



Márcia Cristina Duarte

Expressão de genes relacionados ao ciclo celular e proteção da mucosa gástrica em metaplasia intestinal e úlcera gástrica em comparação com câncer gástrico.

Orientadora: Profa. Dra. Ana Elizabete Silva

**Tese apresentada para
obtenção do Título de Doutor
em Genética.**

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Tese apresentada para obtenção do título de Doutor em Genética, junto ao Programa de Pós-Graduação em Genética do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de São José do Rio Preto.

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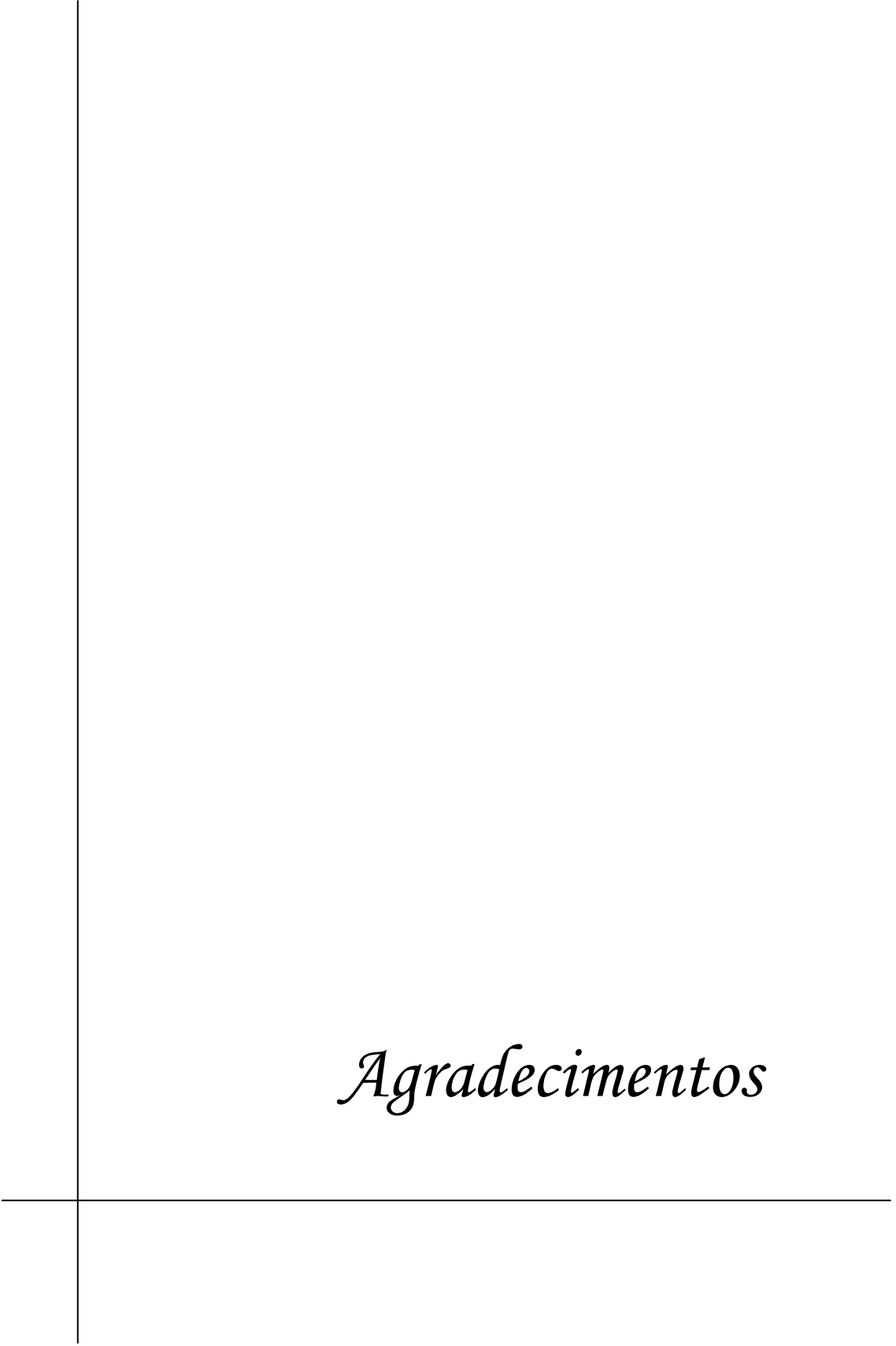
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e ao Marlon*

*por tantas razões
e pela maior das emoções:
o amor.*



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A Deus pelo mais sublime dos dons.... a vida!

Epígrafe

“A mente que se abre a uma nova idéia jamais volta ao seu tamanho original.”

Albert Einstein

A Pedra

*O distraído nela tropeçou...
O bruto a usou como projétil.
O empreendedor, usando-a, construiu.
O camponês, cansado da lida, dela fez assento.
Para meninos, foi brinquedo.
Drummond a poetizou.
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E em todos esses casos, a diferença não esteve na pedra, mas no homem!
Não existe "pedra" no seu caminho que você não possa aproveitá-la para o seu próprio crescimento.*

Autor desconhecido

“É preciso amar as pessoas como se não houvesse amanhã...”

Renato Russo

Lista de abreviaturas

Lista de abreviaturas de genes

AHR	Aryl Hydrocarbon Receptor
APC	Adenomatous Polyposis Coli
BCL-2	B-cell CLL/lymphoma 2
c-ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
c-FOS	FBJ murine osteosarcoma viral oncogene homolog
c-JUN	Jun oncogene
CD44	CD44 molecule
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CDKN1C	Cyclin-Dependent Kinase Inhibitor 1C
CGRP	Calcitonin-Related Polypeptide alpha
CLDN18	Claudin 18
COL4A1	Collagen, type IV, alpha 1
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CTSB	Cathepsin B
DCC	Deleted in Colorectal Carcinoma
EGFR	Epidermal Growth Factor Receptor
EGR-1	Early Growth Response 1
EPHB1	Ephrin Receptor EphB1
FAS	Fas (TNF receptor superfamily, member 6)
GNK1	Gastrokine 1
HER2-neu	c-erb B2/neu protein
HGF	Hepatocyte Growth Factor

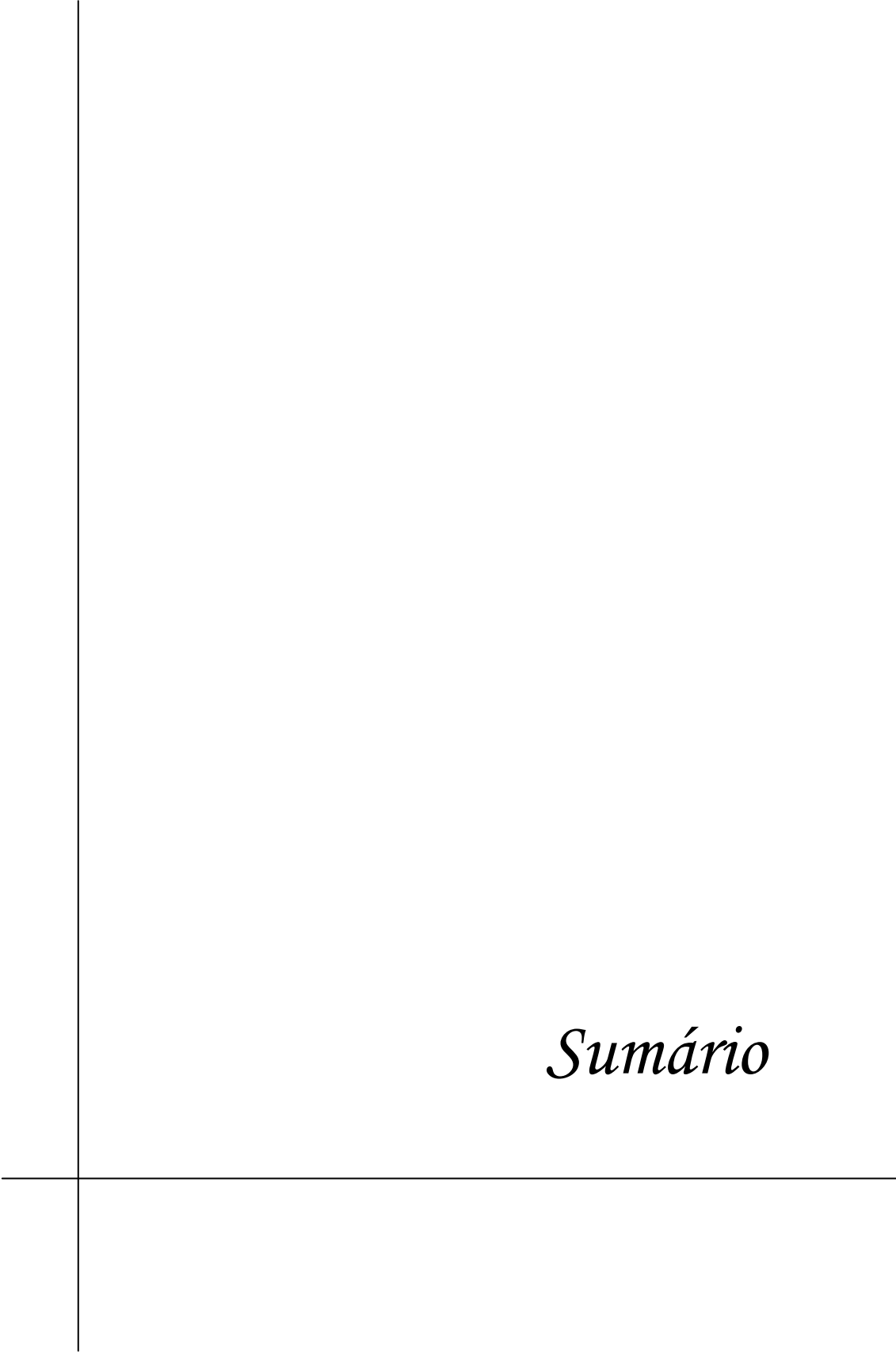
KRAS	v-Ki-Ras2 Kirsten rat sarcoma viral oncogene homolog
MET	Met proto-oncogene (hepatocyte growth factor receptor
MMP2	Matrix Metalloproteinase 2
MSH2	MutS homolog 2
MUC	Mucin 1, cell surface associated
MYC	v-myc myelocytomatosis viral oncogene homolog
NOS2	Nitric Oxide Synthase 2
OCT4	Octamer-binding transcription factor 4
P16	Cyclin-Dependent Kinase Inhibitor 2A
P27	Interferon, alpha-inducible protein 27
PDX1	Pancreatic and Duodenal Homeobox 1
PTEN	Phosphatase and Tensin Homolog
RAR β	Retinoic Acid Receptor, beta
SOX2	SRY (sex determining region Y)-box 2
SP-1	Sp1 transcription factor
SPK2	Protein Kinase 2
SRF	Serum Response Factor
TERC	Telomerase RNA component
TFF1	Trefoil Factor 1
TFF2	Trefoil Factor 2
TFF3	Trefoil Factor 3
TFIZ1/GNK2	Gastrokinase 2
TGF β	Transforming Growth Factor, beta 1
TP53	Tumor Protein p53
TP73	Tumor Protein p73

VIM

Vimentin

XAF1

XIAP Associated Factor 1



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Sumário

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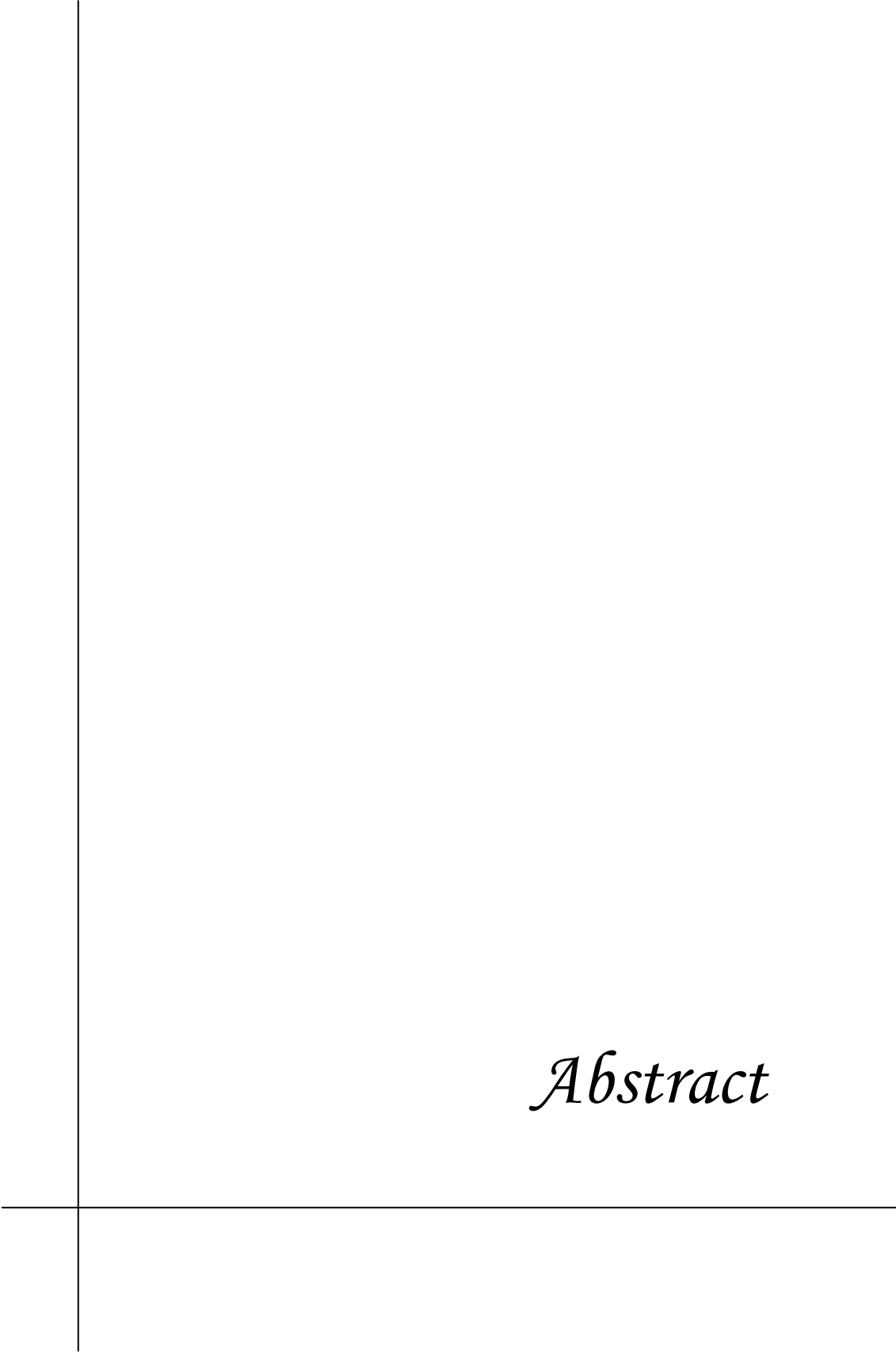
Resumo

