ANA ELIZIA MASCARENHAS MARQUES

NOVOS ALVOS TERAPÊUTICOS NO TRATAMENTO DO CÂNCER DE CABEÇA E PESCOÇO

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Tese apresentada como requisito parcial para a obtenção do título de Doutor em Ciências da Saúde pelo Programa de Pós-Graduação em ciências da Saúde da Universidade de Brasília.

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Coorientadora: Profa. Dra. Cristiane Helena Squarize

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Dedico este trabalho a todos que lograram comigo esta jornada,
especialmente aos meus amados pais!

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RESUMO

O campo em evolução da medicina personalizada desempenha um papel cada vez mais relevante na prevenção, diagnóstico, prognóstico e terapêutica do câncer. Diversas vias de sinalização celular, que são fundamentais na regulação do metabolismo das células, encontram-se desreguladas no câncer e estão diretamente relacionadas ao desenvolvimento, crescimento, proliferação e metástase tumoral. Dentre elas, a via de sinalização PI3K-AKT-mTOR encontra-se frequentemente alterada em várias malignidades e foi constatado ser a via mais desregulada no carcinoma de células escamosas de cabeça e pescoço (HNSCC). Além disso, evidências científicas sugerem que modificações epigenéticas estão frequentemente envolvidas na carcinogênese, progressão tumoral e resistência à terapia de cabeça e pescoço. O objetivo deste estudo foi analisar por meio da técnica de imuno-histoquímica, o perfil de expressão das proteínas da via PI3K-AKT-mTOR em pacientes com HNSCC, e relacionar os níveis de expressão proteica com características clínico-patológicas dos pacientes, bem como avaliar, *in vitro*, o perfil de expressão dessa via em linhagens celulares de HNSCC. Simultaneamente, realizou-se um estudo de revisão sistemática, a fim de verificar se a via do mTOR está superexpressa em amostras de pacientes com HNSCC, e se a superexpressão está associada a um pior prognóstico dos pacientes. Ademais, objetivou-se investigar pela primeira vez, os efeitos *in vitro* do Entinostat, um inibidor de histona desacetilase (HDAC), em linhagens celulares de câncer oral. A revisão sistemática constatou que proteínas da via do mTOR estão superexpressas no HNSCC, e que a alta expressão está relacionada à redução da sobrevida total (OS) e da sobrevida livre de doença (DFS) dos pacientes. A análise imuno-histoquímica observou que as proteínas da via PI3K-AKT-mTOR estão superexpressas em pacientes com HNSCC e, inversamente, a proteína PTEN, inibidora da via, foi encontrada em baixa expressão na maioria dos casos. O estudo *in vitro* também demonstrou alta expressão gênica e proteica da via. Na análise clínico-patológica, observou-se significativa associação entre a expressão de AKT e o gênero, como também entre a expressão de AKT e a expressão de PTEN. O estudo com Entinostat demonstrou que esta droga é citotóxica para as células de câncer oral, por diminuir a proliferação e a viabilidade celular. O Entinostat induziu a interrupção do ciclo celular, o aumento da geração de espécies oxigênio reativas (ROS), reduziu os níveis quantitativos de células-tronco do câncer, inibiu HDAC e aumentou os níveis de expressão de proteínas histonas acetiladas. Foi demonstrado ainda que esta droga levou ao aumento da proteína supressora p21, importante reguladora do ciclo celular. Concluiu-se que a via PI3K-AKT-mTOR apresenta-se superexpressa no HNSCC, consistindo em um importante alvo na terapia desse tipo de câncer. Concluiu-se, também, que o Entinostat é eficaz na redução de células tumorais orais, sugerindo constituir um agente terapêutico promissor no câncer oral.

Palavras-chave: medicina personalizada; câncer de cabeça e pescoço; revisão sistemática; via do mTOR; via PI3K-AKT-mTOR; HDAC; inibidor de HDAC; Entinostat.

ABSTRACT

The evolving field of personalized medicine plays an increasingly important role in cancer prevention, diagnosis, prognosis and therapy. Several cell signaling pathways, which are fundamental in regulating cell metabolism, are deregulated in cancer and are directly related to tumor development, growth, proliferation and metastasis. Among them, the PI3K-AKT-mTOR signaling pathway is frequently altered in several malignancies and was found to be the most unregulated pathway in the head and neck squamous cell carcinoma (HNSCC). In addition, scientific evidence suggests that epigenetic modifications are often involved in carcinogenesis, tumor progression, and resistance to head and neck therapy. The aim of this study was to analyze the protein expression profile of the PI3K-AKT-mTOR pathway by the immunohistochemistry technique in patients with HNSCC, and to relate the levels of protein expression with clinicopathological characteristics of the patients, as well as to evaluate, *in vitro*, the expression profile of this pathway in HNSCC cell lines. Simultaneously, a systematic review study was performed to verify if the mTOR pathway is overexpressed in samples from HNSCC patients, and whether overexpression is associated with a poorer prognosis of patients. In addition, the objective of this study was to investigate for the first time the *in vitro* effects of Entinostat, a histone deacetylase inhibitor (HDAC), on oral cancer cell lines. The systematic review found that mTOR pathway proteins are overexpressed in HNSCC, and that high expression is related to the reduction of patients' overall survival (OS) and disease-free survival (DFS). Immunohistochemical analysis observed that PI3K-AKT-mTOR pathway proteins are overexpressed in HNSCC patients and, conversely, the PTEN protein, pathway inhibitor, was found to be poorly expressed in most cases. The *in vitro* study also demonstrated high gene and protein expression of the pathway. In clinicopathological analysis, a significant association between AKT expression and gender was observed, as well as between AKT expression and PTEN expression. The study with Entinostat has shown that this drug is cytotoxic to oral cancer cells by reducing proliferation and cell viability. Entinostat induced cell cycle disruption, increased generation of reactive oxygen species (ROS), reduced quantitative levels of cancer stem cells, inhibited HDAC, and increased levels of acetylated histone protein expression. It has also been shown that this drug led to the increase of suppressor protein p21, an important regulator of the cell cycle. It was concluded that the PI3K-AKT-mTOR pathway is overexpressed in HNSCC, constituting an important target in the therapy of this type of cancer. It was also concluded that Entinostat is effective in reducing oral tumor cells, suggesting that it is a promising therapeutic agent in oral cancer.

Keywords: personalized medicine; head and neck cancer; systematic review; mTOR pathway; PI3K-AKT-mTOR pathway; HDAC; HDAC inhibitor; Entinostat.

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1 INTRODUÇÃO

A medicina personalizada é uma recente e interessante abordagem na conduta médica e nas indústrias de saúde. É um conceito que tem o potencial de transformar intervenções médicas, fornecendo estratégias terapêuticas efetivas e personalizadas com base no perfil genômico, epigenômico e proteômico do indivíduo, atentando-se à condição particular do paciente. O poder da medicina personalizada reside não só no tratamento, mas também na prevenção. A utilização crescente da estratificação molecular de pacientes, por exemplo, analisando mutações que podem causar resistência ou sensibilidade a certos tratamentos, ainda fornecerá aos profissionais de saúde evidências claras sobre as quais fundamentarão estratégias de tratamento para os pacientes individualmente. Com isso, haverá diminuição ou ausência dos efeitos adversos que ocorrem em resposta aos tratamentos decorrentes de métodos inespecíficos de diagnóstico e prescrição (Vogenberg et al., 2010).

Na oncologia, a medicina personalizada é particularmente importante, pois apresenta maior ênfase na prevenção de toxicidades significativas a curto prazo e implicações funcionais a longo prazo, associadas a estratégias de tratamento cirúrgico, quimioterápico e/ou radioterápico. A seleção apropriada de pacientes ao tratamento para maximizar a eficácia e minimizar a toxicidade, tem sido uma parte fundamental da prática clínica de rotina, mas até recentemente os clínicos tiveram ferramentas limitadas para determinar quais pacientes se beneficiarão e quais podem sofrer toxicidades evitáveis. Importantes descobertas para o câncer no campo da medicina personalizada, incluindo o reconhecimento de biomarcadores prognósticos e preditivos, que conferem a capacidade de direcionar os tratamentos para os pacientes com maior probabilidade de benefício, estão melhorando a sobrevida e estão se tornando, rapidamente, parte fundamental da prática clínica de rotina (Jackson e Chester, 2015).

O carcinoma de células escamosas, também denominado no Brasil como espinocelular e ainda epidermóide, é o tipo mais comum de malignidade decorrente das células epiteliais da região de cabeça e pescoço (Dahiya e Dhankhar, 2016). O carcinoma de células escamosas de cabeça e pescoço (HNSCC) é o sétimo câncer mais comum e a oitava principal causa de morte por câncer em todo o mundo. Com grande preocupação para a saúde pública, frequentemente apresentando-se como doença localmente avançada, o HNSCC desenvolve-se na cavidade oral, laringe e

faringe, afetando cerca de 600.000 pacientes por ano, dos quais aproximadamente 300.000 mortes são relatadas (Ferlay et al., 2015; Shield et al., 2017). A taxa de sobrevida global (OS) para esta neoplasia melhorou nas últimas décadas, mas ainda difere dependendo da sub-região anatômica de HNSCC (Pulte e Brenner, 2010). Nos países em desenvolvimento, a sobrevida para pacientes com esses tumores continua menor que em países desenvolvidos (Wong et al., 2015). Segundo o Instituto Nacional de Câncer (INCA), para o corrente ano de 2018 são estimados no Brasil 11.200 novos casos de câncer da cavidade oral em homens e 3.500 em mulheres. Para o câncer de laringe são estimados 6.390 novos casos em homens e 1.280 em mulheres (INCA, 2018).

Os pacientes com HNSCC possuem grande chance de recorrência e de segundo câncer primário envolvendo particularmente as áreas de cabeça e pescoço, pulmão e esôfago (Jégu et al., 2013). Os principais fatores de risco para o HNSCC incluem o consumo de tabaco e álcool, envolvidos em 75% dos casos, e a infecção pelo papilomavírus humano (HPV) de alto risco, associada a 40-60% dos cânceres orofaríngeos e mais de 20% de todos os casos de HNSCC (Leemans et al., 2011; Suh et al., 2014, Thavaraj et al., 2011). Outros fatores do estilo de vida, como dieta, uso do mate e atividade física foram associados com o prognóstico desses cânceres (Giraldi et al., 2017; Nicolotti et al., 2011; Duffy et al., 2009; Mello et al., 2018). Recentemente, Mello e colaboradores (2018) em uma revisão sistemática, mostraram que de acordo com dados publicados, independentemente da temperatura, o consumo de mate aumentou significativamente as chances de ocorrência de câncer do trato aerodigestivo superior (UADT). Além disso, o status socioeconômico pode estar associado à sobrevida em uma análise univariada. No entanto, o efeito desapareceu após ter considerado idade, gênero, estádio TNM, tabagismo e etilismo em uma análise multivariada (Chu et al., 2016).

O tratamento padrão para HNSCC é uma combinação de cirurgia, radioterapia (RT) e quimioterapia (CT), já que a maioria dos casos de HNSCC apresenta estadiamento avançado no momento do diagnóstico (Kalu e Johnson, 2017). A taxa de sobrevivência de cinco anos de 40-50% é relativamente baixa apesar dos avanços das técnicas cirúrgicas, CT e RT (Sharma et al., 2016). Infelizmente, há uma incapacidade de intensificar ainda mais a terapia atual devido à toxicidade e morbidade inaceitáveis (Li et al., 2014). Tendo em conta a alta taxa de recaída e as

opções terapêuticas limitadas, é de extrema importância investigar os mecanismos moleculares de resistência do HNSCC.

Recentes progressos em biologia molecular e pesquisa translacional iniciaram uma era de medicina personalizada na oncologia clínica de cabeça e pescoço. As informações definidas pela análise de biomarcadores em tumores é indispensável para a administração de agentes alvo moleculares (Yokota, 2014). Avanços no sequenciamento de nova geração, terapias específicas e ensaios de medicina de precisão estão expandindo as opções de tratamento para o câncer de cabeça e pescoço. Portanto, é importante um maior conhecimento sobre esse campo em rápida evolução (Birkeland et al., 2016).

A via de sinalização do receptor do fator de crescimento epidérmico (EGFR) é um alvo terapêutico importante no HNSCC. O anticorpo monoclonal anti-EGFR (mAb), Cetuximab (Cmab), é o único agente alvo para o tratamento do HNSCC aprovado pela Agência de Alimentos e Medicamentos (FDA), com taxas de resposta global como agente único de 10% a 13%. A superexpressão de EGFR varia de 40% a 60% no HNSCC (Colevas, 2006; Szabó et al., 2011). Embora vários biomarcadores, incluindo mutações do gene *KRAS,* tenham sido estabelecidos como potenciais preditivos da eficácia de resposta ao Cetuximab no câncer colorretal (Licitra et al., 2011), pouco ainda se sabe sobre os marcadores preditivos desse anticorpo no câncer de cabeça e pescoço. Eficazes marcadores preditivos e prognósticos são necessários para promover o uso clínico apropriado do Cetuximab e para determinar os fenótipos malignos no câncer de cabeça e pescoço (Vermorken et al., 2014).

Recentemente, o sequenciamento genômico do HNSCC revelou uma multiplicidade e diversidade de alterações genéticas nessa malignidade. Embora moléculas específicas em grande variedade tenham sido encontradas alteradas em cada tumor individual, todas participam de vias de sinalização. Entre elas, a via PI3K-AKT-mTOR é a mais frequentemente ativada, desempenhando um papel central na iniciação e progressão do câncer (Wang et al., 2017). Mutações em PI3K (*PIK3CA*, *PIK3CB*, *PIK3CG* e *PIK3CD*) são as mais frequentes em HNSCC, encontradas em 11% a 33% dos casos e em 30% dos tumores HPV positivos (Amornphimoltham et al., 2017). Além disso, o homólogo da fosfatase e tensina (PTEN), bem conhecido pelo seu papel supressor de tumores e como potente inibidor da via PI3K-AKT-mTOR, é frequentemente mutado em múltiplos cânceres (Li et al., 1997), e apresenta

inúmeras alterações no HNSCC, incluindo redução da expressão (Giudice e Squarize, 2013; Lui et al., 2013).

A via PI3K-AKT-mTOR é uma cascata de sinalização em células de mamíferos, cujas operações coordenadoras promovem importantes atividades celulares. Essa via desempenha um papel crítico na sobrevivência e crescimento de células malignas e sua ativação tem sido foco de trabalho e esforços extensivos de investigação nas duas últimas décadas (Martelli et al., 2010). Através do controle da ativação do mTOR e seus efetores, esta via regula a tradução do mRNA que codifica proteínas pró-oncogênicas e, assim, promovem a sobrevivência de células malignas. A fosfatidilinositol 3-quinase (PI3K) é uma quinase lipídica que controla a formação de diferentes complexos de sinalização sobre a membrana das células (Carnero et al., 2008). A ativação da PI3K leva à ativação da proteína quinase-1 dependente de fosfoinositídeos (PDK1), que fosforila posteriormente a serina-treonina quinase AKT, resultando na ativação de AKT, que por sua vez fosforila múltiplos substratos, inclusive os complexos de mTOR, ocasionando a geração de sinais de proliferação e sobrevivência celular (Wang et al., 2017) (Figura 1).

O progresso substancial no descobrimento das alterações na via PI3K-AKT-mTOR e seus papéis na tumorigênese permitiu o desenvolvimento de novas moléculas alvos, com potencial para desenvolver um eficaz tratamento anticancerígeno. Dois medicamentos anticâncer aprovados, Everolimus e Temsirolimus, exemplificam a inibição direcionada de mTOR na clínica, e no geral, outros estão em desenvolvimento pré-clínico, além de serem testados em ensaios clínicos iniciais para diferentes tipos de câncer (Polivka and Janku, 2014). Os inibidores de PI3K como o PX-866 e os inibidores de mTOR, como o Everolimus e o Temsirolimus, podem representar uma abordagem terapêutica precisa para o HNSCC (Jimeno et al., 2015; Bauman et al, 2013; Grünwald et al., 2015). De fato, a inibição da via PI3K-AKT-mTOR exerce potente atividade antitumoral em sistemas experimentais com HNSCC, e ensaios clínicos têm demonstrado resultados encorajadores (Cai et al., 2017).

