UNIVERSIDADE FEDERAL DO PARÁ
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Neurociências e Biologia Celular

ADRIANO AZEVEDO DE MELLO

Estudo do Papel das Proteínas LYN, CKB e SRC na Carcinogênese Gástrica

Belém – PA
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Tese submetida ao Programa de Pós-Graduação em Neurociências e Biologia Celular da Universidade Federal do Pará como requisito parcial para obtenção do grau de Doutor em Neurocências e Biologia Celular.

Orientador: Prof. Dr. Rommel Mario Rodriguez Burbano
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LISTA DE ABREVIATURAS

CagA	*Cytotoxicity associated gene A*
CG	Câncer Gástrico
CK	*Creatine Kinase*
CKB	Creatina Quinase Tipo Encefálico
DTT	*Dithiothreitol*
EBV	*Epstein-Barr Virus*
FFPE	*Formalin-Fixed and Paraffin-Embedded*
GAPDH	*Glyceraldehyde 3-Phosphate Dehydrogenase*
GC	*Gastric Cancer*
H. pylori	*Helicobacter pylori*
IHC	*Immunohistochemistry*
INCA	Instituto Nacional de Câncer José Alencar Gomes da Silva
MAPK	*Mitogen-Activated Protein Kinase*
MSP	*Methylation-Specific PCR*
NCI	*National Cancer Institute*
PVDF	*Polyvinylidene Fluoride*
RT-qPCR	*Reverse Transcription quantitative PCR*
SDS-PAGE	*Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis*
SFKs	*SRC Family of Kinases*
X^2	*Chi-Squared*

RESUMO

O câncer gástrico (CG) é o quarto tipo câncer mais frequente e a segunda maior causa de mortalidade em todo o mundo. Um melhor entendimento da biologia da progressão dessa neoplasia é crucial para redução da taxa de mortalidade com o desenvolvimento de novas estratégias terapêuticas e de tratamento dos pacientes. Em nosso estudo, foram analisadas amostras de câncer gástrico, encontrando-se expressão elevada de mRNA e de proteínas das quinases SRC e LYN, e níveis diminuídos da quinase CKB. Essas alterações podem ter um papel na invasão e na metástase dos tumores gástricos. A expressão dessas três quinases também foram associadas com a expressão do oncogene *MYC*, um possível biomarcador para o câncer gástrico. Objetivando-se entender os mecanismos que regulam a expressão desses genes, avaliou-se os padrões de metilação dessas três quinases. Assim, descobriu-se que a hipometilação de *SRC* e *LYN* e a hipermetilação de *CKB* estavam presentes apenas nas amostras neoplásicas gástricas. A perda de metilação de *SRC* e *LYN* foi associada com aumento nos níveis de expressão de seus mRNA e proteínas, sugerindo que a metilação do DNA está envolvida na regulação da expressão dessas quinases. A frequência de hipermetilação e metilação parcial de *CKB* foi mais elevada em amostras de câncer gástrico do que em amostras gástricas não-neoplásicas; no entanto, a expressão de *CKB* estava apenas parcialmente regulada por metilação do DNA. Analisando os dados de expressão, descobriu-se que alterações nos padrões de metilação do DNA das três quinases estudadas também estavam associadas com o avanço do câncer gástrico, invasão tumoral mais profunda e a presença de metástase. Portanto, a expressão de *SRC*, *LYN* e *CKB* ou a metilação do DNA, relacionada a esses genes, podem ser marcadores preditivos úteis para a progressão tumoral e alvos estratégicos em terapêutica anticâncer.

Palavras-Chave: Câncer Gástrico; Quinases; Expressão Gênica; Regulação Transcricional; Epigenética; Metilação.

ABSTRACT

Gastric cancer (GC) is the fourth most frequent cancer type and the second ighest cause of câncer mortality worldwide. A better understanding of the biology of the progression of this neoplasia is crucial to reducing the mortality rate with the development of novel patient management and therapeutic strategies. In this study, we analyzed gastric cancer samples and found elevated expression of SRC and LYN kinase mRNA and protein but decreased levels of CKB kinase, alterations that may have a role in the invasiveness and metastasis of gastric tumors. Expression of the three studied kinases was also associated with MYC oncogene expression, a possible biomarker for gastric cancer. To understand the mechanisms that regulate the expression of these genes, we evaluated the DNA methylation patterns of the three kinases. We found that *SRC* and *LYN* hypomethylation and *CKB* hypermethylation were only present in neoplastic gastric samples. The loss of *SRC* and *LYN* methylation was associated with increased levels of mRNA and protein expression, suggesting that DNA methylation is involved in regulating the expression of these kinases. The frequency of hypermethylation and partial methylation of *CKB* was higher in the gastric cancer samples than in the non-neoplastic gastric samples; however, CKB expression was found to be only partly regulated by DNA methylation. In an analysis of the expression data, we found that alterations in the DNA methylation pattern of the three studied kinases were also associated with advanced gastric cancer, deeper tumor invasion and the presence of metastasis. Therefore, SRC, LYN and CKB expression or DNA methylation could be useful markers for predicting tumor progression and targeting in anti-cancer strategies.

Keywords: Gastric Cancer; Kinase; Gene Expression; Transcriptional Regulation; Epigenetics; Methylation.

1. INTRODUÇÃO

1.1. Considerações Gerais

O câncer gástrico é uma doença maligna das mais comuns, apresentando uma das mais elevadas taxas de morbidade e mortalidade de todo o mundo (Martel *et al*, 2013). Estatisticamente é o quarto em incidência mundial, após as neoplasias malignas de pulmão, mama e colorretal (Piazuelo & Correa, 2013), e, segundo a Organização Mundial de Saúde, a terceira principal causa de morte por câncer no mundo (WHO, 2014). Dessa forma, apesar do constante declínio nos países em desenvolvimento, ainda é considerado um grave problema de saúde pública, apresentando uma elevada taxa de incidência no leste asiático, leste europeu e alguns países da América Latina (Souza *et al.*, 2013; Chiurillo, 2014).

Nos Estados Unidos, segundo dados do *National Cancer Institute* (NCI), os óbitos por câncer gástrico, têm sofrido declínio significativo, desde os anos 30 (Figura 1). Hoje, excetuando-se os tumores de pele não-melanoma, essa patologia não configura mais entre os dez tipos principais de cânceres, representando menos de 3% do número estimado de novos casos e de óbitos por câncer em 2015 (Figura 2). Contudo, o quantitativo absoluto ainda é significativo, conforme demonstrado pelas estimativas do NCI – 24.590 novos casos de câncer gástrico em norte-americanos (15.540 ♂ e 9.050 ♀), totalizando 10.720 mortes (6.500 ♂ e 4.220 ♀) (Siegel *et al.*, 2015).

Esses números ainda são superiores aqueles previsto no Brasil para o ano de 2014, onde o câncer gástrico, com exceção dos tumores de pele não melanoma, é o quarto e o sexto mais comum entre homens e mulheresos, respectivamente, causando 13.328 óbitos (8.608 ♂ e 4.720 ♀). Dados nacionais sobre a incidência dessa neoplasia para o referido ano, elaborados pelo INCA (Instituto Nacional de Câncer José Alencar Gomes da Silva), apontam para o surgimento de 20.390 novos casos em todo o país, sendo 12.870 em homens e 7.520 em mulheres (Figura 3), correspondendo a um risco estimado de 13,19 casos novos a cada 100 mil homens e 7,41 a cada 100 mil mulheres (INCA, 2014).

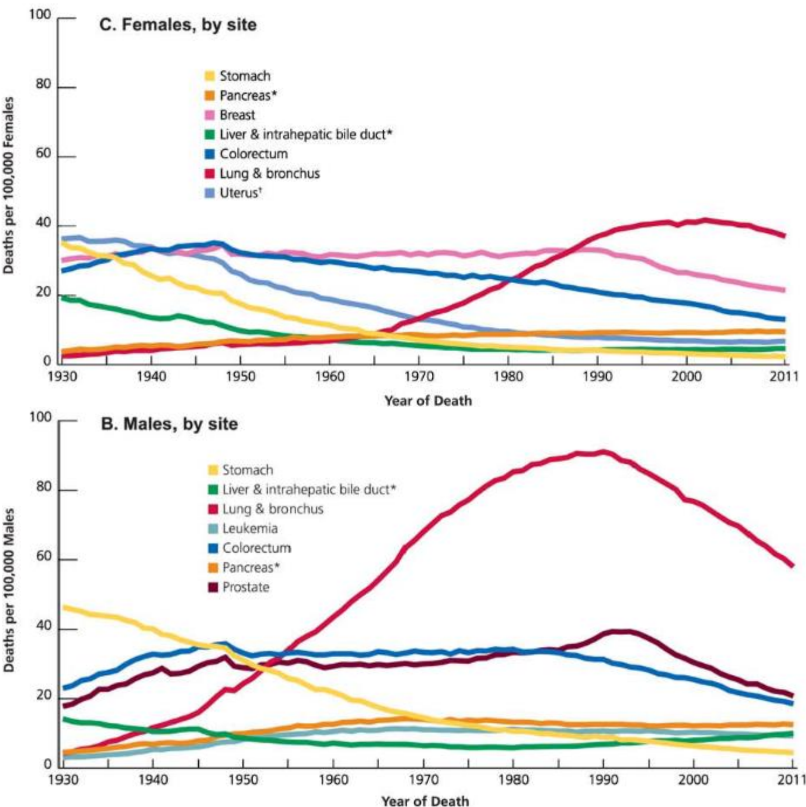
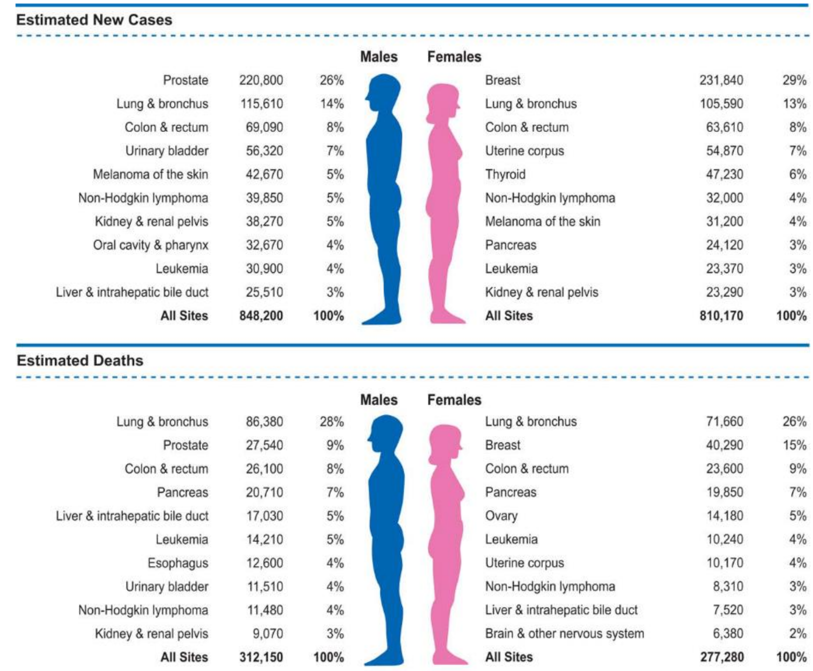


Figura 1 – Tendência da taxa de morte nos Estados Unidos por tipos de neoplasias, de 1930 a 2011. A curva em amarelo representa o declínio dos casos de mortes por câncer gástrico em homens e mulheres (x100.000) ao longo das décadas analisadas.
* As taxas de mortalidade para cânceres hepático e pancreático estão aumentando nos Estados Unidos.
† Útero inclui colo do útero e corpo uterino.
Fonte: Siegel, 2015.



Estimated New Cases

Males			Females		
Prostate	220,800	26%	Breast	231,840	29%
Lung & bronchus	115,610	14%	Lung & bronchus	105,590	13%
Colon & rectum	69,090	8%	Colon & rectum	63,610	8%
Urinary bladder	56,320	7%	Uterine corpus	54,870	7%
Melanoma of the skin	42,670	5%	Thyroid	47,230	6%
Non-Hodgkin lymphoma	39,850	5%	Non-Hodgkin lymphoma	32,000	4%
Kidney & renal pelvis	38,270	5%	Melanoma of the skin	31,200	4%
Oral cavity & pharynx	32,670	4%	Pancreas	24,120	3%
Leukemia	30,900	4%	Leukemia	23,370	3%
Liver & intrahepatic bile duct	25,510	3%	Kidney & renal pelvis	23,290	3%
All Sites	**848,200**	**100%**	**All Sites**	**810,170**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	86,380	28%	Lung & bronchus	71,660	26%
Prostate	27,540	9%	Breast	40,290	15%
Colon & rectum	26,100	8%	Colon & rectum	23,600	9%
Pancreas	20,710	7%	Pancreas	19,850	7%
Liver & intrahepatic bile duct	17,030	5%	Ovary	14,180	5%
Leukemia	14,210	5%	Leukemia	10,240	4%
Esophagus	12,600	4%	Uterine corpus	10,170	4%
Urinary bladder	11,510	4%	Non-Hodgkin lymphoma	8,310	3%
Non-Hodgkin lymphoma	11,480	4%	Liver & intrahepatic bile duct	7,520	3%
Kidney & renal pelvis	9,070	3%	Brain & other nervous system	6,380	2%
All Sites	**312,150**	**100%**	**All Sites**	**277,280**	**100%**

Figura 2 – Distribuição dos dez tipos de câncer mais incidentes nos Estados Unidos, exceto pele não melanoma, por novos casos e mortes, em ambos os sexos, estimados para 2015, segundo estimativas do *Nacional Cancer Institute´s*.
As estimativas foram aproximadas para a casa das dezenas.
Fonte: Siegel, 2015.

Localização primária	casos novos	%		Localização primária	casos novos	%
Próstata	68.800	22,8%	Homens / Mulheres	Mama Feminina	57.120	20,8%
Traqueia, Brônquio e Pulmão	16.400	5,4%		Cólon e Reto	17.530	6,4%
Cólon e Reto	15.070	5,0%		Colo do Útero	15.590	5,7%
Estômago	12.870	4,3%		Traqueia, Brônquio e Pulmão	10.930	4,0%
Cavidade Oral	11.280	3,7%		Glândula Tireoide	8.050	2,9%
Esôfago	8.010	2,6%		Estômago	7.520	2,7%
Laringe	6.870	2,3%		Corpo do Útero	5.900	2,2%
Bexiga	6.750	2,2%		Ovário	5.680	2,1%
Leucemias	5.050	1,7%		Linfoma não Hodgkin	4.850	1,8%
Sistema Nervoso Central	4.960	1,6%		Leucemias	4.320	1,6%

Figura 3 – Distribuição dos dez tipos de câncer mais incidentes no Brasil, exceto pele não melanoma, por novos casos, em ambos os sexos, estimados para 2014.
As estimativas foram aproximadas para a casa das dezenas.
Fonte: INCA, 2014.

Análises, mais cuidadosas dos números brasileiros, demonstram que a distribuição geográfica desses casos, em território nacional, é amplamente desigual (Figura 4). Desconsiderando as neoplasias de pele não melanoma, é o segundo mais frequente nas regiões Norte e Nordeste, o quarto nas regiões Sul e Centro-Oeste, e o quinto nos estados do Sudeste, considerando apenas o gênero masculino. Para as mulheres, é o terceiro mais frequente na região Norte, o quinto nos Estados do Sudeste e Nordeste, e o sexto nas regiões Sul e Centro-Oeste (INCA, 2014).

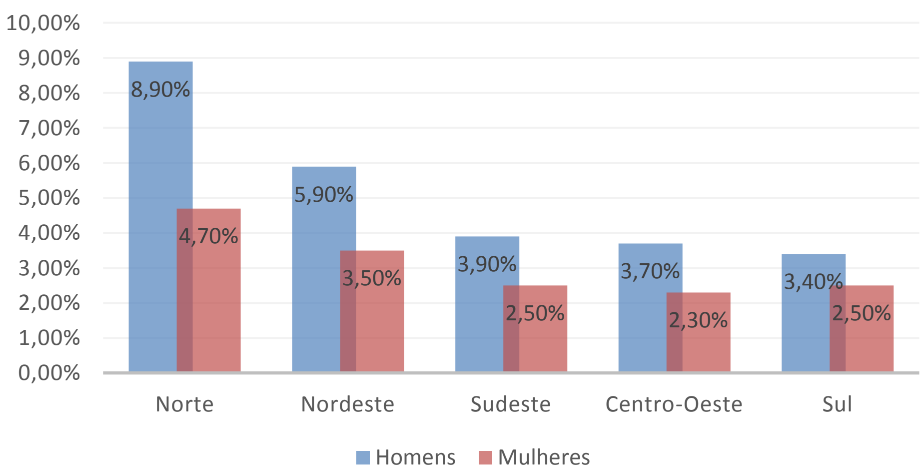


Figura 4 – Representação percentual do câncer gástrico, em relação aos demais cânceres, por região geográfica brasileira, entre homens e mulheres, demonstrando a maior prevalência dessa patologia nas regiões Norte e Nordeste, segundo estimativas do INCA para o ano de 2014.
Não foram considerados as neoplasias de pele não melanoma.

O estado do Pará destaca-se no cenário nacional por apresentar taxas de mortalidade com valores acima da média brasileira (Vinagre *et al.*, 2012), e a sua capital, Belém, já ocupou a décima primeira posição em número de casos de câncer gástrico por habitantes, entre todas as cidades do mundo com registro de câncer (IARC, 2001). Corrobora, ainda, para esta notoriedade, o fato da taxa de sobrevida média cumulativa, cinco anos após o diagnóstico, na cidade de Belém, ser estimada em aproximadamente em cerca de 9-10% (INCA, 2007), contra uma estimativa de aproximadamente 30% nos países desenvolvidos e de 20% nos países em desenvolvimento (Zilberstein *et al*, 2013). Essa baixa sobrevida em relação a países ocidentais deve-se em parte ao diagnóstico dessa malignidade ser realizado em estágio avançado (Brenner *et al.*, 2009; Ali *et al.*, 2012). Quando detectado precocemente e ressecado cirurgicamente, a taxa de sobrevida em cinco anos aumenta significativamente. Contudo, quando a doença encontra-se em metástase o tratamento torna-se largamente paliativo e a média de vida cai para 8-10 meses, dependendo das diferentes combinações de quimioterapia empregadas (Jørgense, 2014).

