UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

TESE DE DOUTORADO

ANÁLISE DO SECRETOMA DE LINHAGENS CELULARES ORIGINADAS DE TUMORES GÁSTRICOS

Identificação de novos potenciais alvos terapêuticos e biomarcadores

BEATRIZ DAL PONT DUARTE

Porto alegre, 2018.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

ANÁLISE DO SECRETOMA DE LINHAGENS CELULARES ORIGINADAS DE TUMORES GÁSTRICOS

Identificação de novos potenciais alvos terapêuticos e biomarcadores

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do grau de Doutor em Biologia Celular e Molecular.

BEATRIZ DAL PONT DUARTE
Orientador: Prof. Dr. Diego Bonatto

Porto alegre, setembro de 2018.

DEDICATÓRIA

Dedico este trabalho ao meu marido Jakson Vassoler,

pelo amor e apoio.

AGRADECIMENTOS

Ao meu orientador, Dr. Diego Bonatto, por me aceitar como orientanda e por me mostrar a profissional que eu quero ser. Muito Obrigada!

Aos membros da banca Dra. Eloiza Helena Tajara, Dr. Paulo Ivo Bittencourt, Dra. Karina Mariante e o Dr. Daniel Pens Gelain por terem aceitado o convite e pelas contribuições feitas a este trabalho.

Aos professores e funcionários do PPGBCM, especialmente a Silvinha que sempre nos recebe com seu lindo sorriso.

Ao CNPq e demais agencias financiadoras que possibilitaram a realização deste trabalho.

Ao professor Dr. Guido Lenz e aos integrantes do seu laboratorio, pelo espaço, conversa e ajuda nas horas de dúvidas.

Ao professor Dr. João Antonio Pêgas Henriques, e aos integrantes do seu laboratório, André, Michelle, Victoria, Tati, Larissa, Iuri, Miriana, José, Temenuga, Priscila, Carol e Jéssica. Um obrigada muito especial a todos vocês por terem me recebido e pelas conversas e risos.

As minhas amigas Renata Fagundes e Raquel Ayres,
por me acolherem na minha chegada ao laboratório, pelas risadas, cafés e desabafos.

Aos meus amigos do eterno 219, Lari, Bruno, Joice, Kendi, Raquel, Gabino, Chapolinha e Gabriel. Vocês são parte fundamental do caminho que eu trilhei até aqui. Nunca vou ter palavras para agradecer por tudo!

Aos meus sogros Valcir (*in memoriam*) e Marisa, ao meu cunhado Ronaldo e a Maira por terem me acolhido tantas vezes.

Aos dois serezinhos que são a pura representação do amor, Alice e Belinha!

Aos meus pais, Américo e Salete. Agradeço todo apoio, amor e dedicação.
Amo muito vocês!

Aquelas que foram minhas primeiras amigas, minhas irmãs, Rosiani e Giseli. Obrigada por todo apoio. Amo vocês demais!

Ao meu marido Jakson Manfredini Vassoler por todo incentivo e amor.
Te amo hoje e sempre!

“I knew who I was this morning, but I've changed

a few times since then.”

Lewis Carroll
Alice's Adventures in Wonderland & Through the Looking-Glass

SUMÁRIO

LISTA DE ABREVIATURAS

5-FU - 5-fluorouracil

ACPO2 - Linhagem celular derivada de adenocarcinoma primário 02

ACP03 - Linhagem celular derivada de adenocarcinoma primário 03

AREG - Anfiregulina

BiP - do inglês *Binding immunoglobulin protein*

BP - do inglês *base pair*

CG - Câncer gástrico

CRISPR – do inglês *Clustered regularly interspaced short palindromic repeats*

CTT - Células tronco tumorais

ECM - do inglês *Extracellular matrix*

EGFR - Receptor de fator de crescimento epidérmico

ER - do inglês *Endoplasmatic reticulum*

ERAD - Sistema de degradação associado ao retículo endoplasmático, do inglês *Endoplasmatic reticulum associated degradation*

FBS - do inglês *Fetal bovine serum*

FDR - do inglês *False discovery rate*

GC - do inglês *Gastric Cancer*

GDF-15- Fator de crescimento transformante 15, do inglês *Growth differential factor 15*

GBM - do inglês *Glioblastoma multiforme*

GO - do inglês *Gene ontology*

gRNA - RNA guia

HB - do inglês *Hub-bottleneck*

HSE - do inglês *Heat shock element*

HSP - Proteína do choque térmico do inglês *Heat shock protein*

IARC - do inglês *International Agency for Research on Cancer*

IL-6 - Interleucina 6

INCA - Instituto Nacional de Câncer

IRE1α - Enzima requerente de inositol 1 alpha, do inglês *Inositol-requiring enzyme 1 alpha*

MC - Meio condicionado

MEC- Matriz extracelular

miR - Micro RNA

MMP - do inglês *Matrix metalloproteinases*

MN01 - Linhagem celular de mucosa normal 01

OMS - Organização Mundial da Saúde

PDI - do inglês *Protein disulfide isomerase*

PDT - do inglês *Population doubling time*

PI – do inglês *Propidium iodide*

PPI - do inglês *Protein-protein interaction*

RE - Retículo endoplasmático

SCCHN - do inglês *Squamous cell carcinoma of the head and neck*

SEM – do inglês *Statistic error of the mean*

TGF-ß - Fatores de transformação do crescimento beta, do inglês *Transforming growth factor beta*

UPR - do inglês *Unfolded protein response*

VEGF - Fator de crescimento endotelial vascular

WHO - do inglês *World Health Organization*

LISTA DE FIGURAS

INTRODUÇÃO

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

CAPÍTULO IV

LISTA DE TABELAS

INTRODUÇÃO

CAPÍTULO I

RESUMO

As neoplasias malignas são responsáveis por milhões de mortes todos os anos, sendo que os problemas acarretados por tumores gástricos representam a terceira causa de morte por câncer a nível mundial. Um dado preocupante em relação ao câncer gástrico é em relação a sobrevida global de 5 anos que é de apenas 30%, aproximadamente. Um dos principais fatores que acarretam em taxas tão alarmantes é que em estágios iniciais o câncer gástrico é assintomático, fazendo com que o seu diagnóstico ocorra normalmente em estágios mais tardios. Além disso, prognósticos ruins mesmo em estágios iniciais da doença são comuns neste tipo de câncer, principalmente devido ao alto índice de resistência das células tumorais aos tratamentos convencionais. Com a necessidade de novos biomarcadores e novas modalidades de tratamentos, o estudo do secretoma de células tumorais vem sendo amplamente explorado. Este tipo de estudo é utilizado como meio de identificar novas moléculas que possam ser utilizadas em diagnósticos mais precoces assim como novos alvos terapêuticos. Por isso, o objetivo desse trabalho foi analisar o perfil do secretoma de células tumorais derivadas de linhagens de câncer gástrico (ACP02 e ACP03), em comparação com uma linhagem de mucosa gástrica normal (MN01), buscando novas moléculas que possam servir de biomarcadores, bem como fonte para novos alvos terapêuticos. Para o alcance desse objetivo o meio condicionado das diferentes linhagens celulares foi coletado, e as proteínas presentes foram concentradas através de ultrafiltração e identificadas através de análises por Q-Tof LC/MS/MS. A análise de espectrometria de massas identificou 333 proteínas secretadas, sendo 86 exclusivamente secretadas por ambas as linhagens de câncer gástrico. Após a identificação, as proteínas foram analisadas através de biologia de sistemas e, devido a sua importância, as proteínas PDIA6, GDF15 e HSP47 foram escolhidas para análises por Western blot e qPCR. A análise de Western blot confirmou que as proteínas GDF15 e HSP47 são secretadas apenas pelas linhagens de adenocarcinomas gástricos. Porém quando analisamos a proteína PDIA6 não conseguimos comprovar a sua secreção diferencial pelas linhagens de adenocarcinomas. Com estes resultados, resolvemos analisar a proteína HSP47 como possível alvo terapêutico, através do efeito do seu silenciamento nas linhagens de câncer gástrico pela técnica de CRISPR. Estas análises demonstraram que o silenciamento da proteína HSP47 reduziu a capacidade migratória, invasiva e de formação de esferas nas células tumorais. Em síntese, os resultados demonstraram que a HSP47 pode ser um possível alvo terapêutico no tratamento do câncer gástrico, e que HSP47 e GDF15 possuem potencial para serem utilizadas como biomarcadores devido a sua secreção diferencial pelas células tumorais.

ABSTRACT

The malignant neoplasms are responsible for millions of deaths every year, and the gastric cancer is the third leading cause of cancer deaths worldwide. An alarming finding regarding gastric cancer is the five-year survival rate of only 30%. One of the main problems related to gastric cancer is that it is asymptomatic in the initial phases of the disease, making their diagnosis usually occur in later stages. Also, poor prognoses even in the early stages of the disease are frequent in this type of cancer, mainly due to the high resistance of tumor cells to conventional treatments. The study of the secretome of tumor cell lines has been extensively explored to discover new biomarkers and new modalities of treatments, that can be used in earlier diagnoses as well as new therapeutic targets. Then, the present study aimed to characterize the secretome of two human gastric cancer cell lines (ACP02 and ACP03) in order to compare their differences concerning the non-neoplastic gastric cell line (MN01). For this purpose, we collected the conditioned medium of these cell lines and concentrated the secreted proteins by ultrafiltration. After that, the identification of the proteins was made by ultra-performance Q-Tof LC/MS/MS. Mass spectrometry-based proteomics identified 333 proteins in the three cell lines of which 86 proteins are identified exclusively from the two carcinoma cell lines. After the identification, the proteins were analyzed through system biology and, due to their importance, the proteins PDIA6, GDF15 e HSP47 were chosen for western blot and qPCR analyzes. The Western blot analysis showed that GDF15 and HSP47 are secreted only by the tumor cell lines. However, when we analyzed de PDIA6 protein, we are not able to confirm the differential secretion by the tumor cell lines. With these results, we decided to analyze the HSP47 protein as a possible therapeutic target, through the effect of its silencing on gastric cancer lines by the CRISPR technique. These analyses showed that HSP47 silencing decreases the migration, invasion, and sphere forming capacity in gastric tumor cells. In summary, the results demonstrated that the HSP47 protein may be a possible therapeutic target in the treatment of gastric cancer and that HSP47 and GDF15 proteins have potential to be used as biomarkers, due to their differential secretion by tumor cells.

1. JUSTIFICATIVA

Apesar dos grandes avanços em relação aos diagnósticos e aos tratamentos disponíveis, as neoplasias malignas continuam sendo uma das principais doenças que apresentam risco de vida. De acordo com os dados fornecidos pela Organização Mundial da Saúde (OMS), 8,8 milhões de pessoas morreram de câncer no ano de 2015 (WHO, 2017), sendo que a previsão é que esta taxa alcance 11 milhões em 2030 (FERLAY et al., 2015). Para o Brasil, estima-se para o biênio 2018-2019 a ocorrência de 1.200.000 novos casos de câncer (INCA, 2017).

Dentre os tipos de câncer, o gástrico é o quinto tipo mais comum de câncer e é a terceira causa de morte por neoplasias a nível mundial. Um dos principais fatores que acarretam taxas tão elevadas é que em estágios iniciais o câncer gástrico é assintomático, fazendo com que o seu diagnóstico ocorra em estágios mais tardios. Outro agravante é que os biomarcadores conhecidos atualmente, relacionados ao câncer gástrico, não apresentam nem especificidade e nem sensibilidade altas (JEREZ; VAN WIJNEN; GALINDO, 2016). Além disso, prognósticos ruins são comuns neste tipo de câncer mesmo em estágios iniciais da doença, principalmente devido ao alto índice de resistência que as células tumorais apresentam frente aos tratamentos convencionais (BARAL et al., 2014). A necessidade de novos biomarcadores e novas modalidades de tratamentos tem acarretado no estudo do secretoma de células tumorais. Este tipo de estudo é utilizado como meio de identificar novas moléculas que possam ser utilizadas em diagnósticos mais precoces, assim como novos alvos terapêuticos (Makridakis; Vlahou, 2010).

O termo secretoma foi introduzido a mais de uma década por Tjalsma e colaboradores para descrever os constituintes da via secretória, bem como os próprios fatores secretados por um grupo de células, por um tecido ou organismo (TJALSMA et al., 2000). As proteínas secretadas pelas células são as principais moléculas da comunicação intercelular, tendo sua participação na maioria dos processos fisiológicos. Estas podem levar a indução da proliferação celular, perda de sinais de morte, assim como acarretar em processos metastáticos (MAKRIDAKIS; VLAHOU, 2010). Apesar de serem secretadas por diferentes vias celulares, as moléculas tem sua regulação altamente controlada. Dessa forma, alterações no secretoma de um tecido pode ser um indício de algum processo patológico, como, por exemplo, processos neoplásicos. Vários estudos já demonstraram que em processos tumorais

as células apresentam alterações no seu secretoma quando comparado ao tecido normal. Essas mudanças podem contribuir para a descoberta de novas moléculas que sirvam como biomarcadores e alvos terapêuticos (PALTRIDGE; BELLE; KHEW-GOODALL, 2013). Além disso, alguns estudos com secretoma de células tumorais vêm descrevendo que a resistência a certas drogas quimioterápicas é devido a capacidade de certas moléculas secretadas metabolizarem os fármacos utilizados. Este fato vem ajudando a direcionar os tratamentos em determinados tipos de câncer tornando o tratamento mais eficaz (EMMINK et al., 2013; TODARO et al., 2007).

O objetivo desse trabalho foi analisar o perfil do secretoma de células tumorais derivadas de linhagens de câncer gástrico, buscando novas moléculas que possam servir de biomarcadores, bem como fonte para novos alvos terapêuticos. Para o alcance desse objetivo esse trabalho foi dividido em duas etapas. Na primeira etapa os secretomas de duas linhagens celulares de câncer gástrico (ACP02 e ACP03) foram analisados em comparação ao secretoma de uma linhagem de mucosa gástrica normal (MN01). Já a segunda etapa do trabalho consistiu na escolha do alvo a ser estudado através dos dados obtidos, sendo que essa molécula teve a sua relação com importantes processos biológicos para o crescimento tumoral, como migração e invasão, analisada.

2. REVISÃO DA LITERATURA

2.1 Câncer

As neoplasias malignas, também conhecidas como câncer, compreendem mais de 100 doenças diferentes, e podem ser caracterizadas por um grupo de células com crescimento e divisão celular anormal, resistência a apoptose, capacidade invasiva e migratória, entre outras características (Figura 1) (HANAHAN; WEINBERG, 2011). Essas características são adquiridas através de mutações que levam a modificações genéticas e epigenéticas, permitindo com que a célula ative mecanismos celulares que normalmente estariam desativados em células de organismos adultos (HAHN; WEINBERG, 2002).

Até algumas décadas, acreditava-se que a tumorigênese e a progressão tumoral ocorria unicamente devido as mutações ocorridas nas células tumorais. Porém com o estudo do microambiente tumoral, foi sendo elucidado a importância da interação entre as células tumorais e as células do estroma que o circundam (MUELLER; FUSENIG, 2004).

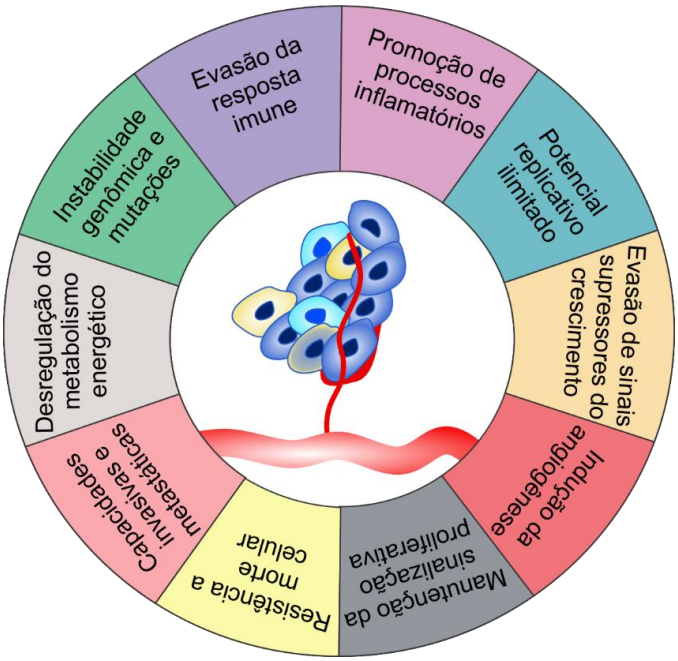

Fig. 1 Principais características apresentadas pelas células tumorais segundo Hanahan e Weinberg (2011).

As células do estroma englobam fibroblastos, células do sistema imune e endoteliais. Através da interação tumor-estroma, as células tumorais induzem a alteração do microambiente ao seu redor tornando-o permissivo e com capacidade de suporte para a progressão tumoral (MUELLER; FUSENIG, 2004). Essas alterações levam, por exemplo, a angiogênese tumoral e a liberação de metaloproteinases capazes de remodelar e degradar a matriz extracelular (MEC), tornando o ambiente receptivo para a migração e invasão das células tumorais (GEIGER; PEEPER, 2009).

A comunicação entre as células tumorais e as células do estroma pode ocorrer tanto por contato direto quanto pela sinalização através de moléculas secretadas, como fatores de crescimento, citocinas e moléculas de DNA/RNA (KOHLHAPP et al., 2015; NASSER et al., 2015). Elucidar quais moléculas estão envolvidas na comunicação tumor-estroma, e como essas atuam na progressão tumoral é essencial para o desenvolvimento de novos alvos terapêuticos mais eficazes, que acarretem em melhores prognósticos para os pacientes e que diminuam as taxas de mortalidade do câncer.

Segundo dados disponibilizados pela Organização Mundial da Saúde (OMS), em 2015 houveram 8,8 milhões de mortes por câncer no mundo, sendo que o gasto estimado em 2010 ao combate a essas doenças chegou a 1,16 trilhões de dólares (WHO, 2017). Do número total de casos de câncer 60% ocorrem na África, Ásia, América Central e do Sul, sendo que estas regiões correspondem a 70% do número total de mortes por câncer. Entre os homens os 5 tipos mais comuns de câncer no ano de 2012 foram pulmão (16.7% do total), próstata (15.0%), colorretal (10.0%), estômago (8.5%), e fígado (7.5%). Já entre as mulheres, para o mesmo ano, os mais incidentes foram câncer de mama (25.2% do total), colorretal (9.2%), pulmão (8.7%), cervical (7.9%) e estômago (4.8%) (STEWART, B., WILD, 2014). A Figura 2 apresenta os tipos de cânceres mais incidentes e com maiores casos de morte em ambos os sexos.

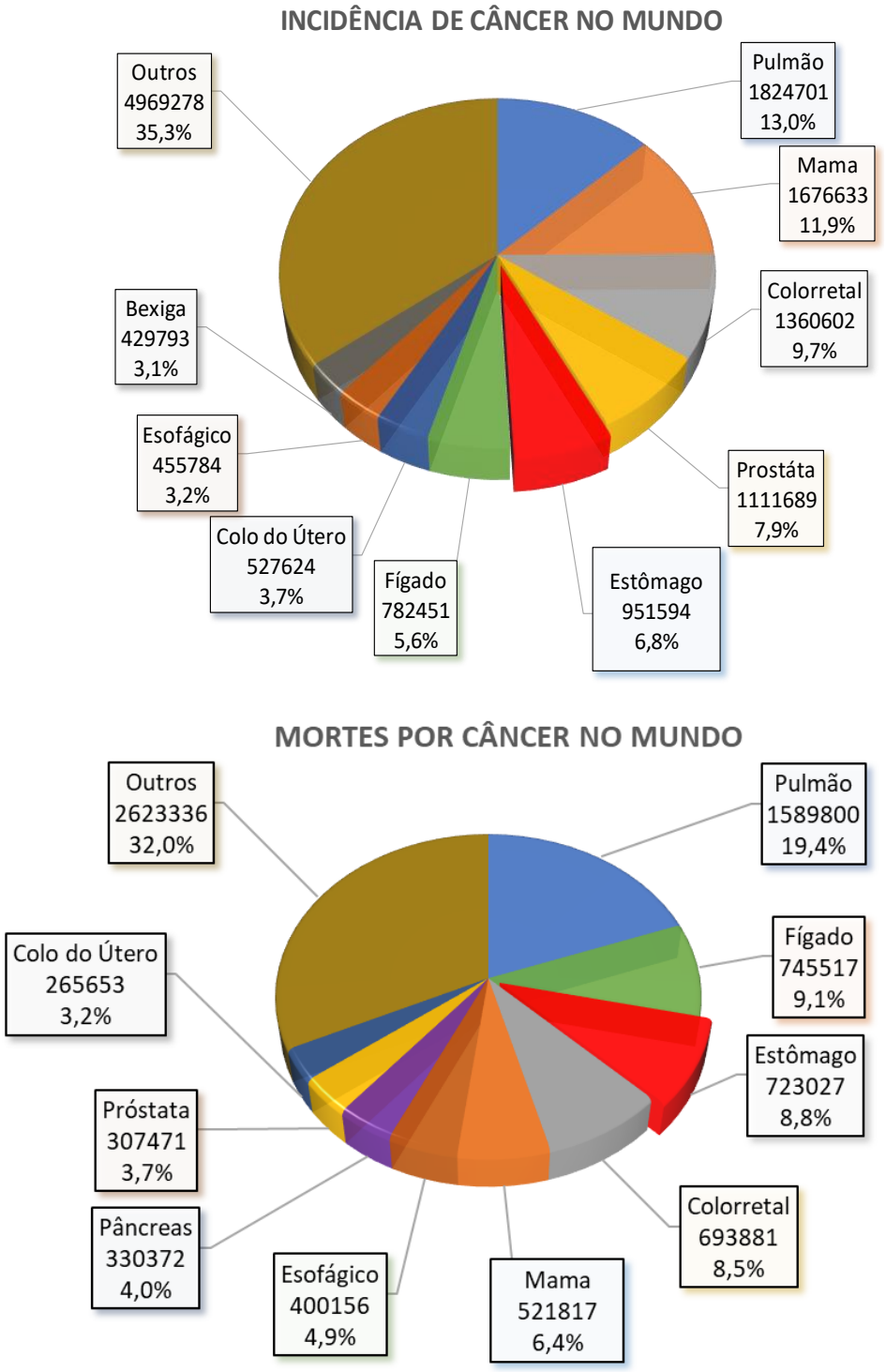

Fig. 2 Incidência e morte por câncer em ambos os sexos no ano de 2012. Abaixo do tipo de câncer encontra-se o número bruto de casos e a porcentagem em relação ao número total de casos. Dados retirados do site IARC/WHO.

2.2 Câncer Gástrico

O câncer gástrico (CG), também denominado câncer estomacal, é um grupo de doenças caracterizadas pelo surgimento de tumores malignos nas diferentes regiões do estômago (VAN CUTSEM et al., 2016). Anatomicamente o estômago pode ser dividido em região da cárdia, que compreende a transição entre o esôfago e o estômago, a região do fundo, formada pela curvatura superior do órgão, a região do corpo, situada entre o fundo e o antro pilórico, e o antro pilórico que une o estômago e o intestino delgado (Figura 3) (VAN CUTSEM et al., 2016).

Além das diferenças anatômicas, quando analisamos as diferenças histológicas podemos separar as regiões do estômago em mucosa, submucosa e muscular, além de um revestimento por uma delgada camada serosa. Na mucosa temos as fossetas gástricas que desembocam as glândulas que secretam o suco gástrico (VAN CUTSEM et al., 2016).

Considerando a classificação histológica o CG pode ser classificado em linfoma, leiomiossarcoma e adenocarcinoma. O subtipo linfoma é originado dos linfonodos associados a mucosa da região estomacal, e o subtipo leiomiossarcoma se origina dos músculos associados ao estômago (KARIMI et al., 2014). Já os tumores classificados como adenocarcinomas, surgem das glândulas da região mais superficial da mucosa estomacal, e correspondem ao maior número de casos registrados de CG (aproximadamente 95%) (INCA, 2017). Os adenocarcinomas, segundo a classificação de Lauren, ainda podem ser subdivididos em difuso e intestinal. De acordo com essa classificação, os tumores do tipo difuso são pouco diferenciados e com ausência de formação glandular. Já o tipo intestinal é caracterizado como moderadamente a altamente diferenciado e, diferentemente do difuso, apresenta estruturas glandulares (VAN CUTSEM et al., 2016).

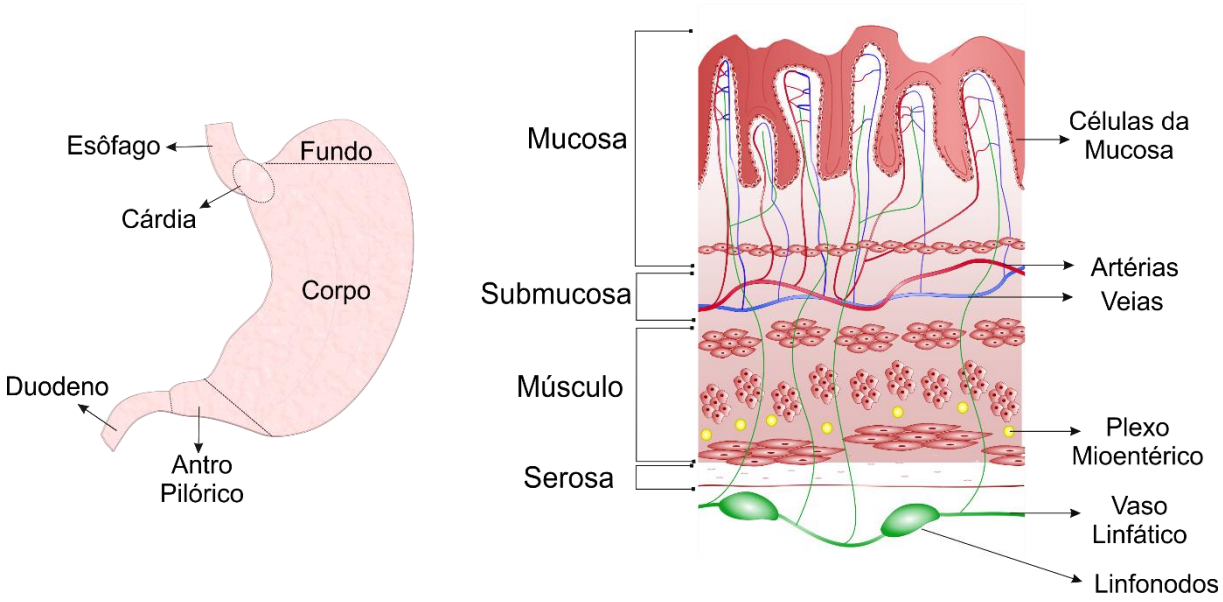

Fig. 3 Diferentes regiões do estômago segundo as características anatômicas e histológicas.

O CG é uma doença multifatorial, sendo que tantos componentes do meio ambiente como componentes genéticos compõem a sua etiologia. Dentre os fatores de risco estão o histórico familiar, tabagismo, alto consumo de carne vermelha, condições socioeconômicas, consumo de álcool, consumo de alimentos defumados e obesidade (KARIMI et al., 2014). Além desses fatores, a infecção pela bactéria *Helicobacter pylori* é considerada a principal causa de risco para o desenvolvimento de CG, sendo que se estima que entre 65 a 85% de todos os casos de CG diagnosticados sejam causados devido a esta infecção bacteriana. Os mecanismos pelos quais a *H. pylori* leva ao desenvolvimento de CG ainda não são totalmente esclarecidos, mas duas possibilidades vêm se destacando nas pesquisas científicas que abordam o assunto. A primeira seria que o desenvolvimento tumoral estaria sendo desencadeado devido ao processo inflamatório causado pela infecção. A segunda hipótese está relacionada com a modulação das células do epitélio gástrico por fatores bacterianos como a citotoxina associada ao gene A (PORMOHAMMAD et al., 2018).

Apesar de alguns países desenvolvidos apresentarem declínio da incidência, altas taxas de mortalidade ainda são registradas na América Latina e no Japão, que é o país com maior incidência de CG mundialmente. O declínio de CG em países desenvolvidos, provavelmente está relacionado com o fato de *H. pylori* ser o principal fator de risco para o desenvolvimento deste tipo de câncer. Nestes países, a população possui um melhor acesso a meios de

sanitização, além do acesso facilitado a antibióticos de última geração, diminuindo os casos de infecção por esta bactéria (KARIMI et al., 2014).

Dados recentes do Instituto Nacional de Câncer (INCA), apontam para 21.290 novos casos para o ano de 2018 no Brasil, sendo 13.540 novos casos em homens e 7.750 novos casos em mulheres, ficando em terceiro lugar na incidência entre homens e em quinto entre as mulheres. O pico de incidência ocorre por volta dos 70 anos e cerca de 65% dos pacientes diagnosticados com CG apresentam mais de 50 anos (INCA, 2017).

Um dos dados mais preocupantes sobre o CG é em relação a sobrevida global de 5 anos que é de apenas 30%, aproximadamente. Essa porcentagem baixa em relação a sobrevida também é encontrada em países desenvolvidos, onde a incidência geral de CG está em declínio, como os Estados Unidos por exemplo (INCA, 2017; WHO, 2017). Esta taxa tem como uma das razões o fato de que a maioria dos casos de CG serem diagnosticados em um estágio mais tardio. Os pacientes em estágios iniciais são assintomáticos, tornando difícil o diagnóstico nesta etapa da doença (VAN CUTSEM et al., 2016). Além disso, o diagnóstico só pode ser feito através de endoscopia, um exame invasivo, ou por tomografia e ressonância magnética, que são exames considerados de alto custo e por esses motivos não realizados em exames de rotina, onde o paciente não apresenta queixa de dores da região abdominal ou outros sintomas como anorexia (BARTH et al., 2014). Devido a estes fatos, é de extrema importância a descoberta de biomarcadores mais específicos e de maior sensibilidade, além de novos alvos terapêuticos que levem a tratamentos mais eficazes, permitindo um aumento na sobrevida dos pacientes com CG.

Uma das maiores causas de morte em pacientes com tumores malignos é a capacidade invasiva e metastática das células que o compõem, levando a formação de tumores metastáticos. As células tumorais que apresentam estas características conseguem modular o meio extracelular e a ativação de vias que levam a transição epitélio-mesenquimal (FIDLER, 1999; HUBER; KRAUT; BEUG, 2005).

O microambiente tumoral exerce um papel fundamental na metástase. As células tumorais são cercadas pelo estroma tumoral, que é composto pela MEC e por vários tipos de células não neoplásicas. Vários estudos vêm demonstrando que a interação entre as células tumorais e o microambiente tumoral é essencial para o complexo processo que desencadeia a metástase (YOKOZAKI et al., 2018; ZUO et al., 2011). Esta interação ocorre

principalmente por proteínas secretadas que podem atuar tanto de maneira autócrina, parácrina assim como endócrina (KARAGIANNIS; PAVLOU; DIAMANDIS, 2010).

2.3 O secretoma

O surgimento da proteômica baseada em espectrometria de massas abriu um novo leque de opções no estudo do câncer, acarretando no surgimento do termo oncoproteômica, e levando ao estudo do secretoma de células tumorais (JAIN, 2008). O estudo do secretoma de células tumorais começou a ascender há um pouco mais de uma década, trazendo resultados promissores no campo dos biomarcadores e de novos alvos terapêuticos.

O termo secretoma foi primeiramente utilizado para definir todos os constituintes da via secretória, bem como os próprios fatores secretados (TJALSMA et al., 2000). Posteriormente o termo secretoma[1] passou a ser utilizado apenas para definir o conjunto de proteínas secretadas pelas células (AGRAWAL et al., 2010). As proteínas secretadas correspondem a aproximadamente 15% das proteínas codificadas em humanos, e incluem fatores de crescimento, citocinas, moléculas de adesão, receptores de superfície celular, proteases, proteínas da MEC e proteínas intracelulares secretadas em vesículas (KARAGIANNIS; PAVLOU; DIAMANDIS, 2010).

