UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Cláudio Marcelo Cardoso

Avaliação de marcadores de hipóxia e proteômica em neoplasias benignas e malignas de glândula salivar

Montes Claros

2023

Cláudio Marcelo Cardoso

Avaliação de marcadores de hipóxia e proteômica em neoplasias benignas e malignas de glândula salivar

Tese apresentada ao Programa de Pós-graduação em Ciências em Saúde (PPGCS) da Universidade Estadual de Montes Claros (Unimontes), como parte das exigências para a obtenção do título de Doutor em Ciências da Saúde.

Área de Concentração**:** mecanismos e aspectos clínicos das doenças.

Orientador: Professor Dr. André Luiz Sena Guimarães

Montes Claros

2023

Cardoso, Cláudio Marcelo.

C268a Avaliação de marcadores de hipóxia e proteômica em neoplasias benignas e malignas de glândula salivar [manuscrito] / Cláudio Marcelo Cardoso – Montes Claros (MG), 2023.

45 f. : il.

Bibliografia: f. 41-45.

Tese (Doutorado) - Universidade Estadual de Montes Claros - Unimontes, Programa de Pós-Graduação em Ciências da Saúde /PPGCS, 2023.

Orientador: Prof. Dr. André Luiz Sena Guimarães.

1. Glândulas salivares - Doenças. 2. Tumores. 3. Hipóxia. 4. Marcadores biológicos de tumor. 5. Imuno-histoquímica. 6. Reação em cadeia de polimerase. I. Guimarães, André Luiz Sena. II. Universidade Estadual de Montes Claros. III. Título.

Catalogação Biblioteca Central Professor Antônio Jorge

UNIVERSIDADE ESTADUAL DE MONTES CLAROS-UNIMONTES

Reitor: Professor Dr. Wagner de Paulo Santiago

Vice-reitor: Professor Dr. Dalton Caldeira Rocha

Pró-reitor de Pesquisa: Professora Dra. Maria das Dores Magalhães Veloso

Coordenadoria de Acompanhamento de Projetos: Professor Dr. Otávio Cardoso Filho

Coordenadoria de Iniciação Científica: Professora Dra. Sônia Ribeiro Arrudas

Coordenadoria Inovação Tecnológica: Professora Dra. Sara Gonçalves Antunes de Souza

Pró-reitor de Pós-graduação: Professor Dr. Marlon Cristian Toledo Pereira

Coordenadoria de Pós-graduação *lato sensu*: Professor Dr. Cristiano Leonardo de Oliveira Dias

Coordenadoria de Pós-graduação *stricto sensu*: Professor Dr. Carlos Alexandre Bortolo

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Coordenadora: Professora Dra.Cristina Andrade Sampaio

Coordenador Adjunto: Professor Dr. Renato Sobral Monteiro Júnior

GOVERNO DO ESTADO DE MINAS GERAIS

Universidade Estadual de Montes Claros

Mestrado e Doutorado em Ciências da Saúde

Anexo nº Folha Aproação Tese CLÁUDIO MARCELO CARDOSO/UNIMONTES/PRPG/PPGCS/2023

PROCESSO Nº 2310.01.0002312/2023-09

FOLHA DE APROVAÇÃO

Data da Defesa: 13/02/2023 - webconferência, via plataforma “Meet”

NOME DO(A) DISCENTE: CLÁUDIO MARCELO CARDOSO

( ) Mestrado Acadêmico em Ciência Da Saúde

(x) Doutorado Acadêmico em Ciências Da Saúde

TÍTULO DO TRABALHO DE CONCLUSÃO DE CURSO (TCC):

"*AVALIAÇÃO DE MARCADORES DE HIPÓXIA E PROTEÔMICA EM NEOPLASIAS BENIGNAS E MALIGNAS DE GLÂNDULA SALIVAR* "

ÁREA DE CONCENTRAÇÃO: Mecanismos e aspectos clínicos das doenças

LINHA DE PESQUISA: Etiopatogenia e Fisiopatologia das Doenças

BANCA (TITULARES)

Prof. Dr. André Luiz Sena Guimarães ORIENTADOR (participação à distância por videoconferência)

Prof. Dr. Alfredo Maurício Batista de Paula (participação à distância por videoconferência)

Prof. Dr. Sergio Henrique Sousa Santos (participação à distância por videoconferência)

Prof. Dr. Gefter Thiago Batista Corrêa (participação à distância por videoconferência)

Prof. Dr. Alexandre Andrade Sousa (participação à distância por videoconferência)

BANCA (SUPLENTES)

Prof.ª Dr.ª Lucyana Conceição Farias

Prof. Dr. João Marcus Oliveira Andrade

Prof. Dr. Antônio Sérgio Barcala Jorge

Prof. Dr. Edimilson Martins de Freitas

Prof. Dr. Danillo Costa Rodrigues

Prof. Dr. Diego Vicente da Costa

A análise realizada pelos membros examinadores da presente defesa pública de TCC teve como resultado parecer de:

[X] **APROVAÇÃO** [ ] **REPROVAÇÃO**

Documento assinado eletronicamente por **André Luiz Sena Guimarães**, **Professor(a)**, em 13/02/2023, às 15:10, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 47.222, de 26 de julho de 2017.

Documento assinado eletronicamente por **Alexandre de Andrade Sousa**, **Usuário Externo**, em 13/02/2023, às 19:56, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 47.222, de 26 de julho de 2017.

Documento assinado eletronicamente por **Gefter Thiago Batista Corrêa**, **Usuário Externo**, em 14/02/2023, às 09:34, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 47.222, de 26 de julho de 2017.

Documento assinado eletronicamente por **Alfredo Mauricio Batista De Paula**, **Professor(a)**, em 16/02/2023, às 15:35, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 47.222, de 26 de julho de 2017.

Documento assinado eletronicamente por **Sergio Henrique Sousa Santos**, **Professor(a)**, em 23/02/2023, às 19:51, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 47.222, de 26 de julho de 2017.

A autenticidade deste documento pode ser conferida no site http://sei.mg.gov.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **60602218** e o código CRC **2DE31B80**.

Referência: Processo nº 2310.01.0002312/2023-09 SEI nº 60602218

DEDICATÓRIA

Dedico este trabalho ao Grande Arquiteto do Universo, inteligência suprema que me guiou até aqui.

Aos meus pais Santo e Mara pelos exemplos de amor e dedicação.

À minha esposa Cristiane e filhas Brenda, Julia e Sofia por darem sentido a todo esforço.

AGRADECIMENTOS

Agradeço imensamente ao meu orientador Prof. Dr. André Luiz Sena Guimarães pela paciência e generosidade em compartilhar seu conhecimento.

Não poderia me furtar a agradecer aos meus colegas de pós-graduação, em especial a Sabrina, Marcela, Eliane, Eloá e Lílian, verdadeiros anjos, sem os quais este trabalho não seria possível.

“o homem é pássaro cativo fadado a grandes voos”[1]

[1] Joana De Ângelis

RESUMO

As neoplasias malignas de glândula salivar são responsáveis por aproximadamente 3%-6% de todos os cânceres de cabeça e pescoço mundialmente e constituem um amplo grupo, histologicamente diverso. Estas neoplasias costumam ter respostas pobres aos tratamentos com quimioterapia e radioterapia. De uma maneira geral, neoplasias malignas bem oxigenadas mostram melhor resposta à radiação e são, portanto, mais radiossensíveis do que as pobremente oxigenadas. A hipóxia é um poderoso estímulo celular que induz a via de sinalização dedicada à adaptação ambiental e está associada a mau prognóstico no câncer. A principal proteína da via da hipóxia é o fator induzido por hipóxia (HIF-1α), que é um fator de transcrição que tem como alvo uma série de genes de adaptação sob condições hipóxicas, dentre eles o miR-210, que é um microRNA regulado positivamente no microambiente hipóxico. Esta tese de doutorado teve como objetivo investigar os marcadores de hipóxia HIF-1α e miR-210 através de imuno-histoquímica e reação em cadeia da polimerase em tempo real (qPCR) em neoplasias benignas e malignas de glândula salivar, além de buscar outros marcadores tumorais nestas neoplasias, através de estudo proteômico. Como resultado, foram observados níveis de expressão similares de HIF-1α e miR-210 entre neoplasias benignas e malignas de glândula salivar, assim como também não foram observadas diferenças significativas na expressão do miR-210 entre neoplasias de glândula salivar e tecido salivar não neoplásico. A proteoma associada à análise com a base de dados Gene Expression Omnibus (GEO) mostrou que a proteína anexina A2 (ANXA-2) foi a mais expressa entre as neoplasias malignas de glândula salivar. Consideramos que os marcadores de hipóxia HIF-1α e miR-210 não parecem diferenciar neoplasias benignas e malignas de glândula salivar. Consideramos ainda que a ANXA-2 pode ser um marcador tumoral de interesse em neoplasias malignas de glândula salivar. O baixo número de amostras é uma limitação do estudo.

Palavras-chave: Neoplasias De Glândula Salivar . Hipóxia . HIF-1α . Mir-210 . ANXA-2 .

ABSTRACT

Malignant salivary gland neoplasms account for approximately 3%-6% of all head and neck cancers worldwide and constitute a large, histologically diverse group. These neoplasms usually have poor responses to chemotherapy and radiotherapy treatments. In general, well-oxygenated malignancies show a better response to radiation and are therefore more radiosensitive than poorly oxygenated ones. Hypoxia is a powerful cellular stimulus that induces the signaling pathway dedicated to environmental adaptation and is associated with poor prognosis in cancer. The main protein of the hypoxia pathway is the hypoxia-induced factor (HIF-1α), which is a transcription factor that targets a series of adaptation genes under hypoxic conditions, including miR-210, which is a microRNA up-regulated in the hypoxic microenvironment. This doctoral thesis aimed to investigate the hypoxia markers HIF-1α and miR-210 through immunohistochemistry and real-time polymerase chain reaction (qPCR) in benign and malignant salivary gland neoplasms, in addition to looking for other tumor markers in these neoplasms, through proteomic study. As a result, similar expression levels of HIF-1α and miR-210 were observed between benign and malignant salivary gland neoplasms, as well as no significant differences were observed in the expression of miR-210 between salivary gland neoplasms and non-neoplastic salivary tissue. . The proteome associated with the analysis with the Gene Expression Omnibus (GEO) database showed that the protein annexin A2 (ANXA-2) was the most expressed among malignant salivary gland neoplasms. We consider that the hypoxia markers HIF-1α and miR-210 do not seem to differentiate between benign and malignant salivary gland neoplasms. We also consider that ANXA-2 may be a tumor marker of interest in malignant salivary gland neoplasms. The low number of samples is a limitation of the study.

Keywords: Salivary Gland Neoplasms . Hypoxia . HIF-1α . Mir-210 . ANXA-2 .

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIATURAS

HIF-1α	Fator induzido por hipóxia 1α
miR-210	MicroRNA 210
qPCR	Reação em cadeia da polimerase em tempo real
GEO	Gene Expression Omnibus
ANXA-2	Anexina A-2
NGSs	Neoplasias de glândula salivar
WHO	World Health Organization
OMS	Organização Mundial de Saúde
PLAG1	Pleomorphic adenoma gene 1
HMGIC	High-mobility-group protein gene IC
CRTC1	CREB Regulated Transcription Coactivator 1
MECT1-MAML2	Mucoepidermoid carcinoma translocated gene 1-mastermind-like gene family
HER2	Human Epidermal Growth Factor Receptor 2
MYB	MYB Proto-Oncogene
NFIB	Nuclear Factor I B
MiRNA	MicroRNA
RNA	Ácido ribonucleico
RISC	Complexo silenciador induzido por RNA
MEC	Carcinoma mucoepidermoide
EGFR	Epidermal Growth Factor Receptor
ACC	Carcinoma adenoide cístico
AVE	Acidente vascular encefálico
IAM	Infarto agudo do miocárdio
GLUT1	Transportador de glicose 1

PGK1	Fosfoglicerato quinase 1
VEGF	Fator de crescimento do endotélio vascular
LOX	Lisil oxidase
CXCR4	Receptor de quimiocina 4
OPN	Osteopontina
TC	Tomografia computadorizada
RM	Ressonância magnética
PET-CT	Tomografia por emissão de pósitrons - Tomografia computadorizada
PAAF	Punção aspirativa por agulha fina
MS	Espectrometria de massa

SUMÁRIO

1 INTRODUÇÃO

1.1 EPIDEMIOLOGIA

As neoplasias de glândula salivar (NGSs) constituem um amplo número de entidades, histologicamente diversas, com histórias naturais variáveis, sendo a vasta maioria de origem epitelial e amplamente categorizadas em neoplasias benignas e neoplasias malignas de baixo e alto grau. NGSs são responsáveis por aproximadamente 3%-6% de todas as neoplasias malignas de cabeça e pescoço mundialmente. (ANDREASEN et al., 2019; MANTRAVADI; MOORE; RASSEKH, 2019; PEDERSON et al., 2011). O número de tipos de carcinoma aumentou de 5 na classificação da Organização mundial de saúde de 1972 para 22 na edição de 2017 e no mesmo período, o número de neoplasias benignas aumentou de 4 para 11, com alguns subtipos descritos (Tabela 1) (ANDREASEN et al., 2019; EL-NAGGAR, 2017; LIN; LIMESAND; ANN, 2018).

