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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

ALTERAÇÕES GENÔMICAS QUANTITATIVAS COM POTENCIAL CLÍNICO NO ADENOCARCINOMA GÁSTRICO

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Belém – Pará
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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFPA como requisito parcial para obtenção do grau de Doutor em Genética e Biologia Molecular.

Orientador: Prof. Dr. André Salim Khayat.

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SUMÁRIO

RESUMO

O câncer gástrico é o quinto tipo tumoral mais frequente e a terceira maior causa de morte por câncer no mundo. A despeito do progresso no tratamento do câncer gástrico avançado, o prognóstico do paciente permanece muito ruim, principalmente em decorrência do diagnóstico tardio. Esse paradigma implica a necessidade de pesquisar e identificar biomarcadores moleculares para o diagnóstico precoce, bem como para o monitoramento da doença, contribuindo ainda para o desenenvolvimento de novas abordagens terapêuticas. Desta forma, o presente estudo objetivou investigar alterações no número de cópias de DNA no adenocarcnoma gástrico através da técnica de Hibridização Genômica Comparativa em array (aCGH) e selecionar genes para a validação em um maior número de amostras, utilizando PCR em tempo real, no intuito de encontrar potenciais biomarcadores moleculares para esse tipo tumoral. Através dos resultados do aCGH, foram identificadas 22 alterações nunca correlacionadas com a carcinogênese gástrica, bem como diversas alterações associadas significativamente com o extravasamento da serosa e com pacientes com idade igual ou inferior a 50 anos. Levando em consideração que a maioria dos genes observados alterados nunca foram descritos como envolvidos no processo de carcinogênese gástrica, foram selecionados para validação genes cujas alterações apresentaram alguma consistência com trabalhos já publicados na literatura em outros tipos de câncer. Assim, foram investigadas por PCR em tempo real as amplificações dos genes *RTEL1*, *B4GALT5*, *TRPV2* e *ABCA13*. Os resultados demonstraram uma frequência elevada de amplificação desses genes, porém as associações estatísticas com os dados clínicopatológicos dos genes *TRPV2*, com pacientes jovens, e *ABCA13*, com o extravasamento da serosa, observadas pelo aCGH, não foram confirmadas. Por outro lado, novas associações significativas foram observadas, tais quais a amplificação recorrente do gene *RTEL1*, que foi associada com idade avançada e com o tipo intestinal do adenocarcinoma gástrico; a amplificação recorrente do gene *B4GALT5*, que foi associada com o tipo intestinal do adenocarcinoma gástrico; a amplificação recorrente do gene *TRPV2*, que foi associada com metástase linfonodal; a amplificação recorrente do gene *ABCA13*, que foi associada com metástase linfonodal e com pacientes do gênero masculino e a co-amplificação dos genes *RTEL1* e *ABCA13*, que foi associada com estadiamento avançado. Desta forma, o aCGH mostrou-se uma ferramenta útil para a investigação de novos genes associados com a carcinogênese. Ademais, a amplificação dos genes *RTEL1*, *B4GALT5*, *TRPV2* e *ABCA13* parecem ter um papel importante no desenvolvimento e na progressão do câncer gástrico, podendo ser considerados potenciais marcadores desta doença.

Palavras-chave: Adenocarcinoma gástrico; variação no número de cópias; marcadores moleculares.

ABSTRACT

Gastric cancer is the fifth most frequent type of cancer and the third cause of cancer mortality worldwide. Despite progression in treatment of advanced gastric cancer, the prognosis of patients remains poor, in part due to the low rate of diagnosis during its early stages. This paradigm implies the necessity to search and identify molecular biomarkers for early gastric cancer diagnosis, as well as for disease monitoring, thus contributing to the development of new therapeutic approaches. Therefore, this study aimed at investigating copy number variations in gastric adenocarcnoma through array Comparative Genomic Hybridization (aCGH) technique and selecting genes for validation in a larger sample size by using real-time PCR, in order to find potential molecular biomarkers for this tumor type. The aCGH results demonstrated 22 gene alterations never described before as correlated with gastric carinogenesis, as well as several alterations significantly associated with serosal extravasation and patients aged less than or equal 50 years. Given that most of the genes had never been described in gastric cancer, we selected for validation four gene alterations that showed some consistency with studies published in the literature for other types of cancer. Thus, we investigated by real-time PCR the amplifications of *RTEL1*, *B4GALT5*, *TRPV2* and *ABCA13* genes. The results showed a high frequency of amplification of these genes, but the statistical associations with clinicopathological data of *TRPV2* gene with younger patients and *ABCA13*, with serosal extravasation, observed by aCGH, were not confirmed. Moreover, new significant associations were demonstrated, including *RTEL1* recurrent amplification associated with advanced age and intestinal type of gastric adenocarcinoma; *B4GALT5* recurrent amplification associated with intestinal type of gastric adenocarcinoma; *TRPV2* recurrent amplification associated with lymph node metastasis; *ABCA13* recurrent amplification associated with lymph node metastasis and male patients and co-amplification of *RTEL1* and *ABCA13* associated with advanced staging. Therefore, the aCGH proved to be a useful tool for the investigating new genes associated with carcinogenesis. Additionally, recurrent amplification of *RTEL1*, *B4GALT5*, *TRPV2* and *ABCA13* seem to have an important role in the development and progression of gastric cancer and can be considered as potential biomarkers for this disease.

Keywords: Gastric adenocarcinoma; copy number variation; molecular biomarkers.

1 INTRODUÇÃO

1.1 CONSIDERAÇÕES GERAIS

Câncer é um termo empregado para designar diferentes doenças que têm em comum o fato de serem uma desordem das células somáticas, ocasionada por um acúmulo de mudanças genéticas e epigenéticas múltiplas. Estas alterações acarretam em características anormais às células, que passam a ter capacidade de crescimento desordenado e invadir o tecido sadio circundante, podendo ainda espalhar-se para outras regiões do corpo (metástase) (Patel *et al.*, 2015; INCA, 2014; Sadikovic *et al.*, 2008; Vogelstein & Kinzler, 1993; Knudson, 1985).

Trata-se de uma doença multifatorial, resultante da interação entre fatores extrínsecos (como o tabaco, agentes infecciosos, produtos químicos ou radiações) e intrínsecos (mutações herdadas, hormônios, condições imunológicas e mutações aleatórias) que podem agir tanto em conjunto como em sequência para iniciar ou promover a carcinogênese (*American Cancer Society*, 2015).

O câncer representa um grave problema de saúde pública mundial. A Organização Mundial da Saúde (OMS) estimou que, no ano 2030, existirão 75 milhões de pessoas vivendo anualmente com câncer, 27 milhões de casos novos de câncer e 17 milhões de mortes por câncer e o maior efeito desse aumento incidirá em países de baixa e média renda (INCA, 2016). De acordo com o Instituto Nacional do Câncer, a estimativa para o Brasil, biênio 2016-2017, aponta a ocorrência de cerca de 600 mil casos novos de câncer. Excetuando-se o câncer de pele não melanoma (aproximadamente 180 mil casos novos), ocorrerão cerca de 420 mil casos novos de câncer (INCA, 2016).

Dentre todos os diferentes tipos de câncer que afetam o homem, o câncer gástrico (CID-1º C16), sem considerar o câncer de pele não melanoma, ocupa a quarta posição quanto ao tipo tumoral mais frequente em homens e a quinta em mulheres e constitui a terceira maior causa de morte por câncer no mundo (Ferlay *et al.*, 2013).

A última estimativa mundial, realizada em 2012 pelo projeto Globocan/Iarc, apontou a ocorrência de cerca de 1 milhão de casos novos de câncer de estômago (6,8% do total de neoplasias malignas) para o ano de 2012, configurando-o como o quinto tumor maligno mais comum no mundo, atrás do câncer de pulmão, de mama, colorretal e de próstata. Do total de casos, 70% ocorreram em países em desenvolvimento e metade do total mundial ocorreu na Ásia Oriental (predominantemente na China) (Ferlay *et al.*, 2013).

Esperam-se 12.920 casos novos de câncer gástrico em homens e 7.600 em mulheres para o Brasil, nos anos de 2016 e 2017. Esses valores correspondem a um risco estimado de 13,04 casos novos a cada 100 mil homens e 7,37 para cada 100 mil mulheres, classificando o câncer gástrico como o quarto tumor mais frequente em homens e o quinto entre as mulheres (INCA, 2016).

Na Região Norte o câncer de estômago é o segundo mais frequente em homens (11,62/100 mil) e o quarto em mulheres (5,82/100 mil). No estado do Pará, o câncer gástrico também apresenta uma elevada incidência, ocupando a segunda posição entre os homens e a quarta entre as mulheres. A estimativa para os anos de 2016 e 2017 é de 690 novos casos no estado e 260 na capital Belém (INCA, 2016).

Vale ressaltar que o número elevado de casos de câncer gástrico se torna um grave problema de saúde pública, uma vez que na maioria dos pacientes a doença é diagnosticada em estágios avançados, com taxas de sobrevida extremamente baixas. O diagnóstico tardio ocorre, principalmente, devido aos pacientes com lesões pequenas serem assintomáticos ou apresentarem apenas sintomas não específicos (Correa, 2013; Mincis, 2009).

Um dos fatores ambientais que tem grande influência na carcinogênese gástrica é a dieta, principalmente o uso excessivo de sal e de comidas contendo o composto N-nitroso, além do tabagismo e da infecção pela bactéria *Helicobacter pylori*, que é considerada o principal fator de risco etiológico para o desenvolvimento dessa neoplasia (de Martel *et al.*, 2013; Konturek *et al.*, 2009; Azarhoush *et al.*, 2008). Adicionalmente, a infecção pelo vírus Epstein-Barr tem sido significativamente associada com o desenvolvimento do câncer gástrico (Shinozaki-Ushiku *et al.*, 2015; Lima *et al.*, 2008).

Indivíduos acometidos por câncer do sistema digestório apresentam frequentemente perda de peso em curto período de tempo, dificuldade de alimentação, dor local, náuseas, vômitos e sensação de plenitude precoce, que contribuem para o agravamento da doença, dificultam os tratamentos propostos e, consequentemente, favorecem o pior prognóstico (Blum *et al.*, 2013; Cecconello & Leite, 2004).

O tratamento desta neoplasia é bastante complexo, sendo a cirurgia de retirada do estômago ou de parte dele, juntamente com linfonodos próximos, a única expectativa de cura real (Takahashi *et al.*, 2013; Dikken *et al.*, 2012; Liakakos & Roukos, 2008; Dicken *et al.*, 2005). No entanto, somente 30 a 50% dos pacientes com esse tumor podem ser operados e

mesmo para os pacientes que são submetidos à ressecção total, a taxa de recorrência ainda é elevada (Hejna *et al.*, 2006).

Apesar de os quimioterápicos adjuvantes serem investigados por mais de 40 anos, pouco benefício na sobrevida dos pacientes com câncer gástrico é observado. Como consequência, essa doença apresenta um prognóstico desfavorável e a sobrevida média cumulativa após 5 anos do diagnóstico no Brasil é estimada em aproximadamente 25% (INCA, 2016; Nagini, 2012). Por outro lado, quando o tumor é detectado e tratado antes de invadir a camada muscular do estômago, a taxa de sobrevida em 5 anos após o diagnóstico da doença pode chegar a 90% (Miyahara *et al.*, 2007).

Anatomicamente, o estômago tem início na junção gastroesofágica e estende-se até o piloro. A parte proximal, que se localiza abaixo do diafragma, é denominada de cárdia. Logo após se encontram o fundo e o corpo e, em seguida, a porção distal conhecida como antro, terminando no piloro, responsável por controlar o fluxo de alimento do estômago para o duodeno (Figura 1) (AJCC, 2004).

Histologicamente, o estômago é constituído por quatro camadas: mucosa, submucosa, muscular e serosa (AJCC, 2004; Sobin & Wittekind, 2004) (Figuras 1 e 2). A camada muscular, por sua vez, é subdividida em muscular oblíqua, muscular circular e muscular longitudinal (Figura 2) (Sobin & Wittekind, 2004).

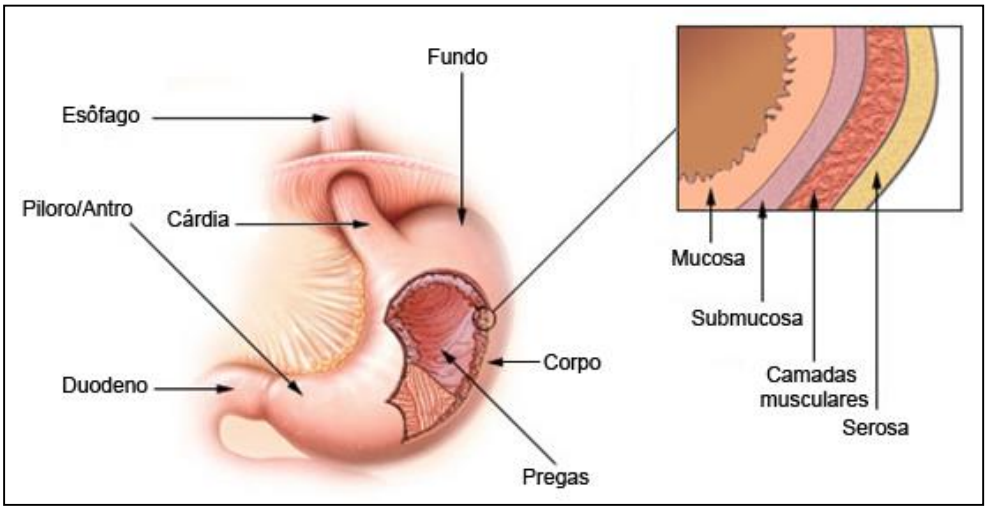

Figura 1: Anatomia do estômago (Adaptado de: *SEER Training Modules*, 2015).

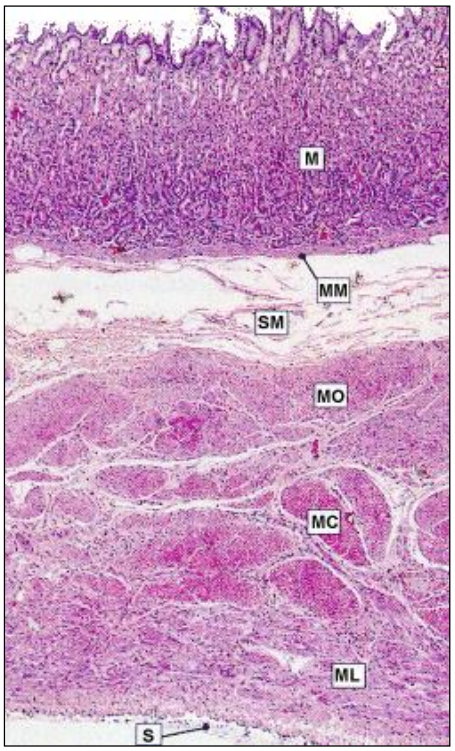

Figura 2: Histologia gástrica normal: M – Mucosa (MM – muscular da mucosa); SM – Submucosa; MO, MC e ML – Musculares oblíqua, circular e longitudinal, respectivamente; S – Serosa (UL, 2014).

O câncer gástrico é uma neoplasia que pode iniciar em qualquer parte do estômago e atingir diferentes camadas de tecidos. Sendo assim, tipos diferentes de câncer podem ocorrer no estômago. O adenocarcinoma, tumor originado na camada mucosa, é o tipo mais comum de câncer do trato digestivo, correspondendo a aproximadamente 95% dos casos que acometem o estômago (McLean & El-Omar, 2014; Shang & Pena, 2005).

Tumores localizados em sítios diferentes devem ser considerados entidades diferentes, uma vez que suas características, tais como epidemiologia, fatores de risco associados e prognóstico, são distintas. Historicamente, o câncer gástrico do tipo distal apresentava-se mais frequente; entretanto, um aumento contínuo no número de casos de câncer gástrico acometendo a região da cárdia e a diminuição da incidência de câncer gástrico na região distal modificaram este panorama e, atualmente, a incidência de adenocarcinomas na região proximal é superior (Anderson *et al.*, 2010; He *et al.*, 2008; Maeda *et al.*, 2008). Esta modificação na epidemiologia é preocupante, uma vez que o câncer gástrico da cárdia geralmente apresenta um pior prognóstico (Peng *et al.*, 2014; Maeda *et al.*, 2008).

Segundo a classificação histológica de Laurén (1965), os adenocarcinomas gástricos podem ser subdivididos nos tipos intestinal e difuso. O tipo intestinal exibe um padrão de crescimento expansivo, células com núcleos grandes e irregulares e uma coesão celular que favorece a formação de estruturas tubulares do tipo glandular (Figura 3, A e B). Por outro lado, o tipo difuso é constituído de pequenas células não coesas, difusamente dispersas, que não formam estruturas glandulares, podendo apresentar células com núcleos periféricos (anel de sinete) devido à elevada produção de mucina (Figura 3, C e D) (Yakirevich & Resnick, 2013; Piazuelo & Correa, 2013; Espejo & Navarrete, 2003; Correa, 1995).

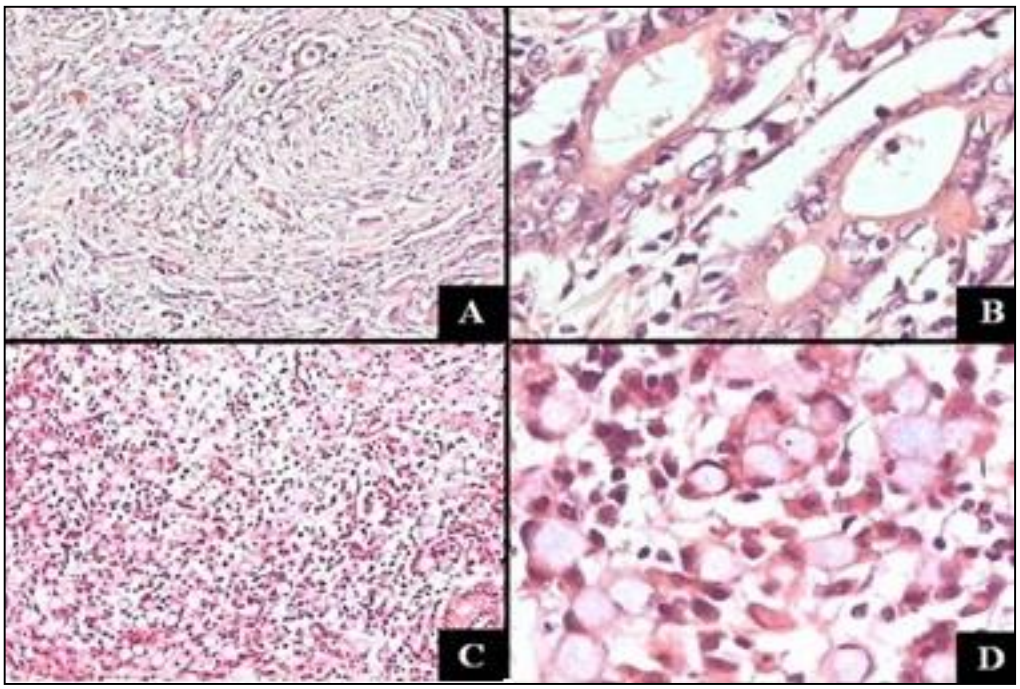

Figura 3: Tipos histológicos do adenocarcinoma gástrico. A e B: Tipo intestinal, em objetivas de 10x e 40x, respectivamente. C e D: Tipo difuso, em objetivas de 10x e 40x, respectivamente.

