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REBECA BARROS NASCIMENTO

ESTUDO DO EFEITO DA MODULAÇÃO DE CAVEOLINA-1 EM CARCINOMA EPIDERMÓIDE DE BOCA

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REBECA BARROS NASCIMENTO

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Tese apresentada ao Programa de Pós Graduação em Odontologia e Saúde, Faculdade de Odontologia, Universidade Federal da Bahia, como requisito para obtenção do grau de Doutora em Odontologia e Saúde.

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TERMO DE APROVAÇÃO

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"EFEITO DA MODULAÇÃO DE CAVEOLINA-1 EM CARCINOMA EPIDERMÓIDE DE BOCA".

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“O correr da vida embrulha tudo, a vida é assim: esquenta e esfria,
aperta e daí afrouxa, sossega e depois desinquieta.
O que ela quer da gente é coragem.”

Guimarães Rosa

APRESENTAÇÃO

Esta tese será apresentada no formato de artigo científico, sendo desenvolvidos 2 artigos para publicação acerca do tema “Modulação da Caveolina-1 em carcinoma epidermóide de boca”.

LISTA DE ABREVIATURAS

ATP – adenosina trifosfato

CAV1 – Caveolina-1

CEB – Carcinoma epidermóide de boca

DNA – Ácido desoxirribonucleico

ECAD – E-caderina

GAPDH – gliceraldeído 3-fosfato desidrogenase

MβCD – Metil-β-Ciclodextrina

MET – Transição mesenquimal-epitelial

NCAD – N-caderina

pTEM – parcial transição epitelial-mesenquimal

RNA – Ácido ribonucleico

siRNA – silenciamento de RNA

siCAV1 – silenciador de Caveolina-1

TEM – Transição epitelial-mesenquimal

βCAT – β-catenina

RESUMO

Introdução: O Carcinoma epidermóide bucal (CEB) apresenta capacidade invasiva e metastática como consequência de alterações fenotípicas e genotípicas, como o processo de transição epitelial-mesenquimal (TEM). Alterações nas membranas das células podem afetar a estrutura de cavéolas, compostas por colesterol e caveolinas (CAV), estando associadas ao processo de tumorigênese. A depleção de colesterol e alteração da expressão de CAV1 podem afetar células tumorais e interferir na carcinogênese. Objetivo: Avaliar a expressão de CAV1 em tumores de CEB e o efeito da depleção de colesterol e do silenciamento de CAV1 (siCAV1) em linhagens celulares de CEB de língua. Material e métodos: Hibridização por microarray, expressão de mRNA e imunohistoquímica foram realizadas em amostras de CEB e de tecido não tumoral (margem). Os tumores foram divididos nos grupos: mais (T1 / T2 N +, n = 14) e menos (T3 / T4 N0, n = 19) agressivos. O efeito da depleção de colesterol e do silenciamento de CAV1 foram analisados em linhagens celulares SCC-25, com perfil não metastático, e HSC-3, com perfil metastático, e foram avaliados viabilidade celular, fluidez da membrana, expressão gênica e protéica de CAV1 e dos marcadores de TEM (E-caderina, N-caderina, β-catenina e Vimentina) e suas capacidades migratória e invasiva. Resultados: CAV1 foi 1,77 vezes mais expressa em tumores do que em tecidos não tumorais e cerca de 2 x mais expresso em tumores mais agressivos do que em menos agressivos. qRT-PCR não mostrou diferença na expressão de CAV1 em nenhuma comparação feita. A proteína CAV1 foi localizada tanto no epitélio tumoral quanto no estroma, em três padrões diferentes. Positividade no estroma foi associado a tumores de maior tamanho. Além disso, as células epiteliais tumorais positivas para CAV1 tenderam a estar associadas a CAV1 baixo ou negativo no estroma tumoral. A depleção de colesterol reudziu a viabilidade celular e fluidez de membrana nas células SCC-25, enquanto que a viabilidade das células HSC-3 foi menos afetada pela depleção e sem alterações na fluidez da membrana. A depleção de colesterol interferiu na expressão de CAV1 e de marcadores de TEM em ambas as células, e diminuiu sua capacidade migratória, sendo a SCC-25 mais afetada. A capacidade invasiva das células metastáticas também reduziu, mas a das células não metastáticas aumentou com a depleção de colesterol. O siCAV-1 aumentou a viabilidade celular apenas em SCC-25 e a expressão gênica de marcadores de TEM (N-caderina e β-catenina), mas não os níveis de proteína, apenas em HSC-3. Além disso, o siCAV1 estimulou a capacidade de invasão de células metastáticas. Conclusão: A mudança da expressão de CAV1 de células epiteliais tumorais para células estromais tumorais pode ser útil para prever a agressividade do CEB. Tanto a depleção de colesterol quando o siCAV1 alteram a viabilidade de células tumorais e afetam a expressão de marcadores de TEM, o que interfere nas capacidades migratória e invasiva. A resposta celular à depleção de colesterol foi diferente da resposta ao silenciamento de CAV1, o que também variou entre as células não metastáticas e metastáticas.

Palavras-chave: carcinoma epidermóide de boca; transição epitelial-mesenquimal; caveolina-1, depleção de colesterol

SUMÁRIO

REVISÃO DE LITERATURA

1.1 CARCINOMA EPIDERMÓIDE DE BOCA (CEB)

O câncer de boca é um dos tipos de câncer mais comuns no mundo. De acordo com o último estudo mundial realizado pelo projeto GLOBOCAN em 2018, estimou-se, para este mesmo ano, 354,9 mil novos casos de câncer de língua e cavidade bucal em ambos os sexos, com estimativa de 177, 4 mil mortes (FERLAY et al., 2019). No Brasil, segundo o Instituto Nacional do Câncer José de Alencar Gomes da Silva (INCA), para o triênio 2020 – 2022 foram estimados 11.180 novos casos de câncer bucal entre homens, sendo o 5º câncer mais comum neste gênero, e 4.010 novos casos em mulheres, correspondendo ao 13º tipo de câncer mais comum. As regiões sudeste, sul e nordeste do Brasil apresentam as maiores incidências desta neoplasia por número de habitantes (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA (INCA), 2019). Diferenças nas taxas de incidência e mortalidade podem ser observadas em países diferentes ou mesmo em regiões distintas dentro de um mesmo país devido a variações na constituição genética de cada indivíduo, associadas ao estilo de vida e à influência de fatores sociais, econômicos, culturais e ambientais, bem como no acesso a cuidados médicos específicos (LIU et al., 2016).

O tipo histológico mais comum é o carcinoma epidermóide, também denominado de carcinoma de células escamosas ou carcinoma escamocelular, e compreende cerca de 90% de todas as neoplasias malignas diagnosticadas em boca e orofaringe (SLOAN et al., 2017). A língua é o sítio bucal de maior prevalência e está associado a alta mortalidade quando comparado com CEB em outros sítios bucais (ALMANGUSH et al., 2020; FARHOOD et al., 2019). A incidência deste tipo específico de câncer tem crescido em muitos países, especialmente entre o grupo mais jovem (DU et al., 2020; NG et al., 2017), mas em geral acomete principalmente homens em faixa etária de 40 anos, sem distinção étnica, podendo estar associado a fatores de risco como exposição ao tabaco, consumo excessivo de álcool, betel, radiação solar (específico para o CEB de lábio), à presença de infecções como o papilomavírus humano (HPV, no caso de orofaringe), menos comumente devido à imunossupressão (transplantes ou síndrome da imunodeficiência adquirida) e, ainda de maneira controversa, por determinados tipos de dieta e infecção fúngica (KUMAR et al., 2016).

2.1.1 Características clínicopatológicas e moleculares

O CEB é caracterizado pela Organização Mundial da Saúde (OMS) como uma neoplasia epitelial invasiva, com diferentes graus de diferenciação escamosa e grande propensão a metástases linfáticas precoces. Pode se apresentar clinicamente, em estágios iniciais, com aspecto eritematoso, leucoplásico ou eritroleucoplásico, e ulcerado, frequentemente assintomático, o que contribui com o diagnóstico tardio. Em estágios avançados, podem ser observados crescimentos tumorais ulcerados, com bordas elevadas, eventualmente com sangramento, podendo levar a dificuldades na fala, mastigação e deglutição. Com o avanço da doença, podem ser observadas metástases linfonodais, na região do pescoço, e metástases à distância. Esta neoplasia pode ser precedida ou estar associada a lesões potencialmente malignas, mais comumente as leucoplasias e eritroleucoplasias (SLOAN et al., 2017).

A transformação maligna dos queratinócitos que revestem a cavidade bucal ocorre através de mutação espontânea do ácido desoxirribonucleico (DNA) (SLOAN et al., 2017). Microscopicamente, as células neoplásicas apresentam diferenciação escamosa, caracterizada pela queratinização intra ou extracelular com ou sem a presença de pontes intercelulares. Os carcinomas epidermóides podem ser subdivididos em bem diferenciados, pouco diferenciados ou moderadamente diferenciados, segundo OMS (2017). Tumores bem diferenciados apresentam queratinização abundante e pontes celulares evidentes. No entanto, em tumores pouco diferenciados, a origem escamosa do epitélio é menos evidente porque as células encontram-se bastante pleomórficas. Tumores moderadamente diferenciados apresentam características intermediárias entre os descritos anteriormente. O padrão morfológico desta neoplasia pode ser de um arranjo em cordões, ninhos, massas, pequenos agrupamentos celulares ou ainda células individuais. As áreas de invasão são caracterizadas pela presença de pequenas ilhas e cordões celulares irregulares ou células individuais (SLOAN et al., 2017).

O câncer é amplamente caracterizado pelo crescimento descontrolado de células anormais que escapam do sistema imunológico, resistência à apoptose e capacidade de desencadear angiogênese e metástase. As mudanças moleculares responsáveis pela célula maligna são causadas por mutações genéticas e mecanismos epigenéticos mediadas principalmente pela metilação do DNA e modificações nas histonas. A interrupção do epigenoma também é um mecanismo fundamental no câncer, onde a regulação do desenvolvimento do tumor é controlada por um equilíbrio entre oncogenes e supressores de tumor (GUPTA; TOUFAILY; ANNABI, 2014).

Do ponto de vista molecular, o CEB apresenta alvos que são explorados como biomarcadores pelo seu potencial para a detecção precoce deste tumor, visando aumentar a taxa de sobrevivência dos pacientes (SINEVICI; O'SULLIVAN, 2016). Múltiplos eventos genéticos e epigenéticos podem ocorrer durante o desenvolvimento desta neoplasia maligna e tais mecanismos podem atuar como promotores oncogênicos ou supressores tumorais. Mecanismos como a expressão aumentada de fatores estimuladores de crescimento (EGFR, ErbB2, FGFR1), ativação de múltiplas cascatas de sinalização (RAS/RAF-MAPK, STAT-3, PI3K/Akt, PTEN), aumento de fatores de crescimento endotelial vascular (VEGF), alterações em fatores de transcrição (MYC, FOS, JUN, CMYC), reguladores do ciclo celular (ciclina D1), inibidores da apoptose (BCL-2, BAX), mutações em genes supressores de tumor (p16, p21, p27, p53, FAT1, NOTCH1, SMAD4, CDKN2A), perda de heterozigosidade (principalmente nas regiões 3p, 8p, 9p, 13q, 17p), polimorfismos de nucleotídeo único (que podem afetar a expressão de genes supressores de tumor ou reguladores do ciclo celular), além de hipermetilação na região promotora do gene CDKN2A e de outros genes que codificam proteínas supressoras de tumor, expressão de marcadores associados a invasão e metástase como caderinas e metaloproteinases de matriz (MMPs), resultando em uma variedade de fenômenos biológicos como modulação da apoptose, crescimento celular, angiogênese, adesão, motilidade, invasão celular e metástase (KARUNAKARAN; MUNIYAN, 2020; LAWRENCE et al., 2015; SCULLY; BAGAN, 2009; SINEVICI; O'SULLIVAN, 2016). Apesar da grande quantidade de estudos e do avanço na pesquisa moleculares do CEB, ainda há muitas lacunas a serem preenchidas e não há, até o presente momento, marcadores moleculares isolados que possam ser usados para a estimativa do prognóstico clínico desta neoplasia.

2.1.2 Tratamento e Prognóstico do CEB

Comumente o CEB é diagnosticado em estágios avançados, o que contribui para um decréscimo nas chances de sobrevida do paciente (WARNAKULASURIYA, 2009). O estadiamento TNM, baseado no tamanho do tumor primário (T), no envolvimento de linfonodo loco regional (N) e na presença de metástase a distância (M) é utilizado clinicamente para estadiar a doença, selecionar terapia apropriada, estimar o risco de recorrência e avaliar a sobrevida. Por outro lado, os dados obtidos deste tipo de classificação não são suficientes para prever a agressividade do tumor ou individualizar o tratamento, uma vez que avalia apenas estruturas anatômicas (ALMANGUSH et al., 2020; RIVERA, 2015).

Após a classificação de grau histológico na previsão do prognóstico de pacientes com CEB, autores sugeriram que há deficiências neste sistema que podem não indicar o verdadeiro

cenário do tumor e consequentemente afetar o prognóstico (ALMANGUSH et al., 2020). Dentro deste contexto, sabe-se que a progressão da doença e a resposta ao tratamento ainda diferem visivelmente entre os pacientes que apresentam estágio TNM semelhante (HUBERT LOW et al., 2015; LINDENBLATT et al., 2012; SAWAZAKI-CALONE et al., 2015), o que torna importante identificar modelos prognósticos mais confiáveis com base nas características histológicas e moleculares do tumor para desenvolver planos de tratamento mais adequados para pacientes com CEB. Em 2018, dois novos parâmetros foram adicionados para o estadiamento CEB: profundidade de invasão tumoral no estágio T e a disseminação extracapsular do linfonodo no estágio N (KATO et al., 2020). Em 2015, Almangush et al. introduziram um modelo simples para CEB que analisa e correlaciona 2 parâmetros: profundidade de invasão tumoral e o brotamento tumoral (budding) (ALMANGUSH et al., 2015) Após revisão sistemática realizada por Wahab et al. (2020), os autores concluíram que este modelo tem um forte poder prognóstico para a sobrevida livre de doença em pacientes com CEB e sugeriram que futuramente esta análise deve ser adicionada a outros modelos histopatológicos práticos, como relação tumor-estroma para possivelmente melhorar o poder prognóstico das características histopatológicas no CEB (WAHAB et al., 2020).

O tratamento de CEB varia com o estadiamento TNM da doença, do seu sítio primário de origem e na histologia tumoral (EL-NAGGAR et al., 2017; WAHAB et al., 2020). O tratamento cirúrgico é o de escolha para a maioria dos pacientes com CEB, sendo o padrão para tumores em estágios iniciais (T1–T2, N0) e geralmente não associado a radioterapia pós-operatória, apesar desta ser muitas vezes recomendada para controle locoregional. O envolvimento linfonodal é o mais importante fator prognóstico desta neoplasia. O tratamento cirúrgico acompanhado de radioterapia e/ou quimioterapia é indicado para os casos mais avançados, que apresentam extenso tamanho, e indicado também para o controle regional e/ou tumores que já envolvam linfonodos cervicais, com alto risco de recorrência (HUANG; O´SULLIVAN, 2013; MARUR; FORASTIERE, 2016). Por outro lado, a quimioterapia paliativa e/ou de suporte é indicada para casos em que já há presença de metástases à distância no momento do diagnóstico, no entanto, muitos casos podem não apresentar evidência clínica ou por imagem de disseminação metastática (HAIGENTZ et al., 2012). Quimioterapia concomitante apresenta melhores resultados na sobrevida quando comparada com a radioterapia como único tratamento (MARTA et al., 2015).

O tratamento das neoplasias malignas, principalmente as metastáticas, é dificultado pela heterogeneidade biológica das suas células e pela capacidade que elas têm de desenvolver uma

rápida resistência a muitos tratamentos anticâncer (CREE; CHARLTON, 2017; FIDLER; ELLIS, 1994). Apesar do avanço nas descobertas de drogas e protocolos quimioterápicos, a porcentagem de pacientes que sobrevivem no mínimo 5 anos após o diagnóstico, cerca de 50%, permanece inalterada por décadas (KUMAR et al., 2016; WARNAKULASURIYA, 2009). Além disso, o índice terapêutico das abordagens associadas à quimioterapia é estreito, significando que as drogas podem atingir níveis tóxicos com pequenas doses (JUNG et al., 2015). Surge desta limitação a importância da pesquisa de biomarcadores que possam predizer a recorrência, a metástase e a resposta do tumor ao tratamento (RIVERA, 2015).

1.2 TRANSIÇÃO EPITELIAL-MESENQUIMAL (TEM)

A principal característica do câncer é a capacidade de invadir tecidos adjacentes e de causar metástases. A metástase do câncer envolve diferentes etapas, começando com invasão local, seguida de invasão a vaso sanguíneo, sobrevivência na circulação, extravasamento do vaso e finalmente colonização do sítio secundário e crescimento naquele sítio. Este processo é promovido por eventos genéticos e epigenéticos que aumentam o potencial oncogênico das células, previnem a morte celular, religam as vias metabólicas e contornam o sistema de vigilância imunológica (CAMPOS et al., 2019). Este processo depende da ativação da transição epitelial-mesenquimal (TEM), um evento dinâmico e reversível em que as células epiteliais perdem sua polaridade e reorganizam seu citoesqueleto, adquirindo um fenótipo mesenquimal típico (THIERY, 2002).

A TEM e seu processo reverso, transição mesenquimal-epitelial (MET), são processos fisiológicos que desempenham papéis importantes durante o desenvolvimento embrionário, tratamento de feridas e reparo de tecidos, entretanto, também está associada a migração aumentada, potencial invasivo e resistência a apoptose (GRIGORE et al., 2016; HUBER; KRAUT; BEUG, 2005; PELTANOVA; RAUDENSKA; MASARIK, 2019; THIERY, 2002). Além disso, as células tumorais sofrem alterações nos contatos célula-célula (BALZAC et al., 2005), nas interações célula-matriz e na sinalização celular (ENGELMAN; ZHANG; LISANTI, 1998). Assim, a promoção da TEM está relacionada à ativação de diferentes vias de sinalização envolvidas na adesão, migração, motilidade, e morfogênese; diferenciação, desenvolvimento, crescimento, proliferação celular; transdução de sinal e fatores de transcrição (SMITH; TEKNOS; PAN, 2013).

A TEM tem sido tradicionalmente vista como um processo binário envolvendo uma conversão completa do estado epitelial para o mesenquimal. Entretanto, tem sido cada vez mais

reconhecido que a TEM também abrange uma variedade de estados híbridos, um fenótipo que foi referido como "TEM parcial" (p-TEM). Como o p-TEM não é bem definido em termos moleculares, não se sabe se esse status híbrido significa uma fase intermediária durante uma transição mesenquimal ou representa seu próprio estado final. Da mesma forma, não está claro se os mesmos mecanismos de repressão transcricional que conduzem um "TEM completa" também operam durante o p-TEM (AIELLO et al., 2018). Durante a TEM, normalmente é visto que as células que cruzam a membrana basal e invadem os vasos sanguíneos se apresentam de forma isolada, porém, em alguns tumores foi notado um padrão migratório coletivo, em que as células mantém o contato célula-célula, notando-se aglomerados de células tumorais multicelulares, que, entretanto, apresentam capacidade de células mesenquimais (CHEUNG; EWALD, 2016). Aparentemente parece haver mecanismos diferenciados em tumores individualmente, isto é, alguns tumores seguem um programa TEM clássico, envolvendo repressão transcricional, e outros seguem um programa alternativo, no qual o fenótipo epitelial é perdido pós transcricionalmente. Além disso, tem sido proposto que células em estado híbrido podem estar relacionadas a uma maior capacidade metastática quando comparadas às células que sofreram a TEM e estão em um estado totalmente mesenquimal (AIELLO et al., 2018; LING; CHENG; TAO, 2020)**.** Devido a isto, tem-se associado esse perfil de células em TEM parcial com a formação de brotamento tumoral (ou tumor budding) em amostras de CEB (LING; CHENG; TAO, 2020)**.**

O processo de TEM está, sobretudo, associado com a regulação negativa de marcadores epiteliais como as proteínas E-caderina, β-catenina, Citoqueratina 8, 9 e 18, Mucina-1, Claudina-1, dentre outros; e concomitantemente com a regulação positiva de marcadores mesenquimais, incluindo a N-caderina, vimentina, fibronectina, α-actina de músculo liso (α-SMA), proteína 1 específica de fibroblasto (FSP1), Colágeno tipo I e tipo IV, Fibronectina e Zônula Ocludens-1 (ZO-1) (FUCHS et al., 2008; PELTANOVA; RAUDENSKA; MASARIK, 2019; TSUKITA; FURUSE; ITOH, 2001; VALCOURT et al., 2005). A superexpressão de fenótipos mesenquimais e outras interações da célula com a matriz extracelular alterada são induzidas por fatores de transcrição indutores de TEM (LING; CHENG; TAO, 2020), como ZEB1, ZEB2, SNAI1, SNAI2 (SLUG), TWIST e FOXC2 (CRAENE; BERX, 2013; GRIGORE et al., 2016).