Resumo

A carcinogênese gástrica apresenta um modelo de múltiplas etapas, que pode iniciar a partir de uma gastrite crônica, frequentemente associada à infecção pela bactéria *Helicobacter pylori*, e progredir para atrofia gástrica, metaplasia intestinal, displasia e câncer gástrico. Outra via, trata do surgimento do câncer gástrico a partir de um sítio de úlcera péptica benigna. Há relatos de algumas alterações genéticas bem estabelecidas nos estágios iniciais e avançados da carcinogênese gástrica, mas em lesões benignas precursoras como a metaplasia intestinal e a úlcera gástrica, relativamente pouco é conhecido. Deste modo, estudos genéticos destas lesões poderão fornecer informações importantes sobre os eventos iniciais da carcinogênese do estômago e contribuir para estratégias de diagnóstico precoce e prevenção. A partir de dados da literatura foram selecionados genes envolvidos com a carcinogênese do estômago como *TERT*, *COX-2*, *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* e *CLDN18*, que atuam na manutenção dos telômeros, processos celulares e proteção da mucosa gástrica. Diante do exposto, este trabalho teve por objetivos avaliar mudanças de expressão gênica e protéica destes genes selecionados, em metaplasia intestinal (MI - 37 casos) e úlcera gástrica (UG - 30 casos), comparadas com suas respectivas mucosas normais (MN) e com adenocarcinoma gástrico (CG - 22 casos) e verificar possíveis correlações entre a expressão destes genes nos três grupos estudados, bem como associações entre os níveis de expressão gênica e protéica e fatores como infecção pela *H. pylori* e tipo histológico de MI e CG. A expressão relativa do RNAm dos referidos genes foi analisada pela técnica de PCR em tempo real, enquanto a expressão das respectivas proteínas foi avaliada por imuno-histoquímica. A avaliação da expressão gênica revelou níveis médios relativos do RNAm aumentados em CG comparado com MN para *TERT* (17,3 x), *COX-2* (27,6 x), *NOS2* (12,8 x), *HGF* (1,8 x), *MET* (3,5 x) e *KRAS* (1,7 x). Para *TFF1* não foi observada variação significativa no nível médio de RNAm (0,968) em comparação a MN, apesar de 68,2% dos casos de CG apresentarem expressão reduzida do RNAm. Enquanto a expressão de *CLDN18* apresentou uma redução de 1,7 x, com nível médio de RNAm de 0,605, comparado com MN. A análise das proteínas mostrou positividade (moderada/forte) em 54,5%, 81,8%, 30% e 70% das amostras de CG para *TERT*, *COX-2*, *NOS2* e *KRAS*, respectivamente, enquanto perda de expressão ou marcação fraca de *TFF1* e *CLDN18* foi observada em 100% e 90% dos casos de CG em comparação com MN. Não foram obtidos resultados satisfatórios da imuno-histoquímica para as proteínas *HGF* e *MET* nos grupos avaliados, após vários testes. No grupo de MI, os níveis de expressão do RNAm não apresentaram diferenças significantes aos do grupo de CG para *TERT* (2 x), *NOS2* (13,6 x), *HGF* (2,6 x) e *MET* (2,8 x). Contudo, foi verificada uma diferença estatisticamente significativa entre MI e CG para a expressão gênica de *COX-2* (2,063 vs. 27,594, $P=0,0290$), *TFF1* (1,842 vs. 0,968, $P=0,0060$) e *CLDN18* (1,639 vs. 0,605, $P=0,0065$), enquanto a expressão de *KRAS* não diferiu significativamente da MN (1,146). A expressão das proteínas *TERT*, *COX-2* e *NOS2* foi positiva em 38,2%, 79,4% e 92,6% dos casos de MI, respectivamente, enquanto que para *KRAS*, *TFF1* e *CLDN18* a expressão mostrou-se fraca ou ausente em 100%, 62,9% e 100% dos casos, respectivamente. Os níveis de expressão do RNAm em UG foram significativamente aumentados para *TERT* (2,7 x), *COX-2* (2,5 x), *NOS2* (4,5 x), *MET* (2,8 x) e *KRAS* (2,6 x) e diminuídos para *TFF1* (1,9 x, média=0,531) comparado com MN, enquanto *HGF* (1,051) e *CLDN18* (0,934) não apresentaram variações significantes nos níveis médios do RNAm. Comparações entre UG e CG revelaram diferenças significantes apenas para os níveis médios de expressão de *MET* ($P=0,0001$), que foi mais expresso em CG. A análise protéica

revelou positividade (moderada/forte) em 34,8%, 17,4%, 61,5%, 38,5% e 100% das amostras de UG para TERT, COX-2, NOS2, KRAS e TFF1, respectivamente, enquanto que para CLDN18 a marcação foi fraca ou ausente em 100% dos casos. Comparado com CG, houve uma diferença estatisticamente significativa apenas para as proteínas COX-2 ($P=0,0030$) e TFF1 ($P=0,0001$), sendo COX-2 imunomarcada principalmente em CG e TFF1 em UG. A análise de correlação mostrou associações entre os níveis de expressões gênicas de *HGF*, *MET* e *KRAS* no grupo de MI; *NOS2*, *HGF* e *KRAS* no grupo de UG e *NOS2* e *HGF* no grupo de CG. Enquanto a análise de associação entre expressão gênica e protéica com fatores de risco e clinicopatológicos revelou apenas associação entre tabagismo e expressão reduzida do RNAm de *TFF1* no grupo de MI, e entre infecção por *H. pylori* e níveis de expressão do RNAm reduzidos para *NOS2* e *HGF* e aumentados para *CLDN18* no grupo CG. Para as demais variáveis não foram verificadas associações com os níveis de expressão gênica e protéica. Considerando estes resultados, sugerimos que MI e UG compartilham alterações de expressão gênica em comum com CG, evidenciada principalmente nos níveis de expressão gênica e protéica de *TERT*, *COX-2*, *NOS2*, *HGF*, *MET* e *CLDN18*. A expressão gênica de *KRAS* não parece ser alterada em MI, mas em UG apresentou expressão elevada, que também foi observada para a proteína TFF1. Tais alterações em úlcera sugerem um papel de proteção e reparação da mucosa danificada, não podendo desconsiderar a hipótese de iniciação do CG a partir de UG, pois as mudanças de expressão desses genes também estão associadas com os mesmos processos envolvidos na progressão maligna. Portanto, alterações de expressão de genes com atuação em diferentes processos celulares podem contribuir para maior risco de câncer gástrico a partir de lesões precursoras como a metaplasia intestinal e úlcera gástrica.

Palavras-chave: metaplasia intestinal, úlcera gástrica, câncer gástrico, *H. pylori*, expressão gênica, expressão protéica.



Abstract

Abstract

Gastric carcinogenesis presents a model of multiple steps, which can be triggered by a chronic gastritis, often associated with infection caused by the bacterium *Helicobacter pylori*, and progress to gastric atrophy, intestinal metaplasia, dysplasia and gastric cancer. However, another pathway has attracted interest in recent decades and refers to origin of gastric cancer from one site of benign peptic ulcer. There are reports of some well-established genetic alterations in the early stages and advanced gastric carcinogenesis, however, in precursor benign lesions as intestinal metaplasia and gastric ulcer, relatively little is known. Thus, genetic studies of these lesions may provide important information regarding the initial events of carcinogenesis of the stomach and contribute to strategies for early diagnosis and prevention. The genes selected for this study *TERT*, *COX-2*, *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18*, act, usually, in cell cycle processes, telomere maintenance and protection of the gastric mucosa. So, this study aimed to evaluate changes in gene and protein expression of these genes, altered in intestinal metaplasia (IM- 37 cases) and gastric ulcer (GU- 30 cases), compared with their corresponding normal mucosa (NM) and gastric cancer (GC - 22 cases) and to verify possible correlations between the expressions of these genes among the three groups studied, and also examine associations between gene and protein expression levels and factors such as *H. pylori* infection and histological type of IM and GC. The relative mRNA expression of these genes was analyzed by real time PCR, while the expression of respective proteins was assessed by immunohistochemistry. Evaluation of gene expression showed mRNA relative mean levels, increased in GC compared to NM to *TERT* (17.3-fold), *COX-2* (27.6-fold), *NOS2* (12.8-fold), *HGF* (1.8-fold), *MET* (3.5-fold) and *KRAS* (1.7-fold). For *TFF1*, there was no significant change in the mRNA mean level (0.968) compared to NM, despite the fact that 68.2% of GC cases had a low mRNA expression, whereas *CLDN18* expression was 1.7-fold decreased, with mRNA mean level of 0.605, compared to NM. Protein analysis showed positivity (moderate or strong) in 54.5%, 81.8%, 30% and 70% of GC samples for *TERT*, *COX-2*, *NOS2* and *KRAS*, respectively, while loss of expression or weak staining for *TFF1* and *CLDN18* was observed in 100% and 90% of GC compared to NM. No satisfactory results were obtained by immunohistochemistry for *HGF* and *MET* proteins after several tests. In the IM group, the gene expression levels were not significantly different from the GC group for *TERT* (2-fold), *NOS2* (13.6-fold), *HGF* (2.6-fold) and *MET* (2.8-fold). However, there was a statistically significant difference between IM and GC for *COX-2* (2.063 vs. 27.594, $P = 0.0290$), *TFF1* (1.842 vs. 0.968, $P = 0.0060$) and *CLDN18* (1.639 vs. 0.605, $P = 0.0065$) gene expressions, whereas the *KRAS* expression did not significantly differ of the NM (1.145). The protein expression of *TERT*, *COX-2* and *NOS2* was positive in 38.2%, 79.4% and 92.6% of IM cases, respectively, while *KRAS*, *TFF1* and *CLDN18* immunostaining was weak or absent in 100%, 62.9% and 100% of cases, respectively. The gene expression levels in GU were significantly increased for *TERT* (2.7-fold), *COX-2* (2.5-fold), *NOS2* (4.5-fold), *MET* (2.8-fold) and *KRAS* (2.6-fold) and decreased for *TFF1* (1.9-fold) compared to NM, while *HGF* (1.051) and *CLDN18* (0.934) showed no significant changes in the mRNA mean levels. Comparisons between GU and GC revealed significant differences only for the mean levels of *MET* expression ($P = 0.0001$) that was more expressed in GC. The protein analysis was positive (moderate or strong) in 34.8%, 17.4%, 61.5%, 38.5% and 100% of GU samples for *TERT*, *COX-2*, *NOS2*, *KRAS* and *TFF1*, respectively, while to *CLDN18* weak or absent staining was

observed in 100% of the cases. Compared to GC, there was a statistically significant difference only for the proteins COX-2 ($P = 0.0030$) and TFF1 ($P = 0.0001$), with COX-2 and TFF1 immunostaining mainly in GC and GU, respectively. Correlation analysis showed associations between the gene expressions levels of *HGF*, *MET* and *KRAS* in the IM group, *NOS2*, *HGF* and *KRAS* in the GU group and *NOS2* and *HGF* in the GC group. The association analysis between gene and protein expressions with clinicopathological and environmental factors showed only an association between smoking and reduced mRNA expression of *TFF1* in the IM group, and between *H. pylori* infection and decreased mRNA expression levels to *NOS2* and *HGF* and increased to *CLDN18* in GC group. For the other variables, associations with gene and protein expression levels were not found. These results suggest that IM and GU share gene expression changes with GC, mainly observed in the gene and protein expression levels of *TERT*, *COX-2*, *NOS2*, *HGF*, *MET* and *CLDN18*. *KRAS* does not seem to be altered in IM, but in GU, it showed increased expression that also was observed for TFF1 protein. These alterations in GU, suggest a role in the protection and repair of damaged mucosa, however, it should be considered the hypothesis of initiation and progression of gastric cancer from GU, because the expression changes of these genes are also associated with the same processes involved in malignant progression. Therefore, imbalances in cell signaling pathways, where there is interaction of several genes, may contribute to increased risk of gastric cancer development from precursor lesions such as intestinal metaplasia and gastric ulcer.

Keywords: intestinal metaplasia, gastric ulcer, gastric cancer, *H. pylori*, gene expression, protein expression.



Introdução

I – Introdução

I.1- Carcinogênese do estômago

O câncer gástrico se destaca no modelo de múltiplas etapas da carcinogênese, que pode iniciar a partir de uma gastrite crônica, geralmente acompanhada de infecção pela bactéria *Helicobacter pylori*, e progredir para atrofia gástrica, metaplasia intestinal, displasia e carcinoma (CORREA, 2004). Outra via da carcinogênese gástrica, também induzida pela *H. pylori*, pode ser por meio da úlcera péptica, que constitui uma lesão pré-cancerosa importante do estômago (TODD et al., 2004).

A metaplasia intestinal, mudança potencialmente reversível de um tipo de célula diferenciada para outro, é frequentemente encontrada no trato gastrointestinal, principalmente no estômago e esôfago, como resultado da infecção por *H. pylori*, refluxo biliar crônico, ou induzida experimentalmente por irradiação e agentes mutagênicos. No estômago, é caracterizada pela substituição da mucosa gástrica por epitélio semelhante ao do intestino delgado, geralmente iniciada pela irritação persistente da mucosa provocada pela infecção da *H. pylori*. Esta lesão pode aumentar a suscetibilidade a carcinogênese gástrica em até 10 vezes, via sequência metaplasia-displasia-carcinoma, sendo considerada uma lesão pré-neoplásica (FILIPE et al., 1994).

A metaplasia intestinal pode ser classificada em tipo completo e incompleto. O tipo completo ou tipo I é caracterizado pela presença de células absortivas, células de Paneth, com grânulos eosinofílicos no seu citoplasma, encontradas na base das glândulas, células secretoras de sialomucinas e células neuroendócrinas, que corresponde ao fenótipo do intestino delgado. O tipo incompleto compreende os tipos II e III e é caracterizado por células colunares e células secretoras de sialomucinas e/ou sulfomucinas (LEUNG; SUNG, 2002; GUTIÉRREZ-GONZÁLEZ; WRIGHT, 2008). A

metaplasia intestinal tipo I está associada com um risco menor de câncer gástrico, enquanto o tipo III, também denominado colônico, apresenta uma associação mais forte, pois indivíduos com este tipo apresentam um risco quatro vezes maior de desenvolver câncer que aqueles com o tipo I (FILIPE et al., 1994).

A úlcera péptica é uma doença heterogênea que afeta cerca de 10% da população (CARVALHO, 2000), devido um defeito na parede gastrintestinal envolvendo toda a espessura da mucosa e penetrando através da parede muscular. É resultante de necrose do tecido, originada por isquemia da mucosa, formação de radicais livres e cessação de liberação de nutrientes, todos causados por dano vascular ou microvascular como trombos, constrição e outras oclusões (TARNAWSKI, 2005). Histologicamente, a úlcera consiste de duas estruturas principais: uma margem distinta formada por mucosa não-necrótica adjacente (componente epitelial) e o tecido de granulação na base da úlcera (componente de tecido conectivo). Este consiste de fibroblastos, macrófagos e células endoteliais proliferativas formando microvasos (CRAWFORD, 2005).