Entre todas as neoplasias de glândula salivar, a glândula parótida é a localização mais comum. Embora os tumores da glândula parótida sejam geralmente benignos, a frequência global faz com que este seja o sítio mais comum das neoplasias malignas (MANTRAVADI et al., 2019). As NGSs ocorrem em uma frequência de 100 neoplasias da glândula parótida para cada 10 de glândula submandibular e aproximadamente 1 de glândula sublingual. Neoplasias de glândula salivar menor ocorrem com aproximadamente a mesma frequência das de submandibular e podem ocorrer na cavidade oral, faringe ou em restos de glândula salivar menor no espaço parafaríngeo. Aproximadamente 20% das NGSs que surgem na parótida, 50% das de submandibular e 47%-87% das de glândula salivar menor são malignas (ENEROTH, 1971; LUNA, 1986; MANTRAVADI et al., 2019; SPIRO, 1986). A maioria das neoplasias de glândula salivar menor surge no palato, principalmente próximo à transição dos palatos mole e duro, devido à alta concentração destas glândulas nesta localização. Neoplasias de glândula salivar menor que surgem no espaço parafaríngeo, são menos comumente malignas do que as que surgem em glândulas salivares menores submucosas (COPELLI et al., 2008).

A distribuição das NGSs entre os sexos é bastante uniforme, com uma proporção de 1,04:1 entre homens e mulheres e com idade variando dos 12 aos 85 anos, com média de 47 anos (ETIT et al., 2012).

Classificação histológica das neoplasias de glândulas salivares – OMS

Neoplasias epiteliais malignas	Neoplasias epiteliais benignas
Carcinoma de células acinares	Adenoma pleomórfico
Carcinoma mucoepidermoide	Mioepitelioma
Carcinoma adenoide cístico	Adenoma de células basais
Adenocarcinoma polimórfico de baixo grau	Tumor de Warthin
Carcinoma epitelial-mioepitelial	Oncocitoma
Carcinoma de células claras, sem outras especificações	Adenoma Canalicular
	Adenoma sebáceo
Adenocarcinoma de células basais	Linfo-adenoma
Carcinoma sebáceo	Papiloma ductal
Linfadenocarcinoma sebáceo	Cistadenoma
Cistadenocarcinoma	
Cistadenocarcinoma cribiforme de baixo grau	
Adenocarcinoma mucinoso	
Carcinoma oncocítico	
Carcinoma de ducto salivar	
Adenocarcinoma sem outras especificações	
Carcinoma mioepitelial	
Carcinoma ex-adenoma pleomórfico	
Carcinosarcoma	
Adenoma pleomórfico metastático	
Carcinoma de células escamosas	
Carcinoma de células pequenas	
Carcinoma de células gigantes	
Carcinoma linfoepitelial	
Sialoblastoma	
Carcinoma secretor análogo ao tecido mamário	

Tabela 1: Classificação histológica dos tumores de glândulas salivares de acordo com a Organização Mundial de Saúde (OMS). 2017 (EL-NAGGAR AK., 2017).

1.2 FISIOPATOLOGIA E HISTOPATOLOGIA

O limitado número de casos, associado à escassa disponibilidade de linhas celulares e modelos animais autenticados, traduzem a restrita compreensão da fisiopatologia do câncer de glândula salivar (WARNER et al., 2013). A análise citogenética das NGSs tem revelado translocações cromossômicas nos pontos de interrupção 8q12, 3p21 e 12q13-155 correspondentes aos genes *PLAG1*, β-*catenina* e *HMGIC,* respectivamente. Há evidência de que o *PLAG1* é um proto-oncogene na tumorigênese destas neoplasias (DECLERCQ et al., 2005). Evidências sugerem que uma nova classe de moléculas chamadas microRNAs (miRNA) desempenham um papel na tumorigênese humana. MiRNAs são pequenas sequencias de RNA não codificantes, de 19 a 25 nucleotídeos que estão envolvidos em diversos processos celulares tais como a diferenciação, proliferação e sobrevivência através da ligação de sequências complementares na região 3’ não traduzida de múltiplos no RNA mensageiro (mRNA) alvo, levando à sua degradação ou repressão da tradução. São inicialmente transcritos como parte de transcritos primários muito mais longos (pri-miRNAs). No núcleo, este transcrito primário é primeiro processado através de um tipo de RNA III endonuclease Drosha a pré-miRNA, que tem de 60 a 70 nucleotídeos de comprimento. Posteriomente, ocorre o transporte do pré-miRNA do núcleo para o citoplasma através da 5 Exportina e a atuação de outra RNA III endonuclease Dicer, com a geração do miRNA maduro. O miRNA maduro forma um complexo RNA-proteína conhecido como Complexo silenciador induzido por RNA (RISC) com proteínas da família Argonauta. Este complexo regula a expressão gênica através da inibição da tradução ou através da degradação de alvos no RNA mensageiro (Figura 1) (HONG et al., 2012; HUANG; LE; GIACCIA, 2010; MATSE et al., 2013; NOHATA et al., 2013; PALMERO et al., 2011).

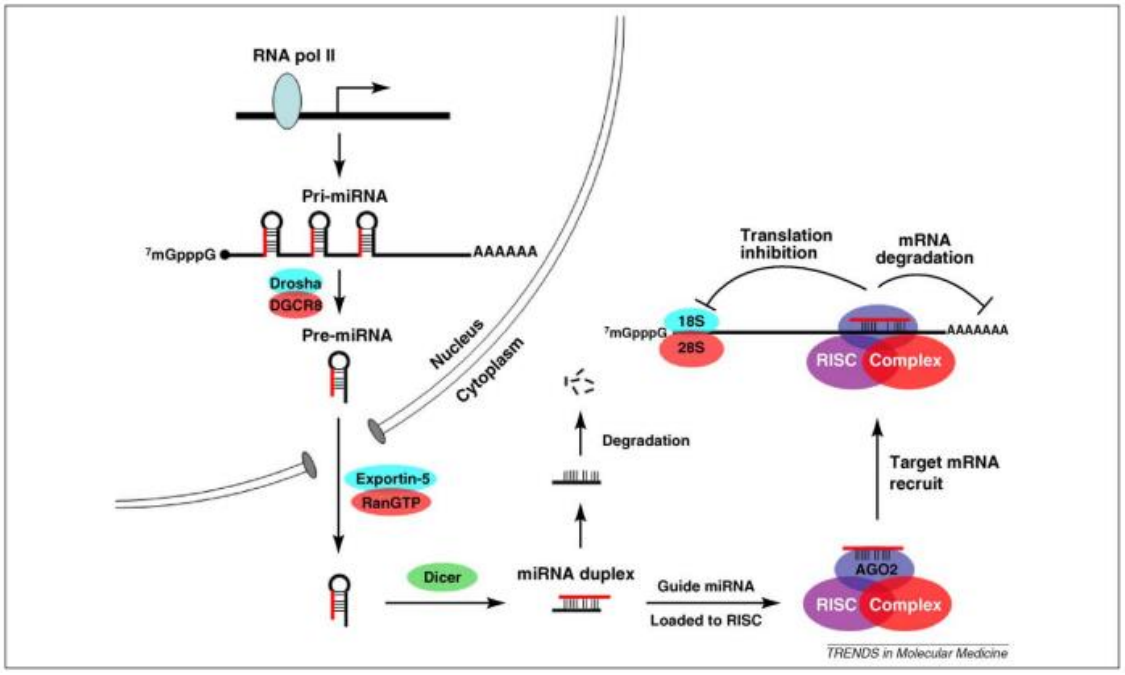


Figura 1: Visão esquemática da biogênese do miRNA e vias de funcionamento. Os genes que codificam miRNAs são transcritos em pri-miRNAs pela RNA polimerase II (RNA Pol II). O pri-miRNA é processado pela primeira vez pela RNA endonuclease Drosha tipo III em pré-miRNAs que têm 60 a 70 nucleotídeos de comprimento e têm uma estrutura haste-alça. O pré-miRNA é exportado para fora do núcleo pela exportina-5 e é posteriormente processado por outra endonuclease de RNA tipo III Dicer para gerar a fita de miRNA maduro de 22 nucleotídeos em de comprimento. Esta é então ligada a proteínas da família Argonauta formando o Complexo silenciador induzido por RNA (RISC). O RISC regula a expressão gênica através da inibição da tradução de RNA ou degradação do RNA mensageiro (HUANG et al., 2010).

Adenoma pleomórfico é a neoplasia de glândula salivar mais comum, representando aproximadamente 50% de todos os casos e 70% daquelas que surgem na parótida. A taxa de recorrência é relatada em mais de 15%. Apesar de ser uma neoplasia benigna, a transformação maligna para carcinoma ex-adenoma pleomórfico é incomum, mas bem descrita e ocorre em 3,3%-8,5% dos casos, com o risco aumentando com o tempo e a idade do paciente (ANDREASEN et al., 2016; ZHANG et al., 2009).

Estudo revelou um aumento na expressão de componentes da maquinaria de processamento de miRNA (Dicer, Drosha, DGCR8 e p68) no adenoma pleomórfico sugerindo que pode haver desregulação da expressão de miRNAs em resultado do aumento de sua biogênese. Identificou um conjunto de 22 miRNAs diferencialmente expressos entre esta neoplasia e tecidos normais das glândulas salivares, com maior destaque para membros como hsa-miR-376a, hsa-miR-301 e hsa-miR-21, que é uma

regulação positiva comum em outros tumores sólidos. O oncogene *PLAG1* foi superexpresso e pode ser potencialmente regulado por esses miRNAs (ZHANG et al., 2009).

O tumor de Warthin é a segunda neoplasia benigna mais comum das glândulas salivares, mais comum na parótida e está quase sempre associado ao tabagismo.

O carcinoma mucoepidermoide (MEC) é a neoplasia maligna primária de glândula salivar mais comum tanto em adultos como em crianças, representando 30 a 35% de todas estas neoplasias, acometendo principalmente as glândulas salivares maiores. É caracterizado por componente produtor de mucina, tipo intermediário e células escamoides com padrão de crescimento sólido e cístico (CHIOSEA et al., 2008; LIN et al., 2018).

Radiação ionizante pode ser um fator de risco para estas neoplasias e um intervalo de 15 anos ou mais tem sido visto entre a exposição e a sua manifestação (BOUKHERIS et al., 2008; MANTRAVADI et al., 2019).

A fusão do gene *CRTC1* (ou *MECT1-MAML2*) é identificada em um grande número de MEC e pode estar associado a neoplasias de baixo grau e um prognóstico favorável. Em contraste, a amplificação do gene de *HER2* ou um número aumentado de cópias do gene de *HER2* ou o receptor do fator de crescimento epidérmico (EGFR) está associado a MEC de alto grau (ALAMRI et al., 2018; LIN et al., 2018; WARNER et al., 2013).

Parece haver uma maior frequência de expressão anormal do Dicer em MEC de alto grau e estádios avançados quando comparado aqueles de graus baixo/intermediário e estádios iniciais. Quase dois terços de MEC mostram expressão anormal de Dicer e sua imunorreatividade anormal (superexpressão e subexpressão combinadas ou separadamente) está correlacionada com pior sobrevida (CHIOSEA et al., 2008).

O carcinoma adenoide cístico (ACC) compreende aproximadamente 10% das neoplasias de glândula salivar e 21-24% de todas as malignidades destas glândulas, em humanos. É caracterizado por crescimento lento e persistente, invasão local, crescimento perineural e metástase à distância precoce (LIN et al., 2018).

A translocação do proto-oncogene *MYB* com fator de transcrição NFIB, como demonstrado pela transcrição de fusão *MYB-NFIB*, foi detectada em 86% das amostras de ACC em um estudo (DECLERCQ et al., 2005).

A expressão de dois miRNAs (miR-4487 e miR-4430) foi significativamente regulada positivamente em linha de células de ACC com características de maior agressividade,

enquanto a expressão de dois outros miRNAs (miR-5191 e miR-3131) foi significativamente regulada negativamente nestas mesmas células (CHEN et al., 2014).

1.2.1 HIPÓXIA

A maioria dos mamíferos vive em uma atmosfera composta por 21% de oxigênio, que é considerado um ambiente fisiológico. No entanto, existem algumas circunstâncias em que a célula pode se tornar hipóxica, isto é, submetida a uma baixa tensão de oxigênio. Por exemplo, um organismo pode ser exposto a condições extremas tais como alta altitude. Acidente vascular encefálico (AVE) e infarto agudo do miocárdio (IAM) são exemplos de doenças isquêmicas. A tensão de oxigênio também é fortemente reduzida no câncer, principalmente por causa da arquitetura caótica dos vasos sanguíneos que leva a uma menor perfusão e proliferação intensa das células cancerosas que se desenvolvem distantes dos vasos sanguíneos (SEMENZA, 2003).

A hipóxia é rara em tecido normal, mas é característica comum em tecidos neoplásicos, onde é detectada em aproximadamente 50% de todas as neoplasias sólidas, independentemente do tamanho ou de características histológicas (HOOGSTEEN et al., 2007). Ela exerce papel na promoção de instabilidade genética, invasividade das células neoplásicas, metástases e está associada a fenótipos malignos mais agressivos e resultados clínicos adversos (BRIZEL et al., 1997; JONATHAN et al., 2006; WIJFFELS et al., 2009).

Em 1955, Thomlinson analisando cortes histológicos de neoplasias de pulmão, levantou a hipótese de que a radio-resistência destas neoplasias estava associada a um gradiente decrescente na tensão de oxigênio entre a periferia e o centro de cada cordão tumoral (THOMLINSON; GRAY, 1955).

As neoplasias de glândula salivar são geralmente consideradas menos sensíveis à radioterapia e à quimioterapia e tem sido sugerido que a hipóxia pode ser um fator causal relacionado a esta alegada radio-resistência, porém, qual o papel que ela exerce neste tipo de tumor é desconhecido (WIJFFELS et al., 2009).