A variante histológica comumente presente em populações de alto risco é o tipo intestinal, sendo resultado da ação de vários fatores ambientais, incluindo a infecção por *H. pylori*. O tipo intestinal é geralmente precedido de lesões pré-neoplásicas progressivas, como a gastrite crônica, a atrofia gástrica, a metaplasia intestinal e a displasia (Brawner *et al.*, 2014; Takenaka *et al.*, 2007; Khan & Shukla, 2006; Fenoglio-Preiser *et al.*, 2003; Correa 1995).

O adenocarcinoma gástrico do tipo difuso não apresenta uma sequência de lesões pré-neoplásicas identificáveis e está geralmente associado a fatores hereditários (Brawner *et*

al., 2014; Yakirevich & Resnick, 2013). Além disso, esta variante histológica apresenta uma taxa mitótica menor em relação aos tumores do tipo intestinal (Piazuelo & Correa, 2013; Piazuelo *et al.*, 2010; Fenoglio-Preiser *et al.*, 2003; Hamilton & Aaltonen, 2000).

Do ponto de vista da distribuição por gênero e idade, o tipo intestinal é mais frequente em homens, sobretudo em faixas etárias mais avançadas. Por outro lado, o tipo difuso apresenta as taxas de casos entre homens e mulheres próximas à igualdade (Piazuelo & Correa, 2013; Piazuelo *et al.*, 2010; Dicken *et al.*, 2005; Henson *et al.*, 2004).

Para relatar a fisiopatologia do adenocarcinoma é necessário um sistema de classificação que inclua todos os seus atributos patológicos, tais como extensão do tumor primário (pT), ausência ou presença de metástases para linfonodos regionais (pN) e ausência ou presença de metástases à distância (pM). Este sistema resulta na combinação de T, N e M em grupos específicos que irão designar a extensão anatômica do câncer e se relacionarão com a história natural do seu tipo particular. A adição de categorização numérica para os componentes do sistema pTNM (TNM patológico) indica progressivamente a extensão da doença (Washington, 2010).

Na tabela 1 estão classificados os tipos de acordo com a profundidade do tumor (em Tumor Primário), a presença e/ou ausência de linfonodos comprometidos (em Linfonodos Regionais) e a presença e/ou ausência de metástase à distância (em Metástase à Distância).

Tabela 1: Classificação do TNM patológico (Washington, 2010).

Pt	**Tumor Primário**
TX	O tumor primário não pode ser avaliado.
T0	Não há evidência de tumor primário.
Tis	Carcinoma *in situ*: tumor intraepitelial sem invasão da lâmina própria.
T1	Tumor invade a lâmina própria, muscular da mucosa ou submucosa.
T1a	Tumor invade a lâmina própria ou muscular da mucosa.
T1b	Tumor invade a submucosa.
T2	Tumor invade a muscular própria.
T3	Tumor penetra no tecido conjuntivo subseroso sem invasão do peritônio visceral ou estruturas adjacentes. Também incluem aqueles que se estendem ao gastrocólico ou ligamentos gastrohepáticos, ou para o omento

	maior ou menor, sem perfuração do peritônio visceral que cobre estas estruturas.
T4	Tumor invade a serosa (peritônio visceral) ou invade estruturas adjacentes.
T4a	Tumor invade a serosa (peritônio visceral).
T4b	Tumor invade estruturas adjacentes como baço, cólon transverso, fígado, diafragma, pâncreas, parede abdominal, glândula adrenal, rim, intestino delgado e retroperitônio.
pN	**Linfonodos Regionais**
NX	Os linfonodos regionais não podem ser avaliados.
N0	Ausência de metástase em linfonodos regionais.
N1	Metástase em 1 a 2 linfonodos regionais.
N2	Metástase em 3 a 6 linfonodos regionais.
N3	Metástase em 7 ou mais linfonodos regionais.
Pm	**Metástase à Distância**
MX	A metástase não pode ser avaliada.
M0	Ausência de metástase à distância.
M1	Metástase à distância.

Na tabela 2, encontra-se o estadiamento do tumor, que está relacionado ao prognóstico do paciente e é resultante da combinação da classificação observada na tabela 1 (Washington, 2010).

Tabela 2: Grupamento por estadios (Washington, 2010).

Estadiamento	**Combinações TNM**		
Estadio 0	Tis	N0	M0
Estadio IA	T1	N0	M0
Estadio IB	T1	N1	M0
	T2	N0	M0
Estadio IIA	T3	N0	M0
	T2	N1	M0
	T1	N2	M0
Estadio IIB	T4a	N0	M0

	T3	N1	M0
	T2	N2	M0
	T1	N3	M0
Estadio IIIA	T4a	N1	M0
	T3	N2	M0
	T2	N3	M0
Estadio IIIB	T4b	N0-1	M0
	T4a	N2	M0
	T3	N3	M0
Estadio IIIC	T4a	N3	M0
	T4b	N2-3	M0
Estadio IV	Qualquer T	Qualquer N	M1

O câncer gástrico é definido como precoce quando o adenocarcinoma está restrito à mucosa e submucosa, independentemente de sua extensão em superfície e da presença ou não de metástase ganglionares. Esse tipo de adenocarcinoma possui um bom prognóstico (Kim *et al.*, 2011; Tan *et al.*, 2011; Dekker & Op Den Orth, 1977).

O tumor vai ser considerado avançado quando atingir as camadas posteriores à submucosa, podendo ou não apresentar metástase nos linfonodos e órgãos como o pulmão, as glândulas adrenais, o fígado, o osso e a cavidade peritoneal (Kim *et al.*, 2011; Tan *et al.*, 2011; MacDonald, 1992).

Esses fatos reforçam a gravidade dessa patologia e a necessidade de desenvolvimento de novos estudos que possam ajudar a modificar esse panorama, por meio da identificação de características genéticas do tumor, o que poderia ampliar a capacidade de prever o comportamento dessa neoplasia e possibilitar o estabelecimento de uma conduta terapêutica mais precisa (Assumpção & Burbano, 2005).

1.2 CITOGENÉTICA DE NEOPLASIAS

De acordo com Hare & Singh (1979), citogenética é a ciência que estuda a estrutura e as propriedades dos cromossomos, o seu comportamento durante a mitose e a meiose, bem

como a sua influência no fenótipo. Nesse contexto, a citogenética é uma ferramenta muito útil para o estudo das aberrações cromossômicas estruturais e numéricas no câncer.

Caspersson *et al*. (1970) desenvolveram uma das primeiras técnicas de bandeamento cromossômico, o bandeamento Q, que envolve a coloração de cromossomos com fluorocromos, como a quinacrina, e sua visualização em microscópio de fluorescência. Posteriormente, diversas outras técnicas de bandeamento foram desenvolvidas, incluindo os bandeamentos G, R, C e NOR, marcando o início da citogenética clássica (Rooney, 2001).

As análises citogenéticas clássicas de tumores obtiveram considerável incremento nos anos 90, em função das análises de banda de alta resolução e da hibridização fluorescente *in situ* (FISH). O aprimoramento tecnológico da hibridização *in situ* deu origem a técnicas de citogenética molecular, tais como a multiplex FISH e a Hibridização Genômica Comparativa (CGH), idealizadas para realizar análises rápidas e precisas do genoma de tumores (Kallioniemi *et al.*, 1992).

A citogenética clássica continua sendo muito útil na identificação de rearranjos cromossômicos em leucemias e linfomas, entretanto, tem-se alcançado um menor sucesso em tumores sólidos, em função da dificuldade da obtenção de preparações metafásicas representativas de alta qualidade, além do elevado número de rearranjos cromossômicos, que dificultam a interpretação dos cariótipos (Guillaume *et al.*, 2001; Gray & Collins, 2000). Essas dificuldades laboratoriais são superadas por ferramentas de citogenética molecular, como por exemplo a Hibridização Genômica Comparativa em array (aCGH), visto que essa técnica não utiliza metáfases tumorais para avaliação de alterações cromossômicas e, através de sua utilização, é possível quantificar as alterações cromossômicas em escala genômica com uma alta resolução (Wan & Ma, 2012; Chun *et al.*, 2000; Rao *et al.*, 1995; Seruca *et al.*, 1993).

A caracterização das aberrações cromossômicas tem contribuído para o entendimento dos mecanismos tumorais através da identificação de genes relacionados à tumorigênese, colaborando para a implementação clínica de ferramentas de prognóstico e de diagnóstico e auxiliando no estabelecimento de novas estratégias terapêuticas (Chia & Tan, 2016; Yap *et al.*, 2015; Grade *et al.*, 2015). Nesse contexto, técnicas de citogenética molecular que permitam a identificação dessas aberrações são extremamente relevantes para o estudo e a compreensão da genética do câncer, uma vez que podem revelar sítios de

mudanças recorrentes em regiões que envolvem genes importantes para o processo de carcinogênese (Ried *et al.*, 2004).

1.3 HIBRIDIZAÇÃO GENÔMICA COMPARATIVA EM ARRAY (aCGH)

A análise por citogenética clássica pode detectar alterações cromossômicas visíveis, como uma banda cromossômica extra, mas pequenos ganhos e perdas no genoma não são observados. Por outro lado, a aCGH é uma ferramenta que permite uma análise em grande escala no genoma, detectando ganhos e perdas de DNA nos cromossomos anteriormente indetectáveis pelas técnicas de citogenética clássica. Essa técnica é similar ao CGH convencional, que permite a análise de todo genoma sem nenhuma prévia informação sobre as aberrações cromossômicas que podem ser encontradas (Shao *et al.*, 2010; Carter, 2007; Rogatto & Rainho, 2004; Kallioniemi *et al.*, 1992).

Uma diferença importante entre essas duas tecnologias consiste na resolução. Na tecnologia de *array*, a resolução depende do número, da distribuição e do tamanho das sondas, que podem ser construídas a partir de sequências que variam de 40-200 kb (*large insert clones*), de 1,5-4,5 kb (*small insert clones*), de 100 pb-1,5 kb (produtos de PCR) e de 25-80 pb (oligonucleotídeos) (Ylstra *et al.*, 2006). Na aCGH, a alta resolução permite a identificação de deleções de até 1 kb (Askree *et al.*, 2013; Bittel *et al.*, 2009). Por outro lado, a técnica de CGH possui uma baixa resolução, que varia de 5-10 Mb, o que não permite detectar alterações inferiores a esse limite (Möhlendick *et al.*, 2013).

Além disso, a aCGH baseia-se na utilização de sondas genômicas imobilizadas em lâminas de vidro em arranjos ao invés de metáfases cromossômicas como alvo para hibridização. As sondas imobilizadas hibridizam com a amostra marcada, levando à uma alta resolução na identificação do número de cópias no genoma, permitindo uma melhor análise do genoma em um único experimento (Affymetrix, 2016; Pinkel *et al.*, 1998; Solinas-Toldo *et al.*, 1997).

A técnica pode ser realizada com DNA extraído de biópsia tumoral fresca ou congelada, de células em cultura e linhagens celulares ou até mesmo de fragmento fixado em formol e embebido em parafina (FFPE), ou seja, alterações na quantidade de material genético podem ser determinadas em amostras que poderiam não ser mais analisáveis por citogenética clássica (Affymetrix, 2016).

A aplicação da aCGH em amostras extraídas de vários tipos de tumores revelou recorrentes ganhos e perdas cromossômicas que foram significativamente corelacionados com as características clinicopatológicas dos pacientes (Mühlbacher *et al.*, 2016; Kreisel *et al.*, 2011; Tomioka *et al.*, 2010; Buffart *et al.*, 2009; Han *et al.*, 2006; Weiss *et al.*, 2004). Nesse contexto, trabalhos desenvolvidos com aCGH em tecido de origem gástrica demonstraram algumas alterações recorrentes com relevância clínica, como a perda do braço curto do cromossomo 5 e o ganho do braço longo dos cromossomos 8 e 20 (Cheng *et al.*, 2012; Deng *et al.*, 2012; Uchida *et al.*, 2010; Kim *et al.*, 2009; Nakamura *et al.*, 2009; Buffart *et al.*, 2009; Tsukamoto *et al.*, 2008; Buffart *et al.*, 2007).

1.4 INSTABILIDADE CROMOSSÔMICA NO CÂNCER GÁSTRICO

A descoberta de que um mesmo gene está alterado em diferentes etapas da carcinogênese em vários tipos tumorais levou à conclusão de que o câncer se desenvolve através de um modelo de múltiplos passos (Vogelstein & Kinzler, 1993). Diversas alterações genéticas e epigenéticas em proto-oncogenes, genes supressores de tumor, genes de reparo de DNA, moléculas de adesão celular e fatores de crescimento (e seus receptores), estão envolvidas no curso das várias etapas da conversão da célula gástrica normal para o câncer gástrico (Kang *et al.*, 2014; Busuttil *et al.*, 2014; Calcagno *et al.*, 2013; Cho *et al.*, 2013).

Recentemente, foi publicada uma classificação molecular para o câncer gástrico, dividindo-o em quatro subtipos: tumores positivos para o vírus Epstein-Barr, caracterizados pela presença de mutações recorrentes no gene *PI3KCA*, pela frequente hipermetilação do DNA, pela amplificação dos genes *JAK2*, *CD274* (também conhecido como *PD-L1*) e *PDCD1LG2* (também conhecido como *PD-L2*) e pelo silenciamento do gene *CDK2NA*; tumores com instabilidade de microssatélites, que apresentam taxas elevadas de mutações, incluindo mutações em genes codificantes de proteínas oncogênicas, e silenciamento do gene *MLH1*; tumores com estabilidade genômica, que são mais comuns no tipo histológico difuso e apresentam mutações nos genes *RHOA* e *CDH1* ou fusões que envolvem proteínas ativadoras de GTPases da família RHO; e tumores com instabilidade cromossômica, que são caracterizados pela presença de aneuploidia, pela amplificação de receptores tirosina quinases e por mutação no gene *TP53* e estão associados ao tipo histológico intestinal (Cancer Genome Atlas Research Network, 2014).

A perda da estabilidade genômica representa um evento molecular importante que ocorre no início do processo da carcinogênese gástrica e cria um ambiente permissivo para o acúmulo de alterações genéticas e epigenéticas (Ottini *et al.*, 2006). A instabilidade cromossômica é um tipo de instabilidade genômica frequentemente observada em tumores gástricos (84% dos tumores esporádicos) e é caracterizada por anormalidades cromossômicas, como ganhos ou perdas de cromossomos inteiros e/ou frações de cromossomos (perda de heterozigose -LOH, amplificação, deleção e translocação), que afetam diretamente a expressão de genes importantes para a carcinogênese (Buffart *et al.*, 2011; Martin *et al.*, 2010; Ottini *et al.*, 2006; Grabsch *et al.*, 2004).

Diversas variações no número de cópias de DNA têm sido reportadas em câncer gástrico e subgrupos com diferentes padrões de alterações têm sido associados com idade, prognóstico, tipo histológico, metástase linfonodal e metástase à distância (Kawauchi *et al.*, 2010; Panani, 2008; Buffart *et al.*, 2007; Kang *et al.*, 2006; Morohara *et al.*, 2005; Weiss *et al.*, 2004).

Notavelmente, as evidências sugerem que os adenocarcinomas gástricos intestinal e difuso surgem por vias genéticas distintas, pois são identificadas diferentes alterações nestes dois tipos histológicos (Donner *et al.*, 2015; Mocellin *et al.*, 2015; Jing *et al.*, 2014; Tahara, 2004).

Um estudo realizado por Calcagno *et al.* (2009) relatou a existência de aneussomia dos cromossomos 8, 9, 17 e X no câncer gástrico, enfatizando que a aneussomia do cromossomo 8 é a alteração mais frequente em ambos os tipos histológicos. Além disso, a região 8q24 (onde está localizado o oncogene *MYC*) encontrou-se altamente amplificada em ambos os tipos histológicos, mas em uma frequência menor no tipo difuso.

Outro estudo, também realizado por Calcagno *et al.* (2013), demonstrou a amplificação do oncogene *MYC* em 51,5% das amostras de câncer gástrico e a deleção dos genes supressores de tumor *FBXW7* e *TP53* em 45,5% e 21,2% das amostras, respectivamente. Adicionalmente, demonstrou que a alteração nos genes *MYC* e *FBXW7* estava relacionada com a presença de metástase linfonodal e o estágio avançado da doença (III e IV) e que a expressão da proteína MYC apresentava-se mais frequente no tipo intestinal.

Khayat *et al.* (2009) observaram a deleção do gene *TP53* em todas as amostras de câncer gástrico estudadas e a aneussomia do cromossomo 17 (onde está localizado o gene)

em 85% das amostras, destacando que a imunorreatividade da proteína p53 foi observada apenas em amostras do tipo intestinal.

A trissomia do cromossomo 7 e deleção do braço curto do cromossomo 17 foram observadas em três linhagens celulares de câncer gástrico: ACP02 (tipo histológico difuso), ACP03 (tipo histológico intestinal) e AGP01 (tipo histológico intestinal) (Leal *et al.*, 2009). Outro estudo, que caracterizou citogeneticamente a linhagem de câncer gástrico ACP01 (tipo histológico intestinal), encontrou trissomia dos cromossomos 1, 3, 7, 8 e 9, sendo a trissomia do cromossomo 8 a alteração mais frequente (Lima *et al.*, 2004).

Deng *et al.* (2012) realizaram uma análise genômica de alta resolução para investigar o perfil de alterações no número de cópias no câncer gástrico. As amplificações cromossômicas mais frequentes envolveram as regiões 1q, 3q, 5p, 6p, 7p, 7q, 8q, 13q, 18p, 18q, 19p, 20p, 20q e 21q e as deleções mais frequentes envolveram as regiões 3p, 4p, 4q, 5q, 6q, 8p, 9p, 11q, 12p, 14q, 16q, 17p, 18q, 19p, 21q e 22q, corroborando com estudos publicados anteriormente (Rossi *et al.*, 2011; Tada *et al.*, 2010; Tsukamoto *et al.*, 2008; Kimura *et al.*, 2004; Tay *et al.*, 2003; Peng *et al.*, 2003). Em relação às alterações genômicas focais, foram observadas amplificações nos genes *FGFR2*, *ERBB2*, *EGFR*, *MET*, *KRAS*, *MYC* e *CCND1* (previamente descritos amplificados no câncer gástrico) e deleções nos genes *FHIT*, *RB1*, *CDKN2A/B* e *WWOX* e em genes nunca reportados anteriormente em câncer gástrico, incluindo *PARK2*, *PDE4D*, *PTPRD*, *CSMD1* e *GMDS*.

Cheng *et al.* (2012) também realizaram uma análise genômica do número de cópias no câncer gástrico e identificaram divergências moleculares para os diferentes subtipos histológicos e para os diferentes estágios de TNM. Por exemplo, foi observado que as perdas em 4p16.1, 4p14, 4q13.2, 5q21.1, 9q21.13, 9q22.31, 10q22.1, 12q15, 14q24.2, 22q11.21 e 22q12.2 apresentaram-se significativamente mais frequentes nas classificações T3 e T4.

A integração dos dados de alterações no número de cópias e de expressão gênica e proteica tem sido importante no estudo dos mecanismos moleculares relacionados ao desenvolvimento e à progressão do câncer gástrico. Além disso, investigar o perfil do número de cópias de DNA pode ser muito útil na identificação de novas drogas, bem como na melhoria da avaliação diagnóstica e prognóstica da doença (Cheng *et al.*, 2012; Fan *et al.*, 2012; Kim *et al.*, 2012; Junnila *et al.*, 2010; Myllykangas *et al.*, 2008; Tsukamoto *et al.*, 2008; Yang *et al.*, 2007).

