For each adhesion, a line (1 pixel-wide) was drawn in the long axis of the adhesion and the X (distance) and Y (time) coordinates originated by the kymograph were used to measure the speed of adhesion assembly. All data were calculated using Microsoft Excel® (Microsoft Corporation) and SPSS 21 software (Statistical Package for the Social Science, IBM).

Statistical Analysis

Student t test or One-way analysis of variance (ANOVA) followed by Tukey’s post-test were employed, using SPSS 21 software and differences were considered significant when $p<0.05$.

Results

Fibronectin induces fast single cell migration of highly invasive OSCCs cells

Since the extracellular matrix composition can influence the migratory properties of various cell types, $L^{inv}/H^{E\text{-}cad}$ (Cal27) or $H^{inv}/L^{E\text{-}cad}$ (SCC25) oral squamous cell carcinoma cell lines [20] were plated on 2D- Matrigel®

(50µl/cm^2), laminin (2µg/ml) or fibronectin (2µg/ml)-coated glass bottomed dishes and imaged for 24h. We tracked the migration velocity of individual as well as group of cells. Matrigel and laminin were used to mimic the laminin-rich basement membrane that supports cells in an epithelial sheet, whereas fibronectin was used to challenge the cells with the connective tissue matrix encountered when cells metastasize. On Matrigel, both cell lines migrated collectively (S1 Movie), while on laminin, both cell types exhibited collective as well as single cell migration (S2 and 3 Movie)..While $L^{inv}/H^{E\text{-}cad}$ cells showed no changes in migration speed, $H^{inv}/L^{E\text{-}cad}$ cells exhibited a ~40% increase in migration speed on laminin when compared to Matrigel (Fig 1A). When cells were plated on fibronectin, both cell types migrated faster than on laminin, and exhibited pronounced changes in directionality. Both OSCC lines showed a ~40% increase in migration speed; but $L^{inv}/H^{E\text{-}cad}$ cells migrated collectively in circles (S2 Movie), whereas $H^{inv}/L^{E\text{-}cad}$ cells migrated as single cells with persistent directionality (Fig 1B, S3 Movie).

To complement the observations in a 2D environment, $L^{inv}/H^{E\text{-}cad}$ or $H^{inv}/L^{E\text{-}cad}$ OSCC cells were plated in a 3D matrix, containing collagen (1.2mg/ml), collagen + laminin (1.2mg/ml+10µg/ml) or collagen + fibronectin (1.2mg/ml+10µg/ml), and imaged for 24h. When compared to a 3D collagen only gel, $L^{inv}/H^{E\text{-}cad}$ cells showed a ~50% increase in migration speed when plated in 3D collagen gel containing laminin or fibronectin (Fig 1F, S4 Movie) with a slight increase in directional persistence when plated on collagen+laminin. $H^{inv}/L^{E\text{-}cad}$ tumor cells showed no changes in migration speed when plated in a collagen+laminin 3D environment, but were able to invade the

collagen gel when plated in collagen+fibronectin matrices (Fig 1F, S5 Movie). Both cells migrated poorly when plated in a 3D matrix containing only collagen.

Since OSCC biopsies exhibit tumor islands inside the connective tissue, we developed spheroids from both cell lines, plated them in a collagen (1.2mg/ml) or a collagen+fibronectin (1.2mg/ml+2µg/ml) 3D environment, and imaged for 36h. Supplementary movie 6 shows that small or big spheroids derived from $L^{inv}/H^{E\text{-}cad}$ OSCCs proliferated, but showed little migratory activity. However, spheroids of the $H^{inv}/L^{E\text{-}cad}$ OSCC cells showed cells that migrated out of the spheroid and invaded the surrounding tissue only when plated in a collagen+fibronectin 3D environment.

To summarize, these results in 2D and 3D matrices show that $L^{inv}/H^{E\text{-}cad}$ OSCC cells migrate more directionally when plated using conditions similar to the epithelial and blood vessel basal lamina; whereas $H^{inv}/L^{E\text{-}cad}$ tumor cells switch from a collective to a faster single cell migration when transitioning from a laminin to a fibronectin rich connective tissue-like environment.

OSCCs extracellular matrix-derived migration properties are associated with changes in RhoGTPase signaling

A differential activation of RhoGTPase signaling is a likely mechanism for the ECM-derived differences in cell migration observed in the $L^{inv}/H^{E\text{-}cad}$ and $H^{inv}/L^{E\text{-}cad}$ OSCC cell lines. To address this, we analyzed the Rac1 and RhoA activation levels by pull down and FRET assay of cells plated on either laminin (2µg/ml) or fibronectin (2µg/ml) coated dishes. Consistent with the increased migration speed observed for both cell types on fibronectin, fibronectin

increased Rac1 activation levels when compared to cells plated on laminin, which was accompanied by a FRET signal mainly at the cell borders (Fig 2A); this effect was slightly, but consistently, more pronounced in the $H^{inv}/L^{E\text{-}cad}$ tumor cells. In contrast, RhoA activity was observed mainly at the cell body and showed a decrease in the $L^{inv}/H^{E\text{-}cad}$ cells plated on fibronectin, but was unaltered in the $H^{inv}/L^{E\text{-}cad}$ cell line (Fig 2B). This decreased RhoA activity may reflect the fact that $L^{inv}/H^{E\text{-}cad}$ cells migrate collectively on fibronectin, whereas the $H^{inv}/L^{E\text{-}cad}$ cells migrate as single cells, where RhoA is necessary for formation of the contractile cell rear underlying persistent directional migration [26]. These data indicate that the effects of ECM constitution on tumor invasion process involve a differential activation of RhoGTPases that varies according to the aggressiveness and differentiation level of the tumor cells.

Extracellular-matrix composition interferes with tumor cell adhesion properties

Since $L^{inv}/H^{E\text{-}cad}$ OSCCs migrated collectively, whereas $H^{inv}/L^{E\text{-}cad}$ OSCCs migrated as single cells, we asked whether these effects correlated with the ratio of cell-cell versus cell-ECM adhesions. We hypothesized that $L^{inv}/H^{E\text{-}cad}$ cell line, which exhibit collective cell migration, would exhibit increased cell-cell adhesion markers, notably cadherin, whereas highly invasive single cells would most likely favor cell-ECM adhesions. Consistent with this hypothesis, by western blotting, we observed that both cell lines expressed integrins for fibronectin, but $H^{inv}/L^{E\text{-}cad}$ OSCCs presented an increase in the expression levels of α5 and β1 (Fig 3B) when compared to $L^{inv}/H^{E\text{-}cad}$ OSCCs. Also, $H^{inv}/L^{E\text{-}cad}$ OSCCs show a slightly increased expression of cell-ECM adhesion related proteins, including the nascent adhesion marker paxillin, the mechanosensing

modulator vinculin and the adhesion signaling marker focal adhesion kinase (FAK). Additionally, $H^{inv}/L^{E\text{-}cad}$ OSCCs gained expression of N-Cadherin, a marker of the epithelial to mesenchymal transition (Fig 3A). These data suggest that epithelial-derived tumor cells show a differential expression of cell-cell and cell-ECM adhesion markers according to the differentiation levels.

In order to analyze the effects of ECM on cellular distribution of adhesion markers, we observed by immunofluorescence that $L^{inv}/H^{E\text{-}cad}$ OSCCs showed a localization of E-cadherin preferentially at the cell-cell contacts on both fibronectin and laminin (Fig 4A, S1 Fig) while the ECM adhesion markers, paxillin, vinculin and FAK, all localized primarily to large, elongated adhesions, as is often observed in slow migratory cells. By contrast, $H^{inv}/L^{E\text{-}cad}$ OSCC cells displayed mainly cytoplasmic E-cadherin with only weak staining at cell-cell contacts in both ECM environments (Fig 4B). On laminin, paxillin, vinculin and FAK localized to elongated adhesions similar to $L^{inv}/H^{E\text{-}cad}$ OSCCs. On fibronectin, however, these adhesion markers preferentially redistributed from large adhesions to small adhesions at the cell border, consistent with the observed increase in Rac activity and the faster migration speed. To confirm these effects of ECM composition on the adhesion of $H^{inv}/L^{E\text{-}cad}$ OSCCs, we performed live cell imaging (TIRF microscopy) of cells expressing the nascent adhesion marker paxillin-GFP in order to analyze adhesions properties during protrusion (Fig 4C and S7 movie). While there was no difference in adhesion assembly speed when $H^{inv}/L^{E\text{-}cad}$ OSCCs were plated on fibronectin or laminin, fibronectin decreased adhesion length by ~80% ($p \leq 0.001$, n=43 adhesions, Student T test), and similarly decreased both individual adhesion area by ~50%

($p \leq 0.05$, n=221 adhesions, Student T test) as well as total adhesion area relative to protrusion area by 30% ($p \leq 0.01$, n=17 protrusions, Student T test). These data indicate that the switch from laminin to fibronectin induces smaller adhesions on $H^{inv}/L^{E\text{-}cad}$ OSCCs cells, which is consistent with the phenotype of highly migratory cells. Thus, the increased persistent migration of $H^{inv}/L^{E\text{-}cad}$ OSCCs on fibronectin at least in part reflects a preference for cell-ECM adhesion, particularly nascent signaling adhesions, rather than cell-cell adhesions.

Human oral squamous cell carcinoma biopsies show increased levels of cell-ECM adhesion proteins

Since ECM-associated changes on OSCC invasiveness are likely driven by changes in adhesion, we analyzed the adhesion proteins on biopsies from 10 patients. The sections were stained for fibronectin, E-cadherin, paxillin, vinculin, and FAK and analyzed using confocal microscopy. We compared regions from Tumor Adjacent Epithelia (TAE) (Fig 5, columns 1 and 2; S2 and S3 Figs) with regions at the center of the tumor (Fig 5, columns 3 and 4; S2 and S3 Figs). Fibronectin signal was observed in the connective tissue in both regions (S3 Fig). E-cadherin labeling in TAE cells was strongest at cell-cell contacts and co-localized with actin; while the center of the tumor cells showed a weak signal mainly within the cytoplasm, suggesting protein degradation and/or mislocalization from junctional regions. In TAE regions, paxillin and FAK stained weakly in puncta through the cytoplasm of the epithelia basal layer and weakly co-localized with actin; while vinculin was present mainly at the basal membrane. In the center of tumor, cells that appeared to have detached from

the tumor island showed an increase in the staining of proteins related to cell-ECM adhesion, with paxillin showing increased labeling at the cell border, close to the ECM, while vinculin and FAK were observed at regions of membrane extensions of cells at the periphery of the tumor island, with some co-localization with actin. Thus, human biopsies exhibit changes in the distribution of cell-ECM adhesion proteins, particularly at the invasive front of the tumor, that correspond with changes observed in cell-ECM adhesion of $H^{inv}/L^{E\text{-}cad}$ OSCC plated on fibronectin.

Discussion

Clinical failures in cancer therapies are due in part to the plasticity of tumor cells to a changing microenvironment [14, 15]. For example, tumor cells physically and biochemically alter extracellular matrix organization [27], which appears to impact several aspects of the epithelial-to-mesenchymal transition (EMT) and cell survival [28, 29]. Furthermore, the migration of epithelial-derived tumors can vary from collective to single cell migration, reflecting changes in tumor cell-cell adhesion and the structure of the tissue that the cells are invading [6, 30, 31]. In glioblastomas, for example, targeted depletion of fibronectin modifies collective cell migration, making cancer cells sensitive to ionizing radiation [32].

We have demonstrated that 2D substrates resembling the epithelial basal membrane induce collective cell migration in OSCC cells independently of the levels of the cell-cell adhesion protein E-cadherin. In contrast, fibronectin-enriched 2D or 3D environments induced single cell, mesenchymal-like cell migration but specifically in the $H^{inv}/L^{E\text{-}cad}$ OSCCs. Gaggioli et al (2007) [17]

demonstrated, in a 3D collagen environment, that squamous cell carcinoma cells showed invasive behavior due to the fibroblast-mediated proteolytic ECM remodeling and fibronectin deposition. Fibronectin also developmentally regulates migration during embryogenesis and determines cell fate [33]. Similarly, fibronectin is overexpressed at the invasive zone of OSCC biopsies [18, 19], where it likely contributes to the abnormal invasive behavior of poorly differentiated cells [34].

Here we sought to determine how the ECM composition of different tumor regions affects cell migration and the signaling mechanisms underlying these different migratory properties. Cell migration is regulated mainly by RhoGTPases, where Rac1 stimulates actin polymerization and nascent adhesion formation while RhoA controls cell contractility and adhesion maturation [7, 10]. During tumor invasion, the balance of RhoGTPase activation is disrupted. Rac1 activation results in a loss of cell junctions and polarity and increased cell motility [11, 35]. Chen et al 2013 [36] demonstrated that mammary epithelial cells undergo EMT when plated on fibronectin, through a mechanism that involves Rac1b activation, while laminin suppresses EMT. Consistent with this observation, increased Rac1 activity drives mesenchymal-like, single cell migration of other cancer cells undergoing EMT [6, 12]. Yap, et al 2009 [37] demonstrated that different OSCC cell lines show increased Rac1 activation when plated on fibronectin, indicating that the microenvironment can influence the tumor invasive behavior through the modulation of cell migration-related signaling pathways. In this study, when compared to laminin, we showed that fibronectin induced an increase in Rac1 activation and a rapid

single cell migration phenotype in $H^{inv}/L^{E\text{-}cad}$ OSCCs. Interestingly, while $L^{inv}/H^{E\text{-}cad}$ OSCCs also exhibited increased Rac1 activation on fibronectin, they had lower RhoA activation. This decreased RhoA activation may account for the decreased directionality of $L^{inv}/H^{E\text{-}cad}$ OSCCs, which tended to migrate collectively in circles, since RhoA-mediated myosin activation promotes persistent directional migration [26]. Thus, differential RhoGTPase activity might contribute to migration speed and persistence, as well as collective versus single cell migration on different substrates.

In addition to differential RhoGTPase expression, we observed changes in cell-ECM adhesions on different substrates, with fibronectin favoring the formation of small nascent adhesions in $H^{inv}/L^{E\text{-}cad}$ OSCCs [38-40]. A possible explanation for the selective effect of fibronectin in our study is the higher expression of fibronectin-related integrins observed in $H^{inv}/L^{E\text{-}cad}$ OSCCs when compared to $L^{inv}/H^{E\text{-}cad}$ cells. Integrins are a family of transmembrane proteins that mediate the binding of ECM proteins with intracellular proteins, resulting in biochemical and mechanical signaling pathways that influence several steps on tumor progression [41-44]. Consistent with our results, Chen et al 2012 [45] showed that laminin induced elongated and fluxing adhesions in CHO.K1 cells, while fibronectin induced smaller and more dynamic adhesions. Similarly, we demonstrated that both, $L^{inv}/H^{E\text{-}cad}$ or $H^{inv}/L^{E\text{-}cad}$ OSCCs, when plated on laminin, showed large and elongated adhesions probably due to the epithelial origin of the tumor. However, specifically in $H^{inv}/L^{E\text{-}cad}$ tumor cells, fibronectin induced smaller cell-ECM adhesions with a fast turnover, which reflected in increased Rac1 activation [46] and faster single cell migration. Thus the ability to

metastasize from a laminin to a fibronectin environment might reflect a switch from cadherin-mediated cell-cell adhesions to signaling integrin-ECM adhesions, which promote directional cell migration.

Several reports associate differential activation of adhesion-related proteins with a worse patient prognosis [47-49]. Also, vulvar squamous cell carcinoma tumors silenced for the fibronectin binding protein, integrin β1, show a more encapsulated and less invasive profile [50] indicating that cell-ECM interaction is an important player during tumorigenesis. Consistent with these findings, we demonstrated that human OSCC biopsies show decreased junctional E-cadherin levels at the center of the tumor when compared to the epithelia adjacent to the tumor, while the cell-ECM adhesion proteins paxillin, vinculin and FAK showed a differential distribution in cancer cells at the border of tumor islands close to regions of contact to the fibronectin enriched ECM. Therefore, our results (Fig 6) show that the extracellular matrix composition is able to influence the pattern of tumor invasion and metastasis according to the differentiation level of the tumor cells, probably through modulation of cell signaling and changes in the balance between cell-cell and cell-ECM adhesion. These data suggest that the invasive behavior of OSCC not only relies on intrinsic factors (i.e. mutations and abnormal expression of proteins) but also on extrinsic factors (such as the ECM composition), which could help to understand the failure of some tumor therapies and contribute to development of new anti-tumorigenic approaches.