A principal proteína da via da hipóxia é o fator induzido por hipóxia (HIF), que é um fator de transcrição que tem como alvo uma série de genes de adaptação sob condições hipóxicas. É um hetero-dímero composto por uma subunidade α, regulatória, e uma subunidade β, constitucional (Figura 2) (BRAHIMI-HORN; POUYSSÉGUR, 2007).

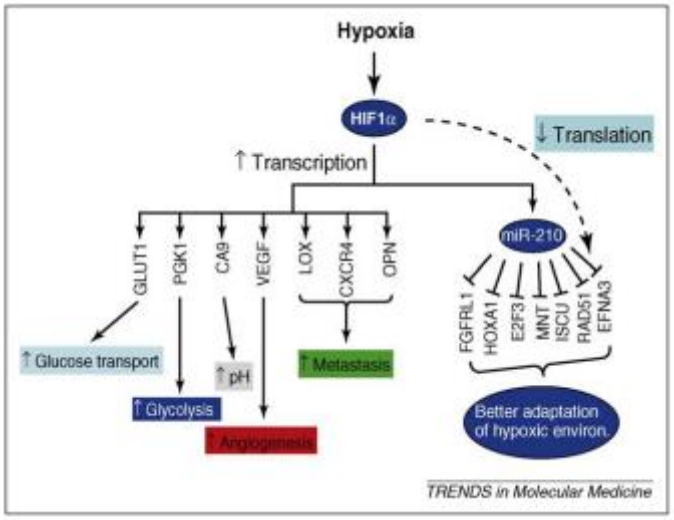


Figura 2: HIF-1α regula um amplo espectro de genes sob hipóxia, como o transportador de glicose 1 (GLUT1), fosfoglicerato quinase 1 (PGK1), fator de crescimento do endotélio vascular (VEGF), lisil oxidase (LOX), 4 receptor de quimiocina (CXCR4), osteopontina (OPN) e o miR-210 suprime a expressão de vários genes envolvidos em uma variedade de funções celulares (HUANG et al., 2010).

A subunidade α do HIF é prontamente detectável na maioria dos tumores humanos enquanto que não é encontrada na análise dos tecidos normais correspondentes (HARRIS, 2002). Entretanto, esta correlação não pode inferir causa, pois embora sua ativação possa tornar os tumores mais agressivos e resultar em pior prognóstico, pode ser que intrinsicamente, tumores mais agressivos levem a uma tensão de O2 mais baixa e portanto com maior ativação do HIF (COMERFORD et al., 2002).

A hiperexpressão do HIF-1 é comum em neoplasias sólidas e suas metástases e está associada à sobrevidas global e livre de doença prejudicadas em cânceres de cabeça e pescoço (HOOGSTEEN et al., 2007).

MiR-210 é um microRNA regulado positivamente em células hipóxicas normais e transformadas. Embora diversos miRNAs sejam regulados por hipóxia, miR-210 é o miRNA mais robustamente e consistentemente regulado para cima em resposta à hipóxia (HUANG et al., 2010). Além disso, age como uma molécula versátil e afeta diversos aspectos da resposta celular à hipóxia. Na iniciação do câncer, miR-210 é induzido pelo HIF-1α e pode ser considerado como um marcador de hipóxia *in vivo* (YING et al., 2011).

Altos níveis de miR-210 estão associados a recidiva tumoral e pior sobrevida global em pacientes com carcinoma de células escamosas de cabeça e pescoço (GEE et al., 2010).

Por causa da íntima relação entre a expressão do miR-210 e hipóxia tumoral, ele pode ser um marcador de prognóstico em pacientes com câncer (GIANNAKAKIS et al., 2008; HE et al., 2013; HONG et al., 2012).

1.3 DIAGNÓSTICO

NGSs benignas ou malignas se apresentam tipicamente como um nódulo indolor, entretanto, história de dor, crescimento rápido ou sintomas que sugerem acometimento de nervos cranianos, como paralisia facial, devem levantar a suspeita de malignidade (MANTRAVADI et al., 2019).

Entre os exames de imagem, a tomografia computadorizada (TC) fornece informações importantes do tumor e sua relação com estruturas adjacentes como mandíbula, porém a ressonância magnética (RM) é preferida para avaliação de neoplasias malignas de glândula salivar por sua maior habilidade em identificar invasão perineural. Outras vantagens da RM são melhor avaliar massa mal definida e/ou de localização obscura (lobo superficial ou profundo), avaliar a extensão extra-glandular e avaliar recorrência. Diversas características vistas na RM têm se mostrado em associação com malignidade, incluindo hipointensidade em T2, margens irregulares, invasão de estruturas locais e baixo sinal em imagens ponderadas por difusão (FRELING; CRIPPA; MAROLDI, 2016; LIU et al., 2015).

Tomografia por emissão de pósitrons-CT (PET-CT) tem mostrado algum benefício na avaliação de metástases à distância em NGSs malignas, entretanto não oferece vantagens adicionais no estadiamento inicial ou na detecção de recidiva loco-regional, quando comparado à TC ou à RM (CERMIK et al., 2007; PARK et al., 2013).

Punção aspirativa por agulha fina (PAAF) é o principal método diagnóstico na maioria dos casos e pode requerer a associação de um método de imagem para guiá-la, se a lesão não for facilmente palpável ou acessível anatomicamente. Apesar da taxa de falso negativo de aproximadamente 10% e de falso positivo de aproximadamente 1% em determinar se a neoplasia é benigna ou maligna, a PAAF tem uma acurácia global suficiente quando combinada com exame de imagem apropriado. Estudos têm demonstrado que a PAAF é confiável para distinguir entre patologias benignas e malignas, mas demonstra utilidade limitada em distinguir os vários subtipos de malignidades (COLELLA et al., 2010; HOWLETT, 2003). Em casos onde a suspeita

de linfoma permanece alta ou quando a PAAF é inconclusiva, biópsia com agulha grossa pode fornecer uma amostra maior para análise histológica e imuno-histoquímica. Entretanto core biopsy está associada com desvantagens como a necessidade de anestesia local, dor, paresia do nervo facial em 0,2% dos casos e hematoma em 1,6% dos casos (DOUVILLE; BRADFORD, 2013).

1.4 TRATAMENTO

Cirurgia inicial é o tratamento mais apropriado para as neoplasias benignas de glândula salivar, assim como em todas as neoplasias malignas ressecáveis e sem metástase à distância (BELL et al., 2005). Estudos indicam que a extensão da neoplasia é mais importante do que o tipo histológico para determinar a extensão cirúrgica. A remoção completa de todo o tumor é crucial para o sucesso, porque a radioterapia não é uma boa opção como modalidade primária de tratamento para estes casos, assim como em casos de doença residual grosseira. A presença de margens positivas e metástase linfonodal impactam negativamente no prognóstico nas NGSs malignas (BELL et al., 2005; BYRD et al., 2013; CERDA et al., 2014; TERHAARD et al., 2005).

1.5 PROTEÔMICA

Biomarcadores são indicadores biológicos de um estado fisiológico e marcas de mudanças durante um processo de doença ou durante terapia. Os biomarcadores têm várias indicações diferentes no câncer, às vezes usados para identificar indivíduos em alto risco de desenvolver câncer, podem ser um indicador precoce de doença, ou podem ser úteis como um monitor da progressão da doença. Biomarcadores clinicamente úteis devem ter o potencial para detectar uma malignidade em um estágio inicial ou para detectar uma recorrência precoce. Eles também podem ser usados como endpoint intermediário na avaliação de terapias desenhadas para prevenir a disseminação ou recorrência de lesões tratadas cirurgicamente. Os biomarcadores de câncer são geralmente proteínas do hospedeiro e peptídeos derivados da circulação periférica ou do microambiente tumoral. Essas proteínas e peptídeos podem representar fragmentos metabólicos resultantes do metabolismo, degradação ou interação proteína-proteína de células cancerosas (LOTZE et al., 2005; STRONCEK et al., 2005).

Técnicas proteômicas estão se tornando um suplemento importante e, em alguns casos, um avanço em relação à genômica. A proteômica tem uma vantagem sobre a genômica em que os próprios biomarcadores de proteínas são endpoints funcionais e, portanto, uma assinatura mais precisa de um estado de doença (INDOVINA et al., 2013; SRINIVAS; KRAMER; SRIVASTAVA, 2001).

Comparada com as classificações genômica e transcriptômica, as características da proteômica parecem ser mais realistas na identificação de alterações moleculares e vias de sinalização relacionadas com o câncer e poderia, portanto, contribuir significativamente para o entendimento do desenvolvimento da carcinogênese. Há diversas razões maiores para se focar na proteômica:

1) Há geralmente uma pobre correlação entre os níveis transcricionais de alguns genes e uma relativa abundância das proteínas correspondentes.
2) Devido ao splicing diferencial e tradução, cada gene pode codificar diversas variantes de proteínas, com diferentes propriedades.
3) As proteínas-chave que impulsionam o comportamento maligno do câncer

   podem sofrer modificações pós-traducionais, incluindo fosforilação e glicosilação (MARTINKOVA et al., 2009).

Proteomas são capazes de monitorar mudanças dinâmicas, por isso podem ser usados no seguimento do curso da doença e avaliação da resposta à terapia (KELLOFF; SIGMAN, 2005).

A espectrometria de massa (MS) desempenha um papel central na proteômica, pois permite a identificação de muitas proteínas com alta sensibilidade, que depende principalmente da eficiência de ionização de um determinado peptídeo. A sensibilidade é alta para peptídeos purificados enquanto diminui com a complexidade das amostras (MARTINKOVA et al., 2009; ZHU; SMITH; HUANG, 2009).

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Investigar marcadores de hipóxia e proteômica em neoplasias benignas e malignas de glândula salivar.

2.2 OBJETIVOS ESPECÍFICOS

I. Investigar os marcadores de hipóxia, HIF-1α e mirR-210 em neoplasias benignas e malignas de glândula salivar, assim como em tecido salivar não neoplásico.
II. Realizar análise proteômica de neoplasias benignas e malignas de glândula salivar, assim como de tecido salivar não neoplásico e investigar a principal proteína nas amostras malignas.

3 PRODUTOS CIENTÍFICOS

PRODUTO 1

Gene 701 (2019) 41–45

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Is HIF1-a deregulated in malignant salivary neoplasms?

Claudio Marcelo Cardoso[b,d,1], Sabrina Ferreira de Jesus[a,1], Marcela Gonçalves de Souza[a], Eloa Mangabeira Santos[a], Cristiane Karine Cordeiro Santos[d], Christine Mendes Silveira[b,d], Sérgio Henrique Sousa Santos[c], Alfredo Maurício Batista de Paula[a], Lucyana Conceição Farias[a], André Luiz Sena Guimarães[a,d,*]

[a] Department of Dentistry, Universidade Estadual de Montes Claros, Montes Claros, Minas Gerais, Brazil
[b] Department of Medicine, Universidade Estadual de Montes Claros, Montes Claros, Minas Gerais, Brazil
[c] Institute of Agricultural Sciences, Food Engineering College, Universidade Federal de Minas Gerais (UFMG), Montes Claros, Minas Gerais, Brazil
[d] Dilson Godinho Hospital, Montes Claros, Minas Gerais, Brazil

ARTICLE INFO

Keywords:
Salivar
Glandular neoplasia
Hypoxia
Chemotherapy

ABSTRACT

Background: There is significant controversy in the literature regarding the relationship between hypoxia and salivary gland neoplasms (SGNs).
Objective: The current study aims to investigate levels of hypoxia markers in both benign and malignant salivary neoplasms.
Patients and methods: The current study sample is comprised of a total of 62 samples. HIF-1α expression was evaluated by immunohistochemistry. Additionally, HIF-1α mRNA and miR-210 levels were assessed using qRT-PCR.
Results: No differences in HIF-1α expression were observed among the control group, benign and malignant SGNs. Similarly, HIF-1α mRNA levels were similar between benign and malignant SGNs. Also, there was no difference in miR-210 expression between case and control groups.
Conclusion: The angiogenic markers, miR-210 and HIF-1α, do not appear to distinguish malignancy in salivary glands.

1. Introduction

Head and neck cancers are associated with poor survival rate (Fonseca et al., 2012; de Oliveira et al., 2009; De Paula et al., 2009). In the case of salivary gland neoplasms (SGNs) a broad spectrum of phenotypic heterogeneity and are divided into five categories, according to the World Health Organization (WHO) (El-Naggar et al., 2017). Prognosis of SGNs is also related to the anatomical location of the neoplasia (Guzzo et al., 2010; Cerda et al., 2014). Recently, a large number of studies have attempted to evaluate the role of hypoxia in cancer development and prognosis (Guimaraes et al., 2016; Fraga et al., 2012). Hypoxia-inducible factor 1-alpha (HIF-1α) is a marker of hypoxia and is activated under hypoxic conditions (Wang and Semenza, 1993).

miRNAs are a class of single-stranded noncoding RNAs 21–22 nucleotides in length which regulate gene expression through the inhibition of RNA translation or degradation of target messenger RNA (mRNA) (Bartel, 2009). Recent evidence has suggested that miR-210 plays a crucial role in the cellular response to hypoxia. HIF-1α can promote isoform-specific stabilization of miR-210 by binding to the Hypoxia Responsive Element (HRE) present in the proximal promoter of miR-210 (Corn, 2008). Similar to HIF-1α, hypoxia induces miR-210 expression, which regulates cellular proliferation, DNA stability, mitochondrial metabolism, apoptosis and angiogenesis (Dang and Myers, 2015). Furthermore, the miR-210 expression is also significantly upregulated in other types of cancer, including non-small cell lung cancer (Zhu et al., 2016).