As células que passam pela TEM adquirem características mesenquimais após a dissociação das junções célula-célula e a supressão de E-caderina, e a diminuição da sua expressão foi correlacionada com tumores pobremente diferenciados, estágios avançados de

TNM, metástases e recorrência de CEBs (LOH et al., 2019; LÓPEZ-VERDÍN et al., 2019; YAO et al., 2017). Menor expressão de E-caderina tem sido observada na fronte invasiva do tumor quando comparado com a sua área central em CEBs (COSTA et al., 2015; OZAKI-HONDA et al., 2017), e também está associada a tumores altamente invasivos, quando comparado com tumores com menor capacidade invasiva (COSTA et al., 2015), enquanto o oposto foi notado em células do centro do tumor (OZAKI-HONDA et al., 2017). Tecidos tumorais de CEBs apresentaram menor imunoexpressão de E-caderina do que tecidos displásicos e tecidos normais (GUO et al., 2018; KAUR et al., 2013). A baixa expressão de E-caderina ainda foi notada em tumores primários e em linfonodos regionais, sendo que a maioria destes tumores com baixa expressão de E-caderina apresentaram alta expressão de N-caderina (PYO et al., 2007).

Durante a TEM, ao contrário da E-caderina, a N-caderina é regulada positivamente, sendo que este "switch" entre caderinas está associado a características migratórias e invasivas aprimoradas, além de ter sido relacionado à taxa de sobrevida inferior em pacientes com câncer (LOH et al., 2019). A N-caderina induz a TEM pela ativação da via ErbB que ocorre por meio da regulação positiva do nível da proteína ligada ao receptor do fator de crescimento 2 (GRB2), da proteína transformadora de SHC e ERK (LOH et al., 2019). Estudos identificaram associação de N-caderina em CEBs. Células de carcinoma epidermóide com alta expressão de N-caderina apresentaram fenótipo fibroblástico e baixa expressão de E-caderina, e esse perfil foi revertido quando as células foram transfectadas com anti N-caderina (ISLAM et al., 1996). A presença de N-caderina foi mais evidente em tecidos tumorais de CEBs do que em tecidos normais, avaliados por imuno-histoquímica, sendo que a sua expressão citoplasmática foi associada a tumores menos diferenciados e a um pior prognóstico (DI DOMENICO et al., 2011). A expressão de N-caderina no centro e no fronte tumoral foi relacionada a um pior prognóstico (OZAKI-HONDA et al., 2017). Alguns estudos notaram ausência de expressão de N-caderina tanto na área central quanto na fronte de invasão tumoral, e notaram expressão aumentada de vimentina em poucos casos de CEB, sem diferença entre área central e invasiva do tumor (COSTA et al., 2015).

As caderinas ainda estão associadas ao citoesqueleto de actina por meio da ligação a cateninas, como a β-catenina. Durante a TEM, a clivagem da E-caderina leva à desestabilização das junções aderentes e à liberação de β-catenina que se acumula no citoplasma e núcleo, enquanto é perdida na membrana. No núcleo, ela se associa a fatores de transcrição, atuando como um ativador transcricional para a proliferação celular (LING; CHENG; TAO, 2020; LOH

et al., 2019). A β-catenina é anormalmente expressa em muitos cânceres, incluindo CEBs. Estudos mostram associação de baixa expressão membranar de β-catenina com o início e progressão tumoral. A expressão de β-catenina foi menor em tecidos de CEB quando comparados a tecidos displásicos e tecidos normais, sendo que a perda de expressão de β-catenina membranar ainda foi relacionada a estágio clínico avançado e metástase nodal (KAUR et al., 2013). Expressão reduzida de E-caderina e de β-catenina e o aumento de N-caderina foi notado em amostras de CEB quando comparado com tecidos bucais normais e este perfil de expressão demonstrou associação significativa com grau histológico menos diferenciado (ANGADI et al., 2016). Expressão diminuída de E-caderina e β-catenina e translocação dessas proteínas da membrana para o citoplasma foram relacionados com tumores pobremente diferenciados, quando comparado a tumores bem diferenciados (ANGADI et al., 2016; MAHOMED; ALTINI; MEER, 2007).

A vimentina é uma proteína de filamento intermediário do tipo III expressa constitutivamente em vários tipos de células mesenquimais, mas não expressa em células epiteliais normais (LING; CHENG; TAO, 2020). Muitos estudos encontraram uma expressão anormal de vimentina em uma variedade de tumores epiteliais, incluindo CEBs. Estudos em CEB que expressam o fenótipo de TEM (baixa expressão de E-Caderina e alta Vimentina) apresentaram o dobro de tumores satélites que os tumores primários que não expressaram o fenótipo TEM (YANG et al., 2011). Notavelmente, o aumento expressão de vimentina está negativamente correlacionada com a expressão de E-caderina no CEB (DMELLO et al., 2018; SAWANT et al., 2014; ZHOU et al., 2015). A diminuição ou perda de expressão de E-caderina e o aumento da expressão de Vimentina também foram associados ao aumento da capacidade migratória de células tumorais e com desenvolvimento de metástases a distância em pacientes com CE de cabeça e pescoço (NIJKAMP et al., 2011). Um desses estudos notou perda de expressão de E-caderina em tumores que tiveram aumento de expressão de Vimentina, e esta combinação mostrou uma significância prognóstica superior em comparação com a expressão proteica individual (DMELLO et al., 2018). Wangmo et al (2020) também mostraram que a análise combinada de E-caderina com Vimentina para mensuração de TEM apresentou uma maior significância para o prognóstico de CEB quando comparado à análise das proteínas de forma isolada. Esses autores notaram que a maioria dos tumores de CEB apresentou um perfil não-TEM (com E-caderina positivo e Vimentina negativo), seguido de tumores com perfil de TEM parcial (Ecaderina e Vimentina positivos ou Ecaderina e Vimentina negativos) e os outros

com perfil de TEM completa (E-caderina negativo e Vimentina positivo) (WANGMO et al., 2020).

Com o estabelecimento da TEM associado a maior expressão de moléculas capazes de degradar a matriz extracelular, as células são capazes de degradar proteoliticamente a membrana basal e a matriz de colágeno subjacente e de migrarem para locais distantes, formando metástase (PELTANOVA; RAUDENSKA; MASARIK, 2019). Sabendo-se que este processo é um fator crítico para progressão e invasividade tumoral e que os tumores podem adquirir diferentes fenótipos (LING; CHENG; TAO, 2020), a compreensão dos eventos biológicos relacionados a este processo é fundamental para identificação de marcadores que possam determinar a progressão da doença e a metástase, bem como para a definição de alvos terapêuticos. Contudo, até o presente momento não está definido para o CEB como ocorre a progressão tumoral a partir dos diferentes fenótipos da TEM e quais aspectos complexos influenciam o comportamento tumoral.

1.3 CAVÉOLA E CAVEOLINA-1

A membrana plasmática celular é uma camada lipido-proteica dupla que funciona como um separador dos ambientes intracelular e extracelular e atua na sinalização e iniciação de eventos celulares, apresentando, portanto, estrutura dinâmica (HEAD; PATEL; INSEL, 2014). Nas membranas são encontradas as balsas lipídicas, invaginações em formato de ômega chamadas cavéolas, caracterizadas por elevada concentração de colesterol e glicoesfingolipídios, que coordenam a atuação de várias proteínas, como membros da família do receptor tirosina quinases (RTK), incluindo o receptor do fator de crescimento epidérmico (EGFR) e outras proteínas, incluindo CD44, uPAR, H-Ras, integrinas, cateninas e caveolinas. Essas proteínas exercem funções celulares na estabilidade membranar, endocitose, tráfego proteico, transdução de sinais, mecano-transdução e homeostase do colesterol, além de estarem associadas a angiogênese e manutenção da polaridade celular (CAMPOS et al., 2019; PATRA, 2008). A estabilidade da cavéola é mantida pela associação do colesterol com proteínas, como as caveolinas, em sua maioria e de maior importância funcional, e as cavinas. A ausência de qualquer um desses elementos leva à perda da estrutura da cavéola (CAMPOS et al., 2019).

Caveolina-1 é uma das três caveolinas conhecidas (CAV1, 2 e 3), sendo a mais expressa. Caveolina-1 e Caveolina-2 são encontrados coexpressos principalmente em células diferenciadas terminalmente como adipócitos, células endoteliais, pneumócitos e fibroblastos, enquanto a expressão de Caveolina-3 é limitada a tipos de células musculares (cardíacas,

esqueléticas e células musculares lisas) (ROUTRAY, 2014). CAV1 atua como uma proteína multifuncional, que além de ser expressa em células, também pode ser encontrada em vesículas endocíticas denominadas caveossomos, no aparelho de Golgi, em gotículas lipídicas e em mitocôndrias, reconhecidas em complexo com chaperones e também secretado no espaço extracelular por algumas células, presentes então em exossomos (CAMPOS et al., 2019; ROUTRAY, 2014). Trata-se de uma proteína de membrana 22 kilodaltons (kDa) que contém 178 aminoácidos, codificada pelo gene CAV1 (CAMPOS et al., 2019; WESTERMANN; STEINIGER; RICHTER, 2005). A Caveolina-1 atua no tráfego membranar e endocitose, transporte de colesterol intracelular, sinalização celular e distúrbios lipídicos (WESTERMANN; STEINIGER; RICHTER, 2005). Pesquisas mais recentes se concentram em esclarecer sua relevância no câncer (NWOSU et al., 2016).

A caveolina-1 é composta por um domínio N-terminal (1-81 aa), um domínio scaffolding (CSD, 82-101 aa), um domínio transmembranar tipo grampo (102-134 aa) e um domínio C-terminal (135 –178 aa). O domínio transmembranar contém duas α-hélices separadas por uma região ligante de três resíduos incluindo uma prolina (P110), que cria um ângulo de $\pm$ 50◦ entre as duas α-hélices, permitindo que esta proteína adote a topologia em gancho de modo que os terminais N e C fiquem voltados para o interior da célula (CAMPOS et al., 2019; GUPTA; TOUFAILY; ANNABI, 2014). Devido à presença de um grande número de resíduos aromáticos, a porção carboxila terminal CSD de CAV1 pode interagir com inúmeras moléculas de sinalização localizadas dentro da cavéola, incluindo moléculas de sinalização modificadas por lipídios (como as quinases da família Src, Ras GTPases, proteínas G e proteínas glicosilfosfatidilinositol (GPI)), proteínas transdutoras de sinalização, como receptor de tirosina quinases (RTKs) , / HER2 (c-erbB2), proteína quinase C, fator de crescimento transformador (TGF) -beta / SMAD, Wnt, proteína quinase p42 / 44 ativada por mitogênio (MAP), proteína G heterotrimérica subunidades α, sintase de óxido nítrico endotelial (eNOS), receptor do fator de crescimento derivado de plaquetas (PDGFR), receptor do fator de crescimento epidérmico (EGFR), proteínas envolvidas no transporte de cálcio e integrinas (GUPTA; TOUFAILY; ANNABI, 2014; PATRA, 2008; ROUTRAY, 2014). A compartimentalização caveolar de moléculas sinalizadoras e sua interação com as caveolinas fornecem um mecanismo para a regulação dos eventos de sinalização, bem como interação entre as diferentes vias de sinalização (GUPTA; TOUFAILY; ANNABI, 2014; PATRA, 2008).

2.3.1 - Caveolina-1 e tumorigênese

A membrana celular das células mutadas desempenha um papel crucial na sobrevivência celular e tem sido implicada no processo de carcinogênese. A presença das cavéolas está relacionada ao processo de tumorigênese por apresentar estrutura que está envolvida na sinalização de células normais e, quando desregulado, promove a transformação celular e a progressão do tumor, além de ter sido associada ao processo de apoptose (ROUTRAY, 2014). Neste contexto, está cada vez mais claro na literatura a implicação desta caveolina na regulação de múltiplos processos associados aos diversos tipos de câncer, como transformação celular, crescimento tumoral, migração, invasão, metástase e resistência multidrogas (CAMPOS et al., 2019).

Warburg propôs que as células tumorais têm função mitocondrial prejudicada, dependendo, portanto, de uma maior demanda pelo metabolismo da glicose através de uma glicólise aeróbica bastante ineficiente, que gera lactato e energia (ATP), mesmo na presença de oxigênio suficiente (NWOSU et al., 2016; PELTANOVA; RAUDENSKA; MASARIK, 2019; WARBURG, 1956). Esta adaptação parece tornar o microambiente tumoral mais ácido e as células utilizam a via da glicólise aeróbica, apesar de menos eficiente, como uma adaptação evolutiva à essas condições adversas do microambiente tumoral, utilizando as cadeias de carbono (do ácido láctico) para a síntese de biomoléculas (ácidos nucléicos, proteínas e lipídios) essenciais para a proliferação celular (DEVIC, 2016). Ou seja, essa condição deixa o microambiente tumoral mais vantajoso para a proliferação, sobrevivência e invasão de células tumorais, e, consequentemente, aumentando o potencial metastático e a resistência ao tratamento (DEVIC, 2016; PELTANOVA; RAUDENSKA; MASARIK, 2019). Embora esta teoria seja notada em vários tumores, uma nova hipótese foi apresentada de que a célula normal se torna cancerosa no momento em que muda o metabolismo da glicose de fosforilação oxidativa para glicólise aeróbia, estando o efeito Warburg relacionado ao início da carcinogênese (DEVIC, 2016). Novas evidências destacam ainda mais a importância do metabolismo lipídico, a via da serina, a secreção autofágica de alanina e o uso de proteínas extracelulares mediado por macropinocitose para sustentar a nutrição do câncer e as atividades de crescimento (NWOSU et al., 2016).

Estudos mostram que a CAV1 está associada ao metabolismo da glicose. Em carcinoma colorretal, Ha et al (2012) mostraram que a depleção de CAV1 diminuiu a captação de glicose, o acúmulo de lactato e reduziu a quantidade de ATP intracelular pois levou à ativação da MAPK. Essa via é ativada quando tensões metabólicas interferem na produção de ATP ou

aceleram o consumo de ATP, e está associada à parada do ciclo celular em condições de baixa glicose e conectada a reguladores do ciclo celular. Estes fatores apoiam que a glicólise aeróbica é induzida pela CAV1, sendo uma vantagem para a proliferação de células tumorais (HA et al., 2012). Além disso, em situações de hipóxia, há um aumento da expressão de CAV1 devido ao aumento da expressão de fatores indutores de hipóxia (HIFs), que atuam como reguladores transcricionais cruciais de genes que codificam enzimas glicolíticas e transportadores de glicose (NWOSU et al., 2016).

Pavlides et al (2009) propuseram que células epiteliais tumorais induzem o efeito Warburg (glicólise aeróbia) em fibroblastos estromais adjacentes, fazendo com que esses fibroblastos associados ao câncer sofram diferenciação mio-fibroblástica e secretem lactato e piruvato (metabólitos de energia resultantes da glicólise aeróbia). As células epiteliais cancerosas poderiam, então, absorver esses metabólitos ricos em energia e usá-los no ciclo mitocondrial, promovendo assim a produção eficiente de energia (geração de ATP por fosforilação oxidativa), resultando em uma capacidade proliferativa maior (PAVLIDES et al., 2009).

A Caveolina-1 atua como um repressor transcricional da ciclina D1 e opera através de uma via dependente de p53 / p21Cip1 induzindo a parada de G0 / G1 quando superexpresso, controla a sinalização ao longo da cascata de quinase Ras-p42 / 44 MAP e funciona como um inibidor endógeno natural da cascata de quinase p42 / 44 MAP e também inibe diretamente a ativação de ERK-1/2, tanto in vitro quanto in vivo (ROUTRAY, 2014). O supressor tumoral p53 regula a transcrição e tradução de CAV1, sendo que a perda desse supressor induziu a regulação negativa de CAV1 (LEE et al., 1998; RAZANI et al., 2000). CAV1 é capaz de inibir a quinase do receptor TGF-β tipo I (associado à TEM) e sua perda de expressão é capaz de induzir um fenótipo miofibroblástico constitutivo (PAVLIDES et al., 2009).

O efeito da CAV1 no desenvolvimento do câncer depende da sua expressão em células neoplásicas ou estromais, sendo a expressão reduzida desta caveolina, ou mesmo sua ausência, em fibroblastos associados ao câncer (CAFs), uma característica mais comum em cânceres agressivos (MARTINEZ-OUTSCHOORN; SOTGIA; LISANTI, 2015). Em contrapartida, a CAV1 pode estar altamente expressa em células estromais, incluindo fibroblastos, e tal expressão pode ser regulada via degradação e autofagia em nichos de hipóxia e má-nutrição do microambiente tumoral (SHEN et al., 2015). Tem-se relatado que expressão da CAV1 varia em resposta a exposições tóxicas durante a diferenciação e migração celular e sob condições de elevadas produções de espécies reativas a oxigênio e nitrogênio (HART et al., 2016).

Em relação à sua função de promoção de tumor, foi relatado que a alta expressão de CAV1 conduz a tumorigênese ao inibir a apoptose, resistência a drogas, bem como metástase (NWOSU et al., 2016). Por outro lado, CAV1 atua como um supressor de tumor em algumas configurações em que sua baixa expressão favorece a progressão do tumor (CAMPOS et al., 2019; NWOSU et al., 2016).

A CAV1 apresenta, portanto, um papel duplo na carcinogênese dependendo do tipo e estágio do tumor. Nos estágios iniciais da doença, a atuação da CAV1 parece ser predominantemente como supressor tumoral, enquanto que em estágios posteriores, a expressão de CAV1 está mais ligada à progressão tumoral e metástase (CAMPOS et al., 2019; QUEST; GUTIERREZ-PAJARES; TORRES, 2008). Como supressor tumoral, foi identificado que a CAV1 está altamente forforilada em tirosina em fibroblastos transformados pelo vírus do sarcoma de Rous (GLENNEY; SOPPET, 1992). A redução dos níveis dessa proteína foi notada em fibroblastos e correlacionada com o aumento do tamanho das colônias formadas por essas células (KOLESKE; BALTIMORE; LISANTI, 1995). Em outro estudo, a reexpressão de CAV1 foi capaz de reverter o fenótipo transformado e prevenir o crescimento independente de ancoragem (ENGELMAN et al., 1997). Além de estudos in vitro, foi identificada a redução da expressão de CAV1 em muitos tipos de câncer em humanos, como em pulmão, mama, cólon, ovário, e sarcomas, como osteossarcoma e glioblastoma (revisado por CAMPOS *et al.*, 2019).

A atuação da CAV1 como promotor tumoral tem sido observada em estágios mais avançados da doença, quando o aumento da sua expressão parece favorecer o desenvolvimento de características celulares relacionadas ao aumento da malignidade, incluindo multirresistência e metástase (QUEST; GUTIERREZ-PAJARES; TORRES, 2008). Em modelos humanos e de camundongos de câncer de próstata, a expressão de CAV1 aumentou com a progressão do câncer, não estando presente em tecido prostático normal (YANG et al., 1998). Estudo in vitro mostrou que a alta expressão de CAV1 promove características metastáticas de células tumorais de próstata (LI et al., 2001). A expressão de CAV1 em melanomas se correlaciona com elevado potencial metastático e baixa sobrevida (LOBOS-GONZALEZ et al., 2014). No câncer de tireoide, a expressão elevada de CAV1 e EGFR combinada com a mutação BRAF V600E está associada a lesões mais agressivas (JANKOVIĆ et al., 2017). Apesar do conhecimento da possível dupla função da CAV1 no câncer, ainda não se sabe como essas distintas funções são desenvolvidas nas células. Estudos mostram que este duplo papel pode estar associado à expressão de E-caderina nas células tumorais (CAMPOS et al., 2019).

2.3.2 Caveolina-1 e a Transição epitelial-mesenquimal

Estudos tem mostrado associação da expressão de CAV1 com a transição epitelial-mesenquimal por interferir com moléculas de adesão celular, causando perda da polaridade celular e consequente mobilidade das células tumorais e desenvolvimento de metástases (BEARDSLEY et al., 2005; GRANDE-GARCÍA et al., 2007). Essa relação ainda é bastante controversa e varia entre estudos e tumores, uma vez que tanto a diminuição quanto o aumento da expressão de CAV1 foram associadas com a indução da TEM. Assim como diferenças na expressão de CAV1 foram notadas em diferentes estágios da tumorigênese, níveis diferentes de expressão de CAV1 parecem estar associadas a diferentes fases do processo de transição epitelial-mesenquimal.