Essa casuística heterogênea, bem como a persistência de taxas de incidência elevadas em determinadas regiões do mundo e do Brasil, encontra-se relacionada a diferenças étnicas e geográficas, bem como, a fatores associados à condições socioeconômicas da população, principalmente aqueles ligados a baixa assistência médica, a infecção por *Helicobacter pylori* e a determinados hábitos alimentares, tais como, consumo de alimentos conservados em sal, o baixo uso de refrigeradores e o pequeno consumo de frutas e vegetais frescos (Guerra *et al.*, 2005; Tsugane & Sasazuku, 2007; Zaterka *et al.*, 2007), sendo, muito desses elementos, fortemente encontrados na população paraense (Vinagre *et al.*, 2012).

1.2. Classificação Histológica

Considerando que 95% dos tumores gástricos são adenocarcinomas, que podem ser classificados em bem-diferenciados (intestinal), indiferenciados

(difuso) e tipos misto (Smith *et al.*, 2006; Shi *et al.*, 2014), utilizaremos a denominação adenocarcinoma como sinônimo de malignidade do estômago.

O câncer de estômago refere-se a qualquer neoplasia maligna que surge na região que se estende entre a junção gastroesofágica e o piloro, classificando-se histologicamente, segundo a Organização Mundial de Saúde e o sistema de classificação de Lauren (1965), em dois subtipos: intestinal e difuso (Smith *et al.*, 2006; Nagini, 2012; Vinagre *et al.*, 2012). Ambos, apresentam morfologia, epidemiologia, patogênese e comportamento clínico diferente, e podem beneficiar-se de distintas abordagens terapêuticas (Tahara, 2004; Khan & Shukla, 2006; Qiu *et al.*, 2013).

O subtipo intestinal, ou "epidêmico", com melhor prognóstico, é o tipo histológico mais frequentemente diagnosticado em populações de alto risco, atingindo mais homens do que mulheres, envolvendo pessoas de mais idade do que jovens, e, provavelmente, é mais esporádico do que hereditário. A gênese tumoral, nesse subtipo, está fortemente associada a infecções por *H. pylori*, e à presença de lesões pré-cancerosas, como gastrite crônica, atrofia gástrica, metaplasia intestinal e displasia (Huntsman *et al.*, 2001; Nagini, 2012; Carcas, 2014).

O subtipo difuso apresenta pior prognóstico, sendo geralmente diagnosticado, principalmente em áreas endêmicas, em paciente mais jovens e sem distinção de gênero (Kunz *et al.*, 2012; Carcas, 2014). Pode ser multifocal e hereditário, não sendo, na maioria das vezes, acompanhada de metaplasia intestinal. Apesar de apresentar, também, uma relação com infecção por *H. pylori*, a gênese tumoral, nesse caso, está mais frequentemente associada com perda de expressão de E-caderina, sem lesão pré-cancerosas definidas (Pilpilidis *et al.*, 2011; Corso, 2012; Carcas, 2014).

A classificação de Lauren é usada por patologistas na rotina prática, e por epidemiologistas e clínicos para avaliar a história natural dos adenocarcinomas gástricos, especialmente no que diz respeito as tendências de incidência e

precursores etiológicos, embora todas as classificações existentes de adenocarcinoma gástrico são de significado limitado em termos de decisões terapêuticas (Yakirevich, 2013).

Geneticamente, ambos os subtipos histopatológicos, apesar de possuírem algumas alterações genéticas comuns (Tamura, 2006), são caracterizados por diferentes padrões de susceptibilidade germinativa e aberrações somáticas (Tan *et al.*, 2012).

1.3. Carcinogênese, Proteômica e Quinases

No ano de 2013, Michael Lodomery, inspirado nos trabalhos de Douglas Hanahan e Robert Weinberg Bob, publicados em 2000 e 2011, sugeriu que todos os cânceres compartilham, pelo menos, dez características relacionadas com o crescimento maligno: (1) auto-suficiência em relação aos sinais de crescimento; (2) insensibilidade aos sinais inibidores de crescimento; (3) aptidão para replicação ilimitada; (4) habilidade para escapar da apoptose; (5) angiogenese sustentanda; (6) capacidade para invadir tecidos e causar metástase; (7) potencialidade para de iludir o sistema imune; (8) presença de inflamação; (9) tendência para instabilidade genômica e (10) metabolismo desregulado (Hanahan & Weinberg, 2000; Hanahan & Weinberg 2011; Ladomery, 2013). Contudo, ressaltamos que a presente lista não atenua o fato do câncer ser, extraordinariamente, uma doença complexa e heterogênea, uma vez que existe uma quantidade significativa de intersecções entre as dez características e que vários genes especificos, associados ao câncer, podem estar envolvidos com mais de um marcador (Ladomery, 2013).

Os supracitados marcadores do câncer são acionados pela aquisição intra e intertumoral de variações genéticas e epigenéticas. Por isso, o entendimento detalhado dos mecanismos de como essas variações individuais contribuem para modificar a expressão gênica e a função proteica é necessário para um melhor

esclarecimento do intricado processo de progressão tumoral e de resposta ao tratamento (Gerdes, 2014).

Evidências epidemiológicas indicam que a etiologia do câncer gástrico é multifatorial. Fatores ambientais, dieta, estilo de vida, condições socioeconômicas e infecção por *Helicobacter pylori*, esse responsável por mais de 80% dos casos, estão entre os principais atores da tumorigênese gástrica. Além disso, o desenvolvimento do câncer gástrico envolve uma evolução em múltiplos passos, através de uma cascata de lesões histopatológicas pré-cancerosas sequenciais, desenvolvidas a partir do acúmulo serial de alterações genéticas – mutações gênicas, amplificação de genes, deleções ou perdas alélicas e instabilidade genômica (microssatélite ou cromossômica) – e epigenéticas – hipermetilações gênicas, alterações em proteínas histonas e microRNAs - que desregulam vias oncogênicas canônicas, causando ganho de função em oncogenes e perda de função em genes supressores de tumor, que, certamente, contribuem para a carcinogênese gástrica (Nagini, 2012; Shi *et al.*, 2014).

Dessa forma, a utilização de novas tecnologias, além das atuais plataformas de genômica e proteômica, para integração e análise dados coletados, são fundamentais para uma compreensão mais abrangente das características do câncer e dos complexos eventos de múltiplos passos relacionados com a carcinogênese (Hood *et al.*, 2004; Gerdes 2014).

Assim, o estudo do proteoma do câncer, apesar de excepcionalmente complexo – uma vez que contém informações de cada processo biológico que existe nas células tumorais, do microambiente e da interação célula tumoral-hospedeiro – é um ponto de partida para a identificação de biomarcadores de diagnóstico e alvos terapêuticos para o câncer. Vários possíveis biomarcadores de câncer, com potencial de aplicação clínica, têm sido descritos graças às tecnologias de proteômica (Alaoui-Jamali & Xu, 2006), cujos objetivos destinam-se aos estudos das proteínas, incluindo detecção, identificação, mensuração de concentração, análises de modificações, caracterização de interação proteína-proteína, bem como de suas respectivas regulações (Chung *et al.*, 2007).

Considerando o grande potencial terapêutico, em virtude de seu envolvimento, direto ou indireto, em mais de quatrocentas doenças humanas, as proteínas quinases destacam-se dentro desse cenário de estudo da proteômica do câncer, tornando-se objeto de intensa atividade de pesquisa. Corrobora, ainda, para essa premissa, o fato dessas proteínas, também denominadas de fosfotransferase, atuarem pela transferência de grupamentos fosfato aos aminoácidos serina (Ser), treonina (Thr), tirosina (Tyr) ou histidina (His) de suas moléculas alvo, modificando-as pós-traducionalmente (Figura – 5). Esse tipo de fosforilação é um componente fundamental de uma miríade de vias de sinalização celular, com papeis cruciais em uma pletora de cascatas de transdução de sinais que controlam do crescimento celular a iniciação e regulação das respostas imunológicas (Patterson *et al.*, 2013; Kelly & Genovese, 2013).

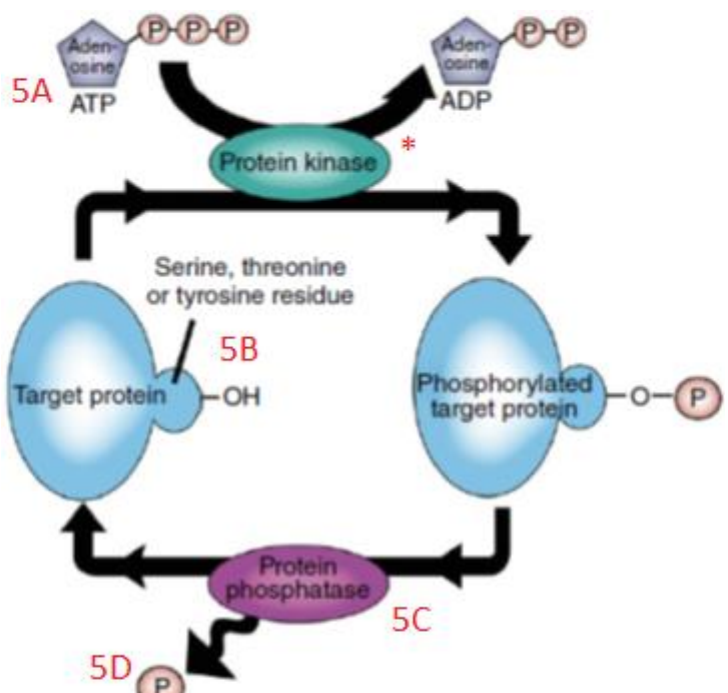


Figura 5 - Ciclo catalítico de fosforilação de proteínas mediada por quinases (*Protein Kinase*) **(*)**, demonstrando a transferência de um grupamento fosfato (P) do ATP **(5A)** para o grupo hidroxila de um resíduo de serina, treonina ou tirosina da proteína alvo (*target protein*) **(5B)**. Esse mecanismo de fosforilação atua como um “interruptor molecular”, que ativa ou inativa as funções da proteína escopo (*target protein*). O ciclo é contraposto por fosfatases (*Protein phosphatase*) **(5C)** que revertem os efeitos da fosforilação, pela remoção catalítica do fosfato da proteína alvo (Fonte: Patterson *et al.*, 2013)

Aproximadamente metade das 518 proteínas quinases codificadas pelo “*kinome*” humano – termo cunhado por Manning *et al* (2002) – é expressa em loci associados a doenças especificas ou a regiões amplificadas em cânceres humanos (Manning, 2009; Zhang *et al.*, 2012). Dessa forma, alterações em

diferentes tipos de quinases, que participam de vias de sinalização, relacionadas ao controle do crescimento e do processo de divisão celular, têm sido relatadas em diversos processos carcinogênicos, tais como, câncer de pulmão (Fan *et al.*, 2013; Ma, 2012), câncer de mama (Fyffe & Falasca, 2013), câncer colorretal (Stintzing & Lenz, 2013), câncer de próstata (Zheng & Tyner, 2013), câncer pancreático (Giroux *et al.*, 2009) e o próprio câncer gástrico (Almhanna *et al.*, 2011).

O câncer gástrico apresenta resistência intrínseca à radioterapia e à quimioterapia, e, provavelmente, a prevenção é o meio mais eficaz para a redução da incidência de mortalidade dessa malignidade (Rocco *et al.*, 2012). Atualmente, apesar dos consideráveis avanços no tratamento do câncer gástrico, a cirurgia ainda é o principal tratamento curativo (Stiekema *et al.*, 2013). A modificação desse panorama depende da identificação de alterações envolvidas na predisposição e na progressão do tumor, o que ampliaria a capacidade de prever o comportamento dessa neoplasia e estabelecer a conduta terapêutica de forma mais precisa (Assumpção & Burbano, 2005). Dentro deste contexto, o desenvolvimento de estudos que permitam identificar proteínas marcadoras de malignidade, para o câncer gástrico precoce, em populações de alto risco, substrato deste estudo, é de grande valia.

1.4. Proteínas LYN, SRC e CKB

Das proteínas escolhidas para o nosso estudo, LYN e SRC, cujos genes pertencem ao mesmo locus (20q11-13.1) (Stein *et al.*, 2002), caracterizam-se por pertencerem à família SRC de tirosinas quinases não receptoras (SFKs), sendo importantes intermediárias de sinalização celular. Membros dessa congregação, que contabilizam um total de nove proteínas estruturalmente similares (SRC, FYN, LYN, YES, BLK, LCK, HCK, FGR e YRK), são moduladores *upstream* de MAPK (*Mitogen-Activated Protein Kinase*) em várias vias de sinalização, estando, portanto, envolvidos em muitos processos que regulam o crescimento, a diferenciação, a apoptose, a migração, a resposta imune, a adesão

e o metabolismo, além de outros processos biológicos. Dessa forma, desregulação individual de SFKs, incluindo LYN e SRC, ocorre em centenas de diferentes tipos de tumores (Ingley, 2012; Su *et al.*, 2012; Liu *et al.*, 2013; Varkaris, 2014).

O envolvimento de quinases da família SRC, incluindo LYN, em várias cascatas de sinalização, está gradualmente sendo elucidado. Incialmente, LYN foi identificada como uma quinase especificamente hematopoiética e a sua expressão, em diversos tecidos, está envolvida na transmissão de sinais de inúmeros receptores, tais como receptores de células B, receptores GM-CSF, FcεR1, Epo-Receptor e c-kit, entre outros, bem como integrinas (Ingley, 2012).

Em virtude de seu papel em estabelecer limites, agindo tanto como um modulador positivo quanto negativo de uma variedade de respostas de sinalização (Su *et al.*, 2012), a atividade alterada de LYN tem sido relacionada com uma série de tumores humanos, incluindo câncer de mama, câncer de próstata, glioblastomas, adenocarcinomas de pulmão e câncer colorretal (Croucher *et al.*, 2013; Sutton *et al.*, 2013; Liu *et al.*, 2013). Atividades anormais de LYN também foram relatadas em tumores gástricos, conforme descritos por Kubo *et al.* (2009) e Kiyose *et al.* (2012). Dessa forma, alterações de LYN podem estar relacionadas ao processo de carcinogênese gástrica.

SRC é uma tirosina quinase que tem um papel chave nas sinalizações intracelulares por meio da interação e fosforilação de várias proteínas ou complexos proteicos (Kim *et al.*, 2009). Além disso, tem sido descrito que a SRC contribui com a transformação, proliferação, sobrevivência e mobilidade de células malignas, assim como no processo de angiogênese em tumores (Yeatman, 2004; Kim *et al.*, 2009). SRC é altamente ativada em uma variedade de neoplasias e estudos clínicos tem mostrado que isso está relacionado à progressão maligna (Irby & Yeatman, 2000). A atividade de SRC também parece estar aumentada na maioria dos tumores gástricos (Takekura *et al.*, 1990; Masaki *et al.*, 2000; Humar *et al.*, 2007; Peng *et al.*, 2009).

Estudos têm demonstrado que inibidores de SRC induzem apoptose ou parada do ciclo celular em vários tipos de neoplasias (Johnson *et al*., 2005; Song *et al*., 2006; Jallal *et al*., 2007; Zhang *et al*., 2007; Chang *et al*., 2008; Park *et al*., 2008; Leung *et al*., 2009; Nautiyal *et al*., 2009). Ao nosso conhecimento, nenhum estudo publicado na literatura procurou investigar o papel de SRC em neoplasias gástricas de indivíduos da população brasileira. Dessa forma, é importante avaliar a expressão de SRC em tumores gástricos de indivíduos brasileiros para investigar se inibidores de SRC podem vir a ser uma estratégia de tratamento na nossa população.

Diferentemente das duas primeiras proteínas, CKB é uma das três isoformas da creatina quinase (CK – *Creatine Kinase*), uma importante enzima dimérica que atua na estocagem temporal e espacial de ATP na forma de creatinafosfato, bem como na regulação metabólica de diversos tipos celulares (Ishikawa *et al*., 2005; Wallimann *et al*., 2011; Arnold *et al*., 2012).

Todas as isoformas da CK são codificadas por genes nucleares separados, e, em muitos tecidos, uma única isoforma de CK citossólica é coexpressada junto a uma única isoforma CK mitocondrial (mtCK). As formas citosólicas, tipo muscular (CKM) e tipo encefálico (CKB), formam homodímeros (CK-MM e CK-BB) ou hetrodímeros (CK-MB), cujos níveis de expressão são particularmente elevados nas células que apresentam grandes demandas energéticas, tais como músculos liso, esquelético e cardíaco, rins, cérebro e células neuronais, células fotorreceptoras da retina, espermatozoides e células sensoriais ciliadas do ouvido interno (Wallimann *et al*., 2011; Arnold *et al*., 2012).

Apesar do epíteto encefálico, cuja láurea se deve a sua principal localização, a CKB possui ampla distribuição no ser humano, ocorrendo principalmente em células alongadas e altamente polares, cujas mitocôndrias estão localizadas a certa distância dos sítios intracelulares de consumo de ATP. Muitas dessas células caracterizam-se por apresentar demanda energética altamente flutuante ou por necessitarem de suporte enérgico para manterem suas elevadas taxas de divisão celular, reabsorção ou atividades de secreção. Nas células

parietais do epitélio estomacal, o sistema CK/Fosfocreatina trabalha em conjunto com a bomba H^+/K^+- ATPase e está envolvido na secreção de ácido gástrico (Wallimann *et al.*, 2011).

Ishikawa *et al.* (2005) relatam o aumento da isoenzima CK-BB em várias malignidades, principalmente tumores sólidos, e em várias desordens hematológicas. Nesse estudo, o ínclito autor demonstra a inopinada relação entre expressão de CKB mRNA e CKB com o processo de transformação blástica do sistema hematopoiético, correlacionando esse fenômeno a um pobre prognóstico. Arnold *et al.* (2012) também inferiram resultados semelhantes ao analisar altos níveis de atividade de CKB em leucócitos e plaquetas humanas.

Diversos autores já relataram a expressão elevada de CKB em vários tipos de neoplasias, incluindo câncer de mama, de colo, colo-retal e ovariano (Balasubramani *et al.*, 2006, Huddleston *et al.*, 2005, Mooney *et al.*, 2011, Wallimann & Hemmer, 1994 and Zarghami *et al.*, 1996). Zhang *et al* (2007), descreveram que CKB é um possível antígeno associado com o carcinoma gástrico após análise de proteômica no soro de pacientes com e sem esse tipo de neoplasia. No entanto, mais estudos são ainda necessários para entender o papel de CKB no processo de carcinogênese gástrica.