Although the first choice treatment for malignant SGNs is surgery (Green et al., 2016), adjuvant therapy has historically been determined based on data from studies focused on squamous cell carcinomas of the upper aerodigestive tract (Cerda et al., 2014). Importantly, there are

Abbreviations: SGNs, Salivary gland neoplasias; HRE, Hypoxia Responsive Element

* Corresponding author at: Universidade Estadual de Montes Claros, Hospital Universitário Clemente Faria, Laboratório de Pesquisa em Saúde, 562 Av. Cula Mangabeira Santo Expedito, Montes Claros, MG 39401-001, Brazil.
E-mail address: andreluizguimaraes@gmail.com (A.L.S. Guimarães).

[1] Authors contributed equally.

https://doi.org/10.1016/j.gene.2019.03.017
Received 23 June 2018; Received in revised form 10 March 2019; Accepted 11 March 2019
Available online 19 March 2019
0378-1119/ © 2019 Elsevier B.V. All rights reserved.

conflicting reports with regards to the relationship between hypoxia and clinical behavior or treatment responsiveness in patients diagnosed with SGNs (Wijffels et al., 2009). Recent studies have demonstrated that hypoxia might promote an increase in radioresistance (Harada, 2016), specifically via miRNA-mediated modulation of the hypoxic response (Gu et al., 2016). These data suggest that the use of chemoradiation might be a valuable alternative treatment option for patients presenting with radioresistant neoplasias (Cerda et al., 2014). Considering the controversial literature regarding the relationship between hypoxia and SGNs, the current study aimed to investigate levels of hypoxia markers in both benign and malignant salivary neoplasms.

2. Methods

2.1. Study design

A retrospective cross-sectional study was designed to evaluate the expression of the main hypoxia targets in SGNs.

3. Patients

Ethical approval for this study was obtained from the relevant Institutional Review Board. A total of 62 samples were included from the databases of the glandular tumor surgery services from 2010 to 2016. Samples were divided into three groups according to the world health organization classification of head and neck tumors (El-Naggar et al., 2017).

Group 1 ($n = 16$) was comprised of glandular tissue without neoplasia. Samples were obtained from mucocele excisions. The inclusion criteria for this group were histopathological diagnosis of mucocele.

Group 2 ($n = 22$) was comprised of patients with Pleomorphic Adenoma. The inclusion criteria for this group were histopathological diagnosis of pleomorphic adenoma.

The group 3 ($n = 24$) was comprised of one sample of Acinar Cell Carcinoma, two samples of Adenocarcinoma not otherwise specified, nine samples of Adenoid cystic carcinoma, ten Mucoepidermoid Carcinoma samples and two Myoepithelial Carcinoma samples.

4. Immunohistochemistry

Immunohistochemical reactions were performed from paraffin blocks of 49 patients. Including 13 controls (normal salivary gland samples), 16 benign tumors (Pleomorphic adenoma) and 20 malignant neoplasms (Acinar Cell Carcinoma $n = 1$, Adenocarcinoma not otherwise specified $n = 2$, Adenoid cystic carcinoma $n = 8$, Mucoepidermoid Carcinoma $n = 10$ and Myoepithelial Carcinoma $n = 2$). Protein expression of HIF-1α was evaluated through immunohistochemical reactions performed on 3.0 μm thick sections. The sections were deparaffinized with xylene and rehydrated with graded alcohol solutions. Antigen retrieval was conducted in an electric pressure cooker at 121 °C for 10 min in Trilogy buffer (Trilogy, Cell Marque, CA, USA). Endogenous peroxidase activity was blocked with 2 baths of 0.3% hydrogen peroxide for 15 min each. Samples were then incubated overnight with primary mouse monoclonal antibodies against HIF-1α (Clone H1a67; Sigma-Aldrich, MO, USA) at 4 °C at a dilution of 1:200. The samples were then incubated with a secondary biotinylated link and streptavidin-biotin-peroxidase complex using the LSAB kit (Dako Labs, Glostrup, Denmark), followed by incubation with the 3′3-diaminobenzidine-tetrahydrochloride (Cat.No.32750, Sigma-Aldrich, MO, USA). Counterstaining was performed using Mayer's hematoxylin (Cat. no. 109249, Merck, MA, USA). The samples were then dehydrated, and slides were mounted in ERV-mount (EasyPath, Brazil). Kidney samples were used as positive controls. Negative controls were performed by replacing the primary antibody with phosphate buffered saline (PBS). Ten microscopic fields of tumor parenchyma were photographed to quantify the staining at microscope FSX100 (Olympus, Center Valley, PA, USA). Cells were then counted using ImageJ software (Rueden et al., 2017). The ratio of positive cells to the total number of cells counted was used to quantify the staining.

5. RNA isolation and real-time PCR

Validation of the immunohistochemical results was performed with qRT-PCR. Samples of 24 patients, including 10 controls (normal salivary gland samples), 7 benign tumors (Pleomorphic adenoma) and 7 malignant neoplasms (Acinar cell carcinoma $n = 1$, Adenoid cystic carcinoma $n = 2$, Mucoepidermoid Carcinoma $n = 2$, Myoepithelial carcinoma $n = 2$) were used for qRT-PCR. RNA was isolated using Trizol (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. Total RNA was treated with DNase I, Amplification Grade (Invitrogen, cat number 18068015, Carlsbad, CA, USA) and 1.5 μg of RNA were reversely transcribed with the SuperScript® First-Strand Synthesis System for qRT-PCR (Invitrogen, cat number 11904018, Carlsbad, CA, USA). Each reaction for SYBR green-based qRT-PCR (total volume 20 μl) contained 10 μl of SYBR Green master mix, 0.25 μM of both forward and reversed primers, 1 μl of cDNA (66 ng/reaction) and 8.5 μl H_2O. Non-Template Control (NTC) was included for each assay. The thermal cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of the following steps: 95 °C for 15 s and 60 °C for 1 min. The specific primers/probes (Life Technologies, Carlsbad, CA, USA) were described previously (Guimaraes et al., 2016). Specifically, the following primers were used for HIF1α: 5′-TCTGCAACATGGAAGGTATTGC-3′ and 5′-CTGAGGTTG GTTACTGTTGGTATCA-3. Beta-Actin was used to normalize HIF1α gene expression and was amplified using the following primers: 5′-TGCCGACAGGATGCAGAAG-3′ and 5′-CTCAGGAGGAGCAATGATCT TGA-3′. qPCR was performed on a StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA, USA).

For miR-210 (ID: Hs04231470_s1, Life Technologies, Carlsbad, CA, USA), a TaqMan assay was performed according to the manufacturer's protocol. RNU44 was used as an endogenous control for mi-210 analysis (ID: 001094, Life Technologies, Carlsbad, CA, USA), All reactions were done in triplicate. Normal salivary glands were used as a control group. The following reaction steps were used for amplification: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. The Cq values of endogenous controls were subtracted from the Ct values of the respective targets to calculate the ΔCq. The ΔCq values from each experimental group were averaged and converted to log base 2 using equation 2^-ΔΔCq to compare expression among different samples.

5.1. Statistical analysis

Analyses were performed using SPSS (Version 18.0, IBM Software Inc., Armonk, New York, USA) and GraphPad Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA, USA). The Kolmogorov-Smirnov and Shapiro-Wilk Tests were carried out to evaluate data distribution. These analyses revealed that the data were non-parametrically distributed; therefore, the Mann-Whitney and Kruskal-Wallis tests were performed. All data are given as means ± S.D. $p < .05$ was considered statistically significant.

6. Results

Considering all individuals, 35 (56%) were male, and 24 (44%) were female (Table 1). There is a significant association between hypoxic markers such as HIF-1α and miR-210 and angiogenesis (Dang and Myers, 2015; Zhang et al., 2017) (Fig. 1). However, the impact of hypoxic markers in SGNs still unknown. Qualitative analyses of HIF-1α immunoexpression is presented in Fig. 2(A–G). Most of the groups presented week staining for HIF-1α. The staining was not homogeneous considering the histological types. Quantitative analyses of HIF-1α immunoexpression and mRNA is shown in Fig. 3 (A and B respectively).

Table 1
Descriptive data of the patient population.

Group 1	Mucocele	Female	miRNA/Protein
Group 1	Mucocele	Female	miRNA
Group 1	Mucocele	Male	miRNA
Group 1	Mucocele	Male	RNA/miRNA/Protein
Group 1	Mucocele	Male	miRNA/Protein
Group 1	Mucocele	Male	miRNA/Protein
Group 1	Mucocele	Male	RNA/miRNA/Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	RNA/miRNA/Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Female	RNA/miRNA
Group 1	Mucocele	Female	miRNA/Protein
Group 2	Pleomorphic Adenoma	Male	miRNA
Group 2	Pleomorphic Adenoma	Female	RNA/miRNA/Protein
Group 2	Pleomorphic Adenoma	Female	RNA/miRNA
Group 2	Pleomorphic Adenoma	Male	miRNA
Group 2	Pleomorphic Adenoma	Female	RNA/miRNA
Group 2	Pleomorphic Adenoma	Male	RNA/miRNA
Group 2	Pleomorphic Adenoma	Female	RNA/miRNA
Group 2	Pleomorphic Adenoma	Female	miRNA/Protein
Group 2	Pleomorphic Adenoma	Female	RNA/Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Male	Protein
Group 2	Pleomorphic Adenoma	Male	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Male	Protein
Group 2	Pleomorphic Adenoma	Male	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 3	Acinar Cell Carcinoma	Female	miRNA/Protein
Group 3	Adenocarcinoma (NOS)	Male	Protein
Group 3	Adenocarcinoma (NOS)	Male	Protein
Group 3	Adenoid Cyst Carcinoma	Male	miRNA/Protein
Group 3	Adenoid Cyst Carcinoma	Female	RNA/miRNA
Group 3	Adenoid Cyst Carcinoma	Female	Protein
Group 3	Adenoid Cyst Carcinoma	Female	Protein
Group 3	Adenoid cystic Carcinoma	Female	Protein
Group 3	Adenoid cystic Carcinoma	Male	Protein
Group 3	Adenoid cystic Carcinoma	Male	Protein
Group 3	Adenoid cystic Carcinoma	Male	Protein
Group 3	Adenoid cystic Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Female	miRNA
Group 3	Mucoepidermoid Carcinoma	Male	miRNA
Group 3	Mucoepidermoid Carcinoma	Female	RNA/Protein
Group 3	Mucoepidermoid Carcinoma	Female	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Myoepithelial Carcinoma	Male	miRNA/Protein
Group 3	Myoepithelial Carcinoma	Male	RNA/miRNA/Protein

HIF-1α immunoexpression did not differ between the case and control groups or between benign and malignant SGNs (Fig. 3A). In the agreement, HIF-1α mRNA levels or immunoexpression did not differ between the case and control groups or between benign and malignant SGNs (Fig. 3B). miR-210 is an essential molecule associated with neoplastic hypoxia (Dang and Myers, 2015). Similarly to HIF-1α, miR-210 levels were similar among control, malignant and benign SGNs (Fig. 3C).

7. Discussion

Previous reports have suggested that hypoxia is an important mechanism related to radioresistance in specific cancers (Barker et al., 2015). Because the indirect actions of radiation depend on oxygen levels, hypoxia is believed to be the central mechanism leading to radioresistance (Cerda et al., 2014; Harada, 2016; Gu et al., 2016; Barker et al., 2015). Furthermore, it is important to highlight that vascular damage, which triggers an immune response, is a consequence of radiation-induced tumor hypoxia (Barker et al., 2015). However, fractionated radiation can induce subsequent tumor revascularization via recruitment of bone marrow-derived cells (BMDCs) in a HIF1α-dependent or independent manner (Kioi et al., 2010).

Traditionally, SGNs were considered radioresistant (Cerda et al., 2014). However, the primary biological mechanism that contributes to the radioresistance observed in SGNs is still not well established. As such, recommendations for the management and treatment of malignant SGNs have changed in recent years (Bell et al., 2005; Laramore et al., 1993). The shift in the treatment of SGNs is due to an improvement in imaging technology, as well as the development of various treatment options, including external beam radiation, neutron beam therapy, and chemotherapy (Bell et al., 2005; Laramore et al., 1993). It has been shown that miR-210 expression is induced by both HIF-1α (Wang et al., 2014; Kulshreshtha et al., 2007) as well as hypoxia (Kulshreshtha et al., 2007). The current study was the first to investigate miR-210 expression in SGNs. This report suggests that there are no significant alterations in miR-210 expression in this specific tumor subtype. Specifically, no differences in miR-210 levels were observed between SGNs and normal, healthy salivary glands. In support of the miR-210 findings reported here, our results regarding HIF-1α demonstrate that salivary gland neoplasms are not hypoxic. Instead, an elegant study has shown that SGNs are well oxygenated, and it is unlikely that hypoxia is a relevant factor in the clinical progression and treatment responsiveness of these tumors (Wijffels et al., 2009). Moreover, repair of sublethal damage during the interval between fractionated radiotherapy doses is minimal in neutron therapy but plays a significant role in external-beam radiotherapy (Hall et al., 1975), thus suggesting that radioresistance of SGNs is independent of HIF1α. This notion is supported by the central mechanism of chemotherapeutic agents that induce cell death in SGNs is DNA damage and not hypoxia (Cerda et al., 2014).

Hypoxia is associated with both radioresistance and tumor progression (Fraga et al., 2012; Dang and Myers, 2015; Wang et al., 2015). However, the importance of the hypoxic response appears to be specific to the tumor subtype. Correctly, a substantial positive correlation has been observed between adenoid cystic carcinomas and hypoxia (Wang et al., 2015). However, HIF-1α levels do not appear to be altered in pleomorphic adenomas, when compared with control samples (Wang et al., 2015).