Acknowledgments

The authors thank Laura de Campos Hildebrand and Ana Luisa Homem de Carvalho for help with the human sample biopsy collection and Karen Newell-Litwa for revision of the article.

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Figure Legends

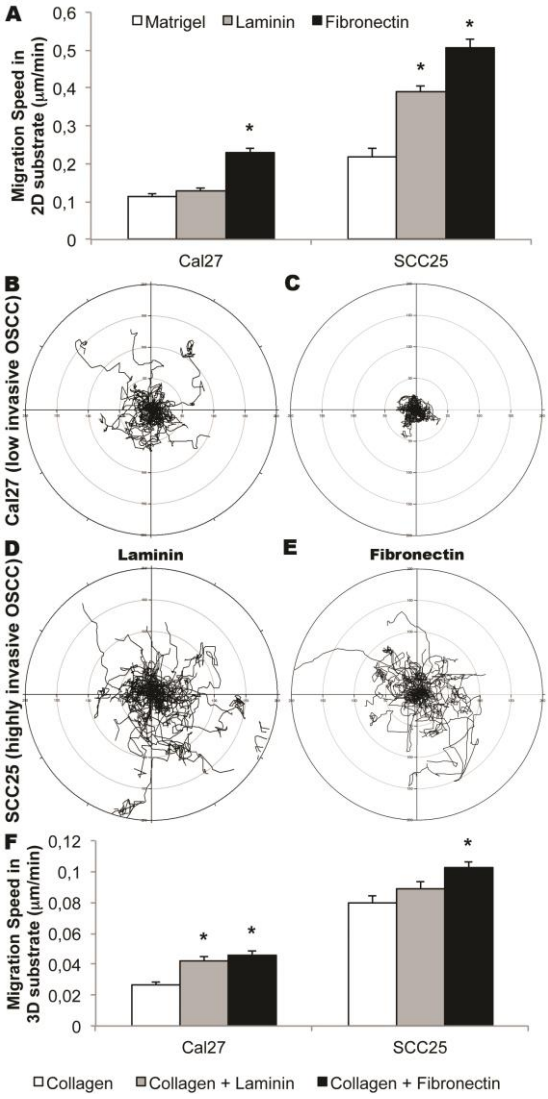


Fig 1. Fibronectin induces faster migration speed in 2D and 3D substrates. (A) Effects of different 2D substrates on migration speed (24h) of $L^{inv}/H^{E\text{-}cad}$ (Cal27) or $H^{inv}/L^{E\text{-}cad}$ (SCC25) OSCC cell lines (n=3); (B-E) Cell migration trajectory of $L^{inv}/H^{E\text{-}cad}$ (B-C) or $H^{inv}/L^{E\text{-}cad}$ (D-E) cells plated on laminin (B and D) or fibronectin (C and E); (F) Effects of different 3D substrates on migration speed of $L^{inv}/H^{E\text{-}cad}$ and $H^{inv}/L^{E\text{-}cad}$ cell lines (n=3). Results are expressed as mean ± SEM. (*) $p<0.05$ according to One-way analysis of variance (ANOVA) followed by Tukey's post-test.

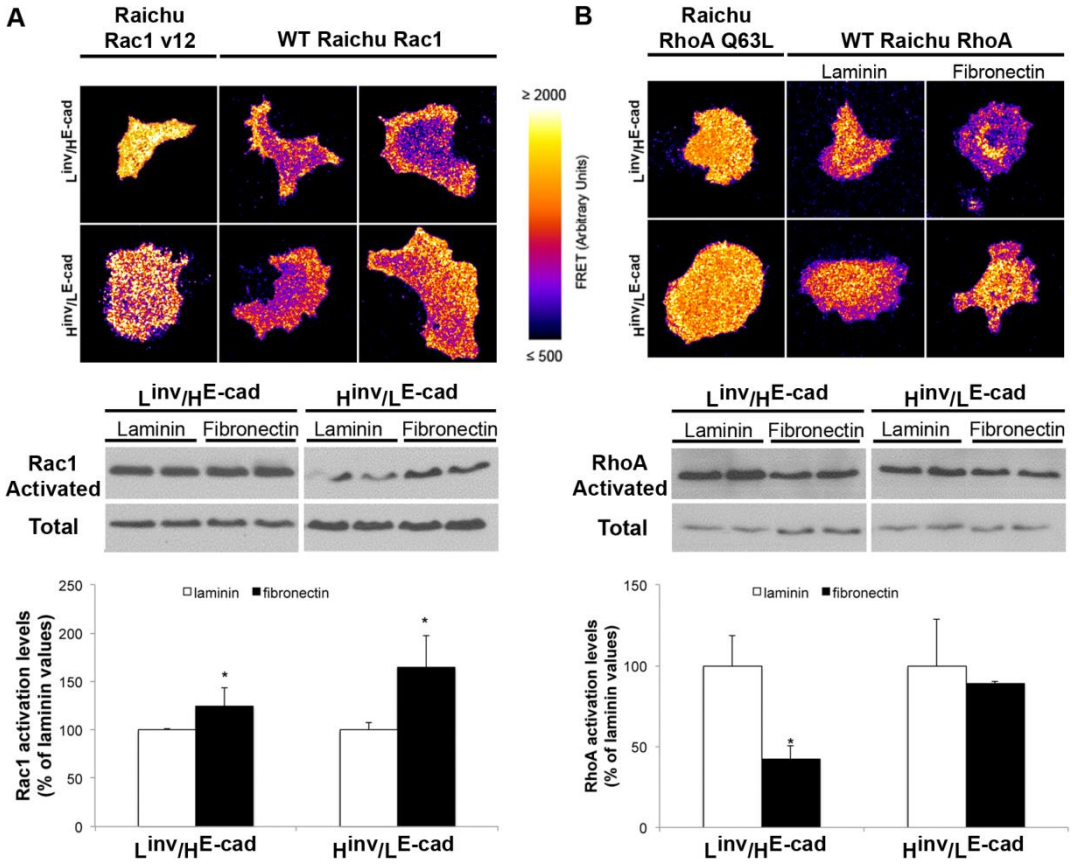


Fig 2. RhoGTPase activation varies according to extracellular matrix composition and tumor differentiation levels. FRET analysis and pull down assay for Rac1 (A) and RhoA (B) of $L^{inv}/H^{E\text{-}cad}$ (Cal27) or $H^{inv}/L^{E\text{-}cad}$ (SCC25) OSCC plated in laminin (2µg/ml) or fibronectin (2µg/ml). Raichu-Rac1-V12 and Raichu-RhoA-Q63L represents the constitutively activated isoform. Results are expressed as mean ± SD. (*) $p<0.05$, n=4

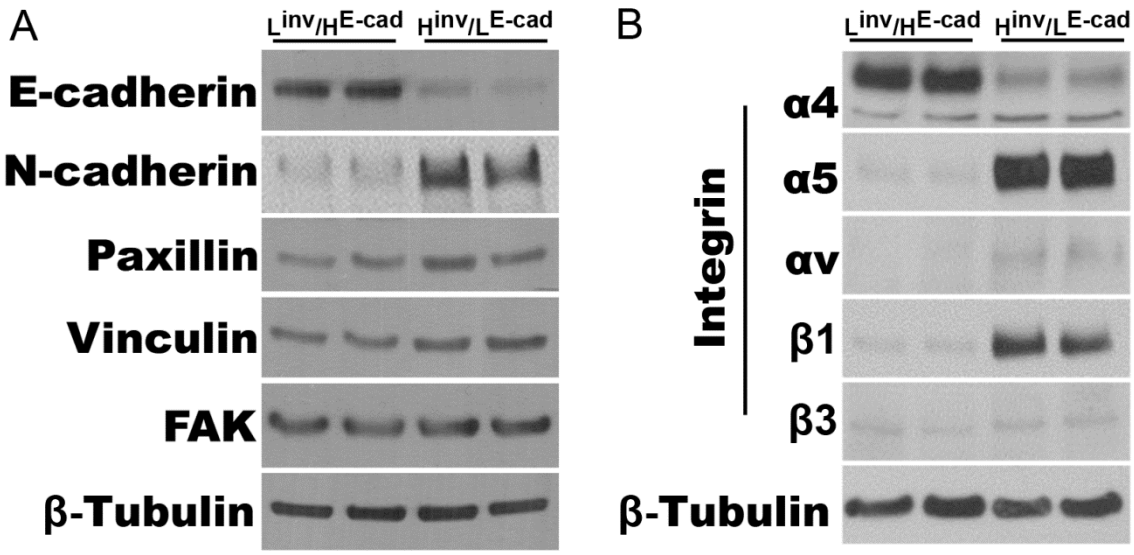


Fig 3. Decreased cell-cell and increased cell-ECM adhesion proteins characterize invasive OSCC. Representative western blotting images of cell-cell (E-cadherin, N-cadherin), cell-ECM (paxillin, vinculin and FAK) and integrins (α4, α5, αv, β1 and β3) from $L^{inv}/H^{E\text{-}cad}$ (Cal27) or $H^{inv}/L^{E\text{-}cad}$ (SCC25) OSCC total cell lysates. Densitometry values for each protein were normalized to the loading control (β□Tubulin).

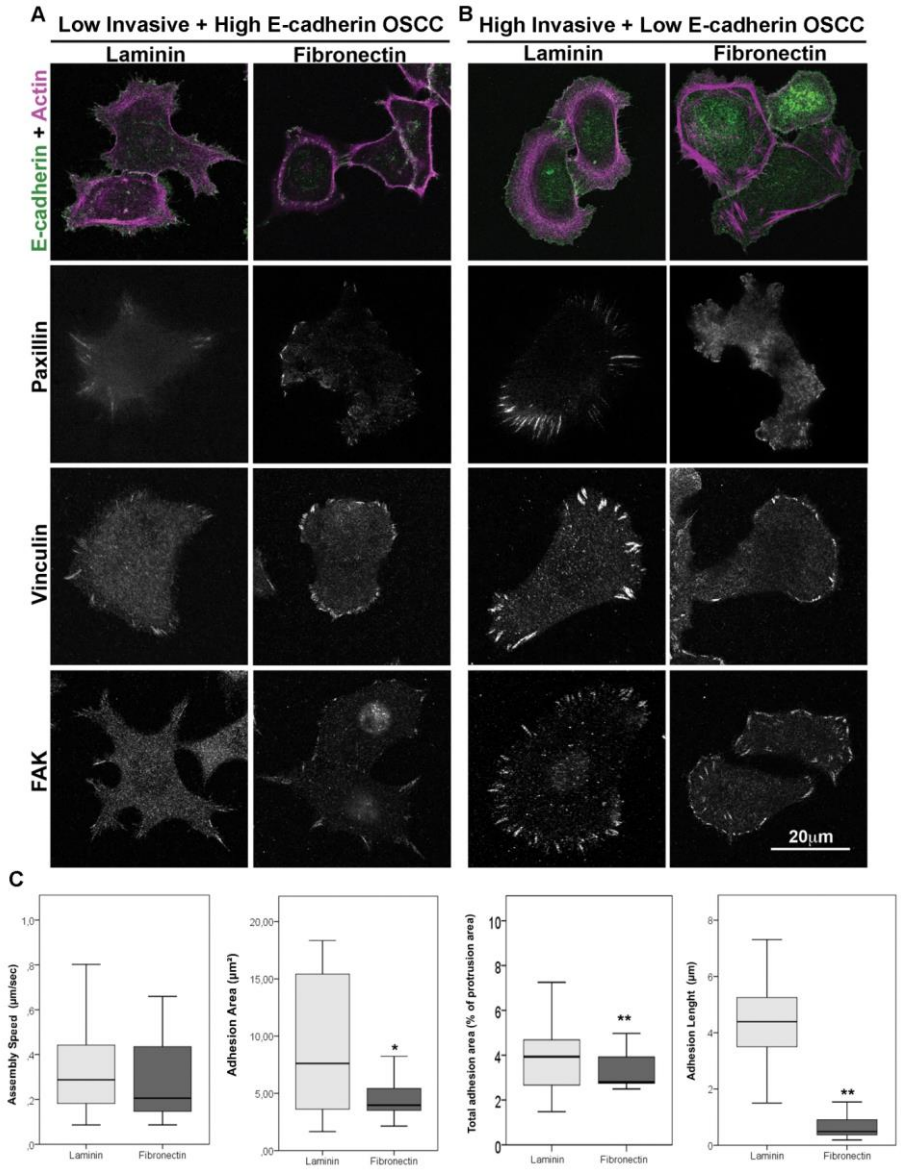


Fig 4. Fibronectin induces smaller adhesion on low E-cadherin expression OSCC cell line. $L^{inv}/H^{E\text{-}cad}$ (A) or $H^{inv}/L^{E\text{-}cad}$ (B) invasive OSCC were plated on laminin or fibronectin, fixed and stained for E-cadherin and actin, paxillin, vinculin and FAK. White arrows indicate the signal of E-cadherin between cells. Scale bar = 20μm. Data regarding adhesion properties (C) were obtained using Total Internal Reflectance Fluorescent microscopy analysis of $H^{inv}/L^{E\text{-}cad}$ OSCC cells expressing paxillin-GFP and plated on laminin (light gray) or fibronectin (dark gray). The data shows the assembly speed (μm/sec),

adhesion area (μm²), total adhesion area (as % of total protrusion area) and adhesion length (μm). Results are expressed as mean ± SEM. (*) p = 0.05; (**) p < 0.01, according to Student T – test.

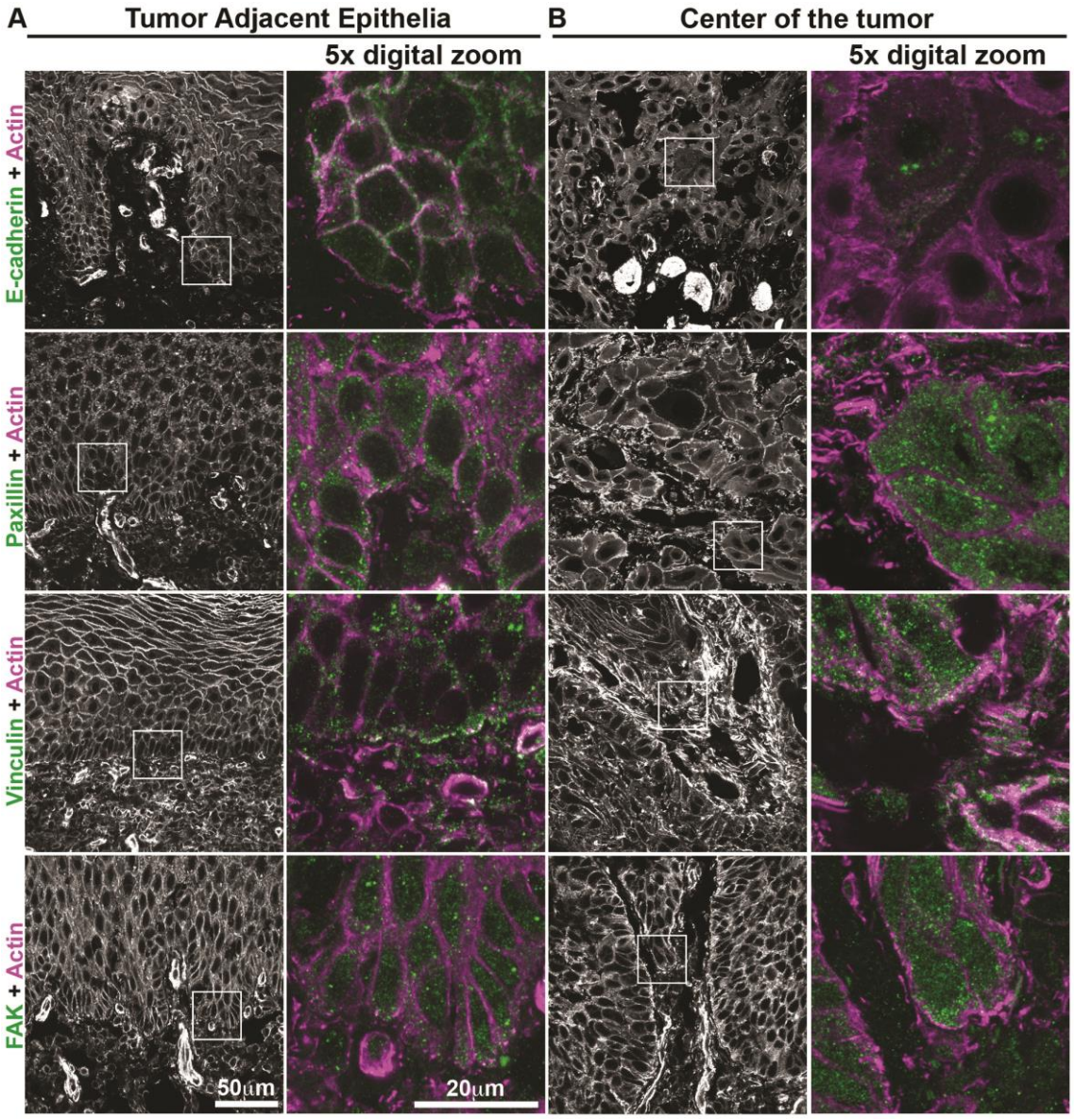


Fig 5. Human oral squamous cell carcinoma biopsies show differential distribution of adhesion proteins between center of the tumor cells and tumor-adjacent epithelia. Regions of biopsies corresponding to the epithelia adjacent to the tumor (A) and from the center of the tumor (B) were

submitted to immunostaining for E-cadherin, paxillin, vinculin or FAK (green) and actin staining (magenta). Inserts demonstrated in actin staining, were digitally magnified (5x) to show intracellular localization. Representative images from different patients (n=10), scale bar = 50μm or 20μm.