2. OBJETIVOS

2.1. Objetivo Geral

- Analisar os padrões de expressão gênica e de metilação nas proteínas quinases LYN, SRC e CKB entre pares de amostras normais e tumorais de tecido estomacal, demonstrando o papel desses eventos na carcinogênese gástrica.

2.2. Objetivos Específicos

- Verificar a expressão de mRNA e das proteínas SRC, LYN e CKB em amostras de câncer gástrico.
- Avaliar se os genes das proteínas SRC, LYN e CKB podem ser regulados por metilação do DNA na carcinogênese gástrica.
- Investigar a possível associação entre expressão das quinases SRC, LYN e CKB, ou a metilação dessas, com variáveis clínicas, bem como a expressão e a metilação do gene MYC.

CAPÍTULO 1

ARTIGO CIENTÍFICO

Deregulated Expression of SRC, LYN and CKB kinases by DNA Methylation and its Potential role in gastric cancer progression

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Keywords

Gastric cancer; kinase; SRC; LYN; CKB; protein expression; gene expression; transcriptional regulation; epigenetics; DNA methylation.

Abbreviations

GC: Gastric cancer; FFPE: formalin-fixed and paraffin embedded; IHC: Immunohistochemistry; RTqPCR: reverse transcription quantitative PCR; MSP: Methyltion-specific PCR; χ2: Chi-square test;

ABSTRACT

Kinases are downstream modulators and effectors of several cellular signaling cascades and play key roles in the development of neoplastic disease. In this study, we analyzed gastric cancer samples and found elevated expression of SRC and LYN kinase mRNA and protein but decreased levels of CKB kinase, alterations that may have a role in the invasiveness and metastasis of gastric tumors. Expression of the three studied kinases was also associated with MYC oncogene expression, a possible biomarker for gastric cancer. To understand the mechanisms that regulate the expression of these genes, we evaluated the DNA methylation patterns of the three kinases. We found that *SRC* and *LYN* hypomethylation and *CKB* hypermethylation were only present in neoplastic gastric samples. The loss of *SRC* and *LYN* methylation was associated with increased levels of mRNA and protein expression, suggesting that DNA methylation is involved in regulating the expression of these kinases. The frequency of hypermethylation and partial methylation of *CKB* was higher in the gastric cancer samples than in the non-neoplastic gastric samples; however, CKB expression was found to be only partly regulated by DNA methylation. In an analysis of the expression data, we found that alterations in the DNA methylation pattern of the three studied kinases were also associated with advanced gastric cancer, deeper tumor invasion and the presence of metastasis. Therefore, SRC, LYN and CKB expression or DNA methylation could be useful markers for predicting tumor progression and targeting in anti-cancer strategies.

INTRODUCTION

Gastric cancer (GC) is the fourth most frequent cancer type and the second ighest cause of câncer mortality worldwide.[1] Treatment of GC at advanced stages remains difficult, and the prognosis is still poor, partly as a result of local recurrence, tumor invasion and/or metastasis. The overall relative 5-year survival rate is currently less than 20%.[2] A better understanding of the biology of the progression of this neoplasia is crucial to reducing the mortality rate with the development of novel patient management and therapeutic strategies.

Phosphotransferases, also known as kinases, are downstream modulators and effectors of several cellular signaling cascades and play key roles in the development of neoplastic disease.[3] To date, several protein kinase-interacting drugs have been registered for clinical trials.[4] We previously performed screening to identify kinase proteins expressed in GC using Capture Compound Mass Spectrometry[5,6] (Supplementary information 1), and 22 kinase proteins, including SRC, LYN and CKB, were detected (Supplementary information 2).

SRC was the first proto-oncogene discovered, and it plays a central role in cellular signal transduction pathways. Aberrant SRC activity is observed in several human cancers, including GC[7-9], and it may be important during tumor development and progression.[10,11] The mitogenic function of SRC is, at least in part, mediated by the induction of MYC, a cell cycle regulator and transcription factor.[12,13] Our group previously described MYC upregulation in human GC and in N-methylnitrosourea- treated non-human primates.[14-19] Because the activation of SRC, as well as that of other kinases, has pleiotropic effects that depend on the cell type and context,[20] it is still important to understand the possible relationship between kinases and MYC expression in gastric carcinogenesis and the molecular mechanism involved in their regulation.

LYN is another member of the SRC family of kinases, and the *LYN* gene is located at chromosome 8q13. Our group previously reported the presence of gains of chromosome 8 (on which the *MYC* gene is also located) in GC cases from Northern Brazil[16,21-23] and in all GC cell lines established from neoplasias in this population.[24,25] Therefore, this chromosome may contain important genes involved in gastric carcinogenesis. To our knowledge, no previous study has investigated the role of LYN and its regulation in GC. However, LYN overexpression has been reported in several cancers.[26-32] In addition, the regulation of *LYN* by DNA methylation was demonstrated in both colorectal cancer and Ewing's sarcoma,[33,34] and *LYN* methylation has been observed in some hematopoietic and non-hematopoietic cell lines.[35] DNA methylation is a molecular modification of DNA that is tightly associated with gene function and cell type-specific gene function.[36] Moreover, DNA methylation may be a robust biomarker, as it is vastly more stable

than RNA or protein and is therefore a promising target for the development of new approaches for the diagnosis and prognosis of cancers.[36]

CKB is one of two cytosolic isoforms of creatine kinase and may participate in metabolic processes involving glycolysis in non-muscle cells.[37] In contrast to normal cells, which primarily generate energy via oxidative phosphorylation, most cancer cells prefer aerobic glycolysis, which is known as the Warburg effect.[38] Interestingly, the MYC oncogene appears to activate several glucose transporters and glycolytic enzymes, thereby contributing to the Warburg effect.[39] Our previous proteomic study revealed that several proteins involved in energy production processes were deregulated in GC samples and reinforced the Warburg effect in this neoplasia.[40] The role of CKB in GC remains poorly understood: some transcriptomic studies reported the upregulation of *CKB* in GC samples,[41,42] whereas another showed *CKB* downregulation.[43] In addition, as for the *LYN* gene, *CKB* methylation was previously described in hematologic and solid cancer cell lines, including GC cell lines. 44 The methylation of *CKB* appears to be related to its reduced level of expression; however, further investigation is still necessary to understand the regulation of *CKB* by epigenetic modifications.

Therefore, we first aimed to evaluate the mRNA and protein expression of SRC, LYN and CKB in a large set of GC samples. Then, we evaluated whether these genes may be regulated by DNA methylation in gastric carcinogenesis. In addition, we investigated the possible association between kinase expression or methylation and clinical variables as well as MYC expression and methylation.

MATERIAL AND METHODS

Tissue samples

Kinase expression and methylation patterns were evaluated in 138 pairs of GC samples and their corresponding non-neoplastic gastric tissue samples obtained from patients who underwent gastrectomy in Northern Brazil. All of the

patients had negative histories of exposure to either chemotherapy or radiotherapy prior to surgery, and there was no co-occurrence of other diagnosed cancers. Informed consent with approval of the ethics committee of João de Barros Barreto University Hospital (Protocol #316737) was obtained from all patients prior to specimen collection.

Part of each dissected tumor sample was formalin-fixed and paraffin-embedded (FFPE). Sections of FFPE tissue were stained with hematoxylin-eosin for histological evaluation or used for immunohistochemistry (IHC) analysis. Additional portions of each tumor and paired non-neoplastic tissue specimens were snap-frozen in liquid nitrogen and stored at -80 °C until protein and nucleic acid purification.

All of the samples were classified according to Laurén,[45] and the tumors were staged according to the TNM staging criteria.[46] The presence of *Helicobacter pylori*, a class I carcinogen, in gastric samples was detected by the rapid urease test, and its virulence factor cytotoxicity associated gene A (CagA gene) was detect by PCR using DNA purified simultaneously with proteins and mRNA, as previously performed by our group.[47] EBV was detected by RNA in situ hybridization.[47]

For 49 of these pairs of neoplastic and non-neoplastic samples, we assessed the MYC immunoreactivity, mRNA expression and methylation status data previously published by our group.[18]

Protein, mRNA and DNA purification

Total protein, mRNA, and DNA were simultaneously isolated from gastric tissue samples using the AllPrep DNA/RNA/Protein Kit (Qiagen, Germany) according to the manufacturer's instructions. The protein pellet was dissolved in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 1% Protease Inhibitor Cocktail (Sigma-Aldrich, USA), and 0.5% each Phosphatase Inhibitor Cocktail 1 and 2 (Sigma-Al drich, USA), as previously performed by our group.[48]

The protein concentrations were determined by the method of Bradford (Sigma-Aldrich, USA). The RNA concentration and quality were determined using a NanoDrop spectrophotometer (Kisker, Germany) and 1% agarose gels, respectively. Samples were stored at -80 °C until use.

Protein immunoreactivity analysis

Tumor tissue sections (3 or 4-mm thick) were deparaffinized in xylene and rehydrated in a graded series of ethanol. After heat-induced epitope retrieval, the tissue sections were incubated with primary mouse monoclonal antibodies against SRC (dilution 1:400; clone 28, Life Technologies, USA), LYN (dilution 1:400; clone C13F9; Life Technologies, USA) or CKB (dilution 1:250; HPA001254, Santa Cruz Biotechnology, USA). A universal peroxidase-conjugated secondary antibody kit (LSAB System, DakoCytomation, USA) was used for detection. We used 3,30-diaminobenzidine/ H_2O_2 (DakoCytomation, Denmark) as the chromogen and hematoxylin as the counterstain. A protein immunoreactivity-positive sample was defined as one having 10% or more neoplastic cells that were positive for the protein.

Protein expression analysis

Western blot analysis was performed as previously described by our group. 49 Reduced protein (25 µg) from each sample was separated by 12.5% homogeneous SDS-PAGE and electro-blotted onto a PVDF membrane (Hybond-P, GE Healthcare, USA). The PVDF membrane was blocked with phosphate-buffered saline containing 0.1% Tween 20, and 5% low fat milk and incubated overnight at 4 °C with the corresponding primary antibodies: anti-SRC (dilution 1:1000; clone 28, Life Technologies, USA), anti-LYN (dilution 1:1000; clone C13F9; Life Technologies, USA), anti-CKB (dilution 1:400; HPA001254, Santa Cruz Biotechnology, USA), and anti-ACTB (dilution 1:250; Ac-15, Life Technologies, USA). After extensive washing, a peroxidase-conjugated secondary antibody was added for 1 h at room temperature. Immunoreactive bands were visualized using

the western blotting Luminol reagent, and the images were acquired using an ImageQuant 350 digital image system (GE Healthcare, Sweden). ACTB was used as a loading reference control.

mRNA expression analysis

First, RNA was reverse-transcribed using the High-Capacity cDNA Archive kit according to the manufacturer's protocol (Life Technologies, USA). Complementary DNA was then amplified by real-time reverse transcription quantitative PCR (RT-qPCR) using TaqMan probes purchased as Assays-on-demand Products for Gene Expression (Life Technologies, USA) and a 7500 Fast Real-Time PCR instrument (Life Technologies, USA). The *GAPDH* gene was selected as an internal control for RNA input and reverse-transcription efficiency. All RT-qPCRs were performed in triplicate for both the target genes (*SRC*: Hs01082246_m1; *LYN*: Hs00176719_m1; *CKB*: Hs00176484_m1) and the internal control (*GAPDH*: NM_002046.3). The relative quantification of gene expression was calculated according to Livak and Schmittgen.[50] The corresponding control sample was designated as a calibrator from each tumor.

DNA methylation analysis

The methylation pattern and frequency of kinase genes were evaluated by Methylation-specific PCR (MSP).[51] The EZ DNA Methylation-Lightning™ Kit (Zymo Research, USA) was used to modify the gDNA by bisulfite treatment, converting unmethylated cytosines into uracils and leaving methylated cytosines unchanged. Specific primers for the gene promoters are described in table 1.

PCR reactions were carried out using 0.1 μmol/L dNTPs, 2 μmol/L $MgCl_2$, 0.5 μmol primers, 1.25 U Taq DNA polymerase, and 100 ng bisulfite-modified DNA. After initial denaturation for 5 min at 94 ºC, 40 cycles at 94 ºC for 45 s, the annealing temperature (Table 1) for 45 s, and 72 ºC for 30 s were carried out,

followed by a final extension for 5 min at 72 ºC. The PCR products were directly loaded onto 3% agarose gels and electrophoresed. The gel was stained with SYBR® Safe DNA Gel Stain (Life Technologies, USA) and directly visualized under UV illumination. As a positive control for all MSP reactions, a gDNA sample was completely methylated using CpG Methylase (SssI, New England Biolabs, USA) following the manufacturer's instructions. Furthermore, primers for detecting the wild-type sequence were used to monitor the complete conversion of DNA obtained in the bisulfite reaction.

The samples were stratified as follows: 1) a sample was defined as hypomethylated when a positive amplification product was detected only in the PCR with specific primers for unmethylated sequences; 2) a sample was defined as hypermethylated when positive amplification was detected only in the PCR with specific primers for methylated sequences; 3) a sample was defined as partially methylated when positive amplification was detected in the PCR with the two primer sets.

The primers' specificity and MSP results were confirmed using a bisulfite sequencing PCR approach[52] in which we randomly selected 14 (10%) samples for each studied gene (data not shown).

Statistical analyses

The data are shown as the frequency, median and interquartile range. The Shapiro-Wilk test was used to evaluate the distribution of the age, mRNA and protein expression data and to determine the appropriate subsequent test for statistical comparisons. The Mann-Whitney test was used to investigate possible associations between kinase mRNA or protein expression and categorical variables, such as immunoreactivity, methylation pattern and clinicopathological features. Na association between categorical variables was analyzed using the Chi-squared (χ^2) test. A Spearman correlation test was used to evaluate the possible correlation between mRNA and protein expression, as well as gene methylation patterns. A *p*-

value less than 0.05 was considered significant. Bonferroni adjustment of the p-value was applied when multiple comparisons were performed, with the alpha level being divided by the number of comparisons.

RESULTS

Kinase expression in gastric tumors

Non-atypical gastric cells did not present SRC or LYN immunoreactivity (Figure 1A and 1C). However, SRC immunoreactivity was observed in dysplastic cells. Cell membrane and cytoplasmic immunoreactivity for SRC and LYN was detected in neoplastic cells (Figure 1B and 1D), and LYN also presented nucleic immunoreactivity. CKB immunoreactivity was detected in the cytoplasm or in the cell membrane in non-neoplastic gastric cells (Figure 1E). In contrast, GC cells did not presente CKB immunoreactivity (Figure 1F).

SRC, LYN and CKB immunoreactivity was detected in 72 (52.2%), 66 (47.8%) and 0 (0%) of the tumor samples. SRC and LYN immunoreactivity were associated with higher mRNA and protein levels in GC samples ($p < 0.001$, for all comparisons; Mann-Whitney test; Figure 2A, 2C, 2E and 2G).

The protein and mRNA levels of SRC were increased at least 1.5-fold (at least a 50% increase in expression) in 67 (48.6%) and 80 (58%), respectively, GC samples in relation to their matched nonneoplastic gastric samples (Figure 2B, 2D and 2K). Moreover, the protein and mRNA levels of LYN were increased at least 1.5-fold in 36 (26.1%) and 72 (52.2%) GC samples, respectively (Figure 2F, 2H and 2K). Conversely, downregulation of CKB protein and mRNA (at least 50% decrease of expression) was detected in 104 (75.4%) and 49 (35.5%), GC samples, respectively (Figure 2I, 2J and 2K). A strong and direct correlation was observed between mRNA and protein expression ($p < 0.001$, $\rho = 0.856$, Spearman correlation test) for SRC, LYN ($p < 0.001$, $\rho = 0.762$) and CKB ($p < 0.001$, $\rho = 0.819$).

The immunoreactivity of SRC was associated with the immunoreactivity of LYN ($p < 0.001$, χ^2 test), with 52 (37.7%) of the GC samples presenting immunoreactivity for both proteins. In addition, a direct correlation was observed between SRC and LYN protein ($p < 0.001$, $\rho = 0.556$) and mRNA ($p < 0.001$, $\rho = 0.779$) expression. The levels of CKB protein and mRNA expression were inversely correlated with SRC ($p < 0.001$, $\rho = -0.734$; $p < 0.001$, $\rho = -0.806$, respectively) and LYN ($p < 0.001$, $\rho = -0.643$; $p < 0.001$, $\rho = -0.703$, respectively).

Table 2 shows the results for SRC, LYN and CKB expression and the clinicopathological characteristics. The tumors of patients with late-onset GC presented significantly higher SRC and LYN protein (by IHC and western blotting) and mRNA (by RT-qPCR) expression, as well as reduced CKB protein expression by western blotting, compared with early-onset CG samples ($p < 0.05$, for all comparisons; Table 2). Increased protein and mRNA expression of SRC and LYN and reduced CKB expression were associated with advanced stage, deeper tumor invasion, and the presence of lymph node and distant metastases ($p < 0.05$, for all comparisons; Table 2).

A gradual significant increase in SRC protein (by western blotting) and mRNA expression was observed corresponding to the tumor stage ($p < 0.008$, for most of the comparisons; Mann-Whitney test followed by Bonferroni correction; Figure 3A and 3B). In contrast, a gradual significant decrease in CKB protein and mRNA expression was observed corresponding to the tumor stage ($p < 0.008$, for most of the comparisons; Figure 3E and 3F). With regard to LYN expression, we did not observe a significant difference between stages I and II or between stages III and IV. However, stages I and II were significantly different from stages III and IV ($p < 0.008$, for these comparisons; Figure 3C and 3D).

Kinase gene methylation patterns in gastric samples

Table 3 shows the methylation pattern of the studied protein kinases in neoplastic and non-neoplastic gastric samples. Approximately 60% and 30% of

the GC samples presented positive amplification with only the unmethylated primer set (hypomethylated samples) for the *SRC* and *LYN* genes, respectively (Figure 4A and 4B). Hypomethylation of these genes was not observed in any nonneoplastic sample. Therefore, the frequency of *SRC* and *LYN* hypomethylation was significantly higher in GC than in non-neoplastic gastric samples ($p < 0.001$, for all comparisons; $\chi 2$ test followed by Bonferroni corrections).