As proteínas que compõem o secretoma de uma célula são secretadas por diferentes vias celulares, sendo que em termos gerais podemos considerar como vias secretórias a via clássica (ou via do retículo endoplasmático - Golgi) e a via não-clássica (ou Golgi-independente). As proteínas secretadas pela via clássica possuem um peptídeo-sinal na sua região N-terminal que é reconhecido pelos constituintes da via. Normalmente estas proteínas são sintetizadas no retículo endoplasmático (RE) e direcionadas ao complexo de Golgi em vesículas COPII. No Golgi as proteínas podem sofrer modificações como glicosilação por exemplo, antes de serem transportadas pela via trans-Golgi. Este transporte ocorre por vesículas que irão se fundir com a membrana plasmática liberando o seu conteúdo ao meio extracelular (LEE et al., 2004).

As rotas e os componentes da via não-clássica ainda não estão totalmente elucidados. Porém sabe-se que através da via não-clássica várias proteínas, que se pensava estarem confinadas no interior da célula ou ancoradas a membrana plasmática, são liberadas ao meio

[1]Apesar de a expressão secretoma atualmente referir-se a todos os fatores secretados pela célula (moléculas de DNA, RNA vesículas, etc.), neste trabalho adotaremos o termo secretoma para designar apenas o conjunto de proteínas secretadas.

extracelular por diferentes tipos de vesículas, como exossomos e nano-vesículas. Após serem secretadas, essas proteínas participam de diversos processos celulares como remodelação da MEC, comunicação célula-célula, modulação do sistema imune, entre outros (CHUA et al., 2012).

Alterações crônicas no secretoma de um tecido podem ser indício da presença de um processo patológico, como, por exemplo, processos neoplásicos. Os tumores apresentam variação do seu secretoma quando comparado aos das células normais do tecido no qual teve origem. Estas proteínas diferencialmente secretadas nas células tumorais, estão envolvidas em vários pontos críticos da agressividade tumoral como aumento da capacidade metastática e indução a quimiorresistência. Devido a estes fatos, a análise do secretoma de células tumorais vem acarretando na descoberta de novas moléculas que podem atuar como biomarcadores de diagnóstico e prognóstico, assim como alvos terapêuticos mais eficazes (PALTRIDGE; BELLE; KHEW-GOODALL, 2013).

2.3.1 O secretoma e o processo metastático

Como citado anteriormente, uma das maiores causas de morte em pacientes com tumores malignos é a formação de metástase (FIDLER, 1999). Para a formação da metástase as células tumorais precisam se desprender do tumor primário, alcançar vasos linfáticos/sanguíneos e implantar-se em outros órgãos. As células tumorais que tem capacidade metastática conseguem modular o microambiente tumoral principalmente através da secreção de proteínas como fatores de crescimento, citocinas e proteínas relacionadas a MEC (YOKOZAKI et al., 2018; ZUO et al., 2011).

Os fatores de crescimento estão envolvidos em várias etapas da progressão do tumor, incluindo no processo metastático. Um desses fatores, é o fator de crescimento de insulina II (IGF-II). O IGF-II é superexpresso em várias linhagens celulares de câncer de esôfago, mediando a progressão e a capacidade de metástase do tumor através da via PI3K/AKT (LI et al., 2014). Outro exemplo de fator de crescimento envolvido na capacidade metastática é o fator de crescimento endotelial vascular (VEGF). Em estudo utilizando anticorpos contra o receptor de VEGF em células de sarcoma, foi observado uma diminuição na capacidade invasiva através da diminuição da secreção de metaloproteinases responsáveis pela remodelação da MEC (ZHANG et al., 2006).

A Interleucina 6 (IL-6) é outra proteína secretada relacionada com aumento da capacidade metastática. A IL-6 é uma citocina pró-inflamatória secretada por células T e macrófagos que age na ativação da resposta imune (BUTERA; PACCHIANA; DONADELLI, 2017). Em estudo com células de tumor de próstata foi observado que IL-6 induz a expressão de SP100, e que a expressão elevada tanto desta proteína como de IL-6 está relacionada com a capacidade metastática desse tipo tumoral (CHUN et al., 2009). Em células originadas de glioblastoma foi demonstrado que a ação de IL-6 também está relacionada com um aumento da capacidade metastática (DENYSENKO et al., 2010).

2.3.2 As proteínas secretadas e a resistência aos fármacos

Os tratamentos contra o câncer englobam a remoção cirúrgica do tumor, radioterapia, quimioterapia e/ou imunoterapia. Em relação a quimioterapia, um dos principais obstáculos é o desenvolvimento de resistência aos fármacos disponíveis como antraciclinas, taxanos, antraquinonas, inibidores tirosina quinase e vinca alcaloides (JAIN, 2008; PLUEN et al., 2001). Atualmente, conhecem-se duas formas nas quais as células tumorais podem obter resistência ao quimioterápico. A primeira forma está relacionada à capacidade de absorção e metabolização do medicamento, diminuindo a disponibilidade do fármaco no sangue e a redução da difusão de drogas para as células da massa tumoral. A segunda maneira seria através de mutações adquiridas pelas células tumorais, acarretando na secreção de proteínas que possuem capacidade de conferir resistência ao tratamento (GOTTESMAN; FOJO; BATES, 2002). Alguns exemplos de grupos de proteínas secretadas que conferem quimiorresistência são os fatores de crescimento, alguns grupos de glicoproteínas e citocinas (BUTERA; PACCHIANA; DONADELLI, 2017).

Os fatores de crescimento, como citado, são um exemplo de substâncias secretadas envolvidas na quimiorresistência. Um exemplo disso pode ser observado através da família de receptores do fator de crescimento epidérmico (EGFR), que compreende 4 receptores e seus vários ligantes. Um desses ligantes é a anfirregulina (AREG), que está envolvida na resistência a diversos quimioterápicos como a sorofenibe, um quimioterápico utilizado principalmente no tratamento do câncer renal. A AREG foi descrita como secretada por células tumorais de câncer hepático, ovariano e pulmão (BLIVET-VAN EGGELPOËL et al., 2012; CARVALHO et al., 2015; ECKSTEIN et al., 2008).

As glicoproteínas são outro grupo de proteínas relacionadas com a quimiorresistência. Dentre essas proteínas podemos citar as proteínas envolvidas na via de sinalização por WNT. Essa via está envolvida em vários aspectos da tumorigênese como nos processos de proliferação, migração e invasão celular (ALBERTS et al., 2010). Em trabalho de Kobune e colaboradores foi demonstrado que a WNT-3 é secretada por células de mieloma, e que essa proteína age de forma autócrina. A ação dessa proteína se dá através da ativação da sinalização WNT/RHOA, fazendo com que as células tumorais tenham uma maior adesão com a MEC, levando a resistência a diversos quimioterápicos (KOBUNE et al., 2007). Outra ação das proteínas dessa família na quimiorresistência pode ser observado através da ação da WNT-6 em células de tumores gástricos. Nessas células, WNT-6 aumenta a resistência a apoptose dirigida por antraciclinas, um grupo de quimioterápicos amplamente usado no tratamento de cânceres (YUAN et al., 2013).

A descoberta dessas e de outras proteínas no processo de quimiorresistência, tem permitido direcionar melhor o tratamento utilizado nos pacientes. Essas proteínas são consideradas biomarcadores de prognósticos, e ajudam na decisão final de qual quimioterápico deve ser utilizado para cada caso.

2.3.3 O secretoma e a descoberta de novos biomarcadores

Um biomarcador pode ser considerado como um indicador mensurável de processos biológicos, tanto em condições normais quanto patológicas. O interesse por biomarcadores vem crescendo devido a capacidade desses de indicarem a presença de uma determinada doença, como, por exemplo, um câncer, assim como também o grau de severidade da doença (STRIMBU; TAVEL, 2011). Os principais benefícios dos biomarcadores, quando pensamos em neoplasias malignas, é a possibilidade de identificação do tumor em um estágio inicial e o diagnóstico poder ser feito através de um exame sanguíneo. Além disso, os biomarcadores podem ser utilizados durante o prognóstico do paciente, direcionando de maneira mais adequada o tratamento da doença (BARTH et al., 2014).

As características consideradas ideais para um biomarcador são a especificidade, a sensibilidade e a reprodutibilidade entre os diferentes gêneros e etnias. Sendo assim, um biomarcador ideal deveria apresentar 100% de sensibilidade e especificidade. No cenário atual, um dos melhores biomarcadores de diagnóstico para tumores é o antígeno prostático

específico (PSA). O PSA é indicativo da presença de tumores na próstata e apresenta 90% de sensibilidade e apenas 25% de especificidade (BARTH et al., 2014).

Um dos primeiros passos para a descoberta de novos biomarcadores é através de análises "ômicas" diferencias, comparando culturas de células tumorais em relação as células sadias do mesmo tecido. Em técnicas de proteômica, a análise do secretoma através do meio condicionado (MC) por exemplo, pode representar o primeiro passo para a descoberta de biomarcadores detectáveis em exames sanguíneos (MAKRIDAKIS; VLAHOU, 2010). A tabela 1 mostra alguns dos atuais candidatos a biomarcadores e a amostra utilizada para o estudo.

Tabela 1 – Exemplos de algumas proteínas candidatas a biomarcadores

Tipo de câncer	Candidato a Biomarcador	Amostra utilizada	Referência
Mama	ALCAM	Meio Condicionado	KULASINGAM; DIAMANDIS, 2007
Mama	IL-8	Tecido	YAO et al., 2011
Mama	TXK, IGFBP3, GNS, BMP1, FKBP-10, CTSL, LOX, PSAP	Plasma	AHN et al., 2010
Colorretal	TFE-3, GDF15	Meio Condicionado	XUE et al., 2010
Colorretal	CRMP-2	Plasma	DIEHL et al., 2007
Gástrico	GRN	Meio Condicionado	LOEI et al., 2012
Gástrico	CTTS, PCSK9, PDAP1	Tecido	YIXUAN et al., 2010
Fígado	CD14	Plasma	WU et al., 2010
Pulmão	A1AT, PARK7	Tecido	CHANG et al., 2012a, 2012b
Próstata	GDF15	Meio Condicionado	CACCIA et al., 2011; SELANDER et al., 2007

2.3.4 O meio condicionado como uma ferramenta de estudo do secretoma

Estudos utilizando o MC vem sendo amplamente utilizados para descoberta de novos biomarcadores de neoplasias, assim como a identificação de moléculas que possa levar a novos tratamentos (PALTRIDGE; BELLE; KHEW-GOODALL, 2013). Apesar do MC de células em cultura não apresentarem exatamente o mesmo microambiente que um tumor *in vivo*, o estudo do secretoma por MC apresenta diversas vantagens. Uma delas é a menor complexidade quando comparada a um biofluído, facilitando a identificação dos componentes secretados. Essa facilidade em relação a identificação pode ser observada principalmente com moléculas que se encontram em baixa concentração. No estudo por MC é possível a coleta de grandes quantidades do meio para posterior concentração dos componentes secretados (NASSER et al., 2015).

Apesar das vantagens apresentadas, o uso do MC para estudo do secretoma também apresenta alguns desafios. Um desses é a presença de contaminantes de componentes derivados do soro utilizado para a manutenção das culturas celulares. Para driblar este problema uma possibilidade é o cultivo das células por um pequeno período sem a presença do soro no meio de cultura. Entretanto, a retirada do soro por um período inapropriado pode levar a indução da morte celular nas células, levando a liberação de proteínas e outros componentes no meio que normalmente não seriam liberados. A minimização deste efeito é possível através da otimização do tempo que as culturas celulares permanecem sem soro, assim como a taxa de confluência que elas atingem durante este tempo, diminuindo as taxas de morte e o descolamento celular da placa de cultura (MBEUNKUI; FODSTAD; PANNELL, 2006).

Apesar dos desafios mencionados, o uso do MC vem sendo considerado de grande eficácia para a determinação do perfil do secretoma de diferentes tipos celulares, assim como para a descoberta de possíveis novos biomarcadores e novos alvos farmacológicos (DOWLING; CLYNES, 2011).

2.3.5 Desenvolvimento de novos alvos terapêuticos

Devido as proteínas secretadas terem grande correlação com o desenvolvimento tumoral, o secretoma se tornou uma rica fonte de descobertas para o desenvolvimento de novas drogas. Como exemplo podemos citar as terapias desenvolvidas contra fatores pró-angiogênicos como bloqueadores de VEGF. A bevacizumabe é um anticorpo monoclonal

capaz de se ligar e inibir a ação de VEGF, impedindo a formação de novos vasos sanguíneos e, por consequência, a progressão tumoral (QIU et al., 2018). Além do VEGF, outra proteína do grupo dos fatores de crescimento normalmente secretado por células tumorais e que vem sendo utilizada como alvo para terapia do câncer é o fator de crescimento transformante 15 (GDF-15). Inibidores dessa proteína são utilizados principalmente para tratar a caquexia causada por processos neoplásicos (LERNER et al., 2015a, 2015b).

Além dos fatores de crescimento, proteínas envolvidas em vias de sinalização de estresse também vêm sendo estudadas como fonte de terapia no câncer. Durante o desenvolvimento do câncer várias sinalizações de estresse, como vias de estresse de RE são ativadas (WU et al., 2016a). O processo pelo qual as células tumorais se adaptam ao estresse celular ainda não é inteiramente compreendido, mas sabe-se que as chaperonas são uma peça chave nessa adaptação. Sendo assim, as proteínas pertencentes a este grupo vem sendo fonte de diversas pesquisas como alvos terapêuticos no tratamento de neoplasias malignas (JOSHI et al., 2018). Alguns exemplos de chaperonas secretadas por células tumorais e que já apresentam fármacos em uso, ou em fase de teste, são as HSP90, HSP70 e HSP47 (MALONEY et al., 2007; SHARBEEN; MCALPINE; PHILLIPS, 2015; SÕTI et al., 2005).

2.3.6 O GDF-15

O GDF-15 é uma citocina membro da superfamília de fatores de transformação do crescimento beta (TGF-ß). Esse fator é secretado na forma de propeptídeo podendo se associar com a MEC ou na sua forma dimérica circulante. Em condições fisiológicas normais, GDF-15 só é expresso em grandes quantidades pela placenta ou em alguns tecidos embrionários (CORRE; HÉBRAUD; BOURIN, 2013). Porém em condições patológicas como em algumas neoplasias, a superexpressão de GDF-15 pode ocorrer independente do tecido (WANG; BAEK; ELING, 2013).

A exata função de GDF-15 no processo neoplásico maligno ainda não é bem esclarecida, mas sabe-se que esse fator está correlacionado com a agressividade tumoral (CORRE; HÉBRAUD; BOURIN, 2013). Um dos efeitos da indução anormal de GDF-15 em pacientes com câncer é o desenvolvimento da caquexia. A caquexia se caracteriza pela perda de peso que não consegue ser reposta através da alimentação (STEWART, B., WILD, 2014). O desenvolvimento da caquexia em pacientes com câncer é responsável por 20 a 30% das mortes por neoplasias malignas. Em análises com pacientes com caquexia em estágio crítico, a utilização de inibidores de GDF-15 foi capaz de impedir o agravamento dos sintomas e,

em vários casos, foi capaz de reverter ao menos em parte o quadro clínico do paciente, levando a reposição da massa corporal (LERNER et al., 2015a, 2015b).

A secreção de GDF-15 em células de tumores colorretal também está correlacionada com a indução a quimiorresistência a diversos tipos de fármacos, como, por exemplo, a oxaliplatina, o 5-fluorouracil e o SN38 (BUTERA; PACCHIANA; DONADELLI, 2017). Além disso, a detecção dessa proteína a nível sérico foi possível em pacientes com tumor de mama, colorretal, pâncreas e próstata, indicando um grande potencial de GDF-15 como biomarcador (BROWN et al., 2010; KOOPMANN et al., 2004; PROUTSKI et al., 2009; WELSH et al., 2010).

2.3.7 A HSP47

As chaperonas são proteínas que ajudam a manter a correta síntese e conformação das proteínas, sendo assim, são moléculas chaves para a manutenção da homeostasia celular. Alterações na funcionalidade das chaperonas estão relacionadas com o desenvolvimento de diversas patologias como osteogenia imperfecta, doenças neurodegenerativas e diversos tipos de neoplasias malignas (ALCANTARA et al., 2014; BARRAL et al., 2004; LEE et al., 2015b; LINDERT et al., 2015). Apesar de não haver um entendimento completo da ação dessas proteínas no câncer, vários estudos vêm demonstrando a importância dessas para a progressão tumoral (MALONEY et al., 2007; SHARBEEN; MCALPINE; PHILLIPS, 2015). Devido a esses fatos, a utilização de chaperonas como possíveis alvos terapêuticos vem sendo considerado um campo promissor (SÕTI et al., 2005).

A proteína de choque térmico 47 (HSP47) é uma chaperona pertencente à família das Serpinas. Uma característica peculiar dessa proteína é que, ao contrário de outras chaperonas, HSP47 possui um único substrato que é o colágeno. A HSP47 participa de várias etapas na síntese do colágeno (NATSUME et al., 1994). Os tipos de colágenos os quais HSP47 tem interação não estão ainda totalmente elucidados, mas sabe-se que ela age durante todo o processo da síntese do colágeno tipo -I e tipo IV (WIDMER et al., 2012a).

Durante a síntese da molécula do colágeno, HSP47 interage com a cadeia polipeptídica em formação, impedindo que o colágeno forme aglomerados na célula. A HSP47 também forma um complexo proteico com LH2, FKBP65, e *Binding immunoglobulin protein* (BiP) que é essencial para a regulação da hidroxilação de lisinas na molécula do colágeno. Esse

padrão de hidroxilação é responsável pela ligação do colágeno a MEC (DURAN et al., 2017).

Estudo recentes também demonstraram que HSP47 tem ação direta durante a ativação do estresse de RE. A HSP47 atua se ligando diretamente à enzima requerente de inositol 1 α (IRE1α), ativando a via do sistema de degradação associado ao RE (ERAD) (SEPULVEDA et al., 2018).

A HSP47 tem sua expressão proteica controlada pelo microRNA(miR)-29 (YAMAMOTO et al., 2013; ZHU et al., 2015a). Estudos com análises de expressão tanto de HSP47 como de miR-29, demonstraram que essas duas moléculas estão relacionadas com a progressão de tumor cervical, mama, pancreático, ósseo, pulmonar, gástrico e glioblastomas (CAO et al., 2005; HIRAI et al., 2006; JIANG et al., 2016; KAMIKAWAJI et al., 2016; KOBAYASHI et al., 2014; POSCHMANN et al., 2009; TSUKIMI; OKABE, 2001; UOZAKI et al., 2000; WU et al., 2016b; YAMAMOTO et al., 2013; ZHAO et al., 2014a). Devido a estes fatos, a HSP47 vem sendo considerada como um potencial alvo para terapias em processos neoplásicos.

3 OBJETIVOS

3.1 Objetivo Geral

Analisar o perfil do secretoma de células tumorais derivadas de linhagens de câncer gástrico (ACP02 e ACP03) em comparação com o secretoma de uma linhagem de mucosa gástrica normal (MN01), buscando novas moléculas que possam servir de biomarcadores, bem como fonte para novos alvos terapêuticos.

3.2 Objetivos específicos

- Analisar comparativamente as proteínas secretadas pelas linhagens de câncer gástrico ACP02 e ACP03 em relação a linhagem de mucosa gástrica normal MN01;

- Avaliar, através de dados já estabelecidos na literatura, possíveis novos biomarcadores e /ou alvos terapêuticos através dos resultados obtidos no secretoma;

- Estabelecer o silenciamento da HSP47 e do GDF15 nas linhagens MN01, ACP02 e ACP03.

- Estudar o efeito do silenciamento da proteína HSP47, diferencialmente expressa no secretoma das linhagens de câncer gástrico, em processos de importância para o desenvolvimento tumoral.

CAPÍTULO I

ARTIGO DE DADOS 1

DIFFERENTIAL SECRETOME ANALYSIS OF HUMAN GASTRIC CANCER CELL LINES AND NON-NEOPLASTIC GASTRIC CELLS

Artigo a ser submetido em periódico internacional da área

Differential secretome analysis of human gastric cancer cell lines and non-neoplastic gastric cells

Beatriz Dal Pont Duarte*, Joice de Faria Poloni, Diego Bonatto

Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul, Departamento de Biologia Molecular e Biotecnologia - Universidade Federal do Rio Grande do Sul, Porto Alegre, RS – Brasil.

***Corresponding author**

Beatriz Dal Pont Duarte

Centro de Biotecnologia da UFRGS – Sala 107

Departamento de Biologia Molecular e Biotecnologia

Universidade Federal do Rio Grande do Sul – UFRGS

Avenida Bento Gonçalves, 9500 – Prédio 43421 – Sala 107

Caixa Postal 15005

Porto Alegre – Rio Grande do Sul

BRAZIL

91509-900

Phone: (+55 51) 3308-7765

E-mail: beatriz.duarte@ufrgs.com.br

Contract/grant sponsor: CNPq

Abstract

Gastric cancer (GC) is the third cause of cancer death in the world. The high mortality of GC can be explained by the fact that this disease is normally detected in late-stages. In the last decade, the study of secretome has become a powerful identification tool for biomarkers that can help in early diagnosis and the direction of treatment. In order to demonstrate the profiling of secreted proteins in GC cells lines in comparison with gastric normal mucosa cells, we established culture conditions to assure minimal autolysis contamination and optimal recovery of the secreted proteins. The mass spectrometry analysis identified 333 proteins, of which 86 proteins were exclusive in two carcinoma cell lines. By combining experimental and system biology approaches, our study shows a comprehensive analysis of the main signaling pathways orchestrated by differentially secreted proteins from gastric cancer cells. Furthermore, western blot analyses were performed to confirm the secretion of targets chosen through bioinformatic analysis. According to our results, we suggest that GDF15 and HSP47 are potentials candidates for gastric cancer biomarker.

1. INTRODUCTION

Gastric cancer (GC) is the third leading causes of cancer-related deaths worldwide. Several factors interfere with the survival rate of cancer, where the stage of the tumor in the time of diagnostic is critical for a good prognosis (FERLAY et al., 2015). The high mortality of patients with GC can be attributed to the fact that the GC is asymptomatic in the initial phases of the disease (VAN CUTSEM et al., 2016). This situation is further aggravated by a lack of biomarkers that can aid in the early detection. The study of secretome is a powerful tool to discover new biomarkers and therapeutic targets that help in early diagnosis and the direction of treatment (PALTRIDGE; BELLE; KHEW-GOODALL, 2013).

The modulation of microenvironment by the tumor cells is a critical mechanism in the progression of cancer. This modulation is necessary for the migration and invasion capacity of the neoplastic cells and is possible through the communication between the tumor cells and the stroma cells (FIDLER, 1999). This communication is mainly mediated by secreted factors that controlling cell–matrix and cell-cell interactions. In this mean, understand the changes in secreted factors in pathologies can help to elucidate the disease progression (YOKOZAKI et al., 2018).

Here, we report the changes found in the secretome of two gastric cancer cells lines (ACP02 and ACP03) in comparison with normal mucosa gastric cells (MN01). We found that the cancer cell lines have 86 proteins in common that were not secreted by the normal gastric cells. To understand the biological mechanisms related to these proteins, we performed a system biology analysis to investigate the nature of proteins involved with the malignant phenotype. In this sense, using a bioinformatic approach, were observed that these proteins were mainly involved in cell cycle, extracellular matrix, cytoskeletal components and cellular metabolism. We also analyzed three differentially secreted proteins by western blot, which leads to the confirmation that the heat shock protein 47 (HSP47/SERPINH1) and

growth differentiation factor 15 (GDF15) are differentially secreted by tumor cells, and they can be potential candidates for gastric cancer biomarker.

2. MATERIALS AND METHODS

2.1 Cell lines and conditioned medium

Gastric adenocarcinoma cell lines (ACP02 and ACP03) (LEAL et al., 2009) and normal gastric mucosa cells (MN01), derived from primary cultures of normal gastric epithelial cells, were cultured in RPMI medium with 10% of fetal bovine serum (FBS) and 1% penicillin/streptomycin. When the cells cultures reached 70% of confluence, they were washed three times with serum-free medium to remove residues coming from FBS, and the cells were cultured for 48 h in serum-free medium. After this period, the conditioned medium was collected, and centrifugation was performed three times (2000 rpm for 5') to removed cellular debris and possible floating cells. The proteins of conditioned medium were concentrated by ultrafiltration with Amicon Ultra-15 centrifugal filter with a nominal molecular weight limit (NMWL) of 3 kDa (®Milipore), according to the manufacturer's instruction.

2.2 Protein samples preparation and LC-MS/MS analysis

The protein samples were separated on a 10% polyacrylamide gel electrophoresis (PAGE) without sodium dodecyl sulfate (SDS). The proteins bands were excised into ten gel slices. The dehydration of the band slices was made with acetonitrile (100%), reduced with dithiothreitol (DTT), and alkylated with iodoacetamide. The proteins were digested with Trypsin Gold Mass Spectrometry Grade (Promega) (diluted in 200 mM NH_4HCO_3 pH 7.8) and incubated overnight at 37 °C. The peptides extraction was carried out with a solution of formic acid (5%) and acetonitrile (50%). The samples were vacuum dried and stored at −20 °C until LC-MS/MS analysis.

The digested peptides were analyzed with Q-Tof Premier API mass spectrometer (MicroMass/Waters), attached to a nanoACQUITYTM ultra performance liquid chromatography (UPLC) system (Waters). MS/MS raw data were processed using Mascot Distiller 2.2.2 and database was performed by Mascot Search engine 2.3. Mascot results were analyzed by Scaffold Q+ 4.6.2 with 1% of false discovery rate (FDR) and protein probability values over 95%.

2.3 Western blot analysis

Analyses of the protein expression were performed using antibodies anti-HSP47 (Sigma-Aldrich 1:250), anti-GDF15 (Sigma-Aldrich 1:250) and anti-PDIA6 (Sigma-Aldrich 1:250). Whole-cell lysate obtained with RIPA buffer was used as a positive control. Protein extracts obtained from the cell lysate and conditioned medium (20 µg of protein loaded per well) were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes. After the incubation with primary antibody, an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) (Cell Signaling 1:1500) was used and visualized with ECL Western blotting detection reagents (Clarity™ Bio-Rad).

2.4 Reverse transcription and quantitative PCR analysis

Total RNA was isolated with PureLink™ RNA kit (Invitrogen), according to manufacturer's instruction. The cDNA synthesis was obtained with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RNA and cDNA were quantified using a NanoDrop spectrophotometer. Relative quantification for expression of *HSP47* (5'TGACTGAGGCCATTGACAAG3'/5'TTGCCATCTGTGTCCAACTC3'), *GDF15* (5'CTCCAGATTCCGAGAGTTGC3'/5'TGTTCGAATCTTCCCAGCTC3'), and *PDIA6* (5'CGGTGATAAGAAGGGGAATG3'/5'TCTTCAACGCTCCTTTCTCG3') was performed on a StepOne™ Real-Time PCR System (Thermo Fisher), using pre-designed

primers. Relative expression was normalized to *GAPDH* levels (5'TGTTCGTCATGGGTGTGAAC3'/5'GTTGTCATGGATGACCTTGG3').

2.5 Protein-protein interaction network construction

To design the protein-protein interaction (PPI) network, the proteomic results from secreted proteins by both tumor lines, ACP02 and ACP03, and the control cells MN01 were used as input in the metasearch engine STRING 10.5 (SZKLARCZYK et al., 2015). The parameters employed were as follow: the predictions methods "experiments", "databases" and "co-expression" enabled; minimum confidence value of interactions of 0.4 and no more than 20 interactors in the first shell and no more than 5 interactors in the second shell. Nonconnected nodes were once again submitted to the PPI network construction protocol mentioned above until no more connections were found. All constructed subnetworks were merged in the software Cytoscape 3.6 (CLINE et al., 2007; KAPHINGST; PERSKY; LACHANCE, 2010).

2.6 Cluster analysis

To assess the presence and number of clusters in the PPI networks, the Cytoscape plugin MCODE 1.51 was employed (BADER; HOGUE, 2003). This analysis was performed using the following pre-processing protocol: degree cutoff, 2; node score cutoff, 0.2; k-core, 2; and maximum network depth, 100; haircut and fluff option were enabled with node density cutoff, 0.1. Thus, each cluster generates a degree of connection for a given group of nodes (the "cliquishness" value, C*i*), which in this case was selected as a threshold C*i* > 5. Only clusters with a score above the threshold were selected for further gene ontology (GO) analysis.

2.7 GO analysis

The major biological processes associated with each cluster were accessed using the Cytoscape plugin BiNGO 3.0.3 (MAERE; HEYMANS; KUIPER, 2005). The degree of functional enrichment for a given GO category was quantitatively assessed (p-value) based on a hypergeometric distribution, following the multiple test correction by applying the FDR algorithm with a significance level of $p < 0.05$ (RIVALS et al., 2007). Clusters with similar GO results were merged.

2.8 Centrality analysis

To evaluate the most important nodes in the network according to their topological relevance, a centrality analysis was performed using the Cytoscape plugin CentiScape 2.1 (SCARDONI et al., 2015; SCARDONI; PETTERLINI; LAUDANNA, 2009). In this analysis, the centrality parameters analyzed were node degree and betweenness, in order to detect hub, bottleneck, and hub-bottleneck (HB) nodes.

Node degree analysis evaluates node connectivity, which was expressed as the number of connections for a given node, whereas betweenness calculates the shortest paths connecting adjacent nodes that pass through each node. Nodes with a high node degree value are called hubs and have a central regulatory role in the cell, while nodes with higher betweenness value are called bottleneck and may have an important role in communication between signaling mechanisms (SCARDONI; LAUDANNA, 2012).

2.9 Statistical analysis

Results were expressed as mean ± statistic error of the mean (SEM), and statistical analyses were performed using SPSS 18.0.0 software. ANOVA and Dunnett tests were used for multiple comparisons among the cells lines. Differences were considered statistically significant at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Secretome analysis

The LC-MS/MS samples were analyzed by Scaffold Q+ 4.6.2, and the results were filtered using protein probability values over 95%. Of these, the total number of proteins in secretome analyses was 333. Being 198 found in MN01, 301 in ACP02 and 301 in ACP03. Of all proteins identified, 86 proteins (Table 1) were found in both adenocarcinoma cell lines and not found in normal mucosa cells. Our findings showed difference in the secretome of gastric tumor cell lines, and these proteins differentially found only in adenocarcinomas could be a source of new biomarkers and therapeutic targets against GC (PALTRIDGE; BELLE; KHEW-GOODALL, 2013).