Aumento da expressão genica e proteica de CAV1 foi observada durante a indução da TEM em células de carcinoma embrionário humano e em células epiteliais de mama de ratas (BAILEY; LIU, 2008). Em outro estudo, a diminuição da expressão de CAV1 e a perda da sua polaridade em células endoteliais impediu que estas células polarizassem e adquirissem capacidade de migração (BEARDSLEY et al., 2005). Associação entre o aumento da expressão de CAV1 e indução de TEM foi notada em carcinoma de bexiga em que células tumorais com expressão aumentada de CAV1 apresentaram baixa expressão de E-caderina e aumento de N-caderina e Vimentina além de apresentarem maior capacidade migratória. Além disto, neste mesmo estudo, a inibição da expressão de CAV1 através de RNA de interferência reduziu a migração celular (LIANG et al., 2014). Foi observada que metástases cerebrais advindas de câncer de pulmão apresentavam maior expressão de CAV1 e de SNAIL (marcador mesenquimal) quando comparadas ao tumor primário e a supressão da expressão desse gene diminuiu a expressão de SNAIL e a migração das células tumorais (KIM et al., 2019).

Salem et al (2011) mostraram que em células pancreáticas, a expressão de CAV1 induziu fenótipo epitelial, e aumentou a expressão de E-caderina e de β-catenina com consequente promoção do contato entre células e diminuiu a expressão de SNAIL, um repressor de E-caderina, além de ter sido notada redução da migração e invasão destas células (SALEM et al., 2011). Em outro estudo, a redução da expressão de CAV1 fez com que células de CEB, SCC-9, mudassem do seu fenótipo cuboidal pra uma aparência fibroblastóide, que foi associada a perda de expressão de E-caderina e β-catenina e aumento de vimentina (JUNG et al., 2015).

2.3.3 – Caveolina-1 e CEB

Em CEBs, a expressão de CAV1 também varia nos diferentes estudos, apresentando função oncogênica ou supressora tumoral. Alguns estudos já foram conduzidos na tentativa de elucidar o papel da CAV1 na carcinogênese de boca e de cabeça e pescoço (AUZAIR et al., 2016; HAN et al., 2004; HUNG et al., 2003; MASOOD et al., 2013; MASUELLI et al., 2012; NAKATANI et al., 2005; VERED et al., 2015; ZHANG et al., 2008). Com esses diferentes estudos, é possível notar que há controversas na expressão e atuação da CAV1 na carcinogênese de OSCC, parecendo haver um duplo papel desta molécula.

A progressão da carcinogênese do CEB foi relacionada à expressão de CAV1. No trabalho de Hung et al (2003) através da análise de tecidos de mucosa bucal normal, tecidos correspondentes não neoplásicos, lesões bucais potencialmente malignas, CEB primário e tecidos metastáticos, a expressão de CAV1 aumentou de maneira gradativa dos tecidos de mucosa bucal normal aos de carcinoma primário, e a maioria das metástases linfonodais ocorreram a partir de CEBs primários positivos para CAV1, indicando uma relação direta entre a sua expressão e a progressão tumoral (HUNG et al., 2003). Por outro lado, em tecidos metastáticos foi notada regulação negativa da CAV1, apresentando, portanto, uma função bifásica. Semelhante a este estudo, Jaafari-Ashkavandi e Aslani (2017) também notaram um aumento gradativo significativo da expressão de CAV1 ao analisar amostras teciduais de Líquen Plano, tecidos com displasia epitelial e tecidos de CEB (JAAFARI-ASHKAVANDI; ASLANI, 2017). Nohata et al (2011) observaram, em linhagens celulares de Carcinoma Epidermóide de Cabeça e Pescoço, relação da diminuição da capacidade migratória e invasiva com o silenciamento de CAV1 (NOHATA et al., 2011). A relação da CAV1 com a progressão pode estar relacionada com a expressão de marcadores associados à adesão celular e ao processo de TEM, como foi observado no estudo de Masuelli et al (2012) que notou relação entre o aumento da expressão de CAV1 com o desarranjo do complexo E-caderina/ β-catenina e com a metástase linfonodal de tumores de cabeça e pescoço. O complexo E-caderina/ β-catenina é essencial para a adesão celular e TEM. Quando expresso anormalmente, esse complexo é associado à progressão tumoral (MASUELLI et al., 2012).

A associação da expressão de CAV1 com prognóstico do CEB foi observado por Auzair et al (2016) e Huang et al (2014). Ao comparar com tecidos de mucosa normal, através de imunohistoquímica, os autores notaram maior expressão de Cav-1 nos tecidos tumorais, sendo que a expressão de CAV1 foi associada significativamente a um pior prognóstico no estudo de Auzair et al (2016). Nesses estudos não foi encontrada relação significativa entre a expressão

de CAV1 e os dados clinico-patológicos do CEB. Vered et al (2015) também detectaram, através de imuno-histoquímica, a expressão de CAV1 em células tumorais e no microambiente tumoral de carcinoma epidermóide de língua, sendo que o microambiente tumoral apresentou maior expressão de CAV1 do que as células tumorais. Em contrapartida, em outro estudo foi observada uma baixa expressão de CAV1 em pacientes com tumores primários de carcinoma epidermóide de cabeça e pescoço que apresentaram um maior risco de desenvolver metástases distantes (JUNG et al., 2015).

Resistência multidrogas também foi relacionada à expressão de CAV1, como no trabalho de Nakatani et al (2005) que notou diminuição na expressão de CAV1 em linhagem de CEB resistente à cisplatina (CDDP), além de observar que espécimes de tumores oriundos de pacientes com melhor resposta à quimioterapia a base de Cisplatina apresentavam maiores níveis de CAV1, sugerindo que esta proteína pode ser marcador de quimiossensibilidade (NAKATANI et al., 2005).

O CEB caracteriza-se, portanto, como uma malignidade que continua a reduzir consideravelmente a sobrevida do indivíduo mesmo após anos de inovações terapêuticas e diagnósticas (WARNAKULASURIYA, 2009). A solução para um tratamento mais eficaz pode consistir na elucidação dos eventos moleculares subjacentes a esse processo e identificação de marcadores prognósticos moleculares, uma vez que os dados clínicos e histopatológicos não são suficientes para indicar com precisão o comportamento tumoral (RIVERA, 2015). A atuação da CAV1 na carcinogênese do CEB parece ter um efeito duplo, atuando como oncogene ou supressor tumoral ou uma função bifásica (FU et al., 2017; HUNG et al., 2003; JAAFARI-ASHKAVANDI; ASLANI, 2017; ROUTRAY, 2014). Porém, ensaios funcionais e estudos para validação clínica são requeridos para elucidação da participação deste gene no CEB.

2.4 DEPLEÇÃO DE COLESTEROL

As cavéolas e a CAV1 estão associadas à homeostase do colesterol, podendo regular os níveis deste componente modulando o influxo e efluxo celular (CAMPOS et al., 2019). O colesterol regula a expressão de CAV1 em nível de transcrição através de elementos de ligação reguladores de esteróides na região promotora de CAV1. Além disso, evidências postulam CAV1 como um regulador chave dos níveis de colesterol em diferentes organelas subcelulares, como acontece com mitocôndrias, em que o acúmulo de colesterol está associado à sua disfunção e também ao aumento da apoptose, notada em fibroblastos de camundongos *knock-out* para CAV1 (BOSCH et al., 2011). Foi observado que células de câncer de próstata humano,

quando negativas para CAV1, apresentam membrana rica em colesterol, o que interferiu na sinalização induzida pelo fator de crescimento epidérmico (EGF) através da via Akt, sendo que quando ocorreu um distúrbio na membrana e essa via foi inibida, a sobrevivência celular foi afetada (ZHUANG et al., 2002).

Substâncias capazes de remover o colesterol das membranas celulares têm sido utilizadas em pesquisa de cultura celular. As ciclodextrinas, sendo a ß-ciclodextrina e metil-ß-ciclodextrina (MßCD), são oligômeros cíclicos de glicose que possuem a capacidade de incluir substâncias lipofílicas em seus poros. A MßCD, em particular, forma complexos de inclusão solúveis em água com colesterol e tem se mostrado mais eficiente do que a ß-ciclodextrina (WESTERMANN; STEINIGER; RICHTER, 2005). A MßCD tem sido aplicada na maioria dos estudos por sua alta sensibilidade em ligar-se ao colesterol, sem interferência nos demais lipídios e componentes da MP (GARCIA et al., 2019). O tratamento de células com agentes ligantes de colesterol resulta no achatamento das cavéolas (ROTHBERG et al., 1992). Em altas concentrações, a ação da MβCD é citotóxica, entretanto, Onodera et al. (2013) mostraram que em concentração de 20mM, esta substância foi capaz de induzir apoptose em células tumorais de CEB, de melanoma e de colangiocarcinoma.

Efeito da depleção de colesterol na proliferação, adesão, invasão e angiogênese foi observada em células de câncer de mama utilizando-se diferentes disruptores de colesterol (MβCD, nistatina e filipina III), em diferentes concentrações: 0.1, 0.2, 0.3, 0.4, e 0.5 mM, por 01, 24 e 48 horas (BADANA et al., 2016). Estes autores notaram que a presença de MβCD removeu de forma eficaz o colesterol da membrana e causou citotoxicidade dependente da concentração (0.05 mM/48 h) quando comparado aos outros agentes. Além desse distúrbio na membrana, houve redução significativa da expressão de CAV1, o que indicou uma interrupção da integridade das balsas lipídicas induzida pela extração do colesterol. A ação da MβCD também promoveu significante inibição na adesão, proliferação e migração celular e afetou a angiogênese (BADANA et al., 2016).

A atuação da MβCD pode variar em cada tipo celular, sendo que, mesmo utilizando as mesmas concentrações e tempo, o grau de depleção de colesterol pode diferir significativamente entre os tipos de células (ZIDOVETZKI; LEVITAN, 2007). Quase todo colesterol celular total é removido quando células são expostas a alta concentração de MβCD (5–10 mM) por um período de tempo prolongado (> 2 horas) e, nessas condições, as células normalmente perdem sua morfologia, arredondam-se e podem se tornar inviáveis. Diminuir a sua concentração e/ou usar tempos de incubação mais curtos resulta em efeitos de depleção mais brandos

(ZIDOVETZKI; LEVITAN, 2007). Baixa concentração e pouco tempo de exposição ao MβCD foi capaz de aumentar a concentração de colesterol celular de linfócitos T de pessoas jovens, efeito que foi revertido quando as células foram expostas a 60 min do reagente na mesma concentração (FÜLÖP et al., 2001). A depender do tipo de célula e da concentração e tempo de exposição ao MβCD, a remoção de colesterol pode acontecer na região de balsas lipídicas ou nas regiões fora das balsas. Em geral, alta concentração (maior ou igual a 10mM) em amplo tempo de exposição (maior ou igual a 30 minutos) leva à depleção de colesterol de todas as partes da membrana. Por outro lado, sob baixo tempo de exposição e/ou sob baixas concentrações, o colesterol preferencialmente removido está presente nas balsas lipídicas, e não deixam as células inviáveis (ZIDOVETZKI; LEVITAN, 2007).

O nível de depleção por MβCD além de variar de acordo com concentração e tempo de exposição à droga, também varia conforme o tipo celular (ZIDOVETZKI; LEVITAN, 2007). Uma taxa de 100% de depleção foi notada em fibroblastos de camundongos após estas células serem submetidas a 10mM de MβCD por 8 horas, entretanto a taxa foi de 80% quando estas mesmas células foram submetidas a 5mM de MβCD por 8 horas (KILSDONK et al., 1995). Por outro lado, nesta mesma concentração, mas com apenas 1 hora de exposição, o nível de depleção de colesterol foi de 60% em mastócitos (SHEETS; HOLOWKA; BAIRD, 1999), enquanto que esta mesma dosagem e tempo levou a uma depleção de 20% em células arteriais (DREJA et al., 2002). A utilização de 5mM de MβCD por 2 horas gerou uma depleção de 60% em células epiteliais de ovário – CHO (ROMANENKO et al., 2004) e de 30 a 40% de depleção em células epidérmicas - A431- submetidas à mesma concentração de MβCD mas em apenas 1 h de exposição (GRIMMER; VAN DEURS; SANDVIG, 2002).

Diversos estudos têm avaliado as consequências da modulação do colesterol de membranas plasmáticas de células neoplásicas, uma vez que tais células apresentam uma maior concentração de colesterol nas balsas lipídicas (SOHN et al., 2018; ZHUANG et al., 2002). Nos primeiros minutos de tratamento com MβCD, observa-se a ativação transitória de várias vias de sinalização, que é seguida de inativação (QUEST; GUTIERREZ-PAJARES; TORRES, 2008).

Westermann et al. (2005) notou que imediatamente após a depleção de colesterol com MßCD em células 3T3 (fibroblastos de camundongo), a estrutura caveolar da membrana muda drasticamente, se achatando, entretanto, a proteína caveolina permanece na membrana plasmática e a distribuição primária, que antes se assemelhava a de um cinturão na parte profunda da cavéola, se desintegra formando um anel e posteriormente um fragmento disperso.

Esta estrutura caveolar ainda se mantém, mas a alça da caveolina restante é encontrada na periferia dos domínios sem partículas. Estes autores ainda observaram que, após ficarem 1 dia em meio livre de MßCD, o processo de achatamento caveolar e redistribuição de caveolina é revertido nas células que foram previamente tratadas, e a estrutura e distribuição de caveolina nas cavéolas se apresentam semelhantes às das células que não passaram por tratamento (WESTERMANN; STEINIGER; RICHTER, 2005).

Os efeitos da manipulação da homeostase do complexo colesterol/CAV1/cavéola com 10mM de MβCD em membranas plasmáticas de células tronco da medula óssea foi observada no estudo de Sohn et al. (2018), onde foi notado alteração na fluidez membranar, também afetando a adesão celular. A depleção do colesterol por 10mM de MβCD reduziu a expressão gênica e proteica de CAV1, o número de cavéolas, as taxas de adesão ao colágeno e fibronectina e a concentração de integrinas de superfície, e, em consequência, o aumento da fluidez da membrana (SOHN et al., 2018).

ARTIGO 1:

CHOLESTEROL-RICH DOMAINS DISRUPTION AFFECT CELL MIGRATION AND INVASION CAPACITY OF NON-METASTATIC AND METASTATIC TONGUE ORAL SQUAMOUS CELL CARCINOMA CELL LINES

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ABSTRACT

Cholesterol is a key lipid molecule within cell membranes and is involved in physiological and pathological process. High expression of cholesterol was seen in different types of tumors, however its roles is not clear. Cholesterol-enriched cell membrane sites are called lipid rafts. When it is associated with Caveolin (CAV) proteins, the membrane invagination is named caveolae. This structure acts in many cell signalling pathways, such as cell adhesion, migration and invasive capacity. Little is known about cholesterol function and its depletion outcomes in Oral squamous cell carcinoma (OSCC). The aim of this study was to evaluate the influence of cholesterol depletion by methyl-β-cyclodextrin (MβCD) on tongue SCC cell lines (SCC-25/non metastatic and HSC-3/metastatic). For that, cell membrane fluidity, cell viability, gene and protein expression of CAV1 and epithelial-mesenchymal transition (EMT) markers (E-cadherin/ECAD, N-cadherin/NCAD, β-catenin/βCAT, and Vimentin/VIM) were evaluated in monolayer culture. Cell migration and invasion were evaluated under extracellular matrix components. Initially, cell viability and membrane fluidity were evaluated by different concentrations of MßCD (7.5, 10, and 15 mM). For SCC-25, cell viability decreased under crescent MβCD concentration and an increase on membrane fluidity was observed. In contrast, HSC-3 cell viability was less affected by depletion and no changes on membrane fluidity. In SCC-25, upregulation of most gene evaluated (except Cav-1) was 6h after depletion, followed by downregulation at 24h (except Cav-1). In HSC-3, only Ecad was upregulated at 6h. No significant changes were seen in protein expression after 24-48h after depletion for both cell lines, except βCAT in SCC-25. The cholesterol depletion dose-dependent decreased cell migration capacity, which SCC-25 was more affected than HSC-3. On the other hand, on the human myoma organotypic model, however, SCC-25 under 7.5 mM MβCD had increased invasion capacity, while HSC-3 invasion capacity decreased under 10 mM. In conclusion, cholesterol depletion affects the expression of CAV1, which may be related to ECAD expression. Depletion effects vary according to the type and stage of the cell, since the invasive capacity was improved in non-metastatic cells while was decreased in metastatic cells.

Key-words: Tongue squamous cell carcinoma cell lines, Caveolin-1, cholesterol depletion, methyl-β-cyclodextrin, epithelial-mesenchymal transition

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is associated with early lymphatic invasion and the development of metastases. [1] Despite the advances in OSCC treatment, recurrence and tumor resistance for treatment are frequent, mainly due to OSCC biological heterogeneity. [2] The definition of OSCC prognosis and treatment is based on cTNM system and tumor histological grade. [3] However, this classification has generated discussion as some patients with a similar cTNM stage present different outcomes and responses to treatment, which led the scientific community to include others reliable parameters on diagnosis based on histological features of the tumor. [4,5] In this context, the evaluation of the tumor budding, defined as cell clusters of fewer than five cancer cells at the invasive front of the tumor, has demonstrated predictive value for high locoregional recurrence risk and shortened survival in OSCC patients. [6,7] Moreover, together with the evaluation of depth of invasion, tumor budding showed strong prognostic power for disease-free survival in OSCC. [6]

Tumor invasion and metastasis is promoted by the epithelial-mesenchymal transition (EMT) process, in which signalling pathways involved in cell adhesion, motility, migration, proliferation and differentiation are activated. [8,9] In this process induced by EMT-inducing transcription factors, negative regulation of epithelial markers, such as E-cadherin and β-catenin are noticed, in parallel to the positive regulation of mesenchymal markers, including N-cadherin and vimentin, along with a loss of polarized function of epithelial cells. [10–13] When the cells reach other sites, they undergo the reverse process, called as mesenchymal-epithelial transition (MET), stablishing a metastatic focus in which the tumor resembles the primary one. It has been increasingly recognized that EMT also encompasses a variety of hybrid states, a phenotype that has been referred to as "partial EMT" (p-EMT). Since p-EMT is not well defined in molecular terms, it is not known whether this hybrid status means an intermediate phase during a mesenchymal transition or represents its own final state. Likewise, it is not clear whether the same transcriptional repression mechanisms that drive a "complete EMT" also operate during p-EMT. [14] Studies have proposed that p-EMT cells have greater metastatic capacity than EMT cells. [14,15] In addition, the role of p-EMT and its association with clinical manifestation and tumor budding has gained recent attention in OSCC and others tumors. [15–17]

Cholesterol is a key lipid molecule classically recognized by its roles on the structure, fluidity, and function of cell membranes. It is also important for membrane organization, which interacting with sphingolipids in specific flat membrane microdomains named lipid rafts. It forms a dynamic and heterogeneous signalling platform due to the aggregation of many

different proteins and signalling molecules and are responsible for intracellular signalling. Lipid rafts can also be enriched with caveolin proteins, forming a cell invagination called caveolae. Cholesterol homeostasis modifications as well high concentration of cholesterol in cell membranes have been reported in several tumor types. [18] Thus, cholesterol is a possible target for cancer therapy. Methyl-β-cyclodextrin (MβCD) is pharmacological agent widely used method for membrane cholesterol-depletion. [19] Caveolin-1 (CAV1) is the main protein in caveolae and alteration on its expression has been reported in different types of malignancies, acting as both tumor promoter or tumor suppressor, depending on the stage and type of tumor. [20] Apparently, in the initial stages of tumorigenesis, CAV1 function as a tumor suppressor, while in later stages, the expression of CAV1 has been linked to tumor progression and metastasis. [21,22] This double function of *CAV1* has also been noted in OSCC, however, the role of this gene in OSCC development is unknown. [23–25] Moreover, some studies have shown an association of CAV1 expression with the EMT by interfering with cell adhesion molecules, causing loss of cell polarity, which favour cellular motility and metastases. [26,27]

Caveolae structure and the expression of CAV1 at the transcription level is regulated by cholesterol through two steroid regulatory binding elements in the CAV1 promoter region. On the other hand, CAV1 can regulate cholesterol membrane levels by modulating cellular influx and efflux. [22] In this context, the effects of cholesterol depletion is variable according to the type of cell and is also able to modulate *CAV1* expression, cell signalling, apoptosis, cell adhesion ability, cell proliferation and migration. [28,29] However, the effects of cholesterol depletion on OSCC cells is unknown. Thus, the aim of this study was to investigate the effects of cholesterol depletion on CAV1 expression as well as its role in the modulation of the expression of EMT markers, migration and invasion capacity of metastatic and non-metastatic oral tongue squamous cell carcinoma (OTSCC) cell lines.