It is important to note that there are limitations to the current study, including a small sample size, which precluded our ability to perform comprehensive analyses of different specimens and histological types. Irrespective of these limitations, the current study is the first to evaluate RNA levels of hypoxia markers in SGNs. Furthermore, the present study validates previously published data as we observed no differences in hypoxia markers between control, benign and malignant neoplasms.

In conclusion, our data suggest that salivary gland neoplasms do not display increased levels of hypoxia markers. Individually, the angiogenic markers, mir210 and HIF-α, do not seem to correlate with malignancy of salivary glands.

Conflict of interest statement

The authors deny any conflicts of interest related to this study.

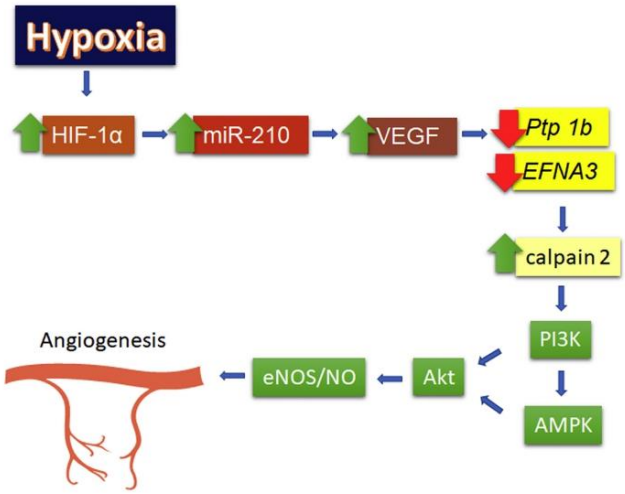


Fig. 1. Schematic representation of angiogenesis induced by Hypoxia.
Hypoxia induces the increase (green arrows) of Hypoxia-inducible factor 1-alpha (HIF-1α), miR-210 and Vascular endothelial growth factor (VEGF). Consequently, there is a transcription inhibition (red arrows) of both genes protein tyrosine phosphatase 1B (*Ptp1b*) and Ephrin A3 (*EPFNA3*). All these molecular events activate (green symbols) Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), 5′ adenosine monophosphate-activated protein kinase (AMPK), AKT serine/threonine kinase (AKT), Nitric oxide synthases (NOS) and Endothelial NOS (eNOS) which induces angiogenesis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

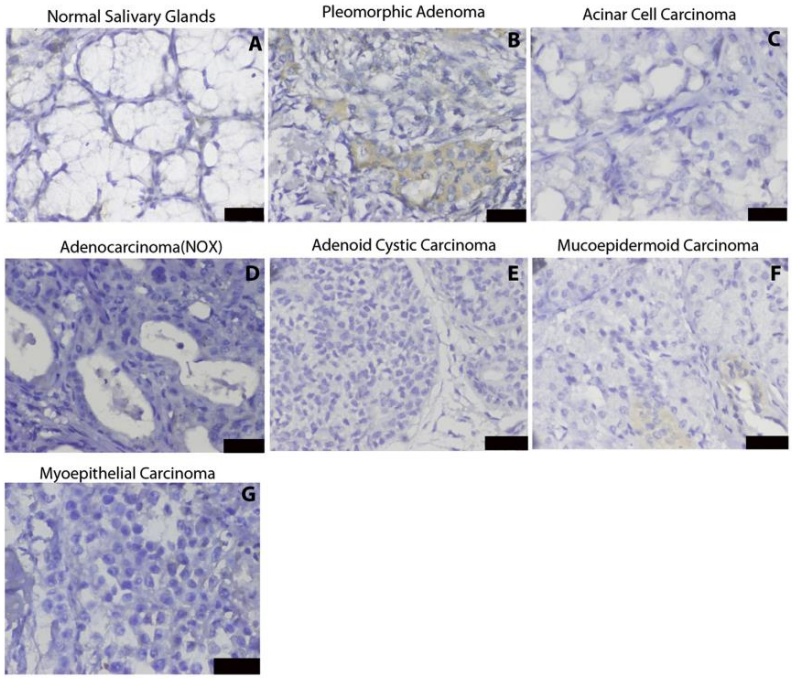


Fig. 2. Qualitative HIF-1α immunoexpression in samples.
HIF-1α expression qualitative results in Control (A), Pleomorphic Adenoma (B), Acinar Cell Carcinoma (C) Adenocarcinoma (NOX) (D), Adenoid cystic carcinoma (E), Mucoepidermoid Carcinoma (F) and Myoepithelial Carcinoma (G).

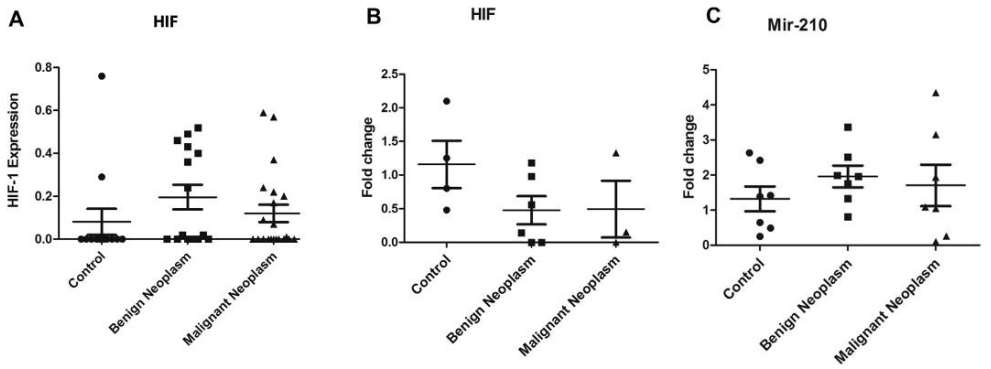


Fig. 3. Quantitative analyses of HIF-1α immunoexpression, HIF-1α mRNA and miR-210.
HIF-1α immunoexpression (A), HIF-1α mRNA (B) and miR-210 (C) in control, benign and malignant SGNs. (A). HIF-1α expression (A) or mRNA levels (B) is not altered in SGNs, compared with control samples. No difference in expression of miR-210 is observed between groups (C).

Role of funding source

The current study was not sponsored by an organization that has a financial interest.

Ethical approval

Ethical approval for this study was obtained from the relevant Institutional Review Board (52767316600005146). All patients signed informed consent.

Acknowledgements

This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG). Dr. Guimarães, Dr. Santos and Dr. de Paula are research fellows of the CNPq.

References

Barker, H.E., Paget, J.T., Khan, A.A., Harrington, K.J., 2015. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nat. Rev. Cancer 15 (7), 409–425.

Bartel, D.P., 2009. MicroRNAs: target recognition and regulatory functions. Cell. 136 (2), 215–233.

Bell, R.B., Dierks, E.J., Homer, L., Potter, B.E., 2005. Management and outcome of patients with malignant salivary gland tumors. J. Oral Maxillofac. Surg. 63 (7), 917–928.

Cerda, T., Sun, X.S., Vignot, S., Marcy, P.Y., Baujat, B., Baglin, A.C., et al., 2014. A rationale for chemoradiation (vs radiotherapy) in salivary gland cancers? On behalf of the REFCOR (French rare head and neck cancer network). Crit. Rev. Oncol. Hematol. 91 (2), 142–158.

Corn, P.G., 2008. Hypoxic regulation of miR-210: shrinking targets expand HIF-1's influence. Cancer Biol. Ther. 7 (2), 265–267.

Dang, K., Myers, K.A., 2015. The role of hypoxia-induced miR-210 in cancer progression. Int. J. Mol. Sci. 16 (3), 6353–6372.

de Oliveira, F.A., Duarte, E.C., Taveira, C.T., Maximo, A.A., de Aquino, E.C., Alencar Rde, C., et al., 2009. Salivary gland tumor: a review of 599 cases in a Brazilian population. Head Neck Pathol. 3 (4), 271–275.

De Paula, A.M., Souza, L.R., Farias, L.C., Correa, G.T., Fraga, C.A., Eleuterio, N.B., et al., 2009. Analysis of 724 cases of primary head and neck squamous cell carcinoma (HNSCC) with a focus on young patients and p53 immunolocalization. Oral Oncol. 45 (9), 777–782.

El-Naggar, A.K., Chan, J.K.C., Grandis, J.R., Takata, T., Slootweg, P.J., 2017. WHO Classification of Head and Neck Tumours. International Agency for Research on Cancer.

Fonseca, F.P., Carvalho Mde, V., de Almeida, O.P., Rangel, A.L., Takizawa, M.C., Bueno, A.G., et al., 2012. Clinicopathologic analysis of 493 cases of salivary gland tumors in a Southern Brazilian population. Oral Surg Oral Med Oral Pathol Oral Radiol 114 (2), 230–239.

Fraga, C.A., de Oliveira, M.V., de Oliveira, E.S., Barros, L.O., Santos, F.B., Gomez, R.S., et al., 2012. A high HIF-1alpha expression genotype is associated with poor prognosis of upper aerodigestive tract carcinoma patients. Oral Oncol. 48 (2), 130–135.

Green, B., Rahimi, S., Brennan, P.A., 2016. Current management of the neck in salivary gland carcinomas. J. Oral Pathol. Med. 46, 161–166.

Gu, H., Liu, M., Ding, C., Wang, X., Wang, R., Wu, X., et al., 2016. Hypoxia-responsive miR-124 and miR-144 reduce hypoxia-induced autophagy and enhance radiosensitivity of prostate cancer cells via suppressing PIM1. Cancer Med. 5 (6), 1174–1182.

Guimaraes, T.A., Farias, L.C., Fraga, C.A., Feltenberger, J.D., Melo, G.A., Coletta, R.D., et al., 2016. Evaluation of the antineoplastic activity of gallic acid in oral squamous cell carcinoma under hypoxic conditions. Anti-Cancer Drugs 27 (5), 407–416.

Guzzo, M., Locati, L.D., Prott, F.J., Gatta, G., McGurk, M., Licitra, L., 2010. Major and minor salivary gland tumors. Crit. Rev. Oncol. Hematol. 74 (2), 134–148.

Hall, E.J., Roizin-Towie, L., Theus, R.B., August, L.S., 1975. Radiobiological properties of high-energy cyclotron-produced neutrons used for radiotherapy. Radiology 117 (1), 173–178.

Harada, H., 2016. Hypoxia-inducible factor 1-mediated characteristic features of cancer cells for tumor radioresistance. J. Radiat. Res. 57, i99–i105.

Kioi, M., Vogel, H., Schultz, G., Hoffman, R.M., Harsh, G.R., Brown, J.M., 2010. Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. J. Clin. Invest. 120 (3), 694–705.

Kulshreshtha, R., Ferracin, M., Wojcik, S.E., Garzon, R., Alder, H., Agosto-Perez, F.J., et al., 2007. A microRNA signature of hypoxia. Mol. Cell. Biol. 27 (5), 1859–1867.

Laramore, G.E., Krall, J.M., Griffin, T.W., Duncan, W., Richter, M.P., Saroja, K.R., et al., 1993. Neutron versus photon irradiation for unresectable salivary gland tumors: final report of an RTOG-MRC randomized clinical trial. Radiation Therapy Oncology Group. Medical Research Council. Int. J. Radiat. Oncol. Biol. Phys. 27 (2), 235–240.

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., et al., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinf. 18 (1), 529.

Wang, G.L., Semenza, G.L., 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc. Natl. Acad. Sci. U. S. A. 90 (9), 4304–4308.

Wang, H., Flach, H., Onizawa, M., Wei, L., McManus, M.T., Weiss, A., 2014. Negative regulation of Hif1a expression and TH17 differentiation by the hypoxia-regulated microRNA miR-210. Nat. Immunol. 15 (4), 393–401.

Wang, W.M., Zhao, Z.L., Zhang, W.F., Zhao, Y.F., Zhang, L., Sun, Z.J., 2015. Role of hypoxia-inducible factor-1alpha and CD146 in epidermal growth factor receptor-mediated angiogenesis in salivary gland adenoid cystic carcinoma. Mol. Med. Rep. 12 (3), 3432–3438.

Wijffels, K.I., Hoogsteen, I.J., Lok, J., Rijken, P.F., Marres, H.A., de Wilde, P.C., et al., 2009. No detectable hypoxia in malignant salivary gland tumors: preliminary results. Int. J. Radiat. Oncol. Biol. Phys. 73 (5), 1319–1325.

Zhang, Y., Liu, N.M., Wang, Y., Youn, J.Y., Cai, H., 2017. Endothelial cell calpain as a critical modulator of angiogenesis. Biochim. Biophys. Acta, Mol. Basis Dis. 1863 (6), 1326–1335.

Zhu, W., Zhou, K., Zha, Y., Chen, D., He, J., Ma, H., et al., 2016. Diagnostic value of serum miR-182, miR-183, miR-210, and miR-126 levels in patients with early-stage non-small cell lung Cancer. PLoS One 11 (4), e0153046.