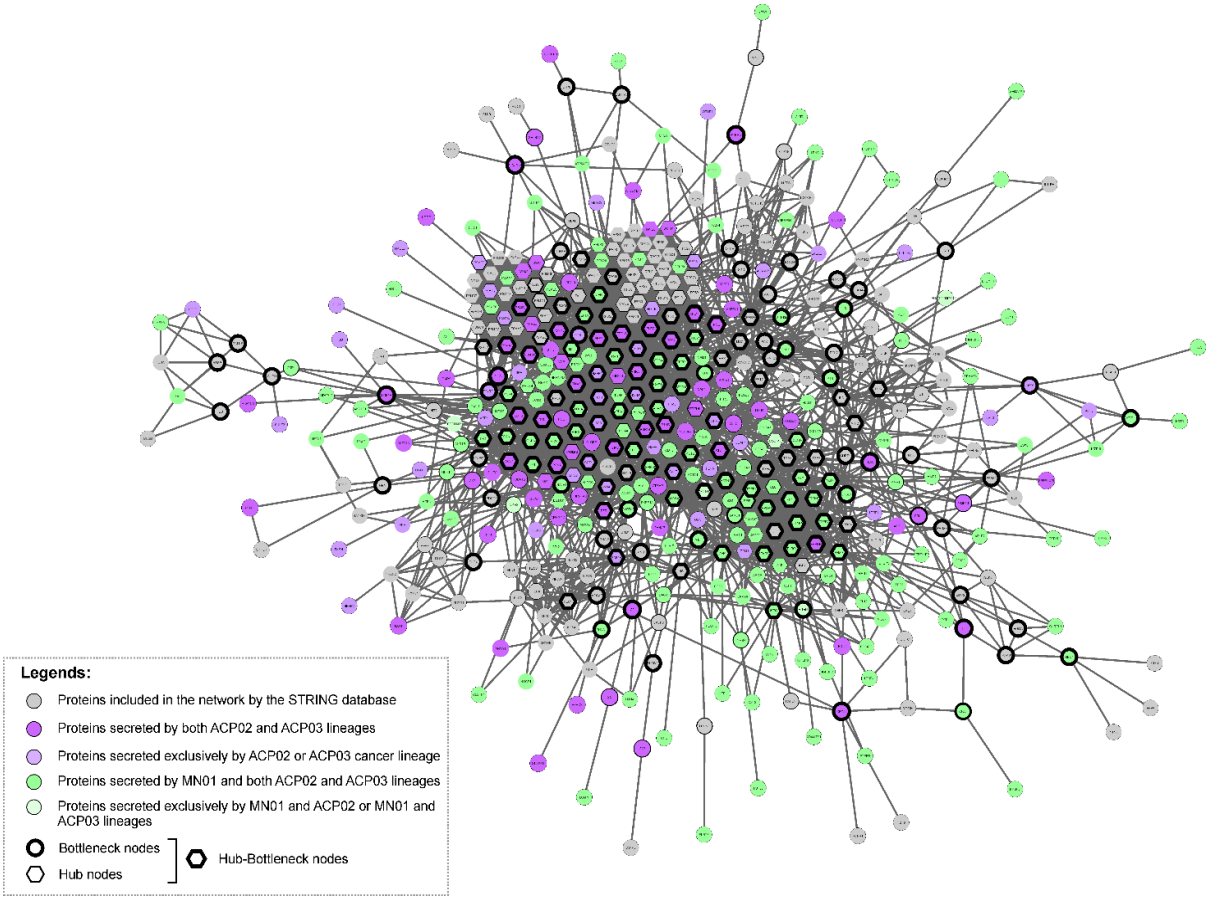

Fig. 1 PPI network representing 308 proteins identified by proteomic analysis in tumor cells as colored nodes. Purple nodes represent the 126 proteins secreted exclusively by tumor cells and green nodes represent the 182 proteins secreted in common with normal mucosa cells. The nodes shape and thickness represent the topological importance of the protein in the network.

Table 1 Proteins secreted in both gastric adenocarcinoma cell lines

Accession number	Protein name	Accession number	Protein name
P08865	40S ribosomal protein SA	**P25788-2**	Proteasome subunit alpha type-3 - Isoform 2
P39687	Acidic leucine-rich nuclear phosphoprotein 32 family member	**Q16181-2**	Septin-7 - Isoform 2
Q01518	Adenylyl cyclase-associated protein 1	**O14773-2**	Tripeptidyl-peptidase 1 - Isoform 2
O43488	Aflatoxin B1 aldehyde reductase member 2	**P68036-2**	Ubiquitin-conjugating enzyme E2 L3 - Isoform 2
P42330	Aldo keto reductase family 1 member c3	**P00749-2**	Urokinase-type plasminogen activator - Isoform
P08697	Alpha-2-antiplasmin	**P02774-2**	Vitamin D-binding protein - Isoform 2
P02771	Alpha-fetoprotein	**Q9NTK5-3**	Obg-like ATPase 1 - Isoform 3
P09525	Annexin A4	**P11413-2**	Glucose-6-phosphate 1 - Isoform Long
P00918	Carbonic anhydrase 2	**P25786-2**	Proteasome subunit alpha type-1 - Isoform Long
Q9Y696	Chloride intracellular channel protein 4	**P07195**	L-lactate dehydrogenase B chain
P10909	Clusterin beta chain	**Q14697**	Neutral alpha-glucosidase AB
P20908	Collagen alpha-1 chain 5	**P19338**	Nucleolin
P01024	Complement C3	**B7ZMG0**	PTPRK protein
Q07021	Complement component 1 Q subcomponent-binding protein	**P36955**	Pigment epithelium-derived factor
Q9NZV1	Cysteine-rich motor neuron 1 protein	**P05154**	Plasma serine protease inhibitor
Q96C19	EF-hand domain-containing protein D2	**Q10471**	Polypeptide N-acetylgalactosaminyltransferase 2
P26641	Elongation factor 1-gamma	**P0CG38**	POTE ankyrin domain family member I
P13639	Elongation factor 2	**P12004**	Proliferating cell nuclear antigen
P15311	Ezrin	**P25789**	Proteasome subunit alpha type-4
P35555	Fibrillin-1	**P60900**	Proteasome subunit alpha type-6
P41250	Glycine--tRNA ligase	**P20618**	Proteasome subunit beta type-1
Q99988	Growth/differentiation factor 15	**P49721**	Proteasome subunit beta type-2
P34932	Heat shock 70 kDa protein 4	**P28070**	Proteasome subunit beta type-4
P50502	Hsc70-interacting protein	**O75629**	Protein CREG1
Q14974	Importin subunit beta-1	**Q96LR4**	Protein FAM19A4
P08195-2	4F2 cell-surface antigen heavy chain - Isoform 2	**P31949**	Protein S100-A11
P62913-2	60S ribosomal protein L11- Isoform 2	**P30101**	Protein disulfide-isomerase A3
P53396-2	ATP-citrate synthase - Isoform 2	**Q15084-2**	Protein disulfide-isomerase A6
Q13510-2	Acid ceramidase - Isoform 2	**P07237**	Protein disulfide-isomerase
P23526-2	Adenosylhomocysteinase - Isoform 2	**P55786**	Puromycin-sensitive aminopeptidase
P54819-2	Adenylate kinase 2 - Isoform 2	**P00352**	Retinal dehydrogenase 1
Q00610-2	Clathrin heavy chain 1 - Isoform 2	**P52565**	Rho GDP-dissociation inhibitor 1
Q93063-2	Exostosin-2 - Isoform 2	**P50454**	Serpin H1
P06744-2	Glucose-6-phosphate isomerase - Isoform 2	**P37108**	Signal recognition particle 14 kDa protein
P08107-2	Heat shock 70 kDa protein 1A/1B - Isoform 2	**P09238**	Stromelysin-2
P51858-2	Hepatoma-derived growth factor - Isoform 2	**P10599**	Thioredoxin
Q99729-2	Heterogeneous nuclear ribonucleoprotein A/B -	**P10646**	Tissue factor pathway inhibitor
P61978-2	Heterogeneous nuclear ribonucleoprotein K -	**P37837**	Transaldolase
P01130-2	Low-density lipoprotein receptor - Isoform 2	**P37802**	Transgelin-2
Q8NCW5-2	NAD(P)H-hydrate epimerase - Isoform 2	**P55072**	Transitional endoplasmic reticulum ATPase
Q13765-2	Nascent polypeptide-associated complex subunit	**P68363**	Tubulin alpha-1B chain
Q8N2Q7-2	Neuroligin-1 - Isoform 2	**P09936**	Ubiquitin carboxyl-terminal hydrolase isozyme
Q15149-2	Plectin - Isoform 2	**Q15904**	V-type proton ATPase subunit S1

3.2 PPI network and GO analyzes

To explore the functional role and signaling pathways associated with the proteins secreted by tumor lines, and how these proteins may contribute to tumorigenesis, we took all identified secreted proteins in the proteomic analysis into consideration. From 333 proteins identified by proteomic analysis, 308 are present in the PPI network, in which 126 are exclusively secreted by tumor cells, and 182 are also found in the control cells (Figure 1).

Cluster analysis indicated that the PPI network is composed of six major clusters. However, many clusters overlap each other and have similar GO processes. Thus, Clusters 1 and 2, Cluster 3 and 4, and Clusters 6 and 7 were merged (Figure 2A, 2C and 2E), resulting in three final subnetworks. The biological processes associated with the clusters are cell cycle, proteolysis, regulation of apoptosis, response to wounding, cell adhesion, regulation of cell migration, inflammatory response and glycolysis (Figure 2B, 2D and 2F). Exclusively, proteins secreted by tumor cells corresponded to the processes proteolysis, glucose catabolic process, mitotic cell cycle, cell redox homeostasis and positive regulation of ubiquitin-protein ligase activity (Figure 3).

According to GO and centrality analysis results, we looked for which processes were more involved with proteins secreted exclusively by the tumor cells in order to highlight the essential aspects and functionalities associated with the gastric tumor, which will be discussed below.

Cell Cycle

According to the centrality analysis, were found different HB proteins. Some of those are proteasome subunits secreted in both cancer lines, but not in the control cells, such as PSMA3, PSMA6, PSMB1, and PSMB2. A recent study showed that PSMA3 and PSMA6

are upregulated in the lung, breast, gastric and bladder cancers, being correlated with patient survival outcome (LI et al., 2017). Deregulation in the expression of genes that encode to proteasome subunits is frequently observed in cancer, being involved in the proteolysis of several tumor suppressor proteins associated to cell cycle, contributing to the abnormal cell proliferation and malignancy (RASTOGI; MISHRA, 2012).

Moreover, interacting with PSMA3 we found PCNA, another HB protein secreted only in the studied tumor cells. PCNA is a cell proliferation-associated protein considered a molecular marker of proliferating cells, whose expression is increased in gastric cancer (HU et al., 2017). PCNA is a necessary factor for DNA replication, but also has a role in DNA damage repair, cell cycle control, cell survival, chromatin assembly, gene transcription, epigenetic maintenance and sister-chromatid cohesion (WANG, 2014). Although PCNA is known to be a nuclear protein, some studies have been shown that it could be found in exosome secreted by malignant cells (BANDARI et al., 2018; CHENG et al., 2017b; HALVAEI et al., 2018).

Cellular redox homeostasis

The HB proteins secreted only by cancer lines PDIA3, PDIA6 and P4HB, are members of the protein disulfide isomerases (PDI) family. These enzymes are important regulators of redox balance and cell homeostasis by catalyzing oxidative protein folding (LEE; HEE LEE, 2017). Additionally, both PDIA3 and P4HB have major roles in the regulation of the proteostasis in the endoplasmic reticulum (LEE; HEE LEE, 2017). Furthermore, PDIA3 is a member of the damage recognition complex, being related to DNA repair (RAMOS et al., 2015). PDI members are involved in different human disorders, including cancer, playing a role in the proliferation, survival, and metastasis (LEE; HEE LEE, 2017). *PDIA3* and *PDIA6* expression are higher in breast cancer when compared with normal tissue, being associated with neoplastic progression and both are suggested to be prognostic markers of

aggressiveness (RAMOS et al., 2015). P4HB is overexpressed in hepatocellular carcinoma and is correlated with poor prognosis and advanced disease stage (XIA et al., 2017). In hepatocellular carcinoma, P4HB promoted cell growth, migration, and invasion, possibly through enhancing epithelial-mesenchymal transition *in vitro* assays and tumor formation *in vivo* assays (XIA et al., 2017).

Curiously, P4HB is also required for the proper collagen proline hydroxylation, necessary for the folding of the procollagen polypeptide chain (GILKES et al., 2013). In this sense, involved with the collagen folding, we found the hub protein HSP47 interacting with PDIA6 and P4HB. HSP47 is an endoplasmic reticulum-resident chaperone required to the assembling of triple-helical procollagens, which is a prerequisite to its extracellular secretion (WIDMER et al., 2012b). *HSP47* overexpression increases the expression of genes related to extracellular matrix (ECM) and is associated with tumor formation *in vivo* (JIANG et al., 2016). The inhibition of HSP47 activity is considered an alternative cancer treatment for blocking collagen deposition and ECM remodeling (JIANG et al., 2016).

ECM-proteins related and cytoskeletal components

Collagen is the main component of ECM, creating a network that connects the cells, serving as a reservoir for growth factors and composes a framework through which the cells are able to move (WIDMER et al., 2012b). Collagen components deposition in the microenvironment may stimulate tumor progression, promoting cell growth, migration, and metastasis (XIONG; XU, 2016; ZHOU et al., 2017). We found the proteins COL18A1, COL4A2, COL6A1, COL6A2 and COL7A1 secreted by the two tumor cell lines and control cells, whereas COL5A1 is secreted only by tumor cells lines. In gastric cancer, different architectural parameters (alignment, density, width, length, and straightness) of collagen was already observed to be altered and reorganized in the tumor microenvironment, thus becoming a potential prognostic indicator for gastric cancer (ZHOU et al., 2017). In the

network, we found that above mentioned HSP47 and P4HB interacting with the collagens proteins, in addition to PLOD1 and PLOD3 that are required for the normal assembly and collagen crosslink.

During cancer progression, several steps are necessary for the malignant cells to invade and metastasize, including loss of cell-to-cell and cell-to-matrix adhesion and ECM remodeling. In this sense, matrix metalloproteinases (MMP) are a family of zinc-dependent proteinases involved in these processes as regulators of ECM turnover. MMP-1, MMP-7 was secreted by the tumor and control cells, but both tumor cell lines secreted only MMP-10. The protein MMP-10 has a vital role in invasion and tumor progression in different cancers (DERAZ et al., 2011; JIANG et al., 2014; ZHANG et al., 2014).

The HB protein EZR is a member of the Ezrin-radixin-moesin (ERM) cytoskeleton-associated proteins and provides the link of the actin cytoskeleton to cell membrane proteins, and the transmission signals in response to extracellular stimulus (LI et al., 2015). In this sense, EZR function is essential for cell shape maintenance, cell adhesion, motility and microvilli formation (LI et al., 2015). EZR overexpression is correlated with a poor prognosis outcome, been observed in different cancer types, such as breast, gastric, osteosarcomas and others (LI et al., 2015). In the network, EZR is directly connected with the cancer-associated proteins ARHGDIA, CLIC4, CLTC, P4HB, and TAGLN2.

TAGLN2 is a hub protein that binds to actin and regulates cytoskeleton dynamic via stabilization of actin filaments (DVORAKOVA; NENUTIL; BOUCHAL, 2014; MENG et al., 2017). Increased levels of TAGLN2 is associated with cancer progression, and it is limited to tumor cells, being reported in different tumors, including gastric cancer (DVORAKOVA; NENUTIL; BOUCHAL, 2014; MENG et al., 2017).

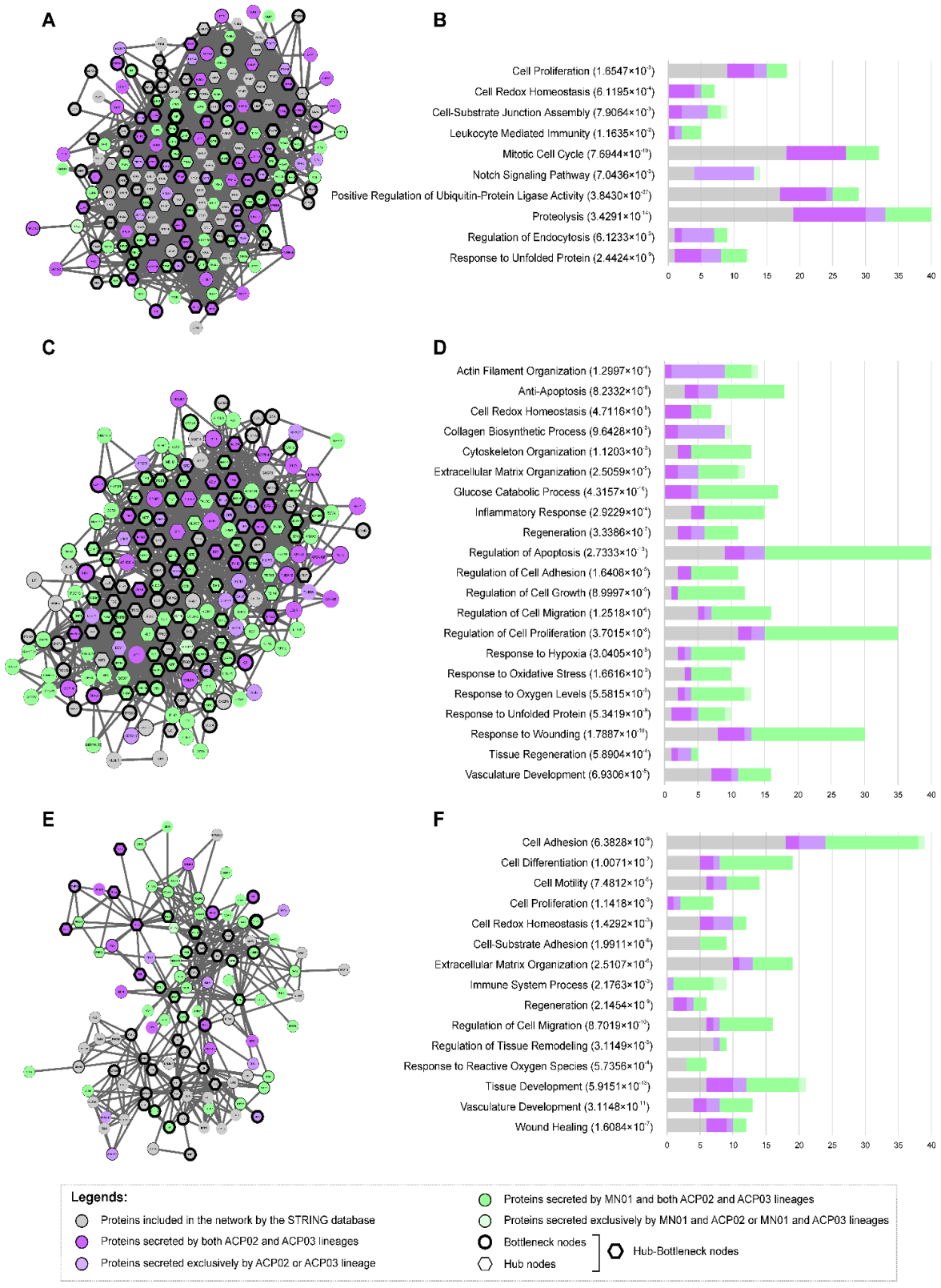

Fig. 2 Representation of the subnetworks resulting from the merge of similar clusters found in the main network and the significant biological processes related with each subnetwork. Clusters 1 and 2 (A and B); Cluster 3 and 4 (C and D); Clusters 6 and 7 (E and F). Purple and green colors represent proteins secreted exclusively by tumor cells and proteins secreted in common with normal mucosa cells, respectively.

Metabolism

Altered metabolism is commonly observed in cancer, and aerobic glycolysis is frequently correlated with higher aggressiveness and poor prognosis (ZAIDI; SWINNEN; SMANS, 2012). In this sense, increased glycolysis activity results in excess of its end product pyruvate, that is converted to lactate and secreted to the extracellular environment (ZAIDI; SWINNEN; SMANS, 2012). Furthermore, a minor part of pyruvate enters in the mitochondria, where is decarboxylated into acetyl-CoA by the enzyme pyruvate dehydrogenase (ZAIDI; SWINNEN; SMANS, 2012). Several important proteins involved in cellular metabolism were secreted by the studied tumor cell lines and are found in the network as topologically relevant nodes, such as ACLY, GPI, LDHB, and ALDH1A1.

The protein ACLY is an HB in the network, acting in the conversion of citrate to acetyl-CoA. In turn, acetyl-CoA is a substrate for synthesis of fatty acids and mevalonate pathways (ZAIDI; SWINNEN; SMANS, 2012). In cancer, ACLY is frequently upregulated and is considered a poor patient prognosis (MIGITA et al., 2008; ZAIDI; SWINNEN; SMANS, 2012). ACLY inhibition decreased cell proliferation and led to cell growth arrest (MIGITA et al., 2008).

The HB protein ALDH1A1 catalyzes the oxidation of retinaldehyde to retinoic acid (TOMITA et al., 2016). ALDH1A1 upregulation is associated with cancer stem cells, contributing to tumor initiation, self-renewal, and differentiation (MIYATA et al., 2017; YANG et al., 2014b). Its activity is observed in different cancer types, being related to drug resistance, invasion and metastasis (JANUCHOWSKI et al., 2016; LANDEN et al., 2010; TOMITA et al., 2016; YANG et al., 2014b).

LDHB is an HB protein, whose function is catalyzing the bidirectional conversion of pyruvate and lactate. LDHB relation with tumorigenesis is not entirely understood since in

some cancers types this protein is silenced by hypermethylation, whereas it is overexpressed in other cancers (DOHERTY; CLEVELAND, 2013). In gastric cancer tissue, a study observed *LDHB* promoter hypermethylation in 3 of 20 patients but not in the healthy mucosa (MAEKAWA et al., 2003). However, 4 of 6 gastric cancer cell lines showed hypermethylation of the *LDHB* promoter (MAEKAWA et al., 2003).

The HB protein GPI is a phosphoglucose isomerase that is a ubiquitous enzyme essential in the glycolytic pathway. This protein can be secreted by tumor cells, acting as autocrine motility factor (AMF), leading to mitogenic, motogenic and differentiation stimulus (HERLING et al., 2011). G6PD, secreted exclusively by the studied tumor cell lines, interacts with GPI and LDHB in the PPI networks. G6PD is the first and rate-limiting enzyme of the pentose phosphate pathway. This pathway is frequently upregulated in different cancer types, impacting in processes, such as proliferation, apoptosis, invasion, drug resistance, and metastasis.

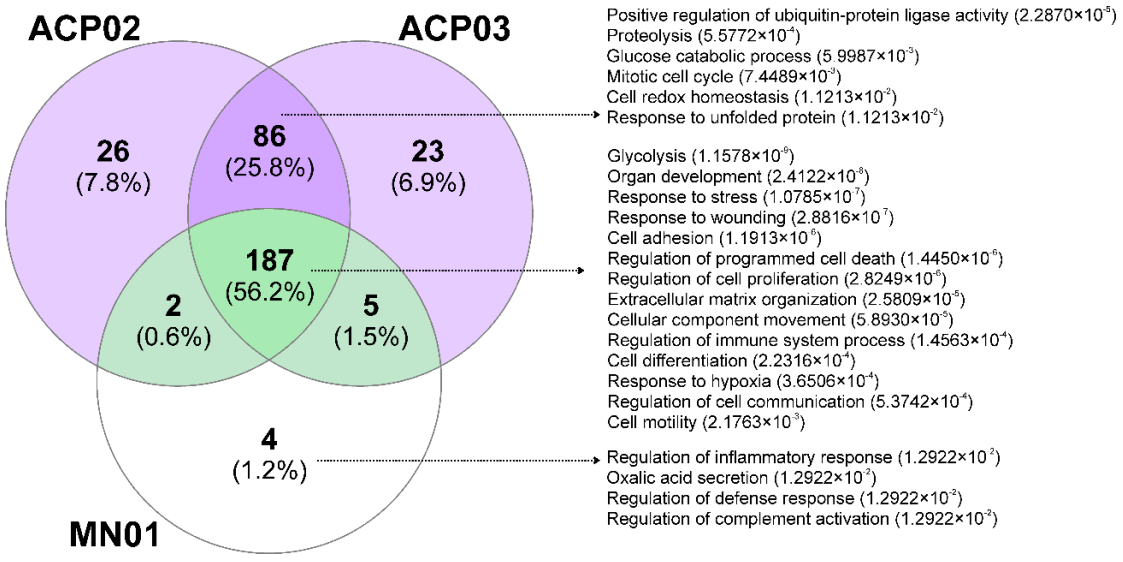

Fig. 3 Overlap between all secreted proteins identified by proteomic analysis in the normal and tumor cell lines and the significative biological processes associated.

3.3 Gene expression

Based on the GO analyzes and topological relevant nodes, we choose PDIA6 and HSP47 to validate gene and protein expression levels (Figure 4A, 4B, 4D and 4E). We also investigate the expression of GDF15 (Figure 4C and 4F), a protein secreted only by tumor cell lines ACP02 and ACP03. GDF15 is not showed interaction in PPI network, regardless it is mainly described as a biomarker in several types of cancer (ZHANG et al., 2016; LIU et al., 2016; WANG et al., 2017; WINDRICHOVA et al., 2017; YANG et al., 2014a).

The expression of *PDIA6* increases in tumor cell lines in comparison with normal mucosa (Figure 4A). The increase in expression of *PDIA6* and its current status in the secretome of gastric cancer was also detected in a SILAC-based proteome approach in the work of Marimuthu and collaborators (MARIMUTHU et al., 2013). PDIA6 has a different role in different types of cancer. In bladder cancer, the downregulation of PDIA6 decreases the proliferation and invasion capacity (CHENG et al., 2017a). However, a study with glioblastoma cells reported that downregulation of PDIA6 increases the migration and invasion capacity of these cells (KIM; RYU; LI, 2017). Therefore, more studies are necessary to clarify the role of PDIA6 in tumorigenesis.

In the analysis with *HSP47*, we detected a significant increase in the expression of this gene in ACP02 and ACP03 lines in comparison with MN01 (Figure 4B). Overexpression of *HSP47* was also observed in analyses of glioma cell lines, and in an *in silico* study using gastric cancer samples (Wu et al. 2014, 2016; Zhao et al. 2014; ZHANG et al. 2010). Knockdown of HSP47 inhibited migration and invasion capacity in glioma cells lines *in vitro* and decreased tumor volume *in vivo* (Zhao et al. 2014; Wu et al. 2016). These data could indicate a role of HSP47 in aggressiveness of the tumor.

GDF15 overexpression is detected in several types of tumor (HUSAINI et al., 2015; ZHANG et al., 2018). However, in our results the expression of *GDF15* in tumor cells lines were a significant lower when compared with control cells (Figure 4C). This fact could be related to the stage of the tumor that originated the cell lines ACP02 and ACP03. In a study with glioblastomas, it was shown significantly lower levels of *GDF15* expression in primary glioblastomas and higher expression levels in secondary glioblastomas (STRELAU et al., 2008). A variance in the *GDF15* expression, among different stages of the tumor, was also described by Yong and collaborators (YONG et al., 2014). In this work, the authors demonstrated that *GDF15* is upregulated during the process of metastasis, which increased production and secretion of *GDF15*.

3.4 Biomarkers

To analyze PDIA6, HSP47, and GDF15 as potential candidates for gastric cancer biomarkers, we realized western blot against these three proteins. Our results demonstrated that HSP47 and GDF15 were detected only in conditioned medium of tumor cell lines (Figure 4E and 4F respectively), but not in the conditioned medium of normal gastric mucosa cells. Unfortunately, when we examined PDIA6 we were not able to obtain a specific result because we continually found multiple bands in our western blot results (Figure 4D). Despite this fact, the band patterns that we found was different among the tumor cell lines and normal gastric mucosa cells. This could indicate a different pattern of PDIA6 isoforms between gastric tumor and normal gastric mucosa.

4. CONCLUSION

In conclusion, our findings showed a significant difference in the secretome of gastric tumor cell lines, and that the proteins differentially secreted are involved in the crucial biological process, like regulation of cell cycle, metabolism, and modulation of ECM. Our

results also confirmed that HSP47 and GDF15 are differentially secreted by tumor cells, and they can be potential candidates for gastric cancer biomarker.

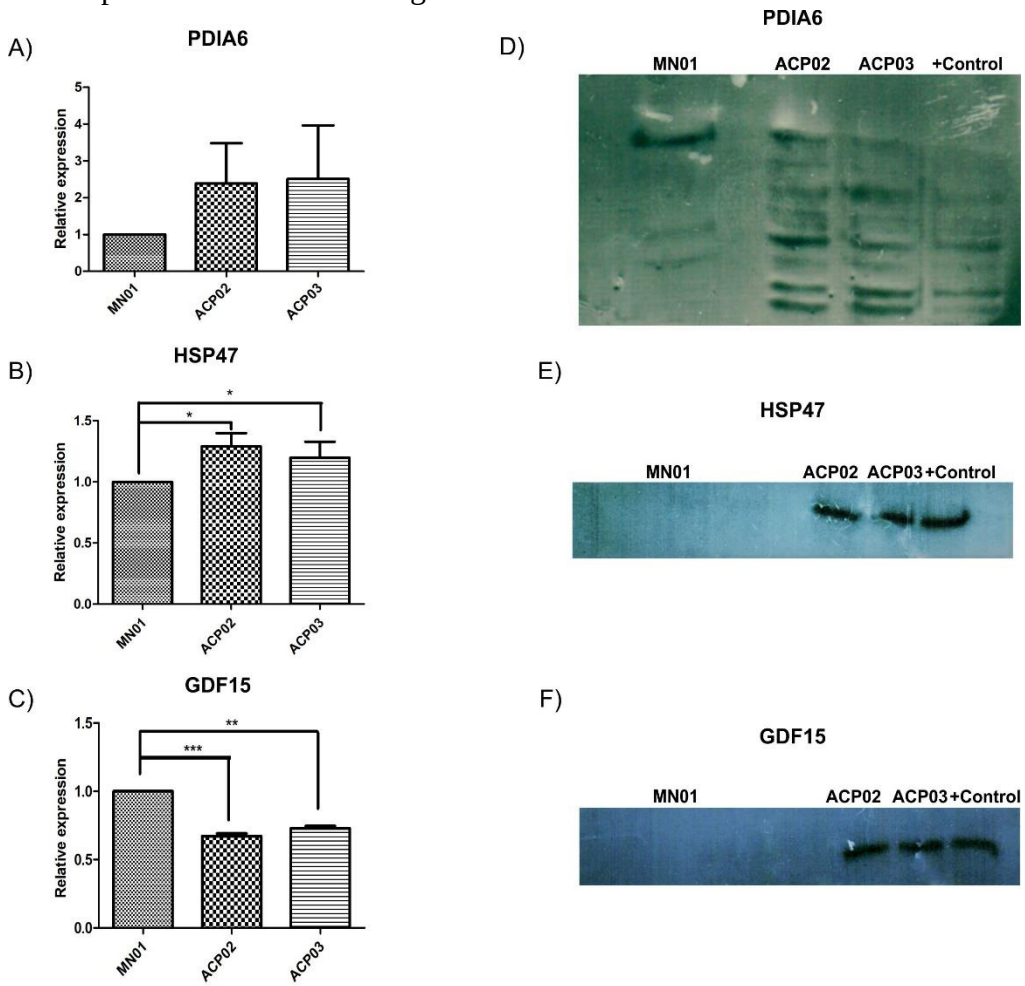

Fig. 4 Relative expression of the gene *PDIA6* (A), *HSP47* (B) and *GDF15* (C). GAPDH were used as internal control gene (2^ΔΔCT). Data are expressed as mean ± SEM. Significant differences are represented by * $p<0.05$, ** $p<0.001$ and *** $p<0.0001$. Analyses were made by one-way analysis of variance followed by Dunnett's multiple comparison test. Analyses of protein in conditioned medium against proteins PDIA6 (D), HSP47 (E) and GDF15 (F). Control + is a mix of total protein extract of the three cell lines.

Acknowledgments

We would like to thank Dr. Rommel Mario Rodríguez Burbano (Federal University of Pará) for supplying the cells used in this work. We also thank Mass Spectrometry Facility at Brazilian Biosciences National Laboratory (LNBio), CNPEM, Campinas, Brazil for their support on mass spectrometry analysis.

Conflicts of interest

There are no conflicts of interest to declare.

REFERENCES

BADER, G. D.; HOGUE, C. W. V. An automated method for finding molecular complexes in large protein interaction networks. **BMC bioinformatics**, [s. l.], v. 4, p. 2, 2003.

BANDARI, S. K. et al. Chemotherapy induces secretion of exosomes loaded with heparanase that degrades extracellular matrix and impacts tumor and host cell behavior. **Matrix Biology**, [s. l.], v. 65, n. 2018, p. 104–118, 2018.