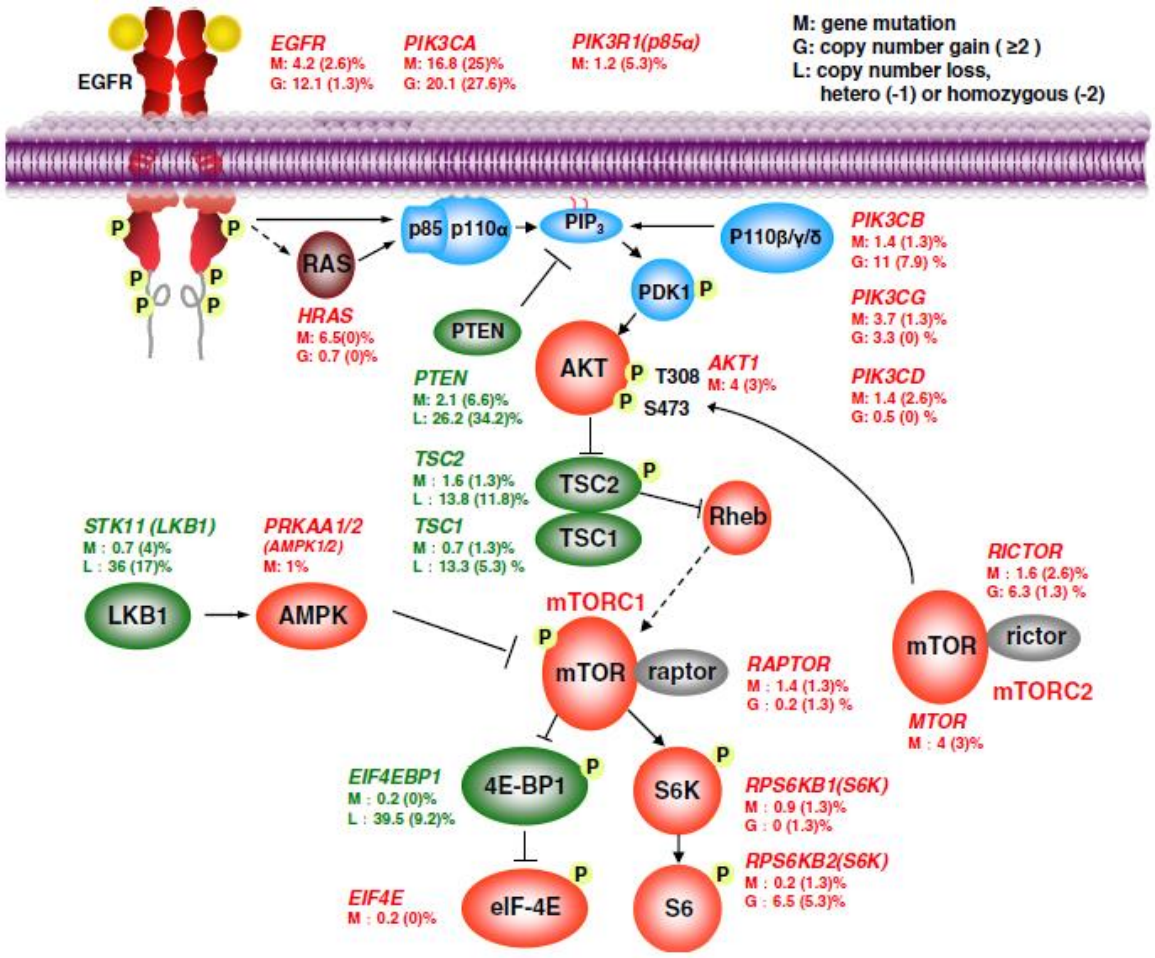


Figura 1: Representação esquemática das alterações genéticas frequentes da via de sinalização PI3K-AKT-mTOR em HNSCC. As alterações identificadas em cada gene-chave são mostradas, percentuais fora e entre parênteses representam HNSCC HPV (-) e HPV (+), respectivamente. Vermelho representa mutações e amplificações do oncogene, e verde representa mutações e perdas de cópia do gene supressor de tumor. A perda de cópia refere-se à deleção homozigótica e heterozigótica de genes (Adaptado de Wang et al., 2017).

Seguindo a abordagem em terapia molecular, a epigenética, bem como modificações pós-traducionais de proteínas estão emergindo como novos alvos atrativos para a terapia anticancerígena (Budillon et al., 2007). É bem conhecido que o genoma dos mamíferos encontra-se comprimido na cromatina, que consiste em DNA juntamente com proteínas histona e não histona. O nucleossomo, unidade básica da cromatina, é formado por 147 pb de DNA envolvido em torno de histona, um octâmero contendo duas cópias de H2A, H2B, H3 e H4 (Khorasanizadeh, 2004). Mudanças no status da cromatina de genes específicos podem levar à sua repressão ou ativação (Bannister e Kouzarides, 2011).

Histonas acetiltransferases (HATs) e histonas desacetilases (HDACs) são duas classes de enzimas que regulam a acetilação de histonas e suas atividades alteradas foram identificadas em vários tipos de câncer. Em particular, o desequilíbrio na acetilação de histonas pode levar a mudanças na estrutura da cromatina e na desregulação transcricional de genes envolvidos no controle da proliferação, progressão do ciclo celular, diferenciação e/ou apoptose (Caponigro et al., 2016). As HDACs surgiram como correpressores transcricionais cruciais em sistemas fisiológicos e patológicos diversos. Mutação e/ou expressão aberrante de várias HDACs têm sido frequentemente observadas em doenças humanas, em particular no câncer, tornando-as importantes alvos terapêuticos para muitos cânceres humanos (Figuras 2 e 3). Portanto, o padrão global de acetilação de histonas está desregulado no câncer. (Fraga et al., 2005; Haberland et al., 2009). Uma vez que as alterações epigenéticas são dinâmicas e geralmente reversíveis, a manipulação epigenética surgiu como um novo e atrativo tratamento anticancerígeno (Caponigro et al., 2016).

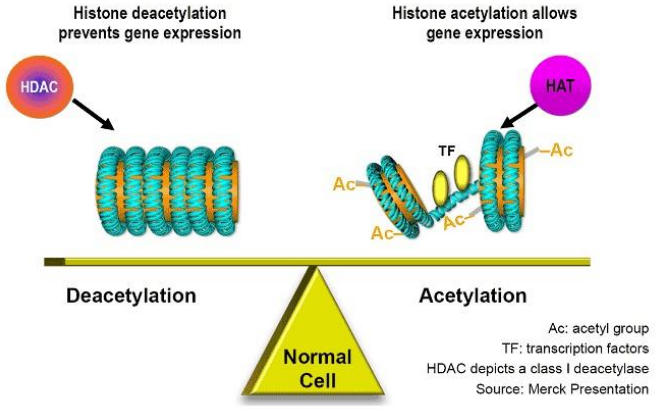


Figura 2: Representação ilustrativa da ação das enzimas HDAC e HAT na cromatina de uma célula normal. Regulação equilibrada da acetilação de histonas (Adaptado de http://www.crystalgenomics.com/en/clinical/anticancer.html?ckattempt=3).

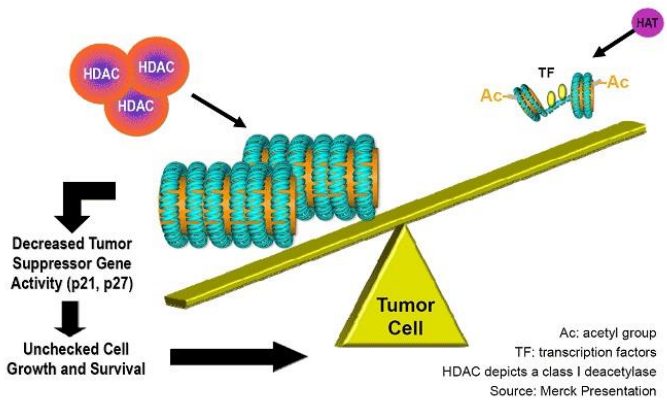


Figura 3: Representação ilustrativa da ação das enzimas HDAC e HAT na cromatina de uma célula tumoral. Aumento da desacetilação de histonas (Adaptado de http://www.crystalgenomics.com/en/clinical/anticancer.html?ckattempt=3).

Os inibidores de histona desacetilase (HDACi) são emergentes agentes antitumorais epigenéticos, com potência e especificidade variadas, cujos principais alvos são as HDACs. Eles demonstraram induzir o acúmulo de histonas acetiladas e fatores de transcrição, inibir o ciclo celular, reduzir a angiogênese, promover a diferenciação e apoptose das células, e modular a resposta imunológica. Por isso, parecem ser drogas anticancerígenas promissoras, particularmente na combinação com outras drogas anticâncerígenas e/ou radioterapia. No entanto, os mecanismos de ação permanecem incompletamente conhecidos e provavelmente envolvem múltiplos alvos moleculares, dependendo do tipo de câncer (Dokmanovic and Marks, 2005; Iglesias-Linares et al., 2010). Estudos *in vitro* mostraram efeito antiproliferativo de inibidores de HDAC em células de HNSCC. Outros estudos mostraram que uma combinação de inibidores de PI3K e AKT causou um aumento da citotoxicidade induzida por HDACi em células de HNSCC *in vitro* (Erlich et al., 2012). Os inibidores de HDAC Vorinostat, Romidepsina e o Belinostat foram aprovados para alguns linfomas de células T, e o Panobinostat para o mieloma múltiplo. Outros inibidores de HDAC estão em ensaios clínicos para o tratamento de malignidades hematológicas e sólidas. Os resultados de tais pesquisas são promissores, mas são necessárias maiores investigações (Eckschlager et al., 2017).

O presente estudo teve como objetivo central compreender a participação de algumas vias de carcinogênese na relação clinico-patológica de pacientes portadores de HNSCC, a fim de responder algumas perguntas clínicas em relação ao perfil de alterações na expressão gênica e proteica encontradas no câncer e seus efeitos na sobrevida e tratamento de pacientes com HNSCC. Além disso, visou-se investigar os efeitos *in vitro* do Entinostat, um inibidor de HDAC e novo agente terapêutico no câncer oral, com a finalidade de conhecer os mecanismos de ação da droga e sua eficácia na terapia desse tipo de câncer.

2 PROBLEMAS E HIPÓTESES

Problema 1: Considerando o papel da via do mTOR na progressão do câncer, a expressão imuno-histoquímica de proteínas da via do mTOR encontra-se elevada no HNSCC? A superexpressão da via influencia no prognóstico dos pacientes com câncer de cabeça e pescoço? O que a literatura aborda sobre isso?

Hipótese 1: Espera-se encontrar, por meio de um estudo de revisão sistemática, redução da sobrevida total (OS) e da sobrevida livre de doença (DFS) dos pacientes com câncer de cabeça e pescoço que apresentam alta expressão tumoral de proteínas da via do mTOR comparados aos pacientes com baixa expressão dessas proteínas.

Problema 2: Diversos tipos de câncer apresentam atividade desregulada de proteínas da via de sinalização PI3K-AKT-mTOR. Essas proteínas estão expressas no câncer de cabeça e pescoço de um grupo de população brasileira? Há associação entre a intensidade de expressão dessas proteínas e as condições clínico-patológicas dos pacientes com câncer de cabeça e pescoço? Há associação inversa de intensidade de expressão com a proteína supressora PTEN?

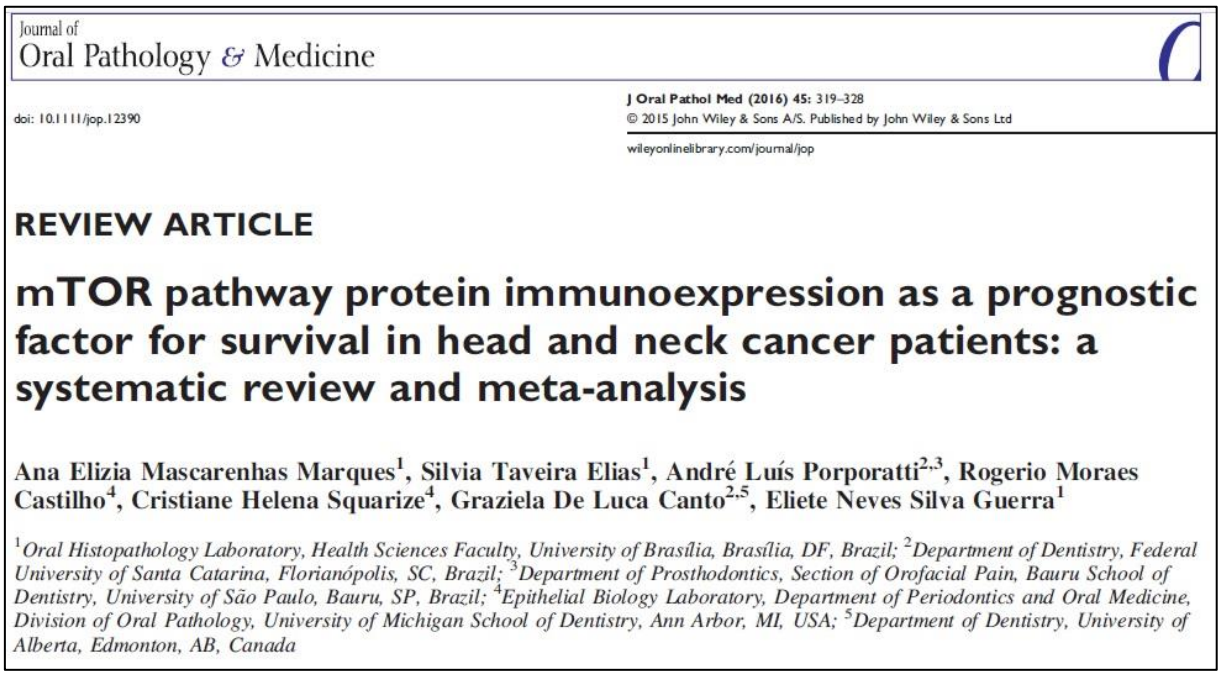
Hipótese 2: Especula-se haver superexpressão de proteínas da via PI3K-AKT-mTOR no câncer de cabeça e pescoço da população brasileira estudada, e uma relação direta entre a intensidade de expressão e o comportamento clínico-patológico de pacientes com câncer de cabeça e pescoço. Além disso, supõe-se observar expressão reduzida de PTEN inversamente à alta expressão de proteínas da via PI3k-AKT-mTOR.

Problema 3: Quais os efeitos *in vitro* do inibidor de HDAC, Entinostat, sobre a viabilidade celular, expressão de histonas e HDAC, ciclo celular, espécies reativas de oxigênio (ROS) e células-tronco, em linhagens celulares de câncer oral?

Hipótese 3: Entinostat tem o potencial de inibir a proliferação e reduzir a viabilidade celular nas células de câncer, considerada sua função citotóxica. Espera-se observar um aumento nos níveis de expressão de histonas acetiladas e alterações na expressão de proteínas reguladoras do ciclo celular. Além disso, espera-se uma interrupção do ciclo celular e aumento da produção de ROS. Espera-se, também, uma redução nos níveis quantitativos de células-tronco analisadas separadamente, após o tratamento de linhagens celulares de cavidade oral com Entinostat.

3 ARTIGOS

3.1 Manuscrito 1



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REVIEW ARTICLE

mTOR pathway protein immunoexpression as a prognostic factor for survival in head and neck cancer patients: a systematic review and meta-analysis

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Referência:

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3.2 Manuscrito 2

Clinicopathologic significance of the PI3K-AKT-mTOR pathway expression in Head and Neck Squamous Cell Carcinoma: data from Brazilian population

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ABSTRACT

Background: PI3K-AKT-mTOR signaling pathway is an important regulator of cell proliferation, survival, and motility. The gain or loss of function of proteins related to this pathway results in the neoplastic transformation in several types of cancers, making it an attractive target in oncology therapy. The objective of this study was to evaluate the immunohistochemical expression profile of the PI3K-AKT-mTOR pathway proteins in patients with head and neck squamous cell carcinoma (HNSCC) and correlate with clinicopathological variables. *In vitro* gene and protein expression of the PI3K-AKT-mTOR pathway was also verified in HNSCC cell lines.

Methods: The study involved twenty-six tissue samples from patients with HNSCC. The material was obtained from tissue removed for biopsy, fixed in formalin and immersed in paraffin. PI3K, AKT, p-mTOR and PTEN proteins expression was investigated. The analysis of immunohistochemical expression was performed by a quantitative assessment. The *in vitro* gene and protein expression evaluation was performed by real-time PCR and western blot assay, respectively, in the cell lines SCC-9 (from tongue squamous cell carcinoma) and FaDu (from hypopharynx squamous cell carcinoma).

Results: High levels of PI3K, AKT and p-mTOR were found in most HNSCC tumors. Conversely, low expression or absence of PTEN expression, suppressor protein of PI3K-AKT-mTOR, was observed in most samples. A statistically significant association ($p \leq 0.05$) was observed between AKT expression and gender, as well as between AKT expression and PTEN expression. PI3K-AKT-mTOR pathway was also overexpressed in HNSCC SCC-9 and FaDu cells.

Conclusions: The results demonstrated *in vivo* and *in vitro* high expression of the PI3K-AKT-mTOR pathway in HNSCC. The loss of PTEN expression may be as well associated with the increased expression of proteins PI3K, AKT, and mTOR.

INTRODUCTION

Squamous cell cancer is the most common malignancy of epithelial origin in the head and neck region (Dahiya and Dhankhar, 2016). Head and neck squamous cell carcinoma (HNSCC) is the seventh most common cancer and the eighth leading cause of cancer death worldwide. Regarded as a public health concern, often diagnosed as a locally advanced disease, HNSCC occurs in the oral cavity, larynx and pharynx, affecting approximately 600,000 patients per year (Ferlay et al., 2015; Shield et al., 2017). Tobacco and alcohol are the most important risk factors so far identified in HNSCC patients, and their consumption combined potentiates the risk of developing the disease (Scully and Bagan, 2009). A subgroup of HNSCC, particularly those of the oropharynx, is caused by high-risk human papillomavirus (HPV) infection (Thavaraj et al., 2011). HPV-positive and HPV-negative tumors are different entities in terms of clinicopathology and molecular biology (Leemans et al., 2011), being HPV-positivity associated with improved clinical outcome and better response to therapy (Albers et al., 2017). Alterations in p16 and p53 genes are frequently observed in head and neck cancers (Reed et al., 1996). Although decrease in p16 with consequent loss of function is frequently observed in tumors, studies report overexpression of such protein in 13% to 50% of OSCC cases (Nemes et al., 2006; Muirhead et al., 2006). This overexpression is frequently related to high-risk HPV infections and therefore, p16 expression has been used as a substitute marker for HPV presence (König et al., 2007; Lewis, 2012).

The main therapeutic modalities currently include surgery, radiation, and chemotherapy, which are nonselective treatments and may lead to associated morbidity and mortality. Cetuximab, a monoclonal antibody and epidermal growth factor receptor (EGFR) inhibitor, is the only target-specific agent approved for HNSCC,

although only 10% of HNSCC patients respond to it, and often for a short period of time (Specenier and Vermorken, 2013). Resistance to EGFR-targeted therapy may be mediated by various extracellular signaling mechanisms, including alternative receptors or constitutively active downstream components.