PRODUTO 2

Received: 15 April 2019 | Revised: 27 May 2019 | Accepted: 17 June 2019
DOI: 10.1111/jop.12932

ORIGINAL ARTICLE

Journal of Oral Pathology & Medicine WILEY

High levels of ANXA2 are characteristic of malignant salivary gland tumors

Claudio Marcelo Cardoso[1,4] | Sabrina Ferreira de Jesus[2] | Marcela Gonçalves de Souza[2] | Lorena dos Reis Pereira Queiroz[2] | Eloa Mangabeira Santos[2] | Erivelton Pereira dos Santos[2] | Luis Paulo Oliveira[2] | Cristiane Karine Cordeiro Santos[4] | Sérgio Henrique Sousa Santos[3] | Alfredo Maurício Batista de Paula[2] | Lucyana Conceição Farias[2] | Andre Luiz Sena Guimaraes[2,4]

[1]Department of Medicine, Universidade Estadual de Montes Claros, Montes Claros, Minas Gerais, Brazil

[2]Department of Dentistry, Universidade Estadual de Montes Claros, Montes Claros, Minas Gerais, Brazil

[3]Institute of Agricultural Sciences, Food Engineering College, Universidade Federal de Minas Gerais (UFMG), Montes Claros, Minas Gerais, Brazil

[4]Dilson Godinho Hospital, Montes Claros, Minas Gerais, Brazil

Correspondence
André Luiz Sena Guimaraes, Laboratório de Pesquisa em Saúde, Hospital Universitário Clemente Faria, Universidade Estadual de Montes Claros, 562 Av. Cula Mangabeira Santo Expedito, Montes Claros, MG, Brazil 39401-001.
Email: andreluizguimaraes@gmail.com

Funding information
This study was supported by grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, The Conselho Nacional de Desenvolvimento Científico e Tecnologico, and Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG). Dr. Guimaraes, Dr. Santos and Dr. de Paula are research fellows of the CNPq. The current study was not sponsored by an organization that has a financial interest.

Abstract

Objective: Malignant salivary gland tumors (MSGTs) present different phenotypic characteristics and various clinical outcomes, which proved to be a diagnostic challenge. Considering the heterogeneity of MSGT, this study aims to identify molecule related to the nature of MSGT.

Methods: For screening, proteomic analysis comparing MSGT with pleomorphic adenoma (PA) and salivary gland was performed. The MSGT-associated protein which presented in the higher number in the Gene Expression Omnibus (GEO) database was selected. To validate the data, immunohistochemistry (IHC) was performed in 14 patients with PA, 22 patients with MSGT, and 14 controls.

Results: 16 proteins were associated with MSGT. ANXA2 was the primary protein, according to GEO database analyses. ANXA2 was most expressed in the cell membrane. However, some ANXA2 staining was also observed in the cytoplasm and nucleus. ANXA2 was highly expressed in MSGT in comparison with control. Also, ANXA2 has a higher expression in adenocarcinoma not otherwise specified (ANOS) and myoepithelial carcinoma (MC) in comparison with PA.

Conclusion: In conclusion, this study demonstrated that MSGT presented higher levels of ANXA2 in comparison with normal salivary glands. Also, ANXA2 might be interesting as a molecular marker of ANOS and MS.

KEYWORDS
gland, malignancy, metastasis, neoplasm, oral cancer

Claudio Marcelo Cardoso and Sabrina Ferreira de Jesus contributed equally.

© 2019 John Wiley & Sons A/S.
Published by John Wiley & Sons Ltd

1 | INTRODUCTION

Benign salivary gland neoplasms are most common than malignant.[1] On the other hand, the clinical appearance of malignant and benignant salivary gland neoplasms is very similar, specifically in the hard palate.[2] It is necessary to highlight that MSGT presents different phenotypic characteristics and various clinical outcomes.[3] Some MSGT such as myoepithelial carcinomas (MCs) is locally aggressive, while acinic cell carcinoma is highly metastatic.[3,4] The distant metastases are frequent event related to MSGT, and 62% of the metastasis was not associated with a local and regional recurrence.[4] The challenges encountered in therapeutics and general care are still relevant because of MSGT heterogeneity and complexity.[1] The comparison of MSGT and the normal salivary gland is useful to identify new biomarkers for various types of cancer.[1,5] Additionally, proteomic analysis is a high-throughput accurate method for exploring the specific proteins, which are potential biomarkers.[6]

Annexin family proteins have different members, which may perform various roles in specific types of cancer.[7] Among them, Annexin A2 (ANXA2) induced microdomains that are likely to be essential for many fundamental biological processes requiring membrane dynamics ranging from cell differentiation to cell migration and secretion.[8,9] The ANXA2 expression is an independent prognostic biomarker for evaluating the malignant progression of laryngeal cancer[10] and is associated with invasive cervical carcinoma, prostate cancer, and lung cancer.[11] However, ANXA2 is expressed in both benign and malignant vascular neoplasms.[8,12] Considering the heterogeneity of MSGT, this study aims to differentiate MSGT using molecular tools.

2 | METHODS

Ethical approval (52767316.6.0000.5146) from the relevant ethics committees was obtained.

2.1 | Patients

The written informed consent form was obtained from all participants. A total of 50 samples were collected from 2010 to 2016. All diagnoses were reviewed by the same pathologist. Three groups were designed to investigate changes in MSGTs. The frequency of benignant SGNs (70%) and MSGTs (30%) was based on previous data.[13] The sample size calculation was performed as described before[14] to have α .05, β .4, and study power 0.6.

Group 1 (n = 14) was comprised of patients with pleomorphic adenoma (PA). The inclusion criteria were a PA. The exclusion criteria were any feature in anamneses, which suggests the presence of neoplasia.

Group 2 (n = 22) was comprised of acinic cell carcinoma (AcCC, n = 1), adenocarcinoma not otherwise specified (NOS, n = 3), adenoid cystic carcinoma (AdCC, n = 9), mucoepidermoid carcinoma (MEC, n = 7), and MC (MC, n = 2).

Group 3 (n = 14) was comprised of glandular tissue without neoplasia. Samples were obtained from mucocele excisions. The inclusion criteria for this group were the absence of neoplastic feature and absent history of any neoplasia during the anamneses. The exclusion criteria were any feature in anamneses, which suggests the presence of neoplasia.

2.2 | Proteomic analysis

2.2.1 | Samples

For proteomic analysis, a total of 4 samples comprised 1 PA, 1 MC, and 2 normal salivary glands. Written informed consent was obtained from all individual participants included in the survey.

2.2.2 | Mass spectrometry: Preparation of samples and analysis

Samples were treated with urea (Cat. No.51457, Sigma-Aldrich) at final concentration of 1.6 M, followed by reduction with dithiothreitol (DTT) (Cat. No. D9779, Sigma-Aldrich) at 5 mmol/L for 25 minutes at 56°C, alkylation with Iodoacetamide (Cat. No.16125, Sigma-Aldrich) at 14 mmol/L for 30 minutes at room temperature protected from light, and digestion with Trypsin (Cat. No. V5280, Promega) for 16 hours at 37°C (enzyme: substrate ratio, 1:50). The reaction was stopped with formic acid (Cat. No.100264, Merck) (up to 0.4% of acid in the sample), and after desalination using Sep-Pak C18 Cartridges (Cat. No. WAT054945, Waters technologies LC-MS), the samples were dried in a vacuum concentrator model SPD1010 SpeedVac™ Systems (Thermo Scientific™). The samples were stored at −20 for subsequent analysis in a mass spectrometer. All obtained peptides were suspended in 80 µL of a solution containing 20 mmol/L of ammonium formate (Cat. No. 70221, Sigma-Aldrich) and 150 fmol/µL of Enolase (Waters Corporation) (MassPREP™ Protein).

Nanoscale LC separation of tryptic peptides was performed using an ACQUITY UPLC®M-Class System (Waters Corporation) equipped with an XBridge® Peptide 5 µm BEH130 C18 300 × 50 mm precolumn; Trap, 2D Symmetry® 5 µm BEH100 C18, 180 µm × 20 mm column, and Peptide CSH™ BEH130 C18 1.7 µm, 100 µm × 100 mm analytical reversed-phase column (Waters Corporation). The peptides were separated using a gradient of 3% at 45% of acetonitrile (Cat. No. 271004, Sigma-Aldrich), with a flow rate of 2.000 µL/min. The lock mass was used for calibration of the apparatus, using a constant flow rate of 0.2 µL/min at a concentration of 200 fmol protein GFP ([Glu1]-Fibrinopeptide B human (Sigma-Aldrich, F3261)). Mass spectrometry analysis was performed on a Synapt G1 MSTM (Waters Co) equipped with a nanoelectrospray source and two mass analyzers: a quadrupole and a time-of-flight (TOF) analyzer operating in TOF V-mode. Data were obtained using the instrument in the MSE mode, which switches the low energy (6 V) and high-power (40 V) acquisition modes every 0.4 seconds. Samples were analyzed from three replicates.[15]

2.2.3 | Data processing and protein identification

The mass spectrometer data that were obtained from the LC-MSE analysis were processed and searched using the ProteinLynx Global

Server version 3.0.2 (Waters). The data were subjected to automatic background subtraction, deisotoping, and charge state deconvolution. After processing, each ion comprised an exact mass/retention time (EMRT) that contained the retention time, intensity-weighted average cost, inferred molecular weight based on charge and m/z, and the deconvoluted intensity. Then, the processed spectra were searched against Homo sapiens (Human) protein sequences (http://www.uniprot.proteomes/query=homo+sapiens&sort=score) together with reverse sequences. The protein identification criteria also included the detection of at least two fragment ions per peptide, the detection of five fragments per protein, and the determination of at least one peptide per protein; the identification of the protein was allowed with a maximum 4% false-positive discovery rate in at least three technical replicate injections. The searches were performed with a fixed modification of carbamidomethyl (C), and variable adjustments were phosphorylation of serine, threonine, and tyrosine. One missed cleavage site was allowed. Protein tables were generated by the Protein Lynx Global Server.

After the protein tables were generated, the qualitative data were exported to Excel®. The qualitative analysis was done by comparing the groups by the Venn diagram.

2.3 | Target selection

After the Venn diagram, the proteins exclusively associated with MSGT were selected and evaluated. To choose the target molecule, data obtained from Gene Expression Omnibus (GEO) (Home-GEO-NCBI) were used to validate data from the current study. For each protein, the GEO search was done using the following keywords: Homo sapiens, the name of protein, and cancer. A heat map was performed, and the protein that was presented in the higher number of GEO database was selected.

2.4 | Immunohistochemical reactions

For immunohistochemical analysis, samples archived tissue blocks from surgically resected epithelial tumors, first specimens and control: benign tumor: PA (n = 14), MSGT: MEC (n = 7), AcCC (n = 1), AdCC (n = 9), MC (n = 2), adenocarcinoma NOS (n = 3), and normal salivary gland (n = 14). Each resected tissue specimen was fixed in formalin, embedded in paraffin, and cut into 3.0-µm thick sections. The following primary mouse monoclonal antibodies were used: ANXA2 (clone C-10; Santa Cruz Biotechnology) was incubated at 4°C for 18 hours at a dilution of 1:300. Incubation blocked endogenous peroxidase with 3% hydrogen peroxide and Protein Blocker EP-12-20532 (Kit Easy Link One, Easy Path, Immunobioscience Corp, EUA). For antigen retrieval, sections were heated in a steam cooker for 10 minutes at 121°C in Trilogy (Cat. No. 920P-06; Cell Marque Corporation). The primary antibodies were detected using the HRP EP-12-20502 of Kit Easy Link One (Easy Path, Immunobioscience Corp, EUA). Signals were developed with 3′3-diaminobenzidine-tetrahydrochloride (Cat. No. 32750, Sigma-Aldrich) for 3 minutes and counterstained with Mayer's hematoxylin (Cat. No. 109249, Merck) for 60 seconds. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline (PBS).

2.5 | Assessment of immunoreactivity

Ten microscopic fields of tumor parenchyma were photographed at 400× magnification (Olympus; FSX-100) to quantify the staining; cells were then counted using ImageJ software.[16] The ratio of positive cells to the total number of cells counted was used to quantify the staining.

2.6 | Statistical analysis

Analyses were performed using SPSS (Version 18.0) and GraphPad Prism software (version 5.0; GraphPad Software Inc). The Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to evaluate data distribution. Immunolocalization analysis assumed a parametric distribution, and comparisons between three groups, PA, malignant gland salivary neoplasia, and normal salivary glands, were performed using one-way ANOVA. When comparing several types of MSGTs, immunolocalization analysis that assumed a non-parametric distribution was performed using the Mann-Whitney test. P values <.05 were considered significant.

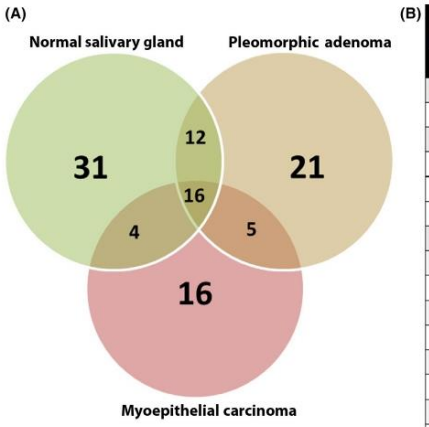
3 | RESULTS

3.1 | Proteomic profile of the pleomorphic adenoma, myoepithelial carcinoma, and normal salivary gland

A comparative proteomic analysis was performed on PA, MC, and normal salivary gland samples using LC-MSE. A total of 105 proteins were identified in all groups. It was detected by the presence of 16 proteins present only in MC, 21 proteins just in PA, and 31 proteins just in normal salivary gland. The Venn diagram is shown in Figure 1A. 16 proteins in MC inserted in GEO (Home-GEO-NCBI) and the number of databases in GEO are shown in Figure 1B. ANXA2 presented as the primary target related to MSGT in general.