METHODS

Cell culture

The human OTSCC primary tumour cell line SCC-25 (ATCC, Wesel, Germany, CRL-1628) and metastatic cell line HSC-3 (JCRB, Osaka, Japan, JCRB0623) were cultured in 1:1 Dulbecco's modified Eagle's Medium (DMEM)/Ham's Nutrient Mixture F-12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL ascorbic acid, 250 ng/mL amphotericin B and 0.4 ng/mL hydrocortisone (all from Sigma Aldrich; Merck KGaA).

Cells were maintained at 37°C with 5% CO_2. Cells were regularly mycoplasma tested with EZ-PCR Mycoplasma test kit (Biological Industries, Beit-Haemek, Israel).

Cholesterol depletion by Methyl-β-cyclodextrin

Cells were washed with warm PBS and depletion was achieved during 1 hour of incubation with Methyl-β-cyclodextrin (MβCD) (Sigma-Aldrich) in DMEM/HEPES pH 7.4 (Gibco; Thermo Fisher Scientific, Inc.) at a final concentration of 7.5, 10 or 15 mM at 37°C. After MβCD treatment, cholesterol-depleted cells were washed with warm PBS and cultured inin their normal culture medium and used to the experiments below. Control cells were incubated with DMEM/HEPES pH 7.4 only for the same time period.

Cell viability analysis

For checking cell viability, 7.5 x 10^3 cells of SCC-25 and HSC-3 cell lines were treated with 7.5, 10 or 15 mM MβCD for 1 h. After 24 h, 48 h and 72 h of treatment, cell viability was evaluated by Alamar Blue (Resazurin sodium salt, R7017, Sigma-Aldrich, Missouri, USA) according to the manufacturer's protocol. Absorbances after 3 h were analysed using 544/15 and 595/60 nm filter in the Victor3V 1420 Multilabel Counter equipment (Perkin Elmer Life & Life Technologies, Waltham, Massachusetts, USA). Results represent the average of three independent experiments, performed in triplicate.

Membrane fluidity

Cholesterol depletion affects membrane fluidity, therefore, in order to confirm the membrane disturbance by the removal of cholesterol, membrane fluidity of OTSCC cells was measured by Membrane Fluidity kit (Abcam)according to the manufacturer's protocol. Briefly, 1.5 x 10^4cells were cultured in a 96-well plate and treated with 0 mM (control) and 10 mM of MβCD for 1 h. The membrane fluidity was evaluated immediately (0 h) and 24 h after treatment according to manufacturer's protocol. Fluorescence was obtained using a 405/10nm (excitation - monomer) and 460/30nm (emission - excimer) filters on the VICTOR® Nivo ™ system equipment (PerkinElmer, United Kingdom) and analysed by measuring the ratio of excimer to monomer.

mRNA extraction and Real-time reverse transcription polymerase chain reaction

The analysis of gene expression was performed on control cells and cells treated with 10 mM MβCD for 1 h and checked 6 h and 24 h after cholesterol depletion. Total RNA was

extracted from control and using PureLink RNA MiniKit (Ambion, Life Technologies, California, EUA) according to the manufacturer's instructions. RNA quantity and quality were measured with NanoDrop 2000 (Thermo Scientific) confirming its purity by checking the ratio 260/280 and 260/230. cDNA was synthesized from 1 µg of DNAse I (ThermoFisher, Massachusetts, USA) treated RNA using the RevertAid First Strand cDNA Synthesis Kit (K1622, ThermoFisher, Massachusetts, USA), according to manufacturer's protocol. qPCR analysis was performed with FastStart MasterMix with ROX (Roche Diagnostics) according to manufacturer's instructions on Rotor-Gene 3000 (Corbett Research, Sydney, Australia) machine. The primers were delineated from the sequences of the respective mRNA on Primer bank ID from GenBank (NCBI - National Center for Biotechnology Information - NIH, USA- http://www.ncbi.nIm.nih.gov/) using a final concentration of 0.3 µM. Reverse and forward primers were evaluated for *CAV1* (forward 5'-CAGGGACATCTCTACACC-3' and reverse 5'-TCAAAGTCAATCTTGACCAC-3'), *ECAD* (forward 5'-CGAGAGCTACACGTTCACGG-3' and reverse 5'-GGGTGTCGAGGGAAAAATAGG-3'), *NCAD* (forward 5'-TCAGGCGTCTGTAGAGGCTT -3' and reverse 5'-ATGCACATCCTTCGATAAGACTG-3'), *βCAT* (forward 5'-CATCTACACAGTTTGATGCTGCT-3' and reverse 5'-GCAGTTTTGTCAGTTCAGGGA-3') and *VIM* (forward 5'-AGTCCACTGAGTACCGGAGAC-3' and reverse: 5'-CATTTCACCGCATCTGGCGTTC-3' – all primers from Sigma-Aldrich). The endogenous gene used was *GAPDH* (Sigma-Aldrich: forward: 5'-CACCAACTGCTTAGCACCC and reverse: 5'-GCAGGGATGTTCTGGA). Relative gene expression analysis was performed according to the 2-ΔΔCT method based on 3 to 5 different experiments.

Western Blotting

The analysis of protein expression was performed on control cells and cells treated with 10 mM MβCD for 1 hour and observed 24 h and 48 h after cholesterol depletion. Cells were lysed elution buffer (50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (v/v) Brij-35 (Sigma Aldrich)) including Complete EDTA-free protease inhibitor cocktail (Roche). The cell debris was removed by centrifugation and protein concentrations were measured with a DC Protein assay (Bio-Rad). Thirty micrograms of soluble protein were separated under reducing conditions on a 10% or 12% SDS-PAGE gel and them proteins were transferred to an Immobilon-P membrane (Millipore). The membrane was blocked with 5% milk powder (Bio-Rad) in Tris-buffered saline/0.1% Tween 20. Membranes were incubated overnight at 4 °C with the following antibodies CAV1 (dilution 1:1,000, ab32577 3238, Abcam), E-cadherin (dilution

1:1,000, 24E10, Cell Signalling), N-cadherin (dilution 1:1,000, 18-0224, Invitrogen), β-catenin (dilution 1:2,500, ab32572, Abcam), vimentin (dilution 1:750, M0725, Dako) or anti-β-actin (dilution 1:2,000, ab8226, Abcam), followed by a biotinylated anti-rabbit IgG (dilution 1:5,000, code E035301-2, Dako) or anti-mouse IgG (dilution 1:5,000, code E035401-2, Dako) secondary antibodies. Immunocomplexes were visualized using a Pierce ECL Western blotting substrate (Thermo Scientific, Waltham, MA, USA) and the Luminescent image analyser LAS-3000 (Fujifilm, Tokio, Japan). Quantification of protein levels was performed with Fiji software 1.51w [30] and β-actin was as endogenous protein for data normalization. The results represent the average of three to five independent experiments.

Horizontal cell migration assays

Plates of 96-well were coated with 50 µl of 0.3 mg/ml Myogel [31] and incubated overnight. Then, 2.5 x 10^4 cells were seeded on wells and 24 h later treated with indicated concentration of MβCD for 1 h. Cell layers were scratched with WoundMaker™ tool (Essen BioScience). The cell migration was followed in IncuCyte® S3 (Essen BioScience) supplied with the Scratch Wound assay module. Cells were kept in normal media supplemented with 1% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.). Cell migration of both cells was analysed during 96 hours. The figures were analysed with Fiji software 1.51w where the wound areas were measured and calculated as a percentage, considering zero time as 100%. The results reflect the average of 3 independent experiments in triplicate.

Myoma disc organotypic invasion assay

Cells treated with 7.5 or 10 mM MβCD for 1 h and control cells (7 x 10^5 cells) were seeded on top of the myoma discs and allowed to invade for 2 or 3 weeks, during which the media was changed every 3 to 4 days [32,33]. At day 15 for HSC-3 and at day 21 for SCC-25, the myomas were fixed in 4% formalin solution, embedded in paraffin and stained with haematoxylin and eosin. Images were taken from the slides and invasion depth, invasion area, number and average size of islands were analysed with Fiji software 1.51w [32]. The results represent the average of three independent experiments in triplicate.

Statistical analysis

Data were analysed using GraphPad Prism 5 software. One-way analysis of variance (ANOVA) with post hoc comparisons based on the Tukey's multiple comparisons test were applied to cell viability and invasion capacity analysis. Student t-tests or Mann-Whitney U test were applied to membrane fluidity and protein expression analysis. Gene expressions were

analysed by student t test, while association between genes was analysed through Kruskall-Wallis test. Two-way-ANOVA with Bonferroni correction used for post-hoc comparison was applied to evaluate migration capacity. The level of significance considered was 5% ($p \leq 0.05$). Results with a p-value < 0.05 were considered significant (*), and those with a p-value < 0.01 (**), or <0.001 (***), very significant.

RESULTS

Cholesterol depletion affects cell viability and membrane fluidity

The cellular viability of SCC-25 and HSC-3 cells decreased significantly ($p< 0.001$) with the increasing concentration of MβCD in all measured timepoints. From SCC-25 cells (Figure 1 A), cell viability decreased under crescent MβCD concentration. In HSC-3 cells, the cell viability was less affected over 24 h, and cells under 15 mM MβCD recovered their cell viability to 50-75% at 72 h (Figure 1B). Thus, the concentration of 10 mM was selected to investigate the gene and protein expression as well as membrane fluidity.

Membrane fluidity in SCC-25 cells (Figure 1C) was significantly higher immediately after cholesterol depletion with 10 mM MβCD ($p< 0.01$) and after 24 h it was slightly higher than the control. However, the membrane fluidity of HSC-3 cells was not affected by cholesterol depletion (Figure 1D).

Cholesterol depletion affects mRNA and protein levels of CAV1 and EMT markers

mRNA expression was evaluated 6 and 24 h after treatment with 10 mM MβCD in SCC-25 and HSC-3 cells (Figure 2A and B). In SCC-25 and HSC-3 cell lines, *CAV1* mRNA expression decreased after 6 h of cholesterol depletion ($p<0.001$ and $p<0.05$) compared with untreated control. However, *CAV-1* expression increased after 24 h only in SCC-25 cells ($p< 0.01$). In SCC-25 cells, *ECAD* mRNA expression level was upregulated at 6 h and downregulated at 24 h ($p< 0.001$ and $p< 0.05$, respectively). In these cells, cholesterol depletion also induced an overexpression of *NCAD* and *βCAT* mRNAs, but not in *VIM* expression, 6 h after treatment ($p< 0.05$, $p< 0.01$, respectively). In HSC3, *ECAD* was overexpressed only after 6 h of treatment ($p<0.01$,) and the expression of the others markers was not affected by cholesterol depletion in different times in these cells.

Furthermore, in SCC-25 cells comparison of the expression of the EMT markers (*ECAD, NCAD* and *βCAT)* with *CAV1* showed differences after 6 h cholesterol depletion ($p= 0.0001$) and 24 h between *CAV1* and *ECAD*, *ECAD* and *βCAT*, and *ECAD* and *VIM* ($p< 0.01$, Figure

2A). In HSC-3, differences between gene expression of EMT markers were noted 6 h after cholesterol depletion between *CAV1* and *ECAD* and between *ECAD* and *βCAT* (p= 0.001, Figure 2B).

At the protein level, 10 mM MβCD cholesterol depletion increased significantly β-catenin expression ($p<0.05$) after 48 h in SCC-25 cells and changed, although not significantly, the expression of CAV-1 and of some EMT markers in both evaluated timepoints (Figure 2C). In HSC-3 cells, changes on protein levels of CAV-1 and of EMT markers were observed but they were not significantly affected by cholesterol depletion and interestingly β-catenin was not detected even in HSC-3 control cells (Figure 2D).

OTSCC cell migration and invasion are affected by cholesterol depletion

A MβCD dose-dependent reduction in SCC-25 cell migration was seen in scratch wound healing assay (Figure 3A). Between control and 7.5 mM, a statistical difference was noted only after 1 day of depletion. On the other hand, 10mM and 15mM showed a statistical difference at all times when compared to control ($p <0.001$, Figure 3A and B). In HSC-3 cells, a significant reduction of migration capacity was noted with 15 mM MβCD treatment ($p<0.001$, Figure 3C and D).

OTSCC cell invasion was assessed using a 3D-myoma organotypic model. Control and treated SCC-25 cells showed a uniform invasion pattern, forming invasive tumor islands in the stroma and both large and small (< 5 cells) tumor islands were observed (Figure 4 A). The invasion depth was higher in cells treated with 7.5 mM MβCD compared to the control ($p< 0.05$, Figure 4B). However, the invasion of HSC-3 showed some islands in the stroma and also isolated cells in all evaluated MβCD concentrations (Figure 4C). Cells treated with 10 mM MβCD showed smaller invasion area then the control cells ($p< 0.05$, Figure 4D).

DISCUSSION

Tumor cells have a high cholesterol content in membrane caveolar structures, which are mostly stabilised by caveolin 1 (CAV) protein. Caveola have structural and functional importance, especially in cell signalling, and absence of any element leads to loss of the caveola. [22,34,35]

In this study, cholesterol depletion with MβCD affected the expression of CAV1, first noted as a reduction in gene expression and later as an increase. In addition, cholesterol depletion increased expression of both epithelial (*ECAD* and *βCAT*) and mesenchymal (*NCAD*) markers in low invasive SCC-25 cells, which suggests hybrid or partial EMT. This EMT profile

was associated with a greater invasive capacity in 3D myoma model. On the other hand, highly invasive HSC-3 cells showed increase in the expression of the epithelial marker *ECAD* and decrease of the invasion area in myoma assay.

It is known that almost all cellular cholesterol is removed when cells are exposed to a high concentration of MβCD (5–10 mM) for an extended period of time (> 2 hours) and, under these conditions, cells change their morphology and die. On the other hand, with short exposure time and/or low concentration, the cholesterol is present in the lipid rafts and does not induce cytotoxicity. [36]. In the present study, 1 h cholesterol depletion with MβCD reduced cell viability in both SCC-25 and HSC-3 cell lines. When 15 mM treated, few cells remained viable, while at 10 mM, about half of the cells maintained their viability. Thereby, 7.5 and 10 mM MβCD treatment was used to study effects on OTSCC cell functions.

In general, removal of cholesterol raises the membrane fluidity [37], and this was also noted in SCC-25 cells immediately after treatment with MβCD and kept 24 h later. Interestingly, a reduction in *CAV1* mRNA expression was noted 6 h after depletion and its expression increased 24 h later. Cholesterol depletion therefore appears to immediately affect the cell membrane fluidity and *CAV1* expression. Additionally, an increase in *CAV1* expression 24 h after depletion and a consequent decrease in *ECAD* expression could affect cell adhesion signalling, increasing cell mobility and tumor progression. Bailey and Liu (2008) observed overexpression of *CAV1* with the downregulation of ECAD and upregulation in NCAD expression in human embryonic carcinoma. [38] In the study of Park *et al* (2009), the disruption of caveolae led to the down-regulation of cell signalling FAK, Src activation, tyrosine phosphorylation of Caveolin-1 and mobilization of caveolae markers from the cell surface to the cytoplasm, which were also recovered by cholesterol addition. [39] Our results showed an increase of invasion after cholesterol depletion in SCC-25, on the other hand a decrease on invasion of mestastatic HCS-3 cell line, showing a probably differences during tumor stage. In prostate cancer cells, cholesterol depletion blocked AKT signalling and reduced cell survival. [40] This signalling pathway has been reported as up-regulated in a number of invasive and metastatic cancers, and many reports implicate the FAK / Src signalling pathway in the progression through the EMT and generation of a more highly invasive phenotype in many types of cancer. [38]

Changes on CAV1 expression has been associated with cell transformation, tumor growth, migration, invasion, metastasis and multidrug resistance. [22] In general, studies show that CAV1 acts as a tumor suppressor in early stages of carcinogenesis, since it has been noted that its low expression induces tumor progression. On the other hand, in later stages, the

expression of CAV1 has been linked to tumor progression and metastasis. [20–22] It can be explained by the complex structure of Caveolin-1 that is implicated in several protein-protein and protein-lipid interactions structure, for instance, the implication of CAV1 in β-catenin pathway (PDK1/PKCα/β-catenin). In addition, Caveolin-1 protein is detected at many locations throughout the cell, it is related to a wide variety of processes, including cholesterol homeostasis and regulation of signal transduction. [21] The interaction with scaffolding domains of Caveolin-1 blocks many signalling events. On the other hand, the protein is implicated as a positive regulator, for instance, integrin, insulin and progesterone signalling. This ambiguous relationship combined with variations in subcellular distribution, provide a potential rationale to understanding how Caveolin-1 presence in tumour cells may in some cases be associated with tumour suppression, but in others, with more malignant phenotypes including multidrug resistance and metastasis. [21] Here, the invasive capacity of SCC-25 was improved by cholesterol depletion, and also those cells acquired a hybrid-like profile (or p-EMT), through the greater expression of both epithelial and mesenchymal markers (*ECAD, NCAD* and *βCAT*). Interestingly, this hybrid EMT profile has been associated to greater metastatic capacity than EMT cells. [14,15] This hybrid profile, however, was not seen 24 hours after cholesterol depletion, which is probably due to the removal of the stimulus. It has been shown earlier that effects of cholesterol depletion on caveola formation are temporary and, around 24 h after removal of the stimulus, the effect is reversed. [41] The caveolin protein remains in the plasma membrane, and its primary distribution, which before resembled that of a belt in the deep part of the caveola, disintegrates forming a ring and later a dispersed fragment.

Aberrant activation of EMT is considered a hallmark of cancer metastasis. [8,10–13]. In the current study, the decrease in CAV1 expression was followed by an increase in ECAD expression 6 h after depletion in the metastatic HSC-3 cells. This inverse expression profile between CAV1 and ECAD was not maintained after 24 h, when there was a stabilization in the gene expression of both molecules. In addition, the migratory capacity was reduced, with significant difference observed only at 15 mM. Moreover, the invasive capacity was significantly reduced at 10 mM MβCD, which could be related to the increase of ECAD expression. Both decrease and increase in CAV1 expression have already been associated with the induction of EMT depending on cell and tumor type, altering adhesion molecules, loss of cell polarity, cell mobility and invasion. [42,43] In Head and neck squamous cell carcinoma (HNSCC) Masuelli et al., 2012 reported an association between CAV1 overexpression and abnormal expression of E-cadherin-α/β-catenin complex in the same HNSCC samples. They also related that chronic stimulation of human hypopharyngeal carcinoma cell line (FaDu) with

EGF induces β-catenin and Caveolin-1 internalization, their co-localization with EGFR and an increase in the physical interaction between EGFR/β-catenin/Caveolin-1 and between E-cadherin/β-catenin/caveolin-1. These molecular events were associated with increased directional motility of cells in vitro, enhanced wound closure and lymph node metastases. [27]

In this present study, cholesterol depletion affected migration and invasion of both cell line. The reduction of migration and invasion of metastatic cells HSC-3 was associated with the increase in ECAD 6h after cholesterol depletion. While non-metastatic cells SCC-25 cells had their migratory capacity reduced, but the invasive capacity increased after cholesterol depletion. This reduction in migratory capacity may have been affected by the decrease in the expression of CAV1 and the consequent increase in the expression of ECAD in the first hours, while the invasive capacity may have been stimulated by the increase in the expression of CAV1 and a decrease in the ECAD noticed 24 h after the depletion. Moreover, the invasive assay of both cells showed both large invasive tumor islands after cholesterol depletion but also small islands containing few cells detached, characteristic of the tumor budding. Tumor budding means the presence of clusters of undifferentiated malignant cells in the tumor stroma, which are located mainly (but not exclusively) in close proximity of the invasive tumor front. [13,17] These cells maintain cell adhesion, which hinders migratory capacity, but are able to invade together. They are apparently more aggressive in terms of metastatic potential. [13,44] Co-expression of epithelial and mesenchymal markers was observed in many tumor buds and has been posit tumor budding to be a manifestation of this hybrid epithelial/mesenchymal phenotype displaying collective cell migration. [6] This association was also reported in tongue squamous cell carcinoma by Wang et al. 2011. The authors observed tumor budding in the most of OTSCC samples and a significant association among tumor budding and the deregulation of E- cadherin and Vimentin. Besides that, tumor budding was associated with tumor size, differentiation, clinical stage, lymph node metastasis, and correlated with reduced overall survival. [44] Comparing to our study, cholesterol depletion means to induce a hybrid invasive profile of non-metastatic cells, while affect the metastatic capacity of metastatic cells, showing different performances to different cells.