Aberrations in various cellular signaling pathways are instrumental in regulating cellular metabolism, tumor development, growth, proliferation, metastasis and cytoskeletal reorganization. The phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K-AKT-mTOR) pathway is crucial and intensively investigated in tumorigenesis, as well as its regulator, the phosphatase and tensin homolog (PTEN) (Engelman, 2009; Courtney et al., 2010). Efforts in uncovering PI3K-AKT-mTOR alterations and their roles in tumorigenesis have enabled the development of novel targeted molecules with potential for developing efficacious anticancer treatments (Polivka and Janku, 2014; Patel et al., 2011). However, the antitumor effects of mTOR inhibitors in HNSCC still remain unclear. Clinical studies suggest that selective inhibitors of mTOR, such as everolimus and temsirolimus, may restore sensitivity to EGFR inhibitors in resistant cell lines and may enhance the antitumor activity of Cetuximab in HNSCC (Bianco et al., 2008; Saba et al., 2014; Grünwald et al., 2014).

Understanding the genomic alterations that drive HNSCC initiation and progression instigates the development of novel precise and efficient therapeutic options. Thus, to ascertain the effects of mTOR inhibitors in HNSCC, investigating the expression levels of PI3K-AKT-mTOR pathway proteins in HNSCC is essential (Wang et al., 2017). The aim of this study was to identify and characterize the immunohistochemical expression of proteins related to the PI3K-AKT-mTOR signaling pathway in HNSCC patients, and correlate with clinicopathological parameters. In

addition, we analyzed the gene and protein expression profiles of the PI3K-AKT-mTOR pathway in HNSCC cell lines.

MATERIAL AND METHODS

PATIENTS

The present retrospective study included samples (n=26) of HNSCC patients from the central-western region of Brazil. The samples were obtained from tissues removed by incisional and excisional biopsies fixed in 10% formaldehyde and embedded in paraffin. The patients were diagnosed and treated at University Hospital of Brasília (HUB/UnB) from 2004 to 2014. Eighteen tumor samples were obtained from the oral cavity, six from the larynx and two from the oropharynx. The paraffin blocks were selected after analysis of biopsy records and collected from the archives of the HUB Anatomopathology Laboratory.

CLINICAL DATA COLLECTION

Clinical-pathological variables of the patients were identified in the archive research sector of the HUB. All patients had their medical records examined. Data regarding gender, age, location and histological grade were collected. Most patients were treated with surgery and/or radiochemotherapy. This study was approved by the Committee of Ethics in Research with Human Beings of the Faculty of Health of the University of Brasilia, Brazil (Registry Nº 410.815).

IMMUNOHISTOCHEMISTRY

Unstained 5-µm tissue sections of formalin-fixed, paraffin-embedded specimens were mounted on silanized glass slides. Immunohistochemistry was performed by the

streptavidin-biotin-peroxidase method. Tissue sections were dewaxed in xylene PA (100%) and rehydrated using a graded alcohol series. Antigen retrieval at high temperature, ranging from 95°C to 99°C, was performed in 0.002 M Citrate buffer (99.5% granular citric acid, Alfa Aesar, MA, USA), pH 6.0, in Steamer vaporizer for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (10 volume) for 15 min and non-specific protein binding was blocked with 2.5% Normal Horse Serum (Vectastain Kit, Cat. PK-7200, Vector Laboratories, CA, USA) incubated for 20 minutes at room temperature. The slides were incubated overnight at 4 °C with the primary antibodies against PI3K p110α (Rabbit mAb, Cat #4249S, Cell Signaling Technology, MA, USA) at 1:300 dilution, AKT (Rabbit mAb, Cat #4691S, Cell Signaling Technology, MA, USA) at 1:300, p-mTOR (59.Ser2448) (Mouse mAb, sc-293133, Santa Cruz Biotechnology, TX, USA) at 1:50, PTEN (Rabbit mAb, Cat. #9559S, Cell Signaling Technology, MA, USA) at 1:200, and Ki-67 (SP6 mAb, Cat. M3064, Spring Bioscience, CA, USA) at 1:50. Dilution of primary antibodies against PI3K p110α, AKT, p-mTOR and PTEN was done on 0.05 mol/L TRIS-HCL buffer (Tris (hydroxylamine) aminomethane, HCl acid) containing 0.1% Tween-20. For Ki-67, dilution was on 1% albumin buffer.

Specimens were then washed with phosphate-buffered saline (PBS, pH 7.4), incubated with secondary antibody (Vectastain Kit, Cat. PK-7200, Vector Laboratories, CA, USA) at room temperature for 20 min and stained with chromogen solution (DAB Peroxidase Substrate Kit, Cat. SK-4100, Vector Laboratories, CA, USA) from 1 to 5 min at room temperature. After washing with distilled water, the slides were counterstained with Harris Hematoxylin for 1 min. A negative control with the primary antibody substituted by PBS was included in all experiments.

IMMUNOHISTOCHEMICAL ANALYSIS

Slides were scanned and visualized using the Aperio ScanScope slide scanner (Leica Biosystems, Germany), connected to a computer with Aperio ImageScope (Leica Biosystems, Germany). For each slide, 10 fields were analyzed at 20x magnification. Nuclear and cytoplasmic markers of all antibodies were analyzed.

Quantitative analysis adapted from previous studies was performed (Putti et al., 2002; Hiraishi et al., 2006; Hsu et al., 2015), in which the percentage of positive tumor cells (PP) was classified as follows: 0, absence of stained tumor cells; 1, ≤ 25% stained cells; 2, 26 – 50% stained cells; and 3, > 50% stained cells. The staining intensity (SI) was also evaluated and classified as follows: 0, without immunostaining; 1, weak immunostaining; 2, moderate immunostaining; and 3, strong immunostaining. The immunoreactive score (IRS) was calculated by multiplying the percentage of positive tumor cells (PP, labeling 0-3) by the staining intensity (SI, labeling 0-3). Protein expression in tumors with an IRS < 4 was considered low (negative or weak) and high expression (moderate and strong) was defined by an IRS ≥ 4.

CELL LINES AND CULTURE CONDITIONS

Two immortalized human cell lines were used in the study: SCC-9, derived from tongue squamous cell carcinoma, and FaDu, derived from hypopharynx squamous cell carcinoma. All cell lines are described in the American Type Culture Collection (ATCC). Cells were incubated under ideal conditions in 5% CO2 and 37 °C.

SCC-9 cell line was cultured in Dulbelcco's Modified Eagle Medium (DMEM) culture medium supplemented with F12 medium in the proportion 1:1, 10% fetal bovine serum, penicillin and streptomycin at final concentrations of 100 ng/mL and 1 µg/mL, respectively, and hydrocortisone at 400 ng/mL. FaDu cell line was cultured in DMEM

supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin. All reagents used were purchased from Sigma Aldrich (MO, USA).

REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (REAL-TIME RT-PCR)

The expression of genes related to the PI3K-AKT-mTOR pathway in SCC-9 and FaDu cell lines was assessed through Real-Time RT-PCR. Total RNA from the cells was extracted using the guanidine/phenol/chloroform thiocyanate method as recommended by the manufacturer (TRIzol®, Invitrogen, CA, USA). Quantification and purity of total RNA samples were determined by spectrophotometry using NanoVue Plus (GE Healthcare Life Sciences, UK), in the wavelength of 260 nm and expressed in µg/µL. To eliminate possible contaminations by genomic DNA, 400 ng of total RNA were treated with 1 unit/µL of DNAse I (Sigma Aldrich, MO, USA) according to the protocol of the enzyme manufacturer. Then the cDNA was synthesized by reverse transcription (RT) reaction using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA), following manufacturer's instructions.

The sequences of forward and reverse primers used for amplification of genes of interest were obtained from Harvard PrimerBank (https://pga.mgh.harvard.edu/primerbank/) and provided by the IDT® (Integrated DNA Technologies, IA, USA). The specificity of the primers was verified *in silico* by comparing with the available sequences in the non-redundant NCBI database (National Center for Biotechnology Information) with Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). To guarantee amplification efficiency of 100% (± 10%), each pair of primers was submitted to a validation experiment consisting of a serial dilution curve (dilution factor of 5) of a cDNA sample,

containing 5 dots (in triplicate). Validation and melting curves, generated for each pair of primers by the StepOne v2.1 software (Applied Biosystems, CA, USA), are shown in Appendix 1.

The expression of *PI3KCA*, *AKT1*, *mTOR* and *PTEN* was determined by the SYBR® Green system using the PowerUp® SYBR® Green Master Mix kit (Applied Biosystems, CA, USA), following manufacturer's instructions. The final volume for all reactions was 10 ml. All qPCR experiments were performed on StepOnePlusTMReal-Time PCR Systems (Applied Biosystems, CA, USA) and data generated by the StepOne v2.1 software (Applied Biosystems, CA, USA). The gene encoding β-ACTIN, expressed constitutively, was used as endogenous control and normalizer of the reaction (reference gene). Target gene levels relative to *β-ACTIN* levels were calculated by comparative cycle threshold (Ct) method by using the formula defined as $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008). Each experiment was performed in biologic triplicate with technical duplicate for each sample. Sequences of used primers are shown in Table 1 of the supplemental material (Supplemental Table S1).

WESTERN BLOT ANALYSIS

Proteins were extracted with RIPA buffer (protease and phosphatase inhibitors added). and quantified by the Lowry method. A standard curve to each cell line was constructed with bovine serum albumin (BSA), the absorption values obtained from the protein samples were interpolated to the BSA curve, and the concentration of proteins in each sample was determined.

Samples were prepared (30µg of protein and sample buffer with betamercaptoethanol, and sufficient distilled water to complete 30µL), the proteins were denatured at a temperature of approximately 96 °C for 5 minutes and applied on

a 10% acrylamide gel. Then 30µL of sample was applied to each well and the system was subjected to a 100V current for approximately 2 hours for proper separation of proteins according to molecular weight. The proteins were transferred from the gel to a PVDF membrane at a 24V current for 2 hours. The membrane was blocked with blocking solution (5% skim milk) for 1 hour and then incubated overnight at 4°C with the primary antibodies against PI3K p110α (Rabbit mAb, Cat#4249S, Cell Signaling Technology, MA, USA) at 1:1000 dilution, AKT (Rabbit mAb, Cat#4691S, Cell Signaling Technology, MA, USA) at 1:1000, p-AKT (Ser473) (Rabbit mAb, Cat#4060S, Cell Signaling Technology, MA, USA) at 1:1000, mTOR (Rabbit mAb, Cat#2983S, Cell Signaling Technology, MA, USA) at 1:1000, p-mTOR (Ser2448) (Rabbit mAb, Cat#5536S, Cell Signaling Technology, MA, USA) at 1:1000, PTEN (Rabbit mAb, Cat#9559S, Cell Signaling Technology, MA, USA) at 1:1000, and GAPDH (constitutive) (Mouse mAb, Cat#sc47724, Santa Cruz Biotechnology, TX, USA) at 1:1000. After washing with Tris-saline buffer (0.2 M) and 0.1% Tween 20 (TBST), the membranes were incubated for 1 hour at 4°C with secondary antibody (Goat Anti-Rabbit IgG-HRP, Cat#ab6721, Abcam, MA, USA; Goat Anti-Mouse IgG-HRP, Cat#sc2005, Santa Cruz Biotechnology, TX, USA) at 1:5000. After washing, the bands were visualized using a chemiluminescence detection kit (Amersham ECL Prime, GE Healthcare Life Sciences, UK) in a chemiluminescence imager (Amersham Imager 600, GE Healthcare Life Sciences, UK). The experiment was reproduced at least three times independently.

STATISTICAL ANALYSIS

Kruskal-Wallis statistical test followed by Dunn's multiple comparison test were used to analyze the results of protein expression by immunohistochemistry. For the

analysis of the association between protein expression and clinicopathological variables, the statistical significance was determined by Fisher's Exact Test at the 5% level. All statistical analyzes and graphical representations were performed with the program GraphPad version 5.0 for Windows. Values with $p \leq 0.05$ were considered statistically significant.

RESULTS

A total of 26 patients were involved in this study. Patients were aged between 25 and 77, with a media age of 59. Nineteen patients (73%) were male and 7 (27%) were female. Eighteen (69%) tumor samples were obtained from the oral cavity, 6 (23%) from the larynx and 2 (8%) from the oropharynx. Among the samples of the oral cavity, 9 (50%) were tongue tumors, 4 (22%) lip, 3 (17%) buccal floor and 2 (11%) gum. As to the histological degree of differentiation (Anneroth et al., 1987), 15 (58%) tumors were moderately differentiated and 11 (42%) were well differentiated.

HIGH EXPRESSION OF PI3K-AKT-mTOR PATHWAY PROTEINS IN HNSCC

High and low expression patterns of PI3K-AKT-mTOR pathway proteins were observed in the HNSCC samples. Protein expression was observed predominantly in the cell cytoplasm. Some cases showed nuclear and plasma membrane marking. Representative images of strong, moderate and weak marking intensity found for the PI3K, AKT and p-mTOR proteins are presented in Figure 1. Strong marking intensity of PTEN protein was not observed in any of the cases (Figure 2).

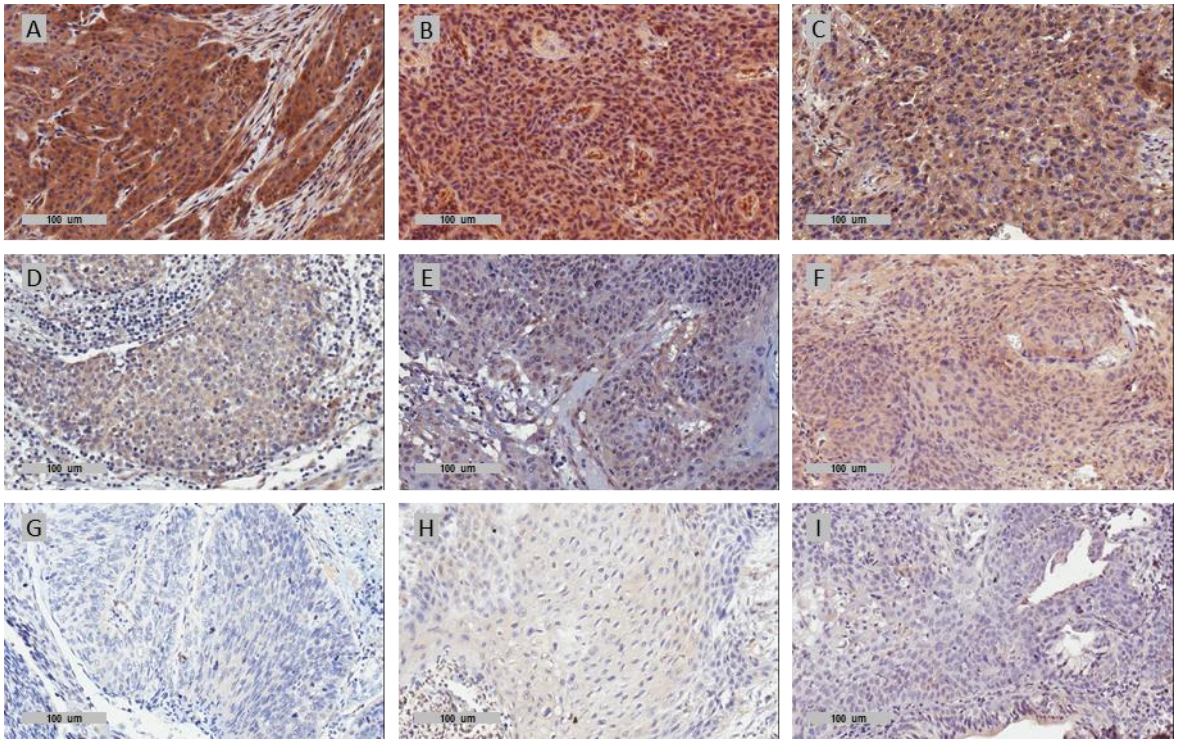


Figure 1: Representative images of the immunohistochemical marking intensity of PI3K-AKT-mTOR pathway proteins in the HNSCC samples. **A, B and C -** Representative images showing strong marking intensity of PI3K, AKT and p-mTOR, respectively. **D, E and F -** Representative images showing moderate marking intensity of PI3K, AKT and p-mTOR, respectively. **G, H and I -** Representative images showing weak marking intensity of PI3K, AKT and p-mTOR, respectively.

