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ATIVAÇÃO DA VIA PI3K/AKT/mTOR EM QUEILITES ACTÍNICAS E CARCINOMAS ESPINOCELULARES DE LÁBIO

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Dissertação de mestrado apresentada ao programa de Pós-graduação em Odontologia da Universidade Federal do Rio Grande do Sul como requisito à obtenção do título de mestre em Odontologia.

Área de concentração: Clínica Odontológica - Estomatologia

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“Foi o tempo
que dedicastes
à tua rosa
que a fez
tão importante.”

Antoine de Saint-Exupéry

RESUMO

ARIOTTI, Carla. **Ativação da via PI3K/AKT/mTOR em queilites actínicas e carcinomas espinocelulares de lábio.** 2019. Dissertação (Pós-graduação em Clínica Odontológica com ênfase em Estomatologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2019.

Alterações epiteliais observadas na queilite actínica (QA) e no carcinoma espinocelular (CEC) de lábio inferior foram estudadas com diferentes marcadores, a fim de observar fatores diagnósticos e prognósticos para ambas as lesões. O objetivo do estudo foi analisar a ativação do PI3K, pAkt^{Ser473} e pRPS6, importante cascata da via PI3K/Akt/mTOR, na mucosa oral normal (MON), QA e CEC de lábio comparando com aspectos clínico-demográficos e histopatológicos. Nove casos de MON, 38 de QA e 40 de CEC de lábio foram incluídos. Reações imunohistoquímicas para PI3K, pAkt^{Ser473} e pRPS6 foram realizados em todos os casos. Para cada caso, a extensão total da lesão foi analisada e um escore imunorreativo (IRS) foi designado. O IRS foi calculado multiplicando a porcentagem de células positivas (PP) (coradas 0-2) pela intensidade da coloração (SI) (coradas 0-3). O PI3K demonstrou um aumento gradual e significativo de MON, QA para CEC de lábio. O marcador pAkt^{Ser473} foi significativamente maior na QA e CEC de lábio quando comparado a MON, no entanto, não foi observada diferença entre QA e CEC de lábio. A ativação do pRPS6 diminuiu significativamente no CEC de lábio quando comparado a MON. De acordo com nossos resultados, a modificação no PI3K/Akt/mTOR esteve significativamente associada ao CEC de lábio. A identificação dessa modificação da via de sinalização no câncer labial é importante não apenas para entender o processo de carcinogênese, mas também para identificar pacientes que podem se beneficiar da terapia-alvo, especialmente aqueles com QA.

PALAVRAS-CHAVE: Câncer oral. Prognóstico. Biomarcadores. Imuno-Histoquímica.

ABSTRACT

ARIOTTI, Carla. **PI3K/AKT/mTOR pathway activation in actinic cheilitis and lip squamous cell carcinomas.** 2019. Dissertação (Pós-graduação em Clínica Odontológica com ênfase em Estomatologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2019.

Epithelial changes observed in actinic cheilitis (AC) and lower lip squamous cell carcinoma (LLSCC) have been studied using different markers in order to observe diagnostic and prognostic factors for both lesions. The aim of the study is to analyses the activation of PI3K, pAkt^{Ser473} and pRPS6, important downstream of Pi3K/Akt/mTOR pathway, in normal oral mucosa (NOM), actinic cheilitis (AC) and lower lip squamous cell carcinoma (LLSCC) comparing with clinico-demographic and histopathological aspects. Nine cases of NOM, 38 of AC and 40 of LLSCC were included. Immunohistochemical for PI3K, pAkt^{Ser473} and pRPS6 were performed in all cases. For each case, the full extent of the lesion was analyzed and an immunoreactive score (IRS) was designated. The IRS was calculated by multiplying percentage of positive cells (PP) (stained 0-2) by staining intensity (SI) (stained 0-3). PI3K demonstrated a significant gradual increase from NOM, AC to LLSCC. pAkt^{Ser473} marker was significantly higher in AC and LLSCC when compared to NOM, however, no difference was observed between AC and LLSCC. Activation of pRPS6 was significantly decreased in LLSCC when compared to NOM. According to our results modification in PI3K/Akt/mTOR were significantly associated with LLSCC. Identification of modification on this signaling pathway in lip cancer is important not only for understand the carcinogenesis process but also for identifying patients who may benefit from target therapy especially with AC.

Keywords: Lip Cancer. Actinic Cheilitis. Potentially Malignant Disorders. Biomarker. Prognosis. Immunohistochemistry.

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LISTA DE ABREVIATURAS E SIGLAS

Lista de abreviaturas e siglas da Introdução

Akt	Proteína quinase B
CCND1	Ciclina D1
CEC	Carcinoma Espinocelular
DPM	Desordem Potencialmente Maligna
EGFR	Receptor do Fator de Crescimento Epidérmico
mTOR	Proteína alvo da rampamicina em mamíferos
mTORC2	Proteína reguladora de mamíferos para rampamicina C2
PI3K	Fosfatidilinositol 3-quinase
PDK-1	Proteína dependente de 3-fosfoinosídeo quinase-1
PH	Proteínas de domínio homólogo a pleclestrina
PIP2	Fosfatidilinositol-4,5-bifosfato
PIP3	Fosfatidilinositol-3,4,5-trifosfato
QA	Queilite Actínica
S6K1	S6-quinase-1
UV	Ultra violeta

SUMÁRIO

1 INTRODUÇÃO

O carcinoma espinocelular (CEC) de cabeça e pescoço é o sexto tipo de câncer mais comum em todo o mundo, representando 2,5% de todos os novos casos de câncer e 1,9% de todas as mortes causadas por câncer anualmente (ALI *et al.,* 2017; BRAY *et al*., 2018; HUSSEIN *et al.,* 2017). Estima-se que, no mundo em 2018, ocorreram aproximadamente 400.000 casos de câncer de boca (BRAY *et al.,* 2018). No Brasil, no ano de 2017, estimava-se 11.140 casos novos de câncer de boca em homens e 4.350 em mulheres (INCA, 2016).