CASTAÑÓN, M. J. et al. Plectin-intermediate filament partnership in skin, skeletal muscle, and peripheral nerve. **Histochemistry and Cell Biology**, [s. l.], v. 140, n. 1, p. 33–53, 2013.

CHEN, J. et al. Candidate genes in gastric cancer identified by constructing a weighted gene co-expression network. [s. l.], p. 1–19, 2018.

CHEN, R. et al. Low expression of LDHB correlates with unfavorable survival in hepatocellular carcinoma: Strobe-compliant article. **Medicine (United States)**, [s. l.], v. 94, n. 39, p. e1583, 2015.

CHENG, H.-P. et al. The Inhibitory Effect of PDIA6 Downregulation on Bladder Cancer Cell Proliferation and Invasion. **Oncology Research**, [s. l.], v. 25, n. 4, p. 587–593, 2017. a.

CHENG, L. et al. A comprehensive overview of exosomes in ovarian cancer: Emerging biomarkers and therapeutic strategies. **Journal of Ovarian Research**, [s. l.], v. 10, n. 1, p. 1–9, 2017. b.

CLINE, S. M. et al. Integration of biological networks and gene expression data using Cytoscape. **Nature protocols**, [s. l.], v. 2, n. 10, p. 2366–2382, 2007.

DERAZ, E. M. et al. MMP-10/stromelysin-2 promotes invasion of head and neck cancer. **PLoS ONE**, [s. l.], v. 6, n. 10, 2011.

DOHERTY, J.; CLEVELAND, J. Targeting lactate metabolism for cancer therapeutics. **The Journal of clinical investigation**, [s. l.], v. 123, n. 9, p. 3685–3692, 2013.

DVORAKOVA, M.; NENUTIL, R.; BOUCHAL, P. Transgelins, cytoskeletal proteins implicated in different aspects of cancer development. **Expert Review of Proteomics**, [s. l.], v. 11, n. 2, p. 149–165, 2014.

GILKES, D. M. et al. Collagen prolyl hydroxylases are essential for breast cancer metastasis. **Cancer Research**, [s. l.], v. 73, n. 11, p. 3285–3296, 2013.

GROGAN, T. M. et al. Thioredoxin, a putative oncogene product, is overexpressed in gastric carcinoma and associated with increased proliferation and increased cell survival. **Human Pathology**, [s. l.], v. 31, p. 475–481, 2000.

HALVAEI, S. et al. Exosomes in Cancer Liquid Biopsy: A Focus on Breast Cancer. **Molecular Therapy - Nucleic Acids**, [s. l.], v. 10, n. March, p. 131–141, 2018.

HERLING, A. et al. Enzymatic features of the glucose metabolism in tumor cells. **FEBS Journal**, [s. l.], v. 278, n. 14, p. 2436–2459, 2011.

HONG, W. et al. Inhibition of glucose-6-phosphate dehydrogenase reverses cisplatin resistance in lung cancer cells via the redox system. **Frontiers in Pharmacology**, [s. l.], v. 9, n. JAN, p. 1–11, 2018.

HU, L. et al. Clinical significance of expression of proliferating cell nuclear antigen and E-cadherin in gastric carcinoma. **World Journal of Gastroenterology**, [s. l.], v. 23, n. 20, p. 3721–3729, 2017.

HU, T. et al. Variant G6PD levels promote tumor cell proliferation or apoptosis via the STAT3/5 pathway in the human melanoma xenograft mouse model. **BMC Cancer**, [s. l.], v. 13, n. 163, p. 251, 2013.

HUSAINI, Y. et al. Macrophage inhibitory cytokine-1 (MIC-1/GDF15) gene deletion promotes cancer growth in TRAMP prostate cancer prone mice. **PLoS ONE**, [s. l.], v. 10, n. 2, p. 1–12, 2015.

JANUCHOWSKI, R. et al. Inhibition of ALDH1A1 activity decreases expression of drug transporters and reduces chemotherapy resistance in ovarian cancer cell lines. **International Journal of Biochemistry and Cell Biology**, [s. l.], v. 78, p. 248–259, 2016.

JIANG, H. et al. Helicobacter pylori infection promotes the invasion and metastasis of gastric cancer through increasing the expression of matrix metalloproteinase-1 and matrix metalloproteinase-10. **Experimental and Therapeutic Medicine**, [s. l.], v. 8, n. 3, p. 769–774, 2014.

JIANG, X. et al. HSP47 promotes glioblastoma stemlike cell survival by modulating tumor microenvironment extracellular matrix through TGF-β pathway. **ACS Chemical Neuroscience**, [s. l.], v. 8, n. 1, p. 128–134, 2016.

JOSHI, A. A. et al. Plectin-1 as a novel biomarker for pancreatic cancer. [s. l.], v. 55, n. 20, p. 6197–6214, 2011.

KAPHINGST, K. A.; PERSKY, S.; LACHANCE, C. **BIOLOGICAL NETWORK EXPLORATION WITH CYTOSCAPE 3**. [s.l: s.n.]. v. 14

KATADA, K. et al. Plectin promotes migration and invasion of cancer cells and is a novel prognostic marker for head and neck squamous cell carcinoma. **Journal of Proteomics**, [s. l.], v. 75, n. 6, p. 1803–1815, 2012.

KIM, T.; RYU, H.; LI, S. PDIA6 regulation of ADAM17 shedding activity and EGFR-mediated migration and invasion of glioblastoma cells. **J Neurosurg**, [s. l.], v. 126, n. June, p. 1829–1838, 2017.

LANDEN, C. N. et al. Targeting Aldehyde Dehydrogenase Cancer Stem Cells in Ovarian Cancer. **Molecular Cancer Therapeutics**, [s. l.], v. 9, n. 12, p. 3186–3199, 2010.

LEAL, M. F. et al. Establishment and conventional cytogenetic characterization of three gastric cancer cell lines. **Cancer Genetics and Cytogenetics**, [s. l.], v. 195, n. 1, p. 85–91, 2009.

LEE, E.; HEE LEE, D. Emerging roles of protein disulfide isomerase in cancer. [s. l.], v. 50, n. June, p. 401–410, 2017.

LI, J. et al. Prognostic Value of Ezrin in Various Cancers: A Systematic Review and Updated Meta-analysis. **Scientific Reports**, [s. l.], v. 5, p. 1–13, 2015.

LI, Y. et al. The transcription levels and prognostic values of seven proteasome alpha subunits in human cancers. **Oncotarget**, [s. l.], v. 8, n. 3, p. 4501–4519, 2017.

LIM, J. Y. et al. Thioredoxin and thioredoxin-interacting protein as prognostic markers for gastric cancer recurrence. **World Journal of Gastroenterology**, [s. l.], v. 18, n. 39, p. 5581–5588, 2012.

LIN, L. L.; HUANG, H. C.; JUAN, H. F. Revealing the Molecular Mechanism of Gastric Cancer Marker Annexin A4 in Cancer Cell Proliferation Using Exon Arrays. **PLoS ONE**, [s. l.], v. 7, n. 9, p. 1–10, 2012.

LIU, Y.-N. et al. Macrophage Inhibitory Cytokine-1 as a Novel Diagnostic and Prognostic Biomarker in Stage I and II Nonsmall Cell Lung Cancer. **Chinese Medical Journal**, [s. l.], v. 129, n. 17, p. 2026, 2016.

MA, X. et al. miR-486-5p inhibits cell growth of papillary thyroid carcinoma by targeting fibrillin-1. **Biomedicine and Pharmacotherapy**, [s. l.], v. 80, p. 220–226, 2016.

MAEKAWA, M. et al. Promoter hypermethylation in cancer silences LDHB, eliminating lactate dehydrogenase isoenzymes 1-4. **Clinical Chemistry**, [s. l.], v. 49, n. 9, p. 1518–1520, 2003.

MAERE, S.; HEYMANS, K.; KUIPER, M. BiNGO: A Cytoscape plugin to assess overrepresentation of Gene Ontology categories in Biological Networks. **Bioinformatics**, [s. l.], v. 21, n. 16, p. 3448–3449, 2005.

MARIMUTHU, A. et al. SILAC-based quantitative proteomic analysis of gastric cancer secretome. **PROTEOMICS – Clinical Applications**, [s. l.], v. 7, n. 0, p. 355–366, 2013.

MCCLELAND, M. L. et al. An integrated genomic screen identifies LDHB as an essential gene for triple-negative breast cancer. **Cancer Research**, [s. l.], v. 72, n. 22, p. 5812–5823, 2012.

MENG, T. et al. Transgelin-2: A potential oncogenic factor. **Tumor Biology**, [s. l.], v. 39, n. 6, 2017.

MIGITA, T. et al. ATP citrate lyase: Activation and therapeutic implications in non-small cell lung cancer. **Cancer Research**, [s. l.], v. 68, n. 20, p. 8547–8554, 2008.

MIYATA, T. et al. The Clinical Significance of Cancer Stem Cell Markers ALDH1A1 and CD133 in Lung Adenocarcinoma. **Anticancer Research**, [s. l.], v. 37, n. 5, p. 2541–2547, 2017.

MOGAMI, T. et al. Annexin A4 is involved in proliferation, chemo-resistance and migration and invasion in ovarian clear cell adenocarcinoma cells. **PLoS ONE**, [s. l.], v. 8, n. 11, 2013.

PHAN, L. M.; YEUNG, S.-C. J.; LEE, M.-H. Cancer metabolic reprogramming: importance, main features, and potentials for precise targeted anti-cancer therapies. **Cancer biology & medicine**, [s. l.], v. 11, n. 1, p. 1–19, 2014.

RAMOS, F. S. et al. PDIA3 and PDIA6 gene expression as an aggressiveness marker in primary ductal breast cancer. **Genetics and Molecular Research**, [s. l.], v. 14, n. 2, p. 6960–6967, 2015.

RASTOGI, N.; MISHRA, D. P. Therapeutic targeting of cancer cell cycle using proteasome inhibitors. **Cell Div**, [s. l.], v. 7, n. 1, p. 26, 2012.

RIVALS, I. et al. Enrichment or depletion of a GO category within a class of genes: Which test? **Bioinformatics**, [s. l.], v. 23, n. 4, p. 401–407, 2007.

SCARDONI, G. et al. Biological network analysis with CentiScaPe: centralities and experimental dataset integration. **F1000Research**, [s. l.], n. 0, p. 1–9, 2015.

SCARDONI, G.; LAUDANNA, C. Centralities Based Analysis of Complex Networks. **New frontiers in Graph Theory**, [s. l.], n. 2008, 2012.

SCARDONI, G.; PETTERLINI, M.; LAUDANNA, C. Analyzing biological network parameters with CentiScaPe. **Bioinformatics (Oxford, England)**, [s. l.], v. 25, n. 21, p. 2857–9, 2009.

STRELAU, J. et al. Expression and putative functions of GDF-15, a member of the TGF-beta superfamily, in human glioma and glioblastoma cell lines. **Cancer Lett**, [s. l.], v. 270, n. 1, p. 30–39, 2008.

SZKLARCZYK, D. et al. STRING v10: Protein-protein interaction networks, integrated over the tree of life. **Nucleic Acids Research**, [s. l.], v. 43, n. D1, p. D447–D452, 2015.

TOMITA, H. et al. Aldehyde dehydrogenase 1A1 in stem cells and cancer. **Oncotarget**, [s. l.], v. 7, n. 10, 2016.

VOUTSADAKIS, I. A. Proteasome expression and activity in cancer and cancer stem cells. **Tumor Biology**, [s. l.], v. 39, n. 3, p. 1–17, 2017.

WANG, S. C. PCNA: A silent housekeeper or a potential therapeutic target? **Trends in Pharmacological Sciences**, [s. l.], v. 35, n. 4, p. 178–186, 2014.

WANG, X. et al. Circulating MIC-1/GDF15 is a complementary screening biomarker with CEA and correlates with liver metastasis and poor survival in colorectal cancer. **Oncotarget**, [s. l.], v. 8, n. 15, p. 24892–24901, 2017.

WARREN, J. J. et al. Plectin-1 as a Biomarker of Malignant Progression in Intraductal Papillary Mucinous Neoplasms: A Multicenter Study. [s. l.], v. 44, n. 2, p. 319–335, 2017.

WIDMER, C. et al. Molecular basis for the action of the collagen-specific chaperone Hsp47/SERPINH1 and its structure-specific client recognition. **Proceedings of the National Academy of Sciences**, [s. l.], v. 109, n. 33, p. 13243–13247, 2012.

WINDRICHOVA, J. et al. MIC1/GDF15 as a Bone Metastatic Disease Biomarker. **Anticancer Research**, [s. l.], v. 37, n. 3, p. 1501–1506, 2017.

XIA, W. et al. P4HB promotes HCC tumorigenesis through downregulation of GRP78 and subsequent upregulation of epithelial-to-mesenchymal transition. **Oncotarget**, [s. l.], v. 8, n. 5, p. 8512–8521, 2017.

XIONG, G.-F.; XU, R. Function of cancer cell-derived extracellular matrix in tumor progression. **Journal of Cancer Metastasis and Treatment**, [s. l.], v. 2, n. 9, p. 357, 2016.

YANG, C. Z. et al. GDF15 is a potential predictive biomarker for TPF induction chemotherapy and promotes tumorigenesis and progression in oral squamous cell carcinoma. **Annals of Oncology**, [s. l.], v. 25, n. 6, p. 1215–1222, 2014. a.

YANG, D.; ZHAO, D.; CHEN, X. MIR-133b inhibits proliferation and invasion of gastric cancer cells by up-regulating FBN1 expression. **Cancer Biomarkers**, [s. l.], v. 19, n. 4, p. 425–436, 2017.

YANG, L. et al. ALDH1A1 defines invasive cancer stem-like cells and predicts poor prognosis in patients with esophageal squamous cell carcinoma. **Modern Pathology**, [s. l.], v. 27, n. 5, p. 775–783, 2014. b.

YONG, K. M. A. et al. Morphological effects on expression of growth differentiation factor 15 (GDF15), a marker of metastasis. **Journal of Cellular Physiology**, [s. l.], v. 229, n. 3, p. 362–373, 2014.

ZAIDI, N.; SWINNEN, J. V.; SMANS, K. ATP-citrate lyase: A key player in cancer metabolism. **Cancer Research**, [s. l.], v. 72, n. 15, p. 3709–3714, 2012.

ZHANG, G. et al. Matrix metalloproteinase-10 promotes tumor progression through regulation of angiogenic and apoptotic pathways in cervical tumors. **BMC Cancer**, [s. l.], v. 14, n. 1, p. 1–14, 2014.

ZHANG, X. et al. Identification of stem-like cells and clinical significance of candidate stem cell markers in gastric cancer. **Oncotarget**, [s. l.], v. 7, n. 9, p. 9815–9831, 2016.

ZHANG, Y. et al. GDF15 promotes epithelial-to-mesenchymal transition in colorectal carcinoma. **Artificial Cells, Nanomedicine, and Biotechnology**, [s. l.], p. 1–7, 2018.

ZHOU, Z. H. et al. Reorganized collagen in the tumor microenvironment of gastric cancer and its association with prognosis. **Journal of Cancer**, [s. l.], v. 8, n. 8, p. 1466–1476, 2017.

CAPÍTULO II

ARTIGO DE REVISÃO

THE HEAT SHOCK PROTEIN 47 AS A POTENTIAL BIOMARKER AND A THERAPEUTIC AGENT IN CANCER RESEARCH

Artigo publicado em

Journal of Cancer Research and Clinical Oncology

Journal of Cancer Research and Clinical Oncology
https://doi.org/10.1007/s00432-018-2739-9

REVIEW – CANCER RESEARCH

The heat shock protein 47 as a potential biomarker and a therapeutic agent in cancer research

Beatriz Dal Pont Duarte[1] · Diego Bonatto[1]

Received: 19 July 2018 / Accepted: 12 August 2018

Abstract
Heat shock protein 47 (HSP47) is an important chaperone required for the correct folding and secretion of collagen. Several studies revealed that HSP47 has a role in numerous steps of collagen synthesis, preventing procollagen aggregation and inducing hydroxylation of proline and lysine residues. HSP47 is encoded by the SERPINH1 gene, which is located on chromosome 11q13.5, one of the most frequently amplified regions in human cancer. The altered expression levels of HSP47 have been correlated with several types of cancer, such as cervical, breast, pancreatic and gastric cancers. Studies have shown that HSP47 promotes tumor angiogenesis, growth, migration and metastatic capacity. In this review, we highlight the fundamental aspects of the interaction between HSP47 and collagen and the recent discoveries of the role of this chaperone in different types of malignant neoplasias. We also discuss recent treatments using HSP47 as a therapeutic target, and present evidences that HSP47 is an essential protein for cancer biology and a potential molecular target for chemotherapy.

Keywords HSP47 · SERPINH1 · Collagen · Cancer · Therapeutic target

Introduction

The maintenance of cellular protein homeostasis (proteostasis) is essential for the proper function of the cell. The main compartment involved in the correct protein-folding process is the endoplasmic reticulum (ER), where a complex network of molecules assists with proteostasis (Dufey et al. 2015). At the core of this process are the chaperones, which support the correct maturation of new peptide chains (Brandvold and Morimoto 2015).

Heat shock protein 47 (HSP47) is an important chaperone required for the correct folding and secretion of various types of collagen (Natsume et al. 1994). This protein was first described in chicken embryo fibroblasts as an HSP that can bind to collagen type 1 (Nagata and Yamada 1986; Nagata et al. 1986). In 1991, with the determination of its nucleotide sequence, it was demonstrated that HSP47 belongs to the serpin family, despite the lack of serine protease inhibitory activity (Hirayoshi et al. 1991). However, the *HSP47* gene (SERPINH1) is transcriptionally modulated by a heat shock element (HSE) (Natsume et al. 1994).

In several types of cells, the expression of *HSP47* can be correlated with the expression of collagen. *HSP47* is highly expressed in cells such as chick embryo fibroblasts, which also express high levels of collagen (Hirayoshi et al. 1991). In addition, it has also been demonstrated that there are no detectable levels of HSP47 in cells that do not synthesize collagen type I, such as neuroblastoma and erythroleukemic cell lines (Clarke et al. 1993). In fibroblasts of chick embryos transformed by Rous sarcoma virus, the expression of both collagen I and HSP47 is decreased (Nagata and Yamada 1986). This effect is also observed in F9 teratocarcinoma cells. In these cells, the levels of *HSP47* and collagen type I and type IV are not detectable. However, when these cells are differentiated by treatment with retinoic acid, both *HSP47* and collagens show increased expression (TAKECHI et al. 1992).

Several types of cancer are associated with abnormal protein folding. In the last two decades, HSP47 has been described as an important chaperone in the control and maintenance of cellular proteostasis. In this paper, we review the importance of the interaction between HSP47

✉ Beatriz Dal Pont Duarte
beatriz.duarte@ufrgs.br

[1] Departamento de Biologia Molecular e Biotecnologia, Centro de Biotecnologia da UFRGS, Universidade Federal do Rio Grande do Sul-UFRGS, Avenida Bento Gonçalves, 9500, Prédio 43421, Sala 107, Caixa Postal 15005, Porto Alegre, Rio Grande Do Sul 91509-900, Brazil

Published online: 20 August 2018

Journal of Cancer Research and Clinical Oncology

and collagen and the role of this chaperone in different types of malignant neoplasias.

HSP47 and collagen maturation

Collagen is the most abundant protein in the body and the major component of the extracellular matrix (ECM). Collagen is composed of three α-chains, each with a triple-helix domain. These chains have three distinct domains: an N-terminal propeptide, a central collagen domain and a C-terminal propeptide. After translation, the C-terminal domains of the α-chains lead to recognition and the beginning of trimerization of the three α-chains. This process is accelerated by protein disulfide isomerase (PDI), which forms interchain disulfide bonds in this domain (Wilson et al. 1998). Subsequently, the molecule of collagen is transported and secreted via the ER–Golgi pathway (Fig. 1). When procollagen is in the extracellular environment, its N- and C-propeptide domains are cleaved by propeptidases, followed by ligation of collagen in the ECM (Layman and Ross 1973). HSP47 is capable of returning to the ER due to its RDEL ER-retention signal sequence and the KDEL receptors present in the Golgi membrane (Sauk et al. 1998).

HSP47 is essential for the correct folding of a collagen chain. During the formation of the collagen triple helix, regions of the molecule become hydrophobic. HSP47 binds to these regions and prevents aggregation of procollagens in the ER. The interaction between HSP47 and procollagens happens in a pH-dependent manner. In the range between pH 6.4 and 7, the fibril formation of type I collagen is inhibited by HSP47 ligation. When pH is decreased to a value of 6.3 or lower, fibril formation is increased. This occurs because the HSP47 structure is altered at lower pH, and this conformational change inhibits the ligation between HSP47 and collagen I (Thomson and Ananthanarayanan 2000). This explains why HSP47 is associated with procollagen in the ER, which has a neutral pH, and why it is dissociated from procollagen during the transport from the ER–Golgi compartment, which has a low pH (Nakai et al. 1992).

The central collagen domain is composed of 338 Gly–Xaa–Yaa repeats, where Xaa and Yaa are normally a proline or a hydroxyproline. In a previous work, in which various collagen model peptides were synthesized, it was revealed that the dominant binding site of HSP47 in collagen is formed by an Xaa–Arg–Gly triplet. In addition, it was demonstrated that HSP47 was able to bind to a procollagen molecule only when arginine (Arg) residues were incorporated in the Yaa position (Koide et al. 2002).

Several modifications are necessary for the correct formation and secretion of the collagen molecule. One key modification is hydroxylation of proline and lysine residues. These modifications only occur in unfolded chains and involve the three enzyme families prolyl 3-hydroxylases,

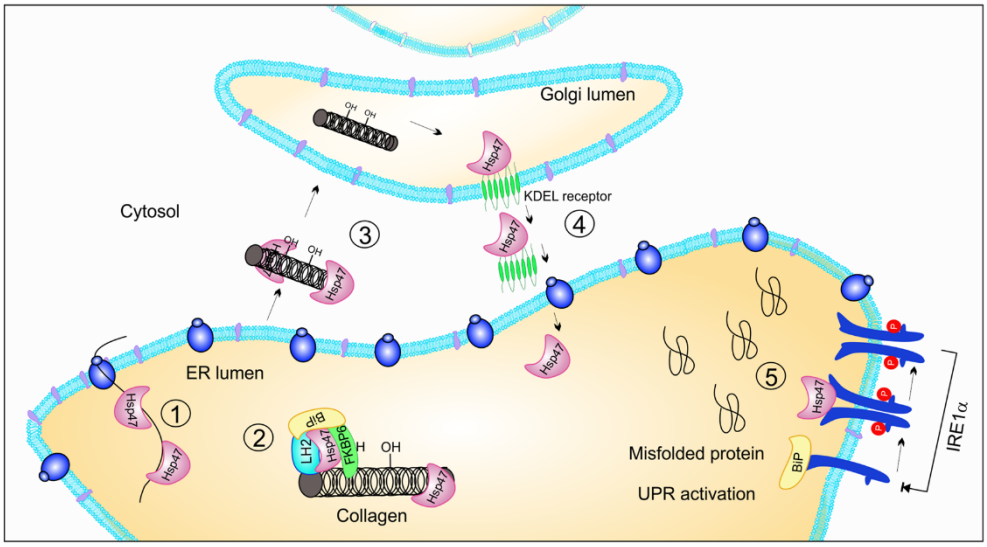

Fig. 1 Collagen synthesis and HSP47: a newly synthesized collagen is released into the ER, HSP47 binds to procollagen and prevents aggregation in the ER (1). A protein complex formed by LH2, FKBP65, HSP47 and BiP regulates lysyl hydroxylation (2). Then, the molecule of collagen is transported and secreted via the ER–Golgi pathway (3). HSP47 returns to the ER due to its KDEL ER-retention signal sequence (4). During ER stress, HSP47 binds with IRE1α, which reduces the association between IRE1α and BiP, leading to the activation of IRE1α (5)

prolyl 4-hydroxylases and lysyl hydroxylases. The activity of lysyl hydroxylase 2 (LH2) produces tissue-specific patterns of hydroxylation. These patterns modulate intra- and intercrosslinking between molecules and hence modulate the ECM (Walker et al. 2005). Alterations of these patterns can lead to changes associated with cancer metastasis (Chen et al. 2015).

In a recent work, Duran et al. (2017) described a chaperone complex that involved LH2 and HSP47. This complex regulated lysyl hydroxylation in type I procollagen, and it was composed of LH2, FKBP65, HSP47 and immunoglobulin heavy-chain-binding protein (BiP) (Fig. 1). BiP allows correct folding of nascent ER polypeptides and regulates the unfolded protein response (UPR). FKBP65 is a collagen chaperone resident in the ER, and the loss of its function leads to a decrease in lysyl hydroxylation. However, in cells with a reduction in HSP47 protein levels, there is an increase in lysyl hydroxylation of type I collagen (Lindert et al. 2015). Finally, the data from Duran et al. (2017) suggest that BiP participates in the formation of and affinity among the proteins in the complex. In addition, they also showed a balance between FKBP65 and HSP47 and that this balance is responsible for regulating lysyl hydroxylation (Duran et al. 2017).

Another important posttranslational modification that ensures collagen stability is glycosylation. Glycosyltransferases (GLT25D1 and GLT25D2) transfer galactose to the hydroxylysine residues of procollagen, preventing collagen from forming interchain crosslinks (Yamauchi et al. 1982).

HSP47 response to ER and Golgi stress

When misfolded/unfolded proteins accumulate in the ER, several signals lead to ER stress, triggering the UPR (Walter and Ron 2011). When the UPR is activated, expression of genes that improve protein folding is induced, and degradation of misfolded proteins is promoted as a way to re-establish ER homeostasis. A continuous ER stress state can lead to cell death, normally by apoptosis (Ma and Hendershot 2004). Chronic ER stress is a relevant factor in the development of pathological conditions, such as neurodegenerative diseases and cancer (Wang and Kaufman 2016).

Inositol-requiring enzyme 1 alpha (IRE1α) is an ER transmembrane protein and the most conserved ER stress signal transducer activated by the UPR. Activation of IRE1α leads to ER-associated degradation (ERAD), protein secretion and the expression of X-box binding protein 1 (XBP1), a transcription factor of genes involved in protein folding (Wang and Kaufman 2016). In a recent study, it was shown that HSP47 increases the activation of IRE1α by binding with the ER luminal domain of IRE1α and reducing the association between IRE1α and BiP (Sepulveda et al. 2018). The binding of BiP with the luminal domain of IRE1α preserves the inactive state of IRE1α (Kimata et al. 2003). Thus, a change in binding from BiP to HSP47 leads to activation of IRE1α (Fig. 1), and this results in ER stress attenuation (Sepulveda et al. 2018).

A major increase in protein secretion can cause insufficiency of Golgi apparatus functions and activation of the Golgi stress response. A disturbance in glycoprotein traffic by inhibition of O-glycosylation is an effective stimulus for the activation of the Golgi stress response (Sasaki and Yoshida 2015). To elucidate the role of HSP47 in the Golgi stress response, the expression of HSP47 was downregulated by small interfering RNA (siRNA) in NIH3T3 cells and treated with O-glycosylation inhibitor. The results showed that induction of Golgi stress in HSP47 knockdown cells caused cell death. HSP47-knockdown cells also exhibited an increase in cleavage of Golgi-resident caspase-2 and activation of mitochondrial caspase-9. Furthermore, the induction of Golgi stress also induced a UPR response. These discoveries could indicate that HSP47 protects the Golgi apparatus from the effects of O-glycosylation inhibition and protects cells from the Golgi stress response (Miyata et al. 2013).

Relationship among cancer, collagen and HSP47

ECM is essential to the support, resistance and organization of tissues. Moreover, it is responsible for numerous biochemical signals that modulate cellular function. The ECM comprises several components, such as collagen, laminin, fibronectin, glycoproteins, proteoglycans, and matricellular proteins. To develop cancer, an extensive reorganization of the ECM is necessary (Schedin and Keely 2011). This reorganization drives tumor progression through prosurvival and proliferative signals, promoting tumor metastasis. Deposition of collagen is higher in breast cancer cells than in nonmalignant mammary cells (Curran and Keely 2013). Furthermore, collagen was identified as a prognostic marker, and its expression is associated with cancer recurrence (Helleman et al. 2008).

HSP47 is encoded by the SERPINH1 gene, located on chromosome 11q13.5. This region is one of the most frequently amplified in human cancer (Schwab 1998). HSP47 promotes tumor growth and invasion, probably through regulation of the ECM network, and may be a possible biomarker and therapeutic target (Zhu et al. 2015). HSP47 has a 3′-UTR region in messenger RNA that is regulated by microRNA(miR)-29. Several works have demonstrated that miR-29 repressed the expression of HSP47, controlling the levels of this protein in cells (Yamamoto et al. 2013; Zhu et al. 2015). The downregulation of miR-29, leading to the upregulation of HSP47, has been correlated with several

types of cancer, such as cervical, breast, pancreatic and gastric cancer (Maitra et al. 2002; Hirai et al. 2006; Yamamoto et al. 2013; Zhu et al. 2015).

Gastric and colorectal cancer

Gastric cancer is the third most common type of malignant neoplasm (Ferlay et al. 2015). Good patient prognosis depends on several factors, and the stage of the tumor detection is critical. Thus, finding good biomarkers for early gastric cancer detection is essential and has been the subject of diverse scientific works that applied proteomic, transcriptomic and in silico analyses.

To determine novel biomarkers for gastric cancer, Zhang et al. (2010) performed an analysis with 22 genes imported into the public Affymetrix gene expression-profiling dataset. Of these genes, eight showed a significant difference ($p < 0.001$) in expression levels between the gastric cancer samples ($n = 22$) and healthy gastric tissue ($n = 8$), and *HSP47* was found to be upregulated in gastric cancer (Zhang et al. 2010).

Ulcerative colitis (UC) is a chronic condition that leads to inflammation and formation of ulcers in the colon and rectum. Patients with a longer disease duration seem to be at higher risk of neoplastic development (Ford et al. 2013). A proteomic approach for UC-associated cancer and sporadic colon cancer cell lines demonstrated elevated expression of HSP47 in UC-associated cancer compared with that in sporadic colon cancer. In addition, immunohistochemical analysis showed an increase in expression of HSP47 with the progression of cancer. Interestingly, HSP47 was also detected in the culture medium through a Western blot technique (Araki et al. 2009).

Association between HSP47 and colorectal cancer is also observed with the use of the isobaric tag for relative and absolute quantification (iTRAQ) method. In this study, Mori et al. (2017) identified proteins associated with lymph node metastasis in patients with colorectal cancer. A bioinformatic analysis demonstrated that HSP47 was the main potential biomarker. In addition to these data, they also validated their results through immunohistochemistry. The analysis also demonstrated that expression of HSP47 was significantly higher in lymph node metastasis than in colorectal cancer without metastasis (Mori et al. 2017). These data indicate a correlation between HSP47 and colorectal tumor aggressiveness, which can also be observed in obstructive colorectal carcinoma (OCC) (Xu et al. 2013).

OCC has a poor prognosis and a higher correlation with aggressive types of cancer (Fitchett and Hoffman 1986). The expression of HSP47 was strongly detected in obstructive carcinoma. OCC showed cancer cells with less differentiation and stromal myofibroblast proliferation leading to a fibrotic process. Coexpression of HSP47 with basic fibroblast growth factor (bFGF) in inflammatory cells may contribute to stromal fibrosis formation (Xu et al. 2013). A relation between HSP47 and fibrotic process is also observed in scirrhous carcinoma of the stomach and in cirrhotic human liver (Hirai et al. 2006; Poschmann et al. 2009). The effect of HSP47 on the fibrotic process may occur through the regulation of endothelin receptors A and B (ETBRA, ETBRB) by HSP47 (Zhao et al. 2017).