The *SRC* and *LYN* methylation patterns of the neoplastic and non-neoplastic samples whereas found to be associated ($p < 0.001$, for both analyses; $\chi 2$ test). We observed that 70/81 (86.4%) of tumors with hypomethylated *SRC* presented partial methylation of this gene in the matched non-neoplastic sample. In addition, we found that 37/41 (90.24%) of tumors with hypomethylated *LYN* presented partial methylation of this gene in the matched non-neoplastic sample. *SRC* and *LYN* partial methylation in non-neoplastic samples was more frequently observed in individuals presenting tumor samples with hypomethylation of this gene compared with tumors with partial methylation ($p < 0.001$, for both analyses) or hypermethylation ($p < 0.001$, for both analyses). Furthermore, partially methylated *LYN* in non-neoplastic samples was also more frequently detected in individuals presenting tumor samples with partial methylation of this gene compared with tumors with hypermethylation ($p = 0.004$).

CKB partial and hypomethylation was observed in both neoplastic and non-neoplastic samples. However, 48 (39%) of GC samples presented *CKB* hypermethylation (Figure 4C), which was not detect in the non-neoplastic samples. Moreover, the frequency of *CKB*-hypermethylated samples was significantly higher in neoplastic compared to non-neoplastic gastric samples ($p < 0.001$), and *CKB* partial methylation was also significantly more frequent in GC than in non-neoplastic samples ($p < 0.001$).

Regarding *SRC* and *LYN*, the *CKB* methylation pattern of the neoplastic and non-neoplastic samples appeared to be associated ($p = 0.014$, by $\chi 2$ test). However, a 2x2 analysis using the $\chi 2$ test revealed that pairs in which the tumor samples presented hypermethylated *CKB* and the matched nonneoplastic

samples presented hypomethylation of this gene were more frequent than pairs of tumors with hypermethylation and matched non-neoplastic samples with partial methylation ($p = 0.0381$). However, this finding did not reach statistical significance if the Bonferroni adjustment was applied (adjusted $\alpha = 0.05/3 = 0.0167$).

A direct correlation was observed between the *SRC* and *LYN* methylation patterns in the nonneoplastic samples ($p < 0.001$, $\rho = 0.416$). In addition, an inverse correlation was detected between *SRC* and *CKB* ($p< 0.001$, $\rho = -0.421$) and *LYN* and *CKB* ($p < 0.011$, $\rho = -0.360$) methylation patterns. However, in GC samples, a direct correlation was observed among the methylation patterns of the three studied kinases: *SRC* and *LYN* ($p < 0.001$, $\rho = 0.567$); *SRC* and *CKB* ($p = 0.001$, $\rho = 0.287$); *LYN* and *CKB* ($p = 0.011$, $\rho = 0.230$).

Methylation regulation of kinases

To elucidate the epigenetic regulation of the studied genes, we evaluated the possible association between the methylation pattern and protein immunoreactivity and mRNA and protein expression (by western blotting).

We observed that tumors with SRC and LYN immunoreactivity more frequently presented a hypomethylated or partially methylated promoter than tumors without immunoreactivity for these proteins ($p < 0.016$, for all comparisons; $\chi 2$ test followed by Bonferroni corrections; Figure 5A and 5D). In addition, we observed that both the protein and mRNA expression of SRC (Figure 5C and 5D) and LYN (Figure 5E and 5F) was significantly increased with the loss of promoter methylation ($p < 0.016$, for all comparisons; Mann-Whitney test followed by Bonferroni corrections).

Concerning CKB regulation, increased CKB protein and mRNA expression was observed in tumors with a hypomethylated *CKB* promoter compared with tumors with a partially methylated promoter ($p = 0.015$, $p = 0.008$, respectively; Mann-Whitney test followed by Bonferroni corrections; Figure 5H and 5I). However, tumors with a hypermethylated *CKB* promoter also presented increased protein and

mRNA expression compared with tumors with a partially methylated promoter ($p < 0.001$, for both comparisons).

Methylation of kinase promoters and clinicopathological variables

In non-neoplastic gastric mucosa, samples with a partially methylated *LYN* promoter were more frequently observed in older individuals compared with samples with a hypermethylated promoter (median ± IQR: 64 ± 19 vs 53 ± 31; $p < 0.001$; by Mann-Whitney test), though no other association was observed between the methylation pattern and gender, *H. pylori* and EBV infection in the nonneoplastic samples ($p > 0.05$, by $\chi 2$ test).

Table 4 shows the associations between the methylation pattern in GC samples and clinicopathological characteristics. The p-values in Table 4 were determined using the $\chi 2$ test. To better understand the role of the different patterns of DNA methylation (hypomethylation, partial methylation and hypermethylation), a 2x2 analysis using the $\chi 2$ test with Bonferroni adjustment was also performed (adjusted $\alpha = 0.05/3 = 0.0167$).

In neoplastic samples, the *LYN* methylation pattern was significantly different between early-onset and late-onset GC samples ($p = 0.01$), and post hoc analyses revealed that *LYN* hypomethylation was more frequent than hypermethylation in samples of late-onset GC ($p = 0.0115$). Moreover, we detected a significant difference in the *SRC* methylation pattern between diffuse and intestinal types ($p = 0.032$); however, no difference between groups was observed after Bonferroni adjustment.

The methylation patterns of *SRC* and *LYN* were significantly different between early and advanced GC ($p = 0.001$, for both analyses). Advanced GC (in relation to early GC) more frequently presented *SRC* hypomethylation ($p = 0.0045$) and *LYN* partial methylation ($p = 0.0067$) than hypermethylation of these genes.

Kinase gene methylation patterns were also associated with tumor invasion and metastasis (Table 4). T3/T4 tumors more frequently presented *SRC* and *LYN* partial methylation ($p < 0.001$; $p = 0.009$, respectively) or hypomethylation ($p = 0.006$; $p < 0.001$, respectively) than hypermethylation. Partial methylation of *CKB* was also more frequently found than hypermethylation in T3/T4 tumors ($p = 0.008$).

In addition, the neoplastic samples from patients with lymph node metastasis more frequently presented *SRC* and *LYN* partial methylation ($p < 0.005$; $p < 0.001$, respectively) and hypomethylation ($p < 0.001$; $p < 0.001$, respectively) than hypermethylation.

The neoplastic samples from patients with distant metastasis more frequently presented *SRC* and *LYN* hypomethylation than partial methylation ($p < 0.001$; $p = 0.007$, respectively) and hypermethylation ($p < 0.001$; $p < 0.001$, respectively). Moreover, in these samples, *LYN* partial methylation was more frequently observed than hypermethylation ($p < 0.001$). *CKB* partial methylation was also more frequent than hypermethylation ($p < 0.001$) and hypomethylation ($p = 0.009$) in tumors from individuals with distant metastasis in relation to tumors from individuals without distant metastasis.

Kinases and MYC relationships

We examined MYC immunoreactivity, mRNA expression and methylation status data for a set of 49 of the studied pairs of neoplastic and non-neoplastic samples.[18]

MYC immunoreactivity was detected in 38 (77.6%) tumors. The immunoreactivity of MYC was associated with the immunoreactivity of SRC ($p < 0.001$, by $\chi 2$ test) and LYN ($p < 0.004$, by $\chi 2$ test), with 2 (4.1%) GC samples presenting only kinase immunoreactivity and 9 (18.4%) GC samples without MYC or kinase immunoreactivity.

The mRNA level of *MYC* was increased at least 1.5-fold in all GC samples in relation to matched non-neoplastic gastric samples. In addition, a direct correlation was observed between the mRNA expression of *MYC* and *SRC* ($p < 0.001$, $\rho = 0.856$; Figure 6A) and *MYC* and *LYN* ($p < 0.001$, $\rho = 0.763$; Figure 6B). In contrast, an inverse correlation was observed between *MYC* and *CKB* mRNA expression ($p < 0.001$, $\rho = -0.890$; Figure 6C).

In non-neoplastic samples, a direct correlation was observed between the *MYC* and *SRC* methylation patterns ($p < 0.001$, $\rho = 0.486$) and between *MYC* and *LYN* methylation patterns ($p < 0.001$, $\rho = 0.647$). In addition, an inverse correlation was detected between *MYC* and *CKB* ($p< 0.001$, $\rho = -0.320$). However, no correlation was observed between *MYC* and the three studied kinases in the GC samples: *MYC* and *SRC* ($p = 0.626$, $\rho = -0.071$); *MYC* and *LYN* ($p = 0.724$, $\rho = 0.052$); *MYC* and *CKB* ($p = 0.820$, $\rho = -0.039$).

DISCUSSION

Kinases are the most intensively studied category of protein drug targets in current pharmacological research, as evidenced by the vast number of kinase-targeting agents enrolled in active clinical trials.[4] In the present study, we evaluated the role of CKB and of two members of the SRC family of kinases, SRC and LYN. We observed that the SRC and LYN kinases were upregulated in approximately 50% of GC samples. Aberrant SRC activity has already been observed in several human cancers, including GC.[7-9] In addition, LYN overexpression has been reported in several cancers, such as chronic myelogenous leukemia,[26] colorectal cancer,[27] breast cancer,[28] prostate cancer,[29] oral cancer,[30] renal cancer[31] and Ewing's sarcoma;[32] nonetheless, no previous study has evaluated the role of LYN in gastric carcinogenesis. Our results suggest that SRC and LYN may be targets of anticancer therapies in GC patients presenting elevated expression of these kinases.

SRC immunoreactivity or elevated protein and mRNA expression was associated with late onset, an advanced stage, deeper tumor extension and the presence of metastasis. Yang et al.[53] demonstrated that SRC regulates migration and invasion in a GC cell line (BGC-823) following treatment of these cells with the SRC inhibitors PP2 and SU6656, which is in part in agreement with our findings in primary GC samples. Moreover, as for SRC, LYN may also have a role in gastric tumor invasiveness, metastasis, and thus aggressiveness. These associations have been suggested for other cancers. LYN upregulation was associated with colorectal tumor grade, stage, and lymph node and distant metastases.[27] In addition, the inhibition of LYN was able to decrease primary tumor growth, reduce metastases in an in vivo model of Ewing's sarcoma, and decrease the invasive capacity of Ewing's sarcoma cells in vitro.[32]

The mitogenic function of SRC is, at least in part, mediated by the induction of MYC.[12,13] Here, we report a direct correlation between SRC and LYN expression, as well as between the expression of these kinases and that of MYC. As for SRC and LYN, MYC immunoreactivity or elevated mRNA expression was previously associated with late onset, advanced stage, deeper tumor extension and the presence of metastasis.[18] We also previously described MYC deregulation in preneoplastic gastric lesions.[15,19,54] Therefore, our results suggest that the observed associations among SRC, LYN and MYC might be necessary for gastric carcinogenesis progression.

In our study, CKB downregulation was observed in GC samples. *CKB* downregulation was previously detected in a transcriptomic study,[43] however, further validation by other methodologies was lacking. This enzyme is overexpressed in a wide variety of cancers,[37,55,56] with the exception of colon cancer.[57,58] Li et al.[57] showed that CKB knockdown inhibits ovarian cancer progression by decreasing glycolysis. Our previous proteomic study suggested the presence of a Warburg effect in GC,[40] and we also reported the upregulation of the *MYC* oncogene,[14-18] which appears to contribute to this effect.[39] Therefore, the downregulation of CKB and its strong inverse correlation with MYC expression is

not in agreement with the role of CKB in the regulation of glycolysis in gastric carcinogenesis.[40]

Mooney et al.[58] suggested that ATP storage is not the most important function of CKB in colon cancer, in which the expression of CKB protein and mRNA are downregulated. These authors showed that the overexpression of CKB-C283S, a dominant-negative construct with effects similar to CKB downregulation, appears to promote the epithelial-to-mesenchymal transition in colon cancer.[58] In addition, the authors showed that although CKB expression may be advantageous to the formation of a solid tumor, it appears to be a hindrance to the metastatic potential of colon cancer cells. Similar to the findings of Mooney et al.[58], our results also showed that reduced CKB in GC may have a role in tissue invasion and metastasis. Moreover, Mooney et al.[58] also showed that colon cancer cells overexpressing CKB-C283S and cultured in medium without glucose presented higher expression of MYC than cells with a wild-type CKB construct. In the present study, we detected a strong inverse correlation between CKB and MYC. Thus, an inverse correlation between CKB and MYC might also be necessary for GC progression.

Interestingly, increased SRC and LYN expression and reduced CKB expression were also associated with late-onset GC. Clinicopathological differences between early-onset and late-onset GC have been described,[59-61] but little is known about the genetic and epigenetic changes associated with the age of onset of GC[62]. Buffart et al.[63] previously demonstrated that young and old patients belong to groups with different genomic profiles. The deregulation of the three studied kinases highlights the heterogeneity of GC.

DNA methylation of CpG islands plays a crucial role in the regulation of gene expression. Our group previously reported alterations in the DNA methylation pattern of several oncogenes and tumor suppressor genes in GC samples of individuals from Northern Brazil.[18,64-70] According to CpG Island Searcher, the *SRC* and *LYN* genes contain a CpG island of more than 1 kb (http://cpgislands.usc.edu/; version: 10/29/04). The first CpG island in the *SRC* gene is between intron 1 and intron 2, and the *LYN* gene has a CpG island in its promoter,

exon 1 and part of intron 1. Among the three studied kinases, *CKB* has the largest CpG island, almost 3 kb, located between its promoter and intron 3. To the best of our knowledge, no previous study has evaluated the methylation patterns of these kinases in gastric tissue samples.

In the present study, we observed that the *SRC*, *LYN* and *CKB* methylation patterns were altered in GC. *SRC* and *LYN* hypomethylation was only detected in GC samples. The loss of *SRC* and *LYN* methylation was associated with increased levels of mRNA and protein expression, suggesting that DNA methylation is involved in regulating the expression of these kinases. Moreover, patients with tumors presenting loss of *SRC* and *LYN* methylation also exhibited altered methylation patterns for these genes in non-neoplastic gastric mucosa, albeit at a lower level. Although further investigation of premalignant GC is necessary, this finding suggests that individuals with the loss of *SRC* and *LYN* methylation in the gastric mucosa may have a higher risk for GC. In contrast, the frequency of hypermethylation and partial methylation of *CKB* was higher in GC samples than in non-neoplastic gastric samples. No neoplastic gastric cells presented CKB immunoreactivity, and we were not able to demonstrate a direct correlation between gain of methylation and reduced CKB expression. Tumors with partial methylation of *CKB* presented reduced protein (by western blotting) and mRNA expression compared to tumors with hypermethylated and hypomethylated *CKB*. Our results are in agreement with the study of Ishikawa et al.[44], which evaluated *CKB* methylation patterns in seven GC cell lines and other solid tumor cell lines. These authors reported that *CKB* mRNA was expressed at higher levels in cells with an unmethylated *CKB* promoter than in cells with a methylated promoter but that this difference in expression was not significant. Therefore, the DNA methylation of other CpGs, as well as other genetic and epigenetic mechanisms, may also have a role in *CKB* gene expression. Moreover, posttranscriptional mechanisms may be involved in CKB regulation in gastric carcinogenesis because we observed that the frequency of samples presenting reduced CKB protein expression was higher than those presenting reduced mRNA expression.

The identification of specific DNA methylation patterns may help in the classification of GC and could be associated with specific clinical outcomes. Here, we report that *SRC* and *LYN* hypomethylation (a pattern only detected in cancer samples) was more frequent in tumors with deeper tumor invasion and with lymph node or distant metastasis. SRC hypomethylation was also more frequent in advanced GC. These findings support the hypothesis that DNA methylation is involved in *SRC* and *LYN* regulation because we also observed that the elevated expression of theses kinases may have a role in GC invasiveness and metastasis. DNA is a stable molecule, and the detection of DNA methylation by the MSP assay (a qualitative method) may be readily used as an approach for GC prognosis in the clinical routine. Therefore, analysis of the *SRC* and *LYN* methylation patterns may help in determining GC prognosis.

Partial methylation of *CKB* was associated with deeper tumor invasion and distant metastasis, which is in agreement with the observation that tumors with partial methylation of *CKB* presented reduced expression of this kinase. Thus, *CKB* methylation might contribute to GC cell migration and invasion.

LYN hypomethylation and its expression were associated with late-onset GC. In addition, partial methylation of *LYN* in non-neoplastic gastric samples was more frequent in older patients. The incidence of GC increases with age, highlighting the association between age-related methylation and GC development.[68,71]

In conclusion, our study provides a basis for the development of a biomarker for the prognosis of GC. Expression of SRC, LYN and CKB in gastric cancer is significantly associated with tumor invasion and lymph node and distant metastases, as well as with MYC expression, which is also a possible biomarker for GC. In addition, these three kinases appear to be regulated, at least in part, by DNA methylation in GC. SRC, LYN and CKB proteins or DNA methylation could serve as markers for predicting tumor progression and target in anti-cancer strategies.

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REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55:74-108.

2. Kimura Y, Noguchi T, Kawahara K, Kashima K, Daa T, Yokoyama S. Genetic alterations in 102 primary gastric cancers by comparative genomic hybridization: gain of 20q and loss of 18q are associated with tumor progression. Mod Pathol 2004; 17:1328-37.

3. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144:646-74.

4. Rask-Andersen M, Zhang J, Fabbro D, Schioth HB. Advances in kinase targeting: current clinical use and clinical trials. Trends Pharmacol Sci 2014; 35:604-20.

5. Koster H, Little DP, Luan P, Muller R, Siddiqi SM, Marappan S, Yip P. Capture compound mass spectrometry: a technology for the investigation of small molecule protein interactions. Assay Drug Dev Technol 2007; 5:381-90.

6. Dalhoff C, Huben M, Lenz T, Poot P, Nordhoff E, Koster H, Weinhold E. Synthesis of Sadenosyl- L-homocysteine capture compounds for selective photoinduced isolation of methyltransferases. ChemBioChem 2010; 11:256-65.

7. Takekura N, Yasui W, Yoshida K, Tsujino T, Nakayama H, Kameda T, Yokozaki H, Nishimura Y, Ito H, Tahara E. pp60c-src protein kinase activity in human gastric carcinomas. Int J Cancer 1990; 45:847-51.

8. Masaki T, Shiratori Y, Okada H, Nishioka M, Taniguchi K, Hatanaka Y, Omata M. pp60c-src activation in gastric carcinoma: a preliminary study. Am J Gastroenterol 2000; 95:837-8.

9. Humar B, Fukuzawa R, Blair V, Dunbier A, More H, Charlton A, Yang HK, Kim WH, Reeve AE, Martin I, et al. Destabilized adhesion in the gastric

proliferative zone and c-Src kinase activation mark the development of early diffuse gastric cancer. Cancer Res 2007; 67:2480-9.

10. Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. Cancer Metastasis Rev 2003; 22:337-58.