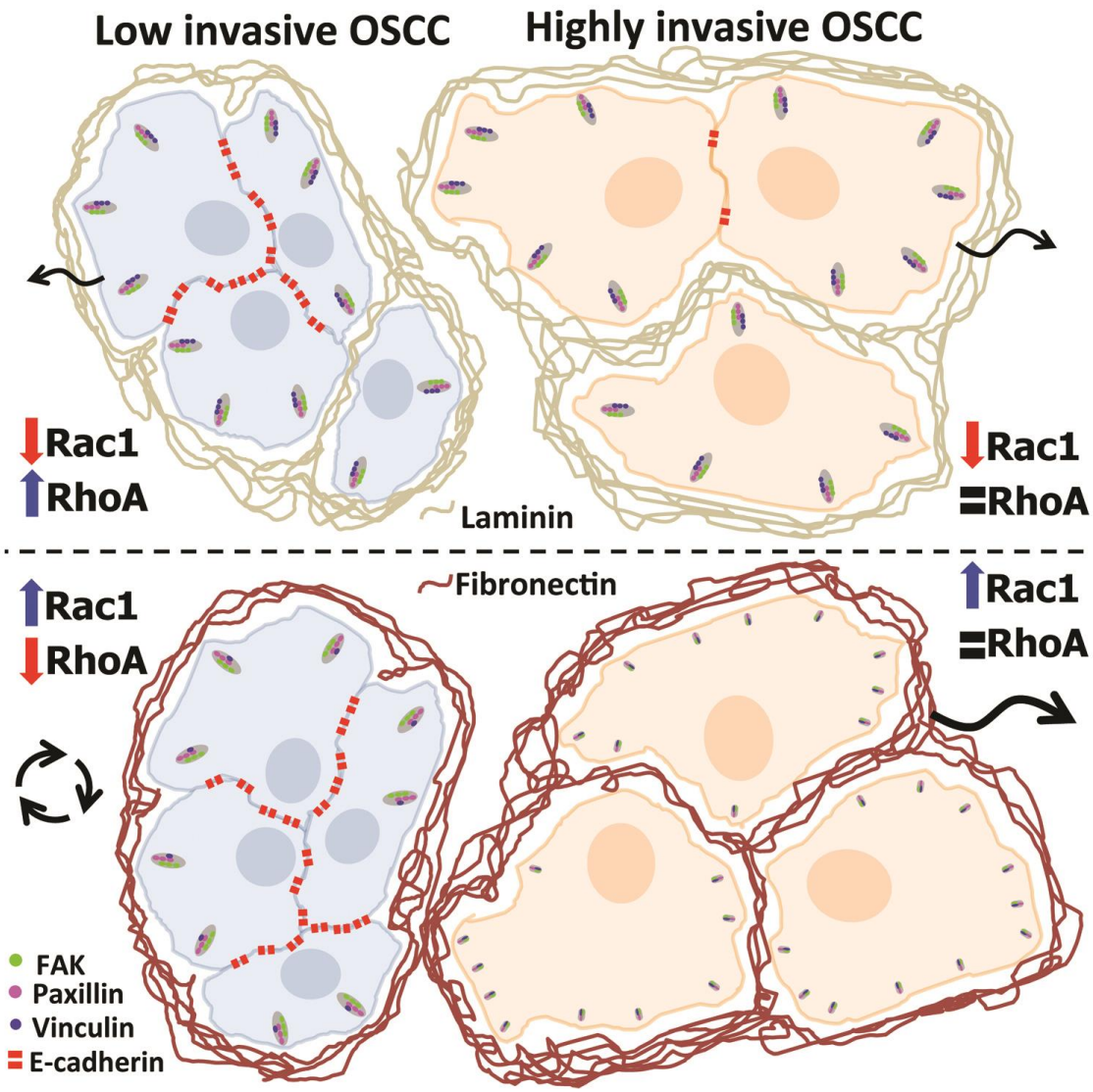


Fig 6. Effects of the differential composition of extracellular matrix on cell adhesion and signaling of Oral Squamous Cell Carcinoma. OSCC with high E-cadherin levels (blue cells) shows collective and single cell migration in the presence of laminin and collective non-directional migration in

fibronectin. This switch correlated to an increase in Rac1 and a decrease on RhoA activation and modulation of the vinculin levels in adhesion, induced by the fibronectin-enriched environment. For OSCCs with low E-cadherin levels (orange cells), fibronectin induced smaller adhesions and increased Rac1 signaling, which correspond to a fast single cell migration phenotype. This model proposes that the ECM composition can trigger the tumor invasive behavior according to differentiation levels of OSCC cells.

Supporting Information

S1 Movie. Migratory properties of low and highly invasive Oral Squamous Cell Carcinoma cell lines plated on Matrigel. Time-lapse images (right column) and cell tracking (left column) of Oral Squamous Cell Carcinoma with $H^{inv}/L^{E\text{-}cad}$ (upper line) or $L^{inv}/H^{E\text{-}cad}$ (lower line) plated for 1h on Matrigel (50µl/cm^2) and imaged for 24h with a 10min time interval.

S2 Movie. Migratory properties of low invasive Oral Squamous Cell Carcinoma plated in laminin or fibronectin. Time-lapse images (left column) and cell tracking (right column) of Oral Squamous Cell Carcinoma with $L^{inv}/H^{E\text{-}cad}$ plated for 1h on laminin (2µg/ml, upper line) or fibronectin (2µg/ml, lower line) and imaged for 24h with a 10min time interval. This movie corresponds to Figure1 B and C.

S3 Movie. Migratory properties of highly invasive Oral Squamous Cell Carcinoma plated in laminin or fibronectin. Time-lapse images (left column) and cell tracking (right column) of $H^{inv}/L^{E\text{-}cad}$ plated for 1h on laminin

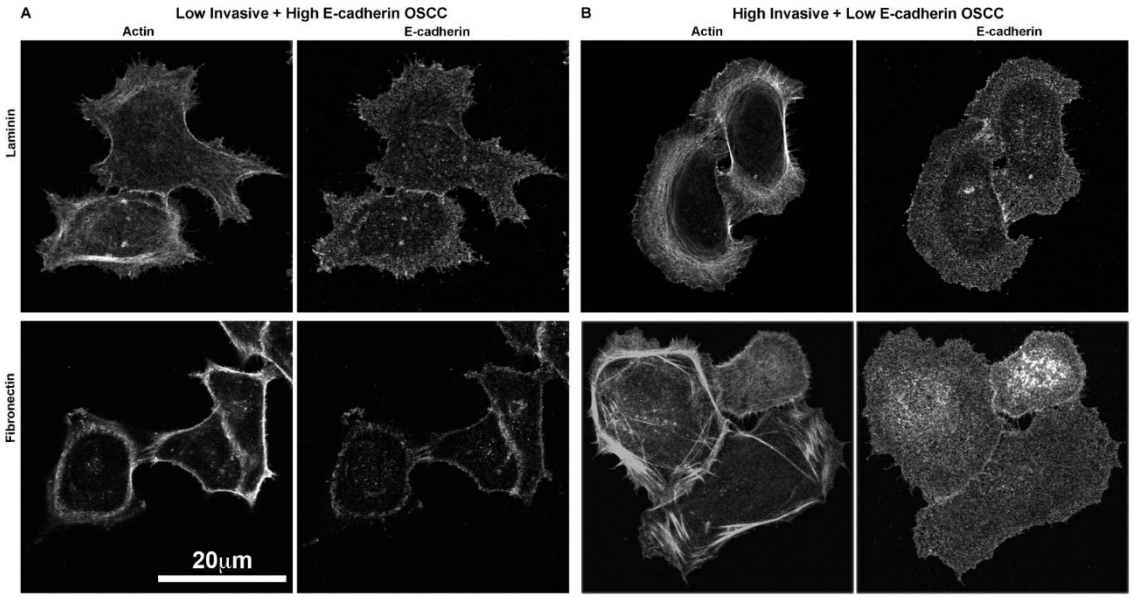
(2μg/ml, upper line) or fibronectin (2μg/ml, lower line) and imaged for 24h with a 10min time interval. This movie corresponds to Figure 1D and E.

S4 Movie. Migratory properties of low invasive Oral Squamous Cell Carcinoma plated in a 3D matrix. Time-lapse images (left column) and cell tracking (right column) of $L^{inv}/H^{E\text{-}cad}$ OSCC were plated for 1h in a 3D matrix of collagen (1.2mg/ml, upper line), collagen+laminin (1.2mg/ml+10μg/ml, center line) or collagen+fibronectin (1.2mg/ml+10μg/ml, lower line) and imaged for 24h with a 10min time interval. This movie corresponds to Figure 1F.

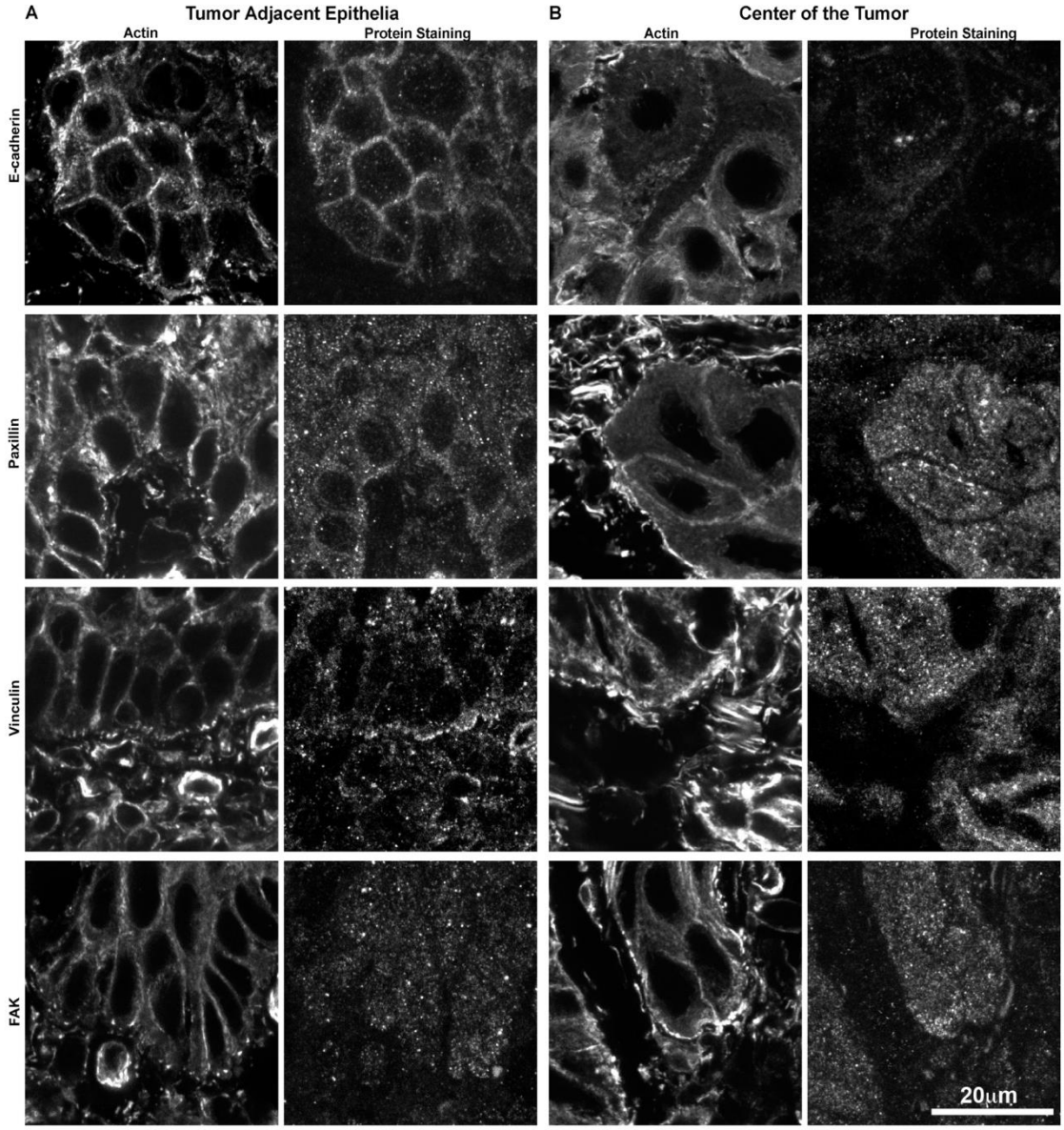
S5 Movie. Migratory properties of highly invasive Oral Squamous Cell Carcinoma plated in a 3D matrix. Time-lapse images (left column) and cell tracking (right column) of $H^{inv}/L^{E\text{-}cad}$ OSCC were plated for 1h in a 3D matrix of collagen (1.2mg/ml, upper line), collagen+laminin (1.2mg/ml+10μg/ml, center line) or collagen+fibronectin (1.2mg/ml+10μg/ml, lower line) and imaged for 24h with a 10min time interval. This movie corresponds to Figure 1F.

S6 Movie. Migratory properties of Oral Squamous Cell Carcinoma cell lines-derived spheroids in a 3D extracellular matrix. Time-lapse images of spheroids obtained from Oral Squamous Cell Carcinoma with $L^{inv}/H^{E\text{-}cad}$ (left column) plated in a 3D matrix containing collagen+fibronectin (1.2mg/ml+10μg/ml) or $H^{inv}/L^{E\text{-}cad}$ (center and right column) plated in a 3D extracellular matrix composed by collagen only (1.2mg/ml, center column) or collagen+fibronectin (1.2mg/ml+10μg/ml, right column) and imaged for 36h with a 10min time interval.

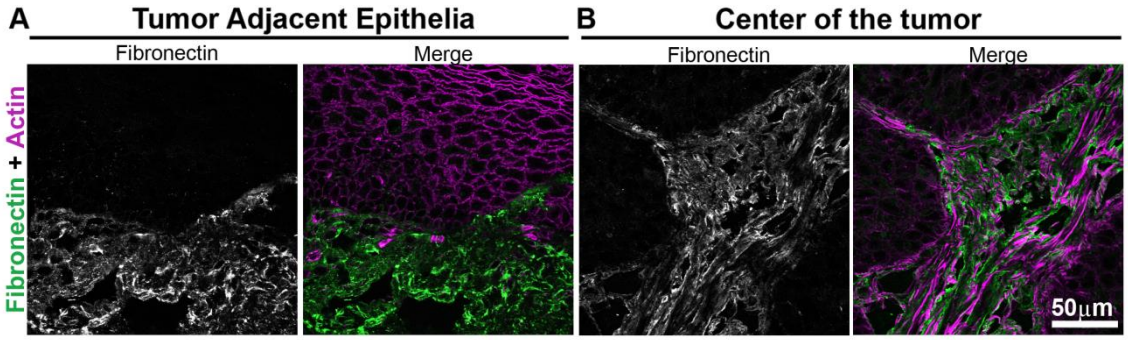
S7 Movie. Extracellular matrix composition affects a**dhesion dynamics of highly invasive Oral Squamous Cell Carcinoma.** Adhesion dynamics of Oral Squamous Cell Carcinoma with low E-cadherin levels transfected with paxillin-GFP, plated for 20min on laminin (2μg/ml, left column) or fibronectin (2μg/ml, right column) and imaged using Total Internal Reflectance Fluorescent (TIRF) microscopy for 10min with a 3s time interval. The black box represents a digital zoom of the original movie showing the details of cell adhesion dynamics in each condition.