Pancreatic cancer

In a study with 57 cases of primary invasive pancreatic ductal adenocarcinomas, the expression of HSP47 was detected in all neoplastic samples. Interestingly, the expression of HSP47 was most intense in the tumor-associated stroma. Only a dispersed immunoreactivity was found in the fibroblast cells of ductal adenocarcinomas, and in 35% of cases, these cells did not express HSP47 (Maitra et al. 2002). In addition, in analyses of stroma of nonductal pancreatic neoplasms, all tested cases were positive for HSP47 (Cao et al. 2005). However, when HSP47 expression was analyzed in the cells of this neoplasm, the results were similar only in cases of pancreatoblastomas, with 75% of cases showing cells positive for HSP47. In acinar cell carcinomas and solid pseudopapillary tumors, the cells were positive for HSP47 in only 23 and 25% of cases investigated, respectively. Nevertheless, 100% of the cases analyzed of osteoclastic-like giant cells showed cells with HSP47 protein expression (Cao et al. 2005).

Gynecological cancers

Cancers in women's reproductive organs are referred to as gynecologic cancers and include cervical, ovarian, uterine, vaginal, and vulvar cancers (Tavassoli and Deville 2003).

In cervical squamous cell carcinoma (SCC), miR-29a is normally found to be downregulated (Li et al. 2011; Yamamoto et al. 2013). When its expression was restored in SCC cell lines, the migration and invasion capacity decreased. Luciferase reporter assays demonstrated that miR-29a acts by directly regulating HSP47 (Yamamoto et al. 2013). In addition to these results, analyses with cervical normal tissue, HPV16-positive SCCs, and HPV16-positive CIN2–3 (abnormal cells found on the surface of the cervix) also demonstrated a relationship between miR-29a and malignant transformation of cervical epithelial cells. Infection by human papillomavirus (HPV) is directly correlated with the development of invasive cervical cancer, and the results showed a small decrease in miR-29a in HPV16-positive CIN2–3 and a significant decrease in HPV16-positive SCCs when compared with that in normal tissue (Li et al. 2011).

17-AAG (17-allylamino-17-demethoxygeldanamycin) is a promising antitumor agent that acts through inhibition of

the molecular chaperone Heat shock protein 90 (HSP90). Despite the antitumor activity, treatment with 17-AAG increased expression of *HSP47* (Maloney et al. 2007). Conversely, 17-AAG disrupted the tumor suppressor pathway LATS–MST2–YAP. These findings may suggest that, although 17-AAG has antitumor activity, it is also involved in signaling pathways that promote tumorigenesis (Huntoon et al. 2010).

Breast cancer

Using gene coexpression network analysis, Zhu et al. (2015) discovered a coexpression network that participates in ECM remodeling from breast cancer tissues. In this network, *HSP47* appears as a nodal hub in the regulation of ECM, and their analysis showed that *HSP47* expression was activated during breast cancer development and progression. To determine the function of HSP47 in breast tumor progression, silencing of *HSP47* was carried out in breast cancer cell lines. Their results showed that HSP47 promotes cancer progression by increasing cell proliferation and invasion. In addition, silencing of HSP47 reduced the levels of collagen I and IV and fibronectin in the conditioned medium but did not alter the protein expression levels in cells, demonstrating the role of HSP47 in secretion of the ECM components (Zhu et al. 2015).

Human breast cancer cells silenced for HSP47 showed restricted tumor growth in xenograft assays. This probably occurs through a reduction in the secretion of collagen and fibronectin and hence their deposition in the ECM. Coexpression network analysis also revealed that levels of microRNA-29b and 29c are associated with expression of HSP47 and ECM network genes (Zhu et al. 2015). TGF-β expression was linked to several molecules that are part of the ECM (Xu and Mao 2011). Treatment with TGF-β also induced HSP47 expression in nonmalignant breast cell lines. In addition, blocking the TGF-β pathway with a TGFBR inhibitor reduced HSP47 expression in breast cancer cell lines. These results could indicate that HSP47 is regulated by the TGF-β pathway (Zhu et al. 2015).

The TGF-β pathway involves the activation of several molecules including SMAD3. SMAD3 is associated with the transcription activation of *GATA3* (Blokzijl et al. 2002), another protein correlated with HSP47 (Wang 1994).

GATA3 is a transcription factor that regulates luminal epithelial cell differentiation in the mammary gland (Kouros-Mehr et al. 2006). In breast cancer cells, there is a loss of GATA3 expression, and this fact is related to poor prognosis in patients. When expression of GATA3 was induced in breast cancer cells, there was suppression of metastasis, modification of the cellular microenvironment and increases in cellular differentiation. These effects are related to the induction of miR-29b expression through GATA3 (Chou et al. 2013). Interestingly, GATA3 is also related to the induction of HSP47 expression (Wang 1994). These data suggest a possible role for GATA3 in the balance of *HSP47* expression.

Lung cancer

Lung tumors are classified into small cell lung carcinoma and non-small cell lung carcinoma. This last group consists of squamous cell carcinoma (SCC), lung adenocarcinoma, and large cell carcinoma (LCC). Cells from normal human bronchial epithelium and squamous cell carcinoma tumors were analyzed in a comparative proteomic approach. The results showed that 32 proteins were differentially expressed between the two groups of cells and that HSP47 was overexpressed in squamous cell carcinoma. In addition to these data, immunohistochemical analysis of the different types of lung cancer revealed a significant coregulation between HSP47 and cytokeratins in squamous cell carcinoma (Poschmann et al. 2009).

Idiopathic pulmonary fibrosis is a chronic lung disease that can lead to lung cancer (Daniels and Jett 2005). Recent studies of miRNA expression demonstrated that miR-29a is downregulated in lung cancer and pulmonary fibrosis. Through a combination of gene expression analysis and in silico analysis of lung cancer cells and lung fibroblast cells lines, 24 possible targets of miR-29a were described. This microRNA is associated with the expression of LOXL2 and HSP47. When the expression of miR-29a is restored in lung cancer cell lines, the aggressiveness and the fibroblast migration capacity were repressed (Kamikawaji et al. 2016).

Osteosarcoma

Collagen I is the principal protein found in mature bone. In addition to the presence of a binding site for HSP47, collagen I also has a binding site for the serine protease inhibitor (serpin) pigment epithelium-derived factor (PEDF). This protein has shown anti-osteosarcoma properties with a protective effect against osteolysis and lung metastasis (Ek et al. 2007b). PEDF was also capable of decreasing the angiogenesis, migration and invasion capacity in experiments in vivo and in vitro with two osteosarcoma cell lines (Ek et al. 2007a). Nevertheless, it was observed that PEDF upregulates collagen I and HSP47 in osteosarcoma cells in vitro (Alcantara et al. 2014).

In a study by Uozaki et al. (2000), the expression of HSP27, HSP47, HSP60, HSP70 and HSP90 was analyzed by immunohistochemical staining in 70 cases of conventional osteosarcoma in the bones of the extremities. The overexpression rate of HSP47 (94%) was higher than that of other HSPs. However, the expression of HSP47 does not

demonstrate a relationship with poor prognosis in patients (Uozaki et al. 2000).

Glioblastoma

Grade IV glioblastoma multiforme (GBM) is the most aggressive form of brain tumor. Overexpression of HSP47 was described in analyses of tissues and glioma cell lines, and this expression was correlated with the grade of the disease (Wu et al. 2014, 2016; Zhao et al. 2014; Jiang et al. 2016). Knockdown of HSP47 using small interfering RNAs inhibited growth, viability, migration and invasion capacity in glioma cells lines in vitro and decreased tumor volume in vivo (Zhao et al. 2014; Wu et al. 2016). The relationship between HSP47 and these cellular mechanisms was also demonstrated through overexpression of *HSP47* by lentivirus infection. In this experiment, it was found that overexpression of *HSP47* promotes glioma formation, invasion and angiogenesis. The effect observed is probably caused by regulation of ECM components through the TGF-β pathway (Jiang et al. 2016).

In addition to the remodeling of the ECM, another fundamental mechanism for tumor growth and metastasis is angiogenesis (Sato 2003; Nishida et al. 2006). Thus, pericytes have an important role in tumor development (Hosaka et al. 2016). Analyses with pericytes in glioma tissues demonstrated that these cells express HSP47 (Wu et al. 2016; Hosono et al. 2017). Knockdown of HSP47 in human umbilical vein endothelial cells decreased the proliferation and migration of the cells and inhibited tube formation. A decrease in HSP47 expression reduced the microvessel density in vivo. Furthermore, gene array and Western blot analyses demonstrated that HSP47 promoted glioma angiogenesis via HIF1α-VEGFR2 signaling (Wu et al. 2016). Therefore, HSP47 can be considered as a potential therapeutic target of GBM.

Tumor immunotherapy treatment with directional targets for cytotoxic T lymphocytes (CTL) leads to tumor regression in several types of neoplasias (Azuma et al. 2016; Verma et al. 2016; Suekane et al. 2017; Wu et al. 2017). The tumor-associated antigen can be a highly differentially expressed gene. In a prospective study, Wu et al. identified two peptides of HSP47 that were candidate epitopes for CTL treatment. The T-cell immune response was analyzed in GBM patients by stimulation with the peptide mixture. The results showed that the GBM patients with a positive CTL response to HSP47 experienced a prolonged progress-free survival time and overall survival (Wu et al. 2014).

Head and neck cancers

Cancers collectively known as head and neck cancers typically begin in the squamous cells and, rarely, in the salivary glands (Huntoon et al. 2010). To understand the role of HSP47 in these types of cancers, analyses of HSP47 were performed in lines of human squamous cell carcinoma of the head and neck (SCCHN) in a comparative mode with a primary gingival fibroblast cell line. The expression of HSP47 was positive for all cell lines, but when the analysis was performed for cell surfaces only, the expression of HSP47 was detected only in SCCHN lines. The invasion capacity of SCCHN cells was evaluated using modified Boyden chambers. The results showed variance among the different cell lines, and this variance could be associated with the level of HSP47 expressed on the cell surface. Unexpectedly, cell lines expressing high levels of HSP47 revealed the lowest migratory index (Hebert et al. 1999).

To understand how HSP47 is anchored in the cell membrane, several immunoprecipitation assays were performed, and the immunoprecipitation assays using anti-CD9 and anti-HSP47 antibodies confirmed that these proteins precipitated together. Reimmunoprecipitation of the CD9 with anti-HSP47 confirmed the interaction between these two proteins in all SCCHN lines. These findings indicated that HSP47 may be anchored in the cell membrane in a complex with CD9 (Hebert et al. 1999). In a previous work, Sauk et al. showed that HSP47 could be recycled and is not permanently anchored to the cell surface (Sauk et al. 2000).

HSP47 as a therapeutic target in cancer

To investigate the potential of HSP47 as a molecular target for chemotherapy, a water-soluble polymeric drug delivery system containing an HSP47-binding peptide sequence and the chemotherapeutic agent doxorubicin (Dox) was tested in SCCHN cell lines. The HSP47-binding peptide sequence WHYPWFQNWAMA (Hebert et al. 2001) and the chemotherapeutic agent Dox were attached to the polymer via a tetrapeptide spacer. The treatment of SCCHN cell lines with the polymer-drug conjugate proved that the drug delivery system is recognized by the HSP47 receptors and that after its recognition, drug internalization and intracellular release occur (Nan et al. 2005).

In the same way, an *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer was bound with a peptide sequence WHYPWFQNWAMA (Hebert et al. 2001) as a targeting ligand and synthesized by a simple synthetic route. Subsequently, 1,3-dimethylol-5-FU, derived from 5-fluorouracil (5-FU), was attached to the polymer. After polymer formation and internalization of the polymer, cytotoxicity and apoptosis assays were evaluated in an SCCHN cell line. All experimental results were compared to those of treatment with 5-FU and a copolymer without the peptide sequence from HSP47. The results showed that the polymer with the HSP47 peptide exhibited the highest cytotoxic efficacy,

the fastest internalization, and an increased apoptotic and necrotic induction of tumor cells when compared to the other conditions (Xiang et al. 2012).

In the development of new chemotherapies, HSP inhibitory molecules emerged as an important strategy. Inhibition of HSP47 is an attractive therapeutic intervention, because unlike other chaperones, HSP47 has collagen as a unique substrate. In this context, a screen with 2,080 compounds identified four molecules that have inhibitory activity against HSP47. After the definition of IC50 values, these compounds were tested in cell culture, and the inhibitory activity against HSP47 was proven (Thomson et al. 2005).

AK778 was described as an inhibitory molecule that competitively inhibited the interaction between HSP47 and collagen. When the cells were treated with AK-778, the molecule of collagen was destabilized. Further experiments demonstrated that AK-778 was degraded into two fragments named Col002 and Col003, and the inhibitory effect on HSP47 was due to Col003. Experiments also demonstrated that Col003 inhibited collagen secretion in vivo by binding with HSP47 in its collagen-binding region (Ito et al. 2017).

Pirfenidone is an antifibrotic drug commonly used for the treatment of idiopathic pulmonary fibrosis (Sharbeen et al. 2015). Pirfenidone exerts its antifibrotic effect by suppressing HSP47 and collagen I expression through downregulation of the TGFβ signaling pathway (Nakayama et al. 2008). Supported by positive clinicals studies, pirfenidone was approved for treatment of idiopathic pulmonary fibrosis. In a recent study by Polydorou et al., it was demonstrated that pirfenidone improves blood vessel perfusion and intensifies the antitumor efficacy of Dox, increasing the drug efficacy in chemotherapy (Polydorou et al. 2017).

Terutroban is a specific antagonist of the thromboxane receptor (TP) that has demonstrated a high antithrombotic efficacy (Siller-Matula et al. 2010). Gelosa et al. demonstrated that terutroban suppresses the expression of HSP47 in the aortic tissues of rats treated with this drug. In addition, quantitative PCR also showed a suppression of TGF-β expression (Gelosa et al. 2011). These discoveries suggest that terutroban could be a possible treatment for diseases that have altered HSP47 expression such as cancer.

Conclusions

HSP47 expression can be directly correlated with several types of cancer, and this protein is emerging as a possible biomarker and therapeutic target in malignant neoplasms. Nevertheless, the expression of HSP47 in different types of cancer has divergent effects. Although in some types of cancer, like glioblastoma and breast cancer, HSP47 is associated with aggressiveness (Zhu et al. 2015; Jiang et al. 2016), in osteosarcoma HSP47 has an indirect protective effect against osteolysis and lung metastasis (Alcantara et al. 2014; Kobayashi et al. 2014). Therefore, more studies are necessary to understand the influence of HSP47 in tumor development.

Funding Contract/grant sponsor: CNPq.

Compliance with ethical standards

Conflict of interest Author Beatriz Dal Pont Duarte declares that she has no conflict of interest. Author Diego Bonatto declares that he has no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

References

Alcantara MB, Nemazannikova N, Elahy M, Dass CR (2014) Pigment epithelium-derived factor upregulates collagen I and downregulates matrix metalloproteinase 2 in osteosarcoma cells, and colocalises to collagen i and heat shock protein 47 in fetal and adult bone. J Pharm Pharmacol 66:1586–1592. https://doi.org/10.1111/jphp.12289

Araki K, Mikami T, Yoshida T et al (2009) High expression of HSP47 in ulcerative colitis-associated carcinomas: proteomic approach. Br J Cancer 101:492–497. https://doi.org/10.1038/sj.bjc.6605163

Azuma M, Takeda Y, Nakajima H et al (2016) Biphasic function of TLR3 adjuvant on tumor and spleen dendritic cells promotes tumor T cell infiltration and regression in a vaccine therapy. Oncoimmunology 5:1–16. https://doi.org/10.1080/2162402X.2016.1188244

Blokzijl A, Ten Dijke P, Ibáez CF (2002) Physical and functional interaction between GATA-3 and SMAD3 allows TGF-β regulation of gata target genes. Curr Biol 12:35–45. https://doi.org/10.1016/S0960-9822(01)00623-6

Brandvold KR, Morimoto RI (2015) The chemical biology of molecular chaperones—implications for modulation of proteostasis. J Mol Biol 427:2931–2947. https://doi.org/10.1016/j.jmb.2015.05.010

Cao D, Maitra A, Saavedra J-A et al (2005) Expression of novel markers of pancreatic ductal adenocarcinoma in pancreatic non-ductal neoplasms: additional evidence of different genetic pathways. Mod Pathol 18:752–761. https://doi.org/10.1038/modpathol.3800363

Chen Y, Terajima M, Yang Y et al (2015) Lysyl hydroxylase 2 induces a collagen cross-link switch in tumor stroma. J Clin Invest 125:1147–1162. https://doi.org/10.1172/JCI74725

Chou J, Lin JH, Brenot A et al (2013) GATA3 suppresses metastasis and modulates the tumour microenvironment by regulating microRNA-29b expression. Nat Cell Biol 15:201–213. https://doi.org/10.1038/ncb2672

Clarke EP, Jain N, Brickenden A et al (1993) Parallel regulation of procollagen-I and colligin, a collagen- binding protein and a member of the serine protease inhibitor family. JCell Biol 121:193–199. https://doi.org/10.1083/jcb.121.1.193

Curran CS, Keely PJ (2013) Breast tumor and stromal cell responses to TGF-β and hypoxia in matrix deposition. Matrix Biol 32:95–105. https://doi.org/10.1016/j.matbio.2012.11.016

Daniels CE, Jett JR (2005) Does interstitial lung disease predispose to lung cancer? Curr Opin Pulm Med 11:431–437. https://doi.org/10.1097/01.mcp.0000170521.71497.ba

Dufey E, Urra H, Hetz C (2015) ER proteostasis addiction in cancer biology: Novel concepts. Semin Cancer Biol 33:40–47. https://doi.org/10.1016/j.semcancer.2015.04.003

Duran I, Martin JH, Weis MA et al (2017) A chaperone complex formed by HSP47, FKBP65, and BiP modulates telopeptide lysyl hydroxylation of type I procollagen. J Bone Miner Res 32:1309–1319. https://doi.org/10.1002/jbmr.3095

Ek ETH, Dass CR, Contreras KG, Choong PFM (2007a) Pigment epithelium-derived factor overexpression inhibits orthotopic osteosarcoma growth, angiogenesis and metastasis. Cancer Gene Ther 14:616–626. https://doi.org/10.1038/sj.cgt.7701044

Ek ETH, Dass CR, Contreras KG, Choong PFM (2007b) Inhibition of orthotopic osteosarcoma growth and metastasis by multitargeted antitumor activities of pigment epithelium-derived factor. Clin Exp Metastasis 24:93–106. https://doi.org/10.1007/s10585-007-9062-1

Ferlay J, Soerjomataram I, Dikshit R et al (2015) Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136:E359–E386. https://doi.org/10.1002/ijc.29210

Fitchett CW, Hoffman GC (1986) Obstructing malignant lesions of the Colon. Surg Clin North Am 66:807–820. https://doi.org/10.1016/S0039-6109(16)43992-7

Ford AC, Moayyedi P, Hanauer SB (2013) Ulcerative colitis. BMJ 346:1–9. https://doi.org/10.1136/bmj.f432

Gelosa P, Sevin G, Pignieri A et al (2011) Terutroban, a thromboxane/prostaglandin endoperoxide receptor antagonist, prevents hypertensive vascular hypertrophy and fibrosis. Am J Physiol Heart Circ Physiol 300:H762–H768. https://doi.org/10.1152/ajpheart.00880.2010

Hebert C, Norris K, Della Coletta R et al (1999) Cell surface colligin/Hsp47 associates with tetraspanin protein CD9 in epidermoid carcinoma cell lines. J Cell Biochem 73:248–258. https://doi.org/10.1002/(SICI)1097-4644(19990501)73:2%3C248::AID-JCB11%3E3.0.CO;2-A

Hebert C, Coletta RD, Norris K et al (2001) Non-natural CBP2 binding peptides and peptomers modulate carcinoma cell adhesion and invasion. J Cell Biochem 82:145–154. https://doi.org/10.1002/jcb.1146

Helleman J, Jansen MPHM, Ruigrok-Ritstier K et al (2008) Association of an extracellular matrix gene cluster with breast cancer prognosis and endocrine therapy response. Clin Cancer Res 14:5555–5564. https://doi.org/10.1158/1078-0432.CCR-08-0555

Hirai K, Kikuchi S, Kurita A et al (2006) Immunohistochemical Distribution of Heat Shock Protein 47 (HSP47) in Scirrhous Carcinoma of the Stomach. Anticancer Res 26:71–78

Hirayoshi K, Kudo H, Takechi H et al (1991) HSP47: a tissue-specific, transformation-sensitive, collagen-binding heat shock protein of chicken embryo fibroblasts. Mol Cell Biol 11:4036–4044. https://doi.org/10.1128/mcb.11.8.4036

Hosaka K, Yang Y, Seki T et al (2016) Pericyte–fibroblast transition promotes tumor growth and metastasis. Proc Natl Acad Sci 113:E5618–E5627. https://doi.org/10.1073/pnas.1608384113

Hosono J, Morikawa S, Ezaki T et al (2017) Pericytes promote abnormal tumor angiogenesis in a rat RG2 glioma model. Brain Tumor Pathol 34:120–129. https://doi.org/10.1007/s10014-017-0291-y

Huntoon CJ, Nye MD, Geng L et al (2010) Heat Shock Protein 90 (HSP90) inhibition depletes LATS1 and LATS2, two regulators of the mammalian Hippo tumor suppressor pathway. Cancer Res 70:8642–8650. https://doi.org/10.1158/0008-5472.CAN-10-1345

Ito S, Ogawa K, Takeuchi K et al (2017) A small-molecule compound inhibits a collagen-specific molecular chaperone and could represent a potential remedy for fibrosis. J Biol Chem 292:20076–20085. https://doi.org/10.1074/jbc.M117.815936

Jiang X, Zhou T, Wang Z et al (2016) HSP47 promotes glioblastoma stemlike cell survival by modulating tumor microenvironment extracellular matrix through TGF-β pathway. ACS Chem Neurosci 8:128–134. https://doi.org/10.1021/acschemneuro.6b00253

Kamikawaji K, Seki N, Watanabe M et al (2016) Regulation of LOXL2 and SERPINH1 by antitumor microRNA-29a in lung cancer with idiopathic pulmonary fibrosis. J Hum Genet 61:1–9. https://doi.org/10.1038/jhg.2016.99

Kimata Y, Kimata YI, Shimizu Y et al (2003) Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins. Mol Biol Cell 14:2559–2569. https://doi.org/10.1091/mbc.E02

Kobayashi E, Satow R, Ono M et al (2014) MicroRNA expression and functional profiles of osteosarcoma. Oncol 86:94–103. https://doi.org/10.1159/000357408

Koide T, Takahara Y, Asada S, Nagata K (2002) Xaa-Arg-Gly triplets in the collagen triple helix are dominant binding sites for the molecular chaperone HSP47. J Biol Chem 277:6178–6182. https://doi.org/10.1074/jbc.M106497200

Kouros-Mehr H, Slorach EM, Sternlicht MD, Werb Z (2006) GATA-3 maintains the differentiation of the Luminal cell fate in the mammary gland. Cell 127:1041–1055. https://doi.org/10.1016/j.cell.2006.09.048

Layman DL, Ross R (1973) The production and secretion of procollagen peptidase by human fibroblasts in culture. Arch Biochem Biophys 157:451–456. https://doi.org/10.1016/0003-9861(73)90661-9

Li Y, Wang F, Xu J et al (2011) Progressive miRNA expression profiles in cervical carcinogenesis and identification of HPV-related target genes for miR-29. J Pathol 224:484–495. https://doi.org/10.1002/path.2873

Lindert U, Weis MA, Rai J et al (2015) Molecular consequences of the SERPINH1/HSP47 mutation in the dachshund natural model of osteogenesis imperfecta. J Biol Chem 290:17679–17689. https://doi.org/10.1074/jbc.M115.661025

Ma Y, Hendershot LM (2004) ER chaperone functions during normal and stress conditions. J Chem Neuroanat 28:51–65. https://doi.org/10.1016/j.jchemneu.2003.08.007

Maitra A, Iacobuzio-Donahue C, Rahman A et al (2002) Immunohistochemical validation of a novel epithelial and a novel stromal marker of pancreatic ductal adenocarcinoma identified by global expression microarrays: Sea urchin fascin homolog and heat shock protein 47. Am J Clin Pathol 118:52–59. https://doi.org/10.1309/3PAM-P5WL-2LV0-R4EG

Maloney A, Clarke PA, Naaby-Hansen S et al (2007) Gene and protein expression profiling of human ovarian cancer cells treated with the Heat Shock Protein 90 inhibitor 17-Allylamino-17-Demethoxygeldanamycin. Cancer Res 67:3239–3253. https://doi.org/10.1158/0008-5472.can-06-2968

Miyata S, Mizuno T, Koyama Y et al (2013) The endoplasmic reticulum-resident chaperone Heat Shock Protein 47 protects the Golgi apparatus from the effects of O-Glycosylation inhibition. PLoS One 8:1–20. https://doi.org/10.1371/journal.pone.0069732

Mori K, Toiyama Y, Otake K et al (2017) Proteomics analysis of differential protein expression identifies heat shock protein 47 as a predictive marker for lymph node metastasis in patients with colorectal cancer. Int J Cancer 140:1425–1435. https://doi.org/10.1002/ijc.30557

Nagata K, Yamada KM (1986) Phosphorylation and transformation sensitivity of a major collagen-binding protein of fibroblasts. J Biol Chem 261:7531–7536

Nagata K, Saga S, Yamada KM (1986) A major collagen-binding protein of chick embryo fibroblasts is a novel heat shock protein. J Cell Biol 103:223–229. https://doi.org/10.1083/jcb.103.1.223

Nakai A, Satoh M, Hirayoshi K, Nagata K (1992) Involvement of the stress protein HSP47 in procollagen processing in the endoplasmic reticulum. J Cell Biol 117:903–914. https://doi.org/10.1083/jcb.117.4.903

Nakayama S, Mukae H, Sakamoto N et al (2008) Pirfenidone inhibits the expression of HSP47 in TGF-β1-stimulated human lung fibroblasts. Life Sci 82:210–217. https://doi.org/10.1016/j.lfs.2007.11.003

Nan A, Ghandehari H, Hebert C et al (2005) Water-soluble polymers for targeted drug delivery to human squamous carcinoma of head and neck. J Drug Target 13:189–197. https://doi.org/10.1080/10611860500065187

Natsume T, Koide T, Yokota S et al (1994) Interactions between collagen-binding stress protein HSP47 and collagen: Analysis of kinetic parameters by surface plasmon resonance biosensor. J Biol Chem 269:31224–31228

Nishida N, Yano H, Nishida T et al (2006) Angiogenesis in cancer. Vasc Health Risk Manag 2:213–219. https://doi.org/10.2147/vhrm.2006.2.3.213

Polydorou C, Mpekris F, Papageorgis P et al (2017) Pirfenidone normalizes the tumor microenvironment to improve chemotherapy. Oncotarget 8:24506–24517. https://doi.org/10.18632/oncotarget.15534

Poschmann G, Sitek B, Sipos B et al (2009) Identification of proteomic differences between squamous cell carcinoma of the lung and bronchial epithelium. Mol Cell Proteomics 8:1105–1116. https://doi.org/10.1074/mcp.M800422-MCP200

Sasaki K, Yoshida H (2015) Organelle autoregulation—stress responses in the ER, Golgi, mitochondria and lysosome. J Biochem 157:185–195. https://doi.org/10.1093/jb/mvv010

Sato Y (2003) Molecular diagnosis of tumor angiogenesis and anti-angiogenic cancer therapy. Int J Clin Oncol 8:200–206. https://doi.org/10.1007/s10147-003-0342-8

Sauk JJ, Norris K, Hebert C et al (1998) Hsp47 binds to the KDEL receptor and cell surface expression is modulated by cytoplasmic and endosomal pH. Connect Tissue Res 37:105–119. https://doi.org/10.3109/03008209809028904

Sauk JJ, Coletta RD, Norris K, Hebert C (2000) Binding motifs of CBP2 a potential cell surface target for carcinoma cells. J Cell Biochem 78(20000801):251–263. https://doi.org/10.1002/(SICI)1097-4644(20000801)78:2%3C251::AID-JCB8%3E3.0.CO;2-G

Schedin P, Keely PJ (2011) Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression. Cold Spring Harb Perspect Biol 3:1–22. https://doi.org/10.1101/cshperspect.a003228

Schwab M (1998) Amplification of oncogenes in human cancer cells. BioEssays 20:473–479. https://doi.org/10.1002/(SICI)1521-1878(199806)20:6%3C473::AID-BIES5%3E3.0.CO;2-N

Sepulveda D, Rojas-Rivera D, Rodríguez DA et al (2018) Interactome screening identifies the ER luminal chaperone Hsp47 as a regulator of the unfolded protein response transducer IRE1α. Mol Cell 69:238–252. https://doi.org/10.1016/j.molcel.2017.12.028

Sharbeen G, McAlpine S, Phillips P (2015) HSP47: the new heat shock protein therapeutic target. In: McAlpine S, A. E (eds) Heat shock protein inhibitors, vol 19. Springer, Cham

Siller-Matula JM, Krumphuber J, Jilma B (2010) Pharmacokinetic, pharmacodynamic and clinical profile of novel antiplatelet drugs targeting vascular diseases: REVIEW. Br J Pharmacol 159:502–517. https://doi.org/10.1111/j.1476-5381.2009.00555.x

Suekane S, Ueda K, Nishihara K et al (2017) Personalized peptide vaccination as second-line treatment for metastatic upper tract urothelial carcinoma. Cancer Sci 108:2430–2437. https://doi.org/10.1111/cas.13404

Takechi H, Hirayoshi K, Nakai A et al (1992) Molecular cloning of a mouse 47-kDa heat-shock protein (HSP47), a collagen-binding stress protein, and its expression during the differentiation of F9 teratocarcinoma cells. Eur J Biochem 206:323–329. https://doi.org/10.1111/j.1432-1033.1992.tb16930.x

Tavassoli F, Deville P (eds) (2003) Pathology and genetics of tumours of the breast and female genital organs, 5th edn. IARC Press, Lyon

Thomson CA, Ananthanarayanan VS (2000) Structure–function studies on Hsp47: pH-dependent inhibition of collagen fibril formation in vitro. Biochem J 349:877–883. https://doi.org/10.1042/bj3490877

Thomson CA, Atkinson HM, Ananthanarayanan VS (2005) Identification of small molecule chemical inhibitors of the collagen-specific chaperone Hsp47. J Med Chem 48:1680–1684. https://doi.org/10.1021/jm049148&%23x002B;

Uozaki H, Ishida T, Kakiuchi C et al (2000) Expression of heat shock proteins in osteosarcoma and its relationship to prognosis. Pathol Res Pract 196:665–673. https://doi.org/10.1016/S0344-0338(00)80118-1

Verma V, Kim Y, Lee M-C et al (2016) Activated dendritic cells delivered in tissue compatible biomatrices induce in-situ anti-tumor CTL responses leading to tumor regression. Oncotarget 7:39894–39906. https://doi.org/10.18632/oncotarget.9529

Walker LC, Overstreet MA, Yeowell HN (2005) Tissue-specific expression and regulation of the alternatively-spliced forms of lysyl hydroxylase 2 (LH2) in human kidney cells and skin fibroblasts. Matrix Biol 23:515–523. https://doi.org/10.1016/j.matbio.2004.11.002

Walter P, Ron D (2011) The unfolded protein response: from stress pathway to homeostatic regulation. Science 334:1081–1086. https://doi.org/10.1126/science.1209038

Wang SY (1994) A retinoic acid-inducible GATA-binding protein binds to the regulatory region of J6 serpin gene. J Biol Chem 269:607–613

Wang M, Kaufman RJ (2016) Protein misfolding in the endoplasmic reticulum as a conduit to human disease. Nature 529:326–335. https://doi.org/10.1038/nature17041

Wilson R, Lees JF, Bulleid NJ (1998) Protein disulfide isomerase acts as a molecular chaperone during the assembly of procollagen. J Biol Chem 273:9637–9643. https://doi.org/10.1074/jbc.273.16.9637

Wu ZB, Cai L, Qiu C et al (2014) CTL responses to HSP47 associated with the prolonged survival of patients with glioblastomas. Neurology 82:1261–1265. https://doi.org/10.1212/WNL.0000000000000290

Wu ZB, Cai L, Lin SJ et al (2016) Heat shock protein 47 promotes glioma angiogenesis. Brain Pathol 26:31–42. https://doi.org/10.1111/bpa.12256

Wu Q, Pi L, Le Trinh T et al (2017) A novel vaccine targeting Glypican-3 as a treatment for Hepatocellular carcinoma. Mol Ther 25:2299–2308. https://doi.org/10.1016/j.ymthe.2017.08.005

Xiang Q, Yang Y, Zhou Z et al (2012) Synthesis and in vitro anti-tumor activity of novel HPMA copolymer-drug conjugates with potential cell surface targeting property for carcinoma cells. Eur J Pharm Biopharm 80:379–386. https://doi.org/10.1016/j.ejpb.2011.10.020

Xu R, Mao J-H (2011) Gene transcriptional networks integrate microenvironmental signals in human breast cancer. Integr Biol 3:368–374. https://doi.org/10.1039/c0ib00087f

Xu CJ, Mikami T, Nakamura T et al (2013) Tumor budding, myofibroblast proliferation, and fibrosis in obstructing colon carcinoma: the roles of Hsp47 and basic fibroblast growth factor. Pathol Res Pract 209:69–74. https://doi.org/10.1016/j.prp.2012.10.008

Yamamoto N, Kinoshita T, Nohata N et al (2013) Tumor-suppressive microRNA-29a inhibits cancer cell migration and invasion via targeting HSP47 in cervical squamous cell carcinoma. Int J Oncol 43:1855–1863. https://doi.org/10.3892/ijo.2013.2145

Yamauchi M, Noyes C, Kuboki Y, Mechanic GL (1982) Collagen structural microheterogeneity and a possible role for glycosylated hydroxylysine in type I collagen. Proc Natl Acad Sci USA 79:7684–7688. https://doi.org/10.1073/pnas.79.24.7684

Zhang X, Yang J-J, Kim YS et al (2010) An 8-gene signature, including methylated and down-regulated glutathione peroxidase 3, of

gastric cancer. Int J Oncol 36:405–414. https://doi.org/10.3892/ijo_00000513

Zhao D, Jiang X, Yao C et al (2014) Heat shock protein 47 regulated by miR-29a to enhance glioma tumor growth and invasion. J Neurooncol 118:39–47. https://doi.org/10.1007/s11060-014-1412-7

Zhao Y, Dang Z, Xu S, Chong S (2017) Heat shock protein 47 effects on hepatic stellate cell-associated receptors in hepatic fibrosis of Schistosoma japonicum-infected mice. Biol Chem 398:1357–1366. https://doi.org/10.1515/hsz-2017-0177

Zhu J, Xiong G, Fu H et al (2015) Chaperone Hsp47 drives malignant growth and invasion by modulating an ECM gene network. Cancer Res 75:1580–1591. https://doi.org/10.1158/0008-5472.CAN-14-1027

CAPÍTULO III

ARTIGO DE DADOS 2

INHIBITION OF HSP47 EXPRESSION REDUCES MIGRATION AND INVASION CAPACITY IN GASTRIC CANCER CELL LINES

Artigo a ser submetido em periódico internacional da área

Inhibition of HSP47 expression reduces migration and invasion capacity in gastric cancer cell lines

Beatriz Dal Pont Duarte*, Diego Bonatto

Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul, Departamento de Biologia Molecular e Biotecnologia - Universidade Federal do Rio Grande do Sul, Porto Alegre, RS – Brasil.