CONCLUSION

Cholesterol depletion affects CAV1 gene expression in OTSCC cell lines. An inverse gene expression profile between CAV1 and ECAD was observed in both cells, with the decrease in *CAV1* followed by an increase in *ECAD* expression. In non-metastatic cells, this inversion was associated with a decrease in its migratory capacity but an increase in its invasive

capacity, in addition to a subsequent increase in the expression of both epithelial and mesenchymal markers. This scenario may be associate with a partial EMT profile that support the development of tumor budding. On the other hand, in metastatic cells, cholesterol depletion and the consequent inverse gene expression profile of increase of CAV1 and decrease of ECAD, decreased the migratory and invasive capacity. Additional studies are needed to understand the mechanisms by which cholesterol depletion contribute for the decrease of these both events in the invasive OTSCC cell line.

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FIGURES

Figure 1 – Effect of cholesterol depletion in cell viability and membrane fluidity. Analysis of % of viable cells by Alamar Blue assay after cholesterol depletion in SCC-25 cells (A) and in HSC-3 cells (B). Evaluation of membrande fluidity by fluorescence assay of Membrane Fluidity kit in SCC-25 (C) and HSC-3 cells after cholesterol depletion. (**p <0.01, *** p <0.001; Error bars represent the standard deviation of the experimental triplicate).

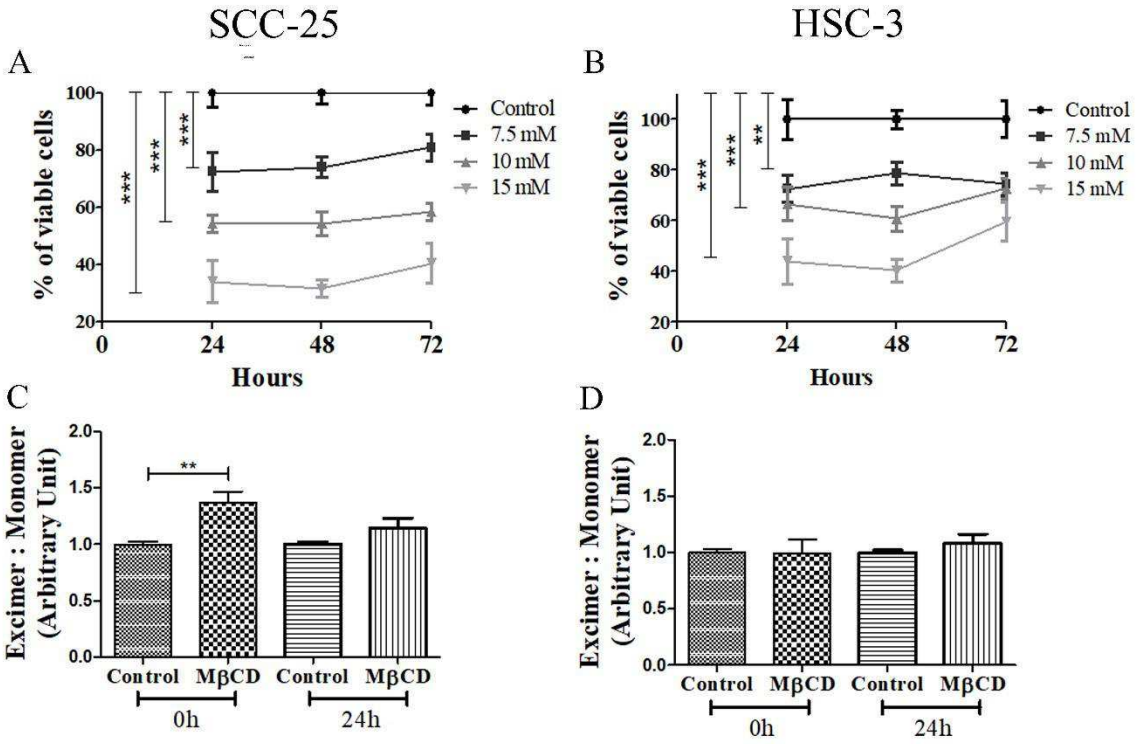


Figure 2 - ***Expression of CAV1 and EMT markers after cholesterol depletion.*** Analysis of gene expression by qRT-PCR in SCC-25 (A) and in HSC-3 cells (B). Experiments of Western Blotting for evaluation of protein expression of CAV1 and EMT markers in SCC-25 (C) and also in HSC-3 cells (D). (* p <0.05, **p <0.01, *** p <0.001; Error bars represent the standard deviation of the experimental triplicate).

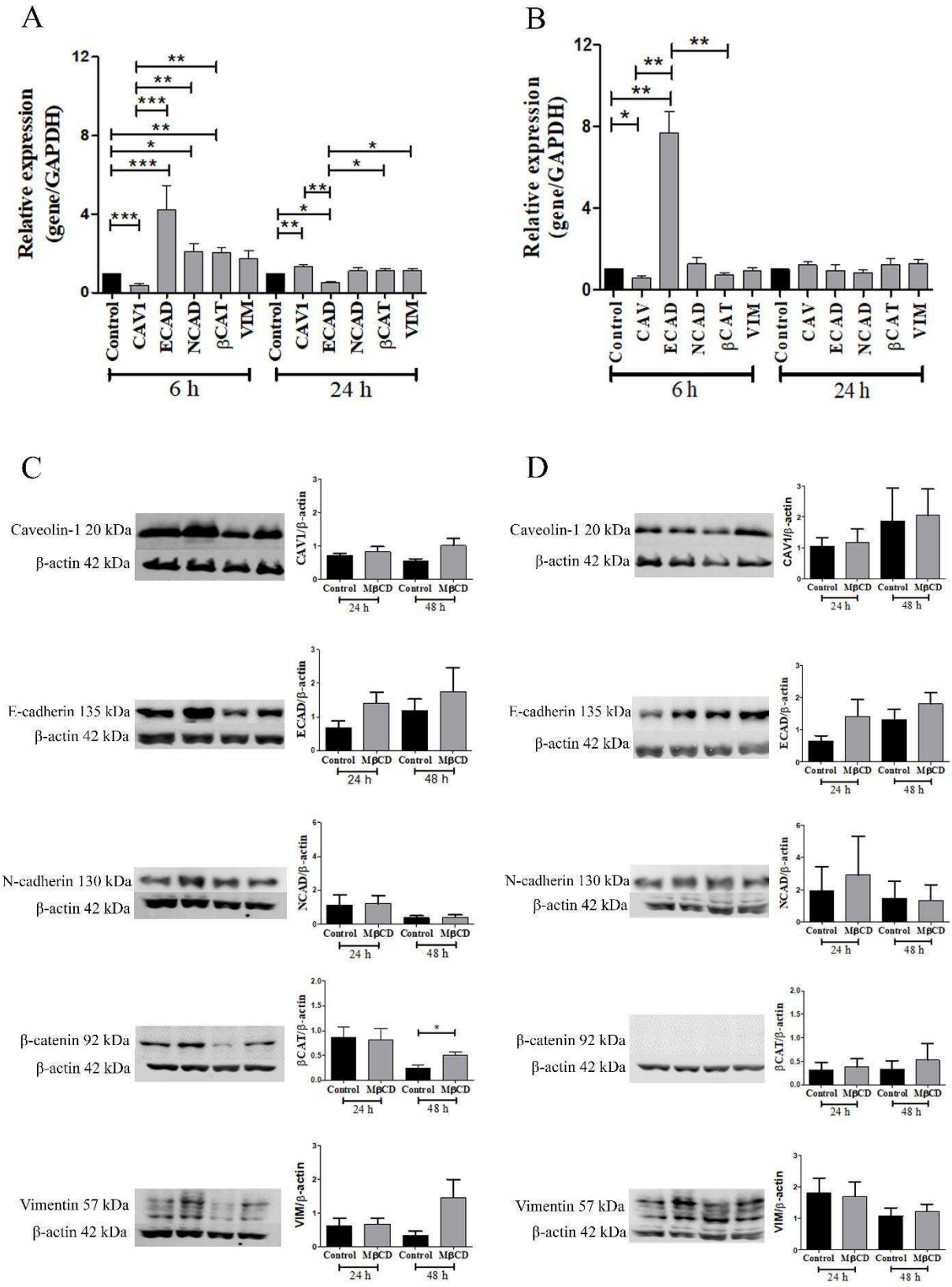


Figure 3– Migration of cells after cholesterol depletion. Migration assay was evaluated by wound healing assay on myogel in both SCC-25 (A,B) and HSC-3 (C, D) cells after 1 h cholesterol depletion and followed up to 96 hours (* $p < 0.05$, *** $p < 0.001$; Error bars represent the standard deviation of the experimental triplicate).

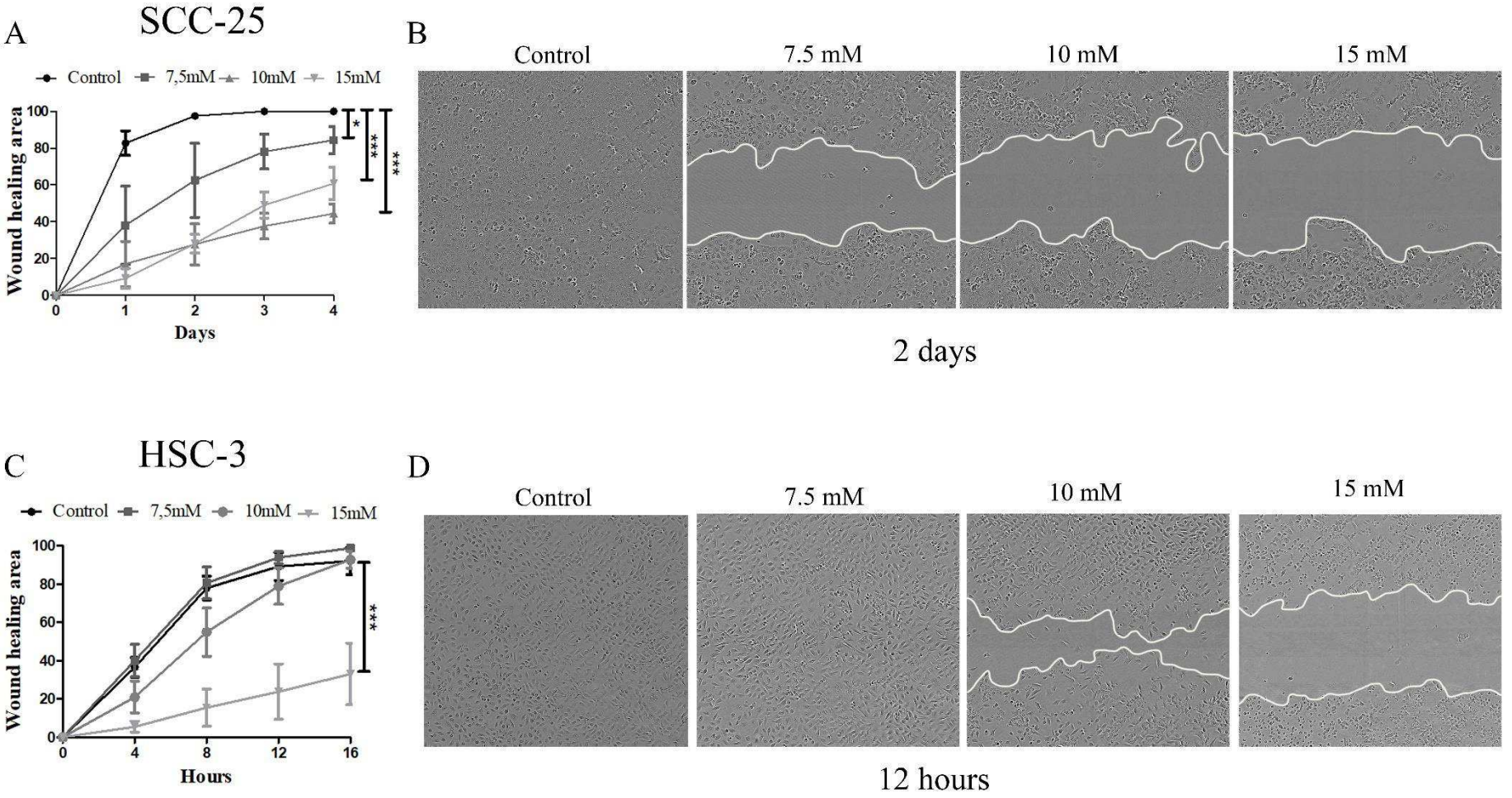
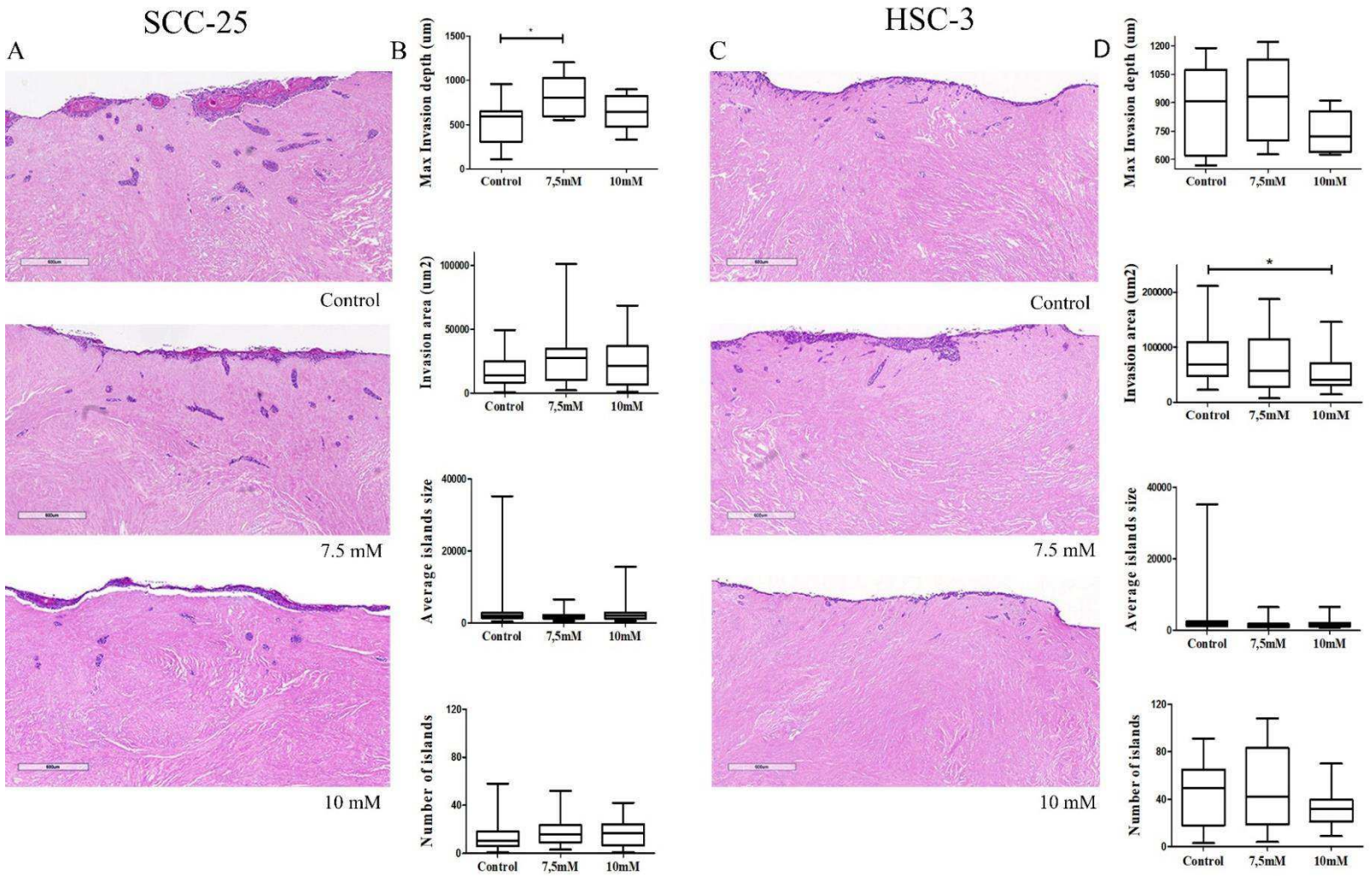


Figure 4 – Invasive capacity after cholesterol depletion. Cells were seeded on the top of and let to invade within the 3D-myoma Organotypic model for up 21 days. Representative images stained with haematoxylin and eosin (scale bars represent 600 µm) of the invaded SCC-25 (A) and HSC-3 (C) cells. Quantification of the invaded cells of SCC-25 (B) and HSC-3 (D) cell lines. Cholesterol depletion with 7.5 mM MβCD induced an increase of SCC-25 depth of invasion, while 10 mM MβCD decreased the invasion area of HSC-3 cells. hours (* $p < 0.05$; Error bars represent the standard deviation of the experimental triplicate).



ARTIGO 2:

OVEREXPRESSION OF CAVEOLIN-1 IS ASSOCIATED WITH TUMOR AGGRESSIVENESS AND ITS SILENCING INDUCES INVASION OF ORAL SQUAMOUS CELL CARCINOMA CELL LINES

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ABSTRACT

Caveolin-1 (CAV1) is a key protein in the cholesterol-enriched caveolar microdomain of cell membrane and participates in many cell signalling pathways. Changes in CAV1 or in caveolae structure are related to tumorigenesis, as a tumor promoter or as a tumor suppressor, depending on the disease stage. This dual role has also been reported in Oral squamous cell carcinoma (OSCC), but it is not fully understood. Our study aimed to understand the CAV1 roles on tumor progression throught the evaluation of OSCC samples and the effect of its silencing on oral tongue squamous cell carcinoma (OTSCC) cell lines (SCC-25/non metastatic and HSC-3/metastatic on lymph node). Methodology: Microarray hybridization, mRNA expression and immunohistochemistry were peformed on samples of OSCC and non-tumoral tissue (margin). Tumors were divided into two groups: (1) more (T1/T2 N+, n=14) vs (2) less (T3/T4 N0, n=19) aggressive tumors. In OTSCC cells were evaluated cell viability, membrane fluidity, gene and protein expression of EMT markers (E-cadherin/ECAD, N-cadherin/NCAD, β-catenin/βCAT, and Vimentin/VIM) and the migratory and invasive capacity of these cells under silencing of CAV1. Results: CAV-1 was 1.77-fold more expressed in tumors than non-tumoral tissues and around 2.0-fold up-regulated in more aggressive than less aggressive tumors. However, qRT-PCR showed no difference in CAV-1 expression in any comparisons done as well as no association with clinicopathological parameters. CAV-1 protein was localized in both tumoral epithelium and stroma, but in three different patterns: (1) positive epithelium vs negative stroma; (2) positivity within epithelium and stroma; and (3) negative epithelium vs positive stroma. CAV-1 positive stromal cells were associated with higher tumor size (T3/T4), presence of disease, independently of epithelium expression. Also, CAV-1 positive tumor epithelial cells tended to be associated with low or negative CAV-1 in tumor stroma. siCAV-1 increased cell viability only in SCC-25 and upregulated EMT markers genes (N-caderin and β-catenin), but not protein levels, only in HSC-3. Also, siCAV-1 stimulates invasion capacity of metastatic cells in myogel. Conclusion: The switch of CAV-1 expression from tumoral epithelial cells to tumoral stromal cells may be useful to predict aggressiveness in OSCC. siCAV-1 of metastatic cells recovered their invasive capacity and also a re-expression of mesenchymal markers, mimicking the reinduction of EMT process.

Keywords: Oral tongue oral squamous cell carcinoma, Oral Squamous Cell Carcinoma Cell Lines, Caveolae, Caveolin-1, epithelial-mesenchymal transition, tumor aggressiveness

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is associated with high mortality rates worldwide and is usually diagnosed in advanced stages, which contributes to lower disease survival. [1,2]. Genetic and epigenetic events occur during OSCC development, leading to the activation of oncogenes and inactivation of tumor suppressor genes [3] Caveolin-1 (CAV1), a protein in caveolar structures in cell membranes, acts on membrane traffic, intracellular cholesterol transport and the caveolar compartmentalization of molecules and their interaction with caveolins provide a mechanism for the regulation of signalling events, as well as interaction between different signalling pathways [4–7].