O sítio mais frequente do CEC em boca é o lábio, representando de 25 a 30% dos casos diagnosticados. Estas lesões ocorrem mais frequentemente em homens brancos idosos e é mais comum no lábio inferior (95% dos casos) (BIASOLI *et al.,* 2016; HAN *et al*., 2016). Os fatores de risco mais associados a este tipo de câncer são a exposição solar crônica, pois a radiação ultravioleta (UV) causa dano ao DNA, tendo assim uma ação carcinogênica, e o consumo de tabaco a longo prazo. Algumas atividades profissionais tem sido mais associadas ao desenvolvimento do CEC de lábio baseado na exposição solar. Portanto, agricultores, pescadores ou indivíduos que possuem atividade ao ar livre tem mais risco de desenvolver este tipo de câncer (BIASOLI *et al*., 2016). Assim como tem sido relatada maior incidência em pessoas residentes em países da América do Sul, como Brasil, especialmente em regiões tropicais, aonde observa-se um alto nível de radiação solar e muitos trabalhadores expostos a radiação UV por longos períodos (BIASOLI *et al*., 2016).

Clinicamente, o câncer de lábio pode se apresentar como manchas brancas, crosta ou ulceração assintomática, até úlceras extensas e lesões infiltrativas (OLIVEIRA *et al.*, 2007). Em geral, os CEC de lábio possuem baixa taxa de metástase cervical e distante. Metástase em linfonodos regionais ocorrem em estágios avançados devido a lenta taxa de crescimento geral do tumor. O local acessível das lesões favorece a ressecção da espessura total da neoplasia que é considerado tratamento curativo para a maioria dos tumores de lábio. O CEC de lábio apresenta uma alta taxa de cura nos estágios iniciais e uma baixa recorrência local em comparação com outros tumores de cabeça e pescoço (taxa de mortalidade de 10 a 15%). Em casos de câncer de lábio bem

diferenciados, observou-se uma taxa de sobrevida de 80 a 90 % em 5 anos, mostrando um melhor prognóstico em comparação com o CEC de outros sítios anatômicos da boca (BIASOLI *et al.*, 2016; CZERNINSKI *et al.*, 2010). No entanto, o tratamento pode causar deformidades em casos mais avançados, impactando na qualidade de vida do paciente.

A maioria dos CECs de lábio é precedida por queilite actínica (QA), uma desordem potencialmente maligna (DPM) que aparece como placas atróficas, erosivas ou queratóticas difusas e mal demarcadas que podem afetar algumas partes ou todo o vermelhão do lábio. A QA tem os mesmos fatores etiológios do câncer de lábio (GONZAGA *et al*., 2018; KERAWALA *et al.,* 2016; LOPES *et al.,* 2015; MELLO *et al.,* 2019). A prevalência de QA é de 12% em populações cronicamente expostas à radiação UV. Histologicamente, os achados da QA incluem hiperceratose, hiperplasia epitelial, acantose ou atrofia do epitélio podendo exibir padrões variados de displasia epitelial (HUBER, 2010; WOOD *et al.*, 2011). O tecido conjuntivo usualmente apresenta elastose solar e inflamação crônica. Não há um consenso em relação à frequência com que a QA evolui para CEC de lábio. A análise morfológica desse tipo de lesão é subjetiva e não é suficiente para prever com certeza quais lesões irão evoluir para CEC (VIEIRA *et al.,* 2012). Todavia, casos com displasia epitelial tem mostrado um risco aumentado de transformação maligna. A QA apresenta uma progressão lenta e muitas vezes imprevisível. Tem sido relatado um risco de 3.07% de transformação para CEC (DANCYNGER *et al*., 2018; GONZAGA *et al*., 2018; KERAWALA *et al.,* 2016; LOPES *et al*., 2015; MELLO *et al.*, 2019). A fotoproteção adequada deve ser a primeira e mais importante medida para prevenir a QA e também para evitar a transformação em CEC. Porém, pouca atenção foi dada no passado aos hábitos de proteção solar focados nos lábios e apenas poucos estudos investigam esse problema (RODRIGUEZ-BLANCO *et al.,* 2019).

O processo de carcinogênese envolve várias etapas, dentre elas a iniciação, promoção, progressão e manifestação clínica do tumor. A iniciação representa a fase na qual ocorre lesão ao DNA por carcinógenos. Na promoção, as células iniciadas, após a ação co-carcinógenos, são estimuladas a proliferar e passar o dano genético as células filhas até que se formem clones de células mutadas que invadam o tecido adjacente caracterizando as

neoplasias malignas (OLIVEIRA *et al.*, 2007). A carcinogênese labial está relacionada a exposição crônica e excessiva à radiação ultravioleta (UV), no qual ocorrem várias alterações genéticas e epigenéticas que levam à desregulação do ciclo celular, alterações de proteínas e transformação maligna. O modelo "patch-field" para a patogênese do câncer de lábio relata a progressão de queratinócitos normais da mucosa que acumulam mutações que levam a modificações de proteínas de maneira gradual, resultando inicialmente em QA sem ou com displasia e, no final, tornam-se um carcinoma com crescimento invasivo e metastático potencial (BOTA *et al*., 2017; DE FREITAS *et al*., 2018; LOPES *et al.,* 2019; PILATI *et al.,* 2017).

O câncer é uma doença que envolve a desregulação de vários genes supressores de tumores, oncogenes e genes de estabilidade, responsáveis por várias vias ligadas à proliferação celular, controle do ciclo celular, apoptose, angiogênese e metástase. No câncer bucal, as principais alterações genéticas descritas estão relacionadas aos genes supressores de tumores, tais como p53, p16, PTEN e Ciclina D1 (CCND1) e oncogenes, tais como o Receptor do Fator de Crescimento Epidérmico (EGFR), ras e Fosfatidilinositol 3-quinase (PI3K). Essas alterações tem sido descritas como fundamentais para a proliferação descontrolada, crescimento e tumorigênese (GARCIA-CARRACEDO *et al.,* 2016; MURUGAN *et al.,* 2013).