1.5 BIOMARCADORES DE PROGNÓSTICO NO CÂNCER GÁSTRICO

Um biomarcador é definido como uma característica mensurável que pode ser utilizada como indicadora de processos biológicos normais, de processos patogênicos e de respostas farmacológicas a uma intervenção terapêutica específica (*Biomarkers Definitions Working Group*, 2001). Biomarcadores podem ser determinados a partir da análise de material genético e proteínas de diversos materiais biológicos, por exemplo, de fluidos corporais facilmente obtidos, tais como plasma, soro e urina, assim como de tecidos, que necessitam de técnicas mais invasivas para sua obtenção (Oldenhuis *et al.*, 2008).

Biomarcadores tumorais são moléculas biológicas que sugerem a presença de câncer em um paciente ou caracterizam tumores já diagnosticados e que podem ser produzidas pelo próprio tumor ou pelo corpo em resposta ao tumor (Shaw *et al.*, 2015). Esses biomarcadores podem ser subcategorizados em biomarcadores de diagnóstico (que determinam a presença de um tipo de câncer), de prognóstico (que geram informações sobre os efeitos das características do paciente ou do tumor no seu quadro clínico) e preditivos (que ajudam na identificação do tratamento mais adequado para o paciente, levando em consideração suas peculiaridades genéticas) (Italiano, 2011; Madu & Lu, 2010; Oldenhuis *et al.*, 2008). Biomarcadores de prognóstico podem ser úteis na seleção de pacientes para um determinado tratamento, mesmo que não sejam capazes de predizer a resposta a esse tratamento (Oldenhuis *et al.*, 2008).

Assim, a utilização de biomarcadores para a classificação tumoral permite que pacientes com o mesmo tipo tumoral, localização e comorbidades recebam uma estimativa individualizada de prognóstico e tratamento, levando em consideração o perfil molecular de seus tumores (Shaw *et al.*, 2015).

Em relação ao câncer gástrico, com exceção do trastuzumab (quimioterápico utilizado em pacientes com superexpressão da proteína HER2 e/ou amplificação do gene *ERBB2*), a quimioterapia de tumores localizados e avançados ainda não é baseada nas características genotípicas do tumor (Warneke *et al.*, 2013; Bang *et al.*, 2010).

Nos últimos anos, diversos estudos têm investigado a base molecular do câncer gástrico, incluindo sua correlação com a patogênese, a invasão e a metástase. Com o desenvolvimento de tecnologias modernas, vários potenciais biomarcadores com valor prognóstico e diagnóstico para o câncer gástrico têm sido encontrados (Jin *et al.*, 2015).

De acordo com Amemiya *et al.* (2002) os mecanismos que levam à metástase hepática podem estar associados com a alta frequência de superexpressão de c-Met no câncer gástrico. Similarmente, Graziano *et al.* (2011) identificaram aproximadamente 10% dos pacientes com um aumento do número de cópias do gene *MET* (5 ou mais cópias) correlacionada significativamente com prognóstico desfavorável.

Um estudo com o inibidor de MET, AMG 337, em 13 pacientes com amplificação de *MET* em amostras de adenocarcinoma gastroesofágico, concluiu que este inibidor constiste em um promissor quimioterápico para esta neoplasia, uma vez que 8 dos pacientes apresentaram respostas parciais ou praticamente completas ao fazer tratamento com este composto (Kwak *et al.*, 2015).

Mutações no gene *PIK3CA* têm sido observadas no câncer gástrico, sendo relacionadas com a ativação constitutiva da via de sinalização PI3K/mTOR. Estudos têm reportado uma frequência de mutações que varia de 5 a 25% (Shi *et al.*, 2012; Lee *et al.*, 2012; Barbi *et al.*, 2010; Velho *et al.*, 2005; Samuels *et al.*, 2004). Adicionalmente, Shi *et al.* (2012) observaram a amplificação de *PI3KCA* em 67% das amostras de câncer gástrico associada a um pior prognóstico. A ativação da via PI3K/mTOR no câncer gástrico foi demonstrada em estudos pré-clínicos (Taguchi *et al.*, 2011; Lang *et al.*, 2007) e sua desregulação foi associada com metástase linfonodal e diminuição da sobrevida em pacientes com câncer gástrico (Xu *et al.*, 2010; Yu *et al.*, 2009).

A amplificação do gene *FGFR2* já foi reportada em diversos estudos, sendo considerada um biomarcador de diminuição da sobrevida global. Bai *et al.* (2010) encontraram uma associação significativa entre a amplificação deste gene e o aumento da proliferação e da sobrevivência de linhagens celulares de câncer gástrico. Kilgour *et al.* (2012) observaram uma amplificação deste gene em 5,9% dos pacientes correlacionada significativamente com metástase linfonodal. Similarmente, Jung *et al.* (2012) demonstraram a presença da amplificação deste gene em 4,5% dos pacientes associada significativamente com estadiamento avançado e diminuição da sobrevida global. O gene *FGFR* é atualmente um alvo de interesse para o tratamento de câncer gástrico e inibidores de FGFR têm sido desenvolvidos (dovitinib e AZD4547) (Xie *et al.*, 2013; Deng *et al.*, 2012).

Lin *et al.* (1999) e Wu *et al.* (2000) identificaram os genes *TIE-1* e *MKK4* como alvos moleculares para a avaliação prognóstica no câncer gástrico. De acordo com Lin *et al.*

(1999), a expressão de TIE-1 é um fator independente que afeta a sobrevida dos pacientes com câncer gástrico. Os resultados de Wu *et al.* (2000), por sua vez, demonstraram que o gene *MKK4* é um poderoso e independente fator prognóstico para a progressão do câncer gástrico, especialmente nos estágios mais avançados do desenvolvimento da doença.

Tomioka *et al.* (2010) realizaram uma análise em larga escala, utilizando a técnica de aCGH em amostras de câncer gástrico, e identificaram quatro loci relacionados ao prognóstico da doença: 6q21 (contendo o gene *FOXO3A*), 9q32 (contendo o gene *UGCG*), 17q21.1-121.2 (contendo o gene *CASC3*) e 17q21.32 (contendo os genes *HOXB3-9*). Suas análises demonstraram que a deleção de pelo menos um desses quatro loci está associada com a piora significativa do prognóstico do paciente.

Como citado anteriormente, a instabilidade cromossômica geralmente é considerada um fator de mau prognóstico para o câncer gástrico (Wiksten *et al.*, 2008). Nesse contexto, a perda de heterozigose de um único braço de alguns cromossomos já foi associada com a diminuição da sobrevida (Suzuki *et al.*, 2003), bem como com a progressão tumoral (Kimura *et al.*, 2004) e presença de metástase linfononal no câncer gástrico (Buffart *et al.*, 2009). Isso se deve ao fato de que esses segmentos cromossômicos incluem genes que estão fortemente associados com a carcinogênese, tais como o *TP53*, o *APC* e o *DCC* (Bamias *et al.*, 2003). Diversos estudos na literatura correlacionaram a perda de diferentes partes de cromossomos com um pior prognóstico do paciente com câncer gástrico (Burbano *et al.*, 2006; Calcagno *et al.*, 2006; French *et al.*, 2004; Koo *et al.*, 2004; Kitayama *et al.*, 2003; Weiss *et al.*, 2003).

É notória a necessidade de identificação de marcadores que auxiliem no diagnóstico ou na avaliação prognóstica, bem como na elaboração de novas abordagens terapêuticas aplicáveis ao câncer gástrico, em função da baixa eficácia de terapias atualmente disponíveis e do diagnóstico tardio, muitas vezes associado a um mau prognóstico.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar alterações genômicas quantitativas em amostras de adenocarcinoma gástrico, com o intuito de identificar potenciais marcadores do processo de carcinogênese.

2.2 OBJETIVOS ESPECÍFICOS

- Validar as alterações no número de cópias observadas no adenocarcinoma gástrico em amostras de carcinoma epidermoide oral;
- Correlacionar individualmente as alterações encontradas com os dados clínico-patológicos dos adenocarcinomas gástricos estudados;
- Avaliar o efeito sinérgico das alterações correlacionado com os dados clínico-patológicos dos adenocarcinomas gástricos estudados;

3 CAPÍTULO I

HIGH-DENSITY ARRAY COMPARATIVE GENOMIC HYBRIDIZATION DETECTS NOVEL COPY NUMBER ALTERATIONS IN GASTRIC ADENOCARCINOMA

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High-density Array Comparative Genomic Hybridization Detects Novel Copy Number Alterations in Gastric Adenocarcinoma

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Abstract. *Aim: To investigate frequent quantitative alterations of intestinal-type gastric adenocarcinoma. Materials and Methods: We analyzed genome-wide DNA copy numbers of 22 samples and using CytoScan® HD Array. Results: We identified 22 gene alterations that to the best of our knowledge have not been described for gastric cancer, including of v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4 (ERBB4), SRY (sex determining region Y)-box 6 (SOX6), regulator of telomere elongation helicase 1 (RTEL1) and UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5 (B4GALT5). The most significant alterations related to peritoneal invasion involved the regions 13q21.1 (gain) and 15q15.1, 17q23.1, 19q13.2 and 20q11.22 (loss of heterozygozity; LOH), where we found LOH of erythrocyte membrane protein band 4.1-like 1 (EPB41L1) gene. In relation to early age of onset, the most significant alterations were gains in the regions Xq26 and Xp22.31 and a loss in the region 11p15.4. Conclusion: These quantitative changes may play a role in the development of this type of neoplasia and may be used as markers in evaluating poor prognosis, as well as act as potential therapeutic targets for gastric cancer.*

Gastric cancer is the fourth most frequent type of cancer (1, 2) and the second cause of cancer mortality worldwide (3). In Northern Brazil, excluding non-melanoma skin cancer, gastric cancer is the second most frequent cancer in men and the third in women (4). The state of Pará has a high incidence of gastric adenocarcinoma and this disease is a public health problem, since mortality rates are above the Brazilian average (5).

This tumor can be classified into two histological types, intestinal and diffuse, according to Laurén (4, 6, 7). The intestinal type predominates in high-risk areas, such as Brazil, and arises from precursor lesions, whereas the diffuse type has a similar distribution in high- and low-risk areas and generally no precursor lesions are identified (8, 9).

Many of these tumors can exhibit features related to aggressiveness and poor outcome, such as early onset (less than 50 years old) (10) and peritoneal invasion (T4 stage), which leads to peritoneal carcinomatosis (11), a disease with a median survival of less than one year with systemic chemotherapy (12). The comprehension of such fundamental processes is very important in reducing morbidity and mortality rates associated with this neoplasia.

The majority of intestinal gastric adenocarcinomas, like other solid tumors, exhibit defects in the maintenance of genome stability, resulting in many DNA copy number alterations that can be analyzed in a genomic approach by array-comparative genomic hybridization (aCGH) (13).

These assays, mainly high-density ones, are a powerful high-throughput technology of molecular cytogenetics for detecting chromosomal copy number aberrations in cancer, aiming at identifying related critical genes from the affected genomic regions (14).

The majority of microarray studies examining gastric adenocarcinoma aim at developing exploratory gene profiles of gastric tumor or gastric cancer cell lines to identify gastric cancer-related genes, delineate molecular phenotypes, demonstrate tumor subtypes, and identify functional gene clusters as potential markers of biological behavior (15-21).

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Table I. *Clinicopathological data and genetic alterations of patients.*

Sample	Gender	Age, years	Localization	TNM/Stage	Laurén classification	No. of genes altered			Mapd*
						Gain	Loss	LOH	
A	Female	46	Antrum and corpus	T3N3a/IIIB	Intestinal	24	337	1	0.22
B	Male	49	Corpus	T3N3b/IIIB	Intestinal	467	1037	5	0.162
C	Male	47	Antrum and pilorus	T3N2/IIIA	Intestinal	136	15	5	0.138
D	Male	49	Antrum and pilorus	T4bN3aM1/IV	Intestinal	355	148	10	0.159
E	Male	35	Antrum	T4bN2/IIIB	Intestinal	314	384	54	0.184
F	Female	36	Antrum, pilorus and corpus	T4aN2/IIIB	Intestinal	9	149	5	0.158
G	Female	50	Antrum	T4N3a/IIIA	Intestinal	122	12	4	0.148
H	Male	35	Corpus	T4bN3aM1/IV	Intestinal	381	144	8	0.152
I	Male	73	Antrum	T4bN2/IIIC	Intestinal	509	470	59	0.162
J	Male	63	Corpus	T4bN3a/IIIC	Intestinal	63	31	5	0.159
K	Male	63	Antrum	T2N1/IIA	Intestinal	264	274	8	0.19
L	Male	61	Corpus	pT4aN3bM1/IV	Intestinal	21	142	12	0.184
M	Male	66	Antrum and pilorus	T3N2/IIIA	Intestinal	316	376	6	0.146
N	Female	55	Antrum and corpus	T3N2/IIIA	Intestinal	8	11	3	0.15
O	Male	52	Corpus	T4aN2/IIIB	Intestinal	48	80	3	0.212
P	Male	68	Corpus	T3N1/IIB	Intestinal	448	500	6	0.156
Q	Male	67	All stomach	T4bN3a/IIIC	Intestinal	451	761	54	0.146
R	Male	64	Corpus and fundus	T4aN3aM1/IV	Intestinal	42	12	4	0.156
S	Male	55	Antrum	T4bN3M1/IV	Intestinal	356	95	14	0.183
T	Male	65	Antrum	T3N2/IIIA	Intestinal	12	30	8	0.156
U	Female	61	Corpus	T3N3a/IIIB	Intestinal	25	25	2	0.157
V	Female	51	Antrum and pilorus	T4bN3b/IIIC	Intestinal	81	1108	4	0.183
Mean						202.4	279.1	12.7	
Standard deviation						182.1	325.1	17.8	

LOH: Loss of heterozygosity. *Quality control for copy number analisys that should be less than 0.25.

Therefore, the objective of this study was to investigate through a high-density aCGH technique, in virtually all quantitative alterations of genome, the most frequent alterations of intestinal gastric adenocarcinoma in an attempt to identify genes that may play critical roles in the carcinogenesis of intestinal-type gastric cancer.

Materials and Methods

Samples. We analyzed 22 samples from patients with intestinal gastric adenocarcinoma, obtained from primary gastric tumors from the João de Barros Barreto University Hospital (HUJBB), located in Pará State, Brazil.

All samples were obtained before administration of chemical treatments or radiotherapy and all individuals signed a Consent Form allowing the use of biological samples and clinical data.

Histopathology. Histopathological data, such as histological sub-type, degree of differentiation, depth of invasion, lymph node involvement and distant metastasis, were taken from pathology reports of the Department of Pathology of HUJBB. The histopathological analysis of tumor fragments was performed according to Laurén's classification (6).

DNA extraction. Genomic DNA extraction was performed using Gentra Puregene Kit (Qiagen®, Germantown, MD, USA), according to the manufacturer's instructions. The CytoScan™ Assay (Affymetrix, Santa Clara, CA, USA) requires a genomic DNA concentration of 50 ng/μl or greater. Therefore, the volume for each sample was adjusted accordingly to achieve the desired concentration, using Low EDTA TE buffer (Affymetrix, Santa Clara, CA, USA).

Array comparative genomic hybridization. We performed high-density microarray analyses through the Affymetrix® CytoScan™ HD Array platform, evaluating the complete genome of all 22 patients. This assay uses over 750,000 Single Nucleotide Polymorphisms probes and 1.9 million non-polymorphic copy number probes with a median spacing of 1.1 kb.

The standard protocol has eight general procedures until scanning: digestion of genomic DNA, ligation of *Nsp*I adapter, amplification of fragments by polymerase chain reaction (PCR), fragmentation of PCR products, labeling, hybridization, washing, staining and scanning.

Firstly, genomic DNA was digested by the *Nsp*I restriction enzyme, then the digested samples were ligated using the *Nsp*I adaptor. The fragments were amplified by PCR and then run on a 2% agarose gel to verify if the PCR product distribution was between 150 bp to 2000 bp.

After PCR product purification and dilution, we performed the quantitation of each sample using a Nanodrop® 1000 Spectophotometer (NanoDrop Technologies, Houston, TX, USA). The average purification yield for each sample was ≥3.0 μg/μl.

The purified samples were then fragmented using DNAse I enzyme, then the products were ran on a 4% agarose gel to verify if the majority of fragment distribution was between 25 to 125 bp.

Labeling was performed using terminal deoxynucleotidyl transferase (TdT) enzyme, which added biotinylated nucleotides at the 3' end of fragmented samples.

During hybridization, each sample was hybridized onto a CytoScan® HD Array and placed in a GeneChip® Hybridization Oven 640 at 50˚C and 60 rpm for 16 to 18 hours.

The processes prior to scanning of arrays, washing and staining, were carried out at a Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA). The arrays were scanned using GeneChip® Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA).

The copy number was deduced from the weighted $\log_2$ ratio and the aberration type was identified and confirmed using the allelic plot.

Statistical analysis. The analysis of copy number variation was performed using Affymetrix® Chromosome Analysis Suite Software v1.2.1 - ChAS (Affymetrix, Santa Clara, CA, USA). The association of results with clinicopathological data of the patients were assessed by Fisher's exact test, using the statistical program BioEstat® v5.0 (22). *p*-Values of ≤0.05 were considered significant.

Results

All samples showed multiple gains, losses and loss of heterozygosity (LOH) (Table I). The most frequent alterations observed in patients were amplifications involving 8q (55.5%), 20q (55.5%), 17q (50%), 1q (41%), 7p (41%), 6p (36.4%), 5p (36.4%), 13q (36.4%), 3q (32%), 7q (32%) e 20p (32%); deletions involving 3p (55.5%), 6q (50%), 2q (50%), 1p (45,5%), 5q (41%), 9p (36,4%), Xq (32%) and Xp (27.3%); and LOH involving 1q (36.4%) and 16p (77.3%).

In relation to the most frequent alterations, we found 29 genes that were altered in at least 50% of patients and which are described in literature as being correlated to carcinogenesis of many types of cancers (23-49), except for *KIAA0125* that has never been cited in cancer literature (Table II). It is noteworthy, 22 of these alterations have not been associated with gastric cancer.

Regarding the comparison between the clinicopathological data, stage (T4 *versus* T1-T3) and age (>50 *versus* ≤50 years old), we found a large number of significantly altered genes (Tables III and IV). We did not find significant results correlating any other clinicopathological data.

The most significant alterations related to peritoneal invasion (observed exclusively in T4 stage, p=0.023) involved the regions 13q21.1 (gain), 15q15.1 (LOH), 17q23.1 (LOH), 19q13.2 (LOH) and 20q11.22 (LOH). The majority of genes found inside these regions have not been described in cancer literature, however, among the alterations, we found LOH of erythrocyte membrane protein band 4.1-like 1 (*EPB41L1*), located at 20q11.22, which is well-discussed in cancer literature and is correlated with aggressiveness of other tumor types (50-51).