11. Kim LC, Song L, Haura EB. Src kinases as therapeutic targets for cancer. Nat Rev Clin Oncol 2009; 6:587-95.

12. Abram CL, Courtneidge SA. Src family tyrosine kinases and growth factor signaling. Exp Cell Res 2000; 254:1-13.

13. Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. Mol Cell Biol 2000; 20:9018-27.

14. Calcagno DQ, Freitas VM, Leal MF, de Souza CR, Demachki S, Montenegro R, Assumpcao PP, Khayat AS, Smith Mde A, dos Santos AK, et al. MYC, FBXW7 and TP53 copy number variation and expression in gastric cancer. BMC Gastroenterol 2013; 13:141.

15. Calcagno DQ, Leal MF, Demachki S, Araujo MT, Freitas FW, Oliveira e Souza D, Assumpcao PP, Ishak G, de Arruda Cardoso Smith M, Burbano RR. MYC in gastric carcinoma and intestinal metaplasia of young adults. Cancer Genet Cytogenet 2010; 202:63-6.

16. Calcagno DQ, Leal MF, Seabra AD, Khayat AS, Chen ES, Demachki S, Assumpcao PP, Faria MH, Rabenhorst SH, Ferreira MV, et al. Interrelationship between chromosome 8 aneuploidy, C-MYC amplification and increased expression in individuals from northern Brazil with gastric adenocarcinoma. World J Gastroenterol 2006; 12:6207-11.

17. Costa Raiol LC, Figueira Silva EC, Mendes da Fonseca D, Leal MF, Guimaraes AC, Calcagno DQ, Khayat AS, Assumpcao PP, de Arruda Cardoso Smith M, Burbano RR. Interrelationship between MYC gene numerical aberrations and protein expression in individuals from northern Brazil with early gastric adenocarcinoma. Cancer Genet Cytogenet 2008; 181:31-5.

18. de Souza CR, Leal MF, Calcagno DQ, Costa Sozinho EK, Borges Bdo N, Montenegro RC, Dos Santos AK, Dos Santos SE, Ribeiro HF, Assumpcao PP, et al. MYC deregulation in gastric cancer and its clinicopathological implications. PLoS One 2013; 8:e64420.

19. da Costa Jde F, Leal MF, Silva TC, Andrade Junior EF, Rezende AP, Muniz JA, Lacreta Junior AC, Assumpcao PP, Calcagno DQ, Demachki S, et al. Experimental gastric carcinogenesis in Cebus apella nonhuman primates. PLoS One 2011; 6:e21988.

20. Okamoto W, Okamoto I, Yoshida T, Okamoto K, Takezawa K, Hatashita E, Yamada Y, Kuwata K, Arao T, Yanagihara K, et al. Identification of c-Src as a potential therapeutic target for gastric cancer and of MET activation as a cause of resistance to c-Src inhibition. Mol Cancer Ther 2010; 9:1188-97.

21. Assumpcao PP, Ishak G, Chen ES, Takeno SS, Leal MF, Guimaraes AC, Calcagno DQ, Khayat AS, Demachki S, Smith Mde A, et al. Numerical aberrations of chromosome 8 detected by conventional cytogenetics and fluorescence in situ hybridization in individuals from northern Brazil with gastric adenocarcinoma. Cancer Genet Cytogenet 2006; 169:45-9.

22. Burbano RR, Assumpcao PP, Leal MF, Calcagno DQ, Guimaraes AC, Khayat AS, Takeno SS, Chen ES, De Arruda Cardoso Smith M. C-MYC locus amplification as metastasis predictor in intestinal-type gastric adenocarcinomas: CGH study in Brazil. Anticancer Res 2006; 26:2909-14.

23. Calcagno DQ, Leal MF, Taken SS, Assumpcao PP, Demachki S, Smith Mde A, Burbano RR. Aneuploidy of chromosome 8 and C-MYC amplification in individuals from northern Brazil with gastric adenocarcinoma. Anticancer Res 2005; 25:4069-74.

24. Leal MF, Martins do Nascimento JL, da Silva CE, Vita Lamarao MF, Calcagno DQ, Khayat AS, Assumpcao PP, Cabral IR, de Arruda Cardoso Smith M, Burbano RR. Establishment and conventional cytogenetic characterization of three gastric cancer cell lines. Cancer Genet Cytogenet 2009; 195:85-91.

25. Lima EM, Rissino JD, Harada ML, Assumpcao PP, Demachki S, Guimaraes AC, Casartelli C, Smith MA, Burbano RR. Conventional cytogenetic characterization of a new cell line, ACP01, established from a primary human gastric tumor. Braz J Med Biol Res 2004; 37:1831-8.

26. Donato NJ, Wu JY, Stapley J, Gallick G, Lin H, Arlinghaus R, Talpaz M. BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. Blood 2003; 101:690-8.

27. Su N, Peng L, Xia B, Zhao Y, Xu A, Wang J, Wang X, Jiang B. Lyn is involved in CD24- induced ERK1/2 activation in colorectal cancer. Mol Cancer 2012; 11:43.

28. Elsberger B, Fullerton R, Zino S, Jordan F, Mitchell TJ, Brunton VG, Mallon EA, Shiels PG, Edwards J. Breast cancer patients' clinical outcome measures are associated with Src kinase Family member expression. Br J Cancer 2010; 103:899-909.

29. Zardan A, Nip KM, Thaper D, Toren P, Vahid S, Beraldi E, Fazli L, Lamoureux F, Gust KM, Cox ME, et al. Lyn tyrosine kinase regulates androgen receptor expression and activity in castrateresistant prostate cancer. Oncogenesis 2014; 3:e115.

30. Bundela S, Sharma A, Bisen PS. Potential therapeutic targets for oral cancer: ADM, TP53, EGFR, LYN, CTLA4, SKIL, CTGF, CD70. PLoS One 2014; 9:e102610.

31. Qayyum T, McArdle PA, Lamb GW, Jordan F, Orange C, Seywright M, Horgan PG, Jones RJ, Oades G, Aitchison MA, et al. Expression and prognostic significance of Src family members in renal clear cell carcinoma. Br J Cancer 2012; 107:856-63.

32. Guan H, Zhou Z, Gallick GE, Jia SF, Morales J, Sood AK, Corey SJ, Kleinerman ES. Targeting Lyn inhibits tumor growth and metastasis in Ewing's sarcoma. Mol Cancer Ther 2008; 7:1807-16.

33. Prabhu JS, Korlimarla A, Banerjee A, Wani S, K P, Sahoo R. Gene-specific methylation: potential markers for colorectal cancer. Int J Biol Markers 2009; 24:57-62.

34. Patel N, Black J, Chen X, Marcondes AM, Grady WM, Lawlor ER, Borinstein SC. DNA methylation and gene expression profiling of ewing sarcoma primary tumors reveal genes that are potential targets of epigenetic inactivation. Sarcoma 2012; 2012:498472.

35. Hoshino K, Quintas-Cardama A, Yang H, Sanchez-Gonzalez B, Garcia-Manero G. Aberrant DNA methylation of the Src kinase Hck, but not of Lyn, in Philadelphia chromosome negative acute lymphocytic leukemia. Leukemia 2007; 21:906-11.

36. Szyf M. DNA methylation signatures for breast cancer classification and prognosis. Genome Med 2012; 4:26.

37. Wallimann T, Hemmer W. Creatine kinase in non-muscle tissues and cells. Mol Cell Biochem 1994; 133-134:193-220.

38. Warburg O. On the origin of cancer cells. Science 1956; 123:309-14.

39. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer 2011; 11:85-95.

40. Leal MF, Chung J, Calcagno DQ, Assumpcao PP, Demachki S, da Silva ID, Chammas R, Burbano RR, de Arruda Cardoso Smith M. Differential proteomic analysis of noncardia gastric cancer from individuals of northern Brazil. PLoS One 2012; 7:e42255.

41. Nair NG, Blessantoli M, Vennila J. Gene Expression in Gastric Cancer for Singapore and UK Population: An Insilico Comparative Approach. Res J Recent Sci 2014; 3:7.

42. Cui J, Chen Y, Chou WC, Sun L, Chen L, Suo J, Ni Z, Zhang M, Kong X, Hoffman LL, et al. An integrated transcriptomic and computational analysis

for biomarker identification in gastric cancer. Nucleic Acids Res 2011; 39:1197-207.

43. Eftang LL, Esbensen Y, Tannaes TM, Blom GP, Bukholm IR, Bukholm G. Up-regulation of CLDN1 in gastric cancer is correlated with reduced survival. BMC Cancer 2013; 13:586.

44. Ishikawa J, Taniguchi T, Takeshita A, Maekawa M. Increased creatine kinase BB activity and CKB mRNA expression in patients with hematologic disorders: relation to methylation status of the CKB promoter. Clin Chim Acta 2005; 361:135-40.

45. Lauren P. The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. an Attempt at a Histo-Clinical Classification. Acta Pathol Microbiol Scand 1965; 64:31-49.

46. Washington K. 7th edition of the AJCC cancer staging manual: stomach. Ann Surg Oncol 2010; 17:3077-9.

47. de Souza CR, de Oliveira KS, Ferraz JJ, Leal MF, Calcagno DQ, Seabra AD, Khayat AS, Montenegro RC, Alves AP, Assumpcao PP, et al. Occurrence of Helicobacter pylori and Epstein-Barr virus infection in endoscopic and gastric cancer patients from Northern Brazil. BMC Gastroenterol 2014; 14:179.

48. Leal MF, Calcagno DQ, Demachki S, Assumpcao PP, Chammas R, Burbano RR, Smith Mde A. Clinical implication of 14-3-3 epsilon expression in gastric cancer. World J Gastroenterol 2012; 18:1531-7.

49. Leal MF, Mazzotti TK, Calcagno DQ, Cirilo PD, Martinez MC, Demachki S, Assumpcao PP, Chammas R, Burbano RR, Smith MC. Deregulated expression of Nucleophosmin 1 in gastric cancer and its clinicopathological implications. BMC Gastroenterol 2014; 14:9.

50. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-8.

51. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996; 93:9821-6.

52. Borges Bdo N, Santos Eda S, Bastos CE, Pinto LC, Anselmo NP, Quaresma JA, Calcagno DQ, Burbano RM, Harada ML. Promoter polymorphisms and methylation of E-cadherin (CDH1) and KIT in gastric cancer patients from northern Brazil. Anticancer Res 2010; 30:2225-33.

53. Yang Y, Bai ZG, Yin J, Wu GC, Zhang ZT. Role of c-Src activity in the regulation of gastric cancer cell migration. Oncol Rep 2014; 32:45-9.

54. Silva TC, Leal MF, Calcagno DQ, de Souza CR, Khayat AS, dos Santos NP, Montenegro RC, Rabenhorst SH, Nascimento MQ, Assumpcao PP, et al. hTERT, MYC and TP53 deregulation in gastric preneoplastic lesions. BMC Gastroenterol 2012; 12:85.

55. Huddleston HG, Wong KK, Welch WR, Berkowitz RS, Mok SC. Clinical applications of microarray technology: creatine kinase B is an up-regulated gene in epithelial ovarian cancer and shows promise as a serum marker. Gynecol Oncol 2005; 96:77-83.

56. Zarghami N, Giai M, Yu H, Roagna R, Ponzone R, Katsaros D, Sismondi P, Diamandis EP. Creatine kinase BB isoenzyme levels in tumour cytosols and survival of breast cancer patients. Br J Cancer 1996; 73:386-90.

57. Li XH, Chen XJ, Ou WB, Zhang Q, Lv ZR, Zhan Y, Ma L, Huang T, Yan YB, Zhou HM. Knockdown of creatine kinase B inhibits ovarian cancer progression by decreasing glycolysis. Int J Biochem Cell Biol 2013; 45:979-86.

58. Mooney SM, Rajagopalan K, Williams BH, Zeng Y, Christudass CS, Li Y, Yin B, Kulkarni P, Getzenberg RH. Creatine kinase brain overexpression protects colorectal cells from various metabolic and non-metabolic stresses. J Cell Biochem 2011; 112:1066-75.

59. Tavares A, Gandra A, Viveiros F, Cidade C, Maciel J. Analysis of clinicopathologic characteristics and prognosis of gastric cancer in young and older patients. Pathol Oncol Res 2013; 19:111-7.

60. Kitamura K, Yamaguchi T, Taniguchi H, Hagiwara A, Yamane T, Sawai K, Takahashi T. Clinicopathological characteristics of gastric cancer in the elderly. Br J Cancer 1996; 73:798-802.

61. Maehara Y, Emi Y, Tomisaki S, Oshiro T, Kakeji Y, Ichiyoshi Y, Sugimachi K. Age-related characteristics of gastric carcinoma in young and elderly patients. Cancer 1996; 77:1774-80.

62. Panani AD. Cytogenetic and molecular aspects of gastric cancer: clinical implications. Cancer Lett 2008; 266:99-115.

63. Buffart TE, Carvalho B, Hopmans E, Brehm V, Kranenbarg EK, Schaaij-Visser TB, Eijk PP, van Grieken NC, Ylstra B, van de Velde CJ, et al. Gastric cancers in young and elderly patients show different genomic profiles. J Pathol 2007; 211:45-51.

64. Gigek CO, Leal MF, Lisboa LC, Silva PN, Chen ES, Lima EM, Calcagno DQ, Assumpcao PP, Burbano RR, Smith Mde A. Insulin-like growth factor binding protein-3 gene methylation and protein expression in gastric adenocarcinoma. Growth Horm IGF Res 2010; 20:234-8.

65. Gigek CO, Leal MF, Silva PN, Lisboa LC, Lima EM, Calcagno DQ, Assumpcao PP, Burbano RR, Smith Mde A. hTERT methylation and expression in gastric cancer. Biomarkers 2009; 14:630-6.

66. Gigek CO, Lisboa LC, Leal MF, Silva PN, Lima EM, Khayat AS, Assumpcao PP, Burbano RR, Smith Mde A. SMARCA5 methylation and expression in gastric cancer. Cancer Invest 2011; 29:162-6.

67. Lima EM, Leal MF, Burbano RR, Khayat AS, Assumpcao PP, Bello MJ, Rey JA, Smith MA, Casartelli C. Methylation status of ANAPC1, CDKN2A and TP53 promoter genes in individuals with gastric cancer. Braz J Med Biol Res 2008; 41:539-43.

68. Leal M, Lima E, Silva P, Assumpcao P, Calcagno D, Payao S, Burbano RR, Smith M. Promoter hypermethylation of CDH1, FHIT, MTAP and PLAGL1 in gastric adenocarcinoma in individuals from Northern Brazil. World J Gastroenterol 2007; 13:2568-74.

69. Lima EM, Leal MF, Motta FJN, Assumpção PP, Harada ML, Smith MAC, Burbano RR, Casartelli C. Study of methylation pattern of de novo DNA methyltransferase genes and its correlation with DNA methylation pattern of RUNX3 in individuals with gastric cancer from Northern region of Brazil. International Journal of Morphology 2008; 25:8.

70. Lima EM, Leal MF, Smith MAC, Burbano RR, Assumpção PP, Bello MJ, Rey JA, Lima FF, Casartelli C. DNA mismatch repair gene methylation in gastric cancer in individuals from northern Brazil. Biocell 2008; 32:7.

71. Waki T, Tamura G, Tsuchiya T, Sato K, Nishizuka S, Motoyama T. Promoter methylation status of E-cadherin, hMLH1, and p16 genes in nonneoplastic gastric epithelia. Am J Pathol 2002; 161:399-403.

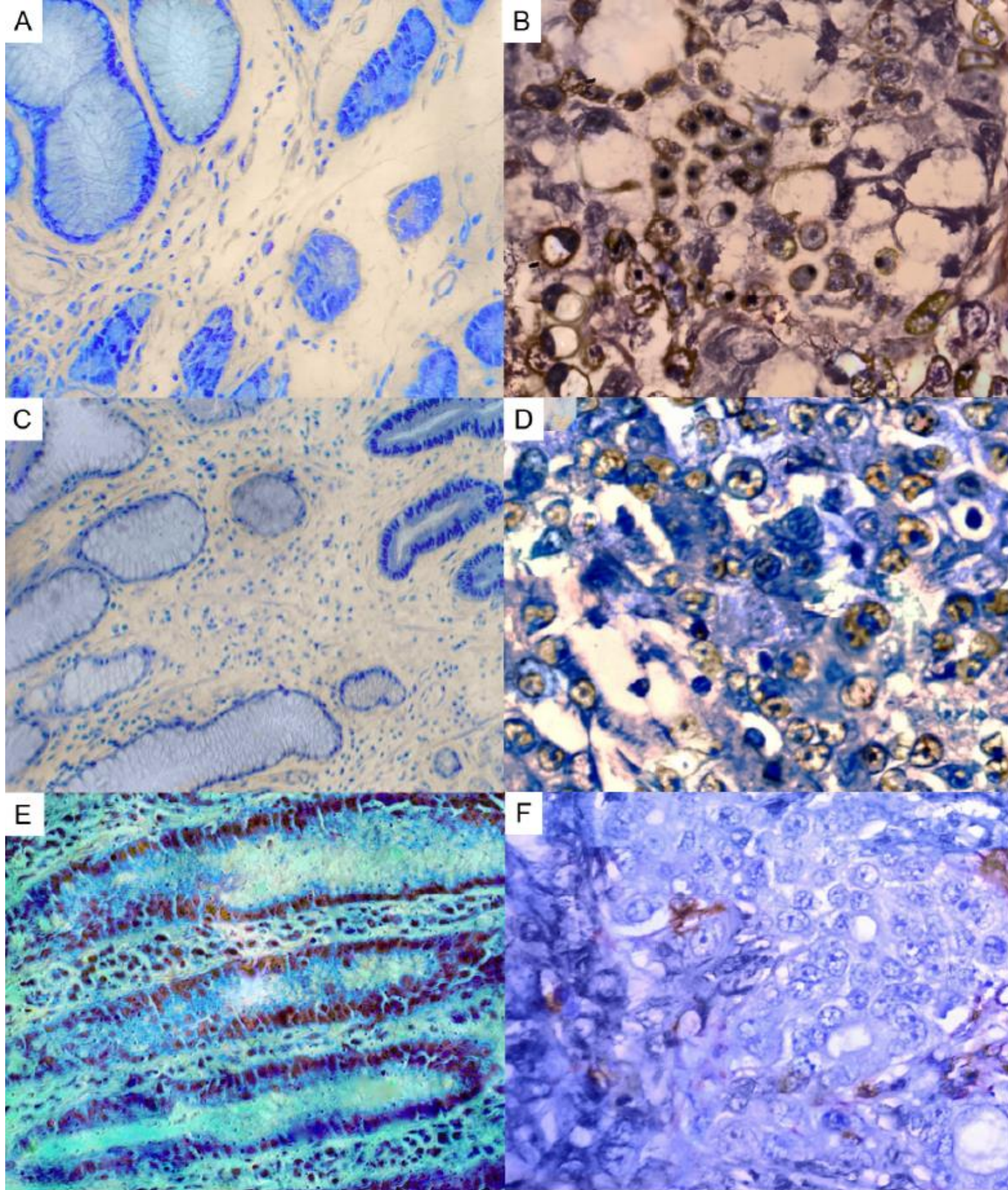

Figure 1. Kinases immunoreactivity in gastric tissue samples. A) gastric mucosa without SRC immunoreactivity; B) diffuse-type gastric cancer presenting cell membrane and cytoplasmic immunoreactivity of SRC; C) non-neoplastic gastric tissue without LYN immunoreactivity; D) intestinal-type gastric cancer presenting LYN immunoreactivity; E) non-neoplastic gastric mucosa showing weak cytoplasmic CKB staining in glandular cells; F) diffuse-type gastric cancer cells without CKB immunoreactivity.