Embora a compreensão da carcinogênese bucal tenha crescido nos últimos anos, os eventos moleculares envolvidos na carcinogênese labial não são totalmente compreendidos. Algumas vias de sinalização têm demonstrado papel importante em diferentes tipos de câncer (LAKSHMINARAYANA *et al.,* 2018; LEEMANS *et al.,* 2018; MATSUOKA, YASHIORO, 2014; YANG *et al*., 2019). Entre eles, a fosfatidilinositol-3-quinase (PI3K) / proteína kinase B (Akt) / proteína alvo da rampamicina em mamíferos (mTOR) são essenciais para o crescimento e sobrevivência celular, em condições fisiológicas e patológicas. Eles estão tão inter-relacionados que poderiam ser considerados como um caminho único e que interage com vários outros caminhos. Alterações na via PI3K/Akt/mTOR é uma das mais frequentes em alguns tipos de DPM e câncer, incluindo câncer bucal (CHANG *et al*., 2013; GARCIA-CARRACEDO *et al.,* 2016; KISHORE *et al*., 2016; KOZAKI *et al.,* 2006; LAKSHMINARAYANA *et al.,*

2018; MARTINS *et al.,* 2016; PORTA *et al*., 2014; ROY *et al*., 2019; YANG et al., 2019).

A via PI3K constitui uma família grande e complexa dividida em três classes com múltiplas subunidades e isoformas. A PI3K é uma família de quinase lipídica intracelular que fosforila o grupo 3'- hidroxil de fosfatidilinositol das membranas celulares. A ativação consiste em catalisar a formação de fosfatidilinositol-3,4,5-trifosfato (PIP3, PhosphatidylInositol (3,4,5)-triPhosphate) a partir de fosfatidilinositol-4,5-bifosfato (PIP2, PhosphatidylInositol (4,5)-bisPhosphate), produto que transduz um sinal por interação com proteínas de domínio homólogo a pleclestrina (PH, Pleckstrin Homology). A serina-treonina quinase Akt (também conhecida como PKB) é uma proteína efetora central do PIP3. A Akt é ativada por um duplo mecanismo regulatório que requer a translocação e ancoramento na membrana plasmática através do domínio PH sendo, logo em seguida, fosforilada em seus aminoácidos treonina 308 e serina 473 pelas quinases proteína dependente de 3-fosfoinosídeo quinase-1 (PDK-1, 3-Phosphoinositide Dependent protein Kinase-1) e proteína reguladora de mamífero para rapamicina C2 (mTORC2, mammalian Target Of RapamyCin 2) respectivamente (KOZAKI *et al*., 2016).

Somente a classe I PI3K está envolvida no processo carcinogênico (MURUGAN *et al*., 2013; RODON *et al*., 2013). A isoforma PI3K classe I p110α é a mais conhecida e está implicada no processo carcinogênico. A desregulação da via PI3K/Akt/mTOR ocorre após mutações oncogênicas da PK3CA (MATSUOKA et al., 2014; MURUGAN et al., 2013). Vários fatores tem sido considerados como alvo de ligação para dar início a via (EGFR, receptor de fator derivado de plaquetas, receptor de fator de crescimento de insulina ou c-Met) ativando PI3Ks classe I. Uma vez ativada a via PI3K, ocorre a fosforilação do fosfatidilinositol para formar diferentes espécies de fosfoinositídeos com funções distintas no processo de transdução de sinal (FRUMAN et al., 2017). A seguir, várias vias diferentes podem ser simultaneamente iniciadas pela ativação do PI3K. Entre elas, os membros da Akt parecem ser universalmente ativados e são considerados como uma leitura substituta da ativação do PI3K classe I (FRUMAN *et al*., 2017). Surgem duas importantes modificações que são: fosforilação de Akt, inicialmente o loop de

ativação do AKT (Thr 308 no AKT1) e após o complexo mTOR-2 (mTORC2) que irá fosforilar a Ser 473 do AKT ($pAkt^{Ser473}$). A via PI3K desempenha um papel importante na regulação de muitas vias de sinalização celular que controlam a proliferação celular, crescimento, apoptose, sobrevivência, adesão, rearranjo citoesquelético e motilidade (MANNING, TOKER, 2017; MATSUOKA *et al.,* 2014; MURUGAN et al., 2013; RODON *et al.,* 2013).

Outro efetor importante da via PI3K/Akt é o mTOR. Compreende dois complexos de proteínas, mTORC1 e mTORC2 com composição de subunidades distinta e seletividade de substrato. O substrato direto mTORC1 é a S6 quinase-1 (S6K1) que promove o metabolismo anabólico para apoiar o crescimento e a proliferação celular. (MAGNUSON et al., 2011). A função mais importante do mTORC2 é a fosforilação e ativação de Akt (SAXTON, SABATINI, 2017).

A incidência de mutação na via PI3K em CEC de boca é relativamente baixa quando comparada com a de outros cânceres humanos, mas ocorrem em 30% a 66% (LORUSSO, 2016). Porém, estudos mostram um aumento na expressão da via PI3K-Akt em DPM, câncer bucal e em outros sítios. No câncer de mama, câncer de cabeça e pescoço, câncer hepatocelular e câncer gástrico, as mutações no PI3K foram relatadas como um evento precoce da carcinogênese (GARCIA-CARRACEDO *et al.*, 2016; MURUGAN *et al.*, 2013; QIU *et al.*, 2006; WATANABE *et al.*, 2009). No câncer de boca, essas mutações são descritas principalmente nos estágios mais avançados, sugerindo que estes são eventos tardios que pode estar envolvido em mecanismos de progressão da doença, como manutenção de tumores e metástases, ao invés de estarem envolvidos na iniciação do tumor (KOZAKI *et al.*, 2006).

A ativação da via de sinalização PI3K/Akt/mTOR tem sido considerada um evento comum na carcinogênese bucal. Alguns estudos demonstraram a modificação da proteína envolvida nessa via no câncer bucal e na DPM (CHANG *et al.,* 2013; GARCÍA-CARRACEDO *et al.,* 2018; KISHORE *et al.,* 2016; MARTINS *et al.,* 2016; MORAES *et al.,* 2019; PONTES *et al.,* 2009; PONTES *et al.,* 2015; ROY *et al.,* 2019; SILVA *et al.,* 2012). No entanto, não foram estudados na carcinogênese labial.