Na maioria dos países, a úlcera duodenal é a forma mais comum de úlcera péptica. Nos Estados Unidos, 8 a 10 % da população sofrem de úlcera duodenal e 1 % de úlcera gástrica (SZABO et al., 2007). Pacientes com úlcera gástrica com redução no nível de ácido gástrico e pangastrite podem ter um risco de 1,8 vezes maior de desenvolver câncer do estômago que indivíduos com úlcera duodenal com níveis elevados de ácido gástrico e gastrite, predominantemente na região antral (HANSSON et al., 1996). É importante salientar que a ressecção gástrica como tratamento da úlcera não diminui este risco (SAFATLE-RIBEIRO; RIBEIRO; REYNOLDS, 1998). Vários fatores ambientais foram relacionados com sua gênese, como o consumo de álcool e cigarro, ácido acetilsalicílico, outros anti-inflamatórios não-esteróides e corticoesteróides, além de fatores de fundo emocional (TAM, 1987; SZABO et al.,

2007). Porém, o principal fator ambiental envolvido na etiologia desta doença é a infecção pela *H. pylori*, que afeta cerca de 90% dos pacientes com úlcera duodenal e 70 a 90% daqueles com úlcera gástrica (TYTGAT et al., 1993).

O estudo de lesões pré-cancerosas como a metaplasia intestinal e a úlcera gástrica podem fornecer informações importantes a respeito das fases iniciais da carcinogênese do estômago, assim contribuindo para estratégias de prevenção e diminuição de sua incidência. Mesmo com o declínio no número de casos novos, o câncer gástrico encontra-se como a quarta causa mais comum, e a segunda causa de óbitos por câncer mundialmente. De modo geral, é cerca de duas e três vezes mais freqüente em países em desenvolvimento, com as maiores taxas de incidência em países asiáticos como Coréia, Japão e China, além da Europa Oriental e parte da América Latina (SMITH et al., 2006; NITTI et al., 2008; INCA, 2009). No Brasil, segundo estimativas do Instituto Nacional de Câncer (INCA), ele ocupa o sexto lugar em incidência com a previsão de 22 mil casos novos (INCA, 2009).

Aproximadamente 90% dos cânceres gástricos são adenocarcinomas, que podem ser classificados em duas entidades histomorfológicas denominadas intestinal e difuso (LAUREN, 1965). O tipo intestinal é caracterizado por tumores bem diferenciados, que formam glândulas, frequentemente associados com gastrite atrófica e metaplasia intestinal pré-existent, sendo comuns na porção distal do estômago, e ocorrem em pacientes mais velhos e em regiões geográficas de alto risco (JOHNSON; EVERS, 2008). O tipo intestinal de câncer gástrico afeta mais os homens em idade avançada do que as mulheres na razão de 2:1. O tipo difuso ou indiferenciado não apresenta lesões precursoras e pode levar a metástase precoce, apresentando um curso clínico mais agressivo (SMITH et al., 2006; JOHNSON; EVERS, 2008). Ele compreende células que perderam a coesão e que não são mais capazes de exercer a função gástrica,

apresentando aspecto típico de células em anel de sinete, afetando grandes porções do estômago. Esta neoplasia surge em indivíduos mais jovens, afetando homens e mulheres na mesma proporção e apresenta um forte componente genético (SMITH et al., 2006; VAUHKONEN; VAUHKONEN; SIPPONEN, 2006; JOHNSON; EVERS, 2008).

A carcinogênese do estômago apresenta etiologia complexa, na qual fatores ambientais e genéticos estão envolvidos. Dentre os fatores ambientais destaca-se a dieta (alimentos salgados, conservados e defumados, peixes e carnes secas e carboidratos refinados), os hábitos tabagista e etilista, acloridria, além da infecção por patógenos como a bactéria *Helicobacter pylori* (SMITH et al., 2006; ROCCO; NARDONE, 2007; LA TORRE et al., 2009). O consumo concomitante de álcool e tabaco está associado com aumento do risco de câncer gástrico em 2,9 % na população japonesa (YAMAJI et al., 2009), enquanto o consumo de fibras, frutas e vegetais frescos, antioxidantes como o ácido ascórbico e a vitamina E, carotenóides e selênio na dieta parecem exercer um papel importante na prevenção desta doença (KELLEY; DUGGAN, 2003; ROCCO; NARDONE, 2007).

A bactéria *Helicobacter pylori*, considerada pela Agência Internacional de Pesquisa em Câncer, como causa definitiva de câncer gástrico (IARC, 1994), aumenta o risco para esta neoplasia em até nove vezes (KUIPERS, 1999). Esta bactéria possui vários fatores que permitem colonizar o ambiente inóspito do estômago e evadir as defesas do hospedeiro, incluindo a resposta imune. A enzima urease produzida pela bactéria hidrolisa a uréia gástrica em amônia e dióxido de carbono, assim mantendo um pH neutro, mesmo na presença de altas concentrações de H^+ externo (SMITH et al., 2006; AMIEVA; EL-OMAR, 2008).

A infecção por esta bactéria causa gastrite crônica em praticamente todos os indivíduos colonizados e pode levar, posteriormente, à perda de glândulas gástricas. A

gastrite atrófica resultante e a metaplasia intestinal aumentam o risco de displasia e câncer gástrico (KUIPERS; SIERSEMA, 2004). Vários mecanismos foram propostos para a carcinogênese associada à *H. pylori*, dentre os quais estão a desregulação do ciclo celular, formação de adutos de DNA, geração de radicais livres e compostos N-nitrosos, alterações na secreção de fatores de crescimento e citocinas e diminuição das secreções gástricas (HEAVEY; ROWLAND, 2004). Os diferentes tipos de cepa e fatores de virulência da bactéria, além de fatores genéticos do hospedeiro, também foram indicados como responsáveis pelo desenvolvimento de neoplasia gástrica (CORREA, 2004; AMIEVA; EL-OMAR, 2008).

O fator de virulência melhor caracterizado é a ilha de patogenicidade *cag* (*cag*-PAI). Ela contém vários genes que codificam citotoxinas que são injetadas nas células epiteliais gástricas e são fosforiladas, induzindo a secreção de IL-8, um potente fator quimiotático e ativador de neutrófilos, produzindo várias transformações fisiológicas, incluindo a diminuição do pH do estômago. Outros fatores de virulência incluem os dos genes *vacA*, que codifica uma citotoxina vacuolizante que induz a formação de vacúolos nas células e que pode levar à apoptose, além de estimular a formação de poros transmembrana que permeabilizam o epitélio gástrico à uréia. Os fatores *babA* que codifica a proteína BabA que promove uma maior adesão entre a bactéria e a célula gástrica; *iceA* que é induzido pelo contato com o epitélio e, em algumas populações, está associado com úlcera péptica; e *oipA* que parece ser importante para a liberação de IL-8 mesmo em cepas *cag*-negativas, sendo relacionado com gravidade da gastrite, úlcera péptica e metaplasia intestinal. (DE LUCA; IAQUINTO, 2004; GILLEN; McCOLL, 2005; PRINZ; SCHWENDY; VOLAND, 2006; SMITH et al., 2006; AMIEVA; EL-OMAR, 2008).

1.2. Alterações genéticas e carcinogênese gástrica.

Alguns modelos de alterações genéticas têm sido propostos acompanhando a progressão do adenocarcinoma gástrico, inclusive com o envolvimento de vias moleculares distintas entre os tipos intestinal e difuso (TAHARA, 2004). Hasegawa et al. (2002) descreveram a expressão aumentada de genes envolvidos na transdução de sinais, coagulação sanguínea (*PROCR*, *SERPINGI* e *HRG*) e transcrição gênica (*NFL3*, *LHX1* e *HOXB7*), enquanto genes que contribuem para o metabolismo de energia, barreira epitelial (*TFF1* e *TFF2*) e absorção apresentaram uma expressão diminuída. Instabilidade genética e hiperplasia de células tronco positivas para *TERT* (transcriptase reversa da telomerase) precedem erros de replicação no locus DS191, hipermetilação no locus D17S5, perda de *pS2*, *RAR β* e *RUNX3*, transcritos anormais de CD44 e mutação de *TP53*, todos os quais acumulam em 30 % das metaplasias intestinais e são eventos comuns nos cânceres tipo intestinal. Cerca de 20 % dos adenomas com mutações no gene *APC* progridem para carcinoma e durante esta progressão observam-se mutações ou perda de heterozigosidade (LOH) de *TP53*, expressão reduzida de *p73*, perda de *RUNX3*, superexpressão de *ciclina-E* e transcrição anormal de *MET*. O carcinoma gástrico tipo intestinal resultante exibe frequentemente perda de *DCC* e da proteína p27, expressão reduzida do receptor *TGF β* e amplificação do gene *c-ERBB2*. A via envolvida na formação de câncer gástrico tipo difuso envolve LOH no cromossomo 17p, expressão anormal de *p73*, mutação ou LOH em *TP53* e *p16*, desacetilação de histonas, expressão de *TERT*, expressão aumentada de *TGF β* , transcritos anormais de *CD44*, perda de *RUNX3* e mutação ou perda de *E-caderina*, entre outros (STOCK; OTTO, 2005; SMITH et al., 2006; JOHNSON; EVERS, 2008).

Estudos moleculares sobre expressão gênica em tumores gástricos têm destacado uma expressão variável em genes relacionados com o ciclo celular, apoptose, reparo do

DNA, metástase e manutenção dos telômeros. Por exemplo, há relatos de expressão aumentada dos genes *ciclina E1*, *AXIN2* (CHEN et al., 2003), *TERT* (HU et al., 2004), *COX-2* (ZHANG et al., 2005), *NOS2* (AUGUSTO et al., 2008), *HER2-neu* (GRAVALOS; JIMENO, 2008), *OCT4* (CHEN et al., 2009) e *AHR* (PENG et al., 2009) e uma baixa expressão dos genes *MUC*, *TTF1*, *CDKN1C* (CHEN et al., 2003) e *XAF1* (TU et al., 2009). Da mesma forma também tem sido relatado aumento de expressão de algumas proteínas como SPK2 (MA et al., 2005), TERT (GULMANN et al., 2005), NOS2 (WANG et al., 2005), EGFR (KIM et al., 2008), COX-2 (CHEN et al., 2006; YAMAC et al., 2008) e survivina (SONG et al., 2009), assim como redução de outras como E-caderina, β -catenina (CHAN et al., 2003), p27, PTEN (MA et al., 2005), c-ERBB-2 (SATEROGLU-TUFAN; BIR; CALLI-DEMISKAR, 2006), EPHB1 (WANG et al., 2007), GNK1, TFIZ1/GNK2 (MOSS et al., 2008) entre outras.

A mudança fenotípica presente na metaplasia intestinal ocorre como resultado da combinação de expressão alterada de fatores genéticos, silenciamento epigenético, fatores de transcrição, vias de sinalização e fatores de crescimento (GUTIÉRREZ-GONZÁLEZ; WRIGHT, 2008). Os eventos genéticos envolvidos no desenvolvimento da metaplasia intestinal são pouco compreendidos, mas destaca-se a expressão do gene homeobox *CDX2*, cuja proteína é mais expressa em metaplasia intestinal do estômago e no esôfago de Barrett (KUIPERS; SIERSEMA, 2004; GUTIÉRREZ-GONZÁLEZ; WRIGHT, 2008), os genes *HOX* e *ParaHOX* envolvidos no desenvolvimento do tubo digestório, a família de fatores *trefoil*, principalmente *TFF3* expresso no intestino normal, *SHH* e *IHH*, a família de genes *SOX* e *POU*, relacionadas com a embriogênese do trato gastrointestinal, além dos genes *OCT-1* e *RUNX3* (TSUKAMOTO et al., 2004; GUTIÉRREZ-GONZÁLEZ; WRIGHT, 2008).

Estudos prévios em metaplasia intestinal também relataram outras alterações genéticas como LOH do gene de reparo *hMSH2* e dos supressores de tumor *APC* e *TP53* (KIM et al., 2001), expressão aumentada de *TERT* com ativação de telomerase (TAHARA, 2004; GULMANN et al., 2005) e mutações no oncogene *KRAS* (TAHARA, 2004). Também há relatos de expressões aumentadas das proteínas Ciclina D2 (YU et al., 2001), p53 (CÉSAR et al., 2004), BCL-2 (ANAGNOSTOPOULOS et al., 2005), SPK2 (MA et al., 2005), COX-2 (SUN et al., 2006), CDX2 (TSUKAMOTO et al., 2004; KIM et al., 2006) e AHR (PENG et al., 2009) e expressão diminuída para as proteínas RUNX3 (OSAKI et al., 2004), p27, PTEN (MA et al., 2005) e BAX (ANAGNOSTOPOULOS et al., 2005).

Meireles et al. (2004), avaliando *arrays* de c-DNA verificaram que o perfil de expressão de metaplasia intestinal era mais semelhante com o tecido tumoral, em que níveis elevados de *MMP2*, *COL4A1*, *FNH1*, *CTSB*, *COLIA2* e *VIM* eram expressos em maior quantidade no tecido tumoral que na metaplasia intestinal, enquanto amostras de gastrite eram mais semelhantes com tecido normal, ocorrendo a expressão aumentada do gene *MYC* e baixa expressão de *CDKN1A*, como característica do grupo.

Em úlcera gástrica, os estudos genéticos são mais escassos e têm mostrado a participação de vários fatores de crescimento e dos fatores de transcrição *c-FOS*, *c-JUN*, *EGR-1*, *SP-1* e *SRF*, dentre outros (TARNAWSKI, 2005). Uma maior expressão da proteína CGRP (TANI et al., 1999), TFF1 (REN et al., 2005; SHI; CAI; YANG, 2006) e TFF2 (SHI; CAI; YANG, 2006) e dos genes *HGF* (HORI et al., 2000), *TGF- β* e *TGF- β R2* (SHIH et al., 2005) e *COX-2* (GUO et al., 2006) foi verificada na mucosa de pacientes com úlcera gástrica em cicatrização.

Alguns estudos desenvolvidos em nosso laboratório nestes tipos de lesões pré-cancerosas, pela técnica FISH, detectaram trissomias dos cromossomos 7, 8 e 9 em 71%

dos casos de metaplasia intestinal em associação com a infecção pela *H. pylori*, assim como deleção do gene *TP53* e expressão aumentada da proteína em 60% e 12% dos casos, respectivamente (CÉSAR et. al., 2004). Em úlcera gástrica foram detectadas principalmente trissomias dos cromossomos 7 e 17 frequentemente nos casos *H. pylori* positivos, mas não foram observadas deleções do gene *TP53*, apesar de apenas 12% dos casos expressarem imunorreatividade positiva para a proteína p53 (CÉSAR et. al., 2006). Estes achados evidenciaram a ocorrência de instabilidade cromossômica em lesões gástricas consideradas ainda benignas associadas com a infecção pela *H. pylori*, que participam do processo de múltiplas etapas da carcinogênese gástrica.