S1 Figure. Differential distribution of E-cadherin on two different cell lines. $L^{inv}/H^{E\text{-}cad}$ (A) or $H^{inv}/L^{E\text{-}cad}$ (B) OSCC were plated on laminin or fibronectin, fixed and stained for E-cadherin and actin. Scale bar = 20μm.



S2 Figure. Distribution of cell-cell and cell-ECM adhesion molecules in OSCC human biopsies. Original images showed in Fig 5 in the manuscript. Biopsies corresponding to the epithelia adjacent to the tumor (A) and from the center of the tumor region (B) were submitted to actin staining (first column) and immunostaining (second column) for E-cadherin, paxillin, vinculin or FAK. Representative images of n=10, digital zoom 5x, scale bar = 20μm.



S3 Figure. Distribution of fibronectin in OSCC human biopsies. Regions of biopsies corresponding to the epithelia adjacent to the tumor (A) and from the center of the tumor (B) were submitted to immunostaining for fibronectin (green) and actin staining (magenta). Representative images from the same patient (n=10), scale bar = 50µm.

7. ARTIGO CIENTÍFICO 2

Artigo formatado nas normas da revista Matrix Biology

MMP activity influences adhesion properties of Oral Squamous Cell Carcinoma in 3D matrix

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Abstract

During invasion, cells need to remodel the extracellular matrix (ECM), which is mediated mainly by matrix metalloproteases (MMP). We hypothesized that abnormal MMP expression in Oral Squamous Cell Carcinoma (OSCC) could contribute to the invasive behavior of highly migratory tumor cells by the exposition of novel adhesion sites due to ECM remodeling. Initially, we analyze the distribution of MMPs in the OSCC by immunohistochemistry staining for MMPs 1, 2, 9 and 14 in human biopsies from regions of the center of the tumor and tumor adjacent epithelia. It was observed differential expression of all MMPs according to the tumor region, with a predominance of MMP 1, 9 and 14 at the invasion zone. Then we performed time-lapse movies with highly invasive OSCC cell line (SCC25) platted in 3D matrix containing fibronectin (10 μg/ml) and different concentrations of collagen (0.6 mg/ml; 1.2 mg/ml; 1.8 mg/ml) in the presence or absence of MMP inhibitor (GM0001, 25 μM) and analyzed the migration performance for each condition. It was observed a decrease on migration speed and directionality when tumor cells were challenged with a denser ECM and the presence of MMP inhibitor pronounced this effect. The role of MMPs on cell migration could be not only associate to the generation of physical space to migrate in denser matrix, but also to changes in the interaction of epithelial-derived tumor cells with a connective tissue like matrix. We transfected highly invasive OSCC cell line (SCC25) with the adhesion marker paxillin-GFP, plated in different 3D matrix in the presence or absence of the MMP inhibitor, imaged in confocal microscope with reflectance and analyzed the adhesion properties. It was observed a decrease in the area of protrusion according to the density of the collagen gel. The inhibition of MMP led to even smaller protrusions in denser matrix, which was probably due to the decrease in the adhesion area and in the ratio adhesion area/protrusion area. Taken together, the increase on MMP expression observed in tumor cells at the invasion zone of OSCC biopsies might contribute to an increase on ECM remodeling, which results in physical space for cells to invade as well as in a better adhesion process to the substrate and a consequent improvement of migration properties for invasive cells.

Keywords

Cell invasion; oral cancer; matrix metaloproteinase; cell migration;

Abbreviations

OSCC – oral squamous cell carcinoma

MMP – matrix metalloproteinase

EMC – extracellular matrix

TAE – Tumor adjacent epithelia

GFP – Green Fluorescence Protein

1. Introduction

Distant metastasis, local recurrences and the development of new tumors are responsible by the high mortality index observed in patients diagnosed with Oral Squamous Cell Carcinoma (OSCC) (1-4). During metastasis, migrating tumor cells interact with a variety of extracellular matrix (ECM) components such as collagen, fibronectin or laminin, which composition and concentration may vary according to the connective tissue region (5, 6). We recently demonstrated that the composition of ECM *per se* affects the migration pattern according to the differentiation level of OSCC, indicating a role of the microenvironment on the tumor aggressiveness (Ramos et al 2016 – *in press*). However, most of the studies regarding migration of tumor cells are performed in a 2D matrix, which neglects the challenging role of the architecture of ECM on tumor cell migration (7-11).

To migrate in a 3D matrix, cells extend pseudopods and protrusions by actin polymerization at the leading edge. Then, cells interact with ECM through integrins, which recruits structural and signaling proteins that compose highly dynamic nascent adhesion structures that may mature into more stable focal adhesions. During the adhesion process, it is possible to occur contact-dependent ECM cleavage by proteases, such as matrix metalloproteinases (MMP) that, besides its role in providing space for cells to migrate, may expose new integrin-binding residues that were hidden in ECM structure. Then, contractility of the cell body mediated by actomyosin increase the longitudinal tension, resulting in rear retraction and translocation of the cell body (7, 10-14).

Overexpression of MMPs may contribute to cell migration, cancer invasion and metastasis by remodeling of ECM. It is know that tumor cells, as well as endothelial cells, inflammatory cells and fibroblasts present in tumor microenvironment, express a variety of MMP (13, 15-19). Twenty-five MMPs were already identified in normal tissues (15, 19, 20), which are classified into membrane type-MMP (MMP-14, 15, 16, 17, 24 and 25) or secreted MMPs. The secreted MMPs are divided in collagenases (MMP-1, 8, 13 and 18), gelatinases (MMP-2 and 9), stromelysins (MMP-3, 10 and 11), matrilysins (MMP-7 and 26)

and others (MMP-12, 19, 20, 21, 22, 23, 27 and 28) (19, 21). Among the MMPs, MMP-1, MMP-2, MMP-9 and MMP-14 are the most important observed during cell invasion of several cancer types, such as prostate (22), OSCC (23), melanoma (24) and others (15, 19, 20) and the blockage of MMP activity impairs the migration process.

We hypothesized that abnormal MMP expression in OSCC could contribute to the invasive behavior of highly migratory tumor cells by the exposition of novel adhesion sites due to ECM remodeling. In OSCC biopsies, it was observed a differential expression of MMP 1, 2, 9 and 14 according to the tumor region with a predominance of 1, 9 and 14 at the invasion zone. The blockage of MMP activity decreased cell migration only in denser ECM, which was associated to a decrease in protrusion area and in the adhesion maturation process. These data indicates that the MMP activity is necessary for a better cell-ECM interaction necessary for epithelial derived tumor cells invasion into the connective tissue.

2. Results

2.1 MMPs show differential patterns according to tumor zone

In order to analyze the distribution of MMPs in the OSCC, we performed immunohistochemistry staining for MMPs 1, 2, 9 and 14 in 15 biopsies of human OSCC from regions of the center of the tumor and tumor adjacent epithelia (TAE). It was observed staining for all MMPs in all samples, but at the tumor regions it was observed a differential staining when compared to the respective TAE (Table 1). MMP-1 labeling was observed at all epithelial layers (Fig 1A) with a stronger signal at basal and suprabasal layers (Fig 1B and C) in TAE regions, while at center of the tumor, it was stronger at the periphery of the tumor islands (Fig 1D, E and F). MMP2 was observed at surface layers in TAE samples (Fig 2A) with no signal at basal and suprabasal layers (Fig 2B and C); at the center of tumor it was observed a stronger MMP2 signal at tumor islands (Fig 2D and E), as well as in stromal cells (Fig 2F). MMP-9 expression was observed in epithelial and connective cells (Fig 3A, B and C) in TAE samples; in tumor islands, MMP-9 expression was stronger at the periphery of tumor islands

(Fig 3D) and in stromal cells (Fig 3 E and F) surrounding tumor islands (Fig 3F, arrow). MMP-14 expression was detected in epithelial cell (Fig 4A and B) and adjacent connective tissue (Fig 4B and C) in TAE; in tumor regions it was observed mainly at the periphery of the tumor islands (Fig 4F) as well as in the surrounding tissue (Fig 4D and E). These data indicates that the expression level of MMPs vary according to the tumor zone, which might contribute to a differential level of ECM remodeling.

2.2 MMP inhibitor changes the migratory behavior in collagen 3D matrix

One of the possible consequences of altered ECM is to influence the migratory properties of tumor cells. To address the potential role of ECM remodeling on cell migration, we initially challenged a highly invasive OSCC cell line (SCC25) with a 3D matrix containing fibronectin (10 µg/ml) and different concentrations of collagen (0.6 mg/ml; 1.2 mg/ml; 1.8 mg/ml), performed time-lapse movies (24h) and analyzed the migration performance for each condition. It was observed a decrease on migration speed and directionality (Fig. 5) when tumor cells were challenged with a denser ECM. In order to analyze the role of MMPs in the migration process, we repeated the assay with the presence of the MMP inhibitor GM6001 (25µM). It was observed that at a low collagen density (0.6 mg/ml) MMPs are not required by tumor cells to invade; at intermediate collagen concentration (1.2 mg/ml), cells maintained the migration speed but cells showed a non-productive trajectory indicating a low directionality (-49%; control n=72, treated n=54, p = 0,000); at a high collagen concentration (1.8 mg/ml) the inhibition of MMPs led to a decrease in both speed (-69%; %; control n=85, treated n=50, p = 0,000) and directionality (-58%; control n=85, treated n=50, p = 0,009). These data indicate that ECM remodeling mediated by MMPs is an important step necessary for the invasion of tumor cells at a denser ECM.

2.3 Migratory behavior in collagen 3D matrix is dependent of changes in cell – matrix adhesion

A possible role of MMPs for tumor cell migration in a more complex ECM is not only to provide physical space for migration, but also to allow cells to

interact with a remodeled ECM and perform new adhesions necessary to initiate the migration process. In order to test this hypothesis, we analyzed the protrusion area, the number and total area of adhesions, and the ratio adhesion area/protrusion area of highly invasive OSCC cells in different 3D matrix in the presence or absence of the MMP inhibitor GM0001 (25µM). Initially, OSCC cells were transfected with the adhesion marker paxillin tagged with GFP. After 24h, cells were plated in a 3D matrix containing fibronectin (10 µg/ml) and different concentrations of collagen (0.6 mg/ml, 1.2 mg/ml or 1.8 mg/ml) for 3h and then imaged in a confocal microscope with reflectance (Fig. 6, 7 and 8).

For the analysis of protrusion, the Z-stack images were flatted for each time point and we measured the total area of membrane projections in 3D gels with different collagen concentration. Under control conditions, it was observed a decrease in the area of protrusion according to the density of the collagen gel (Table 2). When the MMP inhibitor was present in the environment, the protrusion area was smaller for all collagen concentrations and was more evident (~50%) in denser collagen matrix (1.2 – figure 7 - and 1.8 mg/ml – figure 8 – red arrows). These data indicates that the remodeling of ECM by MMPs is an important step for tumor cells overcome denser matrix during invasion by contributing to the formation of broader protrusions.

A possible reason for the decrease in the protrusion area in the absence of MMP activity is that the blockage of ECM remodeling might impair the adhesion process necessary for the maintenance of protrusion stability. Under control conditions, the analysis of adhesion number, total adhesion area and the ratio between total adhesion area/total protrusion area (Table 2) demonstrated that an increase on collagen density results in a slight decrease in the adhesion process. When MMP activity was inhibited, the adhesion process was severely impaired for all collagen concentrations (Table 2). These data suggest that the remodeling of ECM by MMPs is necessary to improve the adhesion of tumor cells to collagen fibers during the invasion process.

3. Discussion

Oral squamous cell carcinoma is a tumor with poor prognosis due to the invasive and metastatic behavior of tumor cells (25). During invasion, cells need to remodel the extracellular matrix, which is mediated mainly by matrix metalloproteases, in order to provide physical space to migrate (9). Also, during the cleavage of ECM constituents present at the tumor microenvironment, it is released several sub products that are able to interfere with the migration properties of tumor cells, such as adhesion to ECM (19). In fact, several MMPs are associated to invasion in almost all cancer types (15, 20). Understanding the level of MMP expression according to tumor region as well as the mechanism by which MMP activity contributes to tumor invasion is a key step in the development of new complementary therapeutic strategies to improve OSCC patient survival.

OSCC is constituted by a heterogeneous tumor cell population that is surrounded by a complex and diverse microenvironment. Several studies already demonstrated the overall MMP expression level in this tumor (26-29), but few of them compare the expression level of different regions of the tumor (27). In this work we demonstrated that the gelatinases MMP2 and MMP9 and the collagenase MMP1 are overexpressed at the invasion zone when compared to tumor adjacent epithelia, indicating that tumor cells need to develop strategies to undergo a collagen based physical barrier during the invasion process. Also, it was observed MMP staining in stromal cells, which reinforces the hypothesis that some of the invasive properties of OSCC are driven by the microenvironment (19, 30). Interestingly, the membrane-associated MMP14 was more evident in cells at the basal layer of the tissue adjacent epithelia when compared to the center of the tumor. According to the cancerization field theory (31), this data suggest that MMP14 is involved in early events of invasion, where tumor cells need to break up the basal membrane in order to reach the connective tissue (11, 32, 33). Taken together, these data suggest a spatial and temporal regulation of MMPs expression during tumorigenesis, which might be regulated by the challenge imposed by ECM constituents that tumor cells have to overcome during invasion.

Cell migration is a key step during invasion and metastasis and it is influenced by the interplay of intrinsic and extrinsic factors. For instance, we demonstrated that the composition of ECM might affect the migration speed and directionality of tumor cells by modulating the adhesion properties and adhesion-mediated signaling pathways (Ramos et al, 2016 *in press*) (34). Additionally, several reports demonstrated that MMP activity might not only remodel ECM but also interfere with the cell migration machinery (14, 18, 34, 35) contributing to the invasion process of tumor cells. Since most of the cell migration studies are performed in 2D models, the interplay of the intrinsic and extrinsic factors during cell migration might be even more intense when cells are challenged by a complex ECM architecture as observed *in vivo*. In fact, in 3D migration models and in living imaging experiments in animals it was already demonstrated that MMP activity is necessary for cells to migrate in denser matrix (9, 10, 36, 37). Also, there are reports that modulation on cell contractility might assist cells to overcome physical barrier in the absence of MMPs (10). Moreover, the adhesion process in 3D models correlates to matrix density and stiffness and is a critical step for the induction of migration (11, 14, 18, 38). However, how the interplay between ECM density, MMP activity and adhesion affects the migratory outcome is still unclear.

Herein we demonstrated that an increase on ECM density reflected in a decrease in migration speed. Since we observed that this effect correlated to a decrease on protrusion area but did not affected adhesion properties, it is possible that the impairment on migration by denser matrices is due to the physical challenge of smaller ECM pores and not to the ability of cells to interact with ECM fibers. However, when tumor cells were plated in a 3D environment in the absence of MMP activity, the effects of ECM density on cell migration were more evident. It was observed that inhibition of MMP resulted in both decrease in the protrusion area and impairment in the adhesion properties in denser matrix (1.2 and 1.8 mg/ml). These data suggests that MMPs is necessary not only to provide physical space for invasion in 3D matrix but also to allow tumor cells to interact with new adhesion sites in the remodeled ECM.

Taken together, the increase on MMP expression observed in tumor cells at the invasion zone of OSCC biopsies might contribute to an increase on ECM remodeling, which results in physical space for cells to invade as well as in a better adhesion process to the substrate. This increase on MMP expression can be related to the aggressive behavior observed in OSCC, especially for highly invasive cells. However, it is still necessary more studies to understand the interplay of intrinsic and extrinsic factors during cell invasion, in order to develop new strategies to improve the survival of OSCC patients.

4. Material and Methods

4.1 Human Biopsies and OSCC cell culture

The experimental design and the informed consent procedures were approved by the Ethical Committee of Federal University of Rio Grande do Sul - Brazil and of Hospital de Clínicas de Porto Alegre (HCPA) - Brazil (GPPG nº 14-0019) and all patients in this study provided written informed consent. Patients (n= 15) with oral lesions were interviewed and submitted to surgery; OSCC diagnosis was confirmed histopathologically by a pathologist and fragments from two regions were collected (39): one was removed from the center of the tumor (without necrosis) and another sample was removed from surgical margin, and it was named as tumor adjacent epithelia (TAE).