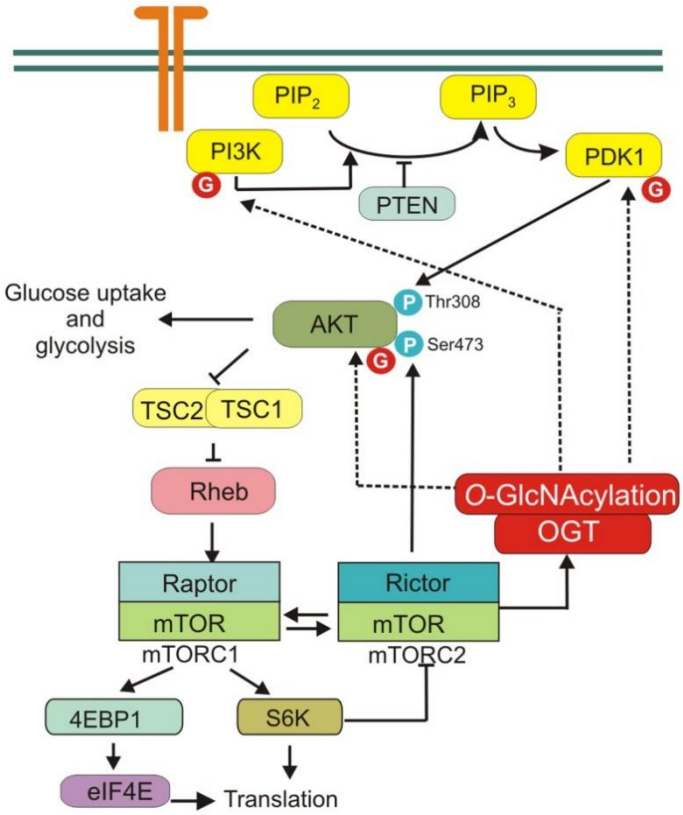

Figura 1: Ilustração da via de sinalização PI3K/Akt/m-TOR.

Fonte: (JOZWIAK *et al.*, 2014)

2 OBJETIVOS

Objetivo Geral

Avaliar a via de sinalização PI3K/Akt/mTOR em mucosa bucal normal, queilite actínica e carcinomas de lábio.

Objetivos Específicos

Correlacionar a imunomarcação do PI3K, pRPS6 (mTORC1) e do $pAkt^{Ser473}$ (mTORC2) com aspectos clínico-demográficos e histopatológicos das QA e CEC de lábio.

3 ARTIGO CIENTÍFICO

O presente artigo, intitulado “**PI3K/AKT/mTOR pathway activation in actinic cheilitis and lip squamous cell carcinomas.”** será formatado de acordo com as normas do periódico Journal of the European Academy of Dermatology and Venereology, ISSN: 1468-3083, Fator de Impacto 5.113 e Qualis A1, ao qual será submetido.

PI3K/AKT/mTOR pathway activation in actinic cheilitis and lip squamous cell carcinomas.

Keywords: lip cancer, actinic cheilitis, potentially malignant disorders, biomarker, prognosis, immunohistochemistry.

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Conflicts of interest

The authors declare that have no conflict of interest.

Abstract

Background: Epithelial changes observed in actinic cheilitis (AC) and lower lip squamous cell carcinoma (LLSCC) have been studied using different markers in order to observe diagnostic and prognostic factors for both lesions. **Objectives:** The aim of the study is to analyses the activation of PI3K, pAkt^{Ser473} and pRPS6, important downstream of Pi3K/Akt/mTOR pathway, in normal oral mucosa (NOM), actinic cheilitis (AC) and lower lip squamous cell carcinoma (LLSCC) comparing with clinico-demographic and histopathological aspects. **Methods:** Nine cases of NOM, 38 of AC and 40 of LLSCC were included. Immunihistochemical for PI3K, pAkt^{Ser473} and pRPS6 were performed in all cases. For each case, the full extent of the lesion was analyzed and an immunoreactive score (IRS) was designated. The IRS was calculated by multiplying percentage of positive cells (PP) (stained 0-2) by staining intensity (SI) (stained 0-3). **Results:** PI3K demonstrated a significant gradual increase from NOM, AC to LLSCC. pAkt^{Ser473} marker was significantly higher in AC and LLSCC when compared to NOM, however, no difference was observed between AC and LLSCC. Activation of pRPS6 was significantly decreased in LLSCC when compared to NOM. **Conclusions:** According to our results modification in PI3K/Akt/mTOR were significantly associated with LLSCC. Identification of modification in this signaling pathway in lip cancer is important not only for understand the carcinogenesis process but also for identifying patients who may benefit from target therapy especially with AC.

Keywords: lip cancer, actinic cheilitis, potentially malignant disorders, biomarker, prognosis, immunohistochemistry.

Introduction

Lower lip squamous cell carcinoma (LLSCC) is one of the most common cancers of the head and neck. It represents around 21.7% to 24.7% of all oral cancer.[1,2] Clinically, it presents as non-healing ulcerative, crusted lesions with variable invasion into underlying tissue, low rate of cervical and distant metastasis with 5-year survival rates of approximately 90% to 92% with surgical treatment. However, the treatment can cause deformity in more advanced cases impacting patient´s quality of life. The majority of LSCC is preceded by actinic cheilitis (AC), a potentially malignant disorder (PMD) that appears as diffuse and poorly demarcated atrophic, erosive or keratotic plaques that may affect some parts of, or the entire vermilion border. AC exhibits a slow and often unpredictable progression.[3-6] Nevertheless, Dancyger et al.[7] reported that 3.07% of AC suffer malignant transformation into LLSCC.