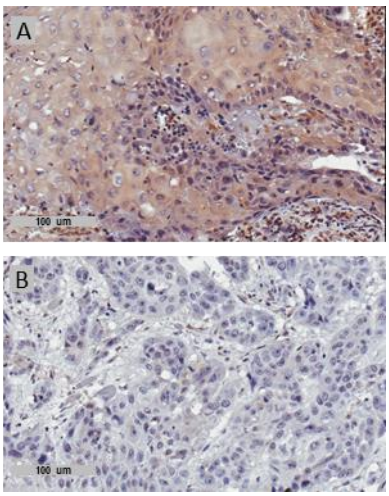
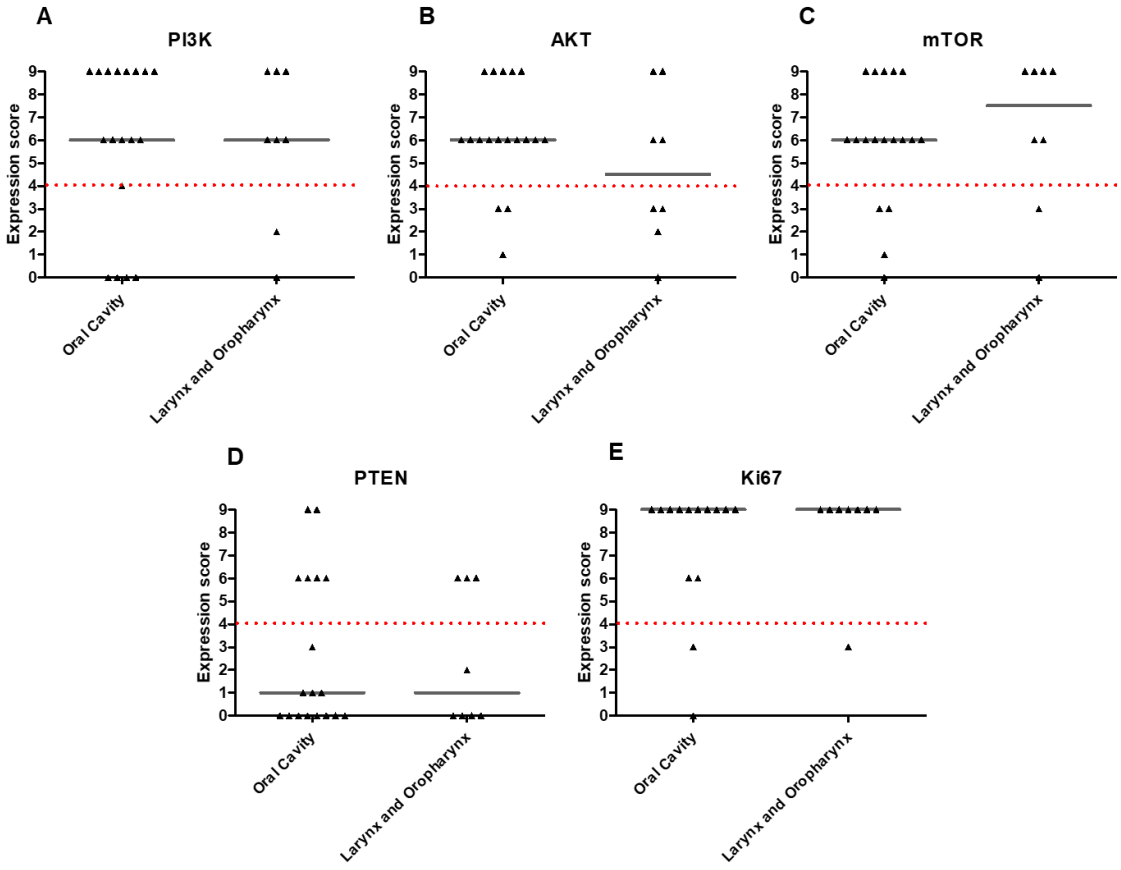


Figure 2: Representative images of the immunohistochemical marking intensity of PTEN protein in the HNSCC samples. **A -** Representative image showing moderate marking intensity of PTEN. **B -** Representative images showing weak marking intensity of PTEN.

Immunohistochemical analysis revealed high expression (IRS ≥ 4) of PI3K and AKT in 20 (77%) and 19 (73%) HNSCC samples, respectively. Fourteen and 15 tumors of the oral cavity presented high PI3K (n = 14, 77.8%) and AKT (n = 15, 83.3%) expression. A total of 6 tumors of larynx and oropharynx presented high PI3K expression (n = 6, 75%) and 4 presented high AKT expression (n = 4, 50%). In Figures 3A and 3B, representative graphs of the expression score of these proteins are presented. Regarding p-mTOR protein, high expression was observed in 20 (77%) cases, namely in 14 oral cavity cases (n = 14, 77.8%) and in most cases of larynx and oropharynx (n = 6, 75%), as demonstrated in Figure 3C. Considering PTEN, high expression was observed only in 9 cases (35%): 6 oral cavity cases (33.3%) and 3 of larynx and oropharynx (37.5%) (Figure 3D), being poorly expressed in most cases. Ki67 cell proliferation marker protein was highly expressed in 23 (88%) samples (Figure 3E). There was no statistically significant association between the PI3K-AKT-mTOR pathway expression pattern and the PTEN expression pattern. However, a statistically significant association was observed between the high expression of Ki-67 and the low expression of PTEN (Figures 4A and 4B).

The graphs in Figures 4A and 4B represent the distribution of the expression score of all evaluated cases of oral cavity and larynx and oropharynx, respectively, for each protein.



Location/High expression	PI3K	AKT	p-mTOR	PTEN	Ki-67
Oral cavity	20/26	19/26	20/26	9/26	23/26
Larynx	(77)	(73)	(77)	(35)	(88)
Oropharynx					
Oral cavity	14/18	15/18	14/18	6/18	16/18
	(77.8)	(83.3)	(77.8)	(33.3)	(89%)
Larynx + oropharynx	6/8	4/8	6/8	3/8	7/8
	(75)	(50)	(75)	(37.5)	(87.5%)

Figure 3: PI3K-AKT-mTOR pathway is overexpressed in the HNSCC. **A, B and C -** High expression score (IR ≥ 4) of PI3K, AKT and p-mTOR proteins in the oral cavity, larynx and oropharynx samples. **D -** Low expression score (IR < 4) of PTEN protein in the oral cavity, larynx and oropharynx samples. **E -** Ki-67 protein is overexpressed in HNSCC samples. *The red dashed line represents the value 4 of the expression score. *The gray line represents the mean of the expression score of samples.

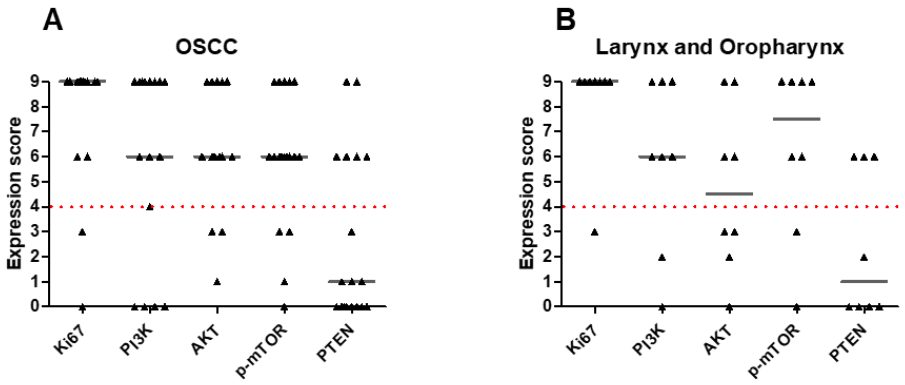


Figure 4: Distribution of the expression score of all evaluated samples for each protein. There was a statistically significant association ($p \leq 0.05$) between high expression of Ki-67 and the low expression of PTEN. **A -** Expression score in oral carcinoma. **B -** Expression score in larynx and oropharynx carcinoma. *The red dashed line represents the value 4 of the expression score. *The gray line represents the mean of the expression score of samples.

ASSOCIATION BETWEEN IMMUNOHISTOCHEMICAL EXPRESSION OF PI3K-AKT-mTOR PATHWAY AND CLINICOPATHOLOGICAL PARAMETERS

The results of the associlation between clinicopathological parameters and immunohistochemical expression of PI3K, AKT and p-mTOR are described in Table 1. The high and low expression of these proteins were correlated with age (< 59 years or ≥ 59 years), gender (male or female), tumor site (oral cavity or oropharynx and larynx), histological grade (well differentiated and moderately differentiated or poorly differentiated), PTEN expression (high or low) and Ki-67 expression (high or low).

A statistically significant association was observed between gender and AKT expression, and between PTEN expression and AKT expression ($p \leq 0.05$), which indicates that the AKT expression is related to gender and PTEN expression in the studied samples. There was no statistically significant association for the other investigated parameters ($p > 0.05$).

Table 1 - Association between clinicopathological parameters and immunohistochemical expression of PI3K, AKT and p-mTOR

Parameter	***PI3K expression***		***p value***	***AKT expression***		***p value***	***p-mTOR expression***		***p value***
	Low	High		Low	High		Low	High	
	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
Age									
<59 y	2 (33)	11 (55)	0.64	3 (43)	10 (52)	1.0	3 (50)	10 (50)	1.35
≥59 y	4 (67)	9 (45)		4 (57)	9 (48)		3 (50)	10 (50)	
Gender									
Male	3 (50)	16 (80)	0.29	3 (43)	16 (84)	0.05*	4 (67)	15 (75)	1.0
Female	3 (50)	4 (20)		4 (57)	3 (16)		2 (33)	5 (25)	
Tumor site									
Oral cavity	3 (50)	15 (75)	0.33	3 (43)	15 (79)	0.14	4 (67)	14 (70)	1.0
Larynx / oropharynx	3 (50)	5 (25)		4 (57)	4 (21)		2 (33)	6 (30)	
Histological grade									
WD / MD	6 (100)	20 (100)	NA	7 (100)	19 (100)	NA	6 (100)	20 (100)	NA
PD	0	0		0	0		0	0	
PTEN									
Low expression	5 (83)	12 (60)	0.37	7 (100)	10 (52)	0.05*	6 (100)	11 (55)	1.0
High expression	1 (17)	8 (40)		0	9 (48)		0	9 (45)	
Ki-67									
Low expression.	2 (33)	1 (5)	0.12	1 (14)	2 (11)	1.0	1 (17)	2 (10)	1.0
High expression.	4 (67)	19 (95)		6 (86)	17 (89)		5 (83)	18 (90)	

WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated.

*P ≤ 0.05 = statistically significant

GENE EXPRESSION OF PI3K-AKT-mTOR PATHWAY

The results of real-time qPCR gene expression indicated that messenger RNAs for *PI3KCA*, *AKT1*, *mTOR* and *PTEN* were ubiquitously expressed in FaDu and SCC-9 cell lines, with similar Ct values (Figure 5). A unique and specific product was confirmed by the presence of only one peak in the *melting* curve (Appendix 1). In FaDu it was observed a highest expression of *PTEN* than the other proteins. The lowest expression was of *PI3KCA*. In SCC-9, the highest expression of *AKT1* and the lowest expression was also of *PI3KCA*. Relatively, *mTOR* and *PTEN* genes were more expressed in FaDu than in SCC-9, and *PI3KCA* and *AKT1* genes were more expressed in SCC-9 than in FaDu (Figure 5).

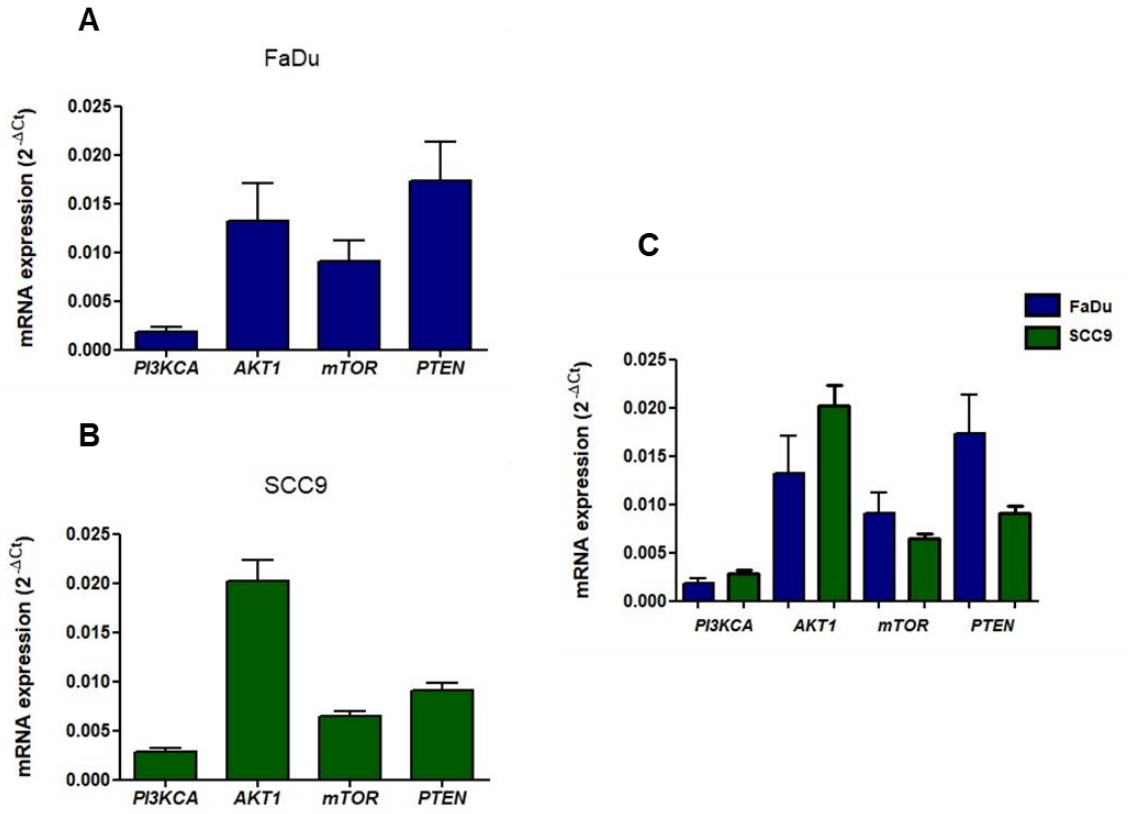


Figure 5: Graphic representation of *PI3KCA*, *AKT1*, *mTOR* and *PTEN* gene expression levels in FaDu and SCC-9 cell lines by qPCR. **A -** Gene expression in FaDu. **B -** Gene expression in SCC-9. **C -** Comparison between gene expression levels in FaDu and SCC-9.

EXPRESSION OF PI3K-AKT-MTOR PATHWAY PROTEINS BY WESTERN BLOT ANALYSIS

The western blot results for PI3K, AKT, p-AKT, mTOR, p-mTOR and PTEN, all proteins related to the PI3K/AKT/mTOR signaling pathway are shown in Figure 6. It is possible to observe that all those proteins were accordingly expressed in both SCC-9 and FaDu cell lines.

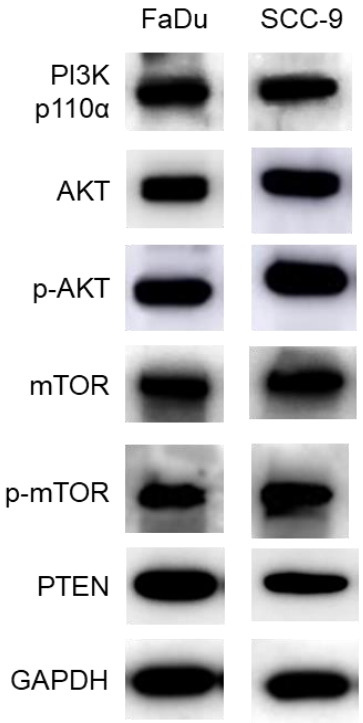


Figure 6: Expression of proteins related to the PI3K-AKT-mTOR pathway in the FaDu and SCC-9 cell lines by western blot.

DISCUSSION

Head and neck squamous cell carcinoma is a heterogeneous disease, involving deregulation of multiple pathways that control cellular differentiation, cell cycle, apoptosis, angiogenesis and metastasis (Leemans et al., 2011). Despite the high diversity of genetic alterations underlying each individual tumor, most molecular alterations converge into a few commonly deregulated pathways (Iglesias-Bartolome et al., 2013). The PI3K-AKT-mTOR signaling pathway is hyperactivated or altered in

many cancer types and regulates a broad range of cellular processes, including survival, proliferation, growth, metabolism, angiogenesis and metastasis (Ersahin et al., 2015). Therefore, proteins related to this signaling pathway have emerged as key targets for treatment of several types of cancer (Lamming et al., 2013).

This pathway is described as one of the most frequently altered in HNSCC (Agrawal et al., 2011; Lui et al., 2013) and multiple upstream and downstream components, such as EGFR, PI3K, AKT, PTEN and mTOR, have been found deregulated, which makes such pathway eligible for the development of molecular-targeted therapies (Courtney et al., 2010).

PI3K is composed of two subunits – an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. It is activated in response to somatic mutation, stimulated receptor tyrosine kinases (RTKs), such as EGFR and insulin-like growth factor receptor 1 (IGF-1R), or G-protein- coupled receptors (GPCRs). PI3K catalytic subunit alpha isoform (*PIK3CA*), the gene that programs for the p110α isoform of PI3K, is one of the most frequently mutated and amplified oncogenes in human cancers (Yuan and Cantley, 2008). Randomized clinical trials using PI3K inhibitors, such as PX-866 and Buparlisib (BKM120), have been performed in combination with other drugs, such as Cetuximab, in HNSCC (Jimeno et al., 2015; Cai et al., 2017).

In normal physiology, PI3K is regulated by the tumor suppressor PTEN. However, inactivating mutation or deletion of PTEN is a frequent alteration in cancer and leads to hyperactivity of the PI3K pathway (Courtney et al, 2010). The PTEN gene is mutated in 3–15% of HNSCC and approximately 30% of HNSCC have loss of PTEN expression (Laurent-Puig et al., 2009; Squarize et al., 2013). The loss of PTEN function by mutation or deletion leads to the increased activation of PI3K and consequent accumulation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which activates a

signaling cascade starting with the phosphorylation of AKT by 3-phosphoinositide dependent protein kinase-1 (PDK1) (Bayascas, 2008). A major downstream effector of AKT is mTOR, the catalytic subunit of mTOR complex 1 (mTORC1). mTORC1 regulates protein translation through phosphorylation of protein synthesis components (Hennessy et al, 2005). Together, the effectors of the PI3K-AKT-mTOR axis interact with and contribute to the regulation of several other signaling molecules in HNSCC, including tumor suppressor protein p53, nuclear factor-kappa B (NF-κB), and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (Vander Broek et al., 2015).

Identification of protein expression profiles is important for understanding the mechanisms of head and neck tumorigenesis, as they could facilitate the development of new tools for the prevention, diagnosis, treatment and prognosis of HNSCCs. For the first time, the present study confirmed that the PI3K-AKT-mTOR pathway is overexpressed in HNSCCs in a Brazilian population. High expression of PI3K, AKT and mTOR proteins was found in tumor samples from the oral cavity, oropharynx and larynx. On the other hand, low expression of PTEN, suppressor of PI3K-AKT-mTOR pathway, was observed in most of the evaluated samples. Our analyzes indicate that these proteins are important markers of HNSCC tumors and, therefore, constitutive targets in their treatment.

Activated mTOR has been associated with poor prognosis in various cancers (Kawasaki et al., 2018). Recently, a systematic review and meta-analysis conducted by our research group supported the evidence that mTOR pathway proteins can be used as predictive markers for survival in patients with HNSCC because their expression was significantly associated with poor overall survival (OS) and short disease-free survival (DFS) (Marques et al., 2016).