OSCC cell lines were obtained from the Tissue Culture Facility at School of Medicine of University of Virginia and checked for mycoplasma by this facility. SCC25 cells (ATCC® CRL-1628™) in DMEM/F12 with 15mM HEPES and 0.5mM sodium pyruvate (Gibco) supplemented with FBS 10% and hydrocortisone (400ng/ml, Sigma), and cells were maintained in incubator (37^{o}C, 5% CO_2). SCC25 cells are highly invasive with low E-cadherin levels (40). For confocal movies, cells were transfected with the plasmids to paxillin + GFP (41) using a TransIT® - 2020 Transfection Reagent (Mirus).

4.2 Experimental conditions

Unless otherwise stated, all reagents were purchased from Sigma Aldrich. For 3D imaging experiments, cells were trypsinized, washed and plated

in a rat tail-derived collagen matrix assembled according to the manufacturer (Gibco, A1048301) in the presence of fibronectin (10µg/ml). It was used 3 different collagen concentrations: 0.6 mg/ml; 1.2 mg/ml; 1.8 mg/ml. For each condition, a thin layer of the respective collagen matrix density was initially plated at the surface of the glass-bottomed dishes. After polymerization, $3x10^4$ cells were embedded in a new collagen matrix and, after 3h, imaged using CCM1 media (Hyclone, Thermo Scientific). For analysis of MMP activity, it was added 25µM (42) of MMP inhibitor (GM6001 – Calbiochen) or vehycle solution (DMSO) during collagen polymerization procedures. To ensure that cells were in the 3D matrix for imaging experiments, it was verified the lower and the upper focus with detectable cells and it was always selected cells for imaging at an intermediate focus position. All images were analyzed using ImageJ software (http://rsbweb.nih.gov/ij) and panels mounted using Adobe Photoshop® 7 software.

4.3 Immunohistochemistry

For tumor staining, human biopsies were fixed immediately after collection (4% formaldehyde, 4h, 4°C), cryoprotected with increasing sucrose concentrations (10-30%, 4°C), embedded in OCT compound, frozen (-20°C), cut using cryostat and seven µm-thick slices were collected in gelatin-covered slides. The human samples were hydrated; block to inhibit endogenous peroxidase activity (3% H_2O_2 in methanol) and were permeabilized (Triton X-100 0.3%, RT 10min), blocked (10% normal goat serum, RT, 1h), incubated with antibodies for MMP – 1, MMP – 2, MMP – 9 and MMP – 14 (ON, 4°C). The Envision dual link kit (Dako Corporation, Carpinteria, CA, USA) was used for the application of the biotinylated link antibody and peroxidase-labeled streptavidin, and the 0,03% 3-3,3'-Diaminobenzidine (DAB, DakoCytomation, Carpinteria, CA, USA) was used as a chromogen, and the sections were counterstained with Harris's hematoxylin. Besides brightness/contrast corrections, no further image editing was performed and figures were prepared using Adobe Photoshop® 7 software. The qualitative analysis was performed based in the

localization of positive cells. Cells with brown cytoplasmic or membrane staining were considered positive.

4.5 Migration and adhesion dynamics assays

Imaging acquisition and analysis was performed as previously described (43). For phase microscopy movies, images were captured at 10min intervals using a Nikon TE300 microscope (10x 0.25 NA CFI Achro DL106 Nikon objective) with a charge coupled device camera (Orca II, Hamamatsu Photonics) using Metamorph software (Molecular Devices). For analysis of migration parameters, it was performed at least 4 independent experiments and the nucleus of each migratory cell was tracked using the “manual tracking” plug-in on ImageJ. To determine migration speed, it was performed the ratio between the total distance traveled (distance) and the number of slices (time) that cell migrated. To analyze the directionality of cell migration, the X and Y coordinates obtained during the tracking of the nucleus of the migratory cell in each slice were normalized to start at a virtual X=0 and Y=0 position and the variation on the position.

For adhesion dynamics, imaging acquisition was performed as previously described (41) using an Olympus Fluoview 1000 laser scanning confocal microscope with a UPlanSApo ×60 (1.20 NA) water-immersion objective and a stage heater that maintained the sample at 37 °C. Paxillin-GFP construction was excited with the 488 nm laser line of an Argon ion laser, while collagen fibers were imaged simultaneously in reflectance mode: exciting with the 488 nm laser and collecting the scattered (reflected) light at the same wavelength. Regions of migrating cells were imaged with a 5x digital zoom and Z slices were acquired every 0.33 μm for 15 slices, which resulted in a total axial size of 5 μm). This procedure was repeated every 30 s during a least 5 min and Laser power was adjusted to minimize photobleaching. All images and movies were analyzed using ImageJ software. For each time point (30 s), the Z stack images were grouped in only one image, using the maximum intensity parameter. With the new images for each time point, it was performed a new movie and then it was analyzed the adhesion length, area, and the percentage of total adhesion

area in each protrusion. Adhesion length and area was determined by measuring, respectively, the long axis or the area of each adhesion that assembled during the movie. The percentage of total adhesion area in each newly formed protrusion was measured by the ratio of the sum of the area of all adhesions that assembled in the protrusion by the total area of the protrusion. The adhesion assembly speed was measured using the “kymograph” plug-in on ImageJ. For each adhesion, a line (1 pixel-wide) was drawn in the long axis of the adhesion and the X (distance) and Y (time) coordinates originated by the kymograph were used to measure the speed of adhesion assembly. All data were calculated using Microsoft Excel® (Microsoft Corporation) and SPSS 21 software (Statistical Package for the Social Science, IBM).

4.7 Statistical analysis

Mann-Whitney was employed, using the SPSS21 software and differences were considered significant when $p \leq 0.05$.

5. Acknowledgements

The authors thank Laura de Campos Hildebrand, Manoel Sant’Ana Filho and Alessandro Menna Alves for help with the human sample biopsy collection and Kris Kubow for the assistance in the preparation of 3D gels and confocal imaging.

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Table and Images

Table 1: OSCC tumor zones show different staining for MMP.

	Tumor adjacent epithelia		**Center of the tumor**	
	Basal and suprabasal layers	Connective tissue cells	Tumor island	Stromal cells
MMP-1	+	-	++	-
MMP-2	-	+	+++	+
MMP-9	+	+	++	+
MMP-14	++	+	+	-

Legend: - negative; + less intensity; ++ mild intensity; +++ high intensity.

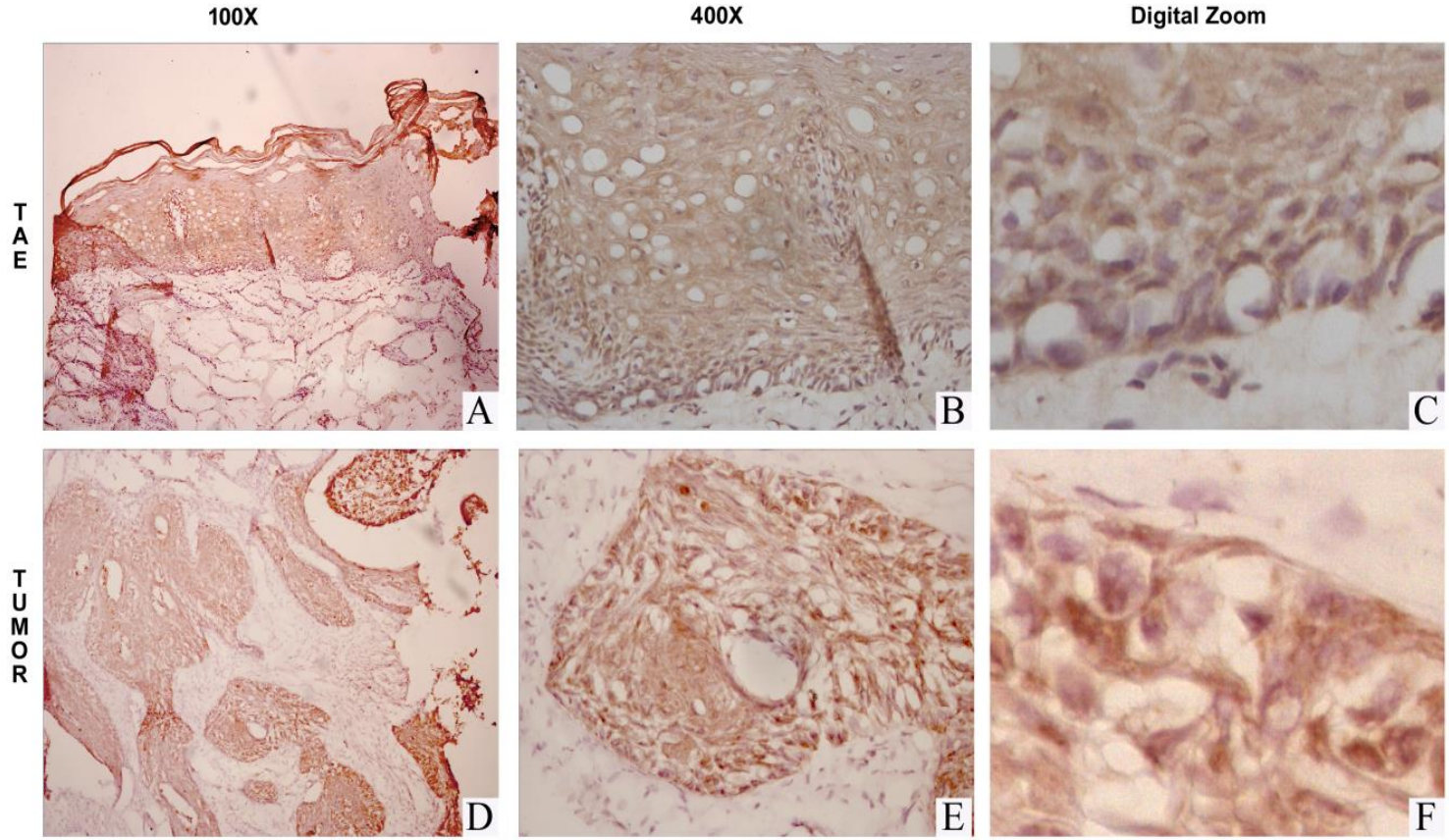

Figure 1 – Immunostaining for MMP-1 in OSCC in different tumor zone: MMP-1 labeling in TAE (A, B and C); center of the tumor (D, E and F). . Legend: TAE – tumor adjacent epithelia.

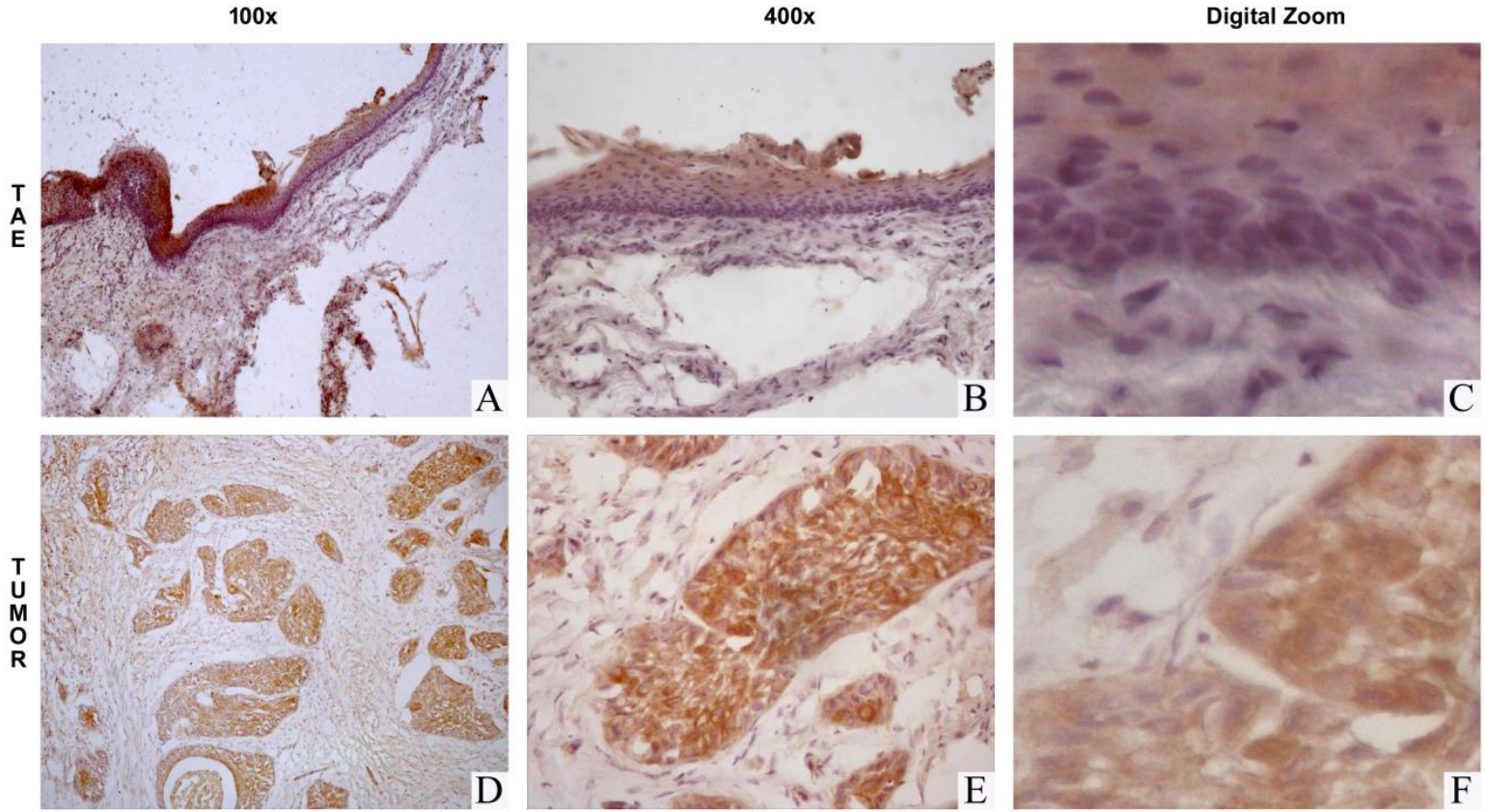

Figure 2 – Immunostaining for MMP-2 in OSCC in different tumor zone: MMP-2 labeling in TAE (A, B and C); center of the tumor (D, E and F). Legend: TAE – tumor adjacent epithelia.

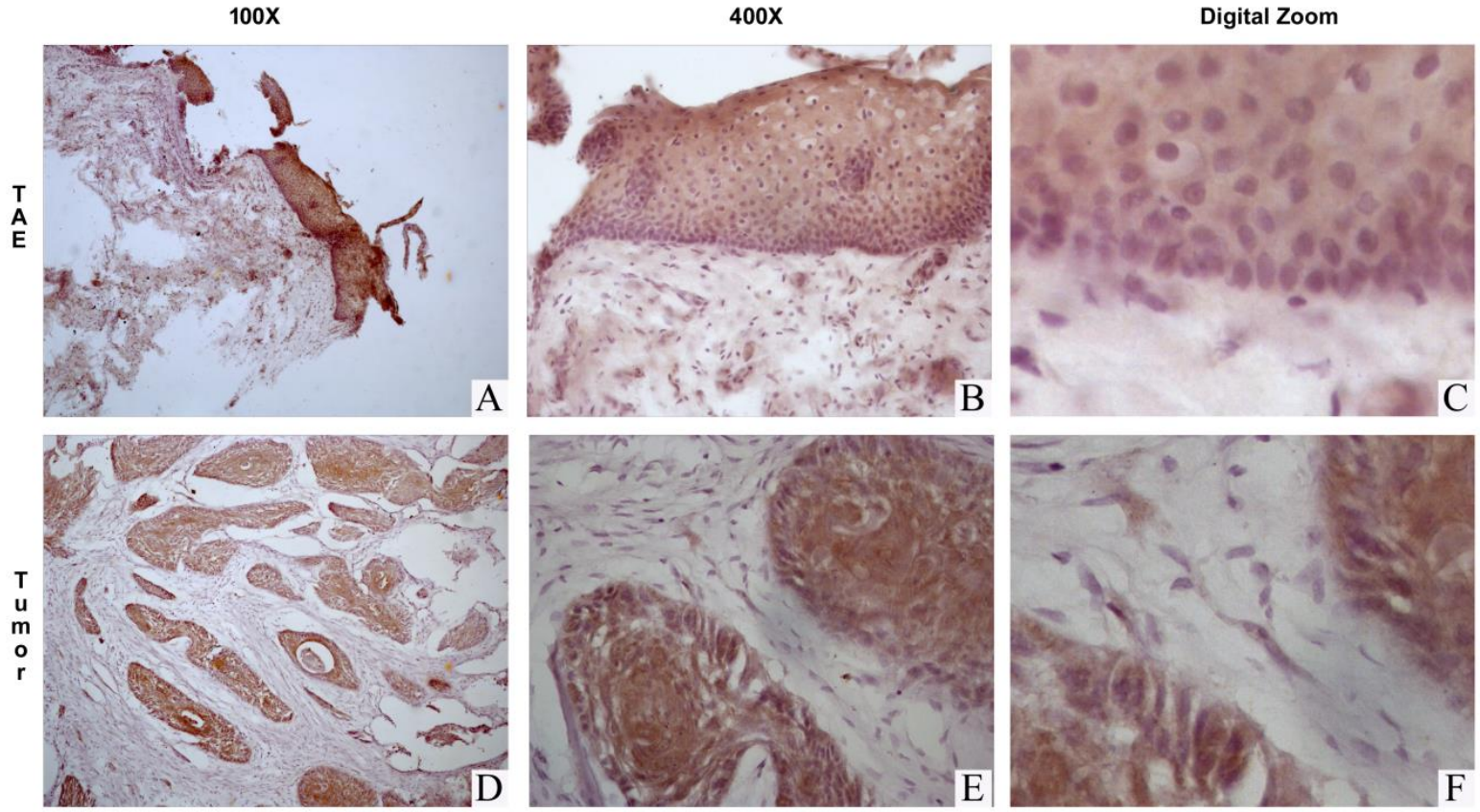

Figure 3 – Immunostaining for MMP-9 in OSCC in different tumor zone: MMP-9 labeling in TAE (A, B and C); center of the tumor (D, E and F).Legend: TAE – tumor adjacent epithelia.