Lip carcinogenesis is a chronic and excessive ultraviolet (UV) radiation-related multistep process in which several genetic and epigenetic changes that lead to the deregulation of the cell cycle, protein alterations and malignant transformation. The “patch-field” model for lip cancer pathogenesis report the progression of normal mucosa keratinocytes that accumulates mutations which lead to proteins modifications in a stepwise manner resulting initially in AC without or with dysplasia and at the end becoming a LLSCC with invasive growth and metastatic potential. In addition, tobacco smoking habit increases the risk of LLSCC development and AC malignant transformation.[8-11]

Although the understanding of the oral carcinogenesis process has grown in recent years, the molecular events involved in lip carcinogenesis are not fully understood. Some signaling pathways have been demonstrated important role in different types of cancer.[11-14] Among them, phosphatidylinositol-3-kinase (PI3K)/ protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathways are essential to cell growth and survival, in physiological and pathological conditions. They are so interrelated that they could be considered as a single, unique pathway which interacts with several other pathways. It has emerged as one of the most frequent alterations in some types of PMD and cancers, including intra-oral cancer.[11,12,15-21] As far as we are concerned, the

expression of PI3K/mTOR pathway proteins has never been investigated in lip carcinogenesis and it's role remains entirely unclear.

PI3K comprises a large and complex family involving of three classes with multiple subunits and isoforms. Class I PI3K isoform p110α is the most well-known and have been implicated in the carcinogenic process. PI3K/Akt/mTOR pathway deregulation occur following to oncogenic mutations of PK3CA.[14,26] In head and neck squamous cell carcinomas the PI3K pathway alterations occur in 30% to 66%.[23] Multiples upstream pathways (EGFR, platelet-derived factor receptor, insulin growth factor receptor, or c-Met are responsible for the activation of class I PI3Ks. Once PI3K pathway is activated occur the phosphorylation of the phosphatidylinositol to form different phosphoinositide species with distinct roles in signal transduction.[24] Several diverging downstream pathways can be simultaneously triggered by PI3K activation. Among them, Akt members seems to be universally activated and are considered a surrogate readout of class I PI3K activation.[24] Two important Akt phosphorylation arises, initially the AKT activation loop (Thr 308 on AKT1) and after mTOR complex-2 (mTORC2) phosphorylates Ser 473 of the AKT ($pAkt^{Ser473}$). Phosphorylated Akt has been shown to promote molecular functions within cell proliferation, metabolism, survival, and motility through several downstream effectors.[14,25]

Another important effector of PI3K/Akt pathway is the mTOR. It comprises two protein complexes, mTORC1 and mTORC2 with distinct subunit composition and substrate selectivity. The direct mTORC1 substrate is S6 kinase-1 (S6K1) that promote anabolic metabolism to support cell growth and proliferation.[26] The most important function of mTORC2 is the phosphorylation and activation of Akt.[27]

The activation of PI3K/ Akt/mTOR signaling pathway have been considered as a common event in oral carcinogenesis. Some studies demonstrated modification of protein involved in this pathway in oral cancer and PMD.[15,16,18,19,21,28-31] However, they were not studied in lip carcinogenesis. Then, the aim of the present study was to analyses the activation of PI3K, $pAkt^{Ser473}$ and pRPS6, important downstream of Pi3K/Akt/mTOR pathway, in

normal oral mucosa (NOM), AC and LLSCC comparing with clinico-demographic and histopathological aspects.

Materials and Methods

This study was approved by the Ethics Committee on Human Research (approval No. 75134417800005327).

Study Population

A manual retrospective search was performed in the archives of the Pathology Laboratory at the Clinics Hospital of Porto Alegre – Brazil to identify cases of LLSCC. A total of forty cases of LLSCC were retrospectively collected and retrieved from the archives of the Head and Neck Department of the Clinics Hospital of Porto Alegre (Brazil). Clinical data were collected from patient's medical files and hospital records like age, sex, tumor location, tumor stage, tumor recurrence, follow-up, survival time (time difference between treatment and either the date of death or last follow-up) and disease-free survival (time between treatment and the date of recurrence).

Thirty-eight cases of AC were selected in the archives of Oral Pathology of the Federal University of Rio Grande do Sul, Additionally, nine cases of normal oral mucosa (NOM) obtained from mucocele specimens were retrieved in the Federal University of Rio Grande do Sul for comparison purposes.

Tissue Microarray (TMA) Construction

LLSCC specimens that were retrospectively collected were arranged into tissue microarray (TMA) blocks for immunohistochemical analysis. TMA construction was performed as previously.[32] Briefly, three representative areas of the invasive front were elected in the H&E slides of each case using an objective marker (Nikon Corp, Tokyo, Japan). A manual tissue arrayer (Sakura Co, Japan) was used to cut the respective cylindrical cores (2.0mm in diameter each) after matching the marked slides over the original paraffin block. Three cores of normal mucosa inserted in the left upper corner of each recipient block for orientation. To enable the interpretation of TMAs, a map indicating the exact position of each case was performed.

Histopathologic Analysis

For the AC cases, histological sections were stained with hematoxylin eosin (HE), and grading was based on the criteria proposed by the World Health Orgnization[33] for determination of the presence and degree of epithelial dysplasia. In LLSCC cases, histological sections were graded according to criteria described by Bryne et al. (1992)[34] which consider the tumour invasive front.

Immunohistochemistry

Immunohistochemical reactions were performed at Experimental Pathology Unit at the Hospital of Clinics at Porto Alegre (HCPA). Briefly, paraffin-embedded tissues were sectioned (3 μm) and placed on sinalized slides. Then, they were subsequently deparaffinized in xylene and hydrated in descending grades of ethanol. Antigen retrieval was performed for 18 hours in a citrate buffer solution heated to 90°C in a water bath. Endogenous peroxidase activity was blocked using 10% hydrogen peroxide in 5 baths during 5 minutes each. The slides were then incubated with the primary antibodies: PI3K Kinase p110 α (1:50, C73F8, Cell Signaling, #4249,), pAkt ^{s473} (1:100, EP2109Y, Abcam,ab81283), pRPS6 antibody (1:200, phospho S235 + S236, Abcam, ab1286). All slides were then exposed to avidin-biotin complex and horseradish peroxidase reagents (LSAB Kit; Dako Cytomation). The reactions were revealed with diaminobenzidine tetrahydrochloride (DAB; Novocastra, Newcastle, UK) and counterstained with Mayer's hematoxylin. Negative controls were obtained through incubation wuth nonimmune serum instead of primary antibodies. Positive controls for pAkt, PI3K and pRPS6, were breast cancer tissue, breast cancer tissue, human skin tissue and colon tissue respectively. Only brown citoplasmatic color regardless of the color intensity will be considered as positive marking.