1.3 – Alterações da expressão gênica e carcinogênese do estômago

Alterações genéticas e epigenéticas que alteram os padrões de expressão de genes que participam da regulação do ciclo celular, reparo do DNA e manutenção dos telômeros têm sido estudadas em uma variedade de neoplasias (TAHARA, 2004; CHEN et. al., 2006; SMITH et. al., 2006; NITTI et al., 2008; JOHNSON; EVERS, 2008). Esses estudos estão sendo incrementados nas últimas décadas e possibilitado a caracterização de tumores em nível genômico, transcriptômico e proteômico. Várias mudanças genômicas observadas em diferentes tumores têm sido relacionadas com o início do processo carcinogênico, enquanto alterações dinâmicas na expressão gênica em nível de RNAm e proteínas podem determinar a progressão da doença (PAGLIARULO et al., 2004; NITTI et al., 2008; JOHNSON; EVERS, 2008).

No processo de carcinogênese do estômago diversos genes têm sido descritos com expressão desregulada como *TERT*, *HGF*, *MET*, *COX-2*, *NOS2*, *KRAS*, *TFF1* e *CLDN18*, os quais desempenham importantes funções na manutenção dos telômeros, processos celulares, como proliferação e apoptose, cascatas de sinalização, integridade

da mucosa gástrica dentre outras (HU et al., 2004; INOUE et al., 2004; SHI; CAI; YANG, 2006; SANADA et al., 2006; AUGUSTO et al., 2007).

O gene *TERT* (Telomerase Reverse Transcriptase), mapeado em 5p15.33 (MEYERSON et al., 1997) codifica uma holoenzima, na qual duas subunidades são essenciais para o desempenho de sua função no processo de manutenção dos telômeros: a subunidade catalítica da enzima telomerase, TERT, que confere sua atividade de transcriptase reversa, adicionando seqüências repetidas as extremidades teloméricas (LINGNER et al. 1997); e o componente RNA, TERC, que consiste de uma seqüência complementar à repetição telomérica, atuando como molde para a extensão dos telômeros (FENG et al. 1995).

Os telômeros são essenciais para a manutenção da integridade do genoma, assim garantindo a estabilidade cromossômica. Essas estruturas compostas de repetições curtas (5'-TTAGGG-3') ligam-se a proteínas específicas e são replicadas a cada ciclo celular pela transcriptase reversa da telomerase (BELGIOVONE; CHIODI, MONDELLO, 2008). Em seres humanos, os telômeros são encurtados a cada ciclo celular, limitando o potencial proliferativo da célula, e levando a um estágio de senescência replicativa (CAMPISI; D'ADDA DI FAGAGNA, 2007). A expressão do gene *TERT* está intimamente associada com a atividade da telomerase, com função na adição de repetições nos telômeros para compensar a perda de seqüências durante a replicação do DNA, participando da imortalização celular pela estabilização da estrutura cromossômica (BLACKBURN, 1992).

A atividade da telomerase é mantida nas células germinativas, mas nos tecidos somáticos normais exibem níveis de atividade muito baixos ou ausentes. De modo contrário é detectada em cerca de 90% das amostras de câncer humano e está associada com a transformação maligna (SHAY; WRITE, 2005). Alguns estudos têm relatado

expressão aumentada de *TERT* em adenocarcinoma gástrico e lesões pré-cancerosas, como a metaplasia intestinal, comparado com tecido gástrico normal (HU et al., 2004, GULMANN et al., 2005). A expressão aumentada da proteína telomerase também foi observada em tumores de estroma gastrointestinal (SABAH et al., 2004), carcinoma de células escamosas de esôfago (HSU et al., 2005), carcinoma de ovário (BRUSTMANN, 2005), câncer de pulmão de células não-pequenas (MAVROGIANNOU et al., 2007), carcinoma oral (CHEN et al., 2007) e astrocitoma pediátrico (WONG; MA; HAWKINS, 2009), e também no tecido e no plasma de pacientes com câncer colorretal (TERRIN et al., 2008). A inativação funcional de *TERT* com inibidores da telomerase (MITTAL et al., 2004; SUN et al., 2007), e mais recentemente, a vacinação baseada na telomerase em células cancerosas (MENNUNI et al., 2008; CHEN; LI; TOLLESFBOI, 2009) consiste em um alvo atrativo para novas estratégias terapêuticas. Vários estudos têm confirmado que a perda de sua atividade resulta no encurtamento progressivo dos telômeros, levando a parada de crescimento e/ou morte celular por apoptose (revisão em CHEN; LI; TOLLESFBOI, 2009).

O gene *HGF* mapeado em 7q21.1 (FUKUYAMA et al., 1991), responsável pelo fator de crescimento do hepatócito, é um fator multifuncional de origem mesenquimal, que atua como mitógeno, morfógeno, motógeno e fator angiogênico, dependendo da célula alvo e do contexto celular (MATSUMOTO; NAKAMURA, 1996). *HGF* também exerce um papel importante no processo de reparo nos rins, mucosa gástrica, pulmão, fígado, tecido miocárdico e retina (CONWAY et al., 2007). Em uma grande variedade de células tumorais, *HGF* estimula a proliferação, dissociação, migração e invasão, além de ser um potente fator angiogênico (JIANG et al., 1999). O receptor de *HGF* é codificado pelo proto-oncogene *MET* (7q31) e consiste de uma glicoproteína transmembrana com atividade de tirosina quinase (PARK et al., 1987). Após auto-

fosforilação dos resíduos de tirosina quando ligada à proteína HGF inicia-se uma cascata de transdução de sinal (CHRISTENSEN; BURROWS; SALGIA, 2005; CIPRIANI et al., 2009). O receptor MET é expresso no epitélio normal da maioria dos tecidos, onde é primariamente localizado nas junções intercelulares junto com moléculas de adesão celular como as E-caderinas (CONWAY et al., 2007).

Considerando a grande diversidade de funções biológicas, existe uma variedade de mecanismos pelos quais *HGF* e *MET* influenciam a tumorigênese. Por exemplo, tanto *HGF* quanto *MET* aparecem com expressão aumentada em tecidos neoplásicos em comparação com o tecido normal adjacente e a intensidade da expressão está relacionada à gravidade e prognóstico da doença. Outros mecanismos incluem mutações, amplificação de *MET*, mecanismos epigenéticos, dentre outros (CIPRIANI et al., 2009). A expressão aumentada de *HGF* e *MET* foram relatadas na carcinogênese gástrica (KONTUREK et al., 2001; INOUE et al., 2004), assim como em outras neoplasias como meningiomas em associação com o índice de proliferação celular e recorrência (MARTÍNEZ-RUMAYOR et al., 2004), tumores de mama (PARR et al., 2004; LINDEMANN et al., 2007), em carcinoma de hipofaringe correlacionado com metástases de linfonodos (KIM et al., 2006), câncer de esôfago (ANDERSON et al., 2006), nos estágios iniciais da carcinogênese pancreática (YU et al., 2006) e rabdomiossarcoma (CHEN et al., 2007). Ainda há relatos em câncer de tireóide papilar, no qual também se revelou um potencial alvo terapêutico (SIRAJ et al., 2007), câncer de pulmão (NAKAMURA et al., 2007) e câncer de bexiga (MIYATA et al., 2009).

O aumento de expressão de ambos os genes também foi observado durante o processo de cicatrização de úlceras gástricas (HORI et al., 2000), em úlceras crônicas de pele (NAYERI et al., 2005; CONWAY et al., 2007) e em esofagite erosiva (LUO et al., 2008). Transcritos de *MET* no sangue periférico foram detectados em 61,5% dos

pacientes com câncer gástrico (UEN et al., 2006) mostrando ser uma ferramenta promissora na detecção de células tumorais circulantes micro-metastáticas. Devido a sua participação em diversas vias metabólicas e interação com vários receptores de membrana como integrinas, plexinas, CD44, FAS e outros, o receptor MET tem sido indicado como alvo terapêutico para dificultar o processo tumorigênico e metastático (CORSO; COMOGLIO; GIORDANO, 2005; TOSCHI; JÄNNE, 2008). Da mesma forma, a terapia molecular tendo *MET* como alvo terapêutico tem mostrado resultados eficazes em testes pré-clínicos para o tratamento de câncer de pulmão (CIPRIANI et al., 2009).

As ciclooxigenases (COX) são enzimas com atividades de oxidase e peroxidase que catalisam a conversão de ácido aracdônico a prostaglandinas. Dentre suas funções em vários processos biológicos, encontra-se a integridade da mucosa gastrintestinal (STACK, 2001). Existem duas isoformas de COX, uma codificada pelo gene *COX-1* (9q32-q33.3) e outra pelo gene *COX-2* (1q25.2-q25.3). O gene *COX-1* é expresso constitutivamente em condições basais na maioria das células, participando da regulação homeostática de vários órgãos. Entretanto, os níveis de *COX-2* são baixos ou indetectáveis em tecidos normais, mas altos em estado inflamatório (MILLER, 2006). Durante a inflamação e estímulo mitogênico, é observado um aumento de 10 a 20 vezes na expressão de *COX-2* e produção de prostaglandinas (BROOKS et al., 1999), que aumentam a infiltração de células inflamatórias, exudação, inchaço e dor nos tecidos afetados.

A expressão de *COX-2* em tumores humanos pode ser induzida por vários fatores de crescimento, citocinas, oncogenes, dentre outros. Em células transformadas, a produção de prostaglandinas via *COX-2* aumenta a proliferação e angiogênese, inibe a apoptose e permite que escapem da vigilância do sistema imune (TSATSANIS et al.,

2006). Alguns estudos têm mostrado uma expressão elevada de *COX-2* em tumores como próstata (UOTILA et al., 2001), esôfago (MÖBUS et al., 2005), tireóide (LO et al., 2005), mama (McCARTHY et al., 2006; LUCCI et al., 2008), oral (PANDEY et al., 2008), estômago (YAMAC et al., 2008), com associação positiva com infecção pela *H. pylori* (ZHANG et al., 2005), e glândula salivar, em que mostrou associação também com a expressão de *HGF* (AOKI et al., 2006). Além do mais, as prostaglandinas derivadas de *COX-2* são importantes para a cicatrização de úlceras gástricas e níveis elevados de expressão podem ser determinados nas bordas e base de úlceras gástricas em cicatrização e em tecidos com gastrite relacionada à *H. pylori* (JACKSON et al., 2000; GUO et al., 2003).

O gene *NOS* (Óxido Nítrico Sintase), mapeado em 17q11.2-q12 (MARSDEN et al., 1994) é membro da superfamília de monoxigenases que incluem a bem caracterizada família do citocromo P450 (CYP) e apresenta três isoformas: *NOS* neuronal (*nNOS*), *NOS* endotelial (*eNOS*) e *NOS* induzível (*iNOS* ou *NOS2*). As duas primeiras são constitutivamente expressas e dependentes de Ca^{+2} , atuando como neurotransmissor nos tecidos neurais e regulando a pressão sanguínea nos tecidos endoteliais, respectivamente. *NOS2* é independente de Ca^{+2} e é induzido em vários tipos de células por lipopolissacarídeos (LPS), endotoxinas, citocinas inflamatórias tais como interleucina-1 (IL-1), fator de necrose tumoral alfa (TNF- α), interferon gama (IFN- γ), hipóxia e outros estímulos (WANG et al., 2005; KEKLIKOGU et al., 2008). A enzima *NOS* é responsável pela conversão de L-arginina para L-citrulina para a produção de óxido nítrico (NO). Devido a não dependência de níveis de cálcio locais, *NOS2* continua a produzir NO durante horas ou mesmo dias após sua indução, diferente das duas formas constitutivas que produzem NO de maneira transiente (BRENNAN et al., 2002). Vários estudos têm mostrado que as três isoformas podem ser detectadas em

vários tipos de células cancerosas e podem estar envolvidas na promoção ou inibição da patologia e fisiologia do câncer (DONCKIER et al., 2006).

O óxido nítrico desempenha várias funções biológicas, destacando seu papel como molécula sinalizadora nos mecanismos de defesa imunológica e carcinogênese (LECHNER; LIRK; RIEDER, 2005). Durante o processo carcinogênico, NO está relacionado à transformação de células normais pela ativação de oncogenes e perda de atividade de supressores de tumor; crescimento de células transformadas (desdiferenciação, proliferação, progressão do tumor de lesões pré-neoplásicas); angiogênese; invasão local e metástase. Como um radical livre, NO pode reagir para produzir peroxidonitritos que podem causar dano direto ou indireto no DNA e, sua produção por longo período de tempo pode levar a mutações e finalmente ao câncer (LIRK; HOFFMANN; RIEDER, 2002; NOMELINE et al., 2008). De modo contrário, a produção de NO também pode exercer um papel anti-tumorigênico, pelo qual o endotélio ativado pode causar lise de células tumorais. Alguns estudos ainda sugerem que NO é citotóxico e citostático contra células malignas e microorganismos, causando apoptose (NOMELINE, et al., 2008).

Estudos sobre a expressão gênica de *NOS2* em tecidos tumorais têm encontrado resultados contraditórios (KEKLIKOGU et al., 2008). A expressão aumentada de *NOS2* foi observada em câncer de mama (VAKKALA et al., 2000), pulmão (MARROGI et al., 2000), próstata (UOTILA et al., 2001), bexiga (HAYASHI et al., 2001), displasia oral (BRENNAN et al., 2002), linfoma de células-B (ATIK et al. 2006), câncer colorretal (YU et al., 2006), tireóide (DONCKIER et al., 2006), fibrose submucosa oral (REJENDRAN; VARKEY, 2007), câncer de ovário (NOMELINI, et al., 2008), tumores de cabeça e pescoço (BRENNAN et al., 2008) e câncer gástrico (WANG et al., 2005; CHEN et al., 2006). Neste último caso, os autores observaram

interação com *COX-2* e uma correlação com invasão, metástase nos linfonodos, infecção com *H. pylori* e invasão vascular. Estudo recente na população brasileira, mostrou associação entre expressão aumentada de *NOS2* em câncer gástrico e gastrite crônica em comparação com a mucosa normal e úlcera gástrica (AUGUSTO, et al., 2007). De modo contrário, estudos prévios evidenciaram uma expressão diminuída da proteína *NOS2* em tumores gástricos e de cólon, assim levantando a hipótese de uma relação entre perda de NO e carcinogênese (RAJNAKOVA et al., 1997; AMBS et al., 1998).

A família Ras de proteínas G monoméricas, denominadas HRAS, NRAS e KRAS atua como “interruptores moleculares” ligando sinais extracelulares, por meio de receptores de membrana, a sinais intracelulares. Estas proteínas alternam de um estado inativo, quando ligada ao GDP, a um estado ativo, quando ligada ao GTP, em resposta a ativação de vários receptores (PATRA, 2008). As proteínas RAS regulam diversas vias celulares que são importantes para o crescimento e dispersão de células malignas, incluindo proliferação celular, regulação do ciclo celular, sobrevivência da célula, angiogênese e migração celular. Sob a influência de EGF e EGFR estimula a proliferação celular pela via de sinalização RAS para promover o reparo de tecidos e cicatrização (FRIDAY; ADJEI, 2005).

Várias mutações de ponto no gene *KRAS* mapeado em 12p12.1 (POPESCU et al., 1985) foram identificadas e resultam em ativação constitutiva do gene em uma grande variedade de tumores como cólon (SAMOWITZ et al., 2000), pâncreas (WANG et al., 2002), tireóide, pulmão (AVIEL-RONEN et al., 2006) e uma forma rara de câncer de pâncreas, o carcinosarcoma pancreático (NAKANO et al., 2008). Recentemente, Watari et al. (2007) correlacionaram a presença de mutações no gene *KRAS* com infecção pela bactéria *H. pylori* em indivíduos com metaplasia intestinal e gastrite

crônica, mostrando que alterações neste gene estão presentes nos estágios iniciais da carcinogênese gástrica e que a erradicação da bactéria diminui a ocorrência das mutações.