We found no statistically significant association between the protein expression patterns and clinicopathological variables such as age, tumor site and histological grade of differentiation. Yet a significant association between the AKT expression and PTEN expression was established. As an important negative regulator of PI3K-AKT-mTOR pathway, the loss of PTEN leads to accumulation of PIP3, which is responsible for the activation of AKT (Giudice and Squarize, 2013). Therefore, it is assumed that the increase in the level of AKT may be closely related to the low PTEN expression.

Among tumor suppressor genes is *CDKN2A*, which encodes the p16 protein and is inactivated in approximately 80% of squamous cell carcinomas (Reed et al., 1996). The p16 protein belongs to the INK4 class of cell cycle inhibitors (Su et al., 2010) and its inactivation has often been found in several tumor types, including HNSCC, and appears to be caused by several mechanisms such as point mutations, homozygous deletion, and DNA methylation (Papadimitrakopoulou et al., 1997). Although there is no consensus about the overexpression or underexpression of p16 in carcinomas, a frequent association between its overexpression and high-risk HPV infection is found in literature. This occurs due to proteolytic degradation of retinoblastoma protein (pRb) induced by E7 viral oncoprotein, with consequent increase of p16 expression through a feedback mechanism (Hennessey et al., 2009; Buajeeb et al., 2009). Previous work conducted by this research group confirmed that the oral and oropharynx tumors involved in the present study are HPV-negative and presented low p16 expression in most cases, when compared with normal epithelium and dysplastic area (Cantarutti et al., 2014). The data suggest the inactivation of p16 tumor suppressor gene in HPV-negative head and neck tumors.

In vitro analysis of the gene and protein expression of the PI3K-AKT-mTOR pathway also demonstrated high expression for *PI3KCA*/PI3K, *AKT1*/AKT and

mTOR/mTOR, in both SCC-9 and FaDu cell lines. Recent studies have identified mutations, amplifications, and overexpression of the different mediators/genes involved in the PI3K-AKT-mTOR pathway. Of these, *PIK3CA* is the most commonly mutated component of this pathway in HNSCC (Cai et al., 2017). In our study, *PI3KCA* gene expression was the lowest in both cell lines. We deduce that possible mutations of this gene have led to decreased expression, not to increase.

Considered our results on the expression profile of PI3K-AKT-mTOR pathway in HNSCC, we conclude that this pathway is overexpressed and that PTEN has a low expression in the studied tumors. These findings reveal the importance of the PI3K-AKT-mTOR pathway in head and neck carcinogenesis, which makes it a promising target for the therapy of this type of tumor. New studies, with a larger sample size and investigation of the mechanisms of action of pathway inhibitors are necessary.

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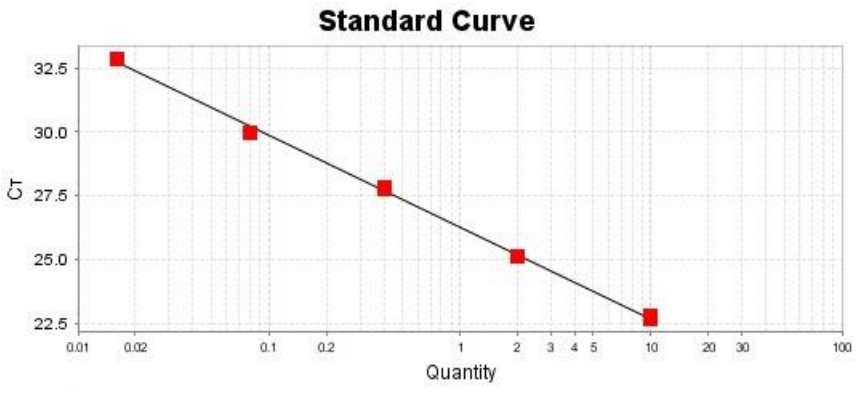
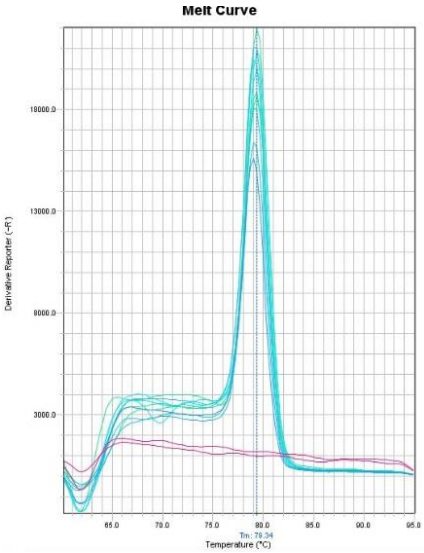
Appendix 1 - VALIDATION AND *MELTING* CURVES

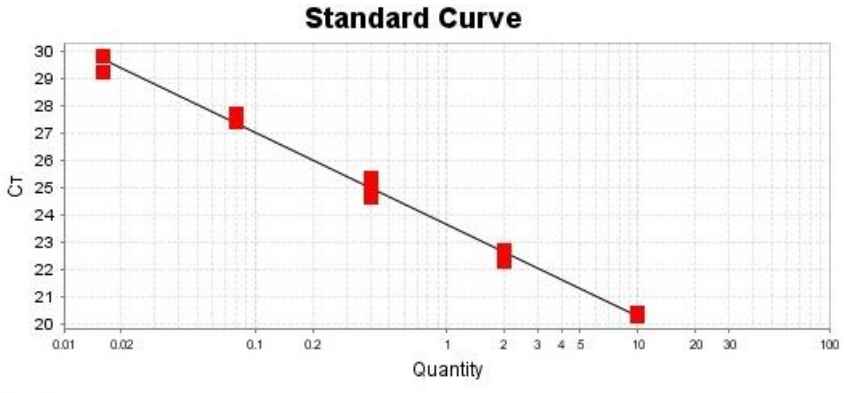
PI3KCA

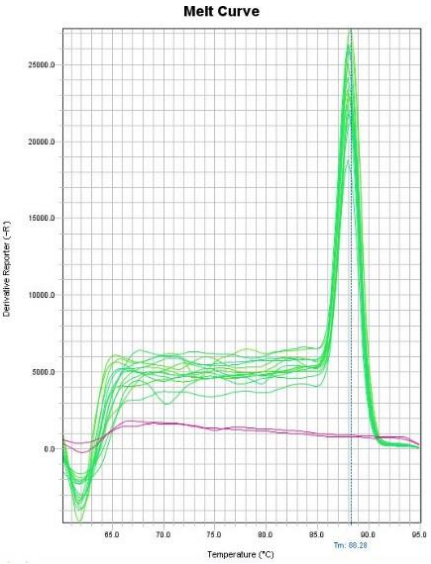




Target: PI3KCA Slope: -3.585 Y-Inter: 26.283 R^2: 0.998 Eff%: 90.085

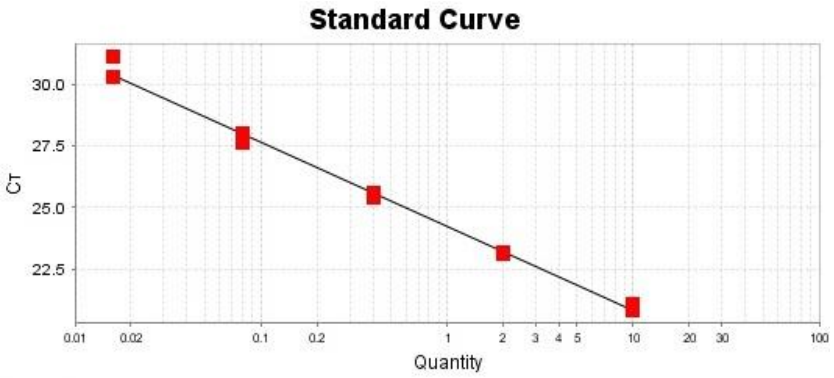
AKT1



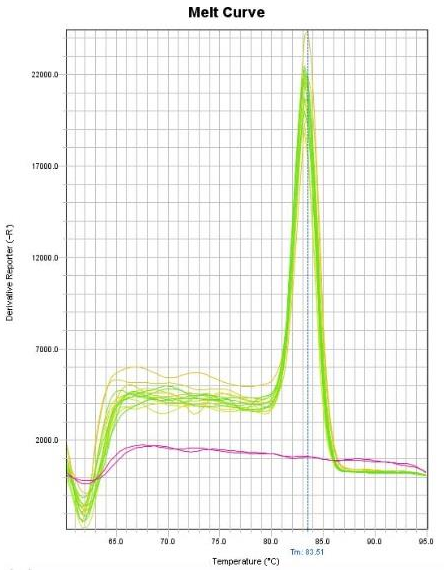


Target: AKT1 Slope: -3.372 Y-Inter: 23.653 R^2: 0.994 Eff%: 97.932

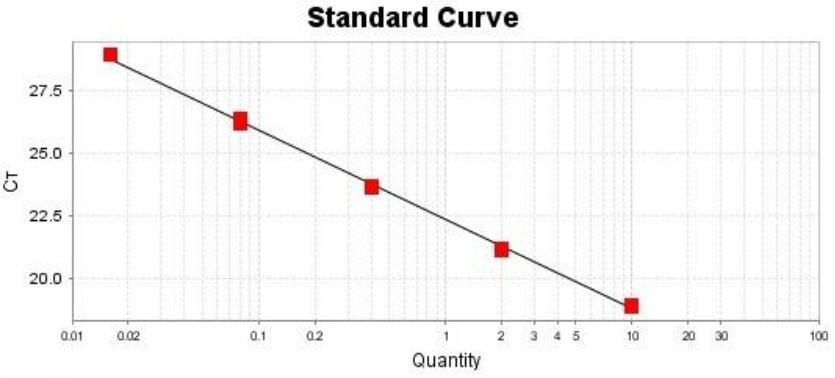
MTOR



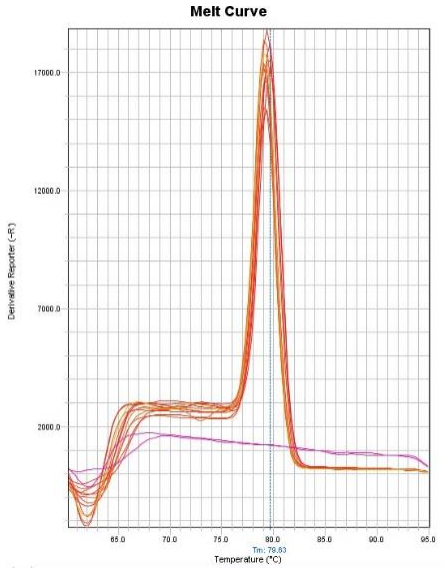
Target: MTOR Slope: -3.423 Y-Inter: 24.231 R^2: 0.995 Eff%: 95.96

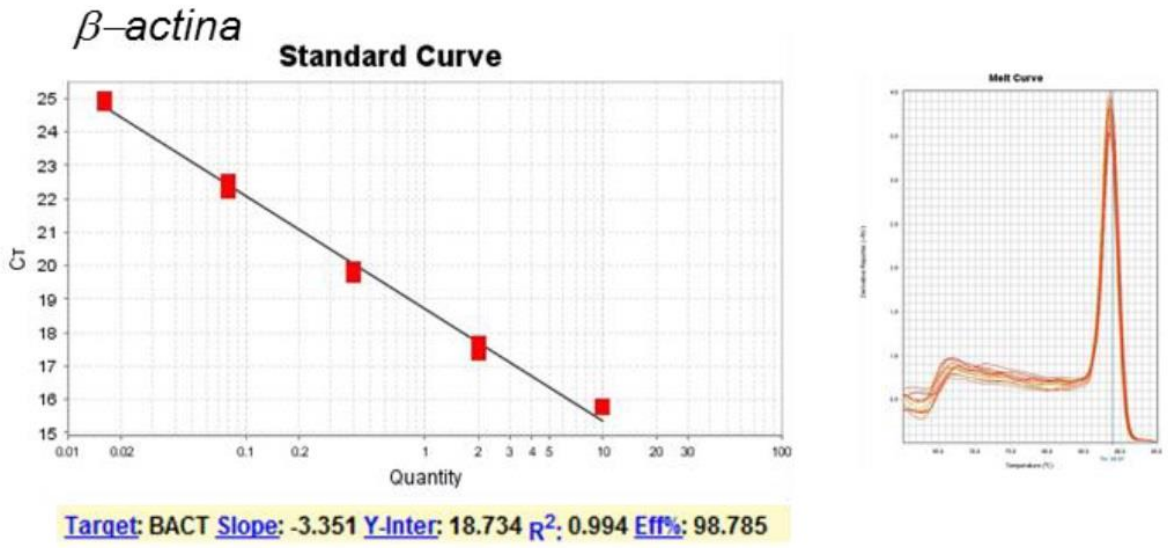


PTEN



Target: PTEN Slope: -3.584 Y-Inter: 22.355 R^2: 0.998 Eff%: 90.129





*Os pares de primers β-actina foram previamente validados pelos alunos Caroline, Maurício e Alexandre, e gentilmente cedidos para utilização neste trabalho.

Supplemental Table S1: Gene sequences of primers used in qPCR

Gene	Sequence (5`- 3`)	Product Size (bp)*
PI3KCA	F: CCACGACCATCATCAGGTGAA R: CCTCACGGAGGCATTCTAAAGT	112
mTOR	F: GCAGATTTGCCAACTATCTTCGG R: CAGCGGTAAAAGTGTCCCCTG	114
AKT1	F: TCCTCCTCAAGAATGATGGCA R: GTGCGTTCGATGACAGTGGT	181
PTEN	F: TTTGAAGACCATAACCCACCAC R: ATTACACCAGTTCGTCCCTTTC	134
β-ACTIN	F: TCACCCACACTGTGCCCATCTACG R: CAGCGGAACCGCTCATTGCCAATG	295

*bp, base pairs

3.3 Manuscrito 3

Entinostat, an HDAC inhibitor, decreases the viability of tumor cells, promotes cell cycle disruption, and reduces stem cells in oral cancer cell lines

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ABSTRACT

Background: Carcinogenesis is determined by various epigenetic events, such as histone deacetylation. Inhibition of histone deacetylase enzymes (HDACs) has been well documented as an attractive target for the development of chemotherapeutic drugs. The purpose of this study was to investigate the effects of the Entinostat, an HDAC inhibitor, on oral squamous cell carcinoma (OSCC) cell lines.

Methods: The cell lines involved in the study were HN6 and HN12 of the OSCC, and normal oral keratinocytes (NOK-SI), used as control. Cell viability was determined by the MTT assay. The action of Entinostat on the distribution of cells in the cell cycle, cancer stem cells (CSCs) and reactive oxygen species (ROS) was analyzed using flow cytometry. In addition, the expression HDAC, histones and cell cycle regulatory proteins were examined by western blot.

Results: Treatment with Entinostat showed a decrease of cell proliferation of OSCC cell lines and it altered cell cycle progression, resulting in a significant increase in the fraction of cells present in the G0/G1 phase at low micromolar concentrations, consistent with the known anticancer effects of the drug. There was a significant reduction in CSCs and an increase in ROS generation. Relevant changes were observed in histone acetylation levels and in the expression of proteins that control the cell cycle, such as overexpression of p21.

Conclusions: This study proved that Entinostat is a promising agent in inhibiting OSCC progression because of its cytotoxic and antiproliferative cellular effects. However, further *in vitro* and *in vivo* studies are needed to deepen the understanding of these findings.

INTRODUCTION

Globally, oral cancer is one of the leading cancers, accounting for 2% of all cancer cases, with a nearly 50% mortality rate (Siegel et al., 2016). Oral squamous cell carcinoma (OSCC) accounts for 95% of oral cavity and lip cancers and represents the eleventh most commonly cancer worldwide, accounting for 300,000 new cases and 145,000 deaths per year (Shield et al., 2017). Smoking, alcohol use, smokeless tobacco use and HPV infection are the major risk factors for OSCC (Hashibe et al., 2009; Thavaraj et al, 2010), and the overall 5-year survival rate is approximately 60%, varying between 80% for stage I cancers and 40% for stage IV cancers (Siegel et al., 2016). Standard therapy for OSCC includes multimodal approaches consisting of surgery, radiation, chemotherapy, and combinations based on the stage at time of diagnosis (Algazi et al., 2017). Active areas of research in head and neck squamous cell carcinoma (HNSCC), of which the OSCC is part, include the identification of novel targets, exploration of resistance mechanisms to current therapies, and identification of combination strategies (Lui et al., 2013). Recent progress in molecular biology and translational research has initiated an era of personalized medicine in head and neck clinical oncology. The genetic information defined by biomarker analysis in tumors and individuals is indispensable for the administration of molecular targeting agents (Jackson and Chester, 2015).

Epigenetics as well as post-translational modifications of proteins are emerging as novel attractive targets for anti-cancer therapy. The regulation in acetylation and deacetylation of the terminal ε-amino group of specific lysine residues of histone proteins, displays a pivotal role in the transcription and control of cell survival (Pazin and Kadonaga, 1997). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two classes of enzymes regulating histone acetylation and whose altered

activity has been identified in several cancers (Budillon et al., 2007). Acetylation is generally associated with elevated transcription, while deacetylated histones are often associated with gene repression. HDACs are critical regulators of gene expression that enzymatically remove the acetyl group from histones (Olsen, 2012). In particular, imbalance in histone acetylation can lead to changes in chromatin structure and transcriptional dysregulation of genes that are involved in the control of proliferation, cell cycle progression, differentiation and/or apoptosis. Since epigenetic issues are dynamic and usually reversible, epigenetic manipulation is a novel and attractive anti-cancer treatment (Caponigro et al., 2016). Epigenetic modifications also contribute to cell plasticity during tumor progression and to the formation of cancer stem cells (CSCs), a small subset of tumor cells with self-renewing ability. CSCs are involved in the development of intrinsic or acquired therapeutic resistance and tumor recurrences. The understanding and characterization of epigenetic modifications associated with head and neck carcinogenesis and the prospective identification of epigenetic markers associated with CSCs maintain the promise of new therapeutic strategies to fight tumors (Castilho et al., 2017).