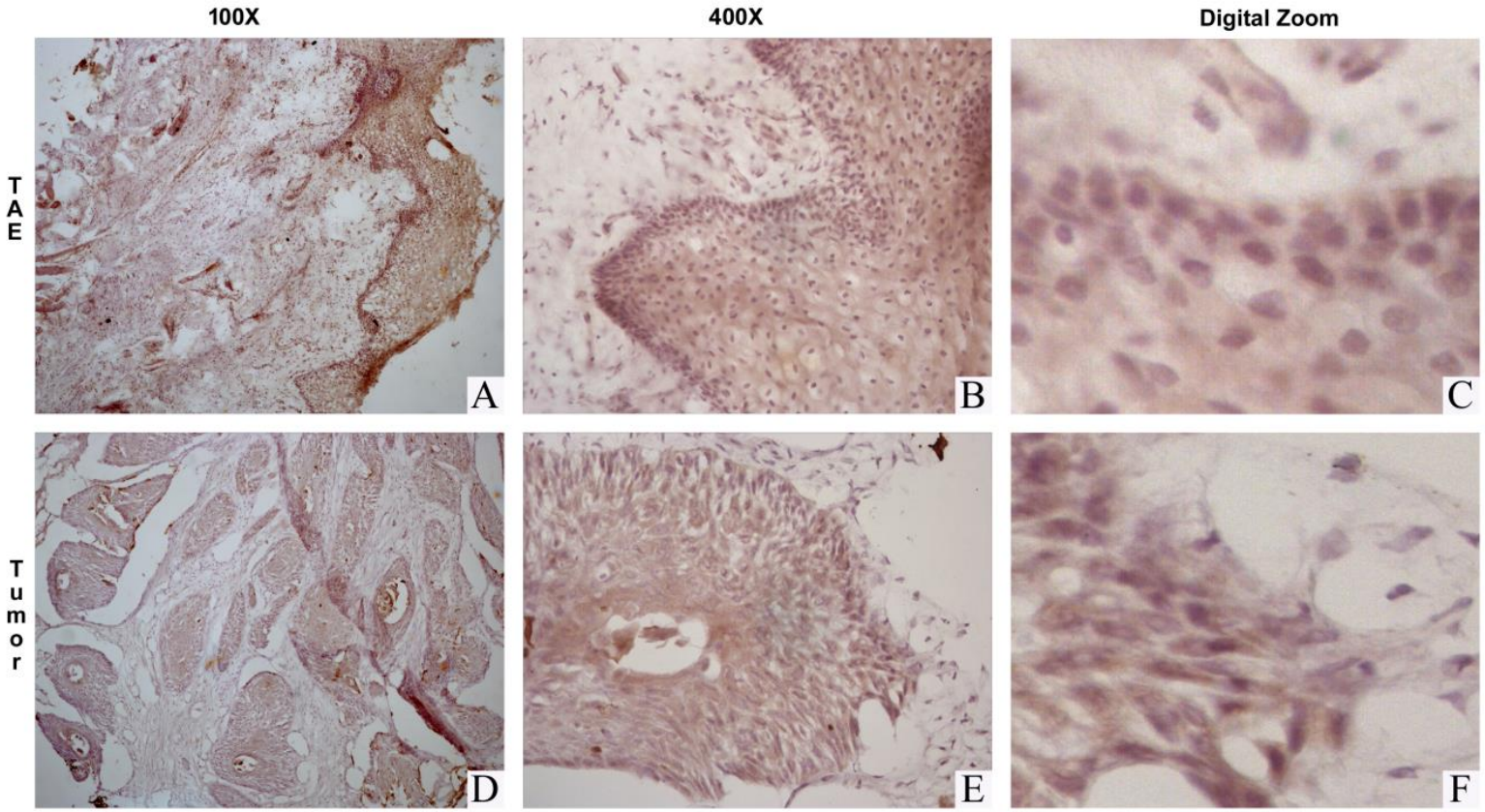

Figure 4 – Immunostaining for MMP-14 in OSCC in different tumor zone: MMP-14 labeling in TAE (A, B and C); center of the tumor (D, E and F).Legend: TAE – tumor adjacent epithelia.

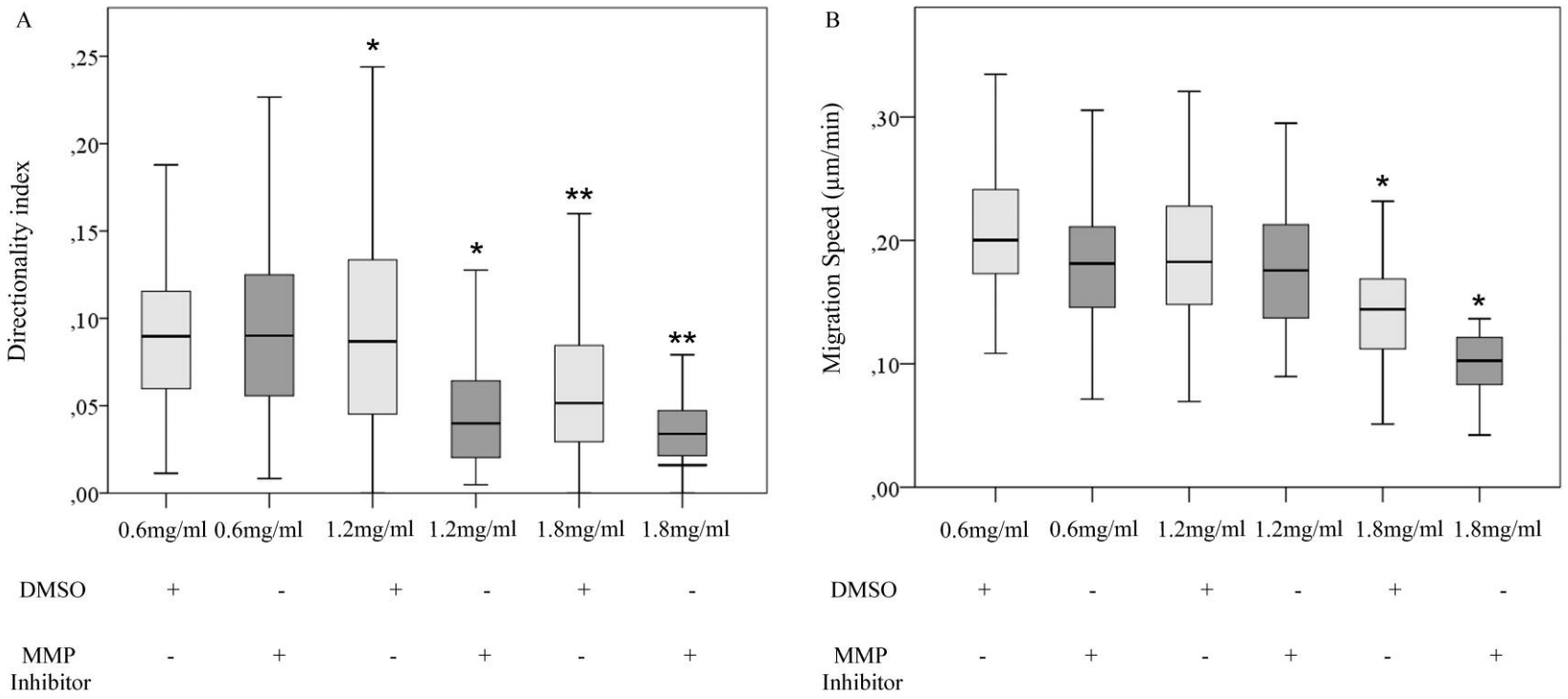

Figure 5: Migratory behavior of OSCC cell line in collagen 3D matrix: (A) cell directionality in different concentrations of collagen; (B) migration speed in different concentration of collagen (B). * – p = 0.000; ** – p = 0.009; Mann Whitney test.

collagen		Number of adhesion per protrusion (mean ± SD)			Sum of total adhesion area per protrusion (mean ± SD)			Ratio adhesion area by protrusion area (%)			Protrusion area (mean ± SD)		
mg/ml		μm	n	P value	μm	n	P value	μm	n	P value	μm	n	P value
0.6	Control (DMSO)	0.6 (±0.2)	14	0.318	0.6 (±0.3)	14	0.000	1.1 (±0.09)	14	0.000	5.2 (±.17)	14	0.002
	GM0001 (25μM)	0.5 (±0.2)	14		0.2 (±0.1)	14		0.4 (±0.03)	14		3.5 (±1.7)	14	
1,2	Control (DMSO)	0.6 (±0.2)	3	0.046	0.7 (±0.3)	3	0.050	1.4 (0.1)	3	0.050	4.5 (±2.0)	3	0.05
	GM0001 (25μM)	0.2 (±0.05)	3		0.06 (±0.02)	3		0.2 (±0.01)	3		2.0 (±0.5)	3	
1,8	Control (DMSO)	0.6 (±0.2)	11	0.106	0.2 (±0.1)	11	0.015	0.9 (±0.07)	11	0.317	2.0 (±1.2)	11	0.282
	GM0001 (25μM)	0.4 (±0.04)	5		0.07 (±0.02)	5		0.5 (±0.04)	5		1.3 (±0.6)	5	

Table 2: Results of adhesion and protrusion analysis of OSCC plated in different 3D matrix

Mann Whitney test, $p \leq 0.05$. Legend: SD – standard deviation; N – number of cells.

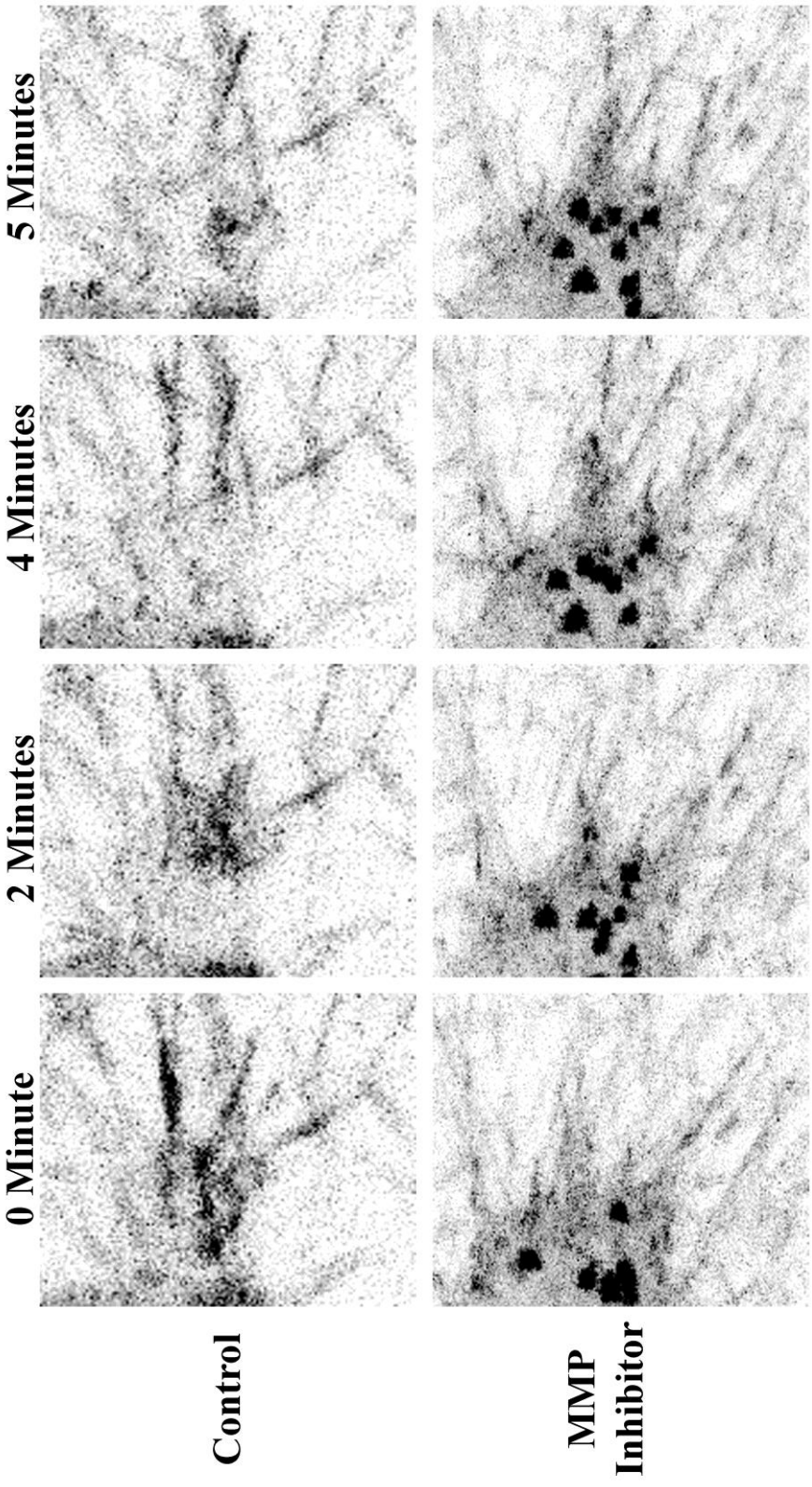

Figure 6: Adhesion behavior of OSCC cells in 3D matrix in low concentration of collagen (0.6 mg/ml): (first line) control conditions; (second line) cells treated with MMP inhibitor (GM6001).