Na ausência de mutações ativadoras, *KRAS* pode ter sua função oncogênica ativada por amplificação e expressão aumentada do gene (GALIANA et al., 1995; VON LINTIG et al., 2000; QIAN et al., 2005). Em câncer gástrico a expressão aumentada da proteína *KRAS* foi correlacionada com metástases nos linfonodos e prognóstico reservado (LI et al., 2006). Devido a sua importância na regulação do ciclo celular, sobrevivência, angiogênese e migração celular, *KRAS* tem sido um dos alvos atrativos para estudos de terapia molecular do câncer (FRIDAY; ADJEI, 2005; PATRA, 2008).

A família dos genes *TFF* (*Trefoil Factor*) humanos é composta por *TFF1*, *TFF2* e *TFF3* que são expressos predominantemente no trato gastrointestinal e interagem com mucinas para estabilizar a barreira de muco e proteger a mucosa contra danos. Também contribui para o reparo da mucosa, promovendo a migração de células epiteliais e reconstituição após dano (SANDS; PODOLSKY, 1996; KORNPRAT et al., 2005). O papel destes fatores no desenvolvimento e progressão tumoral tem sido investigado em vários estudos, que sugeriram funções como fatores de dispersão, agentes pró-invasivos, anti-apoptóticos e angiogênicos (ABDOU; AIAD; SULTAN, 2008). O gene *TFF1*, localizado em 21q22.3 (MOISAN et al., 1985) e também conhecido como *pS2*, atua como um supressor de tumor específico da mucosa gastrointestinal, prevenindo a entrada na fase S do ciclo celular, pelo aumento da expressão da proteína pRb e subsequente diminuição da atividade de *E2F* (TOMASETTO; RIO, 2005).

A proteína *TFF1* também atua como um fator anti-apoptótico, diminuindo a atividade das caspases-3, 6, 8 e 9 e na proteção da mucosa gástrica, onde interage com a mucina gástrica solúvel MUC5AC (LEFEBVRE et al., 1996; RUCHAUD-

SPARAGANO; WESTLEY; MAY, 2004). A inativação do gene *TFF1* está associada com a ocorrência de displasia, adenoma e adenocarcinoma de estômago em ratos (LEFEBVRE et al., 1996). Em humanos, a expressão da proteína TFF1 é perdida em 40-60% dos tumores gástricos, enquanto os tecidos normais adjacentes permanecem positivos (MACHADO et al., 2000; LEUNG et al., 2002; SHI; CAI; YANG, 2006). Os mecanismos envolvidos com a ausência de expressão de *TFF1* em câncer gástrico incluem perda alélica em 28-50% dos casos, metilação do promotor e mutações de ponto que promovem perda da atividade supressora de tumor e ganho de invasividade (YIO et al., 2006). De forma semelhante, os níveis de RNAm de *TFF1* diminuem consideravelmente da mucosa gástrica normal para os adenocarcinomas gástricos (BECKLER et al. 2003). Em lesões pré-cancerosas, como a metaplasia intestinal, a expressão da proteína é menor que no tecido normal, diminuindo consideravelmente do tipo completo para incompleto (KIM et al., 2004), o mesmo ocorrendo para gastrite superficial crônica, gastrite atrófica e úlcera gástrica (SHI; CAI; YANG, 2006).

Saitoh et al. (2000) encontraram níveis aumentados do RNAm de *TFF1* em úlceras gástricas em cicatrização comparados com as úlceras ativas, sugerindo que este gene pode estar relacionado a proteção e diferenciação celular das áreas não-ulceradas da mucosa gástrica. De modo contrário, o gene *TFF1* pode ter atividade oncogênica, atuando como fator de crescimento, pois é altamente expresso em tecidos malignos que normalmente não o expressam, incluindo os tumores de bexiga (KORNPRAT et al., 2005), mama, pâncreas, intestino grosso, pulmão, esôfago, ovário e metástases associadas, e câncer de próstata (TOMASETTO; RIO, 2005; VESTERGAARD et al. 2006; ABDOU; AIAD; SULTAN, 2008).

As claudinas fazem parte de uma família grande de proteínas compreendendo 24 membros que apresentam quatro domínios transmembrana e são expressas nas células

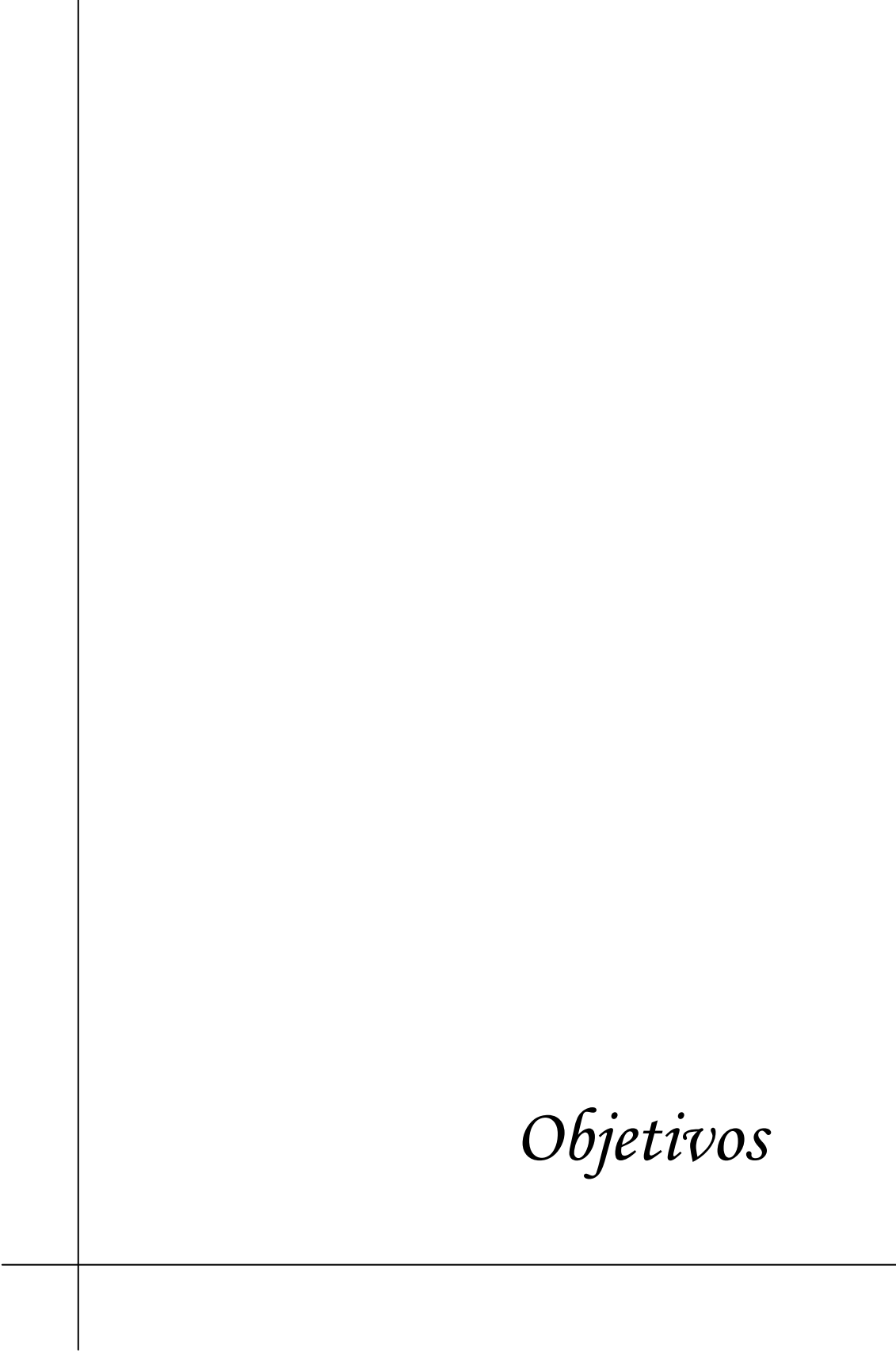
epiteliais de vários tecidos. Estas proteínas interagem entre si formando uma rede que ajuda a criar as junções *tight* das células epiteliais, assim exercendo um papel crítico na polaridade celular (KARANJAWALA et al., 2008). As claudinas também estão envolvidas em vários processos fisiológicos como a permeabilidade e condutância paracelular (SANADA et al., 2006), atuando como uma barreira difusora para o movimento de proteínas e lipídeos através da membrana celular e uma barreira primária para o transporte paracelular de solutos através das células (SEMBA et al., 2008). Deste modo, as quebras das junções *tight*, devido à expressão alterada das claudinas podem resultar na liberação de fatores de crescimento, que pode fornecer o estímulo autócrino e parácrino para a tumorigênese das células epiteliais (VERMEER et al., 2003).

Em humanos, as claudinas exibem padrões específicos de expressão no trato gastrointestinal: na mucosa gástrica a expressão da claudina 18 (*CLDN18*) é normalmente detectada e na mucosa intestinal a expressão das claudinas 3 e 4 é obrigatoriamente observada. É interessante notar que no estágio de gastrite crônica seguida de metaplasia intestinal, as características das claudinas gástricas transformam-se naquelas do tipo intestinal, mostrando-se como bons biomarcadores para determinar a diferenciação do epitélio gástrico (MATSUDA et al., 2007).

O gene *CLDN18*, mapeado em 3q22.3, foi primeiramente descrito como um alvo *downstream* ao fator de transcrição T/EBP/NKX e, em ratos, foram descritas duas variantes: *CLDN18a1* expressa no pulmão e *CLDN18a2* expressa no estômago. Cada isoforma apresenta uma variante de *splicing* alternativo faltando o domínio C-terminal (*CLDN18a1.2* e *CLDN18a2.2*) (NIIMI et al., 2001). A função biológica da claudina 18 é pouco compreendida, do mesmo modo que pouco se sabe sobre o papel das claudinas na tumorigênese humana. Como a variante *CLDN18a2* é expressa somente no estômago normal e células de Paneth do duodeno, as disfunções das junções *tight* causada por

diminuição da expressão de *CLDN18* pode levar a um influxo anormal de fatores de crescimento relacionados ao estômago e células de Paneth. Alguns estudos têm mostrado esta relação, associando a expressão reduzida de *CLDN18* e câncer gástrico (SANADA et al., 2006; SEMBA et al., 2008). Outros estudos têm mostrado o aumento de expressão de *CLDN18* durante a colite experimental e em pacientes com colite ulcerativa (ZWIERS et al., 2008). A expressão aumentada da proteína CLDN18 foi verificada em carcinoma bem diferenciado de pâncreas (KARANJAWALA et al., 2008) e no epitélio colunar especializado do esôfago de Barrett, no qual contribui para a maior resistência ácida deste tipo de lesão (JOVOV et al., 2007).

Considerando o exposto quanto a carcinogênese gástrica e a participação de lesões benignas, e ainda a escassez de estudos genéticos nestas lesões, torna-se necessário à busca de possíveis alterações genéticas, como mudanças na expressão gênica, que possam ser indicadas como biomarcadores para o diagnóstico precoce do câncer de estômago.

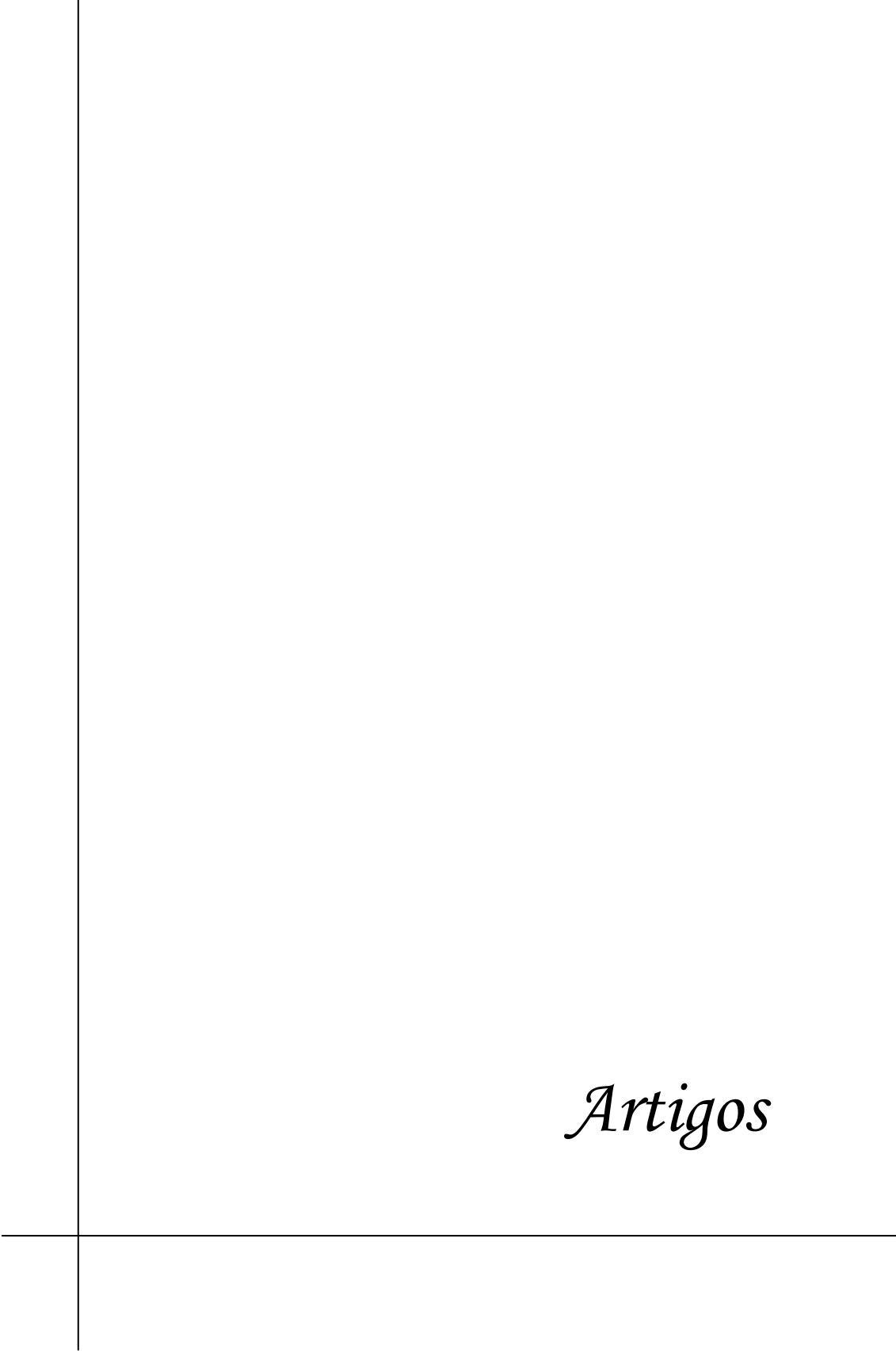


Objetivos

II - Objetivos

Devido ao importante papel das lesões pré-malignas no desenvolvimento do câncer gástrico, há necessidade de estudos para avaliação de mudanças nos níveis de expressão gênica e protéica de genes relevantes que participam de processos celulares como proliferação e apoptose, proteção e reparo do epitélio gástrico, nas etapas iniciais que possam desencadear o processo carcinogênico. Deste modo, este trabalho teve como objetivos:

1. Avaliar os níveis de expressão dos genes *TERT*, *COX-2*, *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* e *CLDN18* em metaplasia intestinal e úlcera gástrica, em comparação com a respectiva mucosa normal e com o câncer gástrico;
2. Avaliar a expressão das proteínas TERT, COX-2, NOS2, HGF, MET, KRAS, TFF1 e CLDN18 nestes mesmos grupos;
3. Verificar a ocorrência de interação entre os genes *NOS-2*, *HGF*, *MET* e *KRAS*, através da correlação entre os níveis de RNA mensageiro nas amostras de metaplasia intestinal, ulcera gástrica e câncer gástrico.
4. Investigar a ocorrência de associação de variáveis demográficas, fatores de risco e clinicopatológicas, tais como sexo, idade, tabagismo, etilismo, infecção pela *Helicobacter pylori* e tipo histológico com os níveis de expressão gênica e protéica de *TERT*, *COX-2*, *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* e *CLDN18* em metaplasia intestinal, úlcera gástrica e câncer gástrico.