Histone deacetylase inhibitors (HDACi) are epigenetic antitumor agents whose main targets are histone deacetylases with varying potency and specificity. They seem to be promising anti-cancer drugs particularly in the combination with other anti-cancer drugs and/or radiotherapy (Eckschlager et al., 2017). HDACi been shown to cause accumulation of acetylated histones and transcription factors, induce cancer cell cycle arrest, differentiation and cell death, reduce angiogenesis, modulate immune response and inhibit cell growth in a variety of preclinical models, however, the mechanisms detailed behind remain incompletely known and probably involve multiple molecular targets depending on development contexts (Iglesias-Linares et al., 2010). Till date,

four drugs, namely Vorinostat (SAHA), Romidepsin (FK-228), Belinostat (PXD-101) and Panobinostat (LBH-589) have been granted FDA approval for cancer and several HDAC inhibitors are currently in various phases of clinical trials, either as monotherapy and/or in combination with existing or novel anti-cancer agents. Regardless of this, today scientific efforts have fortified the quest for newer and novel HDAC inhibitors that show isoform selectivity (Manal et al., 2016). There are several major classes of HDAC inhibitors including hydroxamic acid-based, cyclic tetra/depsipeptides, amino-benzamide-based, and short-chain fatty acid-derived inhibitors (Falkenberg & Johnstone, 2014). A recently discovered hydrazide-based HDAC inhibitor further increased the diversity of the HDAC inhibitors (McClure et al., 2016).

Entinostat, also known as SNDX-275 and MS-275, is the first amino-benzamide-based HDACi to reach clinical trials (Ryan et al., 2005) and it is an orally bioavailable class I HDACi with a long halflife, which is under evaluation in hematological and solid tumour malignancies (Frys et al., 2015). *In vivo* and *in vitro* studies have demonstrated that Entinostat, administered alone and in combination with other agents, is a promising drug for the treatment of cancer by producing antiproliferative and apoptotic effects in several types of tumor cells, such as lymphoma, cancer of breast, lung, kidney cancer, prostate, pancreatic, bladder cancer and bone tumors (Frys et al., 2015; Trapani et al., 2017; Ruiz et al., 2015; Shen et al., 2012; Kato et al., 2007; Yar Saglam et al., 2016; Elmer et al., 2016; Kiany et al., 2017). The action of some HDAC inhibitors such as Valproic acid (VPA), Vorinostat, Panobinostat, CUDC-101, and Resminostat (Kothari et al., 2010; Erlich et al., 2012; Galloway et al.,2015; Enzenhofer et al., 2017) has been investigated in the HNSCC and has shown promising inhibitory effects of cell proliferation. However, there is a gap regarding to the effects of Entinostat in this type of tumor, especially in OSCC.

For the first time, in this present study we investigated the *in vitro* effects of the Entinostat on cell viability, cell cycle, histones, HDAC and regulatory proteins expression of the cell cycle in OSCC cell lines. In addition, we evaluated the action of the Entinostat on cancer stem cells and on the reactive oxygen species (ROS) production in oral cancer cell lines.

MATERIAL AND METHODS

Cell lines and culture

Oral tongue squamous cell carcinoma (HN6 and HN12) (Easty et al., 1981; Yeudall et al., 1994), and a control normal oral keratinocyte (NOK-SI) cell line (Castilho et al., 2010) were culture under the same conditions. They were maintained in a 5% CO2 humidified incubator at 37 °C and cultured in DMEM/HIGH GLUCOSE (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 4.00 mM L-Glutamine (GE Healthcare Life Sciences), 4500 mg/L Glucose (GE Healthcare Life Sciences, 10% Fetal Bovine Serum) and 1% antibiotic.

Cell Proliferation, Viability Assay and IC50 determination

Cell viability was assessed by the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, a yellow reagent which is converted to formazan (a purple dye) by living cells, following the manufacturer's protocol (MTT Cell Proliferation Assay Kit, Trevigen, Gaithersburg, MD, USA) and using a absorbance at 595 nm in a spectrophotometer (TECAN microplate reader, TECAN US, Durham, NC). This assay is a commonly used indicator of metabolic activity, which reports for cell proliferation and viability. Cells were seeded into 96-well plates at the density of 2 ×

10^4 cells/well. Cells were treated with Entinostat (LC Laboratories, Woburn, MA, USA) or vehicle for 24h in the indicated concentrations. Wells with medium without Entinostat were used as negative control. The experiment was carried out in sextuplicate. The dose-response curves for defining the Entinostat concentration that inhibited cell proliferation by 50% (IC50) for each cell line were constructed in logarithm using the GraphPad 7.0 software by PRISM (GraphPad Software, San Diego, CA).

Cell cycle assay

Cells were plated and treated with Entinostat for 24h at IC50 concentration and vehicle (controls). Cells were then washed with phosphate-buffered saline (PBS) and fixed with 70% cold ethanol for 30 min in ice. After centrifugation and removal of ethanol, 0.5 mL PI-RNAse solution (50 µg/mL PI + 100 µg/mL RNAse Type I-A in PBS) was added to the cell pellet and then incubated at room temperature in the dark for f 20 min. Samples were analyzed by flow cytometry using Accuri C6 Flow Cytometer (BD Biosciences, USA) and differences in cell cycle between drug *versus* vehicle treatment were determined using the Accuri CFlow Plus software. Assays were done in triplicates.

Reactive oxygen species (ROS) detection assay

After treatment with Entinostat for 24 h, the cells were trypsinized and centrifuged at 400 x g for 5 minutes. Removed the supernatant, the cells were resuspended in fresh medium at the concentration of 1-5 x 10^5/mL. For the negative control, ROS inhibitor was added (N-acetyl-L-cysteine) to pre-treated the cells prior to induction. For the induction, the same volume of ROS/Superoxide Detection Solution (2x) was loaded with addition of either the vehicle, experimental agent, ROS inducer

(pyocyanin) at the desirable working concentrations. After, the samples were incubated under normal tissue culture conditions for 1h and read using flow cytometry.

Flow cytometry analysis and Stem cell quantification

Cancer stem cells (CSCs) from HN6 and HN12 cells, and NOK-Si were identified by ALDH activity combined with CD44-APC expression (clone G44-26, BD Biosciences) using flow cytometry (Accuri C6 Flow Cytometer, BD Biosciences). The Aldefluor kit (StemCell Technologies, Durham, NC, USA) was used according to the manufacturer's instructions to identify cells with ALDH enzymatic activity. Cells were trypsinized, counted ($1x10^6$ cells) and resuspended in AldeFluor assay buffer containing ALDH substrate. Cells were also centrifuged and incubated with anti-CD44. for 25 min at 4 °C in dark. The controls were cells separately incubated with diethylaminobenzaldehyde (DEAB, a specific ALDH inhibitor), anti-CD44, ALDH substrate, or cells alone (one aliquot was prepared without ALDH substrate, without anti-CD44 and without DEAB). Cell samples with Entinostat or vehicle after 24h treatment. Experiments were done in triplicates. All samples were analyzed using Accuri CFlow Plus software (BD Biosciences, USA).

Western blot analysis

After 24 h treatment with Entinostat or vehicle, cells were washed and lysed in cell lysis buffer containing protease inhibitors. The protein lysate samples with loading buffer were boiled for 5 min at 100°C and run on SDS-Page electrophoresis gel. Westen blotting was conducted using standard techniques. Proteins transferred membrane were blocked with TBS-T containing 5% skim milk for 1h shaking in room temperature, followed by incubation with the primary antibodies overnight at 4 °C

shaking. Primary anti-human antibodies (Table 1 of Appendix S1) were diluted (1:1000) in 5% bovine serum albumin (BSA)/TBS-T. After TBST 1X wash, they were incubated for 1 h with the secondary antibody 5% BSA/TBS-T (1:3000 dilution;Table 1, Appendix S1). After incubation, the membrane was thoroughly washed with TBS-T, treated with ECL western blotting detection system (Thermo Fisher Scientific, Massachusetts, EUA) and exposed for development on film in a dark chamber. 3-phosphate dehydrogenase (GAPDH) was used as loading controls (Table 1, Appendix S1).

Statistical analysis

Statistical analyses for cell viability assays and flow cytometric experiments were performed by unpaired Student`s t-test and Kruskal Wallis using GraphPad 7.0 software by PRISM (GraphPad Software, San Diego, CA). Dose–response curves were generated using GraphPad version 7.0. All flow cytometric experiments were repeated in triplicate or sextuplicate. The p values < 0.05 were considered as statistically significant.

RESULTS

Entinostat treatment reduced tumor viability of OSCC cells

The rates of proliferation of cells after treatment with Entinostat were investigated. The OSCC cell lines, HN6 and HN12, and the NOK-SI cell line were treated with entinostat or vehicle. After 24 hours, a significant dose-dependent inhibition rate was seen in all cell lines tested. IC50 values were calculated and ranged from 0.54 µM to 23.31 µM (IC50 for HN6: 0.54 µM and HN12: 23.31 µM). The IC50

value in NOK-SI cells was 18.74 µM. The graphs with the concentrations used and the dose-response curves of cell viability are shown in Figure 1.

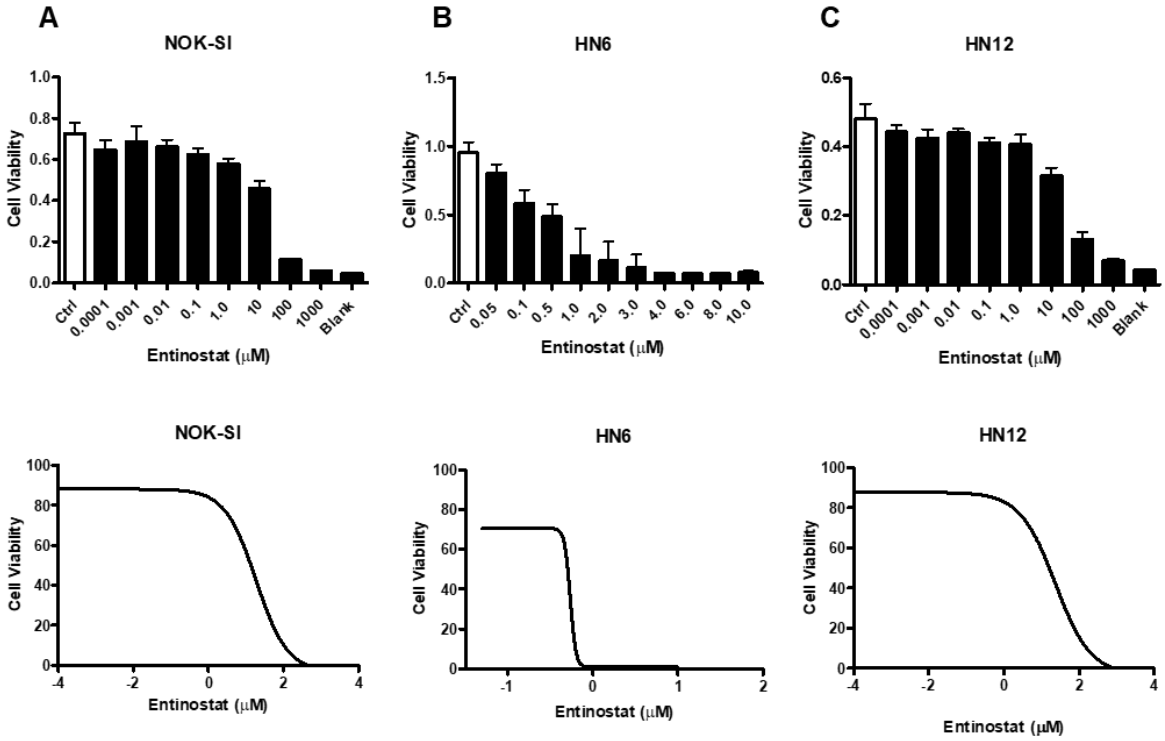

Figure 1: Schematic plots of Entinostat concentrations used in the MTT assay to determine the 50% inhibitory concentration of cell viability (IC50) in each cell line (A: NOK-SI; B: HN6; C: HN12), in 24 h of treatment, and their respective dose-response curves. Entinostat decreased the cell proliferation of OSCC lines and NOK-SI. IC50 for NOK-SI = 18.74 µM; HN6 = 0.54 µM; and HN12 = 23.31 µM.

Effects of Entinostat on cell cycle progression in OSCC

As cell viability assays showed a dose-dependent reduction of cell proliferation, we next investigated the mechanisms underlying the effect of Entinostat on cell growth. We analyzed the cell cycle distribution by flow cytometry to determine cell populations at different phases. As shown in Figure 2, cell cycle analyses showed that more cells were at G0/G1 phase of HN6 and HN12 cells compared with vehicle/control cells with most cells in G2/M. Similarly, NOK-SI normal keratinocyte cells also had higher

numbers of cells at G0/G1 phase after treatment with Entinostat (Figure 2A). These data suggest that the effects of the drug mainly blocked the cells in the G0/G1 phase to enter the S phase, with a decrease in the number of cells in the S and G2/M phases. The results were statistically significant in HN6, HN12, and NOK-SI cell lines, with values of $p < 0.05$.

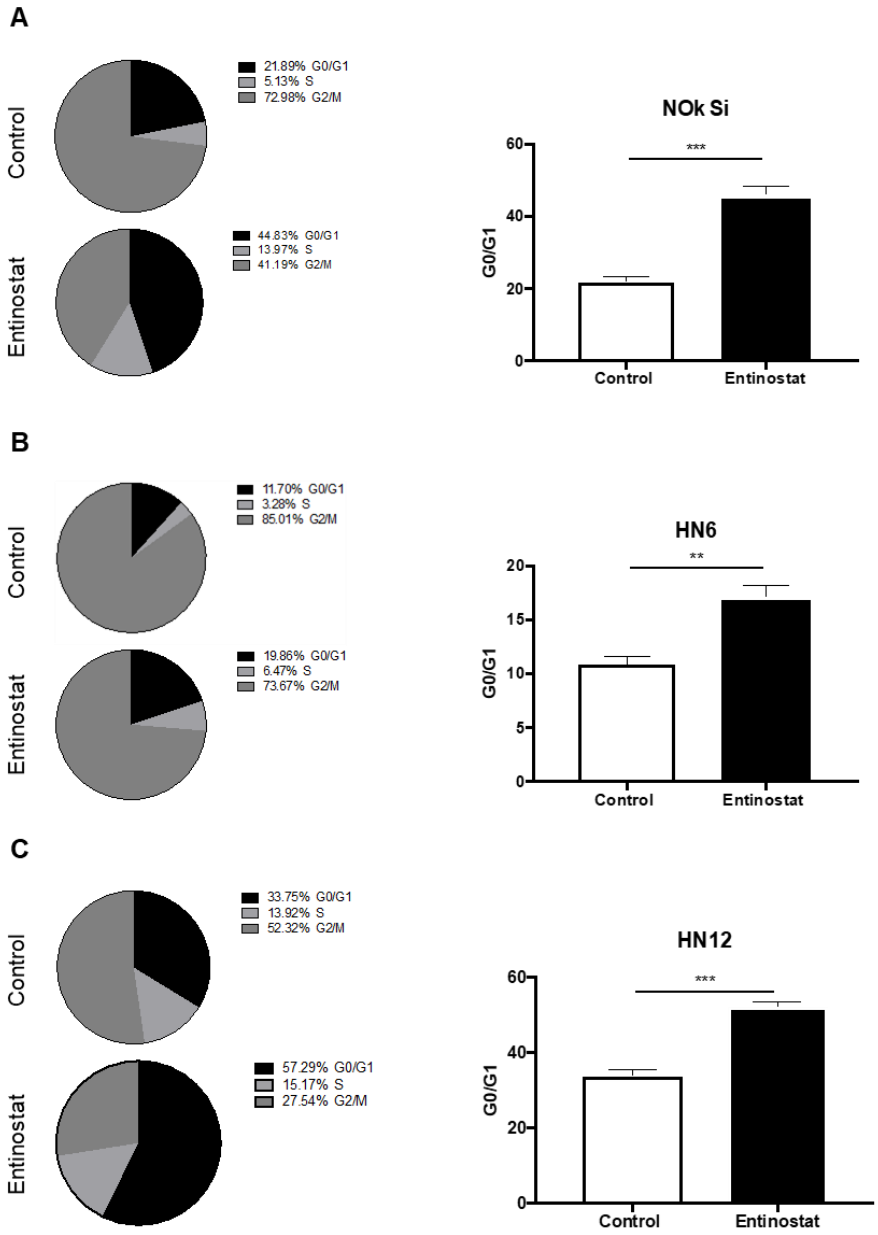

Figure 2: Representation of the cell distribution in the G0/G1, S and G2/M phases of the cell cycle after 24 h of treatment with Entinostat, by flow cytometry (A: NOK-SI; B: HN6; C: HN12). There was a statistically significant increase of G0/G1 phase in NOK-SI HN6, and HN12 cells. ** = $P < 0.005$. *** = $P < 0.0005$.

Entinostat increased ROS production

Previous studies have reported that normal cells have lower reactive oxygen species (ROS) levels compared to cancer cells and that one of the factors by which cancer cells are more vulnerable to HDAC inhibitors is due to their increased sensitivity to production of ROS (Szatrowski and Nathan, 1991; Ungerstedt et al., 2005). Our results also demonstrated that ROS levels are higher in OSCC cells, compared to normal keratonocytes. In addition, Entinostat induced a significant increase ($p < 0.05$) of ROS in HN6 and HN12 cells, as well as in NOK-SI cells. In the specific analysis of the amount of superoxide, a type of ROS, similar results were found in all cell lines, as shown in Figure 3. Only in HN12, the increase of superoxide was not statistically significant ($p > 0.05$).

Entinostat decreased OSCC cancer stem cells

We evaluated the action of Entinostat on cancer stem cells through expression of CD44 and ALDH activity. In NOK-SI, HN6 and HN12 cells, a significant reduction of CSCs ($p < 0.05$) was observed after treatment for 24h, as shown in Figure 4. The results demonstrated that this HDAC inhibitor is effective against OSCC CSCs.