Table II. *The most frequent genetic alterations found in intestinal-type gastric cancer samples (n=22).*

Gene	Localization	Copy number state	N (%)
*TP53TG3B**	16p11.2	LOH	18 (82%)
*TP53TG3**	16p11.2	LOH	18 (82%)
*ZNF267**	16p11.2	LOH	18 (82%)
*ERBB4**	2q34	1	16 (73%)
FHIT	3p14.2	1	13 (59%)
*LUZP2**	11p14.3	1	13 (59%)
*CDH8**	16q21	1	13 (59%)
LRP1B	2q22.2	1	12 (54.5%)
*GBE1**	3p12.2	1	12 (54.5%)
ROBO2	3p12.3	1	12 (54.5%)
*ADAM3A**	8p11.22	1	12 (54.5%)
*NRG3**	10q23.1	1	12 (54.5%)
*SOX6**	11p15.1	1	12 (54.5%)
*GPC5**	13q31.3	1	12 (54.5%)
*KIAA0125**	14q32.33	3	13 (59%)
*ADAM6**	14q32.33	4	13 (59%)
*RTEL1**	20q13.33	3	11 (50%)
TNFRSF6B	20q13.33	3	11 (50%)
*ZGPAT**	20q13.33	3	11 (50%)
*SLC2A4RG**	20q13.33	3	11 (50%)
*ZBTB46**	20q13.33	3	11 (50%)
*TPD52L2**	20q13.33	3	11 (50%)
*PRPF6**	20q13.33	3	11 (50%)
SOX18	20q13.33	3	11 (50%)
*ASXL1**	20q13.33	3	11 (50%)
*RGS19**	20q13.33	3	11 (50%)
*B4GALT5**	20q13.13	3	11 (50%)
CYP24A1	20q13.2	3	11 (50%)
PTPN1	20q13.13	3	11 (50%)

LOH: Loss of heterozygosity. *Alterations that have never been described in gastric cancer.

In relation to early age of onset, the most significant alterations, found in patients aged 50 years or less, were a gain in the regions Xq26 (cancer/testis antigen family 45, member A4 - CT45A4, p=0.0096), Xp22.31 (steroid sulfatase (microsomal), isozyme S - STS, p=0.0096) and a loss in the region 11p15.4 (olfactory receptor, family 52, subfamily N, member 5 - *OR52N5* and *OR52N1*, p=0.0023). However, to our knowledge, there are no studies in literature correlating these genes with carcinogenesis.

Moreover, we found amplification of the genes ubiquitin B (*UBB*) and transient receptor potential cation channel, subfamily V, member 2 (*TRPV2*) (p=0.0364) in patients aged 50 years old or less, which although not among the most significantly altered genes, are correlated is some studies with progression of carcinogenesis (52-56), which leads us to suspect that these alterations may play a key role in the development of this neoplasia in younger individuals, where gastric cancer is not as common.

Table III. *Alterations differentially observed in the patients with T4 tumor versus those with T1-T3 tumor.*

Gene	Localization	Copy number state	No. of cases		*p*-Value
			T4	T1-T3	
ABCA13	7p12.3	3	5	0	0.049
BAIAP2L1, BRI3	7q21.3	3	5	0	0.049
C7orf69	7p12.3	3	5	0	0.049
CD36, GNAI1	7q21.11	4	5	0	0.049
GLI3	7p14.1	3	5	0	0.049
CHRAC1	8q24.3	3	5	0	0.049
ALG5, C13orf36, CCNA1, CSNK1A1L, EXOSC8, FAM48A, RFXAP, SMAD9, SPG20	13q13.3	3	5	0	0.049
ATP8A2, CDK8, GPR12, RNF6, SHISA2, WASF3	13q12.13	3	5	0	0.049
C13orf15	13q14.11	3	5	0	0.049
C13orf23	13q13.3	3	5	0	0.049
CKAP2	13q14.3	3	5	0	0.049
COG6	13q13.3	3	5	0	0.049
ENOX1	13q14.11	3	5	0	0.049
FREM2	13q13.3	3	5	0	0.049
GTF3A	13q12.13	3	5	0	0.049
GUCY1B2	13q14.3	3	5	0	0.049
HMGB1	13q12.3	3	5	0	0.049
HNRNPA1L2	13q14.3	3	5	0	0.049
KIAA0564	13q14.11	3	5	0	0.049
LECT1	13q14.3	3	5	0	0.049
LHFP	13q13.3	3	5	0	0.049
LOC100188949	13q12.3	3	5	0	0.049
MIR4305	13q13.3	3	5	0	0.049
MIR548F5	13q13.3	3	5	0	0.049
MIR759	13q14.3	3	5	0	0.049
MTIF3	13q12.2	3	5	0	0.049
MTMR6	13q12.13	3	5	0	0.049
MTUS2	13q12.3	3	5	0	0.049
NAA16	13q14.11	3	5	0	0.049
NBEA	13q13.2	3	5	0	0.049
NUPL1	13q12.13	3	5	0	0.049
OLFM4	13q14.3	3	5	0	0.049
OR7E37P	13q14.11	3	5	0	0.049
PCDH8	13q14.3	3	5	0	0.049
PDX1	13q12.2	3	5	0	0.049
POLR1D	13q12.2	3	5	0	0.049
PRR20E, PRR20B, PRR20A, PRR20D, PRR20C	13q21.1	3	6	0	0.023
RFC3	13q13.2	3	5	0	0.049
SERP2	13q14.11	3	5	0	0.049
SLC7A1	13q12.3	3	5	0	0.049
SPERT	13q14.13	3	5	0	0.049
STOML3	13q13.3	3	5	0	0.049
SUGT1	13q14.3	3	5	0	0.049
THSD1	13q14.3	3	5	0	0.049
TPTE2P3	13q14.3	3	5	0	0.049
TRPC4	13q13.3	3	5	0	0.049
TSC22D1	13q14.11	3	5	0	0.049
USPL1	13q12.3	3	5	0	0.049
VPS36	13q14.3	3	5	0	0.049
KIAA0125, ADAM6	14q32.33	3	0	6	0.0011
NCRNA00226	14q32.33	3	0	4	0.0172
PCSK5	9q21.13	1	0	4	0.0172
ARNT, SETDB1	1q21.3	LOH	5	0	0.049
CAPN3, GANC, ZFP106	15q15.1	LOH	5	0	0.049
HAUS2, LRRC57	15q15.1	LOH	6	0	0.023
APPBP2, BCAS3, CA4, CLTC, DHX40, DHX40P1, HEATR6, LOC645638, LOC653653, MIR21, PPM1D, PTRH2, RPS6KB1, SCARNA20, RNFT1, TMEM49, TUBD1, USP32	17q23.1	LOH	6	0	0.023
BZRAP1, C17orf47, C17orf64, C17orf71, HSF5, LPO, MIR301A, MIR454, MKS1, MPO, MTMR4, PPM1E, PRR11, RAD51C, RNF43, SEPT4, SKA2, SUPT4H1, TEX14, TRIM37, YPEL2	17q22	LOH	5	0	0.049
CEACAM5, CEACAM6, CEACAM3, LYPD4, CD79A, ARHGEF1, RABAC1, ATP1A3, GRIK5, ZNF574, POU2F2, MIR4323, DEDD2, ZNF526, GSK3A, ERF, CIC, PAFAH1B3, PRR19, TMEM145, MEGF8, CNFN, LIPE, CXCL17, CEACAM1, CEACAM8	19q13.2	LOH	6	0	0.023
PSG1, PSG10, PSG3, PSG6, PSG7, PSG8	19q13.2	LOH	5	0	0.049
PSMC4	19q13.2	LOH	5	0	0.049
CPNE1, RBM12, NFS1, ROMO1, RBM39, PHF20, SCAND1, C20orf152, LOC647979, EPB41L1, C20orf4, DLGAP4, MYL9, TGIF2, C20orf24, SLA2, NDRG3, DSN1, C20orf117	20q11.22	LOH	6	0	0.023

LOH: Loss of heterozygosity.

Discussion

Although gastric cancer is a highly lethal global disease, the causes are not entirely known. What is clear is that gastric cancer initiation and progression are the outcomes of a stepwise accumulation of genetic alterations. Among these, gene amplification and aberrant expression of oncogenic proteins, as well as deletion or inactivation of tumor-suppressor genes, represent hallmark steps (57-61).

Table IV. *Alterations differentially observed in the patients ≤50 years old versus those >50 years old.*

Gene	Localization	Copy number state	Age		*p*-Value
			≤50 Years	>50 Years	
LOC340094	5p15.33	3	3	0	0.0364
PLEKHG4B	5p15.33	3	4	1	0.0393
AQPEP	5q23.1	1	3	0	0.0364
CEP120	5q23.2	1	3	0	0.0364
LOC644100	5q23.1	1	3	0	0.0364
OR52N5, OR52N1 UBB, TRPV2, NCRNA00188, SNORD49B, SNORD49A, SNORD65,	11p15.4	1	6	1	0.0023
C17orf76	17p11.2	3	3	0	0.0364
CT45A4	Xq26.3	3	4	0	0.0096
CXorf56	Xq24	3	3	0	0.0364
CYLC1	Xq21.1	3	3	0	0.0364
DACH2	Xq21.2	3	3	0	0.0364
KIAA2022	Xq13.3	3	3	0	0.0364
MIR651	Xp22.31	3	3	0	0.0364
PNPLA4	Xp22.31	3	3	0	0.0364
SEPT6	Xq24	3	3	0	0.0364
SMS	Xp22.11	3	3	0	0.0364
STS	Xp22.31	3	4	0	0.0096

In the present study, we identified 29 frequently altered genes in a cohort of patients with intestinal gastric cancer using a high-density aCGH method (Table II). Among them, we highlighted the 22 alterations that to our knowledge have never been described in gastric cancer, however, we chose to discuss only the alterations in v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4 (*ERBB4*), SRY (sex determining region Y)-box 6 (*SOX6*), regulator of telomere elongation helicase 1 (*RTEL1*) and UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5 (*B4GALT5*) genes, since there is not sufficient literature regarding the other alterations to develop a consistent discussion.

Although there are many studies regarding *ERBB4* as an oncogene, including of gastric cancer (62-65), we found that 73% of patients had a deletion of this gene. It is noteworthy that *ERBB4* has a controversial role in carcinogenesis, since some studies have reported a tumor-suppressor function of this gene in breast cancer (66, 67). Suo *et al*. demonstrated that ERBB4 expression was associated with favorable outcome in a study of 100 patients with mammary carcinoma, in contrast to ERBB2 (68). Similarly, Witton *et al*., in an analysis of 220 primary breast cancer biopsies, stated that, unlike epidermal growth factor receptor (EGFR), ERBB2 and ERBB3 overexpression, ERBB4 overexpression was associated with estrogen-receptor-positive, lower grade, and significantly better outcome (69).

In a study of 129 cases of ductal carcinoma *in situ*, the absence of ERBB4 predicted recurrence within a 5-year-period, and co-expression of ERBB2 and ERBB4 resulted in a lower risk of recurrence that expression of ERBB2 alone (70).

Taken together, these results support a possible association of ERBB4 overexpression with favorable outcome in breast cancer, and underexpression with a more aggressive tumor phenotype (71). Despite there being no studies showing a tumor-suppressor function of *ERBB4* in gastric cancer, our results, in accordance with the studies cited above, gives new insight of the role of *ERBB4* in intestinal gastric carcinogenesis, which deserves considerably better investigation.

Additionally, we observed a copy number loss of *SOX6* gene in 54.5% of the samples and recent studies have demonstrated that *SOX6* functions either as a tumor suppressor or as an oncogene in different types of human cancer (72). The aberrant expression of *SOX6* has been demonstrated to be involved in tumorigenesis and tumor progression in esophageal squamous cell carcinoma, endometrial cancer, glioma (72-74) and hepatocellular carcinoma (42).

In a recent study, Guo *et al*. observed that both SOX6 mRNA and protein levels were significantly decreased in hepatocellular carcinoma tissues compared to adjacent non-neoplastic liver tissues, conferring a poor prognosis in this type of cancer (42). These findings support the hypothesis that *SOX6* may function as a tumor suppressor in hepatocellular carcinoma (42). Additionally, another study revealed that this gene was frequently down-regulated in primary esophageal squamous cell carcinoma (72).

As cited above, this is the first report of *SOX6* loss in gastric cancer and, since this alteration is related to poor prognosis, it is important to better investigate the impact of the loss of this gene in intestinal gastric carcinogenesis.

In our study, the long arm of chromosome 20 was frequently amplified and several studies have reported the occurrence of this alteration in cervical, gastric, prostate, colon, melanoma, bladder, breast and pancreatic cancer (75-82), suggesting that 20q amplification may play a causal role in tumorigenesis.

According to Tabach *et al*., 20q amplification may induce tumor initiation (83), which leads us to suggest that the frequent gain in the 20q arm (55.5% of patients) may be involved in the onset of gastric cancer in these patients, therefore, the study of the genes involved in such amplification is important in order to investigate the potential of novel biomarkers for early diagnosis.

In this context, it is important to note, due the high density of the assay, that we are the first to identify recurrent amplifications of *RTEL1, TNFRSF6B,* zinc finger, CCCH-type with G patch domain (*ZGPAT*), SLC2A4 regulator (*SLC2A4RG*), zinc finger and BTB domain containing 46 (*ZBTB46*), tumor protein D52-like 2 (*TPD52L2*), pre-mRNA processing factor 6 (*PRPF6*), additional sex combs like

transcriptional regulator 1 (*ASXL1*), regulator of G-protein signaling 19 (*RGS19*) and *B4GALT5* genes, located in the 20q region, in association with intestinal gastric carcinogenesis.

Several recent studies have established an essential role of *RTEL1* in the maintenance of telomere length and genomic stability (82, 32). Given that telomere dysfunction is dramatically mutagenic and plays an important role in tumor initiation and progression (83), *RTEL1* up-regulation is expected to have a tumorigenic function.

The *RTEL1* genomic locus (20q13.3) is frequently amplified in several types of human cancers, including gastric cancer (84-88). Wu *et al.* stated that up-regulation of *RTEL1* activity could also be important for tumorigenesis (32).

We also observed that 50% of the samples had a copy number gain in *B4GALT5* gene. In agreement with these results, Scotto *et al.* identified a total of 26 overexpressed genes as a consequence of 20q gain in cervical cancer, including a number of functionally important genes in cell-cycle regulation, such as *B4GALT5* (30). Furthermore, high gene expression was associated with multidrug resistance in patients with leukemia, probably by regulating the hedgehog pathway and the expression of p-glycoprotein and multidrug resistance-associated protein 1 (43).

In summary, these alterations, being the more frequent, may have an important role in the development and progression of intestinal gastric adenocarcinoma in these patients; therefore, it is imperative to carry-out further studies, to understand the consequence of these alterations in the pathogenesis of this type of neoplasia.

Furthermore, we found that amplification of *TRPV2* and *UBB* genes were significantly associated with patients aged 50 years or less and *EPB41L1* gene was significantly associated with peritoneal invasion. Gastric adenocarcinoma has a peak reported incidence in patients aged from 50 to 70 years. Although the prevalence of gastric cancer has decreased gradually during the last 50 years, the overall trend masks important age-specific characteristics, for example, the proportion of young patients is increasing year-on-year (89). According to Zheng *et al.*, gastric cancer in young patients is highly malignant, with a lower rate of curative resection and poorer prognosis (90). Fewer than 10% of patients present with the disease before 45 years of age and these young patients are thought to develop carcinomas with a different molecular genetic profile from that occurring at a later age (91).

Hierarchical cluster analysis of aCGH data on patients with gastric cancer (including young patients) revealed clusters with genomic profiles that correlated significantly with age (92). Gains in chromosomes 17q, 19q and 20q have been found in young patients with CGH (93) and LOH findings have also shown that losses are infrequent in this group of patients (91).

The vanilloid receptor family (TRPV) is a sub-group of the transient receptor potential (TRP) superfamily of ion channels, and six members (TRPV1-6) have so far been identified (94). TRP channels constitute a novel area of research in oncology. Malignant transformation of cells is the result of enhanced proliferation, aberrant differentiation and impaired ability to die, resulting in abnormal tissue growth, which can eventually turn into uncontrolled expansion and invasion, characteristic of cancer. Such transformation is often accompanied by changes in ion channel expression and, consequently, by abnormal progression of the cellular responses with which they are involved (95).

High expression levels of members of the TRPV family were correlated with the emergence or progression of certain types of epithelial cancer, such as prostate cancer and melanoma (52-55).

Monet *et al.* stated that *TRPV2* transcript levels were 12-times higher in patients with metastatic prostate cancer (stage M1) compared to primary solid tumors (stages T2a and T2b) (96). Moreover, silencing of this channel drastically reduced the migration of prostate cancer cells, whereas its overexpression increased their migration. Monet *et al.* also found *TRPV2* contributes to enhanced cancer cell migration by induction of expression of key metalloproteinases MMP2 and MMP9, and cathepsin B, which are related to the invasive potential of cancer cells (96).

It is noteworthy that alterations involving the *TRPV2* gene in gastric carcinogenesis have never been described in literature, although some studies have reported the amplification of the 17p region, where this gene is located (97, 98).

The *UBB* gene encodes ubiquitin, one of the most conserved proteins known. The ubiquitin system is extremely versatile and can play multiple essential roles in various cellular processes by regulating not only protein stability but also protein interactions, trafficking, and activation. Therefore, it is not surprising that alterations in the ubiquitin system have been observed in many types of human cancers and that many of its components, when de-regulated, have been found to play key roles in cellular processes relevant to tumorigenesis (99, 100).

The elevated level of ubiquitin has been observed in most, if not all, cancer cells (101-105). In addition, a positive relationship between ubiquitin levels and the progression of hepatocellular carcinoma has been reported (106).

Oh *et al.* demonstrated that ubiquitin levels are efficiently reduced by small interfering RNA (siRNA), which effectively inhibited the survival and proliferation of cancer cells (56), suggesting that it has potential as a new therapeutic intervention for cancer treatment.

Therefore, these quantitative changes in the genetic material of tumors may be involved in gastric carcinogenesis, and may have a key role in the development of this neoplasia in younger individuals.

The progression of epithelial tumors to invasive carcinomas involves changes in cell polarity, adhesion and motility that permit the detachment of cancer cells from the epithelial layer, their invasion into adjacent tissue layers, and eventually their spread throughout the body. These processes require reorganization of the cellular cytoskeleton and altered expression of proteins that connect it to the cell membrane as well as remodeling of the extracellular matrix, including changes in the composition and processing of its constituents (107).

The ability of EPB41L1 proteins to bind very structurally diverse interaction partners *via* their different protein domains enables them to participate in many different physiological processes in a variety of cell types and tissues (108). These EPB41L1 proteins contribute to the organization of cell polarity, adhesion, motility, and affect transport through the membrane and responses to growth factors (107). The EPB41L1 protein was detected in various cell types and tissues, however, its functions in non-erythroid cells are not as clear (51).

Zhenyu *et al*. stated that EPB41L1 potentially serves as an inhibitor of migration and invasion by restoring the membrane cytoskeleton (51). Additionally, the expression of EPB41L1 also was moderately, but significantly decreased in prostate cancer tissues (107).

In our study, we frequently found LOH for this gene, which is a clear mechanism for gene inactivation (109). Taken together, these studies and our results suggest that *EPB41L1* may have had an important role in progression and invasion of gastric cancer in the group of patients with peritoneal invasion.

Through the microarray technique, we were able to identify several quantitative changes in the genome of intestinal gastric adenocarcinoma and novel genes associated with gastric carcinogenesis. A better investigation of these findings could provide useful pathway maps for the future understanding of the molecular pathogenesis of this malignancy, which can represent efficient tools in evaluating poor prognosis, as well as potential therapeutic targets for gastric cancer.