Semi-Quantitave Analysis

An analysis of immunohistochemistry slides was performed blindly by two experienced and calibrated pathologists. A semi-quantitative analysis was performed and a score was established by consensus. For each case, the full extent of the lesion was analyzed and an immunoreactive score (IRS) was designated. The IRS was calculated by multiplying percentage of positive cells (PP) (stained 0-2) by staining intensity (SI) (stained 0-3). The PP was scored as

follows: 0 – 0% to 10% of stained cells; 1 – 11% to 50% of stained cells; 2 – 51% to 100% of stained cells. The SI was scored as follows: 0 – no staining; 1 – weak staining; 2 – moderate staining; 3 – strong staining.

Statistical Analysis

The immunohistochemical data were analyzed using SPSS software (IBM Corporation, Armonk, NY), version 20.0. Initially, a descriptive analysis of clinic-pathologic features was performed for LLSCC and AC. Differences in the IRS score of PI3K Kinase, pAkt ^{s473} and pRPS6 between the diagnoses (NOM, AC and LLSSC) and between different clinic-pathologic features were assessed by Kruskal-Wallis test followed by Dunn's post-hoc test adjusted for Bonferroni error correction. Spearman correlation test was used to determine the correlation of proteins expression in this cohort. For all tests, $p \leq 0.05$ was considered indicative of statistical significance.

Results

Study population

Thirty-eight cases of AC and forty cases of LLSCC were included in the present study. The clinico-demographic profile of AC and LLSCC patients is presented in Table 1. Both lesions affect mainly lower lip of white men with chronic sun and tobacco exposure. Patients with AC presented predominantly asymptomatic non-ulcerative lesions (spot, plaque, nodule) meanwhile the LLSCC patients are older and exhibited ulcerative lesions, sometimes symptomatic (Table 1). Histopathological AC examination revealed 36 cases (94.7%) classified as non-dysplastic and only 2 cases (5.3%) as dysplastic. LLSCC were classified according Bryne´s criteria (16) in Grade I (52.6%), Grade II (31.6%) and Grade III (15.8%).

PI3K and mTORC2 pathway are more activated in LLSCC

We first sought to understand the patterns of expression of PI3K, mTORC1 (pRPS6) and mTORC2 (pAkt^{Ser473}) in NOM, AC and LLSCC. We observed that all samples were positive for the three proteins evaluated (Fig. 1).

PI3K demonstrated a gradually increase from NOM, AC to LLSCC. It was significantly higher in LLSCC when compared to NOM (p=0.0003) and AC (p=0.0045). Also, there was difference between NOM and AC (p=0.047). The analysis of PI3K according to LLSCC histological classification showed similar (p=0.23) labelling among the different grades (low, intermediate and high).

The mTORC2 activation was evaluated by phosphorylation of Akt at serine 473 ($pAkt^{Ser473}$). $pAkt^{Ser473}$ marker was significantly higher in AC (p=0.0001) and LLSCC (p=0.0001) when compared to NOM. However, no difference was observed between AC and LLSCC. Additionally, no differences were observed between $pAkt^{Ser473}$ and LLSCC histological grading (p=0.34).

By the other hand, activation of mTORC1 that results in phosphorylation of RPS6 was significantly decreased in LLSCC when compared to NOM (p=0.03). pRPS6 labelling was more evidenced in morphologically more differentiated cells. Usually, the basal layer of NOM and AC, as well as, the external cells of tumor islands were less labelled or negative for pRPS6 (Fig 1). In addition, pRPS6 was significantly lower in high grade tumours compared to intermediate grade tumours (p=0.02).

The correlation among PI3K, pRPS6 and $pAkt^{Ser473}$ were analyzed. PI3K expression was significantly correlated with $pAkt^{Ser473}$. An increase in PI3K resulted in the increase of $pAkt^{Ser473}$ (rs=0.35, p=0.001). No correlation was observed among pRPS6 with PI3K (rs=0.123, p=0.262) and $pAkt^{Ser473}$ (rs=0.044, p=0.686).

There was no association of PI3K, pAkt, and PRPS6 expression with smoking status (smoker vs. never smoker: p=0.051, p=0.13, p=0,42, respectively), occupation (exposed to sun vs. non exposed, p=0.68, p=0.68, p=0.62, respectively), clinical aspect (ulcer vs others, p=0.77, p=0.27, p=0.054, respectively) TNM (stage I/II vs. stage III/IV, p=0.06, p=0.18, p=0.30, respectively) and outcome (alive vs. deceased, p=0.30, p=0.88, p=0.87, respectively).

Discussion

Modifications in PI3K/Akt/mTOR signaling pathway have been described in intra-oral carcinogenesis[35-37] and, therefore, have been considered

as possible therapeutic targets for oral cancer.[24,35,38,39] However, no previous studies evaluated the modifications of this pathway in lip carcinogenesis. Here, we analyzed the labelling of PI3K, pAkt^{Ser473} and pRPS6, important downstream of this pathway, in NOM, AC and LLSCC comparing with clinico-demographic and histopathological aspects. Our findings revealed an activation of PI3K and mTORC 2 in LLSCC and a decrease of mTORC1 activation.

Brazil is a tropical country (latitude 10.00ºS) that exhibits high levels of UV rays and several persons have occupational activities with chronic sun exposure like as agriculture, livestock, and fisheries. Especially, in Rio Grande do Sul (latitude 30.00ºS) we have a significant European colonization with predominantly Caucasian ethnicity and a high number of people working in agricultural activities. Based on that, our population has experienced several changes related to chronic sun exposure such as skin carcinomas, melanomas, and keratoses, as well as, AC and LLSCC.[40-46] The AC and LLSCC included in the present study showed clinic- demographic characteristics similar to that previously described in the literature. Both lesions occurred predominantly in males, Caucasian ethnicity, mean age 60 years and with chronic sun and cigarette exposure.[6,47,48] LLSCC patients have more ulcerated and painful lesions when compared to patients with AC. Generally, there are no metastases in the LLSCC, and few patients die due to LLSCC.