Dysregulation of CAV1 has been associated with cell transformation and tumor progression, and its function varies according to the type and stage of the disease. [7–9] In OSCC, there is still controversy regarding the role of CAV1 in tumorigenesis and tumor progression. Previously, studies have shown a dual role, with both oncogenic and tumor suppressor functions, which vary in different stages of the tumor. [10–17] Its role as a tumor promoter has been observed in tumor development, when the increase in its expression seems to favor the development of malignant characteristics of epithelial cells, while its loss of expression was related to a decrease in migratory and invasive capacity in head and neck carcinoma cell lines. [9,18]. On the other hand, CAV1 seems to act as a tumor suppressor, once the negative regulation of CAV1 was observed in OSCC metastatic tissues. [10]

During carcinogenesis, cells undergo the epithelial-mesenchymal transition (EMT) and acquire mesenchymal cell phenotype and properties (negative regulation of epithelial markers, such as E-cadherin and β-catenin, and positive regulation of mesenchymal markers, including N-cadherin and Vimentin). [19–21] In this process, changes are noted in cell signalling pathways that affects cell adhesion, motility, migration, proliferation and differentiation, making cells capable of invading adjacent tissues and developing metastases. [22,23] The EMT process has been associated to the ability to form a bud (tumor budding), defined as fewer than five cancer cells forming a cell cluster at the invasive front of the tumor, has been reported as a predictive features associated with high risk for locoregional recurrence and shortened survival. The analysis of tumor budding associated to depth of invasion has been linked to strong prognostic power for disease-free survival in OSCC patients.[24]

Studies have correlated the EMT process with the expression of Caveolin-1 [8] Both the increase and decrease in the expression of CAV1 were associated with the induction of EMT,

and different levels of CAV1 expression seem to be associated with different phases of EMT. [25,26] However, the pattern of expression of CAV1 in OSCC as well as its role in EMT it is not clear. Thus, the aim of this study was to evaluate the expression of CAV1 in OSCC tumors and the effect of its inhibition on the epithelial-mesenchymal transition process, migratory and invasive capacity of OSCC cells.

MATERIAL AND METHODS

Patient Tissue Samples

Thirty-six OSCC fresh samples (tongue and floor of the mouth) and their corresponding non-tumoral tissues (patient-matched tumor-free margin specimens) were obtained by surgical resection from OSCC patients (male ≥40 years old, smokers) admitted for diagnosis and treatment at Instituto do Cancer Arnaldo Vieira de Carvalho, Hospital Heliópolis, and Hospital das Clínicas (School of Medicine, University of São Paulo, Brazil). All patients have provided written informed consent to participate in this study that was approved by the Brazilian National Ethics Committee (Process #16491) and meets the Declaration of Helsinki.

Samples were immediately snap-frozen in liquid nitrogen upon surgical removal. After histological confirmation, OSCC samples were macrodissected prior to processing and each sample contained at least 70% of tumor cells. Corresponding surgical margins were reported as "non-tumoral tissue". GENCAPO (Head and Neck Genome Project) Consortium was responsible for sample collection and initial processing, clinical data collection, histopathological analysis and informed consent acquisition of each patient. Histopathological diagnosis was performed according to WHO classification [27] for tumours and clinicopathological staging was determined by the TNM classification of the UICC. Samples of OSCC and non-tumoral tissue (margin) were evaluated. Tumors were divided into two groups: (1) more (T1/T2 N+, n=14) vs (2) less (T3/T4 N0, n=19) aggressive tumors.

Microarray Hybridization

Ten OSCC samples and a pool of the corresponding non-tumoral tissues were used for microarray analysis. Experiments were carried out as described by Severino et al [28], using CodeLink Whole Genome Bioarrays (GE Healthcare, Piscataway, NJ, USA) representing 55,000 human transcripts. Slides were scanned in the GenePix 4000B Array Scanner (Axon Instruments), according to the recommended scanning procedures and settings. The data were treated with Code-Link feature extraction software v.4.0. A normalized signal for each

transcript was obtained through quantile normalization. [29] For global gene expression visualization, we performed a hierarchical clustering using the Euclidean distance and the average linkage algorithm (MeV® MultiExperiment Viewer software version 4.1 - Copyright © 2005-2009, Boston, MA, USA). [30,31]

Individual *CAV1* expression profile in OSCC samples and their respective non-tumoral tissues were compared with each other. Results were expressed as fold variation and fold-change > 2.0-fold in mRNA levels was considered as up-regulated and < 0.5-fold as down-regulated.

The array design and raw data files are available at the Gene Expression Omnibus database (GEO) under the accession number GSE9792. To validate the results on *CAV1* gene expression, qRT-PCR was further performed in 36 OSCC samples.

Total RNA extraction, cDNA Synthesis, and qRT-PCR from tissues

Total RNA obtained (1 µg) from tissue samples were incubated with DNase I (Invitrogen) and reverse transcribed to single-stranded cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA, USA). Quantitative RT-PCR (qRT-PCR) was performed using Applied Biosystems 7500 Real-Time PCR System with SYBR Green I Dye (Applied Biosystems). Primer sequences were designed from a specified exon–exon junction (*HPRT1*: F: ccaccaccctgttgctgta and R: tccccgttgactggtcat; *CAV1*: F: ccctaaacacctcaacgatg and R: gccttccaaatgccgtcaaa) using GeneTool 2.0 software (Biotools, Alberta, Canada). All qRT-PCR reactions were performed in a total volume of 25 µL, containing 1 µL of cDNA sample (1:5 dilution), 10 pmol of each primer (400 nM) and 12.5 µL of SYBR Green Master Mix® (Applied Biosystems). The thermal cycling was carried out by starting with 95ºC for 10 min hold, followed by 40 amplification cycles of 95ºC for 10s and 60°C for 1 min. Hypoxanthine guanine phosphoribosyltransferase-1 gene (*HPRT1*) was used as endogenous housekeeping gene for data normalization and relative quantification was performed using Pfaffl mathematic model. [32] For comparisons between tumor and non-tumoral tissues, one sample from normal mucosa was used as calibrator sample, while non-tumoral tissue from each case was the calibrator sample for comparisons between less aggressive and more aggressive tumors.

Immunohistochemistry

Twenty formalin-fixed paraffin-embedded (FFPE) tissue sections (5 μm thickness) were selected for immunohistochemical assay. Paraffin sections were deparaffinized and rehydrated through a graded series of ethanol washes. The endogenous peroxidase was blocked by 3% H_2O_2 solution for 45 min and the free charges were neutralized by 1% borax solution for 15 min. The heat-induced epitope retrieval was performed with phosphate-citrate buffer (93-96°C, pH 6.0 for 20 min - P4809, Sigma-Aldrich) and non-specific binding blocked by incubation with 4% non-fat milk solution for 20 min at room temperature (RT). The slices were incubated with CAV1 rabbit polyclonal primary antibody (3238, Cell Signaling – 1:200) in a humid chamber at 4°C overnight, followed by the detection based in Chain Polymer-Conjugated Technology (EnVision®, K4061 DAKO) for 30 min at RT. Reactions were revealed by incubating the sections with chromogenic substrate mixture (3,3'-Diaminobenzidine) (Dako Liquid DAB Plus, K3468, DAKO). The sections were counterstained with Mayer's haematoxylin. All rinses were performed in 0.1% Triton X-100 in PBS. 0.1% PBS/BSA solution was used for both primary antibody dilution and negative control (primary antibody were replaced by this solution). Human colon tumor biopsies were used as positive control.

Immunohistochemical staining of tumoral epithelia and stroma cells were evaluated by light microscopy at x200 magnification throughout each entire section. Distribution of CAV1 positivity was scored semi-quantitatively according to Koo et al [33] as negative (-, no staining), weak (+, staining of less than 30% of the epithelial or stromal cells), moderate (++, staining of 30–60% of epithelial or stromal cells), and strong (+++, staining of more than 60% of epithelial or stromal cells). The expression of CAV1 was considered positive when more than weak staining was identified. Expression in stromal cells was considered only in stromal spindle cells, excluding endothelial cells (internal control). For statistical analysis, either negative and weak staining were grouped as "CAV1 negative expression" and moderate and strong staining was considered "CAV1 positive expression".

Cell culture

The human TSCC primary tumour cell line SCC-25 (ATCC, Wesel, Germany, CRL-1628) and metastatic cell line HSC-3 (JCRB, Osaka, Japan, JCRB0623) were cultured in 1:1 Dulbecco's modified Eagle's Medium (DMEM)/Ham's Nutrient Mixture F-12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL ascorbic acid, 250

ng/mL amphotericin B and 0.4 ng/mL hydrocortisone (all from Sigma Aldrich; Merck KGaA). Cells were maintained at 37°C with 5% CO_2. Cells were regularly mycoplasma tested with EZ-PCR Mycoplasma test kit (Biological Industries, Beit-Haemek, Israel).

Caveolin-1 siRNA transfection

Silencing of Caveolin-1 gene was performed in SCC-25 and HSC-3 cells with interference RNA (siRNA) using Lipofectamine (Invitrogen, USA). Three validated commercially available CAV1 silencers for humans have been identified (CAV1 Silencer select Validated siRNA ID: s2446 (1), s2447 (2) and s2448 (3), Ref. 4427038, AMbion – ThermoFisher Scientific, Inc). siRNA was incubated for 48 hours in final concentrations of 10 nM and 50 nM. The silencing capacity was evaluated by qPCR and Western Blotting.

Cell viability analysis

For checking the cell viability, 7.5 x 10^3 cells of SCC-25 and HSC-3 cell lines were silenced with siCAV1. After 24 h, 48 h and 72 h of silencing, cell viability was evaluated by Alamar Blue (Resazurin sodium salt, R7017, Sigma-Aldrich, Missouri, USA) according to the manufacturer's protocol. Absorbances after 3 h were analysed using 544/15 and 595/60 nm filter in the Victor3V 1420 Multilabel Counter equipment (Perkin Elmer Life & Life Technologies, Waltham, Massachusetts, USA). Results represent the average of three independent experiments, performed in triplicate.

Membrane fluidity

Membrane fluidity of TSCC cells was measured by Membrane Fluidity kit (Abcam)according to the manufacturer's protocol. Briefly, 1.5 x 10^4 cells were cultured in a 96-well plate and silenced with siCAV1 for 48 h. The membrane fluidity was evaluated immediately (0 h) and 24 h after silencing according to manufacturer's protocol. Fluorescence was obtained using a 405/10nm (excitation) and 460/30nm (emission) filters on the VICTOR® Nivo ™ system equipment (PerkinElmer, United Kingdom).

mRNA extraction and Real-time reverse transcription polymerase chain reaction

Total RNA was extracted from control and siCAV1 TSCC cells using PureLink RNA MiniKit (Ambion, Life Technologies, California, EUA) according to the manufacturer's instructions. RNA quantity and quality were measured with NanoDrop 2000 (Thermo Scientific). cDNA was synthesized from 1 µg of DNAse I (ThermoFisher, Massachusetts, USA)

treated RNA using the RevertAid First Strand cDNA Synthesis Kit (K1622, ThermoFisher, Massachusetts, USA), according to manufacturer's protocol. qPCR analysis was performed with FastStart MasterMix with ROX (Roche Diagnostics) according to manufacturer's instructions on Rotor-Gene 3000 (Corbett Research, Sydney, Australia) machine. The primers (final concentration 0.3 μM) used for *CAV1* (forward 5'-CAGGGACATCTCTACACC-3' and reverse 5'-TCAAAGTCAATCTTGACCAC-3'), *ECAD* (forward 5'-CGAGAGCTACACGTTCACGG-3' and reverse 5'-GGGTGTCGAGGGAAAAATAGG-3'), *NCAD* (forward 5'-TCAGGCGTCTGTAGAGGCTT -3' and reverse 5'-ATGCACATCCTTCGATAAGACTG-3'), *βCAT* (forward 5'-CATCTACACAGTTTGATGCTGCT-3' and reverse 5'-GCAGTTTTGTCAGTTCAGGGA-3') and *VIM* (forward 5'-AGTCCACTGAGTACCGGAGAC-3' and reverse: 5'-CATTTCACCGCATCTGGCGTTC-3'). The endogenous gene used was *GAPDH* (Sigma-Aldrich: forward: 5'-CACCAACTGCTTAGCACCC and reverse: 5'-GCAGGGATGTTCTGGA). Relative gene expression analysis was performed according to the 2-ΔΔCT method based on 3 to 5 different experiments.

Western Blotting

Control and siCAV1 OTSCC cells were lysed elution buffer (50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (v/v) Brij-35 (Sigma Aldrich)) including Complete EDTA-free protease inhibitor cocktail (Roche). The cell debris was removed by centrifugation and protein concentrations were measured with a DC Protein assay (Bio-Rad). Thirty micrograms of soluble protein were separated under reducing conditions on a 10% or 12% SDS-PAGE gel and them proteins were transferred to an Immobilon-P membrane (Millipore). The membrane was blocked with 5% milk powder (Bio-Rad) in Tris-buffered saline/0.1% Tween 20. Membranes were incubated overnight at 4 °C with the following antibodies CAV1 (dilution 1:1,000, ab32577 3238, Abcam), E-cadherin (dilution 1:1,000, 24E10, Cell Signalling), N-cadherin (dilution 1:1,000, 18-0224, Invitrogen), β-catenin (dilution 1:2,500, ab32572, Abcam), vimentin (dilution 1:750, M0725, Dako) or anti-β-actin (dilution 1:2,000, ab8226, Abcam), followed by a biotinylated anti-rabbit IgG (dilution 1:5,000, code E035301-2, Dako) or anti-mouse IgG (dilution 1:5,000, code E035401-2, Dako) secondary antibodies. Immunocomplexes were visualized using a Pierce ECL Western blotting substrate (Thermo Scientific, Waltham, MA, USA) and the Luminescent image analyser LAS-3000 (Fujifilm, Tokio, Japan). Quantification of protein levels was performed with Fiji software 1.51w [34]

and β-actin was as endogenous protein for data normalization. The results represent the average of three to five independent experiments.

Horizontal cell migration assays

96-well plates were coated with 50 µl of 0.3 mg/ml Myogel [35] and incubated overnight. Then, 2.5 x 10^4 cells were seeded and 24 h later they were silenced with siCAV1. Cell layers were scratched with WoundMaker™ tool (Essen BioScience). The cell migration was followed in IncuCyte® S3 (Essen BioScience) supplied with the Scratch Wound assay module. The figures were analysed with Fiji software 1.51w where the wound areas were measured and calculated as a percentage, considering zero time as 100%. The results reflect the average of 3 independent experiments in triplicate.

For invasion assay, after scratching, Myogel/fibrin was added (2.4 mg/mL Myogel, 0.5 mg/mL fibrinogen (Merck, Darmstadt, Germany), 0.3 U/mL thrombin (Sigma-Aldrich), and 33.3 µg/ml aprotinin (Sigma-Aldrich); these reagents were diluted in DMEM/F12 media with 10% FBS). Pictures were obtained every 2 hours on Incucyte® S3 Live-Cell Analysis System microscope and analysed through Image J software, where the wound areas were measured and calculated as a percentage, considering zero time as 100%. The results reflect the average of 3 independent experiments in triplicate.

Statistical Analysis

Wilcoxon nonparametric test was used to assess the differences in CAV1 gene expression levels between "tumor vs non-tumoral tissues", while Mann-Whitney test was performed to analyze CAV1 gene expression levels between "less aggressive vs more aggressive tumors" in the microarray and qRT-PCR analysis. The cut-off was set up at the values < 2.0 (negative) and ≥ 2.0 (positive) for gene expression analysis by qRT-PCR.

The Fisher's exact test was used to estimate statistical difference between *CAV1* gene/protein expression levels and clinicopathological parameters such as mean age, tumor location, tumor size-pT, nodal metastasis-pN, pathological grade, lymphatic and/or perineural invasion and recurrence. For this analysis, only OSCC samples paired with their respective non-tumoral tissue in which *CAV1* exhibited detectable expression by qRT-PCR. Spearman Correlation was used to compare tumoral and stromal protein expression. Kaplan-Meier product-limit estimation with log-rank ($p < 0.05$) was used to survival analysis from life-time data according to gene expression levels and immunoexpression of CAV1 in tumor and stroma cells. Overall survival was defined as time from surgery to the day of death or last follow-up.

One-way analysis of variance (ANOVA) with post hoc comparisons based on the Tukey's multiple comparisons test were applied to cell viability and invasion capacity analysis. Student t-tests or Mann-Whitney U test were applied to Membrane Fluidity and protein expression analysis. Gene expression of each gene compared to the control was analysed by t student test, while association between genes was analysed through Kruskall-Wallis test. Two-way-ANOVA with Bonferroni correction used for post-hoc comparison was applied to evaluate migration capacity.

Numerical data are shown as mean and are based on a minimum of 3 repeats for each independent biological sample. Data were analysed using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA). The level of significance considered was 5% ($p \leq 0.05$). Results with a p-value < 0.05 were considered significant (*), and those with a p-value < 0.01 (**), or <0.001 (***), very significant

RESULTS

Microarray data and CAV1 mRNA expression analysis

General analysis of the microarray data revealed that CAV1 transcripts presented a differential expression pattern greatly variable between OSCC tissue samples. The mean fold-change for CAV1 in OSCC samples in relation to their matched pool of non-tumoral tissue was 1.77. Five OSCC samples (5/10) showed CAV1 up-regulation (≥ 2.0-fold) and the remaining five OSCC samples, CAV1 was considered as not differentially expressed (values between 0.5 and 2.0) (Figure 1A and Table 1). Considering tumor aggressiveness, *CAV1* mean fold change was 1.48 and 2.95 in less aggressive and more aggressive tumors, respectively. mRNA of *CAV1* was highly expressed in three samples (75%) of more aggressive tumors, while in less aggressive tumors only one sample showed *CAV1* up-regulation (Figure 1B and Table 1).

To validate data from microarray analysis, *CAV1* gene expression was also evaluated by qRT-PCR in 36 OSCC samples. Comparing "tumors vs non-tumoral margin", no statistically significant difference was found in *CAV1* expression (median value = 0.42 and 0.68, respectively, p=0.29, Figure 1B). Additionally, there was no significant difference in *CAV1* expression between more and less aggressive tumors (median value = 0.65 and 0.57, respectively, p=0.19 (Figure 1C).

Sample characterization and the association of CAV1 expression levels with clinicopathological features and disease outcome were examined and are shown in Table 2 and

Figure 1D. In general, there was no significant association between gene expression levels and age group, tumor location, pTNM classification, pathological grade, lymphatic and/or perineural invasion and survival.

CAV1 immunoexpression

CAV1 reactivity was detected in membrane and/or cytoplasm. In non-tumoral tissue, CAV1 was detected in epithelial basal cells and in blood vessels (internal reaction control) (Figure 2A).

In OSCC, CAV1 was detected in both tumoral epithelial cells (Figure 2B-C) and in the tumoral stroma cells (Figure 2D). CAV1 protein expression in tumoral epithelial cells was positive in 12 cases (36%), and CAV1 in tumoral stroma cells was positive in 13 cases (39%) (Table 3). Interestingly, different patterns were found for CAV1 protein expression: positive epithelium vs negative stroma (n=4) (Figure 2B), positivity within both epithelium and stroma (n=8) (Figure 2C) and negative epithelium and positive stroma (n= 5) (Figure 2D). Only three samples were negative in both tumoral epithelium and stroma.

The immunoexpression levels of CAV1 in tumor cells and tumoral stroma cells was associated with clinicopathological features and disease outcome (Figure 2E and Table 3). CAV1 expression in tumoral stroma was associated with greater tumor size and disease (p=0.05), Additionally, CAV1 positive tumor epithelial cells tended to be associated with low or negative CAV1 in tumor stroma (p=0.064; Fisher exact test). Negative correlation was identified between CAV1 immunoexpression in tumor epithelial and stroma cells (r= -0.09, *p*=0.69; Spearman correlation) without statistical significance.

Silencing of Caveolin-1 increases cell viability of non metastatic cells but not of metastatic cells.

In order to understand the role of *CAV1* in the OSCC tumorigenesis, OTSCC cell lines were silenced for *CAV1*. Silencing was performed using interference RNA (siRNA) for *CAV1* and the knockdown was confirmed by the evaluation of mRNA and protein expression (Figure 3).

The silencing of CAV1 in non-metastatic cells, SCC-25, increased its cell viability (p= 0.0025, t student test, Figure 4A). Membrane fluidity was not affected immediately after silencing of CAV-1(p= 0,17, t student test) however a tendency of increasing the membrane fluidity was observed after 24 h (p= 0.0650, Mann-Whitney test, Figure 4B). In metastatic cells,

HSC-3, the silencing of *CAV1* did not change cell viability and membrane fluidity (Figure 4 C and D).