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4 CAPÍTULO II

RECURRENT AMPLIFICATION OF RTEL1 AND ABCA13 AND ITS SYNERGISTIC EFFECT ASSOCIATED WITH CLINICOPATHOLOGICAL DATA OF GASTRIC ADENOCARCINOMA

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RECURRENT AMPLIFICATION OF RTEL1 AND ABCA13 AND ITS SYNERGISTIC EFFECT ASSOCIATED WITH CLINICOPATHOLOGICAL DATA OF GASTRIC ADENOCARCINOMA

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Abstract:	Gastric cancer is the fifth most frequent type of cancer and the third cause of cancer mortality worldwide. Despite progression in the diagnosis and treatment of advanced gastric cancer, the prognosis of patients remains poor, in part due to the low rate of diagnosis during its early stages. This paradigm implies in the necessity to search and identify molecular biomarkers for early gastric cancer diagnosis, as well as for disease monitoring, thus contributing to the development of new therapeutic approaches. In a previous study, preformed by array-Comparative Genomic Hybridization, we described for the first time in literature the recurrent amplification of RTEL1 and ABCA13 genes in gastric cancer. Thus, the aim of the present study was to validade the recurrent amplification of RTEL1 and ABCA13 genes observed previously and associate CNV status with clinicopathological data of patients. Results showed RTEL1 and ABCA13 genes amplification in 38% of samples. Statistical analysis showed that RTEL amplification is more common in older patients and more associated with intestinal type and ABCA13 amplification increases the risk of lymph node metastasis and is more common in men. Co-amplification of these genes showed a significative association with advanced stage. Therefore, aCGH is a very useful tool for investigating novel genes associated with carcinogenesis and RTEL1 amplification may be important for the development of gastric cancer in older patients, besides being a probable event contributing for chromosomal instability in intestinal gastric carcinogenesis. Moreover, ABCA13 amplification may be a usefull prognostic biomarker for predicting lymph node metastasis in resected gastric cancer patients in early stage. Lastly, RTEL1 and ABCA13 synergistic effect may be considered as a putative prognostic biomarker for advanced staging in gastric cancer patients.
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Running Title: ***RECURRENT RTEL1 AND ABCA13 AMPLIFICATION IN GASTRIC ADENOCARCINOMA***

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ABSTRACT

Gastric cancer is the fifth most frequent type of cancer and the third cause of cancer mortality worldwide. Despite progression in treatment of advanced gastric cancer, the prognosis of patients remains poor, in part due to the low rate of diagnosis during its early stages. This paradigm implies the necessity to search and identify molecular biomarkers for early gastric cancer diagnosis, as well as for disease monitoring, thus contributing to the development of new therapeutic approaches. In a previous study, preformed by array-Comparative Genomic Hybridization, we described for the first time in literature the recurrent amplification of *RTEL1* and *ABCA13* genes in gastric cancer. Thus, the aim of the present study was to validade the recurrent amplification of *RTEL1* and *ABCA13* genes observed previously and associate CNV status with clinicopathological data of patients. Results showed *RTEL1* and *ABCA13* genes amplification in 38% of samples. Statistical analysis showed that *RTEL* amplification is more common in older patients and more associated with intestinal type and *ABCA13* amplification increases the risk of lymph node metastasis and is more common in men. Co-amplification of these genes showed a significant association with advanced staging. Therefore, aCGH is a very useful tool for investigating novel genes associated with carcinogenesis and *RTEL1* amplification may be important for the development of gastric cancer in older patients, besides being a probable event contributing for chromosomal instability in intestinal gastric carcinogenesis. Moreover, *ABCA13* amplification may be a usefull marker for predicting lymph node metastasis in resected gastric cancer patients in early stage. Lastly, *RTEL1* and *ABCA13* synergistic effect may be considered as a putative marker for advanced staging in gastric cancer patients.

Keywords: Gatric cancer; aCGH; *RTEL1*; *ABCA13*; markers.

INTRODUCTION

Gastric cancer is the fifth most frequent type of cancer and the third cause of cancer mortality worldwide (Ferlay *et al.*, 2013).

The estimate for Brazil (2016 and 2017) indicates the occurrence of about 600,000 new cases of cancer. Except for non-melanoma skin cancer (approximately 180,000 new cases), there will be about 420,000 new cases of cancer. Among all different types of cancer that affect humans, gastric cancer, excluding non-melanoma skin cancer, ranks fourth as the most frequent tumor type in men and fifth in women (INCA, 2016). In the Northern Brazil, excluding non-melanoma skin cancer, gastric cancer is the second most frequent cancer among men and the fourth among women (INCA, 2016).

The incidence rate of gastric cancer have decreased overall in recent years, however, it remains the leading cause of cancer-related mortality in developing countries (Jemal *et al.*, 2011). Despite progression in treatment of advanced gastric cancer, the prognosis of gastric cancer patients remains poor, in part due to the low rate of diagnosis during its early stages (Jin *et al.*, 2015). This paradigm implies in the necessity to search and identify molecular biomarkers for early gastric cancer diagnosis, as well as for disease monitoring, thus contributing to the development of new therapeutic approaches (Wu *et al.*, 2014).

With regard to gastric cancer treatment, with the exception of trastuzumab (therapy based on the overexpression of HER2 protein and/or the amplification of its gene *ERBB2*), chemotherapy of localized and advanced gastric cancer still does not consider genotypic tumor characteristics. This implies that part of the patients, if not the majority, receives medical treatment with suboptimal or even lacking efficacy (Warneke *et al.*, 2013; Bang *et al.*, 2010).

Recently, numerous studies have investigated the molecular basis of gastric cancer involving the alteration of pathogenesis, including the mechanisms of invasion and metastasis. With the development of modern technologies, various novel biomarkers had been identified that appear to possess diagnostic and prognostic value (Jin *et al.*, 2015). Among the types of biomarkers, copy number variation (CNV) in key genes has been found in gastric cancer (Buffart *et al.*, 2007; Kang *et al.*, 2006; Weiss *et al.* 2004).

In a previous study, preformed by array-Comparative Genomic Hybridization (aCGH), we observed genomic alterations in 22 patients and described for the first time in literature the recurrent amplification of *RTEL1* (*Regulator of Telomere Length 1*) and *ABCA13* (*ATP-*

Binding Cassette, Sub-Family A, Member 13) genes in gastric cancer, with a frequency of 50% and 27.5%, respectively (Seabra *et al.*, 2016).

RTEL1 is an essential helicase that has been demonstrated to be required for the maintenance of telomere length and genomic stability. Thus, RTEL1 dysfunction is dramatically mutagenic and plays an important role in tumor initiation and progression (Wu *et al.*, 2012; Barber *et al.*, 2008).

ABCA13 gene is a member of ABC (ATP-binding casset) family of transporters that plays a crucial role in the development of resistance by the efflux of anticancer agents outside of cancer cells (Hlavata *et al.*, 2012). Recently, several studies have associated overexpression of *ABCA13* with poor prognosis of cancer (Nymoen *et al.*, 2015; Hlaváč *et al.*, 2013).

The present study evaluated the copy number status of these genes and identified a significant association between gene amplification and co-amplification with clinicopathological data of patients with gastric cancer.

OBJECTIVE

The aim of the present study was to validade the recurrent amplification of *RTEL1* and *ABCA13* genes observed previously by aCGH and associate CNV status with clinicopathological data of patients.

MATERIAL AND METHODS

Samples

We analyzed gastric adenocarcinoma samples obtained from primary gastric tumors of patients from João de Barros Barreto University Hospital (HUJBB), located in Pará State, Brazil.

All samples were obtained before administration of chemical treatments or radiotherapy. This study was approved by HUJBB ethics committee (CAAE: 42999115.7.0000.5634) and all individuals signed a Consent Form allowing the use of biological samples and clinical data.

For *RTEL1* copy number investigation we used 125 samples, 68% from male and 32% from female patients, with a mean age of 59 years (± 13). Regarding tumor site, 80% were obtained from tumors located outside from cardia region and 20% of tumors located in

the cardia region. Of the total of samples, 57% were collected from patients with advanced stage (III and IV) and 43% from patients with early stage (I and II), 66% of tumors belonging to the intestinal type and 34% to the diffuse type of Laurén. Also with respect to staging, it was observed that 23% of patients presented serosal extravasation (T4), 76% presented lymph node metastasis and 52% presented distant metastasis.

For *ABCA13* copy number investigation we used 117 samples, 68% from male and 32% from female patients, with a mean age of 59 years (± 13). Regarding tumor site, 79% were obtained from tumors located outside from cardia region and 21% of tumors located in the cardia region. Of the total of samples, 57% were collected from patients with advanced stage (III and IV) and 43% from patients with early stage (I and II), 66% of tumors belonging to the intestinal type and 34% to the diffuse type of Laurén. Also with respect to staging, it was observed that 24% of patients presented serosal extravasation (T4), 78% presented lymph node metastasis and 54% presented distant metastasis.

Additionally, we evaluated CNV status of these genes in 47 oral squamous cell carcinoma samples, in an attempt to investigate if the frequency of *RTEL1* and *ABCA13* amplification is also high in other type of neoplasia. Sample composition was 64% male and 36% female patients, with a mean age of 61 years (± 13). Regarding tumor site, 80% were obtained from tumors located in tongue or floor of mouth. Of the total of samples, 56% were collected from patients with advanced stage (III and IV) and 44% from patients with early stage (I and II).

Histopathology

Histopathological data, such as histological subtype, degree of differentiation, depth of invasion, lymph node involvement and distant metastasis were taken from pathology reports of the Department of Pathology of HUJBB. The histopathological analysis of tumor fragments was performed according to Laurén's classification (Laurén, 1965).

Quantitative analysis of Copy Number Variants based on Real-Time PCR

Briefly, 1 µl of 10 ng DNA was added to 5 µL of TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA), with 0.5 µl of *RTEL1* or *ABCA13* probe and 3 µL of water. We measured copy number gain using the following profile: denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. We

determined relative quantification using the 7500 Rreal-time PCR system (Applied Biosystems, Foster City, CA) in quadruplicate. RNaseP (Applied Biosystems, Foster City, CA) was used as a control. After amplification, we imported the experiment results containing threshold-cycle values for the copy number and reference assay into the CopyCaller Software (Applied Biosystems, Foster City, CA) for post-PCR data analysis as previously described by Graziano *et al.* (2011).

Statistics

Statistical analysis for comparisons of categorical variables between groups were done by means of Chi-square test and were performed using PASW Statistics program. Odds Ratio (OR) and Confidence Interval (CI=95%) were also calculated. A two-tailed probability value $p \leq 0.05$ was considered to be statistically significant.

RESULTS

RTEL1 gene amplification was observed in 38% of samples. Statistical analysis showed that this amplification is 2.6 times more common in patients older than 50 years (p=0.045; 95%CI=1.011-5.128) and 2.3 times more associated with intestinal type (p=0.034; 95%CI=1.057-6.3) (table 1).

ABCA13 gene amplification was also observed in 38% of samples. Statistical analysis showed that *ABCA13* amplification increases 3 times the risk of lymph node metastasis (p=0.033; 95%CI=1.057-9.018). Additionally, this amplification is 4 times more common in men (p=0.003; 95%CI=1.526-9.851) and demonstrated an inconclusive association with intestinal type (p=0.079) (table 1).

Synergistic effect of these two amplifications was also evaluated. The results showed 17% of *RTEL1* and *ABCA13* co-amplification and it was 3 times more associated with advanced stage (III and IV) (p=0.05; 95%CI=0.958-10.037) (table 1).

Table 1: Clinicopathological data of patients with and without *RTEL1* and *ABCA13* amplification.

	***RTEL1* CNV**		***p-value***	***ABCA13* CNV**		***p-value***	***RTEL1* and *ABCA13* amplification**		***p-value***
	≥ 3 copies	Others		≥ 3 copies	Others		present	absent	
Gender									
Male	32	53	0.987	38	42	0.003*	17	61	0.031*
Female	15	25		7	30		2	34	
Age									
≤50 years	8	27	0.034*	10	21	0.408	2	29	0.074
>50 years	39	51		35	51		17	66	
Histopathology									
Intestinal	36	46	0.045*	34	43	0.079	15	61	0.214
Diffuse	11	32		11	29		4	34	
Localization									
Cardia	12	12	0.197	9	15	0.901	4	19	0.969
Non-cardia	35	63		35	55		15	73	
Stage									
I-II	17	37	0.218	16	34	0.215	4	43	0.05*
III-IV	30	41		29	38		15	52	
pN									
N0	9	19	0.395	5	19	0.033*	2	23	0.157
N1 or more	37	53		39	48		17	67	
pT									
T1-T3	34	62	0.359	36	53	0.431	13	74	0.375
T4	13	16		9	19		6	21	
pM									
M0	10	15	0.746	10	13	0.741	4	17	0.360
M1	12	15		13	14		8	18	

M: male; F: female. pN: lymph node metastasis status; N0: without lymph node metastasis; N1 or more: metastasis in one ore more lymph nodes. pT: extent of the primary tumor; T1-T3: without serosal extravasation; T4: with serosal extravasation. pM: distant metastasis status; M1: with distant metastasis; M0: without distant metastasis. Others: 2 copies or less. *Significant difference between groups with and without amplification, p≤0.05, Chi-square test.

Subcategorization of samples into intestinal and diffuse types did not result in any significant clinicopathological association.

It is important to note that we observed 51% and 24% of high-level amplifications (≥5 copies) of *ABCA13* and *RTEL1* genes, respectively, but subdivision of samples into high-

level amplifications and other amplifications (3 and 4 copies) or high-level amplifications and all other (1, 2, 3 and 4 copies) did not result in any significant clinicopathological association.

Regarding oral squamous cell carcinoma, we observed 30% and 25% of *RTEL1* and *ABCA13* amplification, respectively, but the presence of amplification was not statistically associated with clinicopathological data of patients.

DISCUSSION

High amplification frequencies observed in the present study corroborate with the previous study performed by aCGH, indicating that this technique is usefull to investigate CNV and find novel genes associated with diseases, even with a low number of cases.

The region 20q, where *RTEL1* gene is located, is amplified in several types of cancer (Gorringe *et al.*, 2015; Scotto *et al.*, 2008; Burbano *et al.*, 2006), but we were the first group to describe *RTEL1* amplification in gastric cancer (Seabra *et al.*, 2014).

Rtel1 is an essential helicase for telomere maintenance and the regulation of homologous recombination (HR) (Wu *et al.*, 2012). HR is one of the major pathways to maintain genomic stability and is involved in the repair of complex DNA damage, DSBs, interstrand crosslinks and DNA gaps. Thus, upregulated Rtel1 function might prevent HR when it is needed as a legitimate means for repair, leading to malfunction of repair system (Uringa *et al.*, 2011).

Another hypothesis is that the excessive activity of Rtel1 would increase Rtel1 helicase activity, leading to disengaging of T-loop structure and, consequently, to telomere deprotection, which may result in end-to-end fusions and exonucleolytic attack (Bailey and Murnane, 2006; Barber *et al.*, 2008).

Several observations suggested a possible role for *RTEL1* during DNA replication. Mouse cells deficient for *RTEL1* exhibit reduced proliferative capacity, and worms and mammalian cells lacking *RTEL1* are particularly sensitive to DNA damaging agents that hinder DNA replication, such as inter-strand crosslinking agents (Uringa *et al.*, 2012; Barber *et al.*, 2008; Ding *et al.*, 2004).

Wu *et al.* (2012) showed that increased expression of Rtel1 in mouse hepatocytes induced the development of liver tumors. This finding is consistent with human genetic data that showed that amplification of *RTEL1* genomic locus is not only a common genetic

alteration in human hepatocellular carcinoma, but also closely associated with its malignancy and progression (Taniguchi *et al.*, 2010; Katoh *et al.*, 2006; Niketeghad *et al.*, 2001; Guan *et al.*, 2000; Wong *et al.*, 1999).

In this study, we found for the first time recurrent *RTEL1* amplification statistically associated with advanced age and intestinal type of gastric adenocarcinoma. Thus, we suggest that amplification of *RTEL1* gene may has age-specific function and an important role in adenocarcinoma of intestinal type, which corroborates with the hypothesis that these two histological types have different genetic pathways (Tahara, 2004; Yasui *et al.*, 2000).

Consistent with these results, El-Rifai *et al.* (1998) and Kokkola *et al.* (1997) found a significant association between 20q amplification and intestinal type of gastric cancer. Interestingly, a molecular classification of gastric cancer, proposed by Cancer Genome Atlas Research Network (2014), categorized intestinal gastric cancer as correlated with chormosomal instability. Thus, *RTEL1* may be a key gene of 20q region, since its upregulation triggers chromosomal instability (Bailey and Murnane, 2006; Barber *et al.*, 2008).

Noteworthy, we observed that the frequency of *RTEL1* amplification is almost equal in all stages of cancer (I-41%, II-30%, III-44% and IV-44%), corroborating with the hypotesis proposed by Tabach *et al.* (2011) that amplification of the 20q chromosomal arm occurs early in tumorigenic transformation and may initiate cancer.

The human ABC transporters are encoded by a large transporter gene superfamily, which is composed of 49 members grouped into seven subfamilies (A–G) according to the sequence homology. ABC proteins facilitate translocation of heterogeneous substrates including metabolic products, lipids and sterols, peptides and proteins, saccharides, amino acids and drugs across the cell membrane. To transport these substrates across extracellular and intracellular membranes against a concentration gradient, ABCs use energy acquired by the hydrolysis of ATP (Higgins *et al.*, 1992).

ABCA13 is a member of ABC gene subfamily A (ABCA) that plays a crucial role in the development of resistance by the efflux of anticancer agents outside of cancer cells (Hlavata *et al.*, 2012) and overexpression of one or more membrane-bound ATP-binding cassette (ABC) transportes has been associated with such mechanism of drug resistance (Szakács *et al.*, 2006).

There are few studies in literature regarding the role of *ABCA13* in cancer, but they demonstrate a positive association between *ABCA13* upregulation and unfavorable outcomes.

Upregulation of *ABCA12*, *ABCA13*, *ABCB6*, *ABCC1*, *ABCC2* and *ABCE1* genes were found by Hlavata *et al.* (2012) in colorectal cancer samples when compared to normal tissues and Nymoen *et al.* (2015) observed that *ABCA13* mRNA overexpression was significantly related to shorter overall survival in metastatic ovarian serous carcinoma.

Importantly, Hlaváč *et al.* (2013) stated that *ABCA13*, *ABCB3* and *ABCC1* levels were significantly higher in patients with grade 3 than in patients with grade 1 or 2 of breast carcinoma, suggesting that overexpression of these genes may be associated with poor prognosis.

In the present study, we found for the first time recurrent amplification of *ABCA13* statistically associated with lymph node metastasis in gastric carcinogenesis. Therefore, we suggest that amplification of *ABCA13* gene has an important role in development of lymph node metastasis, which is associated with poor outcomes (Deng and Liang, 2014).

CONCLUSION

aCGH is a very useful tool for investigating novel genes associated with carcinogenesis. Through this technique, we were able to identify recurrent amplification of *RTEL1* and *ABCA13* and this observation was validated by real-time PCR for copy number analysis on a larger number of samples and in other type of cancer, demonstrating that these genes may have important roles in the carcinogenesis process. Thus, *RTEL1* amplification may be important for the development of gastric cancer in older patients, besides being a probable event contributing for chromosomal instability in intestinal gastric carcinogenesis. Moreover, *ABCA13* amplification may be a usefull marker for predicting lymph node metastasis in resected gastric cancer patients in early stage. Lastly, *RTEL1* and *ABCA13* synergistic effect may be considered as a putative marker for advanced staging in gastric cancer patients.