Artigos

Os resultados referentes aos objetivos desta tese serão apresentados, a seguir, na forma de três artigos científicos, conforme as normas de publicações específicas de cada periódico.

Artigo 1

Título: Expression of *TERT* and *COX-2* in precancerous gastric lesions compared to gastric cancer.

Autores: Márcia Cristina Duarte, Érica Babeto, Kátia Ramos Moreira Leite, Kenji Miyazaki, Aldenis Albanese Borim, Paula Rahal, Ana Elizabete Silva

Periódico: *World Journal of Gastroenterology*, submetido em 27/08/2009.

Artigo 2

Título: Expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in intestinal metaplasia and gastric cancer: a real-time PCR and immunohistochemical analysis.

Autores: Márcia Cristina Duarte, Mabel Tatty de Medeiros Fracassi, Kenji Miyazaki, Ana Elizabete Silva

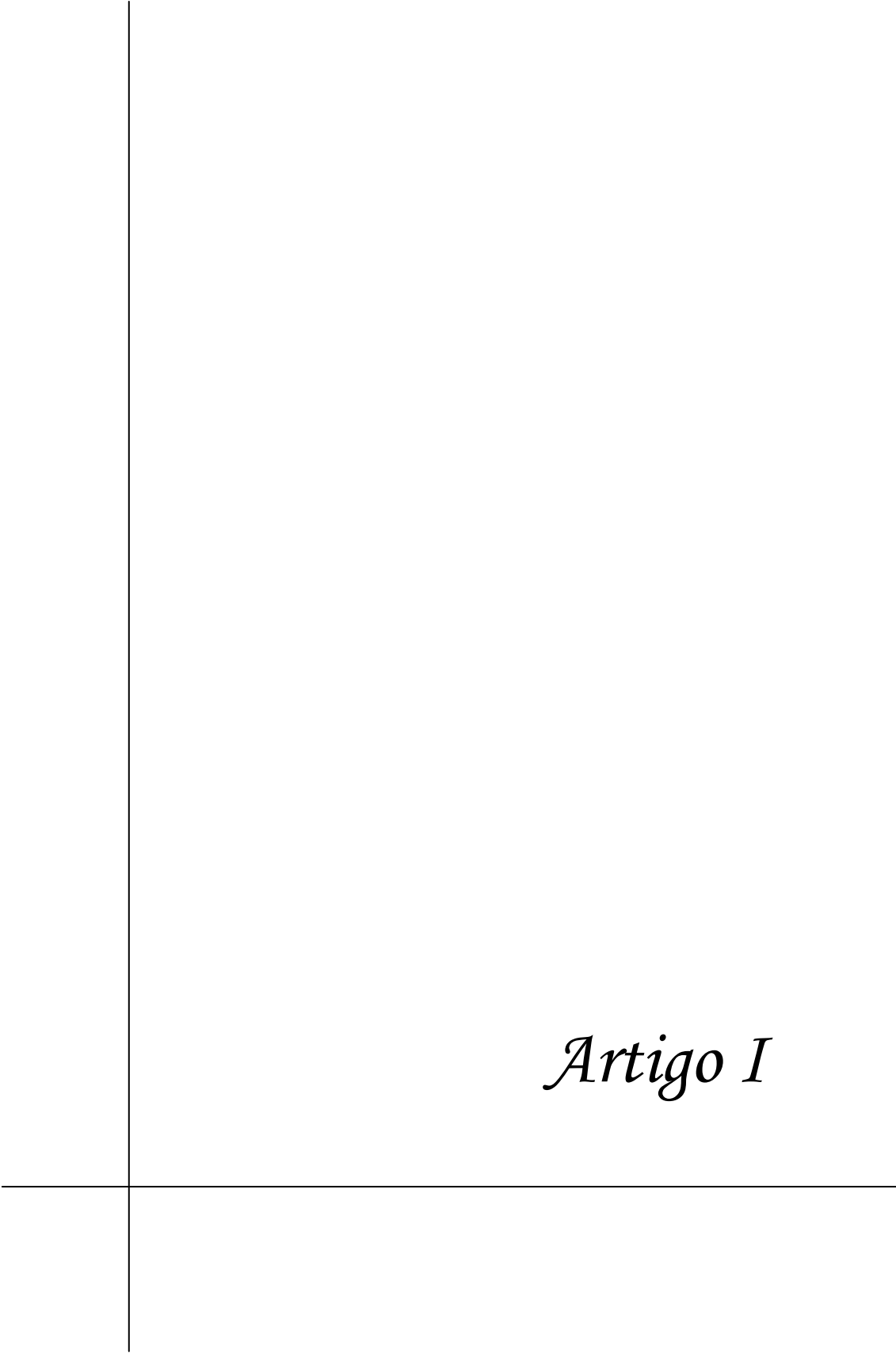
Periódico: *BMC Gastroenterology*, artigo a ser submetido à publicação.

Artigo 3

Título: *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* expression in gastric ulcer and gastric cancer.

Autores: Márcia Cristina Duarte, Mabel Tatty de Medeiros Fracassi, Kenji Miyazaki, Ana Elizabete Silva.

Periódico: *Journal of Gastroenterology and Hepatology*, artigo a ser submetido à publicação.



Artigo I

Artigo I: Expression of *TERT* and *COX-2* in precancerous gastric lesions compared to gastric cancer.

Running title: *TERT* and *COX-2* expression in gastric lesions.

Authorship: Márcia Cristina Duarte, Érica Babeto, Kátia Ramos Moreira Leite, Kenji Miyazaki, Aldenis Albanese Borim, Paula Rahal, Ana Elizabete Silva

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Author contributions: Duarte MC and Silva AE designed the research. Duarte MC performed the research. Rahal P and Babeto E provided the technical support and assistance for Real Time PCR assays. Miyazaki K and Borim AA were responsible for the endoscopic exam and collection of the gastric biopsies. Leite KRM performed immunohistochemical analysis. Duarte MC and Silva AE analyzed the data and wrote the manuscript.

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ABSTRACT

AIM: To investigate *TERT* and *COX-2* mRNA and protein expression in stomach precancerous lesions such as intestinal metaplasia (IM) and gastric ulcer (UG) in comparison to gastric cancer.

MATERIALS AND METHODS: Real-time PCR were performed to detected *TERT* and *COX-2* mRNA expression in 35 biopsies of IM, 30 of GU and 22 of gastric cancer (GC) and their respective normal mucosa. *TERT* and *COX-2* proteins were detected with immunohistochemistry in 68 samples, 34 of IM, 23 of GU and 11 of GC.

RESULTS: The increased mRNA expression levels of *TERT* and *COX-2* were observed respectively in 45.7% and 40% of IM; 50% and 51.7% of GU and 78.9% and 61.9% of GC. Considering all samples (up and down regulated) the relative means levels of *TERT* and *COX-2* mRNA after normalization with housekeeping β -actin gene and comparison with respective adjacent normal mucosa, in the groups of IM, GU and GC were respectively: 2.008 ± 2.605 , 2.730 ± 4.120 and 17.271 ± 33.852 for *TERT*, and 2.063 ± 3.729 , 2.496 ± 4.132 and 27.594 ± 58.952 for *COX-2*. The *TERT* and *COX-2* protein immunostaining was observed, respectively, in 38.2% and 79.4% of IM, 39.1% and 26.1% of GU, and 54.5% and 81.8% of GC. This study showed for the first time an overexpression of *TERT* mRNA and protein in GU. There were no significant differences between the groups regarding *TERT* mRNA and protein expression, whereas for *COX-2* there were significant differences in mRNA ($P=0.0291$ for IM vs. GC) and protein ($P=0.0001$ for IM vs. GU; $P=0.003$ for GU vs. GC). No association between *TERT* and *COX-2* mRNA and protein expression with *H. pylori* infection and other clinicopathological variables was found.

CONCLUSION: This study suggests that *TERT* and *COX-2* may be deregulated expression in intestinal metaplasia and gastric ulcer and play a role in the early events of gastric carcinogenesis.

Key words: intestinal metaplasia, gastric ulcer, gastric cancer, *TERT*, *COX-2*, gene expression, protein expression

INTRODUCTION

Gastric precancerous lesions such as intestinal metaplasia have been associated with the multistep process of well-differentiated gastric or intestinal-type adenocarcinoma that develops from active gastritis, frequently associated with *Helicobacter pylori* infection, to gastric atrophy, intestinal metaplasia, dysplasia, and finally to gastric cancer^[1]. Intestinal metaplasia is characterized as the transformation of the gastric epithelium and glands from secretory to absorptive cells, which closely resemble the mature intestinal epithelium^[2]. It is a well-established pre-malignant condition of the stomach and can produce a 10-fold increase in the risk of this neoplasia^[3]. Another pathway of gastric carcinogenesis includes peptic ulcer, which increases the gastric cancer risk 1.8 times^[4]. Genetic studies in precancerous gastric lesions are still limited, so a better understanding of the mechanisms involved in gene expression in the premalignant steps, which lead to the development of cancer, is necessary. Among the genes with changed gene expression in gastric carcinogenesis, can be detected *TERT* and *COX2*, which can also be overexpressed in premalignant lesions, so participating of early progression of disease.

The *TERT* (human telomerase reverse transcriptase) gene encodes the catalytic subunit of telomerase, which elongates the telomere ends using the RNA subunit hTERC as a template^[5]. The stabilization of the telomere size is a prerequisite for malignant cells to erase the senescence checkpoint and acquire the capacity to proliferate unlimitedly. So, telomerase reactivation is an obligatory event in carcinogenesis and, in fact, increased telomerase activity or *TERT* mRNA expression has been detectable in up to 90% of human cancers^[6] including gastric cancer^[7, 8].

COX-2 (Cyclooxygenase-2) is the rate-limiting enzyme for prostaglandin synthesis^[9], and can be induced by proinflammatory cytokines, growth factors, mitogens and oncoproteins. During inflammation and mitogenic stimulation, a 10 to 20-fold increase in *COX-2* mRNA expression and overproduction of prostaglandins has been observed, which enhance inflammatory cells infiltration, exudation, swelling and pain in the wound tissues^[10]. *COX-2* is

involved in the regulation of a broad range of cellular processes, including angiogenesis, apoptosis and cell proliferation. Recent studies have demonstrated overexpression of COX-2, which plays a role in carcinogenesis and tumor progression in many epithelial tumors including colon^[11], lung^[12], breast^[13], oral^[14], esophagus^[9], and stomach^[15].

Thus, the aim of this study was to investigate changes in the levels of expression of *TERT* and *COX-2* mRNA and protein in intestinal metaplasia and gastric ulcer in comparison to normal mucosa and gastric cancer, and to investigate their potential relationship with *H. pylori* infection and other clinicopathological variables. The findings may indicate changed expression of these genes in precursor lesions, which may confer increased risk of gastric cancer.

MATERIAL AND METHODS

Samples

A total of 87 specimens were evaluated, obtained from 30 patients with gastric ulcer (GU) (mean age 54.90 ± 12.37 years), 35 patients with intestinal metaplasia (IM) (mean age 61.05 ± 12.45 years), all were gastric cancer free, and 22 patients with gastric adenocarcinoma (GC) (mean age 62.45 ± 14.07 years). For each patient were collected three biopsies from lesion area and other three biopsies from their normal gastric mucosa adjacent to lesion, during endoscopic evaluation at the period between March 2006 and March 2008, at the Hospital de Base (São José do Rio Preto, SP, Brazil). The biopsies were collected mainly from the antrum and corpus regions of the stomach. Immediately after, they were stored in RNA later reagent (Ambion) at -20°C until RNA extraction. All specimens were histopathologically diagnosed. Archival paraffin-embedded, formalin-fixed tissues were used for immunohistochemical staining of the *TERT* and *COX-2* proteins. Information about age, gender, smoking and drinking status and the written informed consent was obtained from all patients, and the study was approved by the National Research Ethics Committee (CONEP).

RNA extraction and reverse transcription

Total cellular RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration was determined in a NanoDrop® ND1000 spectrophotometer, and its integrity was visualized on 1% agarose gel. RNA samples were stored in freezers at -80°C and used for reverse transcription. cDNA was synthesized from 5 µg of total RNA using random primers and a High Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. The integrity of all cDNA preparations was tested by PCR assay of the *β-actin* gene and visualized on 2% agarose gel.

Quantitative Real Time PCR

The expression of *TERT* and *COX-2* mRNA was measured by real time PCR, based on the SYBR Green methodology, using an ABI Prism® 7300 (Applied Biosystems). The primer sequences used in the study were as follows: for *TERT*, 5' CGGAAGAGTGTCTGGAGCAA 3' and 5' GGATGAAGCGGAGTCTGGA 3'; and for *COX-2*, 5' CATCCTGAATGGGGTGATG 3' and 5' GATAGCCACTCAAGTGTTGCAC 3'. The real time PCR assays were performed in 10 µL of SYBR™ Green Master Mix (Applied Biosystems), 25 ng of cDNA and 0.9 µM of *TERT* primers or 0.8 µM of *COX-2* primers. Thermal cycling conditions for *TERT* and *COX-2* were: 2 min at 50°C and 10 min at 95°C for initial denaturation, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, and a dissociation step at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Triplicates of the samples were assayed in each run. The relative expressions of *TERT* and *COX-2* were analyzed according to the description by Livak and Schmittgen^[16] and normalized with the housekeeping *β-actin* gene and corresponding normal gastric mucosa.

Immunohistochemistry

The immunohistochemical analysis was performed in 34 samples with IM, 23 with UG and 11 with GC. Tissue sections of 4 µm were cut from paraffin-embedded tissue blocks and mounted on glass slides pretreated with 3-aminopropyl-triethoxysilane/acetone solution and dried overnight at 60°C.