***Corresponding author**

Beatriz Dal Pont Duarte

Centro de Biotecnologia da UFRGS – Sala 107

Departamento de Biologia Molecular e Biotecnologia

Universidade Federal do Rio Grande do Sul – UFRGS

Avenida Bento Gonçalves, 9500 – Prédio 43421 – Sala 107

Caixa Postal 15005

Porto Alegre – Rio Grande do Sul

BRAZIL

91509-900

Phone: (+55 51) 3308-7765

E-mail: beatriz.duarte@ufrgs.com.br

Contract/grant sponsor: CNPq

Abstract

Gastric cancer (GC) is the third leading cause of cancer-related deaths worldwide. Despite the improvement of diagnostic and treatment, the prognosis of the patients usually is poor, mainly due to the high resistance of tumor cells to conventional treatments. Therefore, the study of mechanisms involved in the development of GC is essential to the development of new therapeutic targets. In the last few decades, researches suggested that HSP47 has a crucial role in the development of the tumor. In the current work, we investigated the effects of HSP47 in two gastric adenocarcinomas cell lines (ACP02 and ACP03). To this end, we silenced the HSP47 in these cell lines through the CRISPR technique, and we performed invasion, migration and sphere formation assays. We also analyze if HSP47 induces cell death. Our results showed that loss of HSP47 expression in ACP02 and ACP03 cell lines, decrease cells migration, invasiveness capacity and sphere formation. In conclusion, this study indicates that HSP47 is an important protein in GC development, and might be a novel therapeutic target in GC treatment.

1. Introduction

Gastric cancer (GC) is the fifth most common cancer overall and the third leading causes of cancer-related deaths worldwide (FERLAY et al., 2015). Despite the improvement of diagnostic and treatment of GC in the last years, the prognosis of the patients usually is poor, with a 5-year survival rates less than 30%. Besides the mechanisms involved in the development of GC still unwell understood. In the last few decades, researches suggested that heat shock proteins (HSPs) may have a relationship with GC progress (GIAGINIS et al., 2009; KIMURA et al., 2016; PARTIDA-RODRÍGUEZ et al., 2010).

HSPs are molecular chaperones that facilitate the proper folding of proteins and helping to maintain cellular homeostasis. HSPs are overexpressed in several types of cancer and are correlated with tumor aggressiveness (NAHLEH; TFAYLI; SAYED, 2012). The classification of HSPs are made by the molecular weight, and are usually activated by cellular stress (BARRAL et al., 2004).

HSP47 is an endoplasmic reticulum resident chaperone essential for collagen synthesis (NATSUME et al., 1994). This protein is involved in the prevention of procollagen aggregation and plays an essential role in inducing hydroxylation of proline and lysine residues in collagen molecules (DURAN et al., 2015). The HSP47 is code by SERPINH1 gene located in chromosome 11q13.5, a known cancer hotspot. In several types of cancer, HSP47 is correlated with tumor growth, angiogenesis, migration and metastatic capacity. In GC HSP47 was shown to be upregulated, but the role of this protein in gastric tumors is still unknown (ZHANG et al., 2010).

In the current work, we investigated the effects of HSP47 in gastric adenocarcinomas cell lines, and we show that loss of HSP47 expression decrease cell migration and invasiveness capacity.

2. Materials and methods

2.1 Plasmids and bacterial transformation

Two gRNAs targeting the second exon of *HSP47* were designed to generate a break of 341 base pair (bp) in the gene (5′AAGGACGTGGAGCGCACGGA3′ and 5′CGACACGAGCCCTAGCGACG3′). The gRNAs were designed using benchling platform with a score higher than 90 to off targets. The two gRNAs were individually inserted in a pSpCas9(BB)-2A-Puro (PX459) vector (Addgene), which contains Cas9 nuclease and the puromycin resistance gene. The plasmids were amplified in DH5α-competent cells and then purified using ZR BAC DNA Miniprep (Zymo Research) according to the manufacturers' instruction.

2.2 Cell lines and ablation of the *HSP47* gene

Gastric adenocarcinoma cell lines (ACP02 and ACP03) were cultured in RPMI medium with 10% of fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 ºC in humidified 5% CO_2 incubator. To generate the transfects cells, 1.250 ng of each plasmid were mixed with RPMI medium and Lipofectamine® 2000 (according to the manufacturers' instruction – Invitrogen) and incubated with the cells overnight in culture conditions. To select the cells transfected, the cell culture was incubated for 48 hours with RPMI medium with 10% FBS, 1% penicillin/streptomycin, and 1 mg/ml of puromycin. The confirmation of the deletion in the HSP47 gene was made by PCR. After the confirmation, one cell per well was seeded in a 96 well plate to colonies formation. The colonies were analyzed by PCR and Western Blot to select a clone with homozygote mutation in *HSP47* gene.

2.3 PCR analysis

PCR analysis was made in order to detect the colonies with the deletion of *HSP47*. Total DNA was isolated with PureLink™ DNA kit (Invitrogen) according to manufacturer’s

instruction. DNA was quantified using a NanoDrop spectrophotometer. The expression of *HSP47* was performed on a StepOne™ PCR System (Thermo Fisher), using pre-designed primers (5'GCACTGCGGAGAAGTTGA3' and 5'ACTTCTTGTACCGCAACTGAT3').

2.4 Western blot analysis

Analyses of the protein expression were performed using antibodies anti-HSP47 (Sigma-Aldrich 1:250). Whole-cell lysate was obtained with RIPA buffer resolved in 10% SDS-PAGE (20 µg of protein loaded per well) and transferred to nitrocellulose membranes. After the incubation with primary antibody, an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) (Cell Signaling 1:1500) was used and visualized with ECL Western blotting detection reagents (Clarity™ Bio-Rad).

2.5 Annexin-V/IP double-staining assay

To evaluate cell death in HSP47 silenced cells, Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) was used. The cells were trypsinized and washed twice with PBS before staining. The cell pellet was resuspended in a buffer containing annexin V-FITC conjugate and propidium iodide (PI) and incubated for 10 minutes in the dark. The analysis was performed using Guava EasyCyte flow cytometer and guavaSoft 2.7 software (Millipore/Merck). The cells stained with annexin were considered in early apoptosis, with both annexin and PI in late apoptosis, just with PI in necrosis and without being stained as viable.

2.6 Sphere forming cell assay

The sphere forming assays were performed by coating plates with a thin layer of ECM gel (Sigma-Aldrich E6909) and incubated for 30 min at 37°C. The cells were trypsinized and resuspended in a concentration of $1x10^3$ cells in RPMI medium with 10% FBS and 4% of ECM gel, and seeded in a 24 well plate. The cultures were maintained for ten days with

medium changed every three days. The number of spheres were determined using an inverted microscope and are counted in four random views using ImageJ software (NIH, Bethesda, MA, USA).

2.7 Migration and invasion assay

Migration and invasion assays were carried out in a modified Boyden chamber with transwell inserts (8-µm pores) for 24-well plates (Greiner Bio-one). The cells were seeded at a density of $1x10^5$ cells in a 250 µl of RPMI without FBS in the upper compartment of transwell. In the lower compartment was added 800 µl of RPMI with 10% of FBS. To assess invasion, filters were coated with 80 µl of ECM gel (0,3 mg/ml) (Sigma-Aldrich E6909). After 24 h in culture, for analysis of migration, and 48 h, for analysis of invasion, cells were fixed with 4% paraformaldehyde and post-fixed with methanol and were colored with Giemsa. The cells on the upper compartment of transwell were removed with a cotton swab, and the cells in the lower compartment were photographed and cells in six random views were quantified.

2.8 Population doubling time (PDT)

To plot the growth curve, $2x10^3$ cells were seeded in 24-well culture plate. For 10 consecutive days, cell number was daily determined. The growth curve was plotted using the formula:

$$PDT = \log(\text{number of harvested cells} / \text{number of seeded cells}) / \log(2)$$

2.9 Statistical analysis

All data are shown as mean ± statistic error of the mean (SEM). Statistical comparison was made by analysis of variance followed by Tukey's multiple comparison test. SPSS software version 18 is used in these analyses. P values lower than 0.05 were considered as statistically significant.

3 Results

3.1 Ablation of the HSP47 gene and analyses of proliferation

To examine the role of HSP47 in the gastric adenocarcinoma cell lines, we use the CRISPR technique to lead to *HSP47* gene deletion (Figure 1A). After the transfection of the cell lines with the plasmids, we performed a colony formation assay by seeding one cell per plate. The colonies were analyzed through PCR, and in the two cell lines it was possible to isolate colonies with complete silencing of HSP47 (Figure 1B). The Western blot analysis in these selected colonies proved the completed lack of HSP47 expression (Figure 1C).

To examine if deletion of HSP47 affects the proliferation of the cells, cell population growth was analyzed by PDT. The results showed no differences between the cell lines, indicating that HSP47 has no effect in cellular proliferation in gastric adenocarcinoma cell lines (Figure 2B).

3.2 HSP47 silencing reduced migration and invasion capacity in gastric cancer cells

To further investigate the potential effects of HSP47 silencing in gastric cancer development, we performed analysis of migration and invasion by a modified Boyden chamber, with and without ECM. The migration capacity was significantly decrease in the cell lines silenced for HSP47 (Figure 2A) in comparison with the non-silenced cell lines ($p< 0.0001$ in ACP02 vs. ACP02 HSP47$^{-/-}$, and $p< 0.001$ in ACP03 vs. ACP03 HSP47$^{-/-}$). This effect is also observed in the invasion capacity of these cells (Figure 2C). Where the cell lines ACP02 HSP47$^{-/-}$ and ACP03 HSP47$^{-/-}$ demonstrated a loss of invasion capacity when compared with ACP02 and ACP03 ($p< 0.001$ in ACP02 vs. ACP02 HSP47$^{-/-}$).

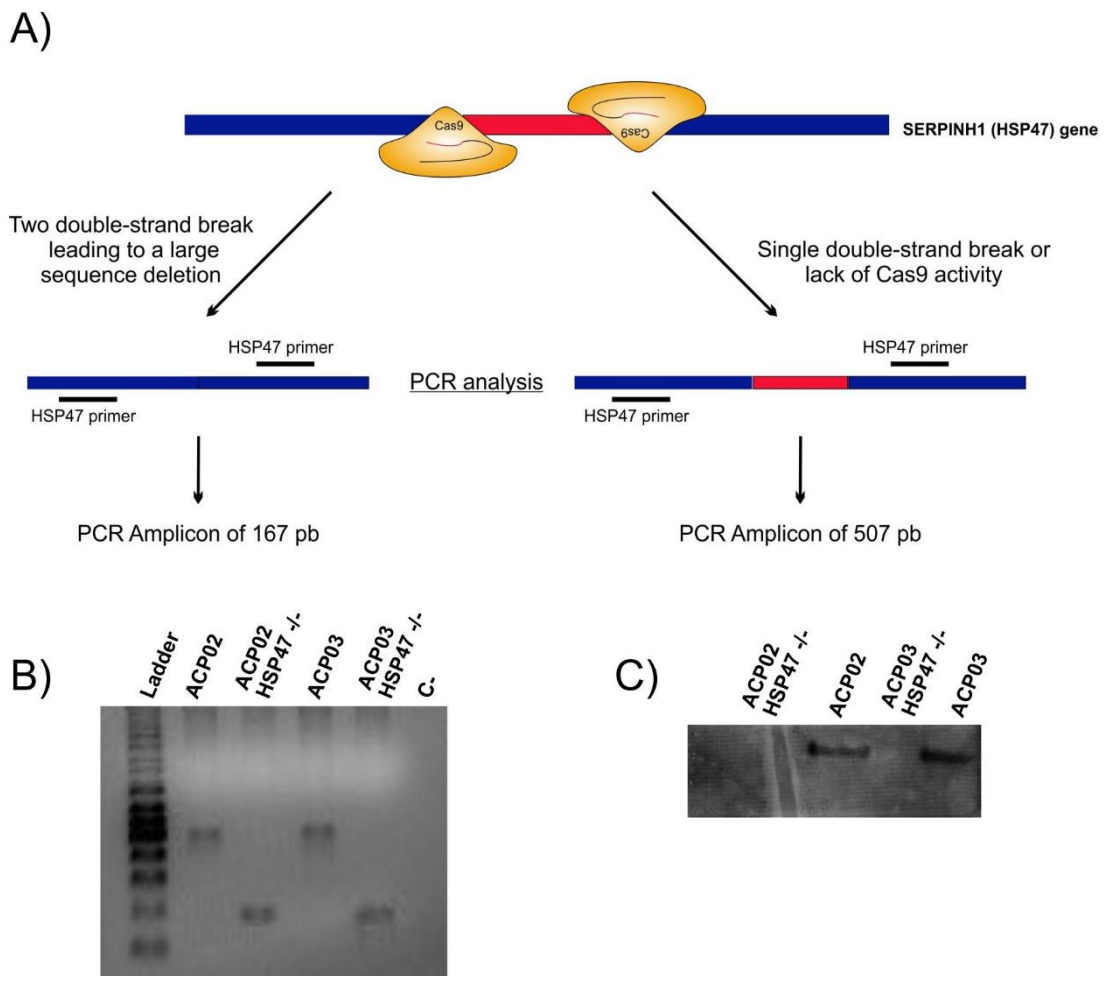

Fig. 1 Experimental design used to select colonies silenced for HSP47 (A). PCR analysis demonstrating a colony silenced for HSP47 in ACP02 and ACP03 cell line (B). Western blot analysis indicating the lack of protein expression in selected silenced colonies (C). Ladder with 100 bp.

3.3 HSP47 do not show effect in cell death

To investigate the action of HSP47 in cell death, fluorescent probe Annexin V-FITC and PI were used, and then apoptotic, early apoptotic and necrotic cells were determined by flow cytometry analysis. As shown on Figure 3, we do not observe differences between the cell lines ACP02 vs. ACP02 HSP47$^{-/-}$, and ACP03 vs. ACP03 HSP47$^{-/-}$, in apoptotic (Figure 3A), early apoptotic (Figure 3C) and necrotic cells (Figure 3B).

3.4 HSP47 silencing has a negative effect on sphere formation

Sphere-forming assays have been widely used to identify stem cells, based on the capacity of this assay to evaluate self-renewal and differentiation at the single cell level. A MEC sphere formation was conducted in this study to illustrate the effect of HSP47 on *in vitro* sphere formation of gastric adenocarcinoma cell lines. Similar to suppression of migration and invasion, the HSP47 silencing induced a decrease in sphere formation in the cell lines (Figure 4A). In the cell line ACP02 HSP47$^{-/-}$ the number of spheres was decreased significantly ($p< 0.001$ in ACP02 vs. ACP02 HSP47$^{-/-}$). However, the reduction in ACP03 HSP47$^{-/-}$ showed no statistical significance.

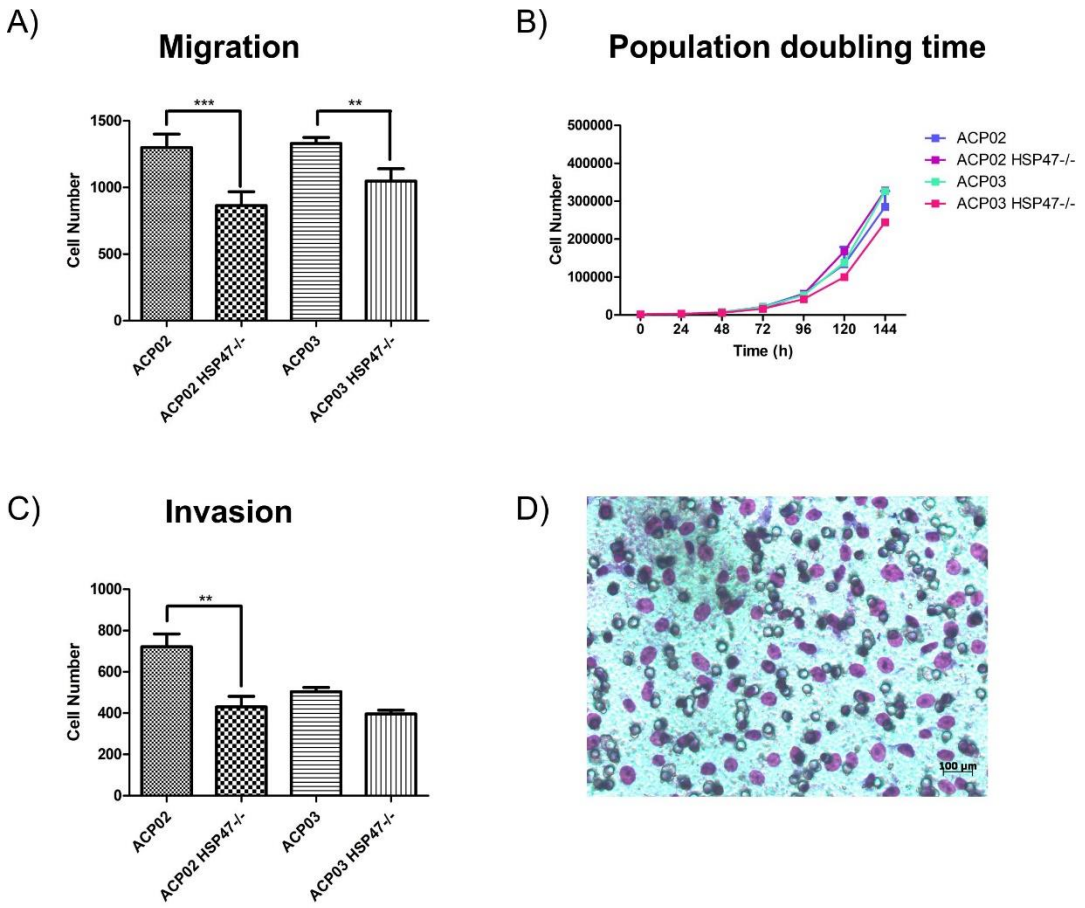

Fig. 2 Graphs show mean ± SEM of migration (A) and invasive cells (C). Comparative analyses are made by cell line vs. silenced cell line. Exponential growth curve of the cell lines ACP02, ACP03, ACP02 HSP47 $^{-/-}$ and ACP03 HSP47$^{-/-}$ (B). Representative result of invasion assay in ACP02 cell line (D). ** $p< 0.001$, *** $p< 0.0001$.

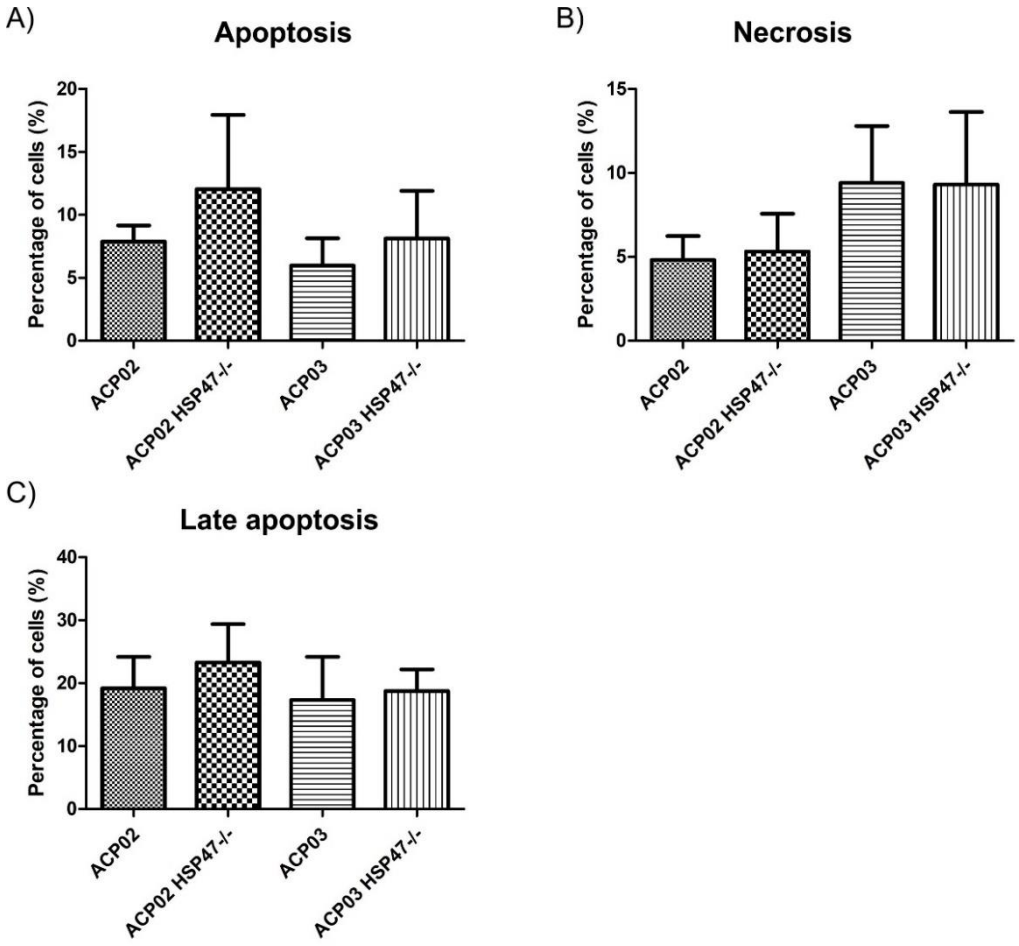

Fig. 3 Graphs show mean ± SEM of apoptosis (A), necrosis (B) and late apoptosis (C). No statistical significance was detected.

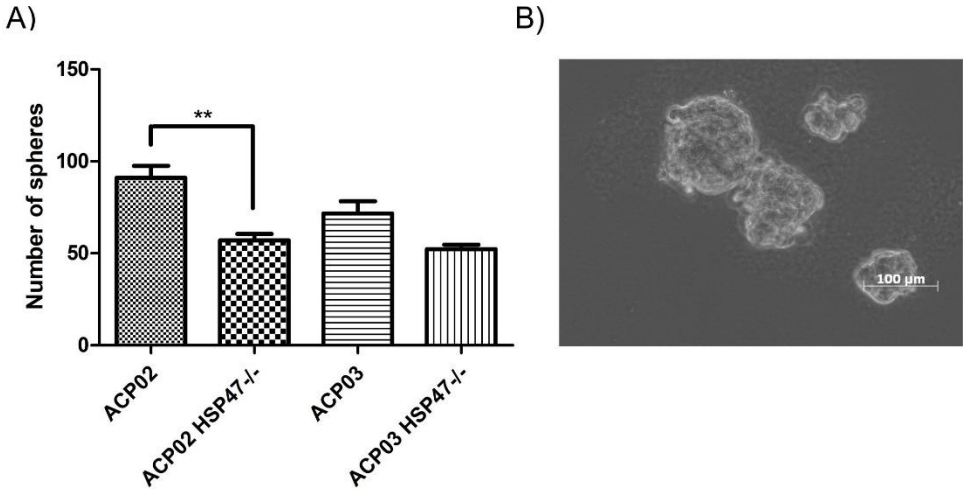

Fig. 4 Graphs show mean ± SEM of sphere formation assay (A). Representative result of ACP02 spheres (D). ** p< 0.001.

4 Discussion

The HSP47 is correlated with several types of cancer, such as pancreatic, lung, breast and osteosarcoma (CAO et al., 2005; HEBERT et al., 2001; HIRAI et al., 2006; JIANG et al., 2016; MAITRA et al., 2002; UOZAKI et al., 2000; XU et al., 2013). However, HSP47 has opposing effects in different types of cancer. In osteosarcoma and laryngeal squamous cell carcinoma, HSP47 present an indirect protective effect (ALCANTARA et al., 2014; SONG et al., 2017), in breast cancer and glioblastoma HSP47 is associated with aggressiveness of tumor (KAMIKAWAJI et al., 2016; ZHU et al., 2015a). In GC was demonstrated that the HSP47 is overexpressed, but the role of this protein in gastric tumors is still unknown (ZHANG et al., 2010). In the present study, we used clustered regularly interspaced short palindromic repeats (CRISPR) technique to conduct deletion of *HSP47* in two gastric adenocarcinomas cell lines, analyzing the effect and therapeutic potential of this protein in GC.

The HSP47 silenced decreased the migration and invasion capacity in the two cell lines. These results are also observed in head and neck epidermoid carcinoma, glioblastoma e breast cancer (YAMAMOTO et al., 2013; ZHAO et al., 2014b; ZHU et al., 2015b). To analyze whether the effect on cell migration and invasion may be correlated with an increase in cell proliferation, we analyze the proliferation capacity of the cells by PDT assay. Our results showed that the suppression of HSP47 expression in the gastric cancer cell lines does not affect the proliferation of ACP02 and ACP03 cells. In a recent work Qi et al. (2018) described an association between the expression levels of *HSP47* and levels of epithelial-to-mesenchymal transition markers. This result may indicate that the effect of HSP47 on migration and invasion capacity can be associated with epithelial-to-mesenchymal transition phenotype.

The HSP47 protect the cells against reticulum endoplasmatic and Golgi stress, leading the cell to apoptosis pathways when cellular homeostasis cannot be recovered (SASAKI; YOSHIDA, 2015; SEPULVEDA et al., 2018). Nevertheless, in our results, the apoptosis and necrotic levels were not altered in HSP47 silenced cells. In analyses with cells knockdown for HSP47, it was observed that cell death occurs when these are treated with a reticulum endoplasmatic or Golgi stress inducer (MIYATA et al., 2013). Therefore, a combination of stress inducer with an HSP47 inhibitor may be a therapeutic approach in the treatment of GC.

Sphere-forming assays is indicative of self-renewal capacity and have been used to determine stem cells population. In our results, the sphere formation was reduced in HSP47 silenced cells. This reduction can be suggested that HSP47 has a direct action in undifferentiated cells. In glioblastoma the overexpression of *HSP47* leads to an increase in sphere formation, consequently increasing the tumor aggressiveness (JIANG et al., 2016).

In conclusion, the findings of the present study indicate that HSP47 is an important protein in GC development, and has a direct effect on migration, invasion and sphere formation capacity in GC cell lines. Therefore, HSP47 might be a novel therapeutic target in GC treatment. Despite, further studies witch therapeutic approaches are required to elucidate the effect of HSP47 in GC.

Acknowledgments

We would like to thank Dr. Rommel Mario Rodríguez Burbano (Federal University of Pará) for supplying the cells used in this work.

Conflicts of interest

There are no conflicts of interest to declare.

References

Alcantara, M.B. et al., 2014. Pigment epithelium-derived factor upregulates collagen I and downregulates matrix metalloproteinase 2 in osteosarcoma cells, and colocalises to collagen i and heat shock protein 47 in fetal and adult bone. *Journal of Pharmacy and Pharmacology*, 66(11), pp.1586–1592.

Barral, J.M. et al., 2004. Roles of molecular chaperones in protein misfolding diseases. *Seminars in Cell & Developmental Biology*, 15(1), pp.17–29.

Cao, D. et al., 2005. Expression of novel markers of pancreatic ductal adenocarcinoma in pancreatic nonductal neoplasms: additional evidence of different genetic pathways. *Modern Pathology*, 18(6), pp.752–761.

Duran, I. et al., 2015. HSP47 and FKBP65 cooperate in the synthesis of type I procollagen. *Human Molecular Genetics*, 24(7), pp.1918–1928.

Ferlay, J. et al., 2015. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer*, 136(5), pp.E359–E386.

Giaginis, C. et al., 2009. Heat Shock Protein-27, -60 and -90 expression in gastric cancer: association with clinicopathological variables and patient survival. *BMC gastroenterology*, 9(14), pp.1–10.

Hebert, C. et al., 2001. Non-natural CBP2 binding peptides and peptomers modulate carcinoma cell adhesion and invasion. *Journal of Cellular Biochemistry*, 82(1), pp.145–154.

Hirai, K. et al., 2006. Immunohistochemical Distribution of Heat Shock Protein 47 (HSP47) in Scirrhous Carcinoma of the Stomach. *Anticancer Research*, 26(1 A), pp.71–78.

Jiang, X. et al., 2016. HSP47 promotes glioblastoma stemlike cell survival by modulating tumor microenvironment extracellular matrix through TGF-β pathway. *ACS Chemical Neuroscience*, 8(1), pp.128–134.