In the currently accepted model of lip carcinogenesis, the early phase of tumor progression is represented by AC that could exhibit histologically intra-epithelial architectural and cellular changes. These changes range from atrophy to hyperplasia of the squamous cell epithelium of the vermilion border, with varying degrees of keratinization, disordered maturation, increased mitotic activity and cytological atypia. Also, the underlying connective tissue presents basophilic degeneration (solar elastosis).[15,48] The LLSCC represents the invasion of connective tissue by malignant epithelial cells with different proliferative profile. The analysis of several proteins involved in the transformation of oral mucosal epithelial cells to malignant LLSCC have been investigated trying to identify biological and prognostic marker and new therapeutic possibilities.[32,47,48] In the present study we focused on PI3K/Akt/mTOR pathway. Here, we evidenced a gradual rise in PI3K (p110α

isoform) from NOM until LLSCC indicating that the increase of this protein was associated to acquisition of malignant phenotype. PI3K pathway alterations occur in 30% to 66% of head and neck squamous cell carcinomas and are also linked to advanced disease.[24] Oncogenic activation of this pathway commonly occurs through activating mutations in the p110α isoform of PI3K or through loss of the PTEN tumour suppressor.[24,50] To our surprise few studies used the immunolabeling with specific antibody for PI3K evaluation in oral cancer or oral carcinogenesis. Most of them reported modification in PI3k/Akt/mTor by the analyses of pAkt and S6 protein [20,29,31,32,37] that represents the downstream of PI3K activation. Won et al. (2012)[51] compared the expression of EGFR, PI3K, Akt, mTOR and PTEN in oral cavity squamous cell carcinoma and oropharyngeal carcinoma. PI3K labelling was similar in both types of cancer. Our results reinforce the important role of PI3K in carcinogenesis, including in lip cancer.

It is well recognized that Akt/mTOR pathway is highly upregulated in oral cancer, associate with its development and aggressive behavior. Akt regulates many proteins related to cellular processes such as cancer cell survival, proliferation, invasion, angiogenesis, and tumor metastasis. More specifically the isoforms Akt1 and 2 have been associated with oral cancer. Here, we used a specific antibody phosphorylated for AKT1 (phospho S473) which detects AKT1 phosphorylated at Serine 473 and has a high degree of similarity to the corresponding regions in AKT2 and AKT3.[19,20] Our results showed an increase of pAkt in LLSCC and AC compared to NOM. It means that AKt modification occur as an early event in lip carcinogenesis. Akt pathway is considered an important element in cell survival and proliferation after UV exposure. Bermudez et al (2015)[52] evaluated the impact of a lower dose of solar-simulated light on key proteins within the PI3K/Akt in normally sub-protected skin of healthy volunteers. Their results showed that sun exposure lead to an increase in pAkt after 5h of exposure. It can justify why AC present high levels of pAkt. Some studies reported that Akt are overexpressed in oral cancer.[19,20,32,51] However, some differences in the literature could be observed regarding the pAkt expression according to site (lip, oral cavity or oropharynx) and tissue morphological alteration (normal tissue, PMD and cancer). Similar to

our findings, Martins et al. (2016)[20] observed an increase in pAkt^{Thr308} in OSCC (excluding lip) and oral epithelial dysplasia compared to non-dysplastic oral tissue. By the other hand, our group observed an increase of pAkt^{Ser473} in OSCC compared to NOM and oral leukoplakia samples[20]. Won et al (2012)[51] showed higher labelling of pAkt^{473} in OSCC compared to oropharyngeal carcinoma. In general, cancer cells presented high expression of pAkt.

Interestingly, our results demonstrated a decrease in mTORC1 activation in LLSCC observed by lower levels of pRPS6 labelling compared to NOM. In addition, pRPS6 was significantly lower in high grade tumours compared to intermediate grade tumours. These finding agree with previous studies,[19,31] Martins et al (2016)[20] reported a decreased number of pRPS6$^{Ser240/244}$ positive cases of OSCC compared to NOM. Also, Moraes et al (2019)[32] detected similar percentage of pRPS6$^{Ser235+236}$ in NOM and oral leukoplakia and lower labelling in OSCC. By the other hand, some studies[53,54] reported higher levels of pRPS6 in oral carcinoma compared to NOM and oral dyplasia supporting the notion that pS6 activation may represent an early event in the oral carcinogenesis process.

pRPS6 immunoexpression is associated with several cellular functions, including protein synthesis, mRNA processing, glucose homeostasis, cell growth and survival. Its expression has been reported both in normal tissues, as well as benign and malignant tumors. Dillenburg et al. (2015)[55] studied PI3K-PTEN-mTOR pathway in lip keratoacanthoma that represents a well-differentiated, well-keratinized tumor that may arise from the derived from pilosebaceous unit. These tumors are characterized by rapid enlargement, maturation of the lesion, and spontaneous regression. They reported a strong positivity for pRPS6 but did not have the same labelling for pAkt^{Ser473}. These authors supported the idea that mTORC1 but not mTORC2 is associated to KA suggesting the absence of pAkt^{Ser473}could be related to low invasive tumor characteristics and the increase of pRPS6 was related to tumor differentiation pattern. Another possible explanation for the reduction in pRPS6 labelling is that mTORC1 is also involved in the initiation of negative feedback regulation of growth factor receptor signaling. So, the inhibition of mTORC1 or pS6K1 leads to elevated activation of PI3K, AKT and the ERK pathway[15,56] In addition,

mTORC1 inhibition also induces autophagy, which can help maintain cancer cell survival in tumor microenvironment with poorly vascularization and nutrition.[56]

According to our results modification in PI3K/Akt/mTOR were significantly associated with LLSCC. Identification of this signaling pathway modification in lip cancer is important not only for understand the carcinogenesis process but also for identifying patients who may benefit from target therapy especially with AC. Some research have been published using topic treatment with chemotherapeutic agents in AC with the drugs like imiquimod, 5- fluorouracil (5-FU), and ingenol mebutate.[57,58] Several PI3K/Akt/mTOR target drugs are available and their use as topic treatment for AC might be investigated.

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Conflict of interest – The authors declare that have no conflict of interest.