After deparaffinization and rehydration, antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 15 min at 120°C, followed by treatment with 3% H₂O₂ for 20 minutes to block the endogenous peroxidase. Then, the sections were incubated for 1 hour at room temperature with specific antibodies: COX-2 mouse monoclonal antibody (clone 4H12, Novocastra, 1:100) or TERT mouse monoclonal antibody (clone 2D8, ABR - Affinity BioReagents, 1:100). After rinsing with Tris-HCl buffer (pH 7.6), the slides were incubated with biotinylated secondary antibody and incubated with streptavidin-biotin peroxidase, following the manufacturer's instructions (Histostain Bulk Kit, Zymed). The immunostain was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin. Negative controls were established by replacing the primary antibody with buffer solution. Colon carcinoma and amygdala were used as positive controls for COX-2 and TERT antibodies, respectively. A single pathologist examined all specimens. All analyses were done under a light microscope (×400 magnification), and the whole tissue extension of all samples was examined. Immunostain for proteins COX-2 (brown cytoplasm staining) and TERT (brown nuclear staining) was graded by staining intensity as negative (-) (absent brown staining) or positive: +1 - weakly stained, +2 - moderately stained, and +3 - strongly stained, as observed in at least 10% of cells.

Statistical Analysis

mRNA relative expression levels were described using the mean as a point estimator and the range of values. Non-parametric Mann-Whitney *U* test were used for comparisons between the groups and mRNA expression level and clinicopathological variables. Fisher's exact test was used to evaluate the protein levels between the groups and the relationship between protein levels and clinicopathological variables. All statistical tests were performed using the GraphPad InStat Software. The level of significance was set at $P < 0.05$.

RESULTS

TERT and COX-2 mRNA relative expression

Real time PCR analysis was performed for all 87 samples and their corresponding adjacent normal gastric mucosa and the results of mRNA expression are summarized in Table I. In the GU and GC groups, no *TERT* expression was detected in 8/30 (26.7%) and 3/22 (13.6%) samples, respectively, while only one sample did not express *COX-2* in both groups. After normalization with housekeeping β -actin gene and comparison with respective adjacent normal mucosa, the relative expression of *TERT* mRNA in the groups was increased in 16 (45.7%) out of 35 IM samples, in 11 (50%) out of 22 GU specimens, and in 15 (78.9%) out of 19 GC samples. To *COX-2* the relative expression of mRNA in the IM, GU and GC groups, was increased in 14 (40%) out of 35, in 15 (51.7%) out of 29, and in 13 (61.9%) out of 21 samples, respectively. Considering all samples (up and down regulated), the mean expression levels of *TERT* and *COX-2* mRNA in the groups of IM, GU and GC were respectively: 2.008 ± 2.605 , 2.730 ± 4.120 and 17.271 ± 33.852 for *TERT*, and 2.063 ± 3.729 , 2.496 ± 4.132 and 27.594 ± 58.952 for *COX-2*. But when we considering only the cases up-regulated the mean expression levels of *TERT* and *COX-2* mRNA in the groups of IM, GU and GC were respectively: 3.833 ± 2.954 , 5.304 ± 4.852 and 21.821 ± 36.987 for *TERT* and 4.908 ± 5.030 , 4.579 ± 5.099 and 44.356 ± 70.663 for *COX-2*. No significant differences were found between the groups for *TERT* mRNA expression, while for *COX-2* there was a significant difference between IM and GC ($P=0.0164$) and GU and GC ($P=0.0066$). The combined analyses of the two genes in each group revealed that 11/35 (31.4%) of cases of IM, 7/22 (31.8%) of GU, and 10/19 (52.6%) of GC showed simultaneous increased levels for *TERT* and *COX-2* mRNA (data not shown).

Immunostaining for TERT and COX-2 proteins

Figure I show representative results of immunohistochemistry for *TERT* and *COX-2* proteins in normal gastric mucosa showing negative expression (Figure

IA and IE) and positive immunostaining in the lesions evaluated. Positive nuclear staining for the TERT protein (Figure 1B-D) was observed in 13 (38.2%) out of 34 IM specimens, in 9 (39.1%) out of 23 GU samples, and in 6 (54.5%) out of 11 GC samples, ranging from weak (+1) to moderate/strong intensity (+2/+3), that was the most common staining (Table II). Both GC and GU showed diffuse staining distribution in most of the cases, while around 50% of the IM cases displayed a focal staining distribution, i.e., exclusively in the metaplastic glandules (globet and non-globet cells). For the COX-2 protein, 27 (79.4%) out of 34, 6 (26.1%) out of 23, and 9 (81.8%) out of 11 cases of IM, GU and GC, respectively, showed positive brown cytoplasmatic staining (Figure 1F-H), with mainly moderate/strong staining intensity. A diffuse distribution was observed in most of the GC and IM samples for COX-2, whereas the GU group showed focal distribution mainly in the regenerative areas. There were no significant differences in the TERT protein expression among the groups, but for the COX-2 protein a significantly increased frequency of positive cases in the GC and IM groups compared to GU ($P=0.003$ and $P=0.0001$, respectively) was found. In addition, when we compared the cases that showed overexpression simultaneously to both proteins, we observed that 11 out of 34 (32.4%) of IM, 3 out of 23 (13%) of GU and 5 out of 11 (45.5%) of GC expressed TERT and COX-2 proteins.

In general for both genes the frequency of cases with overexpression of the mRNA was higher than the frequency of positive cases by immunohistochemistry, except for the COX-2 expression in the IM and GC groups, in which the positive protein immunostaining (79% and 82% of the cases, respectively) was more frequent than the mRNA expression (40% and 62% of the cases, respectively).

Relationship between demographic and clinical variables and *TERT* and *COX-2* expression

The association between demographic and clinical variables such as age, gender, smoking, *H. pylori* infection or the histological type of gastric cancer

with *TERT* and *COX-2* mRNA and protein expression were evaluated, but no association was found (data not shown).

DISCUSSION

In this study, we used real time PCR and immunohistochemistry to examine the *TERT* and *COX-2* mRNA and protein expression in precancerous lesions such as intestinal metaplasia and gastric ulcer and their relationship with expression in gastric cancer. Our findings demonstrated elevated levels of *TERT* and *COX-2* mRNA expression around 40-50% of specimens of IM and GU in comparison with 62-79% of the cases of gastric adenocarcinoma. To our knowledge, there are no studies available in the literature about *TERT* expression in gastric ulcer and our results showed mRNA overexpression in 50% of cases of gastric ulcer with mean level of 5.3 times higher than corresponding normal mucosa. These findings are the first evidence of deregulated expression of *TERT* in this lesion, and together with other studies suggest that both metaplasia and ulcer may present changed expression of genes that participate of initial steps of gastric carcinogenesis.

It has been stated that telomere maintenance due to telomerase activation contributes to cancer cell formation by increasing the cell's life span. This allows the accumulation of additional genetic alterations required for cancer development^[17]. Thus, telomerase activity or *TERT* mRNA expression may play an important role as markers for diagnosis and prognosis in different types of neoplasia^[7].

Recently, increased telomerase activity or mRNA expression has been demonstrated in cancers of the oral cavity^[18], ovaries^[19], uterine cervix^[20], lung^[21] as well as gastric cancer, and there are also some reports about intestinal metaplasia and chronic gastritis^[5, 7, 8, 22, 23, 24]. Earlier clinical studies indicated that telomerase activity and *TERT* are valuable biomarkers for discriminating between normal and malignant gastric tissues^[8].

In gastric cancer telomerase re-expression has been demonstrated in 61 to 90% of cases^[5, 25]. Furthermore, chronic gastritis and intestinal metaplasia have been linked to a higher telomerase activity than that found in normal tissues (23% and 10 to 79%, respectively), albeit lower than that observed in dysplasia or cancer^[5, 23, 24]. Gulmann et al.^[5] found that TERT protein expression in IM and normal mucosa adjacent to gastric cancer was similar (~38% of cases), whereas in carcinoma the TERT expression rates were higher (~50% of cases), as also shown by our results. These authors suggest that IM may harbor molecular abnormalities similar to those in gastric mucosa close to cancer and the early genetic instability require telomerase re-expression in order to overcome telomeres shortening^[5].

Overexpression of COX-2 mRNA or protein has been reported in 43-100% of gastric cancer cases, but is almost undetectable in normal gastric mucosa^[26, 27, 28]. Previous studies have proposed that COX-2 overexpression acts in cellular proliferation, induction of a number of angiogenic factors and resistance to apoptosis, which are important events in the carcinogenic process, rather than in promoting invasion and metastasis^[13, 15]. Our results also revealed expression of COX-2 mRNA and protein in most of the gastric cancer cases (62% and 82% of cases, respectively). Moreover, overexpression of COX-2 mRNA and protein was also observed in GU (52% and 26% of cases, respectively) and IM (40% and 79% of cases, respectively), although with mean levels of relative expression of mRNA lower than in the GC group.

A few studies detected COX-2 mRNA expression in premalignant gastric lesions such as intestinal metaplasia as compared to paired non-metaplastic tissues^[26, 27], in the margin and base of gastric ulcer tissues during healing^[29]. Differently from our study, that found COX-2 protein expression in 79% of the IM samples, a recent report showed COX-2 expression in only 23% of the IM cases in patients without cancer^[28]. However, the same study reported a frequency similar to our data in IM tissue distant from cancer areas (82%). In gastric ulcer, some studies in experimental animals and humans have evidenced the expression of COX-2 with an important event to healing, because

your role in the induction of angiogenic factors^[30, 31]. In our study the positive immunostaining for the COX-2 protein occurred mainly in the regenerative areas, thus not discarding their participation also in the process of healing of the ulcer.

Interestingly, we also observed that around 31% of cases of IM and GU in comparison with 53% of cases of GC evaluated showed overexpression simultaneously of both *TERT* and *COX-2* mRNA, thus suggesting that these precancerous lesions can share different genes with deregulated expression, which together may confer more risk of malignant progression.

No relationship was found in our study between *TERT* and *COX-2* mRNA and protein expression and demographic and clinical variables such as age, gender, smoking, drinking, *H. pylori* infection and histological type of gastric cancer. It is well established that *H. pylori* infection is associate to many gastric lesions, including gastric cancer, so being considered a carcinogen of the stomach^[32]. Previous studies have also rated the relationship between *H. pylori* infection and *COX-2* expression^[33]. Although some of them reported a positive correlation in patients with gastric cancer^[34, 35] and intestinal metaplasia^[28], others^[15, 36] obtained results similar to our findings, not indicating any association between *COX-2* expression and *H. pylori* infection. According of Yamach et al.^[15] the bacterium may have only a minor role in *COX-2* expression in gastric carcinogenesis. In addition, the small number of patients infected by *H. pylori* in the present study also may have had an impact on the results.

In conclusion, our data show that *TERT* and *COX-2* may be up-regulated in precursor lesions such as intestinal metaplasia and gastric ulcer in cancer-free patients in comparison with normal mucosa and can, therefore, act as early events in gastric carcinogenesis. These findings also bring additional information about changed gene expression in gastric ulcer, since this is the first time that *TERT* mRNA and protein expression level are described in this lesion. Thus supporting the need to further investigations in precancerous lesions of

expression levels of other genes involved in initial steps of gastric carcinogenesis.

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Legend of figure:

Figure 1. Immunohistochemical analysis of hTERT protein in normal gastric mucosa showing negative expression (A), TERT nuclear positive immunostaining in intestinal metaplasia (B), gastric ulcer (C) and intestinal-type gastric cancer (D), and of COX-2 protein in normal gastric mucosa, showing negative expression (E), COX-2 cytoplasmatic positive immunostaining in intestinal metaplasia (F), gastric ulcer (G) and intestinal-type gastric cancer (H). (Mayer's hematoxylin stain, x400).

Table I. Relative expression of *TERT* and *COX-2* RNA normalized against β -actin and normal mucosa in intestinal metaplasia (IM), gastric ulcer (GU) and gastric cancer (GC) groups.

Targets		IM			GU		GC		P value		
		N=35			N=30		N=22		IM x GU	IM x GC	GU x GC
<i>TERT</i>	Up-regulated cases	16/35 (45.7%)			11/22 (50%)		15/19 (78.9%)				
	mean±SD	3.833±2.954			5.304±4.852		21.821±36.987		0.8159	0.9224	0.9350
	range	1.042 to 11.484			1.071 to 12.861		1.011 to 129.636				
	Total (up- and down-regulated)										
<i>COX-2</i>	mean±SD	2.008±2.605			2.730±4.120		17.271±33.852				
	range	0.001 to 11.484			0.000 to 12.861		0.005 to 129.636		0.9730	0.0854	0.1227
	Up-regulated cases	14/35 (40%)			15/29 (51.7%)		19/21 (61.9%)				
	mean±SD	4.908±5.030			4.579±5.099		44.356±70.663		0.6848	0.0164*	0.0066*
	range	1.059 to 16.990			1.002 to 18.549		1.132 to 230.720				
<i>COX-2</i>	Total (up- and down-regulated)										
	mean±SD	2.063±3.729			2.496±4.132		27.594±58.952		0.4427	0.0291*	0.0706
	range	0.0009 to 16.990			0.0006 to 18.549		0.023 to 230.720				

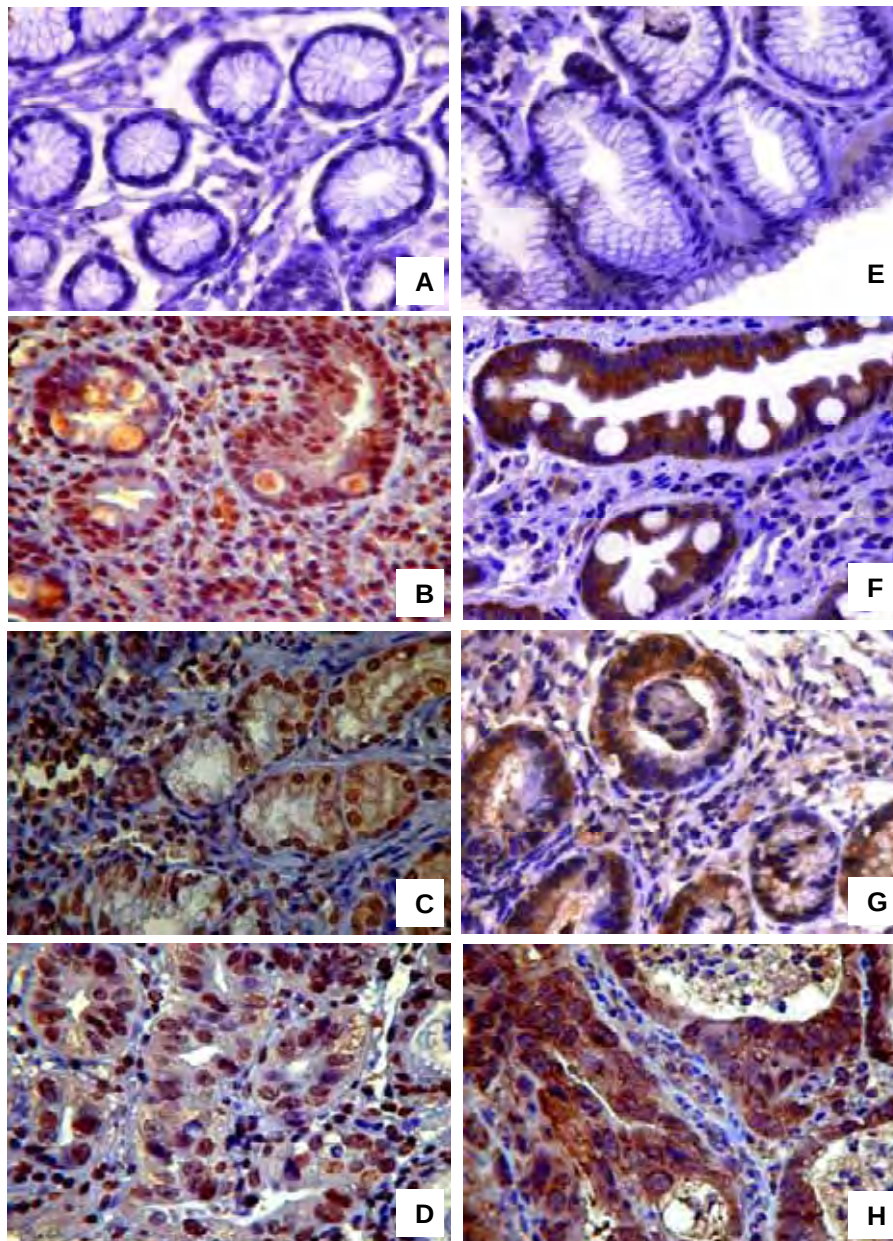
Mann-Whitney *U*-test for comparing mRNA expression levels between the groups; N= number of samples; * = statistically significant difference ($P<0.05$).