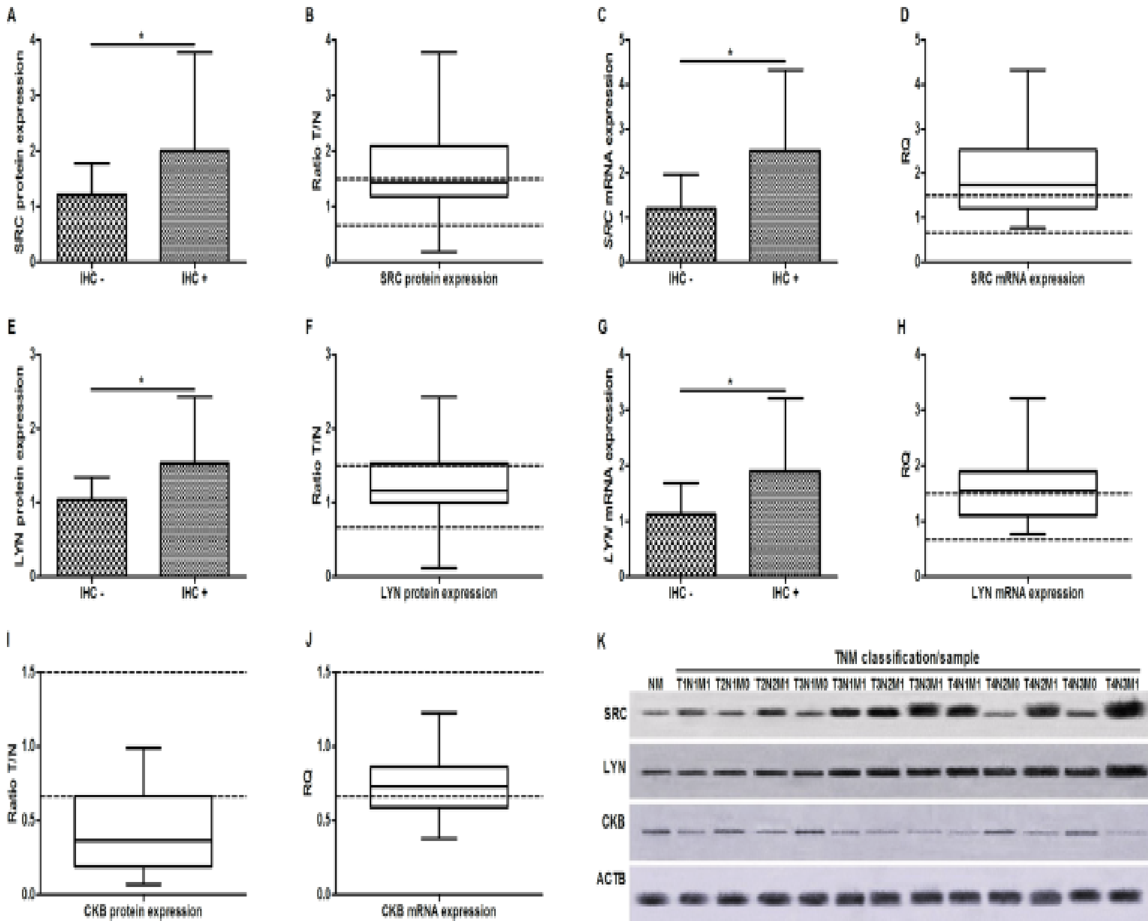

Figure 2. Kinases expression in gastric cancer. A) Association between SRC immunoreactivity and its protein expression; B) SRC protein expression; C) Association between SRC immunoreactivity and its mRNA expression; D) *SRC* mRNA expression. E) Association between LYN immunoreactivity and its protein expression; F) LYN protein expression; G) Association between LYN immunoreactivity and its mRNA expression; H) *LYN* mRNA expression; I) CKB protein expression; J) *CKB* mRNA expression; K) representative image of Western-blot, in each TNM of each sample is show. Protein and mRNA expression were determined by Western-blot and RT-qPCR analysis, respectively. In all graphs, the expression in gastric tumors was normalized by matched non-neoplastic gastric tissue. *Significant difference between groups by Mann-Whitney ($p < 0.05$). IHC+: cases presenting protein immunoreactivity; IHC–: cases without protein immunoreactivity NM: normal mucosa sample.

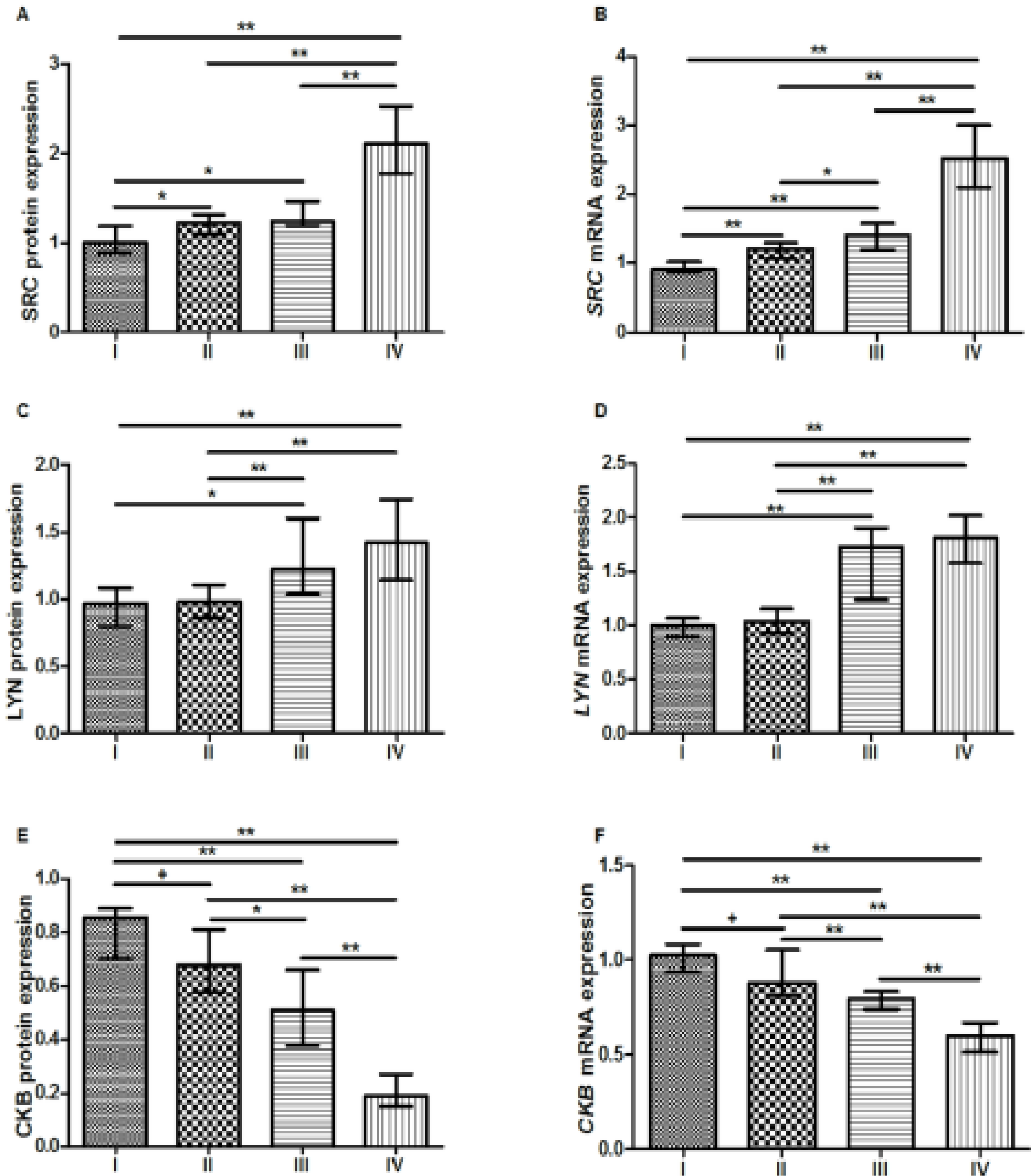

Figure 3. Kinases protein and mRNA expression by tumor stage. A) SRC protein expression; B) *SRC* mRNA expression; C) LYN protein expression; D) *LYN* mRNA expression; E) CKB protein expression; F) *CKB* mRNA expression. Protein and mRNA expression were determined by Westernblot and RT-qPCR analysis, respectively. In these analyses, the expression in gastric tumors was normalized by matched non-neoplastic gastric tissue. *Significant difference between groups by Mann-Whitney test followed by Bonferroni corrections for multiple comparison analysis ($p < 0.008$); **Significant difference between groups by Mann-Whitney test followed by Bonferroni corrections for multiple comparison analysis ($p < 0.001$); +Difference between groups but not statistically significant after Bonferroni adjustment ($p < 0.05$).

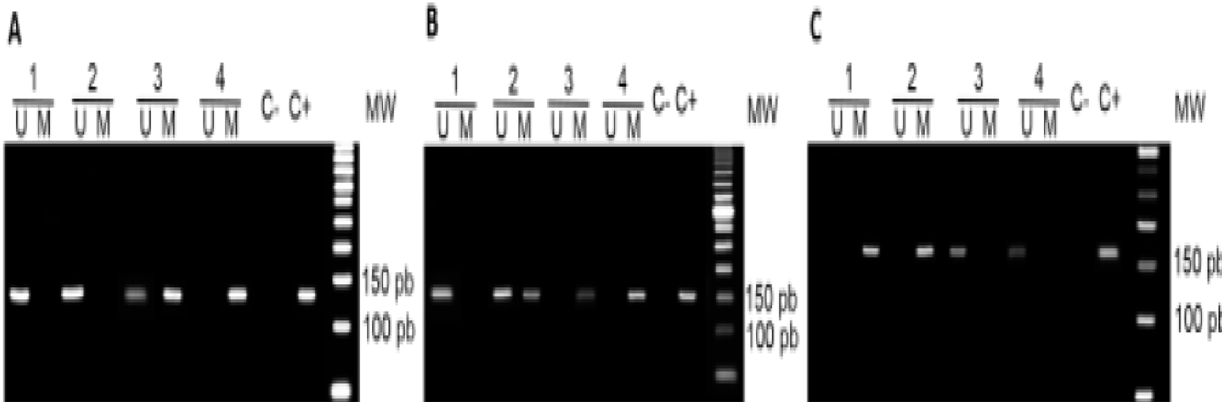

Figure 4. Methylation analysis of the kinases promoters showing methylated and unmethylated bands. A) *SRC* promoted methylation analysis, in which samples 1 and 2 presented hypomethylated promoter, sample 3 presented partial methylation and sample 4 presented hypermethylated promoter; B) *LYN* promoter methylation analysis, in which samples 1 presented hypomethylated promoter, sample 2 presented partial methylation and samples 3 and 4 presented hypermethylated promoter; C) CKB promoter methylation analysis, in which samples 1 and 2 presented hypermethylated promoter, and samples 3 and 4 presented hypomethylated promoter. C–: blank; C+: positive control, gDNA sample completely methylated; U: PCR with unmethylated primer set; M: PCR with methylated primer set; MW: molecular weight marker; bp: base pairs.

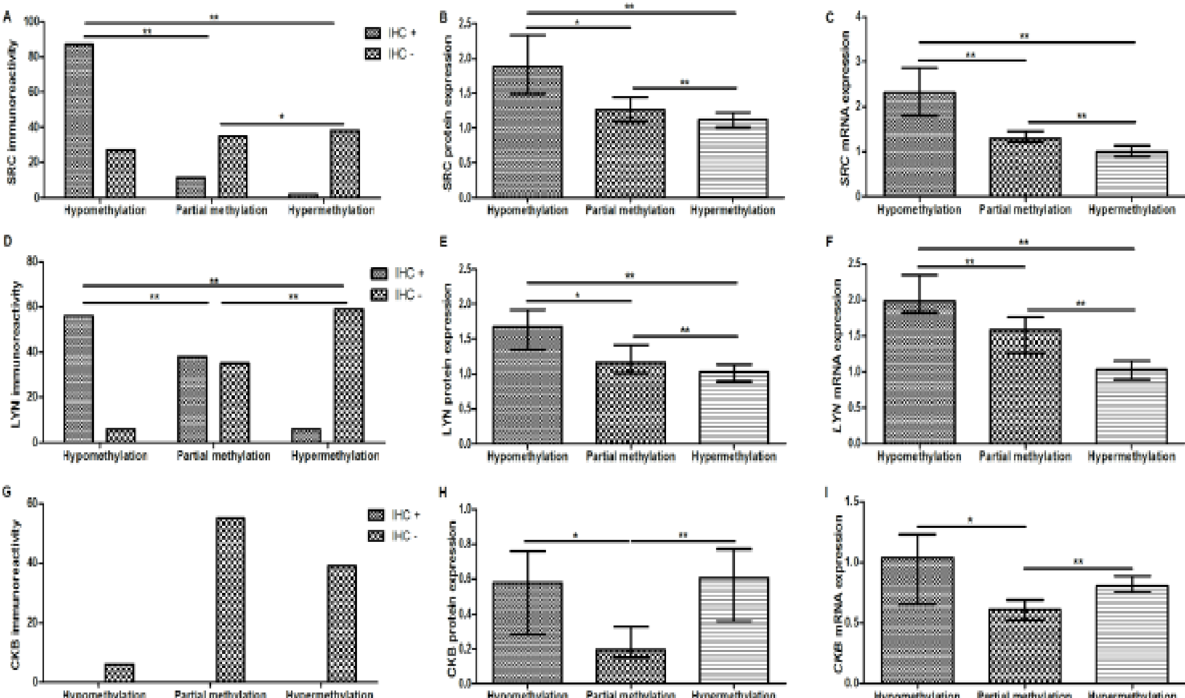

Figure 5. Kinases expression regulation by DNA methylation. A) SRC immunoreactivity; B) SRC protein expression; C) *SRC* mRNA expression; D) LYN protein immunoreactivity; E) LYN protein expression; F) *LYN* mRNA expression; G) CKB immunoreactivity; H) CKB protein expression; I) *CKB* mRNA expression. Protein and mRNA expression were determined by Western-blot and RTqPCR analysis, respectively. In these analyses, the expression in gastric tumors was normalized by matched non-neoplastic gastric tissue. *Significant difference between groups by $\chi 2$ (for analysis involving IHC data) or Mann-Whitney tests followed by Bonferroni corrections for multiple comparison analysis ($p < 0.0167$); **Significant difference between groups by $\chi 2$ (for analysis involving IHC data) or Mann-Whitney tests followed by Bonferroni corrections for multiple comparison analysis ($p < 0.001$). IHC+: cases presenting protein immunoreactivity; IHC–: cases without protein immunoreactivity.

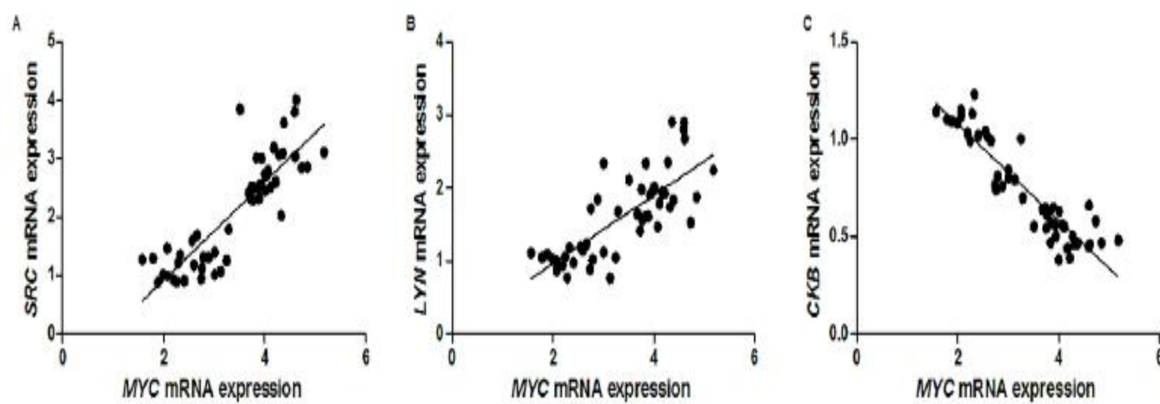

Figure 6. Correlation between *MYC* and kinases mRNA expression. A) *MYC* and *SRC*; B) *MYC* and *LYN*; C) *MYC* and *CKB*.

Table 1: Primer sequences (5'-3') for methylation analysis.

Gene	Type	Sense	Antisense	Anneling temperature (°C)	Product size (bp)
SRC	MSP – M	5' GATTATTTTGGCGTCGGATC 3'	5' ATCACAACAAAAAACCGCG 3'	58	141
	MSP – U	5' GGATTATTTTGGTGTTGGATT 3'	5' CATCACAACAAAAAACCACA 3'	54	143
LYN	MSP – M	5' AGGTTTCGTAGGTGTTCGTC 3'	5' CGACTTCCCCACTATATACGA 3'	55	152
	MSP – U	5' TTGAGGTTTTGTAGGTGTTTGTT 3'	5' CAACTTCCCCACTATATACAAAAA 3'	55	155
CKB	MSP – M	5' CGTTAAGGGATTGGGTTTC 3'	5' ATAAAATCCCAACGACGAAA 3'	56	164
	MSP – U	5' GTGTGTTAAGGGATTGGGTTTT 3'	5' ATAAAATCCCAACAACAAAAAAA 3'	56	167

MSP: primers for methyl-specific PCR; M: primer for methylated sequence by MSP; U: primer for unmethylated sequences by MSP; bp: base pair.