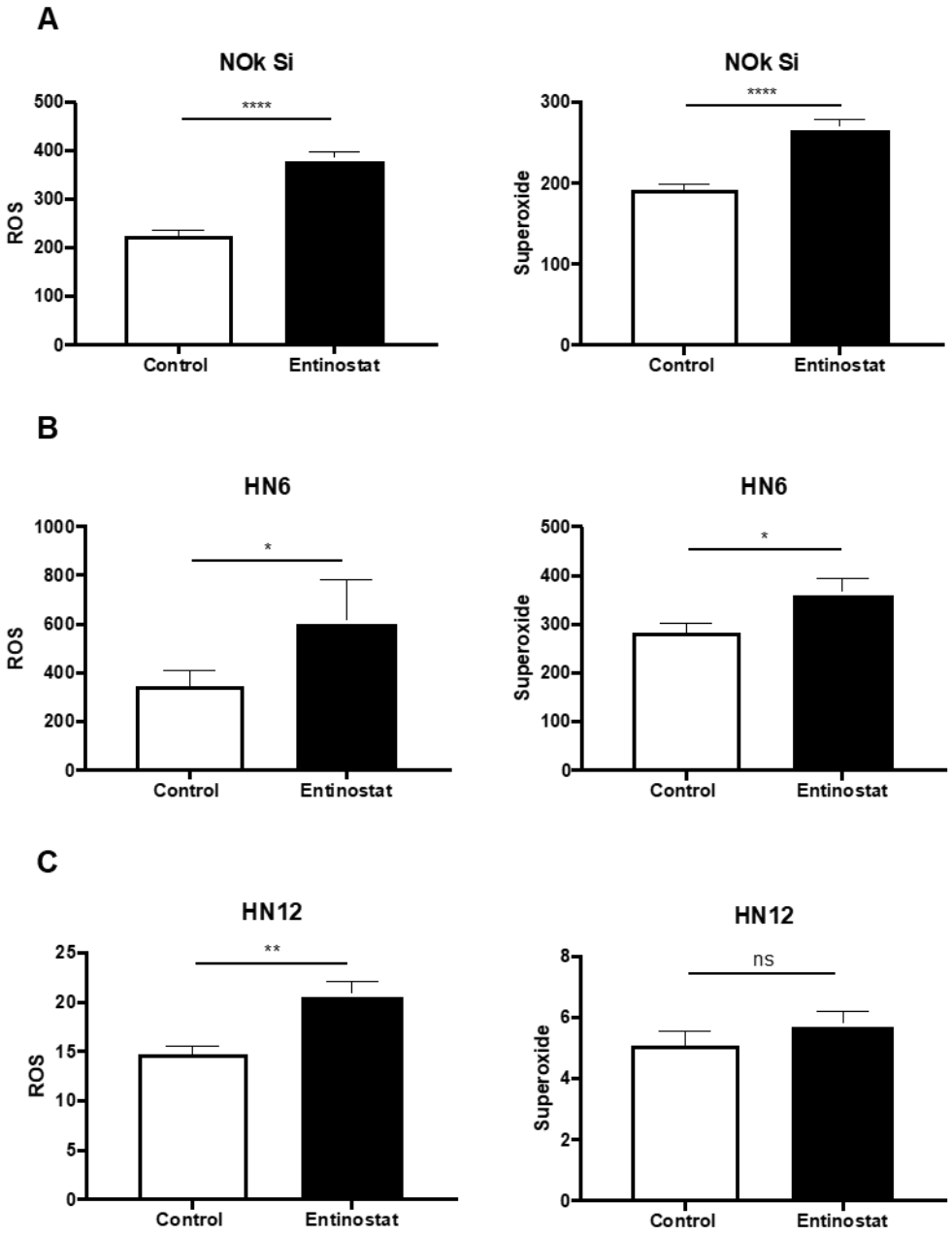

Figure 3: Representation of the action of Entinostat on the production of reactive oxygen species (ROS) and superoxide in cells. After 24-hour treatment, there was an increase in the generation of both ROS and suberoxide in all cell lines. * = $P < 0.05$. ** = $P < 0.005$. **** = $P < 0.00005$. ns = not significant ($P > 0.05$).

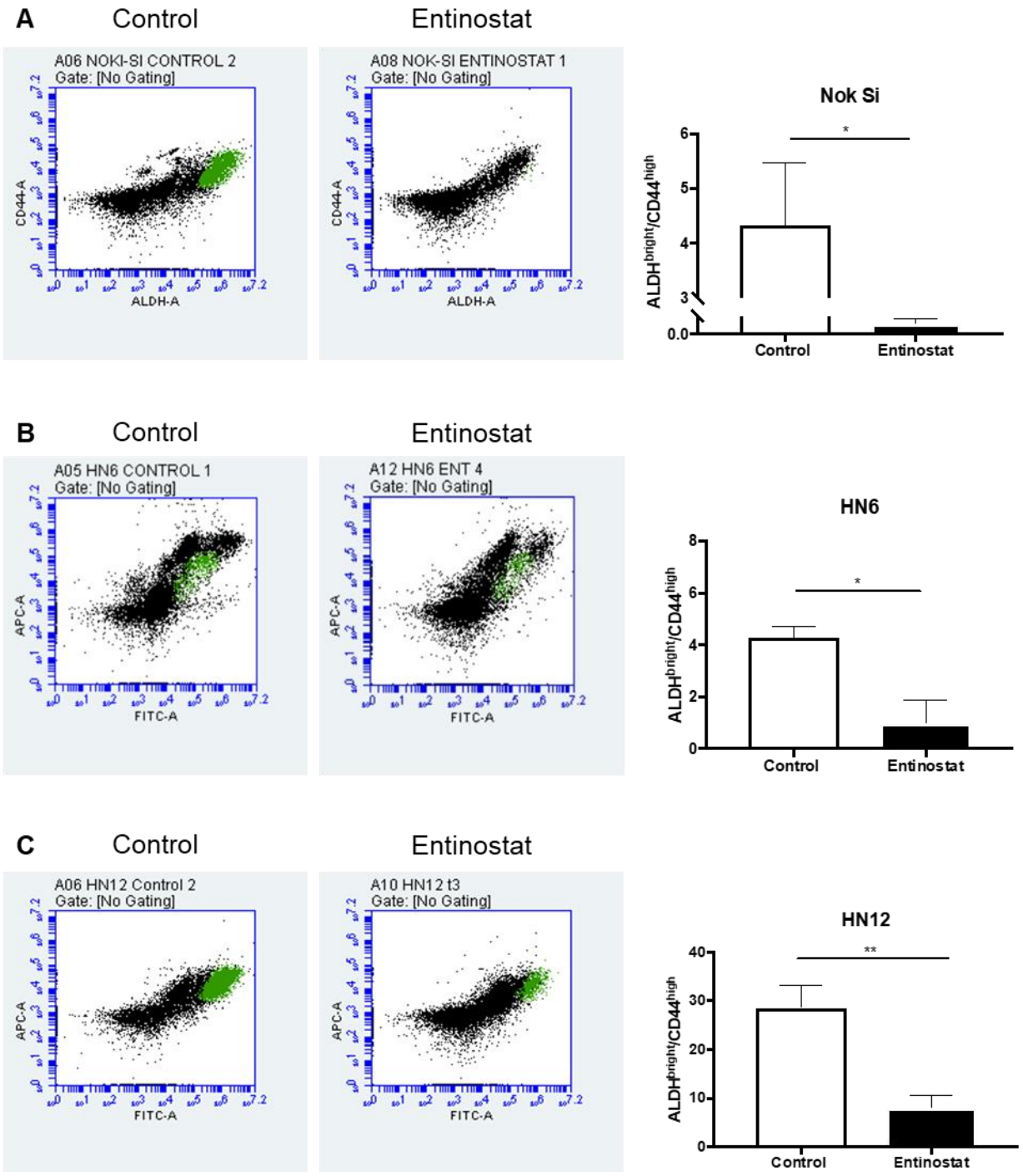

Figure 4: Flow cytometric analysis of NOK-SI (A), HN6 (B) and HN12 (C) cells treated with Entinostat for the expression of ALDH and CD44 (green color). After 24-hour treatment, there was a statistically significant reduction of stem cells in all cell lines. * = $P < 0.05$. ** = $P < 0.005$.

Effect of Entinostat on histones, HDAC, cell cycle regulatory proteins and proteins related to the behavior of CSCs

The action of Entinostat was investigated by western blot analysis, on the expression of acetylated H3 and H4 histones, and Sirtuin 1 (SIRT1) deacetylase enzyme. It was investigated also on cell cycle regulatory proteins, p53 and p21, protein encoded by the phosphatase and tensin homologue gene (PTEN), and on the B lymphoma Mo-MLV insertion region 1 homolog (BMI1).

There was an increase of H3 acetylated histone in NOK-SI, HN6 and HN12 cells compared to the vehicle control. In all cell lines, was observed an increase of H4 acetylated histone after treatment with Entinostat. These findings suggest that the likely inhibition of HDACs caused by the action of Entinostat possibly induced the acetylation of H3 and H4. As expected, unlike acetylated histones, there was a decrease in the expression of the enzyme deacetylase SIRT1 in all cells, confirming the efficacy of Entinostat as a potent inhibitor of HDAC (Figure 5).

As shown in Figure 6, we observed a reduction in the expression of the p53 protein in NOK-SI cells after the treatment, and absence of this protein was observed in the HN12 cells, both in the control and in the treated cells. In consistence with previous reports (Spiegel et al., 2012; Sonnemann et al., 2014), in the present study Entinostat induced overexpression of p21 protein, a major transcriptional target of p53 (Vousden and Prives, 2009). p21 protein was shown to be expressly increased in all cell lines. (Figure 6). As a key cell cycle regulator, increased expression of p21 leads to tumor suppression.

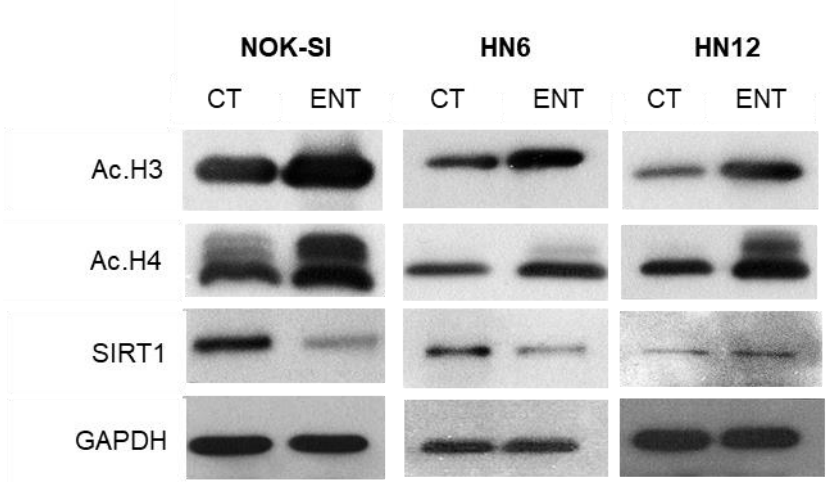

Figure 5: Effect of Entinostat on the expression of histones 3 and 4, and histone deacetylase SIRT1. Entinostat increased the expression of acetylated histones 3 and 4 in all cells. Conversely, Entinostat decreased the expression of SIRT1. CT = control; ENT = Entinostat.

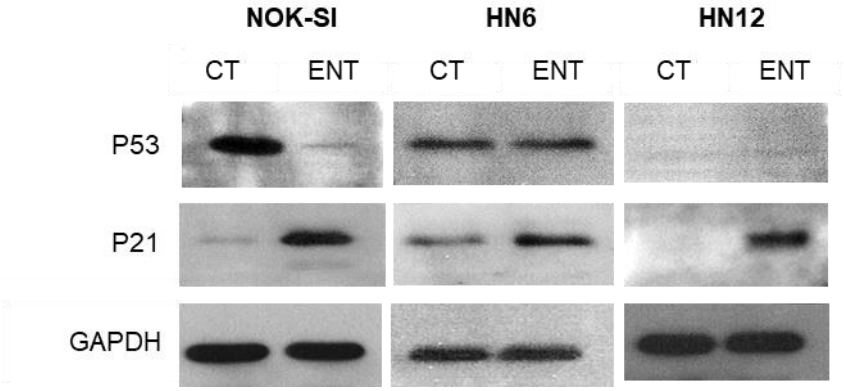

Figure 6: Effect of Entinostat on the expression of the cell cycle regulatory proteins, p53 and p21. Entinostat reduced p53 expression in NOK-SI. Absence of p53 was observed in HN12. Entinostat induced expression of p21 in all cell lines. CT = control/vehicle; ENT = Entinostat.

The phosphatase and tensin homologue deleted on chromosome 10 (PTEN), an important tumour suppressor gene, is mutated, downregulated or dysfunctional in many tumors (Guigon et al., 2009). A recent study by Meng et al. (2016) found that

PTEN activation by inhibiting HDAC6 significantly contributed to tumor inhibition. Therefore, non-selective HDAC or HDAC6-specific inhibitors may be more clinically suitable to treat tumors without PTEN mutations or deletions. Our results showed no change in PTEN expression by Entinostat intervention. Vehicle and entinostat treated cells presented similar PTEN expression, as shown in Figure 7. We also analyzed the expression of BMI1, a Polycomb group repressor, which plays critical and indispensable roles in governing self-renewal capacity of normal and malignant CSCs (Siddique et al., 2012). In our experiments, treatment with Entinostat resulted in decreased BMI1 expression, observed in NOK-SI, HN6 and HN12 cells, consistent with the reduction of CSCs found by flow cytometry (Figure 7).

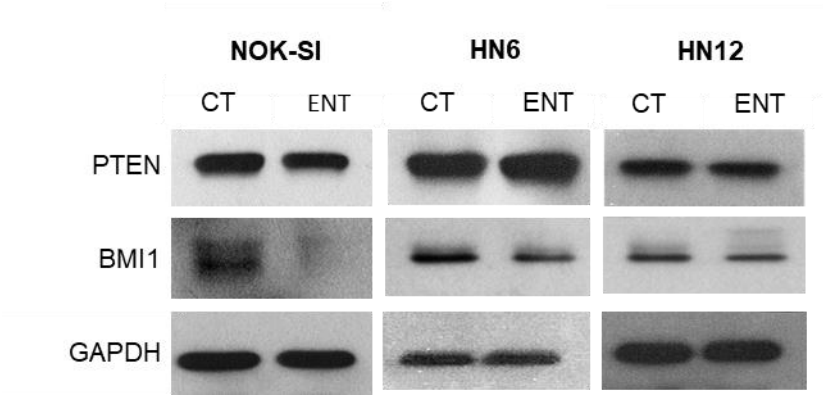

Figure 7: Effect of Entinostat on the expression of PTEN and BMI1. There was no effect on PTEN expression. Entinostat led to the reduction of BMI1 in all cell lines. CT = vehicle/control; ENT = Entinostat.

DISCUSSION

Changes to the epigenome can contribute to the initiation and progression of specific cancers (Feinberg et al., 2016). At the cellular level, these alterations deregulate key cellular processes, like transcriptional control, DNA repair and cell cycle

progression, which can further be modulated by environmental stressors (Song et al., 2011). Chromatin status and histones are regulated by different players and, if altered, may trigger the loss of homeostatic control and tumor proliferation (Kazanets et al., 2016). Enzymes involved in epigenetic processes are often dysregulated in human tumors through mutation, altered expression, or inappropriate recruitment to certain loci. The identification of these enzymes and their partner proteins has driven the rapid development of small-molecule inhibitors that target the cancer epigenome (West and Johnstone, 2014).

Changes in the expression and action of two chromatin enzyme families, histone acetyltransferases (HATs) and histone deacetylases (HDACs), are reported in many types of cancer (Kadoch, 2016; Godoy et al., 2017). By removing acetyl groups from histones, HDAC enzymes increase the binding of these proteins to DNA, thus limiting the access of transcription factors to regulatory regions. Adjusting the balance between HDACs and HATs proved to be a highly effective anti-cancer therapy and led to the development of a family of drugs called HDAC inhibitors (HDACi) (West and Johnstone, 2014). HDAC inhibitors induce cancer cell cycle arrest, differentiation and cell death, reduce angiogenesis, and modulate immune response. HDAC inhibitors Vorinostat, Romidepsin and Belinostat have been approved for some T-cell lymphoma, and Panobinostat for multiple myeloma. Other HDAC inhibitors are in clinical trials for the treatment of hematological and solid malignancies (Eckschlager et al., 2017).

Entinostat (SNDX-275 and MS-275) is a synthetic small-molecule benzamide derivative that has been shown to inhibit HDAC activity (Saito et al., 1999). Previous studies with Entinostat have demonstrated that it effectively inhibits HDACs *in vivo*, resulting in hyperacetylation of histones (Camphausen et al., 2004) and expression of genes that were previously silenced (Duque-Afonso et al., 2011; Kasman et al., 2012).

For the first time investigated in oral carcinoma, the present study proved that even at low concentrations, Entinostat was able to inhibit cell viability in OSCC cells by dose-dependent antiproliferative action, demonstrating its chemosensitivity and cytotoxicity for this type of tumor. These effects corroborate with the findings of other HDAC inhibitors in head and neck cancer (Enzenhofer et al., 2017; Erlich et al., 2012). HN12 cell line presented the highest inhibitory concentration (IC 50 - 23.31 μM), the only one exceeding the inhibitory concentration of the normal keratinocyte control cells (NOK-SI). Interestingly, HN12 cells, in the untreated and treated forms, showed absence of normal and mutated p53 immunosuppressive protein. It is known that conventional chemotherapeutic agents induce apoptosis of cancer cells mostly through p53-dependent pathway. However, p53 mutations and inactivation have been found in more than half of all human carcinoma cells (Vogelstein et al., 2000). Therefore, the outcome of anti-cancer chemotherapy can depend on the p53 status (Soussi and Beroud, 2001). Tumor cells with mutated or deleted p53 tend to be less responsive to several commonly used chemotherapeutic drugs (O'Connor et al, 1997). In agreement with the study of Sonnemann et al. (2014), here we find that p53 null cells are more resistant to the effects of Entinostat.