Kamikawaji, K. et al., 2016. Regulation of LOXL2 and SERPINH1 by antitumor microRNA-29a in lung cancer with idiopathic pulmonary fibrosis. *Journal of Human Genetics*, 61(12), pp.1–9.

Kimura, A. et al., 2016. Nuclear heat shock protein 110 expression is associated with poor prognosis and chemotherapy resistance in gastric cancer. *Oncotarget*, 7(14).

Maitra, A. et al., 2002. Immunohistochemical validation of a novel epithelial and a novel stromal marker of pancreatic ductal adenocarcinoma identified by global expression microarrays: Sea urchin fascin homolog and heat shock protein 47. *American Journal of Clinical Pathology*, 118(1), pp.52–59.

Miyata, S. et al., 2013. The endoplasmic reticulum-resident chaperone Heat Shock Protein 47 protects the Golgi apparatus from the effects of O-Glycosylation inhibition. *PLoS ONE*, 8(7), pp.1–20.

Nahleh, Z., Tfayli, A. & Sayed, A. El, 2012. Heat shock proteins in cancer : targeting the ‘chaperones .’ *Future Med Chem*, 4, pp.927–935.

Natsume, T. et al., 1994. Interactions between collagen-binding stress protein HSP47 and

collagen: Analysis of kinetic parameters by surface plasmon resonance biosensor. *The Journal of biological chemistry*, 269(49), pp.31224–31228.

Partida-Rodríguez, O. et al., 2010. Polymorphisms in TNF and HSP-70 show a significant association with gastric cancer and duodenal ulcer. *International Journal of Cancer*, 126(8), pp.1861–1868.

Qi, Y. et al., 2018. SERPINH1 overexpression in clear cell renal cell carcinoma: association with poor clinical outcome and its potential as a novel prognostic marker. *Journal of Cellular and Molecular Medicine*, 22(2), pp.1224–1235.

Sasaki, K. & Yoshida, H., 2015. Organelle autoregulation - Stress responses in the ER, Golgi, mitochondria and lysosome. *Journal of Biochemistry*, 157(4), pp.185–195.

Sepulveda, D. et al., 2018. Interactome screening identifies the ER luminal chaperone Hsp47 as a regulator of the unfolded protein response transducer IRE1α. *Molecular Cell*, 69(2), pp.238–252.

Song, X. et al., 2017. HSP47 is associated with the prognosis of laryngeal squamous cell carcinoma by inhibiting cell viability and invasion and promoting apoptosis. *Oncology Reports*, 38(4), pp.2444–2452.

Uozaki, H. et al., 2000. Expression of heat shock proteins in osteosarcoma and its relationship to prognosis. *Pathology Research and Practice*, 196(10), pp.665–673.

Xu, C.J. et al., 2013. Tumor budding, myofibroblast proliferation, and fibrosis in obstructing colon carcinoma: The roles of Hsp47 and basic fibroblast growth factor. *Pathology Research and Practice*, 209(2), pp.69–74.

Yamamoto, N. et al., 2013. Tumor-suppressive microRNA-29a inhibits cancer cell migration and invasion via targeting HSP47 in cervical squamous cell carcinoma. *International journal of oncology*, 43(6), pp.1855–1863.

ZHANG, X. et al., 2010. An 8-gene signature, including methylated and down-regulated glutathione peroxidase 3, of gastric cancer. *International Journal of Oncology*, 36, pp.405–414.

Zhao, D. et al., 2014. Heat shock protein 47 regulated by miR-29a to enhance glioma tumor growth and invasion. *Journal of Neuro-Oncology*, 118(1), pp.39–47.

Zhu, J., Xiong, G., Fu, H., Evers, B.M., et al., 2015. Chaperone Hsp47 Drives Malignant Growth and Invasion by Modulating an ECM Gene Network. *Cancer research*, 75(8), pp.1580–91.

Zhu, J., Xiong, G., Fu, H., Evers, B.M., et al., 2015. Chaperone Hsp47 drives malignant growth and invasion by modulating an ECM gene network. *Cancer Research*, 75(8), pp.1580–1591.

CAPÍTULO IV

METODOLOGIA E RESULTADOS ADICIONAIS

METODOLOGIA E RESULTADOS ADICIONAIS

1. Silenciamento de HSP47 na linhagem MN01

A linhagem MN01, utilizada no primeiro capítulo dessa tese, também foi silenciada para HSP47. Porém no decorrer dos experimentos observamos uma mudança morfológica nessas células. Essas mudanças nos levaram a pensar que a linhagem poderia ter sofrido mutações em cultura, levando as células a apresentar um fenótipo *tumor-like*. Realizando alguns testes para marcadores tumorais conhecidos como *NANOG*, *OCT-4*, *SOX2*, *LGR5* e *CD133*, verificamos a expressão desses marcadores nessa linhagem celular. Devido a este resultado, as análises feitas após o silenciamento de HSP47 foram retiradas do artigo correspondente ao Capítulo III, e os resultados obtidos seguem descritos abaixo.

1.1 Metodologia

A expressão dos genes *NANOG, OCT-4, SOX2, LGR5* e *CD133* foi analisada conforme metodologia descrita no Capítulo I. Os primers utilizados para *NANOG* foram 5'TTCCTTCCTCCATGGATCTG3' e 5'TGGGGTAGGTAGGTGCTGAG3', para *OCT4* foram 5'TGCAGAAAGAACTCGAGCAA3' e 5'CTCCAGGTTGCCTCTCACTC3', para *SOX2* foram 5'AGAACCCCAAGATGCACAAC3' e 5'GTTCATGTGCGCGTAACTGT3', para *LGR5* foram 5'ATGTTGCTCAGGGTGGACTG3' e 5'TTGGGGGCACATAGCTGATG3' e para *CD133* foram 5'AGGCGTTGGAGAACATGAAC3' e 5'TGGCGTTGTACTCTGTCAGG3'.

O silenciamento de HSP47 e as análises de migração, invasão, morte celular e formação de esferas, foram realizados segundo metodologia apresentada no Capítulo III. As análises estatísticas foram realizadas através do teste t de *student* com a utilização do software SPSS versão 18. Os resultados estão representados como média ± erro padrão. Valores $p < 0,05$ foram considerados significativos.

1.2 Resultados

Nas análises para os marcadores tumorais *NANOG, OCT-4, SOX2, LGR5* e *CD133*, a linhagem MN01 apresentou expressão positiva para todos os genes analisados (Figura 1). Demonstrando um possível processo de transformação dessas células *in vitro*.

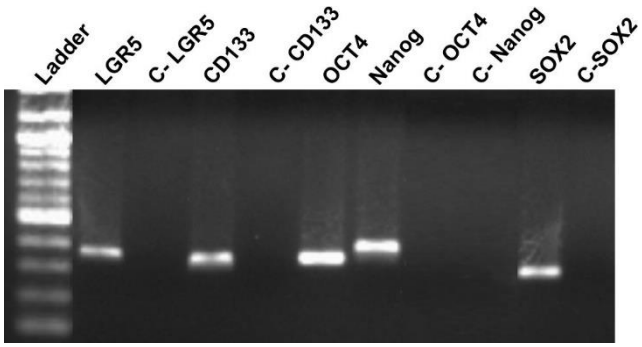

Fig. 1 Análises de marcadores de células tumorais na linhagem MN01. O ladder utilizado apresenta padrão de 100 pb.

O silenciamento do gene *HSP47* através da técnica de CRISPR foi comprovado por análises de Western blot e PCR como demonstrado na Figura 2.

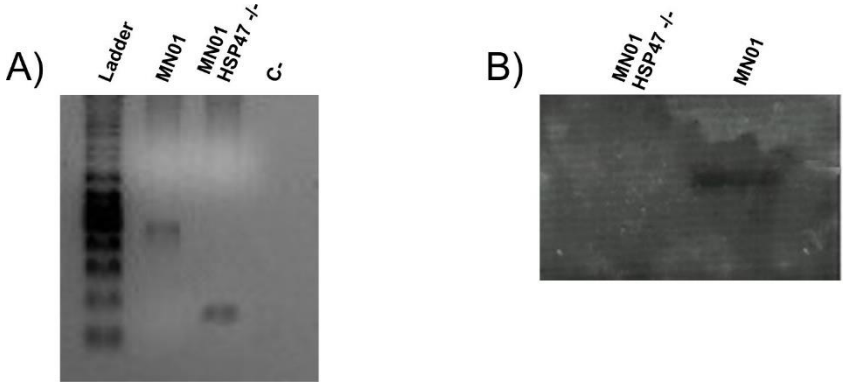

Fig. 2 PCR para análise do perfil gênico de *HSP47* (A) e Western blot para a análise da expressão proteica de HSP47 (B) na linhagem MN01, em células silenciadas e não-silenciadas para HSP47. O ladder utilizado apresenta padrão de 100 pb.

Após o silenciamento, as análises de migração, invasão, morte, PDT e formação de esferas foram realizadas.

O ensaio de migração, assim como o ensaio de invasão demonstrou que o silenciamento de HSP47 diminuiu a capacidade migratória e invasiva dessas células (Figura 3A e 3C). Esses resultados não podem ser atribuídos devido a uma diferença na taxa de crescimento da população celular. Já que estas taxas não sofreram alteração devido ao silenciamento de

HSP47, como demonstrado nos resultados de PDT (Figura 3B). Esses dados corroboram com o efeito visto nas linhagens ACP02 e ACP03 (Capítulo III).

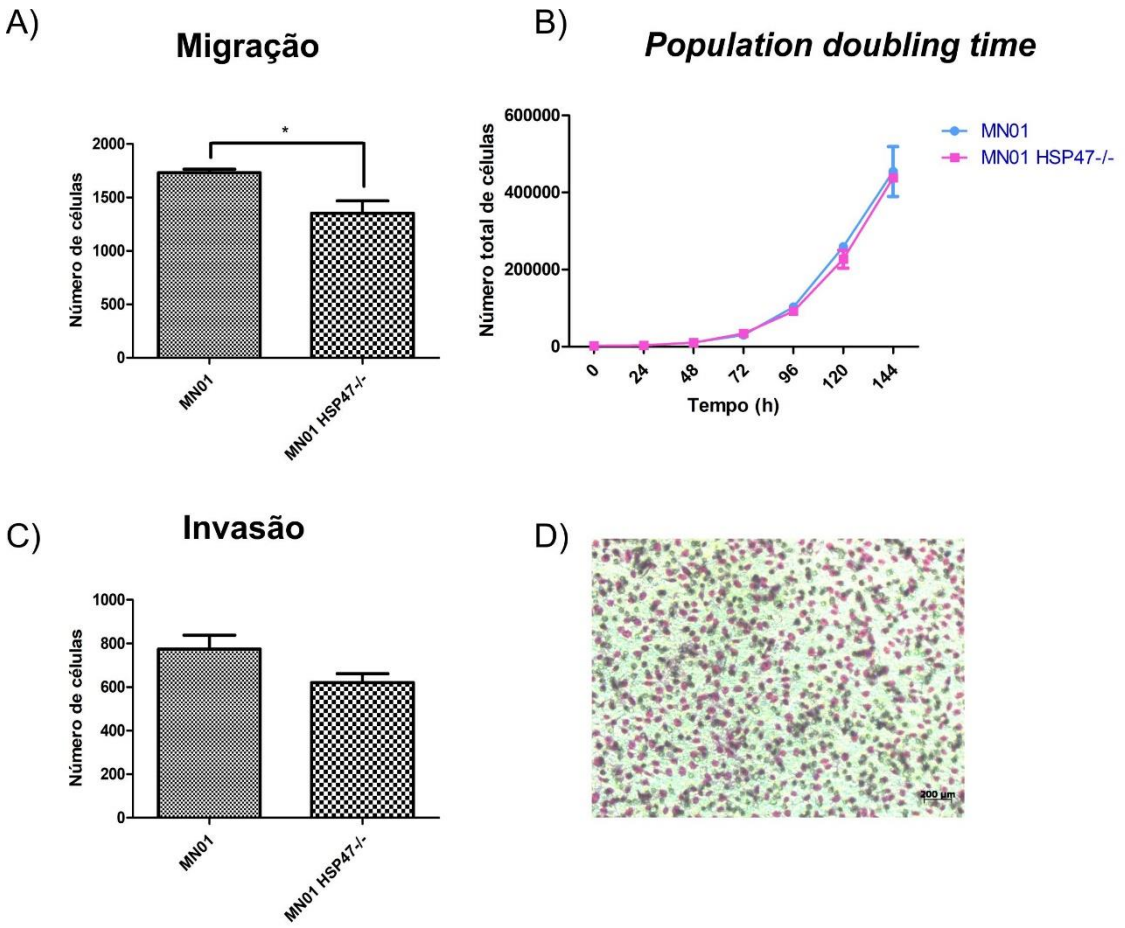

Fig. 3 Análise de migração (A), capacidade invasiva (C) e de *population doubling time* (C) em células silenciadas e não silenciadas para HSP47 na linhagem MN01. Exemplo de uma população com capacidade invasiva em células silenciadas para HSP47 na linhagem MN01 (D). *p< 0,05.

Os dados de morte celular não apresentaram diferenças entre as células silenciadas e a linhagem não silenciada (Figura 4). Interessantemente, nas análises de formação de esferas (Figura 5) não foi observado nenhum efeito do silenciamento de HSP47 nas células. Esses dados são contrários aos observados nas linhagens de tumor gástrico ACP02 e ACP03, visto no Capítulo III, onde houve uma diminuição na capacidade de formar esferas em células silenciadas para HSP47.

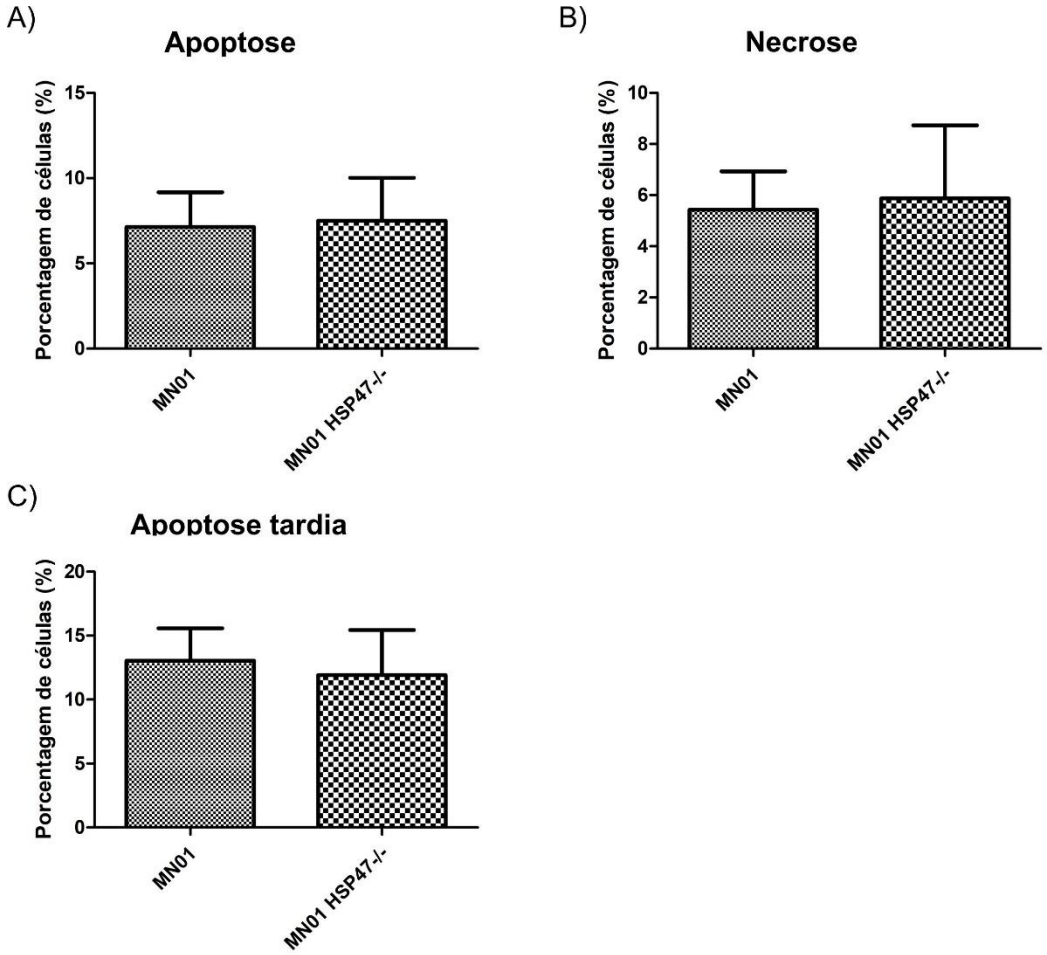

Fig. 4 Ensaio de morte celular por marcação de anexina/iodeto de propídeo em células silenciadas e não silenciadas para HSP47 na linhagem MN01.

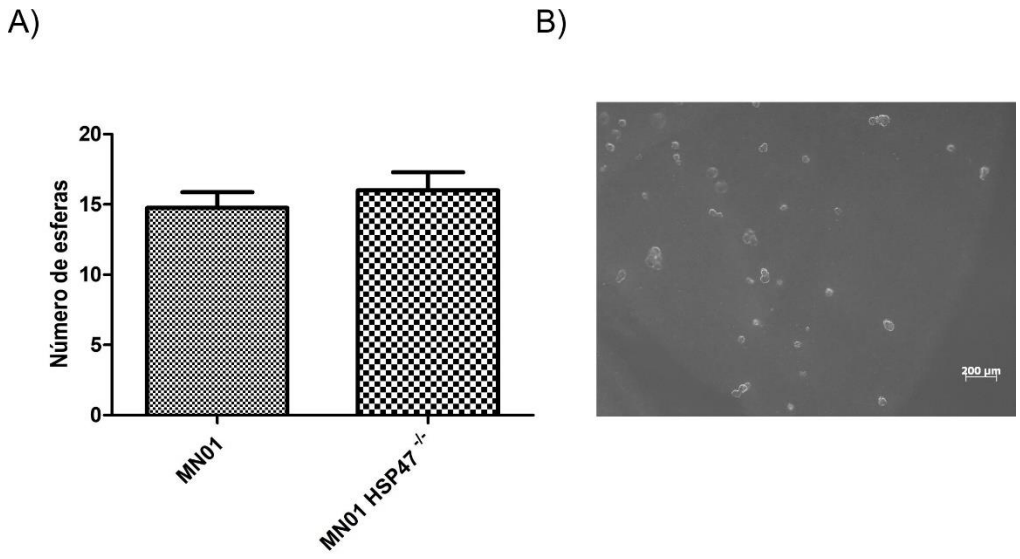

Fig. 5 Ensaio de formação de esferas em modelo tridimensional com uso de MEC em células silenciadas e não silenciadas para HSP47 na linhagem MN01. Número de esferas contabilizadas por poço (A), e foto representativa da morfologia das esferas na linhagem MN01 (B).

2. Silenciamento de GDF15 através da técnica de CRISPR

A proteína GDF15, como descrito no Capítulo I, é diferencialmente secretada pelas linhagens tumorais. Sendo assim, tentamos realizar o silenciamento de GDF15 através da técnica de CRISPR nas linhagens MN01, ACP02 e ACP03 com o intuito de investigar o papel dessa proteína no câncer gástrico. Infelizmente, apesar de conseguirmos detectar a presença da deleção causada no gene GDF15 através da ação da Cas9 nas diferentes linhagens (Figura 7), não foi possível isolar colônias apresentando silenciamento completo de GDF15.

2.1 Metodologia

As linhagens MN01, ACP02 e ACP03 foram incubadas com os plasmídeos contendo os RNAs guias (gRNAs) alvos para *GDF15* como descrito no Capítulo III. Os gRNAs selecionados apresentavam *off-target* com valor mínimo de 83%, sendo que as sequências utilizadas de gRNAs foram 5'GGGACGTGACACGACCGCTG3' e 5'ATGGTCACTTGCACCTCCCG3'. A confirmação da inserção do gRNA no plasmídeo (Figura 6C) foi realizada por PCR com primers específicos para cada sequência (gRNA1 5'CCAGCCGCACTGCTGGTTTTAGAG3, gRNA2 5'CACCGCCGGATCCCAGCCGCACTG3' e primer reverso 5'TGATGTCTGCCAAGTGGGC3'). O controle negativo foi realizado utilizando apenas o plasmídeo original, sem a inserção do gRNA.

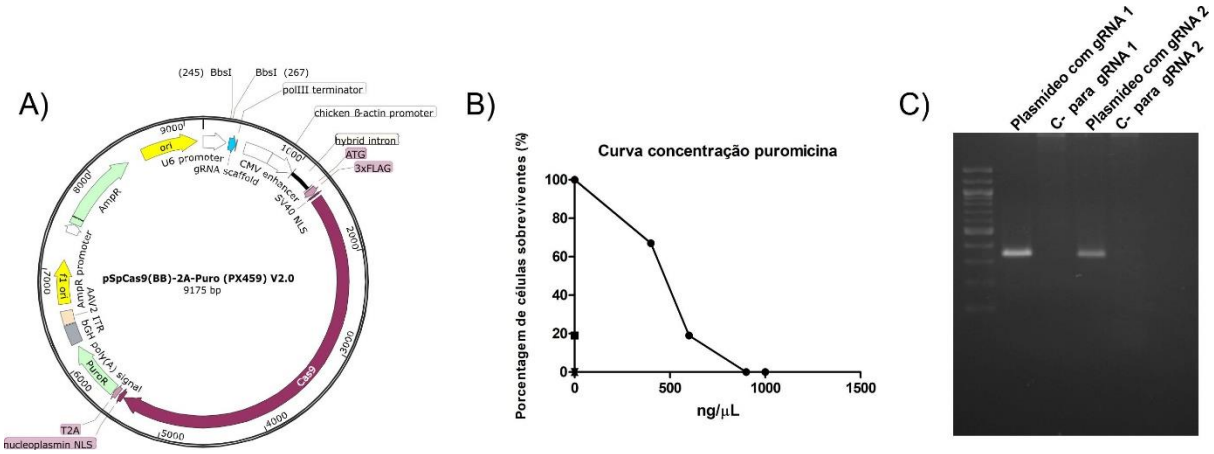

Fig. 6 Imagem representativa do plasmídeo utilizado na execução da técnica de CRISPR (A). Curva de puromicina com as diferentes concentrações testadas para a seleção das células transformadas (B). PCR demonstrando a correta inserção dos dois gRNAs utilizados para o silenciamento de GDF15 no plasmídeo (C).

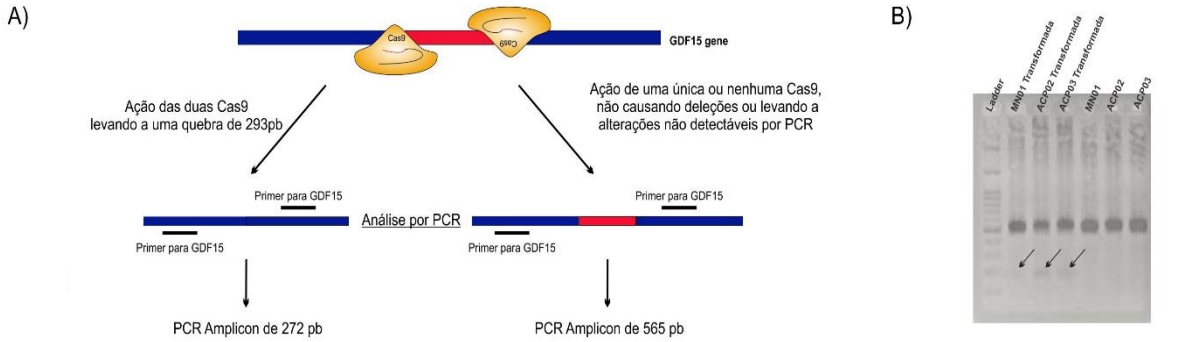

Fig. 7 Desenho experimental para seleção de células silenciadas para GDF15 (A). Análise por PCR das células recém transformadas (B). A seta indica a segunda banda gerada devido a ação da Cas9 no gene.

2.2 Resultados

Como demonstrado na Figura 6C os gRNAs alvos para *GDF15* foram corretamente inseridos no plasmídeo e as três linhagens celulares apresentaram células com deleção no gene *GDF15* (Figura 7B). Entretanto, não foi possível selecionar colônias com silenciamento total para este gene em nenhuma das três linhagens selecionadas, sendo que esta análise foi realizada em aproximadamente 200 colônias para cada linhagem.

5. DISCUSSÃO GERAL

O câncer gástrico (CG) é considerado um dos tipos de câncer com maiores índices de mortalidade na população mundial (WHO, 2017). Apesar do aprimoramento das técnicas de diagnóstico e do número crescente de novas drogas quimioterápicas, a taxa de sobrevida de 5 anos ainda continua abaixo dos 30% na maioria dos países (INCA, 2017; WHO, 2017). Esses dados têm como principais causas o diagnóstico tardio da doença e a resistência aos tratamentos convencionais. Sendo assim, existe uma grande necessidade de desenvolvimento de novos tratamentos e formas de diagnóstico.

As formas de diagnósticos existentes para o CG apresentam alto custo e são consideradas invasivas, sendo apenas utilizadas nos casos em que o paciente já apresenta algum sintoma como dores epigástrica , anemia, perda de peso e náusea. O problema neste quadro é que os sintomas aqui descritos costumam estar presentes numa fase mais adiantada da doença, acarretando em diagnóstico tardio e em prognóstico ruim para o paciente. Devido a este fator, a busca por novos biomarcadores é essencial na luta contra o CG. O estudo do secretoma vem sendo uma ferramenta importante na busca de novos biomarcadores e alvos terapêuticos.

No nosso trabalho, a análise do secretoma de duas linhagens tumorais gástricas em comparação com o secretoma de uma linhagem gástrica normal revelou 135 proteínas secretadas apenas pelas linhagens tumorais, sendo 86 secretadas por ambas as linhagens. Estas proteínas estão envolvidas em processos essenciais para o crescimento tumoral como ciclo e controle da homeostase celular. Dentre as 86 proteínas mencionadas, também foram identificado dois biomarcadores já descritos de CG, a alfa-fetoproteína e a anexina A4 (LIN; HUANG; JUAN, 2012; UCAR et al., 2008), e várias outras proteínas relacionadas com processos tumorais, como HSP47 e GDF15 (BLANCO-CALVO et al., 2014; SONG et al., 2017; WANG et al., 2018; ZHANG et al., 2009; ZHAO et al., 2014b). As proteínas HSP47 e GDF15 tiveram a sua secreção também analisada através da técnica de Western blot, confirmando que estas proteínas podem ser consideradas como candidatas a biomarcadores para CG.

Os altos níveis de expressão de GDF15 estão relacionados com vários tipos de tumores, sendo que altos níveis séricos dessa proteína já foram relatados no soro de pacientes com carcinoma de células escamosas bucais, colorretal, próstata e de mama metastático

(BROWN et al., 2003; SCHIEGNITZ et al., 2012; WANG et al., 2018; YONG et al., 2014). Além disso, os níveis séricos detectáveis de GDF15 costumam estar associados a um alto grau de malignidade (BAUSKIN et al., 2006; BROWN et al., 2003; ZHANG et al., 2009), sendo esse fato também observado em CG (BLANCO-CALVO et al., 2014). Em nossas análises, a proteína GDF15 foi detectável apenas no meio condicionado das células tumorais. Porém quando analisamos os níveis de expressão gênica de *GDF15*, as linhagens ACP02 e ACP03 apresentaram os menores níveis de expressão quando comparados aos da linhagem MN01. Esse fato pode estar relacionado com o grau de desenvolvimento do tumor que originou estas duas linhagens tumorais. Em trabalhos com células originadas de glioblastoma e câncer de próstata, foi demonstrado que os níveis de *GDF15* eram elevados apenas em tumores reincidentes e metastáticos (STRELAU et al., 2008; YONG et al., 2014). As linhagens ACP02 e ACP03, por sua vez, são originadas de tumores primários de pacientes que não apresentavam focos metastáticos (LEAL et al., 2009).

Para analisar a função de GDF15 nas células tumorais gástricas, tentamos realizar o silenciamento dessa proteína nas linhagens tumorais através da técnica de CRISPR. Apesar da ação da Cas9 no gene *GDF15* poder ser comprovada nas análises de PCR (Figura 6, Capítulo IV), não conseguimos isolar colônias com silenciamento completo para GDF15. Este fato pode indicar que o silenciamento desta proteína leva a um fenótipo letal em células derivadas do epitélio gástrico. Infelizmente não encontramos nenhum dado na literatura atual que pudesse explicar a incapacidade do silenciamento total de GDF15 nas nossas linhagens.

A proteína HSP47, que também foi detectada apenas no meio condicionado das células tumorais (Figura 4, Capítulo 1), tem o seu envolvimento relatado com diversos tipos tumorais (BLOKZIJL; TEN DIJKE; IBÁEZ, 2002; HEBERT et al., 1999; KOUROS-MEHR et al., 2006; LEE et al., 2015a; LI et al., 2011; MAITRA et al., 2002; UOZAKI et al., 2000). A HSP47 é uma chaperona envolvida em diversas etapas da síntese do colágeno. O colágeno é a proteína mais abundante que compõem a matriz extracelular (MEC), sendo que a reorganização da MEC é essencial para o processo tumoral e é peça fundamental para a formação da metástase (SCHEDIN; KEELY, 2011). Nos resultados obtidos com o secretoma encontramos várias proteínas envolvidas com a MEC e seus componentes, sendo estas secretadas exclusivamente pelas linhagens tumorais. Dentre estas proteínas podemos ressaltar vários tipos de colágenos, proteínas isomerases e a HSP47. Com estes dados decidimos avaliar a ação da HSP47 nas linhagens tumorais, através do silenciamento dessa proteína.

As linhagens celulares ACP02 e ACP03 apresentaram silenciamento total para HSP47 (Figura 1B e 1C, Capítulo III). Para avaliar o efeito da ausência da HSP47 na progressão tumoral, realizamos ensaios de migração, invasão, indução de morte celular e de formação de esferas.

Na análise de migração celular, nossos resultados demonstraram uma diminuição significativa da capacidade migratória nas linhagens ACP02 e ACP03 silenciadas para HSP47 (Figura 2A, Capítulo III). O efeito da inibição da HSP47 na diminuição da migração celular foi observado em outros tipos tumorais, como em células de carcinoma epidermóide de cabeça e pescoço, glioblastomas e câncer de mama (YAMAMOTO et al., 2013; ZHAO et al., 2014b; ZHU et al., 2015a). A capacidade invasiva dessas células também foi analisada através do uso da MEC em câmeras adaptadas de Boyden. Os nossos resultados demonstraram que, assim como a migração, a capacidade invasiva das células tumorais foi reduzida nas células silenciadas para HSP47 (Figura 2C, Capítulo III). A relação da capacidade invasiva com a proteína HSP47 também foi relatada em linhagens celulares derivadas de câncer de mama, glioma e câncer de pulmão (KAMIKAWAJI et al., 2016; VAN 'T VEER et al., 2002; WU et al., 2016b).

Como forma de analisar se o efeito observado na capacidade migratória e invasiva das linhagens tumorais estava relacionado a um aumento proliferativo, realizamos análises de crescimento populacional nas diferentes linhagens, silenciadas ou não para HSP47. Os nossos resultados demonstraram que o silenciamento de HSP47 não altera a proliferação celular (Figura 2B, Capítulo III). Sendo assim, os resultados observados se devem apenas a uma ação da HSP47 nas vias que induzem a capacidade migratória e invasiva nas células tumorais. Essas vias podem estar relacionadas com a ativação de genes envolvidos na transição epitélio-mesenquimal. Em trabalho com células derivadas de tumor renal, demonstrou-se uma relação direta da expressão de *HSP47* com altos níveis de marcadores mesenquimais e com a redução do marcador epitelial E-caderina (QI et al., 2018).