Table 2:Clinicopathological variables and kinases expression in gastric cancer.

Variable	N	SRC immmnoreactivity		SRC protein		*SRC* mRNA		LYN immunoreactivity		LYN protein		*LYN* mRNA		CKB immmnoreactivity		CKB protein		*CKB* mRNA	
		N (%) of positive cases	p-value[a]	Ratio T/N (median ± IQR)	p-value[b]	RQ (median ± IQR)	p-value[b]	N (%) of positive cases	p-value[a]	Ratio T/N (median ± IQR)	p-value[b]	RQ (median ± IQR)	p-value[b]	N (%) of negative cases	p-value[a]	Ratio T/N (median ± IQR)	p-value[b]	RQ (median ± IQR)	p-value[b]
Gender																			
Female	50	27 (54)	0.442	1.33±0.82	0.285	1.63±1.16	0.674	25 (50)	0.417	1.19±0.48	0.728	1.56±0.77	0.910	50 (100)	-	0.37±0.51	0.529	0.73±0.26	0.750
Male	88	45 (51.1)		1.67±0.99		1.79±1.36		41 (62.1)		1.15±0.53		1.56±0.82		88 (100)		0.36±0.47		0.73±0.31	
Onset																			
< 45 years	35	13 (37.1)	0.031*	1.29±0.69	0.011*	1.46±1.05	0.027*	10 (28.6)	0.007*	1.08±0.27	0.008*	1.23±0.64	0.014*	35 (100)	-	0.49±0.48	0.028*	0.77±0.22	0.132
≥ 45 years	103	59 (57.3)		1.67±1.02		1.82±1.5		56 (54.4)		+1.22±0.62		1.64±0.82		103 (100)		0.32±0.49		0.71±0.30	
Tumor location																			
Cardia	52	22 (42.3)	0.052	1.35±0.77	0.263	1.46±1.2	0.152	21 (40.4)	0.118	1.14±0.57	0.276	1.25±0.82	0.090	52 (100)		0.40±0.47	0.190	0.74±0.24	0.309
Non-cardia	82	50 (58.1)		1.64±0.94		1.85±1.35		45 (52.3)		1.19±0.50		1.62±0.79		86 (100)		0.34±0.47		0.72±0.28	
Histological type																			
Diffuse	64	35 (54.7)	0.353	1.58±0.84	0.665	1.89±1.34	0.838	31 (48.4)	0.515	1.18±0.55	0.785	1.58±0.82	0.629	64 (100)	-	0.33±0.48	0.651	0.71±0.26	0.210

Intestinal	74	37 (50)		1.44±1.03		1.63±1.40		35 (47.3)		1.15±0.45		1.50±0.80		74 (100)		0.39±0.50		0.74±0.27	
Stage																			
Early	12	3 (25)	0.046*	1.04±0.67	0.001*	1.04±0.78	0.002*	0 (0)	<0.001*	1.02±0.19	0.003*	0.89±0.27	<0.001*	12 (100)		0.74±0.58	0.015*	0.95±0.38	0.019*
Advanced	126	69 (54.8)		1.55±0.94		1.80±1.34		66 (52.4)		1.21±0.53		1.61±0.77		126 (100)		0.36±0.47		0.72±0.26	
Tumor invasion																			
T1/T2	43	14 (32.6)	0.002*	1.24±0.64	<0.001*	1.29±0.99	<0.001*	5 (11.6)	<0.001*	1.05±0.26	<0.001*	1.17±0.30	<0.001*	43 (100)	-	0.66±0.54	<0.001*	0.88±0.31	<0.001*
T3/T4	95	58 (61.1)		1.76±1.07		1.99±1.55		61 (64.2)		1.32±0.60		1.75±0.81		95 (100)		0.28±0.40		0.69±0.24	
Lymph node metastasis																			
Absent	16	0 (0)	<0.001*	1.13±0.26	<0.001*	0.93±0.24	<0.001*	1 (6.3)	<0.001*	1.01±0.26	<0.001*	0.99±0.17	<0.001*	16 (100)	-	0.73±0..20	<0.001*	0.95±0.24	<0.001*
Present	122	72 (59)		1.67±0.98		1.89±1.29		65 (53.3)		1.23±0.52		1.63±0.73		122 (100)		0.31±0.40		0.70±0.25	
Distant metastasis																			
Absent	70	11 (15.7)	<0.001*	1.22±0.25	<0.001*	1.22±0.41	<0.001*	16 (22.9)	<0.001*	106±0.28	<0.001*	1.11±0.38	<0.001*	70 (100)	-	0.66±0.31	<0.001*	0.86±0.22	<0.001*
Present	68	61 (89.7)		2.11±0.76		2.53±0.92		50 (73.5)		1.43±0.61		1.81±0.44		68 (100)		0.19±0.12		0.60±0.16	
H. pylori																			
Negative	14	9 (64.3)	0.251	1.47±1.03	0.647	1.71±1.47	0.764	9 (64.3)	0.154	1.20±0.53	0.838	1.60±0.91	0.762	14 (100)	-	0.30±0.34	0.391	0.73±0.18	0.841

Positive	124	63 (50.8)		1.44±0.90		1.73±1.32		57 (46)		1.16±0.52		1.54±0.79		124 (100)		0.37±0.50		0.73±0.29	
CagA																			
Negative	49	26 (53.1)	0.510	1.55±0.84	0.730	1.76±1.17	0.779	24 (49)	0.490	1.15±0.52	0.304	1.41±0.73	0.441	49 (100)	-	0.34±0.44	0.836	0.74±0.30	0.437
Positive	89	46 (51.7)		1.44±0.99		1.70±1.40		42 (47.2)		1.17±0.49		1.58±0.82		89 (100)		0.37±0.51		0.73±0.28	
EBV																			
Negative	117	60 (51.3)	0.399	1.44±0.81	0.259	1.67±1.22	0.371	55 (47)	0.414	1.15±0.53	0.718	1.56±0.79	0.652	117 (100)	-	0.37±0.49	0.397	0.73±0.29	0.543
Positive	21	12 (57.1)		1.77±1.21		2.24±1.67		11 (52.4)		1.18±0.56		1.53±0.95		21 (100)		0.27±0.51		0.70±0.28	

[a]p-value by χ^2 test; [b]p-value by Mann-Whitney test. *Significantly difference between groups ($p < 0.05$). N: number of samples; IQR: interquartile range; EBV: *Epstein-Barr virus*

Table 3: Protein kinases methylation pattern in gastric samples

Methylation pattern	*SRC*			*LYN*			*CKB*		
	Neoplastic	Non-neoplastic	p-value	Neoplastic	Non-neoplastic	p-value	Neoplastic	Non-neoplastic	p-value
Hypermethylated	26 (18.8)	59 (42.8)	$<0.001^{a*}$	46 (33.6)	82 (59.4)	$<0.001^{a*}$	48 (39)	0 (0)	$<0.001^{a*}$
Partial-methylated	31 (22.5)	79 (57.2)	0.8355^{b}	50 (36.5)	56 (40.6)	0.1084^{b}	68 (55.3)	59 (42.8)	$<0.001^{b**}$
Hypomethylated	81 (58.7)	0 (0)	$<0.001^{c,d**}$	41 (29.9)	0 (0)	$<0.001^{c,d**}$	7 (5.7)	79 (91.9)	$<0.001^{c,d**}$

[a]p-value of χ^2 test; [b]p-value of the post-hoc comparison between tissue samples hypermethylated and partial-methylated; [c]p-value of the post-hoc comparison between tissue samples partial-methylated and hypomethylated; [d]p-value of the post-hoc comparison between tissue samples hypermethylated and hypomethylated. *Statistically significant difference between neoplastic and non-neoplastic samples by χ^2 test ($p < 0.05$); **Statistically significant difference between groups by χ^2 test followed Bonferroni adjustment ($p < 0.016$).

Table 4: Clinicopathological variables and kinases methylation in gastric cancer.

Variable	N	*SRC*				*LYN*				*CKB*			
		Hypermethylated [N(%)]	Partial-methylated [N(%)}	Hypomethylated [N(%)]	p-value[a]	Hypermethylated [N(%)]	Partial-methylated [N(%)}	Hypomethylated [N(%)]	p-value[a]	Hypermethylated [N(%)]	Partial-methylated [N(%)}	Hypomethylated [N(%)]	p-value[a]
Gender													
Female	50	10 (20)	10 (20)	30 (60)	0.865	18 (36)	16 (32)	16 (32)	0.709	16 (36.4)	26 (59.1)	2 (4.5)	0.794
Male	88	16 (18.2)	21 (23.9)	51 (58)		28 (32.2)	34 (39.1)	25 (28.7)		32 (40.5)	42 (53.2)	5 (6.3)	
Onset													
< 45 years	35	10 (28.6)	6 (17.1)	19 (54.3)	0.212	19 (54.3)	8 (22.9)	8 (22.9)	0.010*	16 (53.3)	14 (46.7)	0 (0)	0.087
≥ 45 years	103	15 (15.5)	25 (24.3)	62 (60.2)		27 (26.5)	33 (32.4)	42 (41.2)		32 (34.4)	54 (58.1)	7 (7.5)	
Tumor location													
Cardia	52	10 (19.2)	16 (30.8)	26 (50)	0.162	21 (41.2)	17 (33.3)	13 (25.5)	0.341	19 (41.3)	25 (54.3)	2 (4.3)	0.843
Non-cardia	82	16 (18.6)	15 (17.4)	55 (64)		25 (29.1)	33 (38.4)	28 (32.6)		29 (37.7)	43 (55.8)	5 (6.5)	
Histological type													
Diffuse	64	13 (20.3)	8 (12.5)	43 (67.2)	0.032*	19 (29.7)	22 (34.4)	23 (35.9)	0.343	22 (39.3)	33 (58.9)	1 (1.8)	0.223
Intestinal	74	13 (17.6)	23 (31.1)	38 (51.4)		27 (37)	28 (38.4)	18 (24.7)		26 (38.8)	35 (52.2)	6 (9)	
Stage													
Early	12	7 (58.3)	1 (8.3)	4 (33.3)	0.001*	10 (83.3)	1 (8.3)	1 (8.3)	0.001*	5 (50)	5 (50)	0 (0)	0.603
Advanced	12	19 (15.1)	30 (23.8)	77 (61.1)		36 (28.8)	49 (39.2)	40 (32)		43 (38.1)	63 (55.8)	7 (6.2)	

	6												
Tumor invasion													
T1/T2	43	17 (39.5)	8 (18.6)	18 (41.9)	<0.001*	26 (60.5)	14 (32.6)	3 (7)	<0.001*	20 (57.1)	12 (34.3)	3 (8.6)	0.013*
T3/T4	95	9 (9.5)	23 (24.2)	63 (66.3)		20 (21.3)	36 (38.3)	38 (40.4)		28 (31.8)	56 (63.6)	4 (4.5)	
Lymph node metastasis													
Absent	16	12 (75)	3 (18.8)	1 (6.3)	<0.001*	16 (100)	0 (0)	0 (0)	<0.001*	7 (63.6)	3 (27.3)	1 (9.1)	0.147
Present	122	14 (11.5)	28 (23)	80 (65.6)		30 (24.8)	50 (41.3)	41 (33.9)		41 (36.6)	65 (58)	6 (5.4)	
Distant metastasis													
Absent	70	25 (35.7)	27 (38.6)	18 (25.7)	<0.001*	39 (56.5)	23 (33.3)	7 (10.1)	<0.001*	37 (67.3)	13 (23.6)	5 (9.1)	<0.001*
Present	68	1 (1.5)	4 (5.9)	63 (92.6)		7 (10.3)	27 (39.7)	34 (50)		11 (16.2)	55 (80.9)	2 (2.9)	
H. pylori													
Negative	14	3 (21.4)	1 (7.1)	10 (71.4)	0.348	7 (50)	3 (21.4)	4 (28.6)	0.328	5 (45.5)	6 (54.5)	0 (0)	0.665
Positive	124	23 (18.5)	30 (24.2)	71 (57.3)		39 (31.7)	47 (38.2)	37 (30.1)		43 (38.4)	62 (55.4)	7 (6.3)	
CagA													

Negative	49	10 (20.4)	7 (14.3)	32 (65.3)	0.231	17 (35.4)	17 (35.4)	14 (29.2)	0.945	18 (43.9)	20 (48.8)	3 (7.3)	0.568
Positive	89	16 (18)	24 (27)	49 (55.1)		29 (32.6)	33 (37.1)	27 (30.3)		30 (36.6)	48 (58.5)	4 (4.9)	
EBV													
Negative	117	22 (18.8)	27 (23.1)	68 (58.1)	0.917	40 (34.5)	43 (37.1)	33 (28.4)	0.668	40 (38.8)	57 (55.3)	6 (5.8)	0.987
Positive	21	4 (19)	4 (19)	13 (61.9)		6 (28.6)	7 (33.3)	8 (38.1)		8 (40)	11 (55)	1 (5)	

[a]p-value by χ^2 test. *Significantly difference between groups ($p < 0.05$). N: number of samples; EBV: *Epstein-Barr virus*.

3. CONCLUSÃO

Análises cuidadosas dos resultados obtidos, nos permite inferir, com boa margem de segurança, que os padrões de metilação dos genes que codificam as proteínas quinases SRC, LYN e CKB estão alterados em câncer gástrico. A hipometilação de *SRC* e *LYN* foi detectada apenas nas amostras de câncer gástrico, e a perda de metilação dessas proteínas foi associada com aumento dos níveis de mRNA e de expressão proteica, sugerindo que a metilação do DNA está envolvida no processo de regulação dessas quinases. Além disso, pacientes com tumores que apresentaram perda de metilação de *SRC* e *LYN* também exibiram, em nível inferior, padrões de metilação alterados desses genes na mucosa gástrica não-neoplásica. Embora investigações de pré-malignidade para câncer gástrico sejam necessárias, a presente constatação sugere que indivíduos com perda de metilação em *SRC* e *LYN*, na mucosa gástrica, podem apresentar risco mais elevado para o desenvolvimento de malignidade do estômago. Padrões de metilação opostos foram observados para o gene da quinase CKB. A frequência de hipermetilação e de metilação parcial de *CKB* foi superior nas amostras de câncer gástrico, em comparação com as amostras gástricas não neoplásicas, e nenhuma das células gástricas neoplásicas apresentou imunorreatividade para CKB. Contudo, não fomos capazes de demonstrar uma correlação direta entre ganho de metilação e redução da expressão de *CKB*, embora tumores com metilação parcial de *CKB* tenham apresentado redução proteica e de expressão de mRNA, quando comparados com tumores hipermetilados e hipometilados para *CKB*. Além disso, mecanismos pós-transcricionais podem estar envolvidos na regulação de CKB durante a carcinogênese gástrica, pois observamos que a frequência de amostras apresentando redução da expressão da proteína CKB foi superior àquelas que apresentaram redução da expressão de mRNA.

Os padrões de metilação dos genes dessas quinases foram associados com desfechos clínicos específicos. A hipometilação de *SRC* e *LYN*, observada apenas nas amostras tumorais, e a metilação parcial de *CKB* foram mais

frequentes em pacientes com invasão tumoral mais profunda, e com comprometimento de nodos linfáticos ou com metástase distante. A hipometilação de *SRC* também foi mais frequente em pacientes com tumores gástricos mais avançados, ao passo que o mesmo evento em *LYN* esteve associado ao início tardio do câncer gástrico. Esses achados, corroboram para a hipótese de que a metilação do DNA está envolvida na regulação de *SRC, LYN* e *CKB*, uma vez que também observamos que a expressão elevada e/ou diminuição da expressão dessas quinases podem ter um relevante papel na invasão e metástase relacionadas ao câncer gástrico.

Dessa forma, verifica-se que os padrões de metilação das quinases analisadas neste estudo, apresentam elevado potencial para auxiliarem na determinação do prognóstico do câncer gástrico, podendo atuar como biomarcadores do processo de carcinogênese gástrica, atuanda, dessa forma, como marcadores preditivos da progressão tumoral e como alvos potenciais na estratégia anticâncer.

4. REFERÊNCIAS BIBLIOGRÁFICAS

- Alaoui-Jamali MA, Xu YJ. Proteomic Technology for Biomarker Profiling in Cancer: An Update. *J Zhejiang Univ Sci B*. 2006; 7:411-20.

- Ali Z, Deng Y, Ma C. Progress of Research in Gastric Cancer. *J Nanosci Nanotechnol.* 2012; 12:8241-8.

- Almhanna K, Strosberg J, Malafa M. Targeting AKT Protein Kinase in Gastric Cancer. *Anticancer Res.* 2011; 12:4387-92.

- Arnold H, Wienker TF, Hoffmann FF, Scheuerbrandt G, Kemp K, Bugert P. High Levels of Brain-Type Creatine Kinase Activity in Human Platelets and Leukocytes: A Genetic Anomaly With Autosomal Dominant Inheritance. Blood Cells, Molecules and Diseases. 2012; 48:62-67.

- Assumpção PP, Burbano RR. Genética e Cancer Gástrico. In: Linhares E, Laércio L, Takeshi S (editors). Atualização em Câncer-Gástrico. 1º ed. São Paulo: Tecmed Editora, 2005. p95-106.

- ATCC. American Type Culture Collection. Cell Lines and Hybridomas. [Acessado em 02/03/2014]. Disponível em: URL: http://www.atcc.org.

- Balasubramani M, Day BW, Schoen RE, Getzenberg RH. Altered Expression and localization of Creatine Kinase B, Heterogeneous Nuclear Ribonucleoprotein F, and High Mobility Group Box 1 Protein in the Nuclear Matrix Associated With Colon Cancer. *Cancer Res.* 2006; 66:763-9.

- Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia Enables Predictive Modeling of Anticancer Drug Sensitivity. Nature. 2012; 483: 603-7.

- BCRJ. Rio de Janeiro Cell Bank. Human and Animal Cells Catalogue. [Acessado em 02/03/2014]. Disponível em: URL: http://www.bcrj.hucff.ufrj.br/.

- Brenner H, Rothenbacher D, Arndt V. Epidemiology of Stomach Cancer. *Methods Mol Biol.* 2009; 472:467-77.