***Silencing of CAV1 increases NCAD* and *βCAT* expression in metastatic cells.**

Gene expression of the EMT markers (*ECAD, NCAD, βCAT* and *VIM*) was not affected by siCAV1 in SCC-25 cells (Figure 5A). On the other hand, in HSC-3 cells, *NCAD* gene expression increased at 6 h (p= 0.0260, Mann-Whitney) and *βCAT* gene expression increased 24 h after siCAV1 (p= 0.0436, teste t student, Figure 5B). There was no difference in gene expression of the evaluated markers between both cells. (Kruskal-Wallis, Figure 5A and B). The protein expression of all EMT markers remained unaltered after siCAV1 in both cells (Figure 5C and D).

CAV1 knockdown induces invasion of metastatic cells.

There was no difference in the migration ability of SCC-25 and HSC-3 siCAV1 in relation to their correspondent controls, when seeded on top of myogel for 96h (Figure 6A-D). Invasion capacity was also not affected by siCAV1 in SCC-25 cells (Figure 6 E and F). However, the invasion capacity of HSC-3 siCAV1 cells was significantlly increased when compared to control (p=0.0458, Mann-Whitney) (Figure 6 G and H).

DISCUSSION

The caveola system in tumor cell membranes is related to tumorigenesis due its association to cell signaling, and, when it is deregulated, promotes cell transformation, tumor growth, tumor progression through migration, invasion and metastasis, and resistance to antineoplastic drugs. [7,8]. In the present study, the microarray analysis revealed *CAV1* overexpression in OSCC when compared to the non-tumoral samples and also, in more aggressive tumors. In addition, stromal CAV1 expression was associate with the disease and greater tumor size and negatively correlated to the expression in epithelial tumoral cells. Moreover, CAV1 knockdown increased the expression of *NCAD* and *βCAT* genes as well as the invasive capacity of metastatic OTSCC.

In general, normal cells present high CAV1 expression and several studies of oncogenic signalling emerged correlating cell transformation with loss of CAV1 expression. [7,9] Here, the *CAV1* expression assessed by microarray was higher in tumor samples than in patient-matched non tumoral margins, which *CAV1* was negative in the majority of the samples.

Furthermore, *CAV1* gene expression was higher in more aggressive (T1/T2, N+) than less aggressive tumours (T3/T4, N0), although these results were not confirmed in a cohort carried-out by qRT-PCR. Some studies have showed an association between increased *CAV1* expression in the step-wise OSCC carcinogenesis. Jaafari-Ashkavandi and Aslani (2017) observed lower expression of *CAV1* in dysplastic tissue when compared to OSCC tissues, suggesting that its expression decreases in the beginning of carcinogenesis and increases later, in tumor formation. [36]. On the other hand, Hung et al. (2003) reported an increased in *CAV1* expression in the step-wise carcinogenesis from normal oral mucosa, non-cancerous matched tissues, oral potentially malignant lesions and primary OSCC. Interestingly, CAV1 is not frequentlly expressed in metastatic OSCC sample. Thus, in early stages, CAV1 is downregulated and its expression increases gradually during tumor progression. However, during invasion and metastasis, CAV1 expression decreases. [10].

Interestingly, different patterns of Caveolin-1 protein expression observed in our OSCC samples (positive epithelium vs negative stroma, positivity in both epithelium and stroma and negative epithelium vs positive stroma) were also reported in previously studies in others tumors such as breast, melanoma and prostate cancer. [37] It is important to highlight that stromal immunostaining of Caveolin-1 was associated with greater tumor size, suggesting that the Caveolin-1 negativity in tumor cells and Caveolin-1 positivity in the tumor microenvironment (TME) may promote tumoral progession (Figure 7). This positivity in stroma may be an expression of this protein by carcinoma-associated fibroblasts (CAFs), located on stroma. Goetz et al (2011) observed increased numbers of CAV1-expressing CAFs in the stroma of human tumor samples. These authors also related that CAV1 expression by fibroblast, in vitro and in vivo, favors an organized 3D stromal architecture that promotes spindle morphology and facilitates tumor cell invasion. [38]. In general, loss of stromal *CAV1* has been associated with aggressive disease and poor outcomes in different tumors (reviewed by Martinez-Outschoorn et al., 2015). The loss of Caveolin-1 in the stroma of breast cancer samples was correlated with high tumor and nodal stage, shorter disease-free survival and an overall survival, which constitutes in a worse prognosis. [33]

Futhermore, in relation to protein localization, CAV1 expressed by stromal cells was related with disease, indicating an oncogenic role of CAV1 in the TME of the OSCC. CAV1 expression in tumoral stroma was also associated to tumor size (more expressed in T3/T4 than T1/T2 samples). Our results regarding CAV1 positivity in stromal cells, independently of tumor

cell expression, were associated with the presence of disease and they are similar to those described by Vered et al. (2015) Remarkably, these authors found CAV1 expression to be higher in the TME-like myoma environment compared to the cultured cancer cells, but similar to the OSCC samples, even when severe hypoxic conditions were further induced in the myoma assays. The authors also found the triple positivity profile CAV1/ alpha-smooth muscle actin/Twist in CAF (cancer-associated fibroblasts)-like cells surrounding the invading tongue cancer cells, and CAV-TME was positively correlated with CAF density. [16] These findings possibly reflect the role of CAV1 in EMT or in fibroblasts undergoing trans-differentiation to CAFs. Goetz et al (2011) showed that CAV1 expression in CAFs of breast carcinoma correlates with low overall survival and invasiveness *in vitro*. [38]

To investigate the functional role of *CAV1* in cellular viability, expression of EMT markers, cellular migration and invasion in OSCC, we have knockdown CAV1 in two OTSCC cell lines with different metastatic potential. In the non-metastatic cell line SCC-25, the silencing of *CAV1* improved cell viability, although it did not affect cellular migration, invasion and the expression of EMT markers. On the other hand, siCAV1 improved invasion capacity of the metastatic cell line HSC-3, followed by *NCAD* overexpression in the first 6 h. These results suggest a possible association between *CAV1* negative expression and tumor aggressiveness and progression. Jung et al. (2015) observed that the decrease in *CAV1* expression in tumor samples is characterized by high propensity for rapid distant metastasis when compared to tumor samples with less invasive capacity, and that *CAV1* negative expression was associated to poor prognosis. They also reported that *CAV1* negative expression enables cells to undergo EMT process, observed by reduction of ECAD and βCAT expression and increased in migration and invasion capacity in SCC-9 cells. [39] Taken together, these results demonstrate that CAV1 low expression can be associated with EMT process and poor prognosis. Although some studies have shown that changes on cholesterol membrane affect the CAV-1 and the membrane fluidity [40,41], in our study the silencing of CAV-1 did not affect membrane fluidity of both cell. It means that only the change in CAV-1 expression may not be able to make changes on membrane fluidity.

Tumor cells, when going through the EMT process, acquire invasive capacity and colonize distant sites, where they then undergo mesenchymal-to-epithelial transition (MET) to establish a metastatic tumour of the same epithelial character as the parental tumour. [43,44] In this present study, siCAV1 improved invasive capacity and mesenchymal markers expression in already metastatic cells HSC-3, which seems to induce a reprogramming of the MET process

in these cells. In contrast with the results presented here, Nohata et al (2011) showed that CAV1 silencing decreased migratory and invasive capacity of HSC-3 [18]. However, some differences regarding the methodology approach to adress invasion must be taken into account. In the study developed by Nohata et al (2011), the authors used a transwell-precoated Matrigel® membrane filter inserts, while in our study the invasion assay was performed with Myogel derived from human uterus leiomyoma tissue through scratch wound invasion assay. In a recent study, invasion of different SCC cells in distinct matrices indicate that invasion speeds were different regarding the type of matrix and even differ in the same cancer cell. [45] The common matrices, as Matrigel®, are derived from mouse tumour tissue, which confers disadvantage for human studies. Myogel is derived from human tissue and provides a soluble human TME matrix for cancer studies. Compared with the commercial mouse tumour-derived matrix Matrigel®, Myogel contains e.g. latent and active MMP-2, tenascin-C and collagen types XII and XIV, which are not present in Matrigel®. It shows, among other features, a more neutral and stable pH, a more efficiently cell invasive capacity, and when solidified with agarose (Myogel-LMA), is a more viable matrix for invasion assays. [35]

Here, migration capacity in HSC-3 cells was not affected, which can be associated to the significant late increase of *βCAT* expression and the slight increase, but not significant, in *ECAD* expression. In a study evaluating HNSCC tumors tissues, lymph node metastases presented lower levels of Caveolin-1 than in the primary tumours and the restoration of Caveolin-1 protein expression substantially reduced tumour growth and inhibited lung metastasis in a highly metastasis xenograft mouse model and inhibited metastatic cell growth and invasion in vitro. [13]

In summary, we showed that *CAV1* mRNA expression as well as CAV1-TME is associated with tumour aggressiveness and tumour size, respectively. The switch of CAV1 expression from epithelial cells to TME cells may be favorable to tumour growth as well as tumour invasion of OSCC cells. In addition, siCAV-1 of metastatic cells recovered their invasive capacity and also a re-expression of mesenchymal markers, mimicking the reinduction of EMT process.

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TABLES

Table 1 – *CAV1* relative gene expression (fold-change) in ten OSCC samples normalized with the non-tumoral tissue, considering or not the aggressiveness criteria (more and less aggressive tumors), obtained by the microarray analysis.

Criteria	OSCC samples										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Average fold-change**
[a]**Tumors**	2,84[1]	2,34[1]	0,99	3,43[1]	0,76	0,74	2,29[1]	2,13[1]	1,46	0,79	1,77
[b]**Less Aggressive Tumors**	-	-	-	-	0,82	0,81	2,49[1]	2,32[1]	1,59	0,85	1,48
[b]**More Aggressive Tumors**	2,63[1]	2,16[1]	0,91	3,17[1]	-	-	-	-	-	-	2,95

[a]: means that all tumors were normalized with non-tumoral tissue pool
[b]: means that more and less aggressive tumors were normalized with the corresponding non-tumoral tissue pools
[1]: means overexpression (≥ 2-fold)

Table 2 - Association between *CAV1* gene expression and clinicopathological findings and disease outcome.

Clinicopathological features	N° of cases	*CAV1* Expression score (log_2)		P value
		< 2.0	≥ 2.0	
Tumor location *				
Tongue	12	9	3	**0.338**$^{\phi}$
Floor of mouth	20	18	2	
pTNM classification				
T1/2 N+	14	13	1	0.192§
T3/4 N0	19	14	5	
Pathological grade				
Well differentiated	15	13	2	1.00$^{\phi}$
Moderately differentiated	17	14	3	
Lymphatic invasion (LI)*				
LI-	20	17	3	1.00$^{\phi}$
LI+	11	9	2	
Blood invasion (BI)*				
BI-	30	25	5	1.00$^{\phi}$
BI+	2	2	0	
Perineural invasion (PI)*				
PI-	14	12	2	1.00$^{\phi}$
PI+	18	15	3	
Survival*				
Alive	22	3	19	0.731†
Dead of index cancer	10	2	8	

* Missing data, $^{\phi}$ Fisher's exact test, † Log-rank test, § Mann-Whitney

Table 3 - Association between Cav-1 protein localization in tumoral epithelia and stromal compartments and clinicopathological findings and disease outcome.

Clinicopathological features	N° of cases	Cav-1 Expression (Tumoral Epithelia Cells)		P value	N° of cases	Cav-1 Expression (Tumoral Stroma Cells)		P value
		Negative (-/+)	Positive (++/+++)			Negative (-/+)	Positive (++/+++)	
Tumor location								
Tongue	8	4	4	0.648ϕ	8	4	4	0.356ϕ
Floor of mouth	12	4	8		12	3	9	
T classification								
T1/2	14	7	7	0.325ϕ	14	7	7	**0.051ϕ**
T3/4	6	1	5		6	0	6	
N classification								
N0	7	2	5	0.642ϕ	7	2	5	1.00ϕ
N+	13	6	7		20	7	13	
Pathological grade								
Well differentiated	7	3	4	1.00ϕ	8	3	5	1.00ϕ
Moderately differentiated	13	5	8		12	4	8	
Lymphatic invasion (LI)*								
LI-	3	2	1	0.506ϕ	3	1	2	1.00ϕ
LI+	11	3	8		11	5	6	
Blood invasion (BI)*								
BI-	2	1	1	1.00ϕ	2	0	2	0.515ϕ
BI+	15	6	9		15	6	9	
Perineural invasion (PI)								
PI-	7	1	6	0.151ϕ	7	2	5	0.637ϕ
PI+	11	6	5		11	5	6	
Survival								
Alive	11	5	6	0.949†	11	3	8	0.708†
Dead of index cancer	5	2	3		5	3	2	
Disease x Health								
Tumor	20	8	12	0.2451	20	7	13	**0.050ϕ**
Non-tumoral	10	7	3		10	8	2	

* Missing data, ϕ Fisher's exact test, † Log-rank test

Figure 1 - Hierarchical cluster diagram of CAV-1 gene expression in OSCC samples. Gene expression levels in non-tumoral tissues were used as baseline. Data are visualized colorimetrically with heat plots, "red" representing elevated gene expression and "green" decreased gene expression. (A) Tumor vs non-tumoral tissue; (B) Less aggressive vs more aggressive tumors. Relative expression ratio (log10) of CAV-1 mRNA expression analysis by qRT-PCR in OSCC samples. Housekeeping gene: HPRT1. Differential gene expression in Tumor vs non-tumoral tissue (B), and less vs more aggressive tumor (C). Overall survival of OSCC patients according to positive and negative Caveolin-1 gene expression.

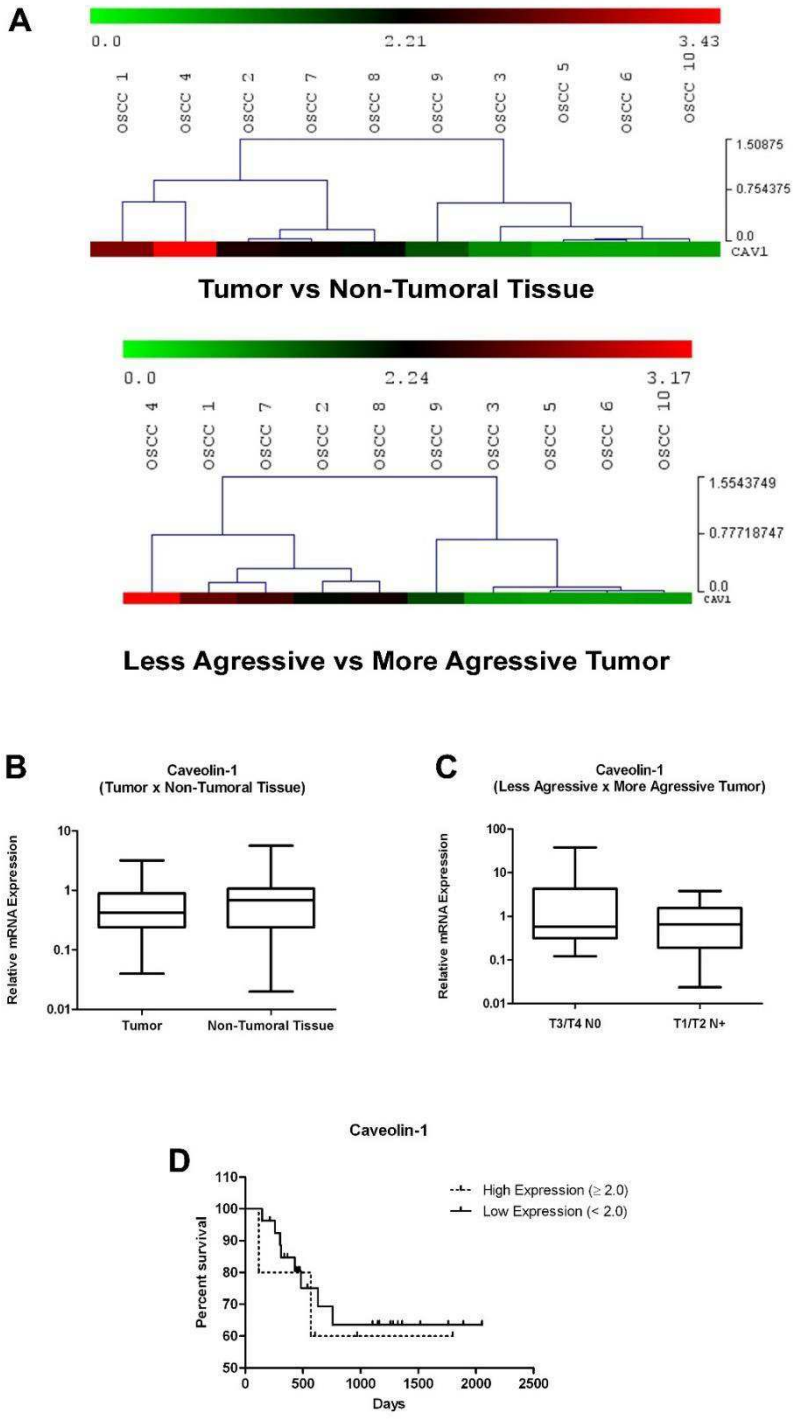


Figure 2. Immunoexpression of Caveolin-1 in OSCC and non-tumoral tissue. Caveolin-1 shows cytoplasmatic immunoexpression in matched non-tumoral tissue in basal /suprabasal layers (A), intense membranous / cytoplasmic immunoexpression in Epithelium (B and C) and Stroma (C and D). Overall survival of OSCC patients according to positive and negative Caveolin-1 in tumoral (E) and stromal (F) immunoexpression.

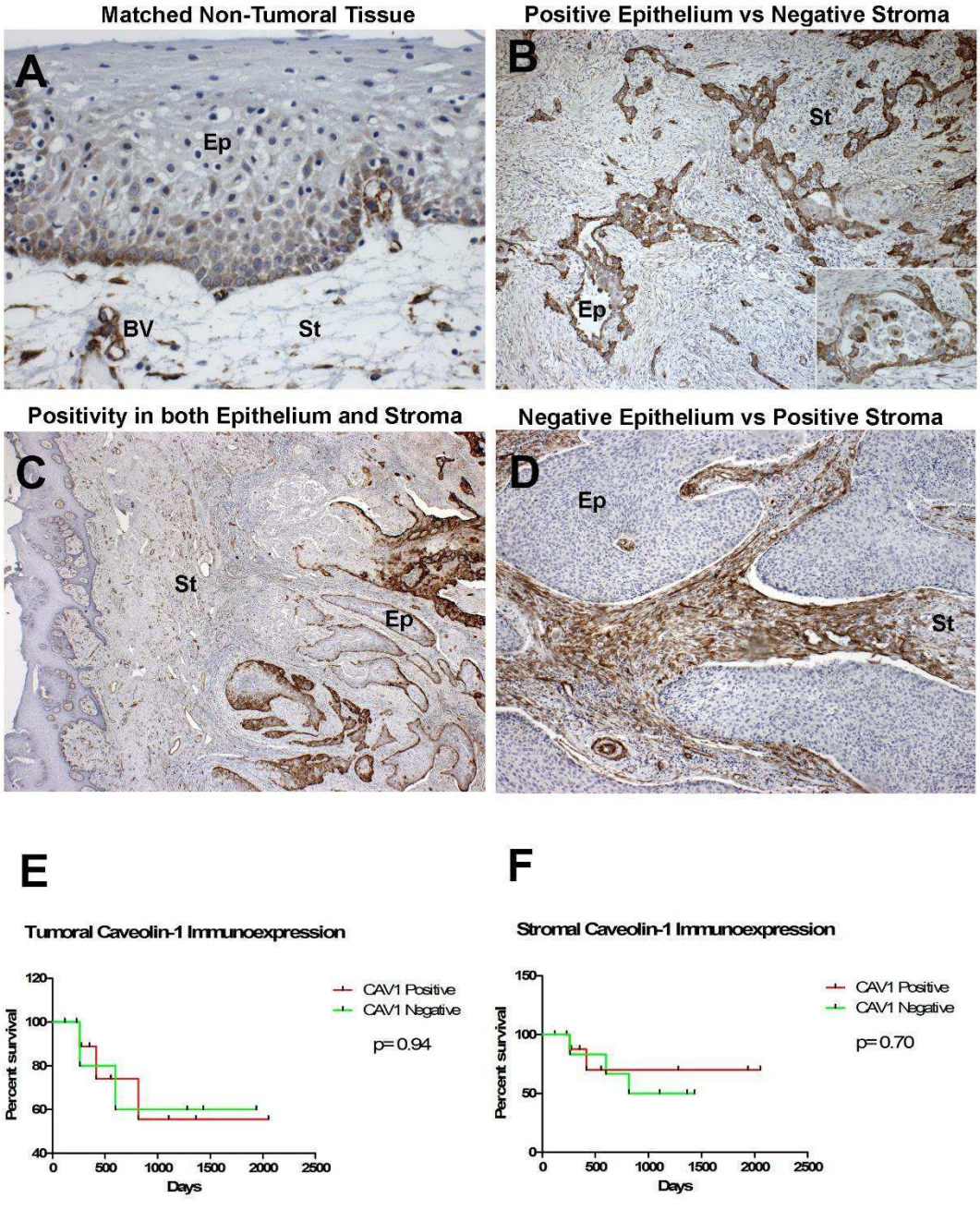


Figure 3– siRNA CAV1 test analysis. Analysis of relative mRNA expression ratio of CAV1 by qRT-PCR in OTSCC cell lines SCC-25 (A) and HSC-3 (C) under different silencers for *CAV1*. Analysis of caveolin-1 expression by Western Blotting from OTSCC cell lines SCC-25 (B) and HSC-3 (D) under different silencers for *CAV1*.