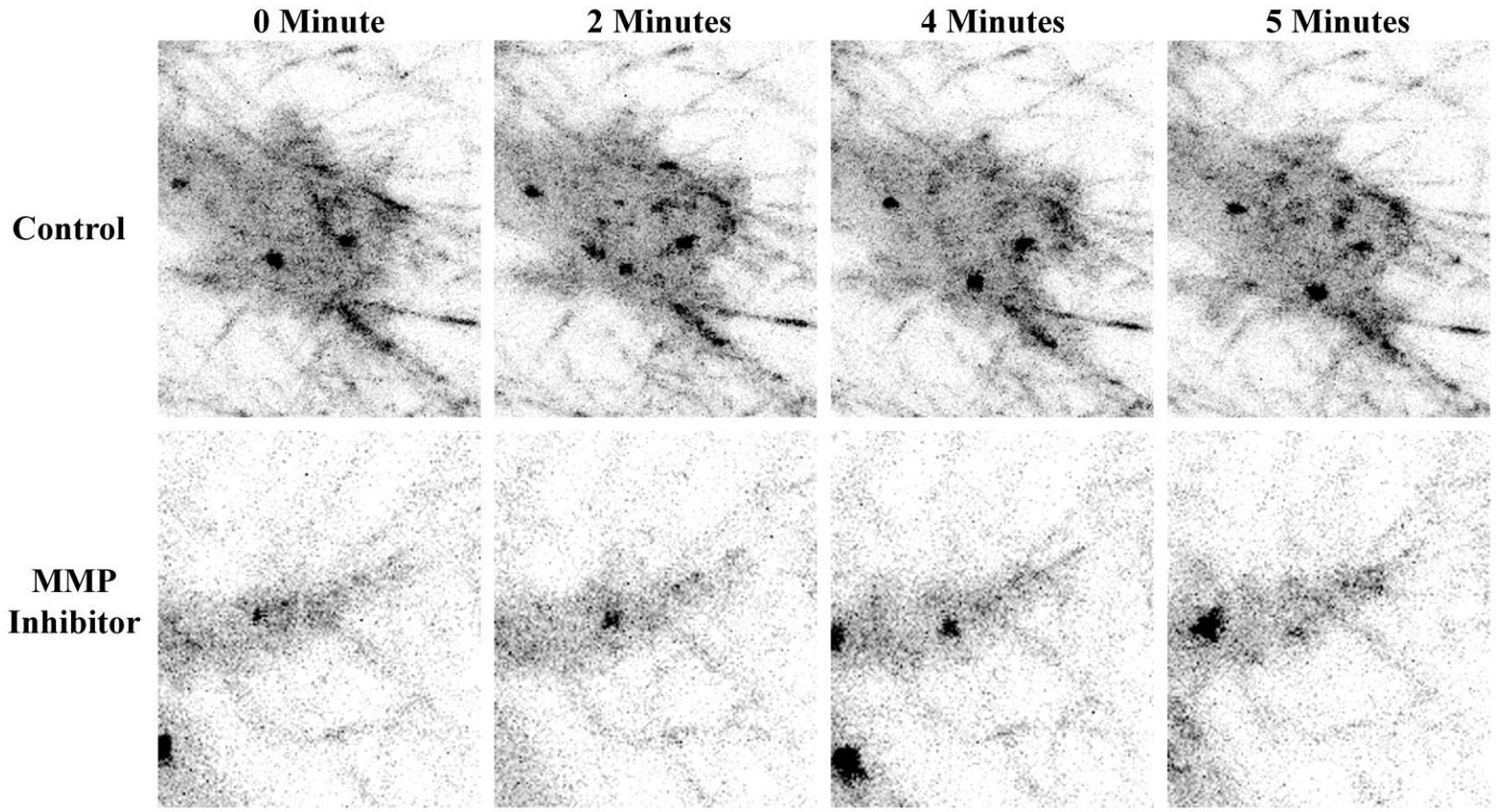

Figure 7: Adhesion behavior of OSCC cells in 3D matrix in intermediated concentration of collagen (1.2 mg/ml): (first line) control conditions; (second line) cells treated with MMP inhibitor (GM6001).

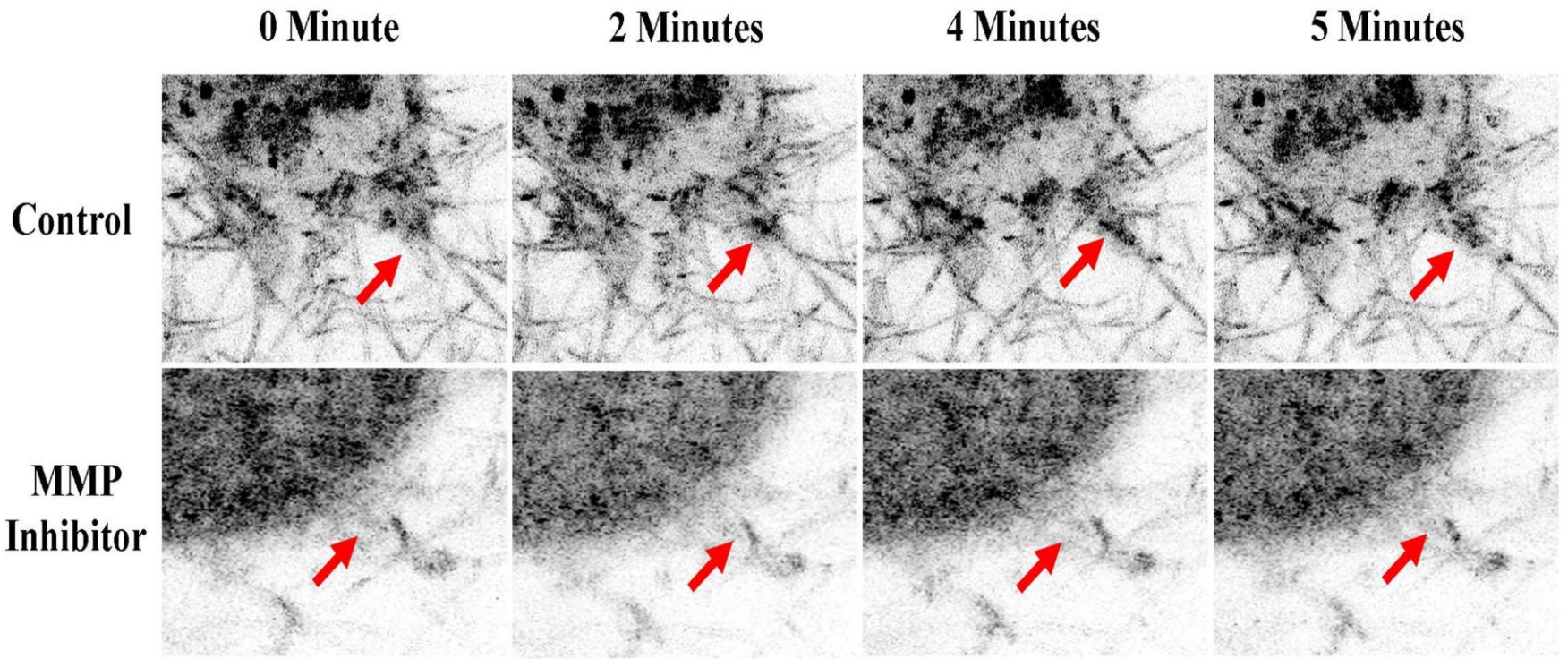

Figure 8: Adhesion behavior of OSCC cells in 3D matrix in high concentration of collagen (1.8 mg/ml): (first line) control conditions; (second line) cells treated with MMP inhibitor (GM6001). Red arrows – new adhesion formation.

8. ARTIGO CIENTÍFICO 3

Artigo formatado nas normas da revista Tumor Biology

Cell contractility modulate migratory behavior in Oral Squamous Cell Carcinoma

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Abstract

Oral squamous cell carcinoma (OSCC) is the most prevalent cancer in oral cavity and presents a high mortality index due to tumor invasion and metastasis. The non-muscle myosin II isoforms (NMII) are involved in the regulation of cell migration, which is the main feature of cell invasion. We hypothesized that the modulation of NMII activity is impaired in OSCC which might contribute to the invasive behavior of this tumor. We analyzed the expression levels of NMIIA, NMIIB and NMIIC in OSCC biopsies and we observed that all NMII isoforms are overexpressed at the invasion zone of

OSCC biopsies and in a highly invasive OSCC cell line (SCC 25). We also analyzed the effects of NMII-related contractility in tumor cell dispersion. By analyzing the cell cohesion in spheroids, it was observed that an increase on contractility resulted in cell detachment from spheroids, while a decrease in contractility resulted in better cell-cell adhesion. Our results indicate that NMII is involved in tumor cell dissemination and that modulation of cell contractility might be an interesting tool for cancer treatment.

Keywords: non-muscular myosin II; cell migration; oral cancer,

Introduction

Oral Squamous cell carcinoma (OSCC) is the most common tumor in oral cavity and represents about 80 – 90% of head and neck cancer [1]. OSCC can occur in any site of oral mucosa, but it is most frequent in tongue and floor of the mouth [1-5]. OSCC is originated from oral keratinocytes with DNA mutation, which might occur spontaneously or by mutagenic agents such as tobacco and alcohol, betel chewing, sunlight exposure, ionizing radiation and human papillomavirus [2, 3, 5, 6]. Despite the evolution in cancer research, patient prognosis remains poor, and ranges around 50% in 5 years. Distant metastasis, local recurrences and the development of new tumors are responsible by the high mortality index [1, 4-6], and the understanding of the tumor invasion process is a key step to improve patient survival.

Cell migration is one of the main events related to tumor invasiveness and the non-muscle myosin II isoforms (NMII) play a key regulatory role in several steps necessary for cell migration [7, 8]. For example, NMIIA participate in lamellipodium formation and adhesion maturation at front of the cell, while NMIIB drives adhesion maturation, nucleus dislocation and detachment from the substrate due to contractility at the cell rear and the role of MIIC in physiological process remains unclear [8, 9]. Also, NMII-mediated contractility might dictate the switch from a slow and collective to a fast and more invasive single cell migration [8, 10], which is observed during epithelial to mesenchyme transition [10]. Besides its role in regulation the migration process, NMII is also involved in other events during tumorigenesis, such as aberrant cell division and cell detachment, and the development of strategies to modulate NMII activity is a promising field in cancer therapy.

NMII is a multimeric protein which structure is composed of a heavy chain, an essential (ELC) and a regulatory light chain (RLC). The heavy chain is composed by a tail region that indicates the subcellular localization of the NMII isoform; a neck region, which binds to ELC and RLC; and a globular head domain that is responsible for F-actin binding, hydrolysis of adenosine triphosphate (ATP) and contraction of actin bundles. The contractile activity of

NMII relies in conformational changes of the head domain, which is mainly controlled by phosphorylation of RLC at residues Thr18 and/or Ser19 [8, 11].

Since NMII is involved in several steps of tumorigenesis, we hypothesized that the modulation of its activity is impaired in OSCC which might contribute to the invasive behavior of this tumor. We observed that NMII isoforms are overexpressed at the invasion zone of OSCC biopsies and in a highly invasive OSCC cell line. Also, changes on NMII activity resulted in modulation in cell contractility and dispersion. Taken together, these results indicate that NMII is involved in tumor cell dissemination by modulation in cell contractility.

Material and Methods

Human Biopsies and OSCC cell culture

The experimental design and the informed consent procedures were approved by the Ethical Committee of Federal University of Rio Grande do Sul - Brazil and of Hospital de Clínicas de Porto Alegre (HCPA) - Brazil (GPPG nº 11-0289; GPPG nº 14-0019) and all patients in this study provided written informed consent. Patients (n=19) with oral lesions were interviewed and submitted to surgery. OSCC diagnosis was confirmed histopathologically by a pathologist and fragments from regions corresponding to the center of the tumor and the carcinoma edge tissue, named as tumor adjacent epithelia (TAE) were collected. Clinical information reveal that the most of patients were male with age around 65,5 years and the lesions appear more in border of the tongue (S. Table 1).

OSCC cell lines were obtained from the Tissue Culture Facility at School of Medicine of University of Virginia and checked for mycoplasma by this facility. Cal27 cells (ATCC® CRL-2095™) were cultivated in DMEM high glucose (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) while SCC25 cells (ATCC® CRL-1628™) in DMEM/F12 with 15mM HEPES and 0.5mM sodium pyruvate (Gibco) supplemented with FBS 10% and hydrocortisone (400ng/ml, Sigma), and cells were maintained in incubator

(37^{o}C, 5% CO_2). Cal27 cells are considered low invasive OSCC cells [12] with high e-cadherin levels (Fig. 1) ($L^{inv}/H^{E\text{-}cad}$), while SCC25 cells are highly invasive with low E-cadherin levels ($H^{inv}/L^{E\text{-}cad}$). Transfection was performed as previously described [13, 14] with 1μg of the plasmids encoding GFP, p-Super RLC + GFP, p-Super RLC + MLC-WT, p-Super RLC + MLC-DD; p-Super RLC + MLC-AA, p-Super RLC + MLC-AD, p-Super RLC + MLC-DA, using a TransIT® - 2020 Transfection Reagent (Mirus).

Experimental conditions

Unless stated otherwise, all reagents were purchased from Sigma Aldrich. Spheroids were performed plating 1x10^4 for SCC 25 or 2X10^4 for CAL 27, in a 96 wells dish covered with 1.5% agarose [15]. The spheroids were treated with DMSO, Blebistatin (1 or 10μM, Calbiochen), calyculin A (1nM or 100pM, Calbiochen) and, after 24h, images were obtained were imaged using an inverted microscope EVOS® FL Cell Imaging System (Live technologies). All images were analyzed using ImageJ software (http://rsbweb.nih.gov/ij) and panels mounted using Adobe Photoshop® 7 software.

Immunoblots

For analysis of NMII isoforms levels, cells (1x10^6) were trypsinized or spheroids were collected, washed and lysed in buffer containing protease and phosphatase inhibitors cocktails. Cell lysates (20μg) were separated in 4-20% SDS gels (Biorad) and proteins transferred to PVDF membranes, blocked (4% BSA) and immunoassayed for E-cadherin, N-cadherin (Cell Signaling), NMIIA, NMIIB, NMIIC (Covance) or β-Tubulin (Sigma) using Pierce ECL Western Blotting Substrate (Thermo Scientific). For the analysis of transfection levels, spheroids were lysed in lysis buffer (1% of NP-40; 10 uM TRIS pH 7.4; 0,1M NaCl; 10mM ATP; 5 mM $MgCl_2$; protease and phosphatase inhibitor). Cell lysates (20μg) were separated in 4-20% SDS gels (Biorad) and proteins transferred to PVDF membranes, blocked (4% BSA) and immunoassayed for GFP, MLC (Cell Signaling) and β-Tubulin using Pierce ECL Western Blotting

Substrate (Thermo Scientific). Densitometry of the bands was performed using ImageJ software.

Immunohistochemistry

For tumor staining, human biopsies were fixed immediately after collection (4% formaldehyde, 4h, 4^{o}C), cryoprotected with increasing sucrose concentrations (10-30%, 4^{o}C), embedded in OCT compound, frozen (-20^{o}C), cut using cryostat and seven µm-thick slices were collected in gelatin-covered slides. The slices were hydrated; blocked to inhibit endogenous peroxidase activity (3% H2O2 in methanol); permeabilized (Triton X-100 0.3%, RT 10min); blocked for unspecific staining (10% normal goat serum, RT, 1h) and incubated with antibodies for NMIIA, NMIIB and NMIIC (ON, 4^{o}C). The Envision dual link kit (Dako Corporation, Carpinteria, CA, USA) was used for the application of the biotinylated link antibody and peroxidase-labeled streptavidin, and the 0,03% 3-3,3'-Diaminobenzidine (DAB, DakoCytomation, Carpinteria, CA, USA) was used as a chromogen, and the sections were counterstained with Harris's hematoxylin. Samples were washed (PBS) and mounted with antifade medium (Vectashield, VectorLab, Burlingame, CA). Besides brightness/contrast corrections, no further image editing was performed and figures were prepared using Adobe Photoshop® 7 software. The qualitative analysis was performed based in the localization of positive cells. The epithelial or epithelial-like cells with brown citoplasmic staining were considered positive.

Results

OSCC show an increase of NMII in invasion zone

In order to analyze the expression levels of NMII in OSCC, biopsies of patients (n=15) were collected and submitted to immunohistochemistry reaction. At the tumor adjacent epithelia, NMIIB was detected mainly at the basal cell layer, while NMIIA and NMIIC showed a weak staining at suprabasal levels (Fig 1). At regions corresponding to the tumor, NMIIA and C were detected at the center of the tumor island, while NMIIB showed a strong labelling in cells at the periphery of the invading island (Fig 1A). We also measured the NMII levels of

two human OSCC cell lines with different levels of differentiation. Interestingly, the less differentiated and more invasive OSCC cell line (SCC25) showed increased expression of MIIA and MIIB when compared to the more differentiated and less aggressive (CAL 27) OSCC cell line. These data suggest that more aggressive OSCC show an increase in NMII expression, especially for the NMIIB isoform at the invasive regions.

Cell contractility modulate the cell migration in OSCC

Since NMII is involved in the regulation of the migration process, we hypothesized that the overexpression of NMIIB observed in cells at the invasion front and in highly invasive OSCC cell lines could increase cell contractility contributing to tumor dissemination. In order to represent the individual epithelial islands observed in the biopsies, we performed spheroids and analyzed the cohesion of the cells. We observed that cells with lower levels of myosin (Cal27) presented a more defined border of the spheroids, indicating a better cohesion, when compared to cells with more NMII (SCC25). To analyze the role of cell contractility on spheroid cohesion, we prepared spheroids of both cell lines and incubated immediately or after 24h of formation with blebstattin or caliculin A, which are respectively a specific inhibitor [16, 17] and an activator [18] of NMII activity. It was observed for both cell lines that when NMII-mediated contractility is inhibited, the spheroids showed a better definition of the borders, however, when NMII activity is increased; there was a dissociation of the spheroids (Fig 2).