We also evaluated the effects of the Entinostat on the cell cycle progression and observed that it was effective in inducing cell cycle arrest in OSCC cell lines. Entinostat altered the distribution of cells in the cell cycle inducing the growth arrest, mainly by leading to cell cycle arrested at phase G0/G1, significantly ($p < 0.05$) in the HN6 and HN12 tumor cell lines, and in the NOK-SI cells. Parallel to these observations, an emphatic increase in p21 tumor suppressor protein (Cdkn1a) levels was also observed in all cells, even without increasing expression of p53. Cell cycle progression in a eukaryotic cell is intricately regulated by the activity of the cyclin-dependent kinase

(Cdk)-cyclin complexes (Obaya and Sedivy, 2002). However, in the event of tumorigenesis, several genetic and epigenetic changes occur that culminate in the deregulation of cell cycle events (MacLachlan et al., 1995). Because the threshold kinase activity of Cdks is a crucial determinant of cell cycle progression, they represent attractive targets for the intervention of sustained proliferation of carcinoma cells (Elsayed and Sausville, 2001). In cancer cells, p21 binds and inactivates cyclin/Cdk complexes primarily mediating a G1 arrest (Roy et al., 2007). The tumor suppressor p53 is perhaps the most frequent target of genetic lesions in human cancer. Following DNA damage, p53 orchestrates biological fates ranging from growth arrest to cell death, and the molecular pathways leading to these various outcomes depend on several factors, including the level and type of stress as well as the cell and tissue type (Mirzayans et al., 2012). Among the p53 target genes, perhaps the best characterized is the cyclin-dependent kinase inhibitor p21, which promotes cell cycle arrest and supports DNA damage repair (Abbas and Dutta, 2009). In our study, the change in Entinostat-mediated p21 showed that it might be p53-independent in these Entinostat-treated cancer cells, or p53 might be mutated. Lee et al. (2001) also found that Entinostat induced the expression of the tumor suppressor p21 in cell lines regardless of the p53 status of the cells, demonstrating an antiproliferative effect in cell lines, which correlates with p21 overexpression.

Histone modifications include H3 and H4 histones lysine deacetylation by HDACs that leads to chromatin decondensation and subsequent gene repression (Li et al., 2010). HDAC inhibition leads to elevated protein lysine acetylation in tumor peripheral-blood cells, which serves as a surrogate potential pharmacodynamic marker of activity (Sabnis et al., 2011). Thus, as an HDAC inhibitor, Entinostat has been shown to be effective in enhancing histone acetylation in OSCC cells. Clearly, in this study we

observed increased expression of acetylated H3 and H4 after treatment with the drug. In addition, Entinostat decreased the expression of the SIRT1, a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase that exerts its biological effects by deacetylating histones and non-histone proteins. Furthermore, its substrates include many proto-oncogenes and tumor suppressors (Smith et al., 2008). Studies report that Sirtuins have emerged as potential therapeutic targets for the treatment of various diseases, such as cancer and cardiovascular (Hu et al., 2014; Borradaile and Pickering, 2009). The effects are due to their regulation of various normal and abnormal cellular and metabolic processes, including tumorigenesis, neurodegeneration, and processes associated with type 2 diabetes and obesity (Aljada et al., 2010). A review produced by Kozako et al. (2014) summarized that the functions of SIRT1 in stimulating cell growth and angiogenesis, and blocking senescence and apoptosis indicate that SIRT1 may play a critical function in tumor initiation, progression and drug resistance. Our findings demonstrated that SIRT1 is chemosensitive to Entinostat, suggesting that this enzyme may be an important anti-cancer target by the action of Entinostat in OSCC.

Entinostat as a single agent or combined with other antitumor agents generates reactive oxygen species (ROS) in certain cancer cells lines (Gao et al., 2008). Similarly, in our study, Entinostat have been shown to increase intracellular ROS levels as single agent. Levels of intracellular ROS were significantly increased in all cells and it was observed a significant increase of superoxide in the HN6 and NOK-SI lines. Considering ROS are markers of cytotoxicity and are associated with the induction of apoptosis and cell cycle arrested, we can suggest that the antitumor effects of Entinostat on cell viability in OSCC cells can be mediated by the increased production of ROS stimulated by Entinostat.

Recent study indicated that HDACs activities are required for maintaining cancer stem cells (CSCs) in head and neck cancer (Giudice et al., 2013). HNSCC stem cells have emerged as an important factor for cancer initiation and maintenance of tumor bulk (Simple et al., 2015). CSCs also possess resistant phenotypes that evade standard chemotherapy and radiotherapy, resulting in tumor relapse. Thus, targeting of CSCs remains an attractive yet elusive therapeutic option, with the goal of increasing specificity and effectiveness in tumor eradication, as well as decreasing off-target or systemic toxicity. Researches into further characterization and targeted therapy toward head and neck CSCs is an active and rapidly evolving field (Birkeland et al., 2015). Common methods to isolate CSCs of head and neck cancer includes identifying high expression of CD44, a cell surface marker, and aldehyde dehydrogenase (ALDH), cytosolic enzyme involved in cell differentiation, detoxification and resistance to drugs by catalyzing the oxidation of aldehydes to carboxylic acids (Dionne et al., 2015). In our experiments, Entinostat demonstrated sensitivity to CSCs populations by significantly reducing these cells in all OSCC lines. In addition to the ALDH and CD44 markers used in the flow cytometric assays, the expression of BMI1, protein related to proliferation and growth of CSCs (Park et al., 2003), was also found to decrease in NOK-SI, HN6 and HN12 cells. These results suggest that chemical inhibition of histone deacetylases (HDAC) reduces the number of CSCs.

Our findings from Entinostat demonstrated that it induces histone acetylation through its HDAC inhibitory role, producing a cytotoxic effect on OSCC cells by decreasing the viability of tumor cells, although this effect was not selective for cancer cells. Entinostat promote cell cycle disruption, enhances ROS generation and reduce CSCs. This indicates that this drug may be a promising agent in the treatment of oral cancer and should be an important focus of future research on this type of tumor.

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Appendix S1 – Table 1 - List of antibodies and their respective manufacturers, used in the western blot assay

Antibody	**Manufacturer**
Acetyl-Histone H3 (Lys9) (C5B11) Rabbit mAb	Cell Signaling Technology
Acetyl-Histone H4 (Lys16) (E2B8W) Rabbit mAb	Cell Signaling Technology
SIRT1 (D1D7) Rabbit mAb	Cell Signaling Technology
Bmi1 (D42B3) Rabbit mAb	Cell Signaling Technology
PTEN (138G6) Rabbit mAb	Cell Signaling Technology
p53 7F5 Rabbit mAb	Cell Signaling Technology
Purified Mouse Anti-p21 Clone SX118 (RUO)	BD Biosciences
Goat anti-rabbit IgG-HRP: sc-2004	Santa Cruz Biotechnology
Goat anti-mouse IgG-HRP: sc-2005	Santa Cruz Biotechnology
Anti-GAPDH Mouse mAb (6C5)	EMD Millipore

4 CONSIDERAÇÕES FINAIS

Considerando toda a abordagem do estudo proposto nesta pesquisa, detalhadamente discutida em cada artigo apresentado, ressaltamos a importância da medicina personalizada para o diagnóstico, prognóstico e terapêutica do câncer de cabeça e pescoço.

A era da medicina personalizada ou medicina de precisão, em que pacientes são individualmente rastreados e combinados com drogas que visam direcionadores moleculares específicos de sua doença, foi prevista desde que os cientistas começaram a decodificar o genoma humano, nos anos 1990. Em nenhuma área tal revolução seria mais bem-vinda do que no tratamento do câncer, uma enfermidade que descreve uma infinidade de doenças causadas por uma vasta gama de mutações (Crow, 2017).

O fato de células normais tornarem-se células neoplásicas por uma sucessão de alterações genômicas, levou ao desenvolvimento de agentes moleculares que inibem as proteínas que são anormalmente ativadas como resultado de alterações genéticas somáticas (Gerlinger et al., 2012; Shaw et al., 2013). Teoricamente, esses agentes são mais específicos para as células malignas que os agentes citotóxicos que visam a replicação celular. Esses agentes molecularmente direcionados, produzem atividade antitumoral, anteriormente inédita, em tipos específicos de tumores na presença da alteração molecular correspondente (Le at al., 2015). Assim, atualmente, a promessa da medicina de precisão para o câncer está sendo realizada com a recente introdução de muitas terapias direcionadas, algumas com testes diagnósticos complementares que identificam pacientes com maior probabilidade de se beneficiarem do tratamento. A utilidade do perfil molecular do câncer para identificar aberrações tem sido sugerida por vários ensaios clínicos conduzidos em pacientes com câncer (Tsimberidou et al., 2014).

O HNSCC representa uma doença multifatorial de prognóstico ruim. Em termos de terapia personalizada, esse tumor teve avanços inferiores, comparado a outros tipos de câncer. Com a expansão e o alto rendimento dos métodos de sequenciamento, estudos recentes e dados do Atlas do Genoma do Câncer identificaram genes relevantes para a carcinogênese e a progressão do HNSCC. Os perfis e taxas de mutação variam muito, dependendo da exposição a carcinógenos, sub-regiões anatômicas e infecção pelo HPV (Thariat et al., 2015).

Como exposto aqui, estudos prévios demonstraram que a via de sinalização PI3K-AKT-mTOR desempenha um importante papel na regulação da proliferação celular e na manutenção das características biológicas das células malignas. Essa via revelou ser a mais predominantemente ativada no HNSCC, dentre a multiplicidade e variedade de alterações genéticas nessa malignidade (Wang et al., 2017). Por essa razão, o objetivo inicial da presente pesquisa foi identificar o perfil de expressão de biomarcadores proteicos de PI3K-AKT-mTOR e da proteína PTEN, potencial supressora dessa via, em uma amostra de pacientes brasileiros com HNSCC, uma vez que não encontramos na literatura estudos realizados no país que abordassem tal temática. Aprofundamos nossa análise investigando possíveis associações entre as características clínico-patológicas da amostra e o padrão de expressão da via PI3K-AKT-mTOR encontrado. Paralelamente às análises experimentais, desenvolvemos um estudo de revisão sistemática, a fim de verificar na literatura uma correlação entre a superexpressão da via do mTOR e um pior prognóstico em pacientes com HNSCC, a partir de análises univariadas e multivariadas. Além disso, também avaliamos, *in vitro,* o perfil de expressão gênica e proteica da via PI3K-AKT-mTOR em células de HNSCC.

O levantamento feito no estudo de revisão sistemática constatou que a alta expressão de proteínas da via do mTOR em tumores de HNSCC está associada à diminuição da sobrevida total e da sobrevida livre de doença dos pacientes. Apesar de não encontrar significância estatística, nossos resultados experimentais de avaliação por imuno-histoquímica demonstraram que as proteínas PI3K, AKT e mTOR apresentam-se altamente expressas na maioria dos casos avaliados e, contrariamente, observou-se baixa expressão de PTEN, na maioria dos tumores. Verificou-se associação estatisticamente significativa entre a alta expressão de AKT e a baixa expressão de PTEN. No aspecto clínico, houve relação estatisticamente significativa entre a expressão de AKT e o gênero. As análises *in vitro* também provaram presença de expressão da via nas linhagens tumorais de HNSCC estudadas.

Nossos achados do estudo da via de sinalização PI3K-AKT-mTOR corroboram sua relevância como potencial alvo terapêutico no câncer de cabeça e pescoço, devido às variações de expressão observadas. Estudos mais aprofundados sobre os mecanismos de sinalização dessa via na carcinogênese e sobre as alterações gênicas que induzem à transformação maligna em HNSCC são necessários.

Com a finalidade de explorar mais a investigação acerca da abordagem da medicina personalizada e, consequentemente, de alvos terapêuticos específicos no câncer de cabeça pescoço, nossa pesquisa abrangeu, também, o estudo da ação de reguladores epigenéticos no processo de carcinogênese.

Além de ser uma condição caracterizada por proliferação celular não controlada e instabilidade genética, o crescimento maligno de células também é determinado por desregulação epigenética. A super-regulação da atividade das enzimas histonas desacetilases (HDACs) está associada à estrutura da cromatina fechada e à subsequente repressão gênica, formando um perfil característico das células malignamente transformadas (De Souza e Chatterji, 2015). A desregulação da desacetilação de histonas por HDACs constitui um processo intimamente relacionado à oncogênese, o que faz das HDACS atrativos alvos para o desenvolvimento de novos agentes anticâncer. Nos últimos anos, inúmeros inibidores de HDAC foram testados em ensaios pré-clínicos e clínicos (Li et al., 2014).

Pela primeira vez, o presente trabalho investigou a ação do inibidor de HDAC, Entinostat, sobre o câncer oral. Assim, nosso objetivo foi analisar os efeitos *in vitro* dessa droga sobre viabilidade celular, ciclo celular, HDAC, histonas, proteínas reguladoras do ciclo celular, espécies oxigênio reativas (ROS) e células-tronco do câncer, em linhagens celulares de OSCC.

Anteriormente elucidado no manuscrito 3, os resultados alcançados nos experimentos realizados foram bastante promissores e revelam o potencial do Entinostat, como um inibidor de HDAC, na redução da proliferação de células tumorais orais. Essa droga demonstrou ser efetiva para a diminuição da viabilidade celular e induziu a interrupção do ciclo celular. O Entinostat também reduziu significativamente células-tronco tumorais, aumentou a produção de ROS e induziu maior expressão da proteína supressora tumoral p21, nas células de OSCC.

As favoráveis respostas obtidas da ação *in vitro* do Entinostat no câncer oral, sustentam a necessidade de prosseguimento dos estudos sobre a atividade dessa droga. A partir deste estudo preliminar, espera-se a continuidade de maiores pesquisas *in vitro* e posteriormente *in vivo*, para que se confirme o impacto desse inibidor de HDAC na terapia do OSCC.

Decorrentes do proposto nesta pesquisa e de todos os experimentos e análises realizados, os resultados encontrados responderam nossas hipóteses e objetivos inicialmente estabelecidos.

5 CONCLUSÕES

- O estudo de revisão sistemática constatou uma associação significativa entre a superespressão de proteínas da via do mTOR (EGFR, AKT, mTOR, 4EBP1 e p70S6K) e a redução da sobrevida total (OS) e da sobrevida livre de doença (DFS) de pacientes com HNSCC, quando comparada aos casos com ausência de expressão ou expressão baixa dessas proteínas, sugerindo que elas podem servir como biomarcadores seguros para identificar subgrupos de HNSCC com alto risco para um prognóstico ruim.

- O estudo do manuscrito 2 demonstrou alta expressão da via PI3K-AKT-mTOR e baixa expressão da proteína supressora PTEN na população brasileira estudada. Houve associação significativa entre a alta expressão da proteína AKT e a baixa expressão de PTEN, bem como entre a expressão de AKT e o gênero. Observou-se também, na análise *in vitro*, que a via PI3K-AKT-mTOR está expressa em linhagens celulares de HNSCC.

- O estudo do manuscrito 3 demonstrou que o Entinostat tem potencial citotóxico sobre linhagens celulares de OSCC, inibindo a proliferação celular e reduzindo a viabilidade celular. Significativamente, o Entinostat induziu as células à parada na fase G0/G1 do ciclo celular, como também aumentou a produção de espécies oxigênio reativas (ROS). Observou-se diminuição significativa dos níveis quantitativos de células-tronco do câncer após o tratamento com a droga. Considerando sua função inibidora de HDAC, o Entinostat reduziu os níveis de expressão da enzima HDAC SIRT1 e aumentou os níveis de expressão das histonas acetiladas H3 e H4. Além disso, demonstrou-se que essa droga estimulou o aumento da expressão da proteína p21, importante reguladora do ciclo celular.

6 REFERÊNCIAS BIBLIOGRÁFICAS

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FACULDADE DE CIÊNCIAS DA SAÚDE DA UNIVERSIDADE DE BRASÍLIA - CEP/FS-UNB

PARECER CONSUBSTANCIADO DO CEP

ANEXO – PARECER NÚMERO 410.815, 11 DE SETEMBRO DE 2013

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EXPRESSÃO IMUNO-HISTOQUÍMICA DA VIA PI3K/AKT/mTOR EM CARCINOMA ESPINOCELULAR DE CABEÇA E PESCOÇO: CORRELAÇÃO CLÍNICO-PATOLÓGICA

Pesquisador: Ana Elizia Mascarenhas Marques

Área Temática:

Versão: 2

CAAE: 16545913.4.0000.0030

Instituição Proponente: Faculdade de Ciências da Saúde da Universidade de Brasília

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer:

410.815 **Data da Relatoria:**

11/09/2013

Apresentação do Projeto:

Idem ao anterior.

Objetivo da Pesquisa:

Idem ao anterior.

Avaliação dos Riscos e Benefícios:

Foi apresentado a análise de riscos e benefícios no projeto da plataforma.

Comentários e Considerações sobre a Pesquisa:

Idem ao anterior.

Considerações sobre os Termos de apresentação obrigatória:

Idem ao anterior.

Recomendações:

Não se aplica.

Conclusões ou Pendências e Lista de Inadequações:

O orçamento no projeto da plataforma foi adequado e esclarecido de que se trata de financiamento próprio.

O cronograma foi adequado e compatibilizado nos projetos (mestrado e plataforma).

Foi corrigido o tempo verbal e também foi apresentado a análise de riscos e benefícios no projeto da plataforma.

Portanto, todas as pendências foram cumpridas.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

BRASILIA, 30 de Setembro de 2013

Assinador por:
Natan Monsores de Sá
(Coordenador)