Declaration of Interest statement

We declare that we have no conflicts of interest.

Ethics, consent and permissions

The patients consent to participate to this study.

Consent to publish

We have obtained consent from the patients to publish and to report individual data.

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5 CAPÍTULO III

RECURRENT AMPLIFICATION OF B4GALT5 ASSOCIATED WITH INTESTINAL TYPE OF GASTRIC ADENOCARCINOMA

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Title: ***RECURRENT AMPLIFICATION OF B4GALT5 ASSOCIATED WITH INTESTINAL TYPE OF GASTRIC ADENOCARCINOMA***

Running title: ***B4GALT5 AMPLIFICATION IN INTESTINAL GASTRIC CANCER***

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ABSTRACT

Studies in literature have identified copy number variations (CNVs) associated with intestinal gastric cancer, suggesting that molecular pathogenesis of each subtype involves a subset of specific genetic alterations. In a previous study, we described for the first time the recurrent amplification of *B4GALT5* gene in gastric cancer. Thus, the aim of the present study was to validate amplification of *B4GALT5* and associate CNV status with clinicopathological data. Results showed that *B4GALT5* gene is amplified in 34% of samples and is significantly associated with intestinal type, suggesting that its recurrent amplification is a probable event related to intestinal gastric carcinogenesis.

Keywords: Gastric cancer; *B4GALT5*; intestinal type.

INTRODUCTION

Gastric cancer continues to be a major health problem by frequency, aggressiveness and low rate of cure in symptomatic stage. Although its incidence is decreasing, globally the gastric cancer is ranked fifth in incidence and mortality remains high[1,2].

In the Northern Brazil, excluding non-melanoma skin cancer, gastric cancer is the second most frequent cancer among men and the fourth among women[3].

Over the past years the histologic classification of gastric carcinoma has been largely based on Laurén's criteria, in which intestinal type and diffuse type of adenocarcinoma are the two major histologic subtypes[4,5]. The intestinal type is more common in high-risk areas and arises secondary to atrophic gastrites and intestinal metaplasia[6], whereas the diffuse type has a similar distribution in high and low-risk areas and arises independently of intestinal metaplasia[7-9]. Each subtype represents distinct clinical and epidemiologic characteristics[10].

A key feature in the pathogenesis of most solid tumors is chromosomal instability, resulting in gains and losses of parts or even whole chromosomes. DNA copy number variations (CNVs) are common in several cancers and other disease endpoints. Variations in DNA copy number might be an indicator of high risk of gastric cancer in individuals, due to gene-dosage effects[11]. Interestingly, many studies in the literature have identified CNVs associated with intestinal type of gastric cancer, suggesting that molecular pathogenesis of each subtype involves a subset of specific genetic alterations[4,12-14].

In a previous study, preformed by array-Comparative Genomic Hybridization, we described for the first time in literature the recurrent amplification of *B4GALT5* (*UDP-Gal:BetaGlcNAc Beta 1,4- Galactosyltransferase, Polypeptide 5*) gene in gastric cancer[15].

β-1,4-Galactosyltransferase gene (B4GALT) family consists of seven members, which encode corresponding enzymes known as type II membrane-bound glycoproteins. These enzymes catalyze the biosynthesis of different glycoconjugates and saccharide structures, and have been recognized to be involved in various diseases, including cancer[16]. There are few studies in literature regarding the role of *B4GALT5* in cancer, but its overexpression has been associated with unfavorable outcome and multidrug resistance[16,17].

The present study evaluated the copy number status of this gene and identified a significant association between gene amplification and intestinal type of gastric cancer.

OBJECTIVE

The aim of the present study was to evaluate the copy number variation of *B4GALT5* gene and associate CNV status with clinicopathological data of patients.

MATERIAL AND METHODS

Samples

We analyzed 123 gastric adenocarcinoma samples obtained from primary gastric tumors of patients from João de Barros Barreto University Hospital (HUJBB), located in Pará State, Brazil.

All samples were obtained before administration of chemical treatments or radiotherapy. This study was approved by HUJBB ethics committee (CAAE: 42999115.7.0000.5634) and all individuals signed a Consent Form allowing the use of biological samples and clinical data.

Sample composition was 68% male and 32% female patients, with a mean age of 59 years (± 13). Regarding tumor site, 78% were obtained from tumors located outside from cardia region and 22% of tumors located in the cardia region. Of the total of samples, 54% were collected from patients with advanced staging (III and IV) and 46% from patients with early stage (I and II), 66% of tumors belonging to the intestinal type and 34% to the diffuse type of Laurén. Also with respect to staging, it was observed that 24% of patients presented serosal extravasation (T4), 76% presented lymph node metastasis and 53% presented distant metastasis.

We also evaluated CNV status of *B4GALT5* gene in 47 oral squamous cell carcinoma samples, in an attempt to investigate if the frequency of this gene amplification is also high in other type of neoplasia. Sample composition was 64% male and 36% female patients, with a mean age of 61 years (± 13). Regarding tumor site, 80% were obtained from tumors located in tongue or floor of mouth. Of the total of samples, 56% were collected from patients with advanced stage (III and IV) and 44% from patients with early stage (I and II).

Histopathology

Histopathological data, such as histological subtype, degree of differentiation, depth of invasion, lymph node involvement and distant metastasis were taken from pathology

reports of the Department of Pathology of HUJBB. The histopathological analysis of tumor fragments was performed according to Laurén's classification[5].

Quantitative analysis of Copy Number Variants based on Real-Time PCR

Briefly, 1 µl of 10 ng DNA was added to 5 µL of TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA), with 0.5 µl of *B4GALT5* probe and 3 µL of water. We measured copy number gain using the following profile: denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. We determined relative quantification using the 7500 Rreal-time PCR system (Applied Biosystems, Foster City, CA) in quadruplicate. RNaseP (Applied Biosystems, Foster City, CA) was used as a control. After amplification, we imported the experiment results containing threshold-cycle values for the copy number and reference assay into the CopyCaller Software (Applied Biosystems, Foster City, CA) for post-PCR data analysis as previously described by Graziano *et al.*[18]

Statistics

Statistical analysis for comparisons of categorical variables between groups were done by means of Chi-square test and were performed using PASW Statistics program. Odds Ratio (OR) and Confidence Interval (CI=95%) were also calculated. A two-tailed probability value $p \leq 0.05$ was considered to be statistically significant.

RESULTS

B4GALT5 gene amplification was observed in 34% of samples. Diffuse and intestinal types demonstrated amplification frequencies of 19% (8/42) and 42% (34/81), respectively and statistical analysis showed that this amplification is 3 times more associated with intestinal type (p=0.011; 95%CI=1.266-7.469) (Table 1).

Table 1: Clinicopathological data of patients with and without *B4GALT5* amplification.

	***B4GALT5* CNV status**		***p-value***
	≥ 3 copies	Others	
Gender			
Male	27	57	0.492

Female	15	24	
Age			
≤50 years	12	22	0.868
>50 years	30	59	
Histopathology			
Intestinal	34	47	0.011*
Diffuse	8	34	
Localization			
Cardia	7	19	0.433
Non-cardia	33	61	
Stage			
I-II	21	35	0.473
III-IV	21	46	
pN			
N0	8	20	0.389
N1 or more	33	55	
pT			
T1-T3	32	61	0.737
T4	9	20	
pM			
M0	4	19	0.89
M1	6	20	

M: male; F: female. pN: lymph node metastasis status; N0: without lymph node metastasis; N1 or more: metastasis in one ore more lymph nodes. pT: extent of the primary tumor; T1-T3: without serosal extravasation; T4: with serosal extravasation. pM: distant metastasis status; M1: with distant metastasis; M0: without distant metastasis. Others: 2 copies or less. *Significant difference between groups with and without amplification, p≤0.05, Chi-square test.

Subcategorization of samples into intestinal and diffuse types did not result in any significant clinicopathological association.

It is important to note that we observed 24% of high-level amplifications (≥5 copies) of *B4GALT5* gene, but subdivision of samples into high-level amplifications and other amplifications (3 and 4 copies) or high-level amplifications and all other (1, 2, 3 and 4 copies) did not result in any significant clinicopathological association.

Regarding oral squamous cell carcinoma, we observed 22.5% of *B4GALT5* amplification, but it was not statistically associated with clinicopathological data of patients.

DISCUSSION

UDP-Gal:BetaGlcNAc Beta 1,4- Galactosyltransferase, Polypeptide 5 (*B4GALT5*) is a member of B4GalT gene family which encodes a set of type II transmembrane glycoproteins that catalyze the biosynthesis of different glycoconjugates and saccharide structures, and have been recognized to be involved in various diseases. *B4GALT5*, as well as *B4GALT4*, has been known for its key roles in the processes of proliferation, invasion, apoptosis suppression and multidrug resistance of cancer cells[16].

There are few studies in literature regarding the role of *B4GALT5* in cancer. However, amplification of chromosomal arm 20q, where this gene is located, occurs in prostate, cervical, colon, gastric, bladder, melanoma, pancreas and breast cancer and some genes have been implicated in inducing the malignant process upon 20q amplification, suggesting that this alteration may play a causal role in tumorigenesis[19].

By integration of array CGH and expression data on 20q13.12–13.33 in hepatocellular carcinoma, Wang *et al.*[17] identified a panel of 19 genes showing overexpression correlated with 20q13.12–13.33 gain. Among the 19 genes, they identified *B4GALT5* as a probable target gene contributing to the unfavorable outcomes due to correlation with progressive cancer behaviors.

Similarly, Scotto *et al.*[20] stated that the acquisition of 20q gain occurs at an early stage in cervical cancer development and the high-grade squamous intraepithelial lesions that exhibit 20q copy number increase are associated with persistence or progression to invasive cancer. Interestingly, they identified a total of 26 overexpressed genes as consequence of 20q gain, including a number of functionally important genes, such as *E2F1*, *TPX2*, *KIF3B*, *PIGT*, and *B4GALT5*.

Zhou *et al.*[16] observed that this gene was highly expressed in four chemoresistant human leukemia cell lines and in leukemia patients with multidrug resistance and stated that altered levels of B4GALT5 were responsible for changed drug-resistant phenotype of HL60 and HL60/adriamycin-resistant cells. Their experiments demonstrated that after *B4GALT5* shRNA transfection, the ability of adriamycin, paclitaxel and vincristine to inhibit the growth of HL60/adriamycin-resistant significantly increased and chemosensitivity was remarkably restored. On the other hand, when nude mice were inoculated with tumor cells HL60/B4GALT5, tumor volumes increased obviously even after adriamycin treatment;

therefore, upregulation of *B4GALT5* gene in HL60 cells led to raised resistance to chemotherapy.

Moreover, in regard to resistance mechanism of *B4GALT5* gene in leukemia cells, Zhou *et al.*[16] showed that gene manipulation of *B4GALT5* could influence the expression of P-gp and MRP1, recognized molecules involved in the development of multidrug resistance, since lower expression levels of P-gp and MRP1 were detected in HL60/ADR-B4GALT5 shRNA cells compared with those in control cells. By contrast, overexpression of B4GALT5 in HL60 cells increased the levels of P-gp and MRP1.

In an attempt to investigate the role of *B4GALT5* on progression and aggressiveness of cancer, Zhou *et al.*[16] also assessed the activity of the hedgehog (Hh) signaling, which activation might be associated with a wide variety of human tumors, by treatment of HL60/ADR cells with B4GALT5 shRNA. They observed that Smo, Shh and GLI-1, key oncoproteins of Hh signaling, transcripts and proteins, were significantly reduced with shRNA transfection. By contrast, overexpression of B4GALT5 in HL60 cells enhanced mRNAs and proteins expression of Smo, Shh and GLI-1.

In this study, we found for the first time recurrent *B4GALT5* amplification statistically associated with intestinal type of gastric adenocarcinoma. Thus, we suggest that amplification of this gene may has an important role in adenocarcinoma of intestinal type, which corroborates with the hypothesis that these two histological types have different genetic pathways[21,22].

Consistent with these results, El-Rifai *et al.*[23] and Kokkola *et al.*[24] found a significant association between 20q amplification and intestinal type of gastric cancer. Interestingly, a molecular classification of gastric cancer, proposed by Cancer Genome Atlas Research Network[25], categorized intestinal gastric cancer as correlated with chormosomal instability. Thus, 20q amplification may be an important event of chromosomal instability in intestinal gastric carcinogenesis.

Noteworthy, we observed that the frequency of *B4GALT5* amplification is very similar in all stages of cancer (I-31%, II-36%, III-37% and IV-28%), corroborating with the hypotesis proposed by Tabach *et al.*[19] that amplification of the 20q chromosomal arm occurs early in tumorigenic transformation and may initiate cancer.

CONCLUSION

Recurrent amplification of *B4GALT5* gene is a probable event related to intestinal gastric carcinogenesis.

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Declaration of Interest statement

We declare that we have no conflicts of interest.

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6 CAPÍTULO IV

RECURRENT AMPLIFICATION OF TRPV2 ASSOCIATED WITH LIMPH NODE METASTASIS IN GASTRIC ADENOCARCINOMA

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Title: RECURRENT AMPLIFICATION OF TRPV2 ASSOCIATED WITH LIMPH NODE METASTASIS IN GASTRIC ADENOCARCINOMA

Article Type: Brief Communication

Keywords: Gastric cancer; TRPV2; gene amplification; lymph node metastasis; biomarker.

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Abstract: Gastric cancer is a major contributor to the global cancer burden, being the third leading cause of cancer death in both sexes worldwide. Dysfunction of oncogenes and tumour suppressor genes contributes to malignant gastric cancer, and various candidate genes had been implicated to serve as biomarkers for gastric cancer. Importantly, lymph node involvement is considered the most reliable prognostic indicator in gastric cancer and many studies have been described prognostic biomarkers for predicting lymph node metastasis. In a previous study, preformed by array-Comparative Genomic Hybridization, we described for the first time in literature the recurrent amplification of TRPV2 gene in gastric cancer. Results demonstrated TRPV2 gene amplification in 18% of samples and statistical analysis showed that this amplification increases in 34% the risk of lymph node invasion. Therefore, we suggest that recurrent amplification of TRPV2 may be a usefull prognostic biomarker for predicting lymph node metastasis in resected gastric cancer patients in early stage.

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Title: ***RECURRENT AMPLIFICATION OF TRPV2 ASSOCIATED WITH LIMPH NODE METASTASIS IN GASTRIC ADENOCARCINOMA***

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ABSTRACT

Gastric cancer is a major contributor to the global cancer burden, being the third leading cause of cancer death in both sexes worldwide. Dysfunction of oncogenes and tumour suppressor genes contributes to malignant gastric cancer, and various candidate genes had been implicated to serve as biomarkers for this type of neoplasia. Importantly, lymph node involvement is considered the most reliable prognostic indicator in gastric cancer and many studies have been described prognostic biomarkers for predicting lymph node metastasis. In a previous study, preformed by array-Comparative Genomic Hybridization, we described for the first time in literature the recurrent amplification of *TRPV2* gene in gastric cancer. Results demonstrated *TRPV2* gene amplification in 18% of samples and statistical analysis showed that this amplification increases in 34% the risk of lymph node invasion. Therefore, we suggest that recurrent amplification of *TRPV2* may be a usefull prognostic biomarker for predicting lymph node metastasis in resected gastric cancer patients in early stage.

Keywords: Gastric cancer; *TRPV2*; gene amplification; lymph node metastasis; biomarker.

INTRODUCTION

Gastric cancer is a major contributor to the global cancer burden, being the third leading cause of cancer death in both sexes worldwide[1].

In Pará State (Northern Brazil), gastric cancer has a high incidence, ranking second among men and fourth among women. The estimate for 2016 and 2017 is 690 new cases in the state and 260 in Belém (capital)[2].

Gastric cancer is the consequence of a multi-step process resulting from different genetic and epigenetic changes in numerous genes. Dysfunction of oncogenes and tumour suppressor genes contributes to malignant gastric cancer, and various candidate genes had been implicated to serve as biomarkers for gastric cancer[3]. Importantly, lymph node involvement is considered the most reliable prognostic indicator in gastric cancer[4] and many studies have been described prognostic biomarkers for predicting lymph node metastasis[5-7].

In a previous study, preformed by array-Comparative Genomic Hybridization, we described for the first time in literature the recurrent amplification of *TRPV2* (*transient receptor potential cation channel subfamily V member 2*) gene in gastric cancer[8].

TRPV2 is a calcium-permeable cation channel belonging to the TRPV channel family. This channel is activated by heat (>52 °C), various ligands, and mechanical stresses and functions as a cation channel[9].

There are plenty of studies in many types of cancer correlating TRPV2 overexpression with poor prognosis, including advanced pT stage, lymph node metastasis and advanced pathological stage[10-13].

The present study evaluated the copy number status of this gene and identified a significant association between gene amplification and lymph node metastasis in gastric cancer.

OBJECTIVE

The aim of the present study was to evaluate the copy number variation of *TRPV2* gene and associate CNV status with clinicopathological data of patients.

MATERIAL AND METHODS

Samples

We analyzed 126 gastric adenocarcinoma samples obtained from primary gastric tumors of patients from João de Barros Barreto University Hospital (HUJBB), located in Pará State, Brazil.

All samples were obtained before administration of chemical treatments or radiotherapy. This study was approved by HUJBB ethics committee (CAAE: 42999115.7.0000.5634) and all individuals signed a Consent Form allowing the use of biological samples and clinical data.

Sample composition was 68% male and 32% female patients, with a mean age of 59 years (± 13). Regarding tumor site, 80% were obtained from tumors located outside from cardia region and 20% of tumors located in the cardia region. Of the total of samples, 56% were collected from patients with advanced stage (III and IV) and 44% from patients with early stage (I and II), 66% of tumors belonging to the intestinal type and 34% to the diffuse type of Laurén. Also with respect to staging, it was observed that 23% of patients presented serosal extravasation (T4), 76% presented lymph node metastasis and 52% presented distant metastasis.

We also evaluated CNV status of *TRPV2* gene in 47 oral squamous cell carcinoma samples, in an attempt to investigate if the frequency of this gene amplification is also high in other type of neoplasia. Sample composition was 64% male and 36% female patients, with a mean age of 61 years (± 13). Regarding tumor site, 80% were obtained from tumors located in tongue or floor of mouth. Of the total of samples, 56% were collected from patients with advanced stage (III and IV) and 44% from patients with early stage (I and II).

Histopathology

Histopathological data, such as histological subtype, degree of differentiation, depth of invasion, lymph node involvement and distant metastasis were taken from pathology reports of the Department of Pathology of HUJBB. The histopathological analysis of tumor fragments was performed according to Laurén's classification Laurén[14].

Quantitative analysis of Copy Number Variants based on Real-Time PCR

Briefly, 1 µl of 10 ng DNA was added to 5 µL of TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA), with 0.5 µl of *TRPV2* probe and 3 µL of water. We measured copy number gain using the following profile: denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. We determined relative quantification using the 7500 Rreal-time PCR system (Applied Biosystems, Foster City, CA) in quadruplicate. RNaseP (Applied Biosystems, Foster City, CA) was used as a control. After amplification, we imported the experiment results containing threshold-cycle values for the copy number and reference assay into the CopyCaller Software (Applied Biosystems, Foster City, CA) for post-PCR data analysis as previously described by Graziano *et al.*[15].