3.2 | ANXA2 expression in benign gland salivary neoplasia, malignant gland salivary, and normal salivary gland by immunohistochemistry

The expression of the ANXA2 protein was analyzed in 14 patients with PA (28%), 22 patients with malignant gland salivary neoplasia (44%), and 14 patients with normal salivary glands (28%) using immunohistochemistry (IHC). MSGT presented higher expression of ANXA2. The profile of ANXA2 expression is shown in Figure 2. A significant difference was observed in between the group's malignant gland salivary neoplasia and control (Figure 2H), so we found a considerable difference in between PA and control (Figure 2H). Most of ANXA2 presented stained in the cell membrane. However, some cytoplasm and nuclear expression were also observed in Figure 2



(B)

Protein	Number of databases in GEO
ANXA2*	1055
ENO1	966
TAGLN2	617
TUBB3	589
PGAM1	350
A1AG1	328
IGLL5	255
IGHA1	42
ALDOA	20
IGLC1	12
TF	8
IGHG3	5
IGLC7	1
IGLC2	0
IGLC3	0
IGLC6	0

FIGURE 1 Comparative proteomic analyses of MC, PA, and normal salivary gland using LC-MSE. A, Venn diagram among MC, PA, and normal salivary gland. Analysis of the Venn diagram shows the amount of protein present in each study group; 16 proteins present only in MC, 21 proteins just in PA, and 31 proteins just in normal salivary gland. B, 16 proteins in MC inserted in GEO (Home-GEO-NCBI) and the number of databases in GEO are shown. ANXA2 was the protein detected more relevant to validate

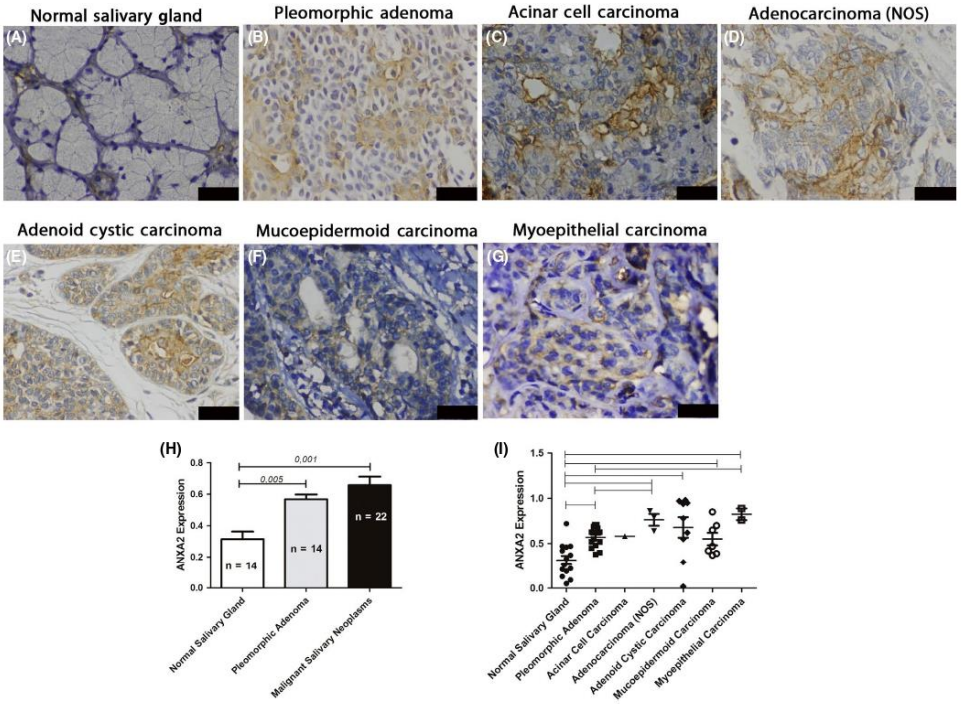


FIGURE 2 The patterns of the immunohistochemical ANXA2 marker. Normal salivary glands (A), pleomorphic adenoma (B), acinic cell carcinoma (C), adenocarcinoma not otherwise specified (NOS) (D), adenoid cystic carcinoma (E), mucoepidermoid carcinoma (F), and myoepithelial carcinoma (G). Graph showing ANXA2 between the group's MSGT, PA, and normal salivary glands, (H) and graph showing ANXA2 expression between different subtypes of MSGTs (I). The scale of 100 µm. Statistical significance $P = <.05$

(A-G). When comparing several types of MSGTs, we saw ANXA2 expression was higher in adenocarcinoma NOS, AdCC, MEC, and MC than in the normal salivary gland. We also noted that there was a significant increase of ANXA2 expression in MC and adenocarcinoma NOS in comparison with PA (Figure 2 I).

4 | DISCUSSION

The precision of the fine-needle aspiration biopsy (FNAB) in the diagnosis of salivary MSGT was established for decades and had good points.[17] Nevertheless, often, parotid carcinomas are diagnosed as a benign process, giving a false-negative diagnosis. Moreover, benign lesions are diagnosed as carcinomas, that is, false positive,[18] leading to unnecessary surgical intervention.[19] The preoperative diagnosis is fundamental for the correct management of these neoplasms. The proteomic analysis suggests that ANXA2 was related to MSGT. Immunohistochemical analysis showed that ANXA2 is expressed in MSGT. ANXA2 expression was associated with a poor prognosis of patients with invasive cervical carcinoma, prostate cancer, and lung cancer.[6] ANXA2 is differentially expressed in many diseases and various tissue types, particularly in several types of cancer. ANXA2 acts as a central player in dynamic reciprocity in tissue homeostasis.[8] ANXs are involved in many cytological processes such as regulation of ion channel activities, endocytotic and exocytotic processes, signal transduction, cellular differentiation, and proliferation.[20] We showed a high expression of ANXA2 in MSGT compared with normal salivary glands; we can be observed significant difference was found in between the MSGT group and the normal salivary gland group. Interestingly, ANXA2 is also shown as a stress-induced antigen, known as a phospholipid-binding protein involved in redox potential regulation, tumorigenesis, and wound healing. Stress-mediated membrane exposure of ANXA2 could thus constitute a danger signal for T cells to recognize various cell dysregulations and protect the host against infections and cancer.[21] In AcCC, adenocarcinoma NOS, AdCC, MEC, and MC, the expression of ANXA2 was high, and the labeling expressed by the IHC reveals an intense and homogeneous marking on the membrane of the cancerous cells. AdCC is relatively rare and is characterized by slow evolution, multiple recurrences, protracted clinical course, and late distant metastasis.[22,23] Due to the AdCC ability to invade adjacent tissues and migrate to blood vessels, the risks of recurrence and metastasis are higher in AdCC than in other oral cancers, such as squamous cell carcinoma (SCC).[24,25] The ANXA2 expression is associated with the metastasis and progression of hepatocellular carcinoma (HCC), and ANXA2 levels in HCC patients are significantly higher compared with the benign liver disease,[26] and also in breast cancer, the exosome-associated ANXA2 plays an essential role in angiogenesis and metastasis.[27] Others, also, such as adenocarcinoma NOS, have a high index of locoregional recurrence and distant metastases.[28] Patients with CM tend to develop metastases due to locally destructive, infiltrative, and progressive behavior.[29,30] The literature very well elucidates the role of ANXA2 in tumor malignancy. Our work opens a light for this protein in MSGTs. Due to our findings in the immunohistochemical analysis, ANXA2 is a potential molecular marker of malignancy for MSGTs. We emphasize that in our study, the number of samples in the malignant groups was our limitation. Thus, a more extensive sampling could better ratify our hypothesis of the identification of ANXA2 as a molecular marker for malignant SGNs. We believe that future large-scale validation studies are needed for further testing to clarify the role of ANXA2 in MSGTs further.

In conclusion, the current study demonstrated that MSGT presented higher levels of ANXA2 in comparison with normal salivary glands. Also, ANXA2 might be interesting as a molecular marker of adenocarcinoma NOS and MC.

ACKNOWLEDGEMENTS

We thank the Laboratório Nacional de Biociências LNBio CNPEM, Brazil, in especial to technical assistance in mass spectrometry analysis given by Bianca A. Pauletti. Dr Guimaraes, Dr De Paula, and Dr Santos are research fellows of CNPq. Dra Farias is a research fellow of FAPEMIG.

CONFLICT OF INTEREST

The authors deny any conflicts of interest related to this study.

ETHICAL APPROVAL

Ethical approval for this study was obtained from the relevant Institutional Review Board (52767316.6.0000.5146). All patients signed informed consent.

ORCID

Lucyana Conceição Farias https://orcid.org/0000-0002-5379-361X

Andre Luiz Sena Guimaraes https://orcid.org/0000-0002-3162-3206

REFERENCES

1. Gana P, Masterson L. Pleomorphic adenoma of the nasal septum: a case report. *J Med Case Rep*. 2008;2:349.
2. Zheng Y, Xiao Z, Zhang H, et al. Differentiation between benign and malignant palatal tumors using conventional MRI: a retrospective analysis of 130 cases. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2018;125(4):343-350.
3. Gobel G, Szanyi I, Revesz P, et al. Expression of NFkB1, GADD45A and JNK1 in salivary gland carcinomas of different histotypes. *Cancer Genomics Proteomics*. 2013;10(2):81-87.
4. Terhaard CH, Lubsen H, Van der Tweel I, et al. Salivary gland carcinoma: independent prognostic factors for locoregional control, distant metastases, and overall survival: results of the Dutch head and neck oncology cooperative group. *Head Neck*. 2004;26(8):681-692; discussion 692–683.
5. Shi F, Wu H, Qu K, et al. Identification of serum proteins AHSG, FGA and APOA-I as diagnostic biomarkers for gastric cancer. *Clin Proteomics*. 2018;15:18.
6. Donadio E, Giusti L, Seccia V, et al. New insight into benign tumours of major salivary glands by proteomic approach. *PLoS ONE*. 2013;8(8):e71874.

7. Wu N, Liu S, Guo C, Hou Z, Sun MZ. The role of annexin A3 playing in cancers. *Clin Transl Oncol*. 2013;15(2):106-110.
8. Hitchcock JK, Katz AA, Schafer G. Dynamic reciprocity: the role of annexin A2 in tissue integrity. *J Cell Commun Signal*. 2014;8(2):125-133.
9. Astro V, de Curtis I. Plasma membrane-associated platforms: dynamic scaffolds that organize membrane-associated events. *Sci Signal*. 2015;8(367):re1.
10. Luo S, Xie C, Wu P, et al. Annexin A2 is an independent prognostic biomarker for evaluating the malignant progression of laryngeal cancer. *Exp Ther Med*. 2017;14(6):6113-6118.
11. Mannelli G, Cecconi L, Gallo O. Laryngeal preneoplastic lesions and cancer: challenging diagnosis. Qualitative literature review and meta-analysis. *Crit Rev Oncol Hematol*. 2016;106:64-90.
12. Syed SP, Martin AM, Haupt HM, Arenas-Elliot CP, Brooks JJ. Angiostatin receptor annexin II in vascular tumors including angiosarcoma. *Human Pathol*. 2007;38(3):508-513.
13. Morais Mde L, Azevedo PR, Carvalho CH, Medeiros L, Lajus T, Costa AL. Clinicopathological study of salivary gland tumors: an assessment of 303 patients. *Cad Saude Publica*. 2011;27(5):1035-1040.
14. Rosner B. *Fundamentals of Biostatistics*. Boston, MA: Brooks/Cole, Cengage Learning; 2011.
15. Aragão AZB, Belloni M, Simabuco FM, et al. Novel processed form of syndecan-1 shed from SCC-9 cells plays a role in cell migration. *PLoS ONE*. 2012;7(8):e43521.
16. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9(7):671-675.
17. Heller KS, Dubner S, Chess Q, Attie JN. Value of fine needle aspiration biopsy of salivary gland masses in clinical decision-making. *Am J Surg*. 1992;164(6):667-670.
18. Fakhry N, Antonini F, Michel J, et al. Fine-needle aspiration cytology in the management of parotid masses: evaluation of 249 patients. *Eur Ann Otorhinolaryngol Head Neck Dis*. 2012;129(3):131-135.
19. Muñoz D, Serrano MK, Hernandez ME, et al. Matrix metalloproteinase and heparin-stimulated serine proteinase activities in post-prostate massage urine of men with prostate cancer. *Exp Mol Pathol*. 2017;103(3):300-305.
20. Benz J, Hofmann A. Annexins: from structure to function. *Biol Chem*. 1997;378(3–4):177-183.
21. Marlin R, Pappalardo A, Kaminski H, et al. Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2. *Proc Natl Acad Sci U S A*. 2017;114(12):3163-3168.
22. van der Wal JE, Becking AG, Snow GB, van der Waal I. Distant metastases of adenoid cystic carcinoma of the salivary glands and the value of diagnostic examinations during follow-up. *Head Neck*. 2002;24(8):779-783.
23. Kokemueller H, Eckardt A, Brachvogel P, Hausamen JE. Adenoid cystic carcinoma of the head and neck–a 20 years experience. *Int J Oral Maxillofac Surg*. 2004;33(1):25-31.
24. Amit M, Binenbaum Y, Sharma K, et al. Incidence of cervical lymph node metastasis and its association with outcomes in patients with adenoid cystic carcinoma. An international collaborative study. *Head Neck*. 2015;37(7):1032-1037.
25. Megwalu UC, Sirjani D. Risk of nodal metastasis in major salivary gland adenoid cystic carcinoma. *Otolaryngol Head Neck Surg*. 2017;156(4):660-664.
26. Al-Khafaji BM, Nestok BR, Katz RL. Fine-needle aspiration of 154 parotid masses with histologic correlation: ten-year experience at the University of Texas M. D. Anderson cancer center. *Cancer*. 1998;84(3):153-159.
27. Maji S, Chaudhary P, Akopova I, et al. Exosomal annexin II promotes angiogenesis and breast cancer metastasis. *Mol Cancer Res*. 2017;15(1):93-105.
28. Spiro RH. Salivary neoplasms: overview of a 35-year experience with 2,807 patients. *Head Neck Surg*. 1986;8(3):177-184.
29. Nagao T, Sugano I, Ishida Y, et al. Salivary gland malignant myoepithelioma: a clinicopathologic and immunohistochemical study of ten cases. *Cancer*. 1998;83(7):1292-1299.
30. Guo X, Watanabe J, Ariyasu S, et al. Myoepithelial carcinoma of the parotid gland: a case of adequate fine-needle aspiration cytology specimens rendering a conclusive diagnosis possible. *SAGE Open Med Case Rep*. 2018;6:2050313X18780842.