- Carcas LP. Gastric Cancer Review. *J Carcinog*. 2014; 13.

- Chang YM, Bai L, Liu S, Yang JC, Kung HJ, Evans CP. Src Family Kinase Oncogenic Potential and Pathways in Prostate Cancer as Revealed by AZD0530. Oncogene. 2008; 27:6365-75.

- Chiurillo MA. Role of Gene Plymorphisms in Gastric Cancer and Its Precursor Lesions: Current Knowledge and Perspectives in Latin American Countries. *World J Gastroenterol.* 2014; 20:4503-4515.

- Chung CH, Levy S, Chaurand P, Carbone DP. Genomics and Proteomics: Emerging Technologies in Clinical Cancer Research. *Crit Rev Oncol Hematol.* 2007; 61:1-25.

- Corso G, Seruca R, Roviello F. Gastric Cancer Carcinogenesis and Tumor Progression. *Ann Ital Chir.* 2012; 83(3):172-6.

- Croucher DR, Hochgräfe F, Zhang L, Liu L, Lyons RJ, Rickwood D, et al. Involvement of Lyn and the Atypical Kinase SgK269/PEAK1 in a Basal Breast Cancer Signaling Pathway. *Cancer Res.* 2013; 73:1969-1980.

- Fan C, li Y, Jia J. Protein Kinase Cs in Lung Cancer: A Promising Target for Therapies. *J Cancer Res Ther.* 2013; Suppl 2:S74-9.

- Fyffe C, Falasca M. 3-Phosphoinositide-Dependent Protein Kinase-1 as an Emerging Target in the Management of Breast Cancer. *Cancer Manag Res.* 2013; 5:271-80.

- Gerdes MJ, Sood A, Sevinsky C, Pris AD, Zavodszky MI, Ginty F. Emerging Understanding of Multiscale Tumor Heterogeneity. *Front Oncol.* 2014; 4:366.

- Giroux V, Dagorn JC, Iovanna JL. A Review of Kinase Implicated in Pancreatic Cancer. Pancreatology. 2009; 6:738-54.

- Guerra MR, Gallo CVM & Mendonça. Risco de Câncer no Brasil: Tendências e Estudos Epidemiológicos Mais Recentes. Revista Brasileira de Cancerologia. 2005; 51:227-234.

- Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000; 100:57-70.

- Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011; 144:646-74.

- Hood LJ, Heath R, Phelps ME, Lin B. Systems Biology and New Technologies Enable Predictive and Preventative Medicine. Science. 2004; 306:640-3.

- Hu Y, Lam KY, Wan TS, Fang W, Ma ES, Chan LC, et al. Establishment and Characterization of HKESC-1, A New Cancer Cell Line From Human Esophageal Squamous Cell Carcinoma. *Cancer Genet Cytogenet.* 2000; 118:112-20.

- Huddleston HG, Wong KK, Welch WR, Berkowitz RS, Mok SC. Clinical Applications of Microarray Technology: Creatine Kinase B is an Up-Regulated gene in Epithelial Ovarian Cancer and Shows Promise as a Serum Marker. *Gynecol Oncol.* 2005; 96:77-83.

- Humar B, Fukuzawa R, Blair V, Dunbier A, More H, Charlton A, et al. Destabilized Adhesion in the Gastric Proliferative Zone and c-Src Kinase

Activation Mark the Development of Early Diffuse Gastric Cancer. *Cancer Res.* 2007; 67:2480-9.

- Huntsman DG, Carneiro F, Lewis FR, MacLeod PM, Hayashi A, Monaghan KG, et al. Early Gastric Cancer in Young, Asymptomatic Carriers of Germ-Line E-Cadherin Mutations. *N Engl J Med.* 2001; 344:1904-9.

- IARC. Intenational Agency for Research on Cancer. Cancer Incidence and Mortality Worldwide in 2001. Disponível em: URL: http://www.iarc.fr.

- INCA, Instituto Nacional de Câncer José Alencar Gomes da Silva. Coordenação Geral de Ações Estratégicas. Coordenação de Prevenção e Vigilância. Estimativa 2014: Incidência de Câncer no Brasil/Instituto Nacional de Câncer José Alencar Gomes da Silva, Coordenação Geral de Ações Estratégicas, Coordenação de Prevenção e Vigilância. – Rio de Janeiro: INCA, 2014.

- INCA, Instituto Nacional do Câncer. Estimativa 2008: Incidência de Câncer no Brasil. Rio de Janeiro: INCA. 2007.

- Ingley E. Review: Functions of The Lyn Tyrosine Kinase in Health and Disease. Cell Communication and Signaling. 2012; 10:21.

- Irby RB, Yeatman TJ. Role of Src Expression and Activation in Human Cancer. Oncogene. 2000; 19:5636-42.

- Ishikawa J, Taniguchi T, Takeshita A, Maekawa M. Increased Creatine Kinase BB Activity and CKB mRNA Expression in Patients With Hematologic Disorders: Relation to Methylation Status of the CKB Promoter. Clinica Chimica Acta. 2005; 361:135-140.

- Jallal H, Valentino ML, Chen G, Boschelli F, Ali S, Rabbani SA. A Src/Abl Kinase Inhibitor, SKI-606, Blocks Breast Cancer Invasion, Growth, and Metastasis In Vitro and In Vivo. *Cancer Res.* 2007; 67:1580-8.

- Johnson FM, Saigal B, Talpaz M, Donato NJ. Desatinib (BMS-354825) Tyrosine Kinase Inhibitor Suppresses Invasion and Induces Cell Cycle Arrest and Apoptosis of Head and Neck Squamous Cell Carcinoma and Non-Small Cell Lung Cancer Cells. *Clin Cancer Res.* 2005; 11:6924-32.

- Jørgensen JT. Role of Human Epidermal Growth Factor Receptor 2 in Gastric cancer: Biological and Pharmacological Aspects. *World J Gastroenterol.* 2014; 20:4526-4535.

- Kelly V, Genovese M. New Molecules for the Treatment os Rheumatoid Arthritis – Novel Small Molecule Therapeutics in Rheumatoid Arthritis. Rheumatology. 2013; 52:1155-1162.

- Khan FA, Shukla AN. Pathogenesis and Treatment of Gastric Carcinoma: “An Up-Date With Brief Review”. *J Cancer Res Ther.* 2006; 2:196-9.

- Kim LC, Song L, Haura EB. Src Kinase as Therapeutic Targets for Cancer. *Nat Rev Clin Oncol.* 2009; 6:587-95.

- Kiyose S, Nagura K, Tao H, Igarashi H, Yamada H, Goto M, et al. Detection of Kinase Amplifications in Gastric Cancer Archives Using Fluorescence In Situ Hybridization. *Pathol Int.* 2012; 62:477-84.

- Ku JL, Kim KH, Choi JS, Kim SH, Shin YK, Chang HJ, et al. Establishment and Characterization of Six Human Gastric Carcinoma Cell Lines, Including One Naturally Infected With Epstein-Barr Virus. *Cell Oncol.* 2012; 35:127-36.

- Kubo T, Kuroda Y, Shimizu H, Kokubu A, Okada N, Hosada F, et al. Resequencing and Copy Number Analysis of the Human Tyrosine Kinase Gene Family in Poorly Differentiated Gastric Cancer. Carcinogenesis. 2009; 30:1857-64.

- Kunz PL, Gubens M, Fisher GA, Ford JM, Lichtensztajn DY, Clarke CA. Long-Term Survivors of Gastric Cancer: A California Population-Based Study. *J Clin Oncol.* 2012; 30:3507-15.

- Ladomery M. Aberrant Alternative Splicing is Another Hallmark of Cancer. International Journal of Cell Biology. 2013; 2013.

- Lauren P. The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification. *Acta Pathol Microbiol Scand.* 1965; 64:31-49.

- Leal MF, Martins do Nascimento JL, da Silva CE, Vita Lamarão MF, Calcagno DQ, Khayat AS, et al. Establishment and Conventional Cytogenetic Characterization of Three Gastric Cancer Cell Lines. *Cancer Genet Cytogenet.* 2009; 195:85-91.

- Leung EL, Tam IY, Tin VP, Chua DT, Sihoe AD, Cheng LC, et al. SRC Promotes Survival and Invasion of Lung Cancers With Epidermal Growth Factor Receptor Abnormalities and is a Potential Candidate for Molecular-Targeted Therapy. *Mol Cancer Res.* 2009; 7:923-32.

- Lima EM, Rissino JD, Harada ML, Assumpção PP, Demachki S, Guimarães AC, et al. Conventional Cytogenetic Characterization of a New Cell Line, ACP01, Established From a Primary Human Gastric Tumor. *Braz J Med Biol Res.* 2004; 37:1831-8.

- Liu WM, Huang P, Kar N, Burgett M, Muller-Greven G, Nowacki AS, et al. Lyn Facilitates Glioblastoma Cell Survival Under Conditions of Nutrient Deprivation by Promoting Autophagy. *PlosOne.* 2013; 8:1-13.

- Ma PC. Personalized Targeted Therapy in Advanced Non-Small Cell Lung Cancer. *Cleve Clin Med.* 2012; Suppl 1:eS56-60.

- Martel C, Forman D, Plummer M. Epidemiology and Risk Factors. *Gastroenterol Clin N Am.* 2013; 42:219-240.

- Manning DB. Challenges and Opportunities in Defining the Essential Cancer Kinome. Science Signaling. 2009; 63pe15.

- Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The Protein Kinase Complet of the Human Genome. Science. 2002; 298:1912-1918.

- Masaki T, Shiratori Y, Okada H, Nishioka M, Taniguchi K, Hatanaka Y, et al. pp60s-Src Activation in Gastric Carcinoma: A Preliminary Study. *Am. J. Gastroenterol.* 2000; 95:837-8.

- Mooney SM, Rajagopalan K, Williams BH, Zeng Y, Christudass CS, Li Y, et al. Creatine Kinase Brain Overexpression Protects Colorectal Cells From Various Metabolic and Non-Metabolic Stresses. *J Cell Biochem.* 2011; 112:1066-75.

- Nagini S. Carcinoma of the Stomach: A Review of Epidemiology, Pathogenesis, Molecular Genetics and Chemoprevention. *World J Gastrointest Oncol.* 2012; 4(7):156-169.

- Nautiyal J, Majumder P, Patel BB, Lee FY, Majumdar AP. Src Inhibitor Dasatinib Inhibits Growth of Breast Cancer Cells by Modulating EGFR Signaling. *Cancer Lett.* 2009; 283:143-51.

- Park JG, Yang HK, Kim WH, Chung JK, Kang MS, Lee JH, et al. Establishment and Characterization of Human Gastric Carcinoma Cell Lines. *Int J Cancer.* 1997; 70:443-9.

- Park SI, Zhang J, Phillips KA, Araujo JC, Najjar AM, Volgin AY, et al. Targeting SRC Family Kinases Inhibits Growth and Limph Node Metastases os Prostate Cancer in an Orthotopic Nude Mouse Model. *Cancer Res.* 2008; 68:3323-33.

- Patterson H, Nibbs R, McInnes I, Siebert S. Protein Kinase Inhibitors in the Treatment of Inflammatory and Autoimmune Diseases. Clinical and Experimental Immunology. 2013; 176:1-10.

- Peng L, Ran YL, Hu H, Yu L, Liu Q, Zhou Z, et al. Secreted LOXL2 is a Novel Therapeutic Target That Promotes Gastric Cancer Metastasis Via the Src/FAK Pathway. Carcinogenesis. 2009; 30:1660-9.

- Piazuelo MB & Correa P. Review Article: Gastric Cancer: Overview. Colombia Médica. 2013; 44:192-201.

– Pilpilidis I, Kountouras J, Zavos C, Katsinelos P. Upper Gastrointestinal Carcinogenesis: *H. pylori* and Stem Cell Cross-Talk. *J Surg Res.* 2011; 166:255-64.

– Qiu MZ, Cai MY, Zhang DS, Wang ZQ, Wang DS, Li YH, et al. Clinicopathological Characteristics and Prognostic Analysis of Lauren Classification in Gastric Adenocarcinoma in China. *J Transl Med.* 2013; 11:58.

– Rocco A, Compare D, Nardone G. Cancer Stem Cell Hypothesis and Gastric Carcinogenesis: Experimental Evidence and Unsolved Questions. *World J Gastrointest Oncol.* 2012; 4:54-9.

– Shi J, Qu YP, Hou P. Pathogenetic Mechanisms in Gastric Cancer. *World J Gastroenterol.* 2014; 20(38):13804-13819.

– Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2015. *CA Cancer J Clin.* 2015; 65:5-29.

– Souza CRT, Leal MF, Calcagno DQ, Sozinho EKC, Borges BN, Montenegro RC, et al. MYC Deregulation in Gastric Cancer and Its Clinicopathological Implications. PlosOne. 2013; 8:1-9.

– Smith MG, Hold GL, Tahara E, El-Omar EM. Cellular and Molecular Aspects of Gastric Cancer. *World J Gastroenterol.* 2006; 12:2979-90.

– Song L, Morris M, Bagui T, Lee FY, Jove R, Haura EB. Dasatinib (BMS-354825) Selectively Induces Apoptosis in Lung Cancer Cells Dependent on Epidermal Growth Factor Receptor Signaling for Survival. *Cancer Res.* 2006; 66:5542-8.

– Stein M, Bagnoli F, Halenbeck R, Rappuoli R, Fanti WJ, Covacci A. cSrc/Lyn Kinase activate *Helicobacter pylori* CagA Through Tyrosine Phosphorylation of the EPIYA Motifs. Molecular Microbiology. 2002; 43:971-980.

– Stiekema J, Cats A, Kuijpers A, van Coevorden F, Boot H, Jansen EP, et al. Surgical Treatment Results of Intestinal and Diffuse Type Gastric Cancer. Implications For a Differentiated Therapeutic Approach? *Eur J Surg Oncol.* 2013; S0748-7983:00271-0.

– Stintzing S, Lenz HJ. Protein Kinase Inhibitors in Metastatic Colorectal Cancer. Let´s Pick Patients, Tumors, and Kinase Inhibitors to Piece the Puzzle Together! *Expert Opin Pharmacother.* 2013; 16:2203-20.

– Su N, Peng L, Xia B, Zhao Y, Xu A, Wang J, et al. Lyn is Involved in CD24-Induced ERK1/2 Activation in Colorectal Cancer. Molecular Cancer. 2012; 11:43.

– Sutton P, Borgia JA, Bonomi P, Plate J. Lyn, a Src Family Kinase, regulates Activation of Epidermal Growth Factor Receptors in Lung Adenocarcinoma Cells. Molecular Cancer. 2013; 12:76.

– Tahara E. Genetic Pathways of Two Types of Gastric Cancer. *IARC Sci Publ.* 2004; 157:327-49.

– Takekura N, Yasui W, Yoshida K, Tsujino T, Nakayama H, Kameda T, et al. pp60c-Src Protein Kinase Activity in Human Gastric Carcinomas. *Int J Cancer.* 1990; 45:847-51.

– Tamura G. Alterations of Tumor Suppressor and Tumor-Related Genes in the Development and Progression of Gastric Cancer. *World J Gastroenterol.* 2006; 12:192-8.

– Tan IB, Ng I, Tai WM, Tan P. Understanding the Genetic Basis of Gastric Cancer: Recent Advances. *Expert Rev Gastroenterol Hepatol.* 2012; 6:335-41.

– Tsugane S & Sasazuki S. Diet and The Risk of Gastric Cancer: Review of Epidemiological Evidence. Gastric Cancer. 2007; 10:75-83.

– Varkaris A, Katsiampoura AD, Araujo JC, Gallick GE, Corn PG. Src Signaling Pathways in Prostate Cancer. *Cancer Metastasis Rev.* 2014; DOI 10.1007/s10555-013-9481-1.

– Vinagre RMDF, Campos BP, Sousa RMP. Case Study of Stomach Adenocarcinoma Conducted at a Cancer Referral Hospital in Northern Brazil. *Arq Gastroenterol.* 2012; 49(2):125-129.

– Wallimann T, Hemmer W. Creatine Kinase in Non-Muscle Tissues and Cells. *Mol Cell Biochem.* 1994; 133-134:193-220.

– Wallimann T, Tokarska-Schlattner M, Schlattner U. The Creatine Kinase System and Pleiotropic Effects of Creatine. Amino Acids. 2011; 40:1271-1296.

– WHO. World Health Organization. Cancer. Fact sheet N°297. Updated February 2014. Disponível em: URL: http://www.who.int/mediacentre/factsheets/fs297/en/.

– Yakirevich E, Resnick MB. Pathology of Gastric Cancer and its Precursor Lesions. *Gastroenterol Clin N Am.* 2013; 42:261-284.

– Yamashita K, Sakuramoto S, Nemoto M, Shibata T, Mieno H, Katada N, et al. Trend in Gastric Cancer: 35 Years of Surgical Experience in Japan. *World J Gastroenterol.* 2011; 17:3390-7.

– Yeatman TJ. A Renaissance for SRC. *Nat Rev Cancer.* 2004; 4:470-80.

- Zarghami N, Giai M, Yu H, Roagna R, Ponzone R, Katsaros D, et al. Creatine Kinase BB Isoenzyme Levels in Tumor Cytosols and Survival of Breast Cancer Patients. *Br J Cancer.* 1996; 73:386-90.

- Zaterka S, Eisig JN, Chinzon D, Rothstein W. Factors Related to *Hecobacter* pylori Prevalaence in an Adult Population in Brazil. Helicobacter. 2007; 12:82-88.

- Zhang H, Photiou A, Grothey A, Stebbing J, Giamas G. The Role of Pseudokinases in Cancer. Cellular Signalling. 2012; 6:1173-84.

- Zhang J, Kalyankrishna S, Wislez M, Thilaganathan N, Saigal B, Wei W, et al. SRC-Family Kinases are Activated in Non-Samll Cell Lung Cancer and Promote the Survival of Epidermal Growth Factor Receptor-Dependent Cell Lines. *Am J Pathol.* 2007; 170:366-76.

- Zheng Y, Tyner AL. Context-Specific Protein Tyrosine Kinase 6 (PTK6) Signalling in Prostate Cancer. *Eur J Clin Invest.* 2013; 4:397-404.

- Zilberstein B, Malheiros C, Lourenço LG, Kassab P, Jacob CE, Weston AC, et al. Consenso Brasileiro sobre Câncer Gástrico: Diretrizes Para o Câncer Gástrico no Brasil. *ABCD Arq Bras Cir Dig.* 2013; 26:2-6.