Statistics

Statistical analysis for comparisons of categorical variables between groups were done by means of Chi-square test and were performed using PASW Statistics program. Odds Ratio (OR) and Confidence Interval (CI=95%) were also calculated. A two-tailed probability value $p \leq 0.05$ was considered to be statistically significant.

RESULTS

TRPV2 gene amplification was observed in 18% of samples. All patients with *TRPV2* amplification had lymph node metastasis and statistical analysis showed that this amplification increases in 34% the risk of lymph node invasion (p=0.003; 95%CI=1.190-1.516).

Table 1: Clinicopathological data of patients with and without *TRPV2* amplification.

	***TRPV2* CNV status**		***p-value***
	≥ 3 copies	Others	
Gender			
Male	18	68	0.43
Female	6	34	
Age			
≤50 years	7	28	0.866
>50 years	17	74	
Histopathology			

Intestinal	17	66	0.569
Diffuse	7	36	
Localization			
Cardia	6	19	0.446
Non-cardia	17	81	
Stage			
I-II	11	44	0.811
III-IV	13	58	
pN			
N0	0	28	0.003*
N1 or more	23	67	
pT			
T1-T3	18	78	0.854
T4	5	24	
pM			
M0	6	19	0.203
M1	2	25	

M: male; F: female. pN: lymph node metastasis status; N0: without lymph node metastasis; N1 or more: metastasis in one ore more lymph nodes. pT: extent of the primary tumor; T1-T3: without serosal extravasation; T4: with serosal extravasation. pM: distant metastasis status; M1: with distant metastasis; M0: without distant metastasis. *Significant difference between groups with and without amplification, $p \leq 0.05$, Chi-square test.

Subcategorization of samples into intestinal and diffuse types did not result in any significant clinicopathological association.

It is important to note that we did not observe high-level amplifications (≥5 copies) of *TRPV2* gene.

Regarding oral squamous cell carcinoma, we observed 22.5% of *TRPV2* amplification, but it was not statistically associated with clinicopathological data of patients.

DISCUSSION

The transient receptor potential proteins (TRPs) is a family of ion channels responsible for a wide array of cellular functions and constitute a novel area of research in oncology[16].

Malignant transformation of cells is the result of enhanced proliferation, aberrant differentiation and impaired ability to die resulting in abnormal tissue growth, which can eventually turn into uncontrolled expansion and invasion, characteristic of cancer. Such transformation is often accompanied by changes in ion channel expression and,

consequently, by abnormal progression of the cellular responses with which they are involved[17].

Transient receptor potential vanilloid type 2 (*TRPV2*), also called *vanilloid receptor like-1* (*VRL-1*), is a member of TRPV cation channel Family[18]. Members of transient receptor potential vanilloid (TRPV) channel family control cellular homeostasis by regulating calcium influx, cell proliferation, differentiation, and apoptosis. Moreover, in the last years, an additional pathophysiological role for TRPV channel family in malignant growth and progression has been demonstrated[19,20].

Monet *et al.*[21] reported that endogenous lysophospholipids such as lysophosphatidylcholine (LPC) and lysophosphatidylinositol (LPI) induce a calcium influx via TRPV2 channel, due to the TRPV2 channel translocation to the plasma membrane. Additionally, it has been suggested that PI3K promotes TRPV2 activity independently of the channel translocation to the plasma membrane[22].

Importantly, at the cellular level, increases in Ca^{2+} trigger a wide variety of physiological processes, including proliferation and migration by cytoskeletal remodeling[23].

In hepatocellular carcinoma, increased expression of TRPV2 was identified in 29% cases and clinicopathologic assessment suggested a significant association between TRPV2 expression and portal vein invasion and histopathologic differentiation, suggesting that this gene plays a role in human hepatocarcinogenesis and might be a prognostic marker of patients with hepatocellular carcinoma[24].

According to Monet *et al.*[13], TRPV2 is expressed in the metastatic androgen-resistant prostate cancer cell lines PC-3, DU-145 and LNCaP C4-2 and, most importantly, *TRPV2* transcript levels were 12 times higher in patients with metastatic cancer (stage M1) compared with primary solid tumors (stages T2a and T2b). They also stated that silencing of this channel drastically reduces the migration of prostate cancer cells, whereas its overexpression increases their migration.

Similarly, Zhou *et al.*[12] observed that *TRPV2* mRNA was overexpressed in esophageal squamous cell carcinoma tissues and cell lines. High expression of *TRPV2* was observed more frequently in patients with advanced pT stage, lymph node metastasis and advanced pathological stage. Additionally, ptients with high expression of *TRPV2* had worse 5-year disease-specific survival and disease-free survival than that with low expression.

Noteworthy, multivariate analysis found that the expression of *TRPV2* mRNA and pN category were independent prognostic factors, so the authors concluded that overexpression of *TRPV2* mRNA might serve as a novel prognostic biomarker for patients with early stage of esophageal squamous cell carcinoma.

Concerning the mechanistic role of *TRPV2* in migration and invasion, TRPV2 silencing with shRNA demonstrated to abolish the stimulatory effect of lysophospholipids on Ca^{2+} entry and, consequently, on migration[13,21]. Therefore, overexpression of TRPV2 would maintain an elevated level of cytosolic Ca^{2+} in cancer cells due to its constitutive channel activity cells [25,26,27].

Another insight regarding TRPV2 role on affecting cancer cell aggressiveness was proposed by Monet *et al.*[13]. They performed small interfering RNA-mediated silencing of TRPV2 and observed a decreasing in growth and invasive properties of prostate tumors established in nude mice xenografts and a decreasing in the expression of invasive enzymes MMP2, MMP9, and cathepsin B. Thus, they stated that activity of this channel would affect cell migration by directly regulating the invasion markers MMP2, MMP9 and cathepsin B used by the cancer cell to invade.

In the present study, we found for the first time recurrent amplification of *TRPV2* statistically associated with lymph node metastasis, as well as observed by Liberati *et al.*[11] and Zhou *et al.*[12]. Moreover, the the massive demonstration that this gene is associated with cell migration in cancer suggests that it should have an important role in the mechanism used by cancer cells to invade lymphatic system.

CONCLUSION

Recurrent amplification of *TRPV2* gene may have an important role in migration and invasion of gastric cancer cells. Moreover, *TRPV2* amplification may be a usefull prognostic biomarker for predicting lymph node metastasis in resected gastric cancer patients in early stage.

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Declaration of Interest statement

We declare that we have no conflicts of interest.

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7 DISCUSSÃO

A despeito do progresso no tratamento do câncer gástrico avançado, o prognóstico do paciente permanece muito ruim, principalmente em decorrência do diagnóstico tardio (Jin *et al.*, 2015). Esse paradigma implica a necessidade de pesquisar e identificar biomarcadores moleculares para o diagnóstico precoce, bem como para o monitoramento da doença, contribuindo ainda para o desenenvolvimento de novas abordagens terapêuticas (Wu *et al.*, 2014).

Como citado anteriormente, à exceção do trastuzumab, a quimioterapia para o câncer gástrico avançado ainda não leva em consideração as características genotípicas do tumor. Dessa forma, uma parte dos pacientes, se não a maioria, recebe um tratamento com baixa eficácia ou muitas vezes ineficaz (Warneke *et al.*, 2013; Bang *et al.*, 2010).

Nos últimos anos, diversos estudos têm investigado a base molecular do câncer gástrico relacionada com a alteração da patogênese, incluindo os mecanismos de invasão e metástase utilizados pelas células durante o processo maligno. Com o desenvolvimento de tecnologias modernas, novos potenciais biomarcadores moleculares com valor prognóstico e diagnóstico têm sido identificados, incluindo genes-chave com alterações no número de cópias no câncer gástrico (Jin *et al.*, 2015; Buffart *et al.*, 2007; Kang *et al.*, 2006; Weiss *et al.*, 2004).

Nesse contexto, a tecnologia de Hibridização Genômica Comparativa em array é uma poderosa ferramenta para identificar regiões cromossômicas com alteração no número de cópias em escala genômica e é largamente utilizada na pesquisa em câncer (Shao *et al.*, 2010). Assim, no presente estudo, a técnica de aCGH foi empregada com o intuito de realizar um *screening* genômico de alterações quantitativas no DNA em amostras de adenocarcinoma gástrico do tipo intestinal.

Todas as amostras apresentaram múltiplos ganhos e perdas, bem como perdas de heterozigose (LOH), com uma média de 202 ganhos (±182), 279 perdas (±325) e 13 perdas de heterozigose (±18) por paciente. As alterações cromossômicas mais frequentes foram amplificações envolvendo as regiões 8q (55,5%), 20q (55,5%), 17q (50%), 1q (41%), 7p (41%), 6p (36,4%), 5p (36,4%), 13q (36,4%), 3q (32%), 7q (32%) e 20p (32%) e deleções envolvendo as regiões 3p (55,5%), 6q (50%), 2q (50%), 1p (45,5%), 5q (41%), 9p (36,4%), Xq (32%) e Xp (27,3%), corroborando com estudos anteriores realizados em câncer gástrico

(Cheng *et al.*, 2012; Deng *et al.*, 2012; Rossi *et al.*, 2011; Tada *et al.*, 2010; Tsukamoto *et al.*, 2008; Kimura *et al.*, 2004; Tay *et al.*, 2003; Peng *et al.*, 2003).

Além disso, foram observadas diversas alterações quantitativas recorrentes em genes envolvidos no processo de carcinogênese de muitos tipos tumorais, assim como em genes pouco descritos na literatura. Entre eles, foram identificados 29 genes que apresentaram-se alterados em pelo menos 50% dos pacientes e estão descritos na literatura como correlacionados a diversos tipos de câncer (Kapitanović *et al.*, 2014; Chiu *et al.*, 2014; Ni *et al.*, 2013; Flossbach *et al.*, 2013; Guo *et al.*, 2013; Zhou *et al.*, 2013; Loo *et al.*, 2013; Lando *et al.*, 2013; Choi *et al.*, 2013; Edsgärd *et al.*, 2013; Song *et al.*, 2012; Wu *et al.*, 2012; Zhou *et al.*, 2012; Tso *et al.*, 2011; Zhang *et al.*, 2011; Narayan & Murty, 2010; Lee *et al.*, 2009; Scotto *et al.*, 2008; Ordway *et al.*, 2006; Dunn *et al.*, 2004; Wu *et al.*, 2003; Boulaiz *et al.*, 2003; Höbaus *et al.*, 2003; Blaschke *et al.*, 2002; Saitoh & Katoh, 2002; Ng *et al.*, 1999). É importante ressaltar que 22 dessas alterações nunca foram descritas em associação com o câncer gástrico.

Em relação aos dados clinicopatológicos estadiamento (T4 x T1-T3) e idade (≤50 e >50 anos) diversas associações significativas foram encontradas com diferentes tipo de alterações.

As alterações mais significantes relacionadas com o extravasamento da serosa (T4) envolveram as regiões 13q21.1 (ganho), 15q15.1 (LOH), 17q23.1 (LOH), 19q13.2 (LOH) e 20q11.22 (LOH). A maioria dos genes encontrados dentro dessas regiões nunca foram descritos na literatura do câncer. Uma das exceções é o gene *EPB41L1*, localizado na região 20q11.22, que apresentou perda de heterozigose nas amostras, corroborando com estudos na literatura que demonstram seu papel supressor correlacionado com agressividade tumoral (Xi *et al.*, 2013; Zhenyu *et al.*, 2012). Entre as amplificações mais frequentes correlacionadas significativamente com o extravasamento da serosa, destaca-se o gene *ABCA13*, cuja superexpressão foi correlacionada com um pior prognóstico no câncer (Nymoen *et al.*, 2015; Hlaváč *et al.*, 2013).

Em relação à idade, as alterações mais significativas identificadas em pacientes com idade inferior ou igual a 50 anos foram os ganhos das regiões Xq26 (gene *CT45A4*), Xp22.31 (gene *STS*) e a perda da região 11p15.4 (genes *OR52N5* e *OR52N1*). Entretanto, apenas o gene *STS* apresenta estudos na literatura e sua expressão foi associada com um bom prognóstico no carcinoma de pulmão de células não pequenas (Iida *et al.*, 2013).

Adicionalmente, foram observadas as amplificações dos genes *UBB* e *TRPV2*, correlacionados significativamente com pacientes com idade inferior ou igual à 50 anos. Apesar de não estarem entre os genes mais significativamente alterados, alguns estudos na literatura têm os correlacionado com a progressão do câncer, o que sugere que possam ser genes-chave no desenvolvimento tumoral de pacientes mais jovens (Oh *et al.*, 2013; Thebault *et al.*, 2006; Tsavaler *et al.*, 2001; Wissenbach *et al.*, 2001; Duncan *et al.*, 1998).

Levando em consideração que a maioria dos genes observados alterados nunca foram descritos como envolvidos no processo de carcinogênese gástrica, foram selecionados para validação genes cujas alterações apresentaram alguma consistência com trabalhos já publicados na literatura em outros tipos de câncer.

Assim, foram investigadas por PCR em tempo real para a avaliação do número de cópias: a) a amplificação do gene *RTEL1*, em 50% dos pacientes, que também apresentou expressão de mRNA e de proteína aumentadas (estudos prévios de proteoma e transcriptoma, ainda não publicados); b) a amplificação do gene *B4GALT5* em 50% dos pacientes, que também apresentou expressão de mRNA aumentada (estudo prévio de transcriptoma não publicado); c) a amplificação do gene *ABCA13*, significativamente associado com o extravasamento da serosa; e d) a amplificação do gene *TRPV2*, significativamente associado com a idade ≤50 anos.

Os resultados demonstraram uma frequência elevada de amplificação desses genes, porém as associações estatísticas com os dados clínicopatológicos dos genes *TRPV2*, com pacientes jovens, e *ABCA13*, com o extravasamento da serosa, não foram confirmadas. Por outro lado, novas associações significativas foram observadas. Essa discordância provavelmente se deve aos diferentes números amostrais utilizados em cada investigação (Tabela 3).

É importante enfatizar que a validação da elevada frequência dessas alterações demonstra que o aCGH é uma ferramenta muito útil para investigar novos genes associados com a carcinogênese, mesmo em um número amostral pequeno.

Vale ressaltar também que, para fins de validação em outro modelo biológico, o número de cópias desses genes foi investigado em amostras de carcinoma epidermoide oral e as frequências observadas também foram elevadas (Tabela 3), reforçando a importância clínica dessas alterações para o câncer.

Tabela 3: Frequências das amplificações gênicas observadas através da utilização das técnicas de aCGH e PCR em tempo real para a avaliação do número de cópias.

Genes (localização)	**Frequência observada no CG por aCGH (n= 22)**	**Frequência observada no CG por PCR em tempo real (n)**	**Frequência observada em CEB por PCR em tempo real (n= 40)**
RTEL1 (20q13.33)	50%	37,6% (125)	30%
TRPV2 (17p11.2)	13,63%	18,4% (126)	22,5%
ABCA13 (7p12.3)	22,7%	38,46% (117)	25%
B4GALT5 (20q13.13)	50%	34,14% (123)	22,5%

*CG: Câncer gástrico; CEB: Carcinoma epidermoide oral.

A amplificação do braço longo do cromossomo 20 já foi observada em diversos tipos de câncer, incluindo o câncer de próstata, o cervical, o de cólon, o gástrico, o de bexiga, o de pâncreas, o de mama e o melanoma e alguns genes presentes nessa região têm sido implicados na indução do processo maligno, o que sugere que o frequente ganho desta região observado pelo aCGH (55,5% dos pacientes) pode estar relacionado com o processo carcinogênese gástrica. Neste contexto, é importante citar que os resultados identificaram pela primeira vez a amplificação recorrente dos genes *RTEL1* e *B4GALT5* em associação com o câncer gástrico.

O gene *RTEL1* (*Regulator of Telomere Elongation Helicase 1*) codifica uma helicase essencial para a manutenção dos telômeros e para a regulação da recombinação homóloga, que é uma das principais vias relacionadas com a manutenção da estabilidade genômica e está envolvida no reparo de danos ao DNA, incluindo quebras de fita dupa e *gaps* de DNA (Wu *et al.*, 2012).

A recombinação homóloga (RH) é um processo conservado essencial para as células em divisão. Durante a mitose, a RH é requerida não apenas para o reparo apropriado das quebras de fita dupla do DNA (DSB), mas também para o início da replicação nas forquilhas. Além disso, a RH também é crucial para o reparo de DSB na meiose, sendo extremamente importante para a segregação dos cromossomos na meiose I. Entretanto, a RH inapropriada pode originar instabilidade genética e câncer, como resultado de rearranjos cromossômicos errôneos e da persistência de estruturas recombinantes intermediárias que não podem ser desfeitas. Desta forma, a RH deve ser rigorosamente regulada e coordenada temporalmente com a progressão celular e a replicação. Nesse sentido, o gene *RTEL1* possui um papel

importante na manutenção da estabilidade genômica, uma vez que previne a RH inapropriada através da disruptura dos D-loops, estruturas que se formam durante a recombinação homóloga (Barber *et al.*, 2008).

Ademais, a atividade de helicase da Rtel1 na manutenção dos telômeros é bem descrita na literatura. Os telômeros são complexos DNA-proteína presentes nas extremidades dos cromossomos que possuem uma função protetora importante. Essa proteção é realizada pela formação de uma estrutura conhecida como T-loop, que é criada pela invasão de uma fita simples de DNA na extremidade do telômero a uma região de fita dupla antecedente, formando também uma outra estrutura denominada D-loop (de Lange, 2004). Além disso, os telômeros são ricos em guanina e são capazes de formar estruturas secundárias estáveis de quadrupexes de guanina (G4) que são importantes para a proteção dessa região contra a ação da telomerase (Sen & Gilbert, 1992). No entanto, durante a replicação, essas estruturas devem ser desfeitas para permitir que a enzima telomerase replique as extremidades dos cromossomos, impedindo o encurtamento acelerado dos telômeros e a consequente perda da estabilidade genética (Uringa *et al.*, 2012).

Nesse sentido, a Rtel1 possui um papel extremamente relevante, uma vez que sua atividade de helicase provoca a desestruturação do D-loop (consequentemente do T-loop) e das regiões G4, provocando a exposição dos telômeros à telomerase e permitindo a replicação eficiente das extremidades cromossômicas (Uringa *et al.*, 2012; Vannier *et al.*, 2012; Ding *et al.*, 2004).

Alguns estudos recentes têm estabelecido um papel essencial do gene *RTEL1* na manutenção dos telômeros e na estabilidade genômica (Uringa *et al.*, 2011; Wu *et al.*, 2012). Uringa *et al.* (2012) observaram que na ausência de Rtel1 os telômeros de células-tronco embrionárias apresentam uma diminuição gradual no seu tamanho, sugerindo que a Rtel1 é essencial para permitir a extensão das extremidades cromossômicas pela telomerase.

Tendo em vista que a disfunção do telômero é altamente mutagênica e seu aumento está relacionado com a iniciação e a progressão tumoral (Maser & DePinho, 2002), a amplificação de *RTEL* deve exercer uma função tumorigênica importante (Wu *et al.*, 2012), principalmente em indivíduos mais velhos, nos quais o processo natural de encurtamento dos telômeros é mais acentuado em relação aos mais jovens, devido ao maior número de divisões celulares ocorrido ao longo dos anos (Valdes *et al.*, 2005).