How to cite this article: Cardoso CM, de Jesus SF, de Souza MG, et al. High levels of ANXA2 are characteristic of malignant salivary gland tumors. *J Oral Pathol Med*. 2019;00:1–6. https://doi.org/10.1111/jop.12932

4 CONCLUSÕES

As avaliações através de RT-PCR e imuno-histoquímica, não demonstraram diferenças significativas na expressão dos marcadores de hipóxia HIF-1α e mirR-210 entre neoplasias benignas e malignas de glândula salivar. Não houve também diferença significativa na expressão de mirR-210 entre neoplasias benignas, malignas e tecido salivar nao neoplásico, o que nos sugere que as neoplasias de glândula salivar não são hipóxicas.

A análise proteômica evidenciou 105 proteínas nos três grupos estudados, com 31 proteínas encontradas exclusivamente em tecido salivar não neoplásico (mucocele), 21 proteínas encontradas exclusivamente na amostra de neoplasia benigna (adenoma pleomórfico) e 16 proteínas encontradas exclusivamente na amostra de neoplasia maligna (carcinoma mioepitelial). A análise com a base de dados Gene Expression Omnibus (GEO) mostrou que a proteína anexina A2 (ANXA-2) foi a mais expressa entre as proteínas específicas encontradas na amostra de neoplasia maligna. Este resultado foi ratificado pela imuno-histoquímica, que evidenciou uma maior expressão de ANXA-2 em neoplasias malignas, quando comparada com neoplasias benignas e tecido salivar não neoplásico. A imuno-histoquímica mostrou ainda uma maior expressão da ANXA-2 em fenótipos mais agressivos como adenocarcinoma SOE e carcinoma mioepitelial. Estes resultados nos faz concluir que a ANXA-2 pode, no futuro, ser um marcador tumoral relevante, necessitando porém de outros estudos, com maior casuística, para confirmarem estes achados.

REFERÊNCIAS

ALAMRI, A. M. et al. Expanding primary cells from mucoepidermoid and other salivary gland neoplasms for genetic and chemosensitivity testing. **Disease models & mechanisms,** v. 11, n. 1, p. dmm031716, 2018.

ANDREASEN, S. et al. An update on head and neck cancer: new entities and their histopathology, molecular background, treatment, and outcome. **Apmis,** v. 127, n. 5, p. 240-264, 2019.

ANDREASEN, S. et al. Pleomorphic adenoma of the parotid gland 1985–2010: a Danish nationwide study of incidence, recurrence rate, and malignant transformation. **Head & neck,** v. 38, n. S1, p. E1364-E1369, 2016.

BELL, R. B. et al. Management and outcome of patients with malignant salivary gland tumors. **Journal of oral and maxillofacial surgery,** v. 63, n. 7, p. 917-928, 2005.

BOUKHERIS, H. et al. Risk of radiation-related salivary gland carcinomas among survivors of Hodgkin lymphoma: a population-based analysis. **Cancer: Interdisciplinary International Journal of the American Cancer Society,** v. 113, n. 11, p. 3153-3159, 2008.

BRAHIMI-HORN, M. C.; POUYSSÉGUR, J. Harnessing the hypoxia-inducible factor in cancer and ischemic disease. **Biochemical pharmacology,** v. 73, n. 3, p. 450-457, 2007.

BRIZEL, D. M. et al. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. **International Journal of Radiation Oncology* Biology* Physics,** v. 38, n. 2, p. 285-289, 1997.

BYRD, S. A. et al. Predictors of recurrence and survival for head and neck mucoepidermoid carcinoma. **Otolaryngology--Head and Neck Surgery,** v. 149, n. 3, p. 402-408, 2013.

CERDA, T. et al. A rationale for chemoradiation (vs radiotherapy) in salivary gland cancers? On behalf of the REFCOR (French rare head and neck cancer network). **Critical reviews in oncology/hematology,** v. 91, n. 2, p. 142-158, 2014.

CERMIK, T. F. et al. FDG PET in detecting primary and recurrent malignant salivary gland tumors. **Clinical nuclear medicine,** v. 32, n. 4, p. 286-291, 2007.

CHEN, W. et al. Identification of microRNA profiles in salivary adenoid cystic carcinoma cells during metastatic progression. **Oncology letters,** v. 7, n. 6, p. 2029-2034, 2014.

CHIOSEA, S. et al. Mucoepidermoid carcinoma of upper aerodigestive tract: clinicopathologic study of 78 cases with immunohistochemical analysis of Dicer expression. **Virchows Archiv,** v. 452, n. 6, p. 629-635, 2008.

COLELLA, G. et al. Fine-needle aspiration cytology of salivary gland lesions: a systematic review. **Journal of Oral and Maxillofacial Surgery,** v. 68, n. 9, p. 2146-2153, 2010.

COMERFORD, K. M. et al. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. **Cancer research,** v. 62, n. 12, p. 3387-3394, 2002.

COPELLI, C. et al. Malignant tumors of intraoral minor salivary glands. **Oral oncology,** v. 44, n. 7, p. 658-663, 2008.

DECLERCQ, J. et al. Salivary gland tumors in transgenic mice with targeted PLAG1 proto-oncogene overexpression. **Cancer research,** v. 65, n. 11, p. 4544-4553, 2005.

DOUVILLE, N. J.; BRADFORD, C. R. Comparison of ultrasound-guided core biopsy versus fine-needle aspiration biopsy in the evaluation of salivary gland lesions. **Head & neck,** v. 35, n. 11, p. 1657-1661, 2013.

EL-NAGGAR, A. K. What is new in the World Health Organization 2017 histopathology classification? **Current treatment options in oncology,** v. 18, n. 7, p. 1-4, 2017.

ENEROTH, C. M. Salivary gland tumors in the parotid gland, submandibular gland, and the palate region. **Cancer,** v. 27, n. 6, p. 1415-1418, 1971.

ETIT, D. et al. An analysis of salivary gland neoplasms: a 12-year, single-institution experience in Turkey. **Ear, Nose & Throat Journal,** v. 91, n. 3, p. 125-129, 2012.

FRELING, N.; CRIPPA, F.; MAROLDI, R. Staging and follow-up of high-grade malignant salivary gland tumours: The role of traditional versus functional imaging approaches–A review. **Oral oncology,** v. 60, p. 157-166, 2016.

GEE, H. E. et al. hsa-miR-210 is a marker of tumor hypoxia and a prognostic factor in head and neck cancer. **Cancer,** v. 116, n. 9, p. 2148-2158, 2010.

GIANNAKAKIS, A. et al. miR-210 links hypoxia with cell cycle regulation and is deleted in human epithelial ovarian cancer. **Cancer biology & therapy,** v. 7, n. 2, p. 255-264, 2008.

HARRIS, A. L. Hypoxia—a key regulatory factor in tumour growth. **Nature reviews cancer,** v. 2, n. 1, p. 38-47, 2002.

HE, J. et al. MiR-210 disturbs mitotic progression through regulating a group of mitosis-related genes. **Nucleic acids research,** v. 41, n. 1, p. 498-508, 2013.

HONG, L. et al. High expression of miR-210 predicts poor survival in patients with breast cancer: a meta-analysis. **Gene,** v. 507, n. 2, p. 135-138, 2012.

HOOGSTEEN, I. J. et al. Tumor microenvironment in head and neck squamous cell carcinomas: predictive value and clinical relevance of hypoxic markers. A review. **Head & Neck: Journal for the Sciences and Specialties of the Head and Neck,** v. 29, n. 6, p. 591-604, 2007.

HOWLETT, D. High resolution ultrasound assessment of the parotid gland. **The British Journal of Radiology,** v. 76, n. 904, p. 271-277, 2003.

HUANG, X.; LE, Q.-T.; GIACCIA, A. J. MiR-210–micromanager of the hypoxia pathway. **Trends in molecular medicine,** v. 16, n. 5, p. 230-237, 2010.

INDOVINA, P. et al. Mass spectrometry-based proteomics: The road to lung cancer biomarker discovery. **Mass spectrometry reviews,** v. 32, n. 2, p. 129-142, 2013.

JONATHAN, R. A. et al. The prognostic value of endogenous hypoxia-related markers for head and neck squamous cell carcinomas treated with ARCON. **Radiotherapy and Oncology,** v. 79, n. 3, p. 288-297, 2006.

KELLOFF, G. J.; SIGMAN, C. C. New science-based endpoints to accelerate oncology drug development. **European Journal of Cancer,** v. 41, n. 4, p. 491-501, 2005.

LIN, H. H.; LIMESAND, K. H.; ANN, D. K. Current state of knowledge on salivary gland cancers. **Critical Reviews™ in Oncogenesis,** v. 23, n. 3-4, 2018.

LIU, Y. et al. Accuracy of diagnosis of salivary gland tumors with the use of ultrasonography, computed tomography, and magnetic resonance imaging: a meta-analysis. **Oral surgery, oral medicine, oral pathology and oral radiology,** v. 119, n. 2, p. 238-245. e2, 2015.

LOTZE, M. T. et al. Workshop on cancer biometrics: identifying biomarkers and surrogates of cancer in patients: a meeting held at the Masur Auditorium, National Institutes of Health. **Journal of Immunotherapy,** v. 28, n. 2, p. 79-119, 2005.

LUNA, B. A. Salivary gland tumors. A review of 2410 cases with particular reference to histological types, site, age and sex distribution: Eveson JW, Cawson RA. Pathol 146: 51, 1985. **Journal of Oral and Maxillofacial Surgery,** v. 44, n. 3, p. 249, 1986.

MANTRAVADI, A. V.; MOORE, M. G.; RASSEKH, C. H. AHNS series: Do you know your guidelines? Diagnosis and management of salivary gland tumors. **Head & Neck,** v. 41, n. 2, p. 269-280, 2019.

MARTINKOVA, J. et al. Challenges in cancer research and multifaceted approaches for cancer biomarker quest. **FEBS letters,** v. 583, n. 11, p. 1772-1784, 2009.

MATSE, J. H. et al. Discovery and Prevalidation of Salivary Extracellular microRNA Biomarkers Panel for the Noninvasive Detection of Benign and Malignant Parotid Gland TumorsmiRNA Biomarkers for the Detection of Parotid Gland Tumors. **Clinical Cancer Research,** v. 19, n. 11, p. 3032-3038, 2013.

NOHATA, N. et al. MicroRNAs function as tumor suppressors or oncogenes: aberrant expression of microRNAs in head and neck squamous cell carcinoma. **Auris Nasus Larynx,** v. 40, n. 2, p. 143-149, 2013.

PALMERO, E. I. et al. Mechanisms and role of microRNA deregulation in cancer onset and progression. **Genetics and molecular biology,** v. 34, p. 363-370, 2011.

PARK, H. L. et al. The value of F-18 FDG PET for planning treatment and detecting recurrence in malignant salivary gland tumors: comparison with conventional imaging studies. **Nuclear medicine and molecular imaging,** v. 47, n. 4, p. 242-248, 2013.

PEDERSON, A. W. et al. Adjuvant chemoradiotherapy for locoregionally advanced and high-risk salivary gland malignancies. **Head & neck oncology,** v. 3, n. 1, p. 1-6, 2011.

SEMENZA, G. L. Targeting HIF-1 for cancer therapy. **Nature reviews cancer,** v. 3, n. 10, p. 721-732, 2003.

SPIRO, R. H. Salivary neoplasms: overview of a 35-year experience with 2,807 patients. **Head & neck surgery,** v. 8, n. 3, p. 177-184, 1986.

SRINIVAS, P. R.; KRAMER, B. S.; SRIVASTAVA, S. Trends in biomarker research for cancer detection. **The lancet oncology,** v. 2, n. 11, p. 698-704, 2001.

STRONCEK, D. F. et al. Advancing cancer biotherapy with proteomics. **Journal of Immunotherapy,** v. 28, n. 3, p. 183-192, 2005.

TERHAARD, C. H. et al. The role of radiotherapy in the treatment of malignant salivary gland tumors. **International Journal of Radiation Oncology* Biology* Physics,** v. 61, n. 1, p. 103-111, 2005.

THOMLINSON, R. H.; GRAY, L. H. The histological structure of some human lung cancers and the possible implications for radiotherapy. **British journal of cancer,** v. 9, n. 4, p. 539, 1955.

WARNER, K. A. et al. Characterization of tumorigenic cell lines from the recurrence and lymph node metastasis of a human salivary mucoepidermoid carcinoma. **Oral oncology,** v. 49, n. 11, p. 1059-1066, 2013.

WIJFFELS, K. I. et al. No detectable hypoxia in malignant salivary gland tumors: preliminary results. **International Journal of Radiation Oncology* Biology* Physics,** v. 73, n. 5, p. 1319-1325, 2009.

YING, Q. et al. Hypoxia-inducible MicroRNA-210 augments the metastatic potential of tumor cells by targeting vacuole membrane protein 1 in hepatocellular carcinoma. **Hepatology,** v. 54, n. 6, p. 2064-2075, 2011.

ZHANG, X. et al. Alterations in miRNA processing and expression in pleomorphic adenomas of the salivary gland. **International Journal of Cancer,** v. 124, n. 12, p. 2855-2863, 2009.

ZHU, W.; SMITH, J. W.; HUANG, C.-M. Mass spectrometry-based label-free quantitative proteomics. **Journal of Biomedicine and Biotechnology,** v. 2010, 2009.