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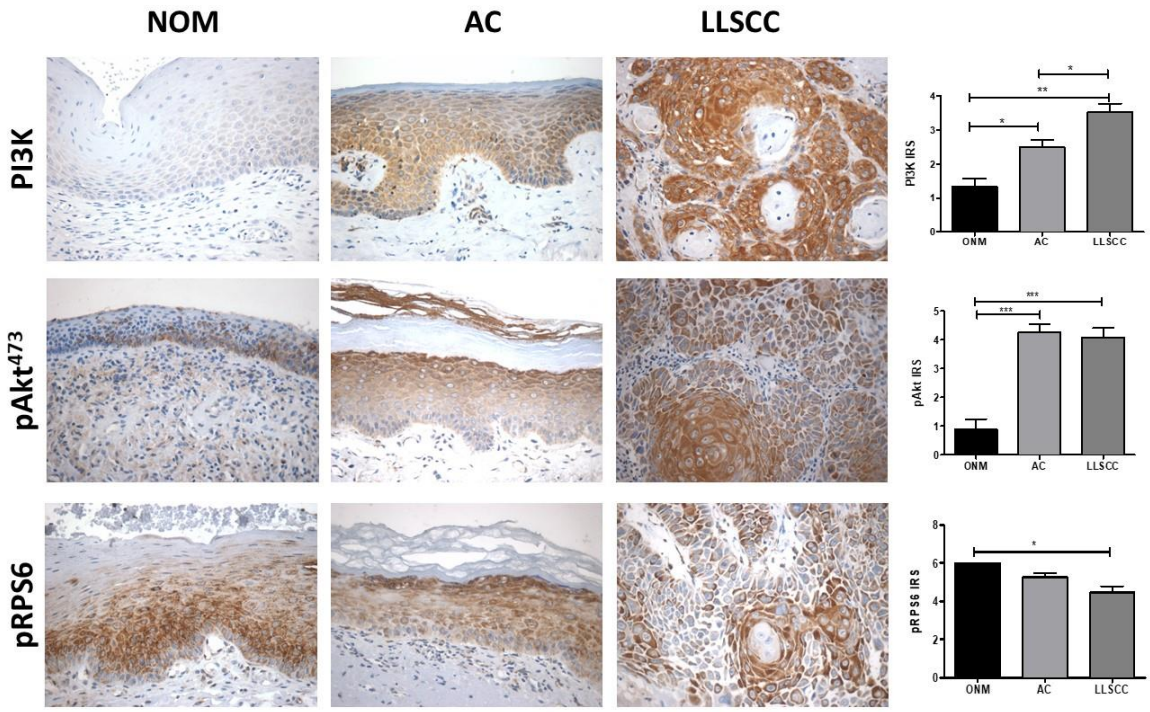
Table 1. Clinico-demographic features of AC and LLSCC patients included in the present study.

Demographic/ Clinical characteristics	AC Frequency (%)	LLSCC Frequency (%)	*p value*
Gender			
Male	89.47%	82.5%	0.376
Female	10.53%	17.5%	
Age (mean ± SD)	58.26 (±8.03)	63.27 (±13.47)	**0.02**
Skincolor			
White	93.3%	100%	0.18
Others	6.7%	0%	
Occupation			
Exposed to the sun	57.14%	84.61%	0.14
Not exposed to the sun	42.86%	15.39%	
Tobacco user			
Yes/former user	70%	66.7%	0.50
Never	30%	33.3%	
Pain			
Yes	0%	34.48%	**0.001**
No	100%	65.52%	
Clinical Aspect			
Ulcer	11.4 %	89.7%	**<0.001**
Spot/Plaque/Nodule	88.6%	10.3%	
Site			
Lower Lip	100%	95%	0.327
Upper Lip	0%	5%	
TNM			
I/II	-	61.5%	-
III/IV	-	38.5%	
Treatment			
Surgery	-	92.3%	-
Others*	-	7.7%	
Recurrence			
Yes	-	5.26%	-
No	-	94.73%	
Outcome			
Alive	-	92.3%	-
Deceased	-	7.7%	

Figure 1. PI3K/Akt/mTOR pathway in NOM, AC and LLSCC. Representative images of immunohistochemical labeling of PI3K, pAkt^{Ser473} and pRPS6 in NOM, AC and LLSCC (original magnification, x400). Mean expression of PI3K, pAkt^{Ser473} and pRPS6 in NOM, AC and LLSCC.



4 CONSIDERAÇÕES FINAIS

A ativação da via de sinalização PI3K/Akt/mTOR tem sido considerada um evento comum na carcinogênese. No câncer de boca essa via tem sido identificada principalmente nos estágios mais avançados, sugerindo que este é um evento tardio que pode estar envolvido em mecanismos de progressão da doença, como manutenção de tumores e metástases, ao invés de estar envolvido na iniciação do tumor. Nosso estudo foi o primeiro a avaliar o papel desta via na carcinogênese labial e sua associação com as características clínico-demográficas. Nossos principais resultados mostram um aumento gradual da immunomarcação do PI3K, quando comparamos MON, AC e CEC de lábio. O $pAkt^{Ser473}$ apresentou um aumento significativo de MON para AC, porém sem diferença estatisticamente significativa. Essa característica de immunomarcação de PI3K e $pAkt^{Ser473}$ nos mostra que suas ativações ocorrem como um evento precoce da carcinogênese labial.

A ativação do pRPS6 diminuiu significativamente do CEC de lábio para a MON e sua marcação foi mais evidenciada em células morfologicamente mais diferencias. Estes resultados merecem ser mais investigados.

De acordo com nossos resultados, a modificação no PI3K/Akt/mTOR esteve significativamente associada ao CEC de lábio. A identificação dessa modificação da via de sinalização no câncer labial é importante não apenas para entender o processo de carcinogênese, mas também para identificar pacientes que podem se beneficiar da terapia-alvo, especialmente com QA. Algumas pesquisas foram publicadas usando o tratamento tópico com agentes quimioterápicos na QA com medicamentos como imiquimod, 5- fluorouracil (5-FU) e mebutato de ingenol. Vários fármacos alvo de PI3K / Akt / mTOR estão disponíveis e seu uso como tópico tratamento para CA pode ser investigado.

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