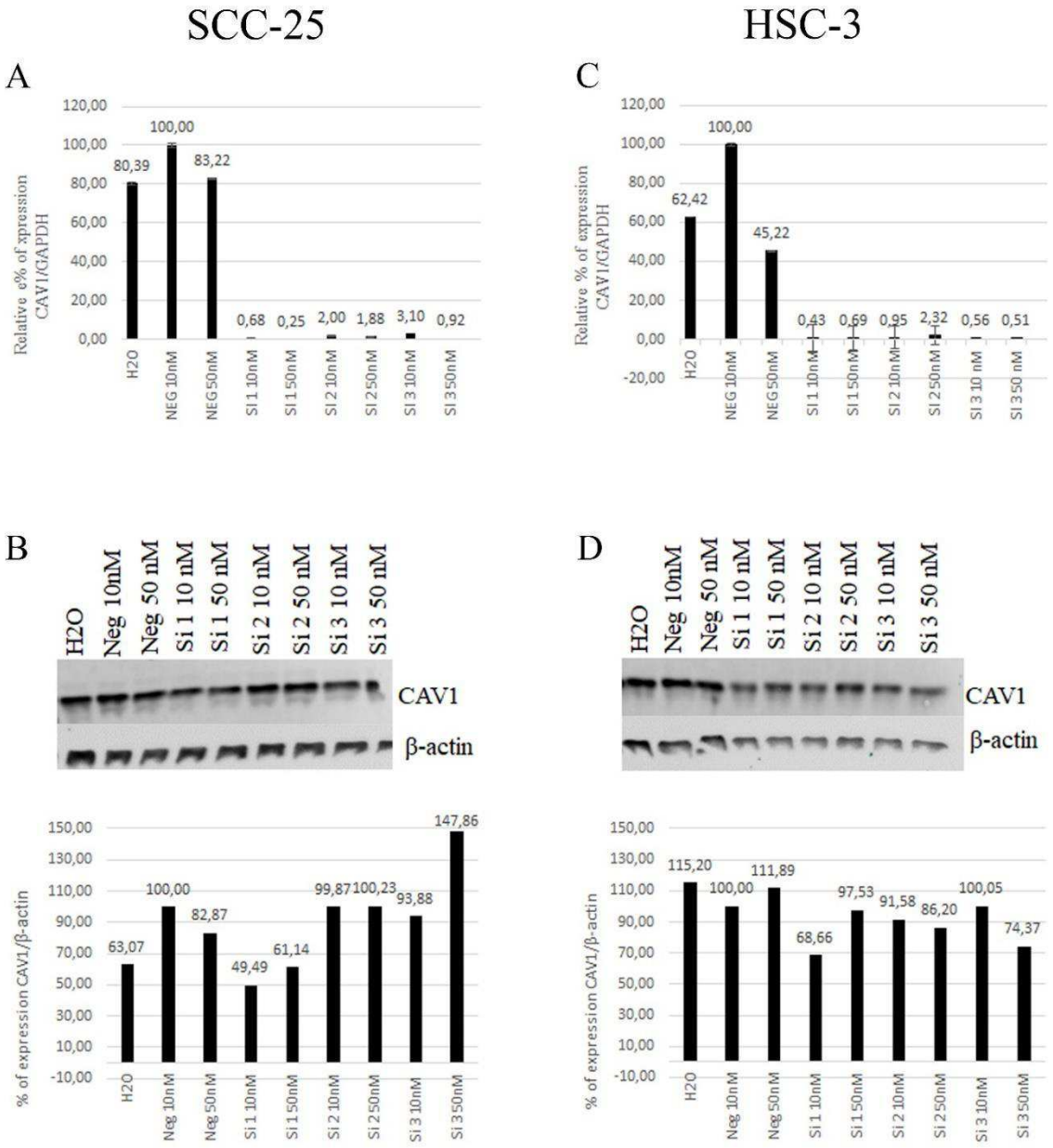


Figure 4: Effect of siCAV1 in cell viability and membrane fluidity. Analysis of % of viable cells by Alamar Blue assay after siCAV1 in SCC-25 cells (A) and in HSC-3 cells (B). Evaluation of membrande fluidity by fluorescence assay of Membrane Fluidity kit in SCC-25 (C) and HSC-3 cells after siCAV1. (* p <0.05, *** p <0.001; Error bars represent the standard deviation of the experimental triplicate).

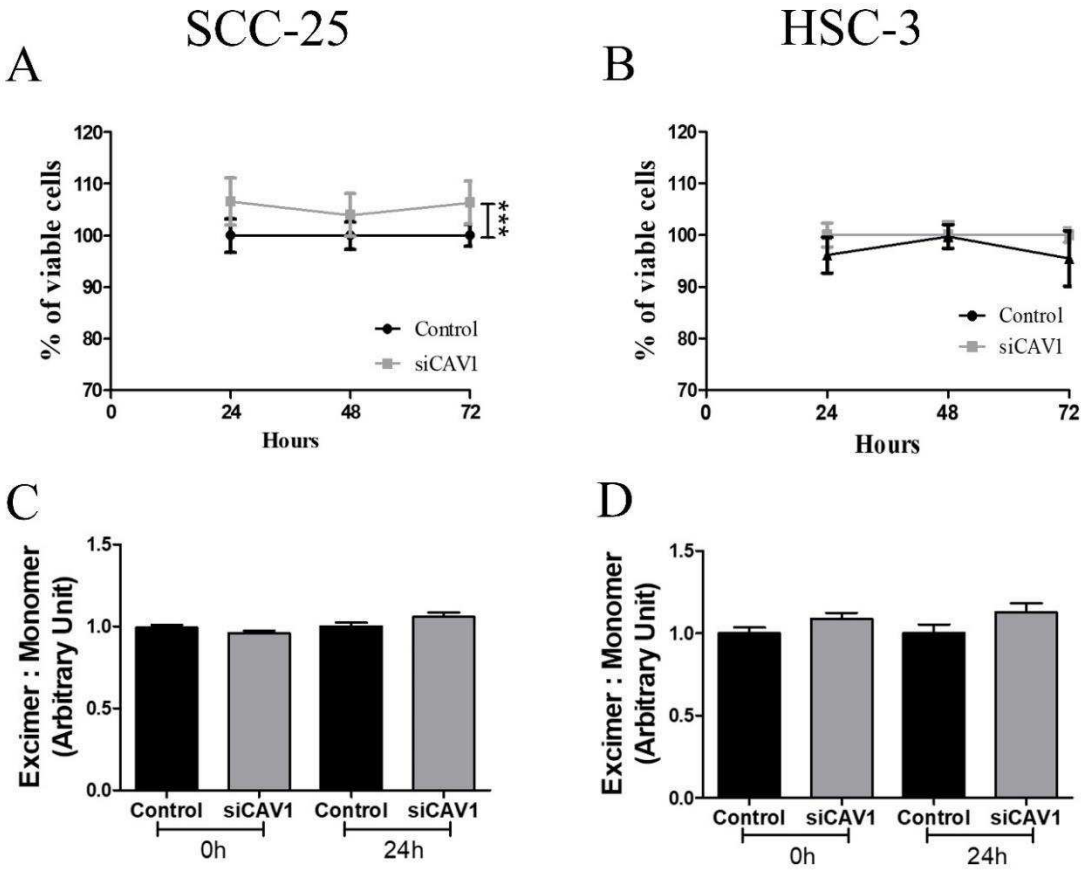


Figure 5: Effect of siCAV1 in gene and protein expression. Analysis of gene expression by qRT-PCR in SCC-25 cells (A) and in HSC-3 cells (B). Experiments of Western Blotting for evaluation of protein expression of EMT markers in SCC-25 cells (C) and also in HSC-3 cells (D). (* p <0.05; Error bars represent the standard deviation of the experimental triplicate).

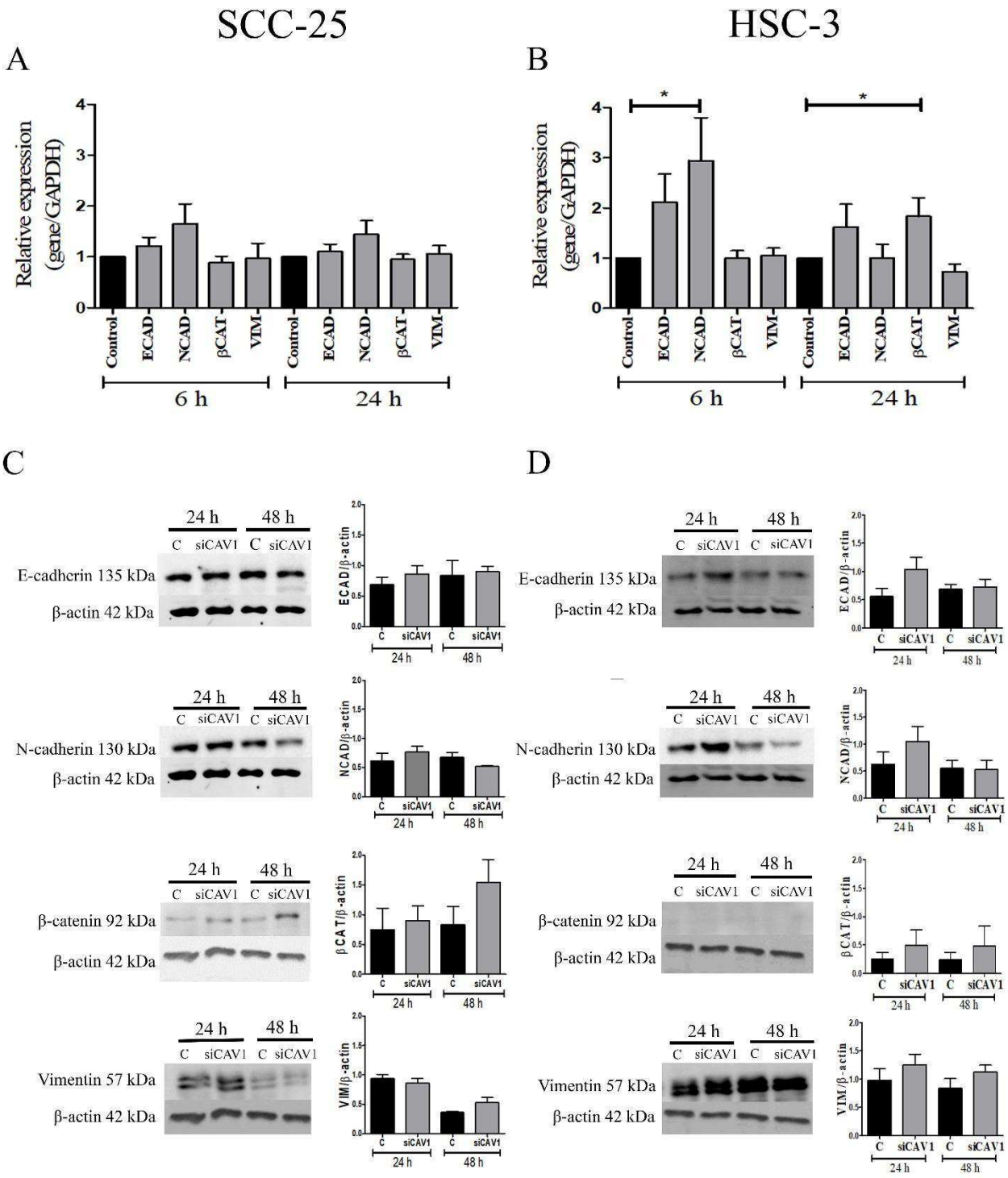


Figure 6: Effect of siCAV1 in cell migration and invasion. Migration assay was evaluated by wound healing assay on myogel in both SCC-25 (A,B) and HSC-3 (C, D) cells after siCAV1. Wound healing assay was performed in myogel/fibrinogen for evaluating the effect of silencing of CAV1 on invasion capacity of SCC-25 (E, F) and HSC-3 (G, H) cells (* p <0.05, ** p <0.01, *** p <0.001; Error bars represent the standard deviation of the experimental triplicate).

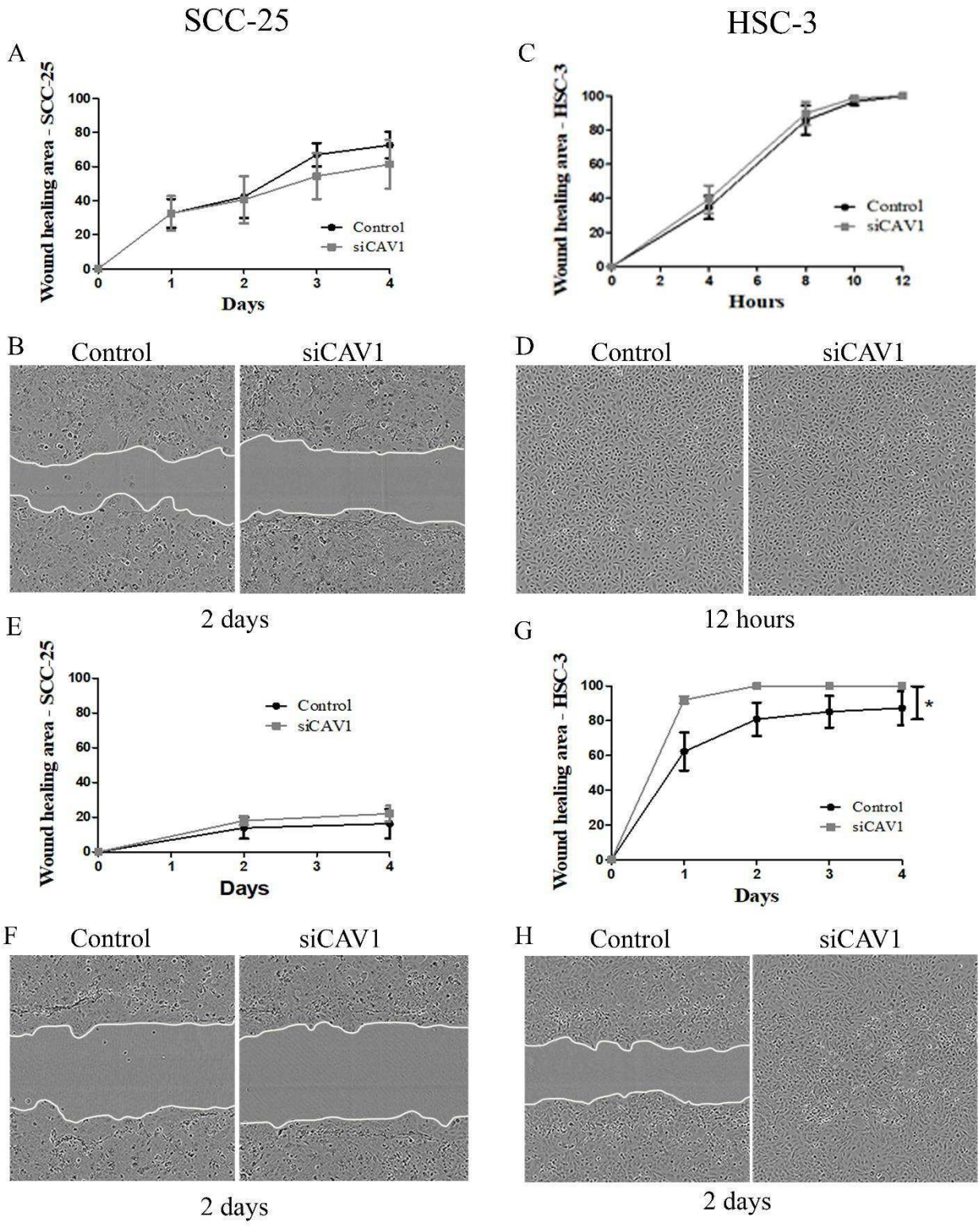
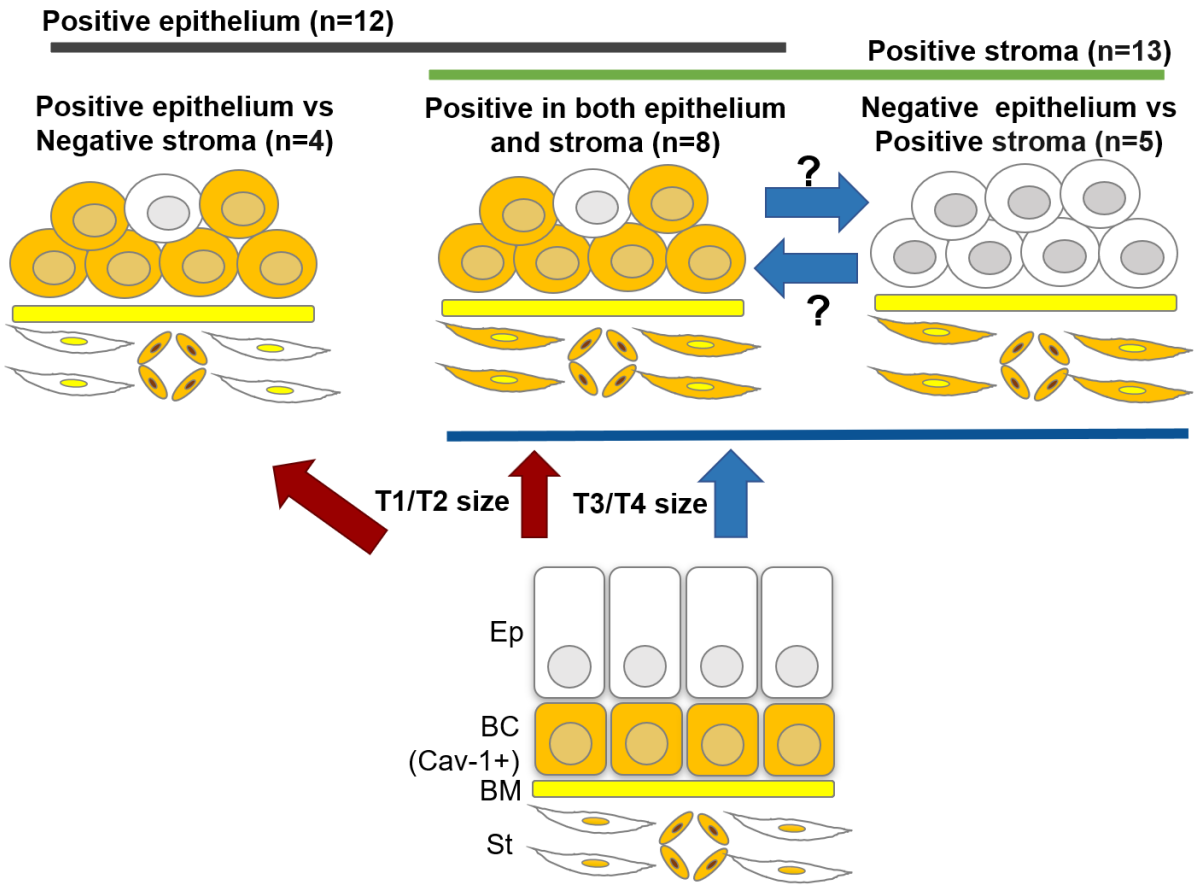


Figure 7: Different caveolin-1 immunoexpression profiles identified in OSCC samples. Immunopositivity in epithelium and negativity in stroma (1), positivity in both (2) and negativity in epithelium and positivity in stroma (3). Stromal immunostaining of caveolin-1 was associated with greater tumor size, and negatively correlated to tumoral cells immunostaining.



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