Ao analisarmos a morte celular nas diferentes linhagens, não observamos diferenças entre as linhagens tumorais silenciadas (Figura 3, Capítulo III). A HSP47 atua em várias etapas protegendo a célula contra estresse de retículo endoplasmático (RE) e de Golgi. Em embriões de camundongos nocautes para essa proteína, ocorre acúmulo de moléculas de colágeno no RE, desencadeando vias de ativação de morte celular e, consequentemente, acarretando na morte do embrião (MARUTANI et al., 2004). O desencadeamento da ativação de vias de morte celular também é observado em células *knockdown* para HSP47

tratadas com inibidores da O-glicosilação. A inibição da O-glicosilação leva ao desencadeamento de estresse de Golgi, levando a ativação de caspase-2 e desencadeando vias de morte celular (MIYATA et al., 2013). Por outro lado, em estudo com carcinomas de células escamosas de laringe, um aumento na expressão de HSP47 foi correlacionado com a indução do apoptose nas células, acarretando em um bom prognóstico para os pacientes (SONG et al., 2017). Já em células originadas de glioma, o *knockdown* para HSP47 induziu a morte celular por apoptose (ZHAO et al., 2014a). Esses dados demonstram uma ação contraditória do efeito da proteína HSP47 na indução da morte celular, podendo estar relacionado com a origem das linhagens celulares analisadas.

O ensaio de formação de esferas é um indicativo da capacidade de auto-renovação celular, indicando a presença de células tronco tumorais (CTT). A presença de CTT tem sido relacionada ao desenvolvimento do tumor, metástase e resistência a diferentes tipos de tratamentos antitumorais (CHANG, 2016). Em trabalho do nosso grupo, foi demonstrado que as linhagens ACP02 e ACP03 apresentavam capacidade de formação de esferas, além de expressarem marcadores de CTT (*LGR5* e *CD24*) indicando a presença de CTT nessas linhagens. Para avaliar o efeito de HSP47 na formação de esferas, realizamos cultivo tridimensional com MEC. Nossos resultados demonstram uma diminuição no número de esferas nas linhagens silenciadas para HSP47 (Figura 4A, Capítulo III) . Esses dados corroboram com os dados encontrados na literatura com células derivadas de gliomas e de carcinomas de células escamosas do esôfago, onde a expressão de HSP47 estava correlacionada com a capacidade de formação de esferas (JIANG et al., 2016; LEE et al., 2015a).

Os dados obtidos com as análises de migração, invasão e morte celular em células silenciadas para HSP47, também puderam ser observados através do silenciamento da HSP47 na linhagem MN01 (Figura 3, 4 e 5, Capítulo IV). Porém ao analisarmos a capacidade de formação de esferas nesta linhagem, não notamos diferença entre as células silenciadas para HSP47 em relação as não silenciadas.

Interessantemente, as análises do silenciamento da HSP47 demonstraram um maior efeito na linhagem ACP02. Nesta linhagem as análises de migração, invasão e formação de esferas tiveram uma redução mais significativa (valor de p) quando comparadas a linhagem ACP03. A linhagem ACP03 também apresentou menor capacidade invasiva quando comparada a ACP02 (Figura 2C, Capítulo III, significância entre as linhagens $p< 0,001$ não apontada no gráfico). Além disso, nas análises de formação de esferas a linhagem ACP03

demonstrou um menor número de esferas em relação a ACP02. Esses dados podem indicar uma relação entre a HSP47 e a agressividade de tumores gástricos, já que esta proteína apresentou um efeito mais significativo na linhagem tumoral que exibiu características mais agressivas. A relação entre HSP47 e a agressividade tumoral vem sendo apontada em diferentes tipos tumorais, como câncer de mama e glioblastomas (KAMIKAWAJI et al., 2016; ZHU et al., 2015b).

Em resumo, os resultados obtidos nessa pesquisa, demonstram que a proteína HSP47 é um potencial candidato como alvo terapêutico no tratamento de CG. A inibição dessa proteína acarreta em redução na capacidade migratória, invasiva e na formação de esferas. Sendo assim, inibidores de HSP47 podem apresentar efeito positivo no controle da agressividade tumoral em CG. Além disso, também demonstramos que a HSP47 e GDF15 são secretadas apenas pelas linhagens tumorais, sendo potenciais candidatas como biomarcadores de CG.

6. CONCLUSÃO

- A caracterização do secretoma das linhagens de adenocarcinomas gástrico demonstrou diferença em relação ao epitélio da mucosa gástrica normal, revelando proteínas secretadas exclusivamente pelas células de adenocarcinomas envolvidas no ciclo celular, regulação da homeostase redox, metabolismo e proteínas relacionas a matriz extracelular e citoesqueleto.

- As proteínas GDF15 e HSP47 apresentam secreção apenas em células de adenocarcinomas gástricos, demonstrando potencial como biomarcador de câncer gástrico.

- O silenciamento de HSP47 ocasionou redução na migração, invasão e na capacidade de formar esferas nas diferentes linhagens celulares.

- O silenciamento de GDF15 não foi possível nas diferentes linhagens. Isso pode representar que o silenciamento de GDF15 acarreta em fenótipo letal nas linhagens gástricas.

7. PERSPECTIVAS

- Investigar a capacidade de detecção de HSP47 e GDF15 em amostras sanguíneas de pacientes com câncer gástrico, para validação dessas moléculas como biomarcadores;

- Analisar as vias de sinalização, relacionadas com a migração e invasão celular, nas quais HSP47 participa;

- Analisar o efeito de indutores de estresse de retículo endoplasmático nas linhagens silenciadas para HSP47;

- Avaliar o efeito de inibidores de HSP47 em linhagens celulares de câncer gástrico comparativamente e em combinação com drogas conhecidas no tratamento quimioterápico dessa doença.

Referências Bibliográficas

AGRAWAL, G. K. et al. Plant secretome: Unlocking secrets of the secreted proteins. **Proteomics**, [s. l.], v. 10, n. 4, p. 799–827, 2010.

AHN, Y. et al. Mining of serum glycoproteins by an indirect approach using cell line secretome. **Molecules and Cells**, [s. l.], v. 29, p. 123–130, 2010.

ALBERTS, B. et al. **Biologia Molecular da Célula**. 5. ed. Porto Alegre: Artmed, 2010.

ALCANTARA, M. B. et al. Pigment epithelium-derived factor upregulates collagen I and downregulates matrix metalloproteinase 2 in osteosarcoma cells, and colocalises to collagen i and heat shock protein 47 in fetal and adult bone. **Journal of Pharmacy and Pharmacology**, [s. l.], v. 66, n. 11, p. 1586–1592, 2014.

BARAL, R. et al. **Advancements of Mass Spectrometry in Biomedical Research**. [s.l: s.n.]. v. 806

BARRAL, J. M. et al. Roles of molecular chaperones in protein misfolding diseases. **Seminars in Cell & Developmental Biology**, [s. l.], v. 15, n. 1, p. 17–29, 2004.

BARTH, D. et al. (EDS.). **Cancer Biomarkers: Minimal and non invasive early diagnosis and prognosis**. Boca Raton: CRC Press, 2014.

BAUSKIN, A. R. et al. Role of macrophage inhibitory cytokine-1 in tumorigenesis and diagnosis of cancer. **Cancer Research**, [s. l.], v. 66, n. 10, p. 4983–4986, 2006.

BLANCO-CALVO, M. et al. Circulating levels of GDF15, MMP7 and miR-200c as a poor prognostic signature in gastric cancer. **Future Oncology**, [s. l.], v. 10, n. 7, p. 1187–1202, 2014.

BLIVET-VAN EGGELPOËL, M. J. et al. Epidermal growth factor receptor and HER-3 restrict cell response to sorafenib in hepatocellular carcinoma cells. **Journal of Hepatology**, [s. l.], v. 57, n. 1, p. 108–115, 2012.

BLOKZIJL, A.; TEN DIJKE, P.; IBÁEZ, C. F. Physical and functional interaction between GATA-3 and SMAD3 allows TGF-β regulation of gata target genes. **Current Biology**, [s. l.], v. 12, n. 1, p. 35–45, 2002.

BROWN, D. A. et al. MIC-1 Serum Level and Genotype : Associations with Progress and Prognosis of Colorectal Carcinoma MIC-1 Serum Level and Genotype : Associations with Progress and Prognosis of Colorectal Carcinoma 1. [s. l.], v. 9, n. July 2003, p. 2642–2650, 2003.

BROWN, D. A. et al. MIC-1 serum level and genotype : associations with progress and prognosis of colorectal carcinoma. **Clinical Cancer Research**, [s. l.], v. 9, p. 2642–2650, 2010.

BUTERA, G.; PACCHIANA, R.; DONADELLI, M. Autocrine mechanisms of cancer chemoresistance. **Seminars in Cell and Developmental Biology**, [s. l.], v. 78, p. 3–12, 2017.

CACCIA, D. et al. Secretome compartment is a valuable source of biomarkers for cancer-relevant pathways. **Journal of Proteome Research**, [s. l.], v. 10, n. 9, p. 4196–4207, 2011.

CAO, D. et al. Expression of novel markers of pancreatic ductal adenocarcinoma in pancreatic nonductal neoplasms: additional evidence of different genetic pathways. **Modern Pathology**, [s. l.], v. 18, n. 6, p. 752–761, 2005.

CARVALHO, S. et al. An antibody to amphiregulin, an abundant growth factor in patients' fluids, inhibits ovarian tumors. **Oncogene**, [s. l.], v. 35, n. 4, p. 438–447, 2015.

CHANG, Y.-H. et al. Secretomic analysis identifies alpha-1 antitrypsin (A1AT) as a required protein in cancer cell migration, invasion, and pericellular fibronectin assembly for facilitating lung colonization of lung adenocarcinoma cells. **Molecular & cellular proteomics : MCP**, [s. l.], v. 11, n. 11, p. 1320–39, 2012. a.

CHANG, Y.-H. et al. Comparative Secretome Analyses Using a Hollow Fiber Culture System with Label-Free Quantitative Proteomics Indicates the Influence of PARK7 on Cell Proliferation and Migration/Invasion in Lung Adenocarcinoma. **Journal of Proteome Research**, [s. l.], v. 11, n. 11, p. 5167–5185, 2012. b.

CHUA, C. E. L. et al. Non-classical membrane trafficking processes galore. **Journal of Cellular Physiology**, [s. l.], v. 227, n. 12, p. 3722–3730, 2012.

CHUN, J. Y. et al. Interleukin-6 Regulates Androgen Synthesis in Prostate Cancer Cells. **Clinical Cancer Research**, [s. l.], v. 15, n. 15, p. 4815–4823, 2009.

CORRE, J.; HÉBRAUD, B.; BOURIN, P. Growth differentiation factor 15 in pathology: A clinical role? **Stem cells translational medicine**, [s. l.], v. 2, p. 946–952, 2013.

DENYSENKO, T. et al. Glioblastoma cancer stem cells : heterogeneity , microenvironment and related therapeutic strategies. **Cell Biochemistry and Function**, [s. l.], v. 28, p. 343–351, 2010.

DIEHL, H. C. et al. A catalogue of proteins released by colorectal cancer cellsin vitro as an alternative source for biomarker discovery. **PROTEOMICS – Clinical Applications**, [s. l.], v. 1, n. 1, p. 47–61, 2007.

DOWLING, P.; CLYNES, M. Conditioned media from cell lines: A complementary model to clinical specimens for the discovery of disease-specific biomarkers. **Proteomics**, [s. l.], v. 11, n. 4, p. 794–804, 2011.

DURAN, I. et al. A chaperone complex formed by HSP47, FKBP65, and BiP modulates telopeptide lysyl hydroxylation of type I procollagen. **Journal of Bone and Mineral Research**, [s. l.], v. 32, n. 6, p. 1309–1319, 2017.

ECKSTEIN, N. et al. Epidermal growth factor receptor pathway analysis identifies amphiregulin as a key factor for cisplatin resistance of human breast cancer cells. **Journal of Biological Chemistry**, [s. l.], v. 283, n. 2, p. 739–750, 2008.

EMMINK, B. L. et al. The secretome of colon cancer stem cells contains drug-metabolizing enzymes. **Journal of Proteomics**, [s. l.], v. 91, p. 84–96, 2013.

FIDLER, I. J. Critical determinants of cancer metastasis: rationale for therapy. **Cancer chemotherapy and pharmacology**, [s. l.], v. 43 Suppl, p. S3–S10, 1999.

GEIGER, T. R.; PEEPER, D. S. Metastasis mechanisms. **Biochimica et Biophysica Acta - Reviews on Cancer**, [s. l.], v. 1796, n. 2, p. 293–308, 2009.

GOTTESMAN, M. M.; FOJO, T.; BATES, S. E. Multidrug Resistance in Cancer: Role of Atp-Dependent Transporters. **Nature Reviews Cancer**, [s. l.], v. 2, n. 1, p. 48–58, 2002.

HAHN, W. C.; WEINBERG, R. A. Modelling the molecular circuitry of cancer. **Nature Reviews Cancer**, [s. l.], v. 2, n. 5, p. 331–341, 2002.

HANAHAN, D.; WEINBERG, R. A. Hallmarks of cancer: The next generation. **Cell**, [s. l.], v. 144, n. 5, p. 646–674, 2011.

HEBERT, C. et al. Cell surface colligin/Hsp47 associates with tetraspanin protein CD9 in epidermoid carcinoma cell lines. **Journal of Cellular Biochemistry**, [s. l.], v. 73, n. 2, p. 248–258, 1999.

HIRAI, K. et al. Immunohistochemical Distribution of Heat Shock Protein 47 (HSP47) in Scirrhous Carcinoma of the Stomach. **Anticancer Research**, [s. l.], v. 26, n. 1 A, p. 71–78, 2006.

HUBER, M. A.; KRAUT, N.; BEUG, H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. **Current Opinion in Cell Biology**, [s. l.], v. 17, p. 548–558, 2005.

INCA. **Estimativa | 2018 Incidência de Câncer no Brasil**. Rio de Janeiro: Instituto Nacional de Câncer - INCA, 2017.

JAIN, K. K. Innovations, challenges and future prospects of oncoproteomics. **Molecular Oncology**, [s. l.], v. 2, n. 2, p. 153–160, 2008.

JEREZ, S.; VAN WIJNEN, A. J.; GALINDO, M. Proteomic Analysis of Exosomes and Exosome-Free Conditioned Media Derived from Human Osteosarcoma Cell Lines Reveal Differential Secretion of Proteins Related to Biological Functions and Tumor Progression. **Journal of cellular biochemistry**, [s. l.], n. June 2016, 2016.

JIANG, X. et al. HSP47 promotes glioblastoma stemlike cell survival by modulating tumor microenvironment extracellular matrix through TGF-β pathway. **ACS Chemical Neuroscience**, [s. l.], v. 8, n. 1, p. 128–134, 2016.

JOSHI, S. et al. Adapting to stress — chaperome networks in cancer. **Nature Reviews Cancer**, [s. l.], 2018.

KAMIKAWAJI, K. et al. Regulation of LOXL2 and SERPINH1 by antitumor microRNA-29a in lung cancer with idiopathic pulmonary fibrosis. **Journal of Human Genetics**, [s. l.], v. 61, n. 12, p. 1–9, 2016.

KARAGIANNIS, G. S.; PAVLOU, M. P.; DIAMANDIS, E. P. Cancer secretomics reveal pathophysiological pathways in cancer molecular oncology. **Molecular Oncology**, [s. l.], v. 4, n. 6, p. 496–510, 2010.

KARIMI, P. et al. Gastric Cancer: Descriptive Epidemiology, Risk Factors, Screening, and Prevention. **Cancer Epidemiol Biomarkers Prev**, [s. l.], v. 23, n. 5, p. 700–713, 2014.

KOBAYASHI, E. et al. MicroRNA expression and functional profiles of osteosarcoma. **Oncology (Switzerland)**, [s. l.], v. 86, n. 2, p. 94–103, 2014.

KOBUNE, M. et al. Wnt3 / RhoA / ROCK signaling pathway is involved in adhesion-mediated drug resistance of multiple myeloma in an autocrine mechanism. **Molecular Cancer Therapeutics**, [s. l.], v. 6, n. 6, p. 1774–1784, 2007.

KOHLHAPP, F. J. et al. microRNAs as mediators and communicators between cancer cells and the tumor micro-environment. **Oncogene**, [s. l.], v. 34, n. 48, p. 5857–5868, 2015.

KOOPMANN, J. et al. Serum Macrophage Inhibitory Cytokine 1 as a Marker of Pancreatic and Other Periampullary Cancers. **Clinical Cancer Research**, [s. l.], v. 10, n. 410, p. 2386–2392, 2004.

KOUROS-MEHR, H. et al. GATA-3 maintains the differentiation of the Luminal cell fate in the mammary gland. **Cell**, [s. l.], v. 127, n. 5, p. 1041–1055, 2006.

KULASINGAM, V.; DIAMANDIS, E. P. Proteomics analysis of conditioned media from three breast cancer cell lines. **Molecular & Cellular Proteomics**, [s. l.], v. 6, n. 11, p. 1997–2011, 2007.

LEAL, M. F. et al. Establishment and conventional cytogenetic characterization of three gastric cancer cell lines. **Cancer Genetics and Cytogenetics**, [s. l.], v. 195, n. 1, p. 85–91, 2009.

LEE, H. W. et al. Overexpression of HSP47 in esophageal squamous cell carcinoma: clinical

implications and functional analysis. **Diseases of the Esophagus**, [s. l.], p. 1–8, 2015. a.

LEE, M. C. S. et al. Bi-Directional protein transport between the ER and Golgi. **Annual Review of Cell and Developmental Biology**, [s. l.], v. 20, n. 1, p. 87–123, 2004.

LEE, Y.-C. et al. Secretome Analysis of an Osteogenic Prostate Tumor Identifies Complex Signaling Networks Mediating Cross-talk of Cancer and Stromal Cells Within the Tumor Microenvironment. **Molecular & Cellular Proteomics**, [s. l.], v. 14, n. 3, p. 471–483, 2015. b.

LERNER, L. et al. MAP3K11/GDF15 axis is a critical driver of cancer cachexia. **Journal of Cachexia, Sarcopenia and Muscle**, [s. l.], 2015. a.

LERNER, L. et al. Plasma growth differentiation factor 15 is associated with weight loss and mortality in cancer patients. **Journal of Cachexia, Sarcopenia and Muscle**, [s. l.], v. 6, n. 4, p. 317–324, 2015. b.

LI, B. et al. Id1-Induced IGF-II and its autocrine/endocrine promotion of esophageal cancer progression and chemoresistance — implications for IGF-II and IGF-IR – Targeted Therapy. **Clinical Cancer Research**, [s. l.], v. 20, n. 10, p. 2651–2663, 2014.

LI, Y. et al. Progressive miRNA expression profiles in cervical carcinogenesis and identification of HPV-related target genes for miR-29. **Journal of Pathology**, [s. l.], v. 224, n. 4, p. 484–495, 2011.

LIN, L.-L.; HUANG, H.-C.; JUAN, H.-F. Revealing the molecular mechanism of gastric cancer marker annexin A4 in cancer cell proliferation using exon arrays. **PloS one**, [s. l.], v. 7, n. 9, p. e44615, 2012.

LINDERT, U. et al. Molecular consequences of the SERPINH1/HSP47 mutation in the dachshund natural model of osteogenesis imperfecta. **Journal of Biological Chemistry**, [s. l.], v. 290, n. 29, p. 17679–17689, 2015.

LOEI, H. et al. Mining the gastric cancer secretome: Identification of GRN as a potential diagnostic marker for early gastric cancer. **Journal of Proteome Research**, [s. l.], v. 11, n. 3, p. 1759–1772, 2012.

MAITRA, A. et al. Immunohistochemical validation of a novel epithelial and a novel stromal marker of pancreatic ductal adenocarcinoma identified by global expression microarrays: Sea urchin fascin homolog and heat shock protein 47. **American Journal of Clinical Pathology**, [s. l.], v. 118, n. 1, p. 52–59, 2002.

MAKRIDAKIS, M.; VLAHOU, A. Secretome proteomics for discovery of cancer biomarkers. **Journal of Proteomics**, [s. l.], v. 73, n. 12, p. 2291–2305, 2010.

MALONEY, A. et al. Gene and protein expression profiling of human ovarian cancer cells treated with the Heat Shock Protein 90 inhibitor 17-Allylamino-17-Demethoxygeldanamycin. **Cancer research**, [s. l.], v. 67, n. 7, p. 3239–3253, 2007.

MARUTANI, T. et al. Accumulation of type IV collagen in dilated ER leads to apoptosis in Hsp47-knockout mouse embryos via induction of CHOP. **Journal of cell science**, [s. l.], v. 117, n. 24, p. 5913–5922, 2004.

MBEUNKUI, F.; FODSTAD, O.; PANNELL, L. K. Secretory protein enrichment and analysis: an optimized approach applied on cancer cell lines using 2D LC-MS/MS. **J Proteome Res**, [s. l.], v. 5, n. 4, p. 899–906, 2006.

MIYATA, S. et al. The endoplasmic reticulum-resident chaperone Heat Shock Protein 47 protects the Golgi apparatus from the effects of O-Glycosylation inhibition. **PLoS ONE**, [s. l.], v. 8, n. 7, p. 1–20, 2013.

MUELLER, M. M.; FUSENIG, N. E. Friends or foes - Bipolar effects of the tumour stroma in cancer. **Nature Reviews Cancer**, [s. l.], v. 4, n. 11, p. 839–849, 2004.

NASSER, M. W. et al. Conditioning solid tumor microenvironment through inflammatory chemokines and S100 family proteins. **Cancer Letters**, [s. l.], v. 365, n. 1, p. 11–22, 2015.

NATSUME, T. et al. Interactions between collagen-binding stress protein HSP47 and collagen: Analysis of kinetic parameters by surface plasmon resonance biosensor. **The Journal of biological chemistry**, [s. l.], v. 269, n. 49, p. 31224–31228, 1994.

PALTRIDGE, J. L.; BELLE, L.; KHEW-GOODALL, Y. The secretome in cancer progression. **Biochimica et biophysica acta**, [s. l.], v. 1834, n. 11, p. 2233–41, 2013.

PLUEN, A. et al. Role of tumor-host interactions in interstitial diffusion of macromolecules: Cranial vs. subcutaneous tumors. **Proceedings of the National Academy of Sciences**, [s. l.], v. 98, n. 8, p. 4628–4633, 2001.

PORMOHAMMAD, A. et al. Risk of gastric cancer in association with Helicobacter pylori different virulence factors: A systematic review and meta-analysis. **Microbial Pathogenesis**, [s. l.], v. 118, p. 214–219, 2018.

POSCHMANN, G. et al. Identification of proteomic differences between squamous cell carcinoma of the lung and bronchial epithelium. **Molecular & cellular proteomics : MCP**, [s. l.], v. 8, n. 5, p. 1105–1116, 2009.

PROUTSKI, I. et al. Prostate-derived factor — a novel inhibitor of drug-induced cell death in colon cancer cells. **Molecular Cancer Therapeutics**, [s. l.], v. 8, n. 9, p. 2566–2575, 2009.

QI, Y. et al. SERPINH1 overexpression in clear cell renal cell carcinoma: association with poor clinical outcome and its potential as a novel prognostic marker. **Journal of Cellular and Molecular Medicine**, [s. l.], v. 22, n. 2, p. 1224–1235, 2018.

QIU, T. et al. Subsequent anti-VEGF therapy after first-line anti-EGFR therapy improved overall survival of patients with metastatic colorectal cancer. **OncoTargets and therapy**, [s. l.], v. 11, p. 465–471, 2018.

SCHEDIN, P.; KEELY, P. J. Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression. **Cold Spring Harbor Perspectives in Biology**, [s. l.], v. 3, n. 1, p. 1–22, 2011.

SCHIEGNITZ, E. et al. GDF 15 as an anti-apoptotic, diagnostic and prognostic marker in oral squamous cell carcinoma. **Oral Oncology**, [s. l.], v. 48, n. 7, p. 608–614, 2012.

SELANDER, K. S. et al. Serum macrophage inhibitory cytokine-1 concentrations correlate with the presence of prostate cancer bone metastases. **Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology**, [s. l.], v. 16, n. 3, p. 532–7, 2007.

SEPULVEDA, D. et al. Interactome screening identifies the ER luminal chaperone Hsp47 as a regulator of the unfolded protein response transducer IRE1α. **Molecular Cell**, [s. l.], v. 69, n. 2, p. 238–252, 2018.

SHARBEEN, G.; MCALPINE, S.; PHILLIPS, P. HSP47: The New Heat Shock Protein Therapeutic Target. In: MCALPINE S.; A., E. (Eds.). **Heat Shock Protein Inhibitors.** Vol. 19 ed. [s.l.] : Springer, Cham, 2015.

SONG, X. et al. HSP47 is associated with the prognosis of laryngeal squamous cell carcinoma by inhibiting cell viability and invasion and promoting apoptosis. **Oncology Reports**, [s. l.], v. 38, n. 4,

p. 2444–2452, 2017.

SÕTI, C. et al. Heat shock proteins as emerging therapeutic targets. **British journal of pharmacology**, [s. l.], v. 146, n. 6, p. 769–80, 2005.

STEWART, B., WILD, C. P. **World Cancer Report 2014**. [s.l.] : International Agency for Research on Cancer - (IARC/WHO), 2014.

STRELAU, J. et al. Expression and putative functions of GDF-15, a member of the TGF-beta superfamily, in human glioma and glioblastoma cell lines. **Cancer Lett**, [s. l.], v. 270, n. 1, p. 30–39, 2008.

STRIMBU, K.; TAVEL, J. a. What are biomarkers? **Curr Opin HIV AIDS**, [s. l.], v. 5, n. 6, p. 463–466, 2011.

TJALSMA, H. et al. Signal peptide-dependent protein transport in Bacillus subtilis: a genome-based survey of the secretome. **Microbiology and Molecular Biology Reviews**, [s. l.], v. 64, n. 3, p. 515–547, 2000.

TODARO, M. et al. Colon cancer stem cells Dictate tumor growth and resist cell death by production of Interleukin-4. **Cell Stem Cell**, [s. l.], v. 1, n. 4, p. 389–402, 2007.

TSUKIMI, Y.; OKABE, S. Recent advances in gastrointestinal pathophysiology: Role of heat shock proteins in mucosal defense and ulcer healing. **Biological & Pharmaceutical Bulletin**, [s. l.], v. 24, n. 1, p. 1–9, 2001.

UCAR, E. et al. Prognostic value of preoperative CEA, CA 19-9, CA 72-4, and AFP levels in gastric cancer. **Advances in Therapy**, [s. l.], v. 25, n. 10, p. 1075–1084, 2008.

UOZAKI, H. et al. Expression of heat shock proteins in osteosarcoma and its relationship to prognosis. **Pathology Research and Practice**, [s. l.], v. 196, n. 10, p. 665–673, 2000.

VAN 'T VEER, L. J. et al. Gene expression profiling predicts clinical outcome of breast cancer. **Letters To Nature**, [s. l.], v. 415, n. 345, p. 530–536, 2002.

VAN CUTSEM, E. et al. Gastric cancer. **The Lancet**, [s. l.], v. 388, p. 2654–2664, 2016.

WANG, T. et al. YAP promotes breast cancer metastasis by repressing growth differentiation factor-15. **Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease**, [s. l.], v. 1864, n. 5, p. 1744–1753, 2018.

WANG, X.; BAEK, S. J.; ELING, T. E. The diverse roles of nonsteroidal anti-inflammatory drug activated gene (NAG-1/GDF15) in cancer. **Biochemical Pharmacology**, [s. l.], v. 85, n. 5, p. 597–606, 2013.

WELSH, J. B. et al. Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. **PNAS**, [s. l.], v. 100, n. 6, p. 3410–3415, 2010.

WHO. 2017. Disponível em: <http://www.who.int/cancer/en/>. Acesso em: 3 abr. 2018.

WIDMER, C. et al. Molecular basis for the action of the collagen-specific chaperone Hsp47/SERPINH1 and its structure-specific client recognition. **Proceedings of the National Academy of Sciences of the United States of America**, [s. l.], v. 109, n. 33, p. 13243–13247, 2012.

WU, C.-C. et al. Candidate serological biomarkers for cancer identified from the secretomes of 23 cancer cell lines and the human protein atlas. **Molecular & cellular proteomics : MCP**, [s. l.], v. 9, n. 6, p. 1100–17, 2010.

WU, J. et al. Heat Shock Proteins and Cancer. **Trends in Pharmacological Sciences**, [s. l.], v. xx, p. 1–

31, 2016. a.

WU, Z. B. et al. Heat Shock Protein 47 promotes glioma angiogenesis. **Brain Pathology**, [s. l.], v. 26, n. 1, p. 31–42, 2016. b.

XUE, H. et al. Identification of serum biomarkers for colorectal cancer metastasis using a differential secretome approach. **Journal of Proteome Research**, [s. l.], v. 9, n. 1, p. 545–555, 2010.

YAMAMOTO, N. et al. Tumor-suppressive microRNA-29a inhibits cancer cell migration and invasion via targeting HSP47 in cervical squamous cell carcinoma. **International journal of oncology**, [s. l.], v. 43, n. 6, p. 1855–1863, 2013.

YAO, L. et al. Discovery of IL-18 as a novel secreted protein contributing to doxorubicin resistance by comparative secretome analysis of MCF-7 and MCF-7 / Dox. **PLoS ONE**, [s. l.], v. 6, n. 9, p. 1–13, 2011.

YIXUAN, Y. et al. Cathepsin S mediates gastric cancer cell migration and invasion via a putative network of metastasis-associated proteins. **Journal of Proteome Research**, [s. l.], v. 9, n. 9, p. 4767–4778, 2010.

YOKOZAKI, H. et al. Cancer as a tissue : The signi fi cance of cancer-stromal interactions in the development , morphogenesis and progression of human upper digestive tract cancer. [s. l.], p. 1–19, 2018.

YONG, K. M. A. et al. Morphological effects on expression of growth differentiation factor 15 (GDF15), a marker of metastasis. **Journal of Cellular Physiology**, [s. l.], v. 229, n. 3, p. 362–373, 2014.

YUAN, G. et al. WNT6 is a novel target gene of caveolin-1 promoting chemoresistance to epirubicin in human gastric cancer cells. **Oncogene**, [s. l.], v. 32, p. 375–387, 2013.

ZHANG, L. et al. Vascular endothelial growth factor overexpression by soft tissue sarcoma cells : Implications for tumor growth , metastasis , and chemoresistance. **Cancer Research**, [s. l.], v. 17, p. 8770–8779, 2006.

ZHANG, L. et al. Expression of growth differentiation factor 15 is positively correlated with histopathological malignant grade and in vitro cell proliferation in oral squamous cell carcinoma. **Oral Oncology**, [s. l.], v. 45, n. 7, p. 627–632, 2009.

ZHAO, D. et al. Heat shock protein 47 regulated by miR-29a to enhance glioma tumor growth and invasion. **Journal of Neuro-Oncology**, [s. l.], v. 118, n. 1, p. 39–47, 2014. a.

ZHAO, D. et al. Heat shock protein 47 regulated by miR-29a to enhance glioma tumor growth and invasion. **Journal of Neuro-Oncology**, [s. l.], v. 118, n. 1, p. 39–47, 2014. b.

ZHU, J. et al. Chaperone Hsp47 drives malignant growth and invasion by modulating an ECM gene network. **Cancer Research**, [s. l.], v. 75, n. 8, p. 1580–1591, 2015. a.

ZHU, J. et al. Chaperone Hsp47 Drives Malignant Growth and Invasion by Modulating an ECM Gene Network. **Cancer research**, [s. l.], v. 75, n. 8, p. 1580–91, 2015. b.

ZUO, J. H. et al. Activation of EGFR promotes squamous carcinoma SCC10A cell migration and invasion via inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin. **Journal of Cellular Biochemistry**, [s. l.], v. 112, n. 9, p. 2508–2517, 2011.