De acordo com Uringa *et al.* (2011), uma possível explicação para a superexpressão de Rtel estar associada à carcinogênese consiste no fato de que a função aumentada de Rtel1 pode desregular o mecanismo de RH, levando à sua incapacidade de prevenir recombinações homólogas inadequadas e ao mau funcionamento do sistema de reparo. Outra hipótese é que a o aumento da expressão da proteína Rtel1 aumentaria sua atividade de helicase, provocando o desestruturamento constitutivo do T-loop nos telômeros e sua consequente desproteção, o que por sua vez, provoca instabilidade cromossômica (Wu *et al.*, 2012; Barber *et al.*, 2008).

Nesse contexto, Wu *et al.* (2012) demonstraram que a expressão aumentada da Rtel1 nos hepatócitos de camundongos induz o desenvolvimento de tumores no fígado. Esse achado é consistente com dados genéticos em humanos que demonstraram que a amplificação do locus genômico do gene *RTEL1* não é apenas uma alteração genética importante, mas está intrinsecamente relacionada com a malignidade da doença (Taniguchi *et al.*, 2010; Katoh *et al.*, 2006; Niketeghad *et al.*, 2001; Guan *et al.*, 2000; Wong *et al.*, 1999).

No presente estudo foi observada pela primeira vez na literatura a amplificação recorrente do gene *RTEL1* associada com idade avançada e com o tipo intestinal do adenocarcinoma gástrico, sugerindo que a amplificação desse gene possa ser especialmente importante para a carcinogênese gástrica de pacientes idosos, devido ao seu encurtamento telomérico mais acentuado, e possa exercer uma função relevante para o desenvolvimento de câncer gástrico do tipo intestinal, corroborando com a hipótese de que os dois tipos histológicos de Laurén possuem vias genéticas diferentes (Tahara, 2004; Yasui *et al.*, 2000).

O segundo gene localizado na região 20q, *β-1,4-galactosiltransferase peptideo 5* (*B4GALT5*), pertence à família β-1,4-galactosiltransferase (B4GALT) que codifica enzimas conhecidas como glicoproteínas do tipo II ligadas à membrana, cuja função bioquímica consiste em catalisar a biossíntese de diferentes glicoconjugados e estruturas de sacarídeo. Membros dessa família têm sido identificados envolvidos em várias doenças. O gene *B4GALT5*, assim como o *B4GALT4* são conhecidos por seus papeis-chave nos processos de proliferação, invasão e resistência de células tumorais a múltiplas drogas (Zhou *et al.*, 2013).

Através da integração de dados de aCGH e expressão da região 20q13.12-13.13 no carcinoma hepatocelular, Wang *et al.* (2015) identificaram o *B4GALT5* como um provável

gene-alvo nessa região contribuindo para respostas desfavoráveis, devido sua correlação com a progressão do câncer.

Similarmente, Scotto *et al.* (2008) relataram que o ganho do braço longo do cromossomo 20 ocorre em um estágio inicial do desenvolvimento do câncer cervical e observaram que lesões intraepiteliais escamosas de alto grau que exibem o ganho dessa região são associadas com a persistência da lesão ou com a progressão para o câncer invasivo. Notavelmente, eles identificaram um total de 26 genes superexpressos como consequência do ganho da região 20q, incluindo o gene *B4GALT5*.

Zhou *et al.* (2013) demonstrarm uma elevada expressão deste gene em quatro linhagens de leucemia com quimiorresistência e em pacientes com leucemia que apresentavam resistência a múltiplas drogas. Níveis alterados de B4GALT5 foram responsáveis por modificar o fenótipo de resistência a drogas das células HL60 e HL60/resistentes à doxorrubicina. Seus experimentos demonstraram que após a transfecção de shRNA para silenciar o *B4GALT5*, a habilidade da doxorruicina, do paclitaxel e da vincristina em inibir o crescimento das células HL60/resistentes à doxorrubicina aumentou significativamente e a quimiorresistência foi restaurada. Por outro lado, quando camundongos nude foram inoculados com células HL60 contendo o gene *B4GALT5*, o volume tumoral aumentou significativamente, mesmo após o tratamento com doxorrubicina, demonstrando, portanto, que a presença de grandes quantidades de *B4GALT5* leva ao aumento da quimiorresistência.

Além disso, em relação ao mecanismo de resistência desse gene em células de leucemia, Zhou *et al.* (2013) demonstraram que a manipulação do gene influencia na expressão de P-gp e MRP1, preoteínas conhecidas por seu envolvimento na resistência a múltiplas drogas, uma vez que baixos níveis de expressão dessas proteínas foram detectados em células HL60/resistentes à doxorrubicina contendo o shRNA para o gene *B4GALT5*. Por outro lado, a superexpressão de B4GALT5 em células HL60 aumentaram os níveis de P-gp e MRP1.

Para investigar o papel do gene *B4GALT5* na agressividade do câncer, Zhou *et al.* (2013) avaliaram o efeito da alteração deste gene na via de sinalização Hedgehog (Hh). Através do tratamento de células HL60/resistentes à doxorrubicina com o shRNA para o *B4GALT5*, eles observaram que os transcritos e proteínas dos genes *SMO*, *SHH* e *GLI-1*, oncogenes-chave na via de Hh, foram significativamente reduzidos. Por outro lado, a

superexpressão de *B4GALT5* em células HL60 aumentou a expressão proteica e de mRNA de *SMO*, *SHH* e *GLI-1*.

É importante ressaltar que a via de sinalização Hh tem sido reconhecida como uma das mais importantes no câncer. Em adultos, a presença de mutações ou a desregulação dessa via exercem um papel importante na proliferação e na diferenciação, levando à tumorigênese ou à aceleração do crescimento tumoral em diversos tipos de câncer, tais como o de pulmão, o de próstata, o de mama e o de pâncreas (Gupta *et al.*, 2010). De acordo com Mimeault & Batra (2007), a cascata de sinalização Hedgehog resulta na ativação da transcrição de diversos oncogenes importantes, incluindo o *MYC*, o *CCND1* e o *SNAI1*, promovendo o crescimento tumoral, a transição epitélio-mesenquimal, através da inibição da E-caderina, e a invasão.

No presente estudo, observou-se pela primeira vez na literatura a amplificação recorrente do gene *B4GALT5* estatisticamente associada com o tipo intestinal do adenocarcinoma gástrico, sugerindo que essa alteração possa ter um papel importante na carcinogênese desse tipo histológico, através da ativação da via Hh.

Consistente com o fato dos genes *RTEL1* e *B4GALT5*, ambos localizados na região 20q, terem sido associados significativamente com o tipo intestinal de Laurén, El-Rifai *et al.* (1998) e Kokkola *et al.* (1997) também observaram uma associação significativa entre a amplificação do braço longo do cromossomo 20 e o tipo intestinal de câncer gástrico. Notavelmente, uma classificação molecular do câncer gástrico, proposta pelo *Cancer Genome Atlas Research Network* (2014), caracterizou o câncer gástrico do tipo intestinal pela presença de instabilidade cromossômica. Assim, a amplificação da região 20q parece ser um evento importante de instabilidade cromossômica para a carcinogênese intestinal e os genes *RTEL1* e *B4GALT5* são possíveis genes-chave dessa região, uma vez que a superexpressão de seus produtos proteicos está relacionada com a agressividade tumoral (Bailey & Murnane, 2006; Barber *et al.*, 2008).

É importante destacar que as frequências das amplificações de *RTEL1* e *B4GALT5* apresentaram-se similar em todos os estágios do câncer (I-41%, II-30%, III-44% e IV-44% e I-31%, II-36%, III-37% e IV-28%, respectivamente), subsidiando a hipótese proposta por Tabach *et al.* (2011) de que a amplificação da região 20q ocorre no início da transformação tumoral e pode estar relacionada com a iniciação do câncer.

O terceiro gene investigado neste estudo foi o *TRPV2* (*Transient receptor potential vanilloid type 2*), um membro da família TRPV de canais de cálcio (Ca^{2+}) (Perálvarez-Marín *et al.*, 2013). Membros dessa família controlam a homeostase celular através da regulação do influxo de cálcio e da proliferação e diferenciação celular. Além disso, nos últimos anos, um papel fisiopatológico da família de canais de cálcio TRPV no crescimento maligno e na progressão tumoral tem sido demonstrado (Bödding, 2007; Prevarskaya *et al.*, 2007).

Nesse sentido, a expressão elevada de membros da família TRPV já foi correlacionada com o surgimento e/ou a progressão de alguns tipos de cancer epiteliais, incluindo o câncer de próstata e o melanoma (Thebault *et al.*, 2006; Tsavaler *et al.*, 2001; Wissenbach *et al.*, 2001; Duncan *et al.*, 1998).

De acordo com Gkika & Prevarskaya (2011), o estudo dos canais TRP constitui uma importante área de pesquisa em oncologia, uma vez que a transformação maligna das células é sempre acompanhada de mudanças na expressão de canais iônicos e, consequentemente, da atividade anormal das respostas celulares que envolvem estes canais.

Monet *et al.* (2009) reportaram que lisofosfolipídeos endógenos, tais como a lisofosfatidilcolina e o lisofosfatidilinositol induzem o influxo de cálcio através do canal TRPV2, através da sua translocação até a membrana plasmática. Adicionalmente, tem sido sugerido que a proteína PI3K promove a atividade de TRPV2 independente da sua translocação para a membrana plasmática (Penna *et al.*, 2006).

É importante ressaltar que, a nível celular, o aumento dos níveis de Ca^{2+} provoca uma variedade de processos fisiopatológicos, incluindo a proliferação e a migração através do remodelamento do citoesqueleto (Clapham, 2007).

O mecanismo de migração através do remodelamento do citoesqueleto envolve atividades coordenadas de protrusão, pequena retração e adesão. O processo se inicia com a protrusão, que requer a polimerização de actina (Vicente-Manzanares & Horwitz, 2011; Ridley *et al.*, 2003; Small *et al.*, 2002). Ao término da protrusão, ocorre uma pequena retração da célula e sua posterior aderência à matriz extracelular (Webb *et al.*, 2002). Durante esse mecanismo, a miosina é recrutada e participa da contração entre os filamentos de actina, favorecendo o deslocamento da membrana e possibilitando a formação de complexos de adesão focal de uma maneira dinâmica (Burnette *et al.*, 2011). Após uma adesão bem sucedida, outro ciclo de protrusão começa com a polimerização da actina. Os ciclos de protrusão-pequena retração-adesão celular são repetidos sequencialmente, possibilitanto a

movimentação celular (Tsai *et al.*, 2015). Para que essas ações procedam e persistam, os componentes estruturais, actina e miosina, são regulados de uma maneira cíclica. Para a regulação de actina, atividades de pequenas GTPases, Rac, RhoA e Cdc4 e proteína quinase A são necessárias (Tkachenko *et al.*, 2011; Machacek *et al.*, 2009). Para a regulação de miosina, sinais locais de Ca^{2+} são necessários (Tsai & Meyer, 2012). Nesse contexto, os canais de cálcio, como o TRPV2, apresentam uma função imporante para o mecanismo de migração celular, contribuindo para a disponibilidade de cálcio intracelular e consequente regulação do complexo miosina/actina (Tsai *et al.*, 2015).

No carcinoma hepatocelular, a expressão aumentada de TRPV2 foi identificada em 29% dos casos e a avaliação clinicopatológica sugeriu uma associação significante entre a expressão dessa proteína e a invasão da veia porta e a diferenciação histopatológica, sugerindo que esse gene exerce um papel importante na hepatocarcinogênese e pode ser considerado um marcador prognóstico para pacientes com esse tipo de neoplasia (Liu *et al.*, 2010).

Monet *et al.* (2010) observaram uma elevada expressão da proteína TRPV2 nas linhagens metastáticas de câncer de próstata e, mais importante, os níveis de transcrito do *TRPV2* apresentaram-se 12 vezes maiores em pacientes com câncer de próstata metastático comparado com pacientes com tumores em estágios iniciais. Além disso, demonstraram que o silenciamento deste canal reduz drasticamente a migração das células de câncer, enquanto que sua superexpressão aumenta a capacidade de migração.

Similarmente, Zhou *et al.* (2014) observaram a superexpressão de mRNA do *TRPV2* em linhagens celulares de carcinoma esofágico de células escamosas e ressaltaram que a expressão elevada foi encontrada mais frequentemente em pacientes com o estágio pT avançado, com metástase linfonodal e com estadiamento avançado. Adicionalmente, pacientes com elevada expressão de *TRPV2* apresentaram uma pior taxa de sobrevivência em relação aos pacientes com baixa expressão. Notavelmente, a análise multivariada demonstrou que a expressão de mRNA de *TRPV2* e a presença de metástase linfonodal são fatores prognósticos independentes, levando os autores a concluírem que a superexpressão desse gene pode servir como um novo biomarcador prognóstico para pacientes com estágios iniciais de carcinoma escamoso esofágico.

Com relação ao mecanismo do *TRPV2* na migração e invasão, o silenciamento deste gene com shRNA demonstrou um efeito inibitório no influxo de cálcio induzido pelos

lisofosfolipídeos, com a consequente inibição da migração (Monet *et al.*, 2010; Monet *et al.*, 2009). Portanto, a superexpressão de TRPV2 deve manter os níveis citosólicos de Ca^{2+} elevados nas células do câncer devido à ativação constitutiva de seus canais (Hao *et al.*, 2007; Raj *et al.*, 2004; Daaka, 2002).

Um outro mecanismo relacionado ao papel do *TRPV2* na agressividade tumoral foi proposto por Monet *et al.* (2010). Seus resultados demonstraram que o silenciamento do *TRPV2* mediado por pequenos RNAs de interferência provoca uma diminuição no crescimento e nas propriedades invasivas de tumores de próstata estabelecidos em camundongos nude, além de uma diminuição da expressão das enzimas invasivas MMP9, MMP2 e catepsina B, sugerindo que a atividade desse canal deve afetar a migração celular através da regulação direta dessas proteases, utilizadas pelas células do câncer para invadir.

No presente estudo foi demonstrada pela primeira vez na literatura a amplificação recorrente do gene *TRPV2* associada estatisticamente com metástase linfonodal, corroborando com os achados de Liberati *et al.* (2014) e Zhou *et al.* (2014). Além disso, a massiva demonstração de que esse gene está associado com a migração e a invasão no câncer sugere que ele deve possuir um papel importante no mecanismo utilizado pelas células para invadir o sistema linfático.

Nesse contexto, é importante destacar que os linfonodos adjacentes ao tumor são muitas vezes o primeiro local de metástase (Sleeman *et al.*, 2009; Sleeman & Thiele, 2009; Alitalo & Carmeliet, 2002) e a detecção de metástase linfonodal possui um grande valor prognóstico para muitos tipos de câncer (Sleeman *et al.*, 2009; Sleeman & Thiele, 2009; Karkkainen *et al*., 2002; Pepper, 2001).

Células tumorais, fatores de crescimento derivados de célular tumorais, citocinas e outras moléculas podem entrar nas vias linfáticas através das células linfáticas endoteliais, que são hiper-permeáveis (Sleeman *et al.*, 2009; Tammela *et al*., 2005; Pepper, 2001). Subsequentemente, essas moléculas trafegam para os linfonodos regionais e linfonodos distantes e, finalmente, chegam até a circulação sanguínea, onde podem metastatizar para órgãos distantes (Alitalo & Carmeliet, 2002; Pepper, 2001). Portanto, os vasos linfáticos associados ao tumor formam uma passagem através da qual as células do tumor podem alcançar os linfonodos e outros órgãos (Datta *et al*., 2010). Metástases à distância, por sua vez, são a causa da maioria das mortes por câncer (Hur *et al*., 2015; Zhang *et al*., 2014; Brower, 2007).

Mais da metade dos pacientes com câncer gástrico apresentam metástase linfonodal quando são diagnosticados ou submetidos à ressecção cirúrgia (Abe *et al*., 2002; Chen *et al*., 2002; Yamaguchi *et al*., 2001; de Manzoni *et al*., 1999). O acometimento dos linfonodos é o indicador de sobrevida global mais importante para os pacientes de câncer gástrico após a ressecção curativa e as taxas de sobrevivência diminuem acentuadamente com o aumento da quantidade de linfonodos acometidos (Cobrun *et al*., 2006; Pan *et al*., 2003; Ding *et al*., 2003; Manfè *et al*., 2000; Yokota *et al*., 2000; Takagane *et al*., 1999). Além disso, muitos pesquisadores demonstraram que a metástase linfonodal é um fator de risco independente para a recorrência de câncer gástrico em pacientes submetidos à ressecção curativa (Nakamura *et al*., 1999; Kodera *et al*., 1998). Notavelmente, a sobrevida global de pacientes linfonodo-negativos é significativamente maior do que a de pacientes linfonodo-positivos. Por outro lado, a taxa de recorrência global é significativamente maior em pacientes linfonodo-positivos em relação aos linfonodo-negativos no câncer gástrico (Nakamoto *et al*., 2007; Sarela *et al*., 2003; Hochwald *et al*., 2000; Guadagni *et al*., 1997).

Por fim, o gene *ABCA13* é um membro da família de cassetes de ligação de ATP (ABC) subfamília A (ABCA) que exerce um papel crucial no desenvolvimento de resistência pelo efluxo de agentes anticancerígenos para o exterior das células de câncer e a superexpressão de um ou mais membros da família de transportadores ABC já foi associada a esse mecanismo de resistência (Hlavata *et al.*, 2012; Szakács *et al.*, 2006).

Existem poucos estudos na literatura abordando o papel do gene *ABCA13* no câncer, porém demonstram uma associação positiva entre a superexpressão desse gene e um pior prognóstico.

Nymoen *et al.* (2015) demonstraram que a superexpressão de mRNA do *ABCA13* estava significativamente relacionada com a diminuição da sobrevida global em carcinoma de ovário metastático e Hlaváč *et al.* (2013) observaram que os níveis de mRNA de *ABCA13*, *ABCB3* e *ABCC1* estavam significativamente mais elevados em pacientes com carcinoma mamário grau 3 em relação a pacientes com os graus 1 e 2, sugerindo que a superexpressão desse gene pode estar associada à agressividade do câncer.

Apesar dessas pesquisas relatarem a correlação da superexpressão do *ABCA13* com a piora do prognóstico, não existem trabalhos que elucidem o papel desse gene nos mecanismos de desenvolvimento e/ou progressão do câncer.

No presente estudo foi observada pela primeira vez na literatura a amplificação recorrente do gene *ABCA13* estatisticamente associada com metástase linfonodal, sugerindo que essa alteração possa exercer uma função importante no desenvolvimento de metástase linfonodal que, como foi ressaltado anteriormente, é uma condição associada a um prognóstico ruim (Deng & Liang, 2014).

8 CONCLUSÃO

A amplificação do gene *RTEL1* é um provável evento contribuinte para a instabilidade cromossômica no tipo histológico intestinal e pode ter um papel chave para o desenvolvimento de câncer gástrico em pacientes mais velhos.

A amplificação do gene *ABCA13* pode ser um marcador útil para predizer metástase linfonodal em pacientes com câncer gástrico ressecados em estágio inicial.

Adicionalmente, a co-amplificação dos genes *RTEL1* e *ABCA13* pode ser considerada um potencial marcador para o estadiamento avançado.

A amplificação recorrente do gene *B4GALT5* é um provável evento relacionado com a carcinogênese do tipo intestinal.

Por fim, o gene *TRPV2* parece exercer um papel importante na migração e na invasão das células de câncer gástrico e sua amplificação pode ser um marcador muito útil para predizer metástase linfonodal em pacientes com câncer gástrico ressecados em estágio inicial.

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