In order to confirm these results, we decreased the expression of endogenous myosin regulatory light chain, and replaced with phosphomimetics forms of RLC [13, 14] representing: - no contractility (MLC-AA), - fully contractile (MLC-DD), and - intermediate level of contractility (MLC-AD or MLC-DA) (Fig 3). After 72hrs of transfection, we performed spheroids for both cell lines and analyzed the morphology of the spheroids. It was observed that cells with low MLC activity showed a better cohesion then cells with increasingly levels of contractility. Taken together, these results indicate that NMII is involved in tumor cell dissemination, where a more contractile state contributes to the invasion of

cells from tumor, while a decrease on its activity favors a more restrained and well defined tumor island.

Discussion

Metastasis and tumor invasion are the main cause of failure in cancer treatment, and one of the most important event in both processes is cell migration. For instance, OSCC is the most prevalent cancer in oral cavity and present a high mortality index [1-3]. Besides its histopathology feature of cohesive epithelial islands [2], cells at the invasion front undergo EMT process resulting in highly migratory and invasive tumor cells that are responsible for the invasive and metastatic behavior of this tumor [1, 4-6]. Besides the current knowledge regarding tumor proliferation and apoptosis [2, 19, 20], the understanding of the mechanisms involved in the invasion process of these migratory cells might contribute to the development of new complementary strategies to improve patient prognosis.

Non-muscle myosin II has a key regulatory role during cell migration [21], which makes NMII a potential candidate for cancer treatment. Under physiological conditions, NMII is involved in several processes such as determination of cell polarization, organization and cellular regulation as well as in mechanotransduction [8, 9, 14, 21-23]. The NMII isoforms (A, B and C) are encoded by three different genes and, besides the high homology, the isoforms present differences in subcellular localization [11]. For instance, MIIA participate in the lamelipodium formation and adhesion maturation at the front of the cell, while MIIB regulates of the cell body and the cellular detachment [9, 24]. The role of MIIC in physiological process remains unclear. However, besides the differences in localization, all isoforms share a similar regulatory pathway, which makes the regulation of NMII a potential tool to regulate several steps associated to tumorigenesis.

Deregulation of NMII activity or expression is associated to a broad spectral of diseases [8, 25-28]. During carcinogenesis, NMII is involved in abnormal cellular division and cell invasion [7, 8]. Also, the expression of NMII

isoforms is altered in several cancer types [28-33] but its role in patient prognosis is still unclear. For instance, NMIIA is associated to a poor prognosis in esophagus, stomach, bladder and chronic lymphocytes leukemia [28, 30, 34-36], but in lung adenocarcinoma, NMIIA is related to a good prognosis [37] while in skin and head and neck squamous cell carcinoma, NMIIA serves as tumor suppressor [31]. The NMIIB expression is increased in oral and vulvar SCC and melanoma and variable expression in glioma [8]; it is related to lymph node metastasis in breast cancer [38] and a worst prognosis in melanoma [39]. NMIIC is overexpressed in breast, pancreas, lung, liver, ovary and prostate tumor cell lines, melanoma and variable expression in glioma [8, 40], and the alteration in NMIIC expression can be related to abnormal cytokinesis [40]. However, most of the studies show changes in the whole tumor tissue with little or no analysis on the expression according to normal tissue or to the region of the tumor. Herein, we demonstrated that NMIIB is predominant in tumor cells at the invasion zone and is overexpressed in a highly invasive/low differentiated OSCC cell line, suggesting a possible role of NNMII, more specifically for MIIB, during invasion and metastasis.

A possible mechanism of action of NMII during metastasis is that NMII-derived contractility might influence the migratory behavior according to the differentiation level of tumor cell. We demonstrated that, in cells with strong cell-cell adhesion, an increase in cell contractility leads to dissociation of cells from spheroids. Also, we demonstrated that a decrease on cell contractility in highly invasive/low differentiated OSCCs results in increase on cell-cell cohesion. These data associated to the overexpression of NMIIB in cells at the invasion zone of patient biopsies, corroborates to the hypothesis of NMII driving the detachment of individual cell from tumor islands. In fact, several *in vitro* studies demonstrated that an increase on NMII-mediated contractility results in impairment of cell-cell adhesions [8, 9] as well as in changes on the mitotic axis favoring the detachment of cells at the basal lamina [8, 41]. Since cell contractility is involved in cell invasion in melanoma [42], glioma [33, 43] and fibrosarcoma [44], the role of NMII in invasion is apparently a conserved mechanism during tumorigenesis of several cancer types.

In summary, we demonstrated that NMII isoforms are overexpressed in OSCC in both the center of the tumor and in highly invasive OSCC cells line. We suggest that NMII-mediated contractility is involved in the invasion of the OSCC, since pharmacological and genetic modulation of cell contractility altered the cohesive state of tumor cells, which might contribute to tumor dissemination. These data indicates that modulation of NMII activity might be considered as a potential therapeutic tool to impair tumor invasion, due to its role in the modulation of cell-cell adhesion properties.

Acknowledgments

The authors thank Laura de Campos Hildebrand, Isabel da Silva Lauxen, Alessandro Menna Alves and the Head and Neck Surgery and Pathology Department from HCPA for help with the human sample biopsy collection.

Compliance with ethical standards

This study was done in consonance with the ethics aspects

Conflicts of interest

None

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Figure Legends

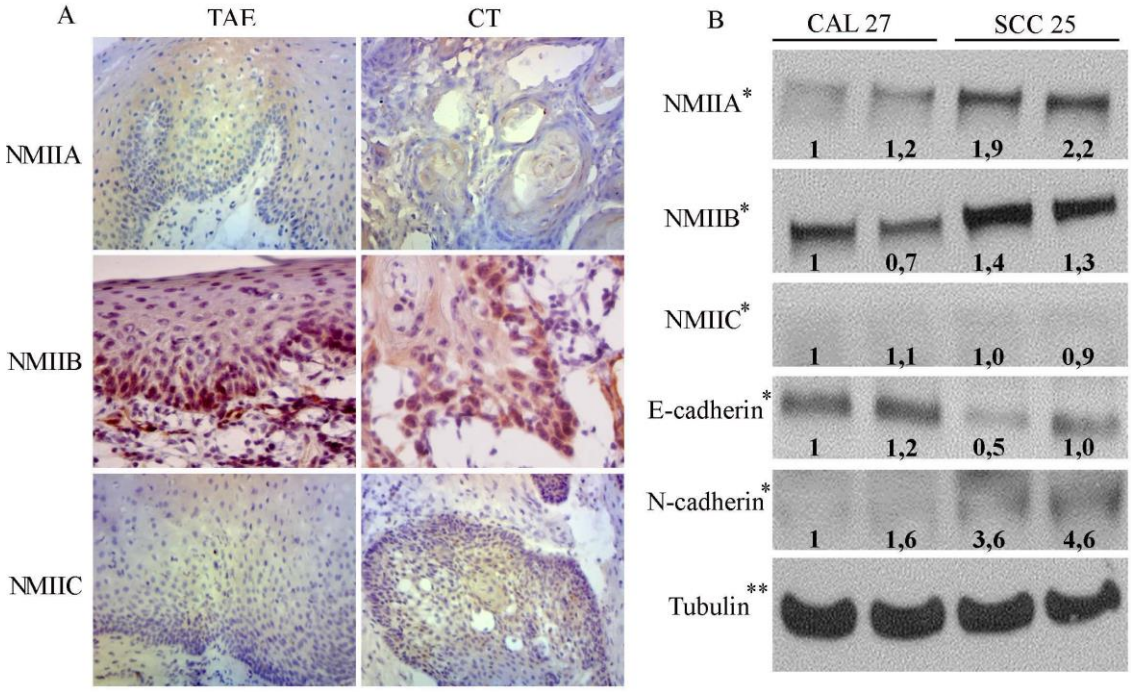

Figure 1 – Characterization of non-muscle myosin II isoforms expression in OSCC biopsies and cell lines: A- Immunohistochemistry staining of NMIIA (Fig1 a, b), NMIIB (Fig 1c, d) and NMIIC (Fig 1e, f) at adjacent epithelia (TAE, Fig 1a, c, e) and center of the tumor (CT, Fig 1b, d, f). B – Representative western blottings for NMIIA, NMIIB, NMIIC, E-cadherin, N-cadherin and tubulin in low (Cal27) or highly (SCC25) invasive OSCC cell lines. * Densitometry values were normalized to the loading control and then compared to the first band (arbitrary value of 1). ** Densitometry values were compared to the first band (arbitrary value of 1).

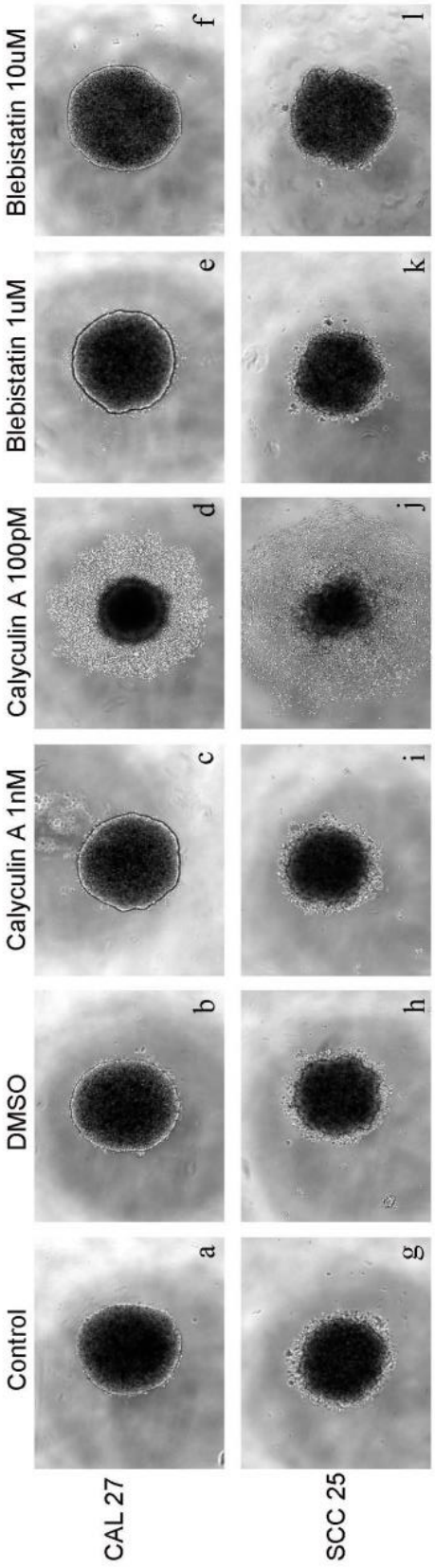

Figure 2 – Cell contractility interferes with cell-cell adhesion dynamics – Representative images of low invasive/high E-cadherin (CAL 27) or highly invasive/low E-cadherin (SCC25) OSCC-derived spheroids incubated in control conditions (Control or DMSO) or with different concentrations of drugs that increase (Calyculin) or blocks (Blebistattin) NMII mediated contractility.

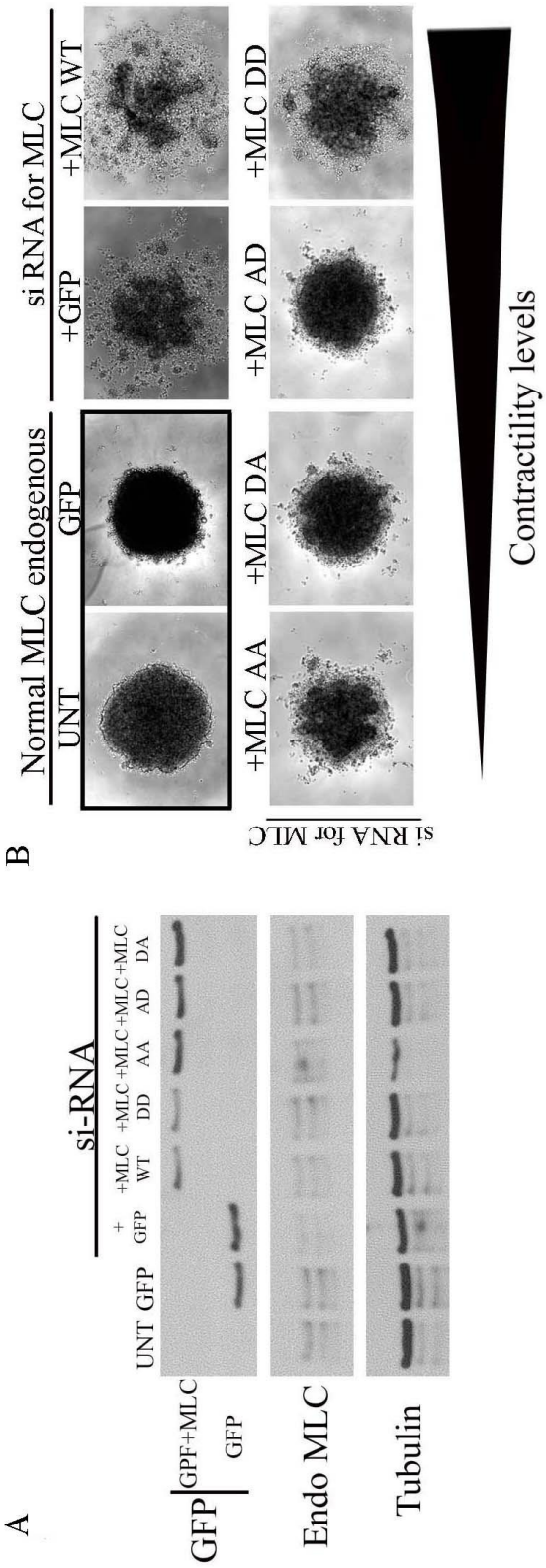

Figure 3 –Myosin Light Chain phosphomimetics mutants reproduces the effects of pharmacological modulation of cell contractility in cell-cell adhesion – A: Representative western blotting of highly invasive OSCC cell line (SCC25) transfected with phosphomimetics-MLC on residues Thr18/Ser19, where A represents not phosphorylated and D represents phosphorylated. B: Images of spheroids formed from untransfected cell, cells transfected with GFP (Black box) and MLC mutants.

Supplementary information

		Number of cases
Gender	Female	5
	Male	14
Localization	Border of tongue	5
	More the one localization	4
	Floor of the month	2
	Others	7
Age	51 – 80 years (mean 65,5)	
Clinical staging	Staging I	4
	Staging II	3
	Staging III	1
	Staging IVa	5
	Staging IVb	1

Supplementary images 1: Clinical information.

9. CONCLUSÕES

- O comportamento das células tumorais pode ser modulado através de fatores extrínsecos como, por exemplo, a alteração no microambiente tumoral, seja ela por mudança no substrato ou na densidade da matriz, e também dos fatores intrínsecos como a alteração nos níveis de miosina.
- As isoformas de miosina II estão superexpressas no Carcinoma Espinocelular Oral, tanto nas amostras de pacientes quanto nas linhagens celulares mais invasivas (SCC25). A contratilidade celular está envolvida no processo de invasão do carcinoma espinocelular oral, e a modulação farmacológica e genética da contratilidade altera o status coesivo das células, o que pode contribuir na disseminação do tumor.
- A composição da matriz extracelular pode induzir a alternância entre a migração coletiva e a migração individual, de acordo com a invasividade tumoral em decorrência das alterações nas adesões entre célula e matriz. Além disso, a composição da matriz extracelular pode influenciar as características de invasão e metástase das células tumorais de acordo com o nível de diferenciação tumoral e provavelmente através de modulação da sinalização celular e mudanças no balanço entre as adesões célula –célula e célula – matriz.

O aumento da expressão de MMP observado nas amostras de Carcinoma Espinocelular Oral pode contribuir no remodelamento da matriz, resultando na criação de espaços físicos para que as células invadam, bem como interferir no processo de adesão ao substrato, resultando em adesões melhores que auxiliem no processo de migração das células tumorais. O aumento da expressão de MMPs pode estar relacionado ao comportamento agressivo observado no Carcinoma Espinocelular Oral, especialmente nas células do fronte de invasão.