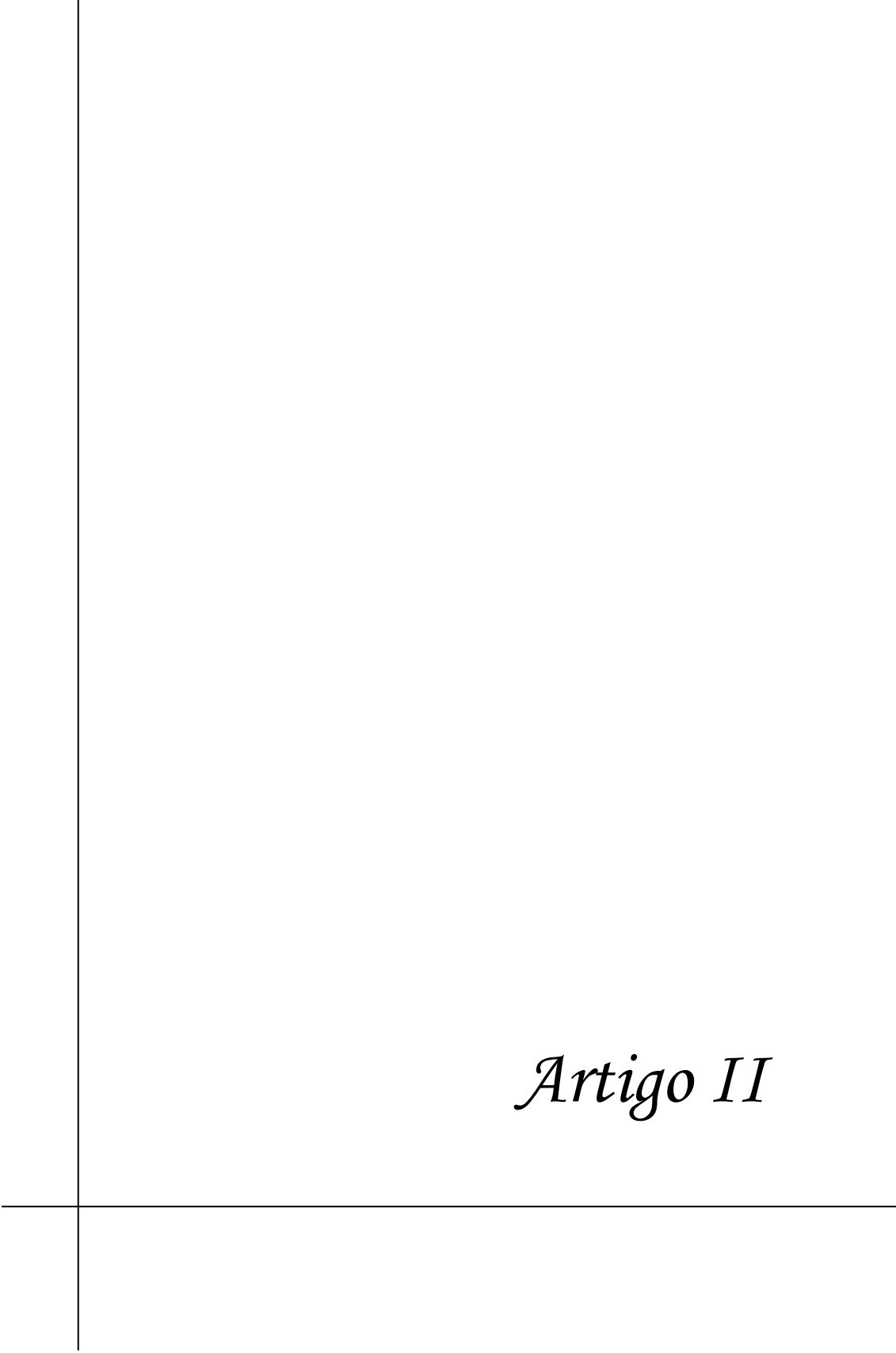
Table II. Protein expression of TERT and COX-2 in intestinal metaplasia (IM), gastric ulcer (GU) and gastric cancer (GC).

Targets	Evaluation	IM <i>n</i> = 34	GU <i>n</i> = 23	GC <i>n</i> = 11	<i>P value</i>		
					IM x GU	IM x GC	GU x GC
Nuclear expression of TERT	(-)	21 (61.8%)	14 (60.9%)	5 (45.5%)			
	(+1)	1 (2.9%)	1 (4.3%)	0 (0%)			
	(+2/+3)	12 (35.3%)	8 (34.8%)	6 (54.5%)	1.000	0.485	0.474
	Total positive cases	13 (38.2%)	9 (39.1%)	6 (54.5%)			
Citoplasmatic expression of COX-2	(-)	7 (20.6%)	17 (73.9%)	2 (18.2%)			
	(+1)	10 (29.4%)	2 (8.7%)	0 (0%)			
	(+2/+3)	17 (50%)	4 (17.4%)	9 (81.8%)	0.0001*	1.000	0.003*
	Total positive cases	27 (79.4%)	6 (26.1%)	9 (81.8%)			

Fisher's exact test for comparing protein expression between the groups. * = statistically significant difference ($P<0.05$).

Figure 1. Immunohistochemistry for TERT and COX-2 proteins.





Artigo II

Artigo II. Expression of NOS2, HGF, MET, KRAS, TFF1 and CLDN18 in intestinal metaplasia and gastric cancer: a real-time PCR and immunohistochemical analysis.

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Abstract

Background: Molecular studies concerning gastric pre-cancerous lesions are scarce and can be important to get a best comprehension about carcinogenic process and to perform prevention strategies. Intestinal metaplasia (IM) is a premalignant lesion of gastric cancer (GC). Thus, changes of expression levels in genes with important roles to cellular cycle progression, cell migration and, gastric mucosa maintenance and protection, can be present in IM, increasing the stomach cancer risk.

Methods: Biopsies from 37 patients with IM (free-cancer) and 22 with GC and their respective normal mucosal were used to RNA extraction and posterior real-time PCR analysis so as to evaluate the mRNA expression level of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18*. *β-actin* was used as reference. Protein expression of these genes was assessed by immunohistochemical tests.

Results: Up-regulated mRNA expression were found for *NOS2*, *HGF* and *MET* in 61.1%, 48.6% and 37.8% of the IM cases (13.6-, 2.6- and 2.8-fold), respectively. In GC, such genes were 12.8-, 1.8- and 3.5-fold up-regulated in 59.1%, 27.3% and 38.1% of the cases, respectively. *KRAS* was up-regulated in 26.7% and 28.6% of IM and GC cases, but the expression mean was not significant in IM (1.1-fold), while in GC, it was 1.7-fold increased. Fifty-four percent and 68.2% of both IM and GC cases were down-regulated to *TFF1* and *CLDN18* mRNA. Expression mean levels were increased 1.8- and 1.6-fold in IM, and decreased to 1.1- and 1.7-fold in GC, respectively. Immunohistochemical analysis for *NOS2* and *KRAS* proteins showed positive immunostaining (moderate/strong) in 92.6% and 0%, respectively of IM cases and 30% and 70%, respectively of GC cases. While for *TFF1* and *CLDN18* proteins, it was observed absent or weak immunostaining in 62.9% and 100% of IM cases, respectively, and in 100% and 90% of GC cases. Associations between clinicopathological variables

and mRNA expression were found for *H. pylori*-positive and *NOS2* and *HGF* (low expression) and *CLDN18* (high expression) in the GC group, and for smokers and *TFF1* (low expression) in IM group.

Conclusions: Our data suggest that intestinal metaplasia shares changes in expression of genes altered in gastric cancer, implying that they may have a key role to the development of this lesion and also in the initial steps of gastric carcinogenesis, so may confer increased risk of malignancy.

Key words: Intestinal metaplasia, gastric cancer, gene and protein expression, *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18*.

Background

Intestinal metaplasia is a pre-cancerous condition leading to stomach carcinogenesis, specifically well-differentiated adenocarcinoma or intestinal type [1]. It is defined as a potentially reversible change from a fully differentiated cell type to another, implying adaptation to environmental stimuli [2]. At this stage, the original glands and the foveolar epithelium are replaced by cells with intestinal phenotype. The type I or complete type of intestinal metaplasia resembles the small intestinal mucosa, with absorptive enterocytes, alternating with mucin-filled goblet cells. Whereas the incomplete type includes types II and III, being characterized by cells that acquire morphologic features of the large intestine, and are lined only by goblet cells of different sizes and shapes [3].

Intestinal metaplasia is involved in the multi-step model of intestinal type of gastric cancer that is triggered after a chronic inflammation of the gastric mucosa, which progresses throughout the years into the premalignant stages of chronic gastritis, leading

to atrophy, intestinal metaplasia, dysplasia and eventually gastric adenocarcinoma [4]. Furthermore, the major risk factors for gastric carcinogenesis consist in *H. pylori* virulence factors, host genetics, and environmental factors, such as diet and smoking [5]. The progression rates to gastric cancer in Western countries are very limited, however, for intestinal metaplasia the progression rates to gastric cancer have varied from 0% to 73% per year [6,7].

The gastric cancer development and progression is driven by an accumulation of genetic alterations that have also been observed in precancerous lesions, such as gastritis, ulcer and intestinal metaplasia [8-12]. The phenotypic shift that appears in intestinal metaplasia occurs as a result of a combination of altered expression of genetic factors, epigenetic silencing, transcription factors, signalling pathways and growth factors [2]. Nevertheless, relatively little is known regarding which key genetic changes in intestinal metaplasia are important in order to initiate tumor progression. Some studies have reported expression changes as in *TERT* [13,14], *PDX1*, *OCT1*, *TFF3*, *SOX2*, *SHH*, *RAR-β* and *RUNX3* [1,2,15] and *KRAS* mutations [13]. Hence, studies of gene deregulation of important genes in the cellular cycle control, growth factors, inflammation and mucosal protection in intestinal metaplasia can help understand and prevent the cancerous process of the stomach. Thus, the aim of this study was to investigate the levels of mRNA and protein expression the genes *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in intestinal metaplasia compared to adjacent normal tissue and gastric cancer, which has been reported with deregulated expression in this neoplasm, and to investigate their potential relationship with *H. pylori* infection and other clinicopathological variables.

Methods

Patients

This study consisted of 37 patients with intestinal metaplasia (mean age 61.05 ± 12.46 years), all were gastric cancer-free, and 22 patients with gastric adenocarcinoma (mean age 62.45 ± 14.07 years), who had undergone endoscopic evaluation between March 2006 and March 2008, at the Base Hospital (São José do Rio Preto, SP, Brazil). Of each patient, three biopsies of lesion region and other three biopsies of apparently normal region were collected, mainly in the antrum and corpus of the stomach. Immediately after this procedure, they were stored in RNA-later reagent (Ambion) at -20°C until RNA and DNA extraction. All specimens were histopathologically diagnosed. Archival paraffin-embedded formalin-fixed tissues were used for immunohistochemical staining of the proteins. Information about age, gender, smoking and drinking habits and the written informed consent was obtained from all patients, and the study was approved by the National Research Ethics Committee (CONEP).

RNA and DNA extraction and reverse transcription

The extraction of total cellular RNA and DNA was carried out by using the Trizol reagent (Invitrogen), according to the manufacturer's protocol. RNA and DNA concentration was determined in a NanoDrop[®] ND1000 spectrophotometer, and RNA integrity was visualized on 1% agarose gel with 2 bands of 18S and 28S rRNA. DNA samples were maintained in freezers at -20°C and used for molecular diagnosis of *H. pylori*. RNA samples were stored in freezers at -80°C and used for reverse transcription. cDNA was synthesized from 5 μg of total RNA using random primers and a High Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. The integrity of all cDNA preparations was tested by PCR assay of the β -*actin* gene and visualized on 2% agarose gel.

Quantitative Real Time PCR

The relative expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* mRNA was measured by real time PCR, based on the SYBR Green methodology, using an ABI Prism[®] 7300 (Applied Biosystems). The primer sequences used in the study were obtained using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with exception of *CLDN18* sequence, obtained of Sanada et al. [16] and they are shown in Table 1. An initial validation step with 10-fold dilution series was performed to obtain the amplification efficiencies of the primers (E), calculated according to the equation $E = 10^{[-1/\text{slope}]}$. The efficiencies of amplification ranged between 90% and 97%. After the validation step, the real time PCR assays were performed in 10 μL of SYBR[™] Green Master Mix (Applied Biosystems), 25 ng of cDNA and 0.6 μM of *β -actin*, *TFF1* and *CLDN18* primers, 0.7 μM of *KRAS* primer and 0.8 μM of *NOS2*, *HGF* and *MET* primers. Thermal cycling conditions for all the genes were: 2 min at 50°C and 10 min at 95°C for initial denaturation, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, and a dissociation step at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Triplicates of the samples were assayed in each run. *β -actin*, *α -tubulin* and *β 2-microglobulin* housekeeping genes were evaluated to case and control samples, to verify which contained the lowest variation of amplification between the samples. After this, *β -actin* gene was used as reference because it showed the lowest variation. The relative expressions of the genes were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method, according to the description by Livak and Schmittgen [17]. First, the mean of cycle number at the threshold level of fluorescence (Ct) for each sample was determined. Next, the ΔCt value was calculated as being the difference between the Ct value of target gene and the Ct value of *β -actin*: $\Delta\text{Ct} = \text{Ct}(\text{target gene}) - \text{Ct}(\beta\text{-actin})$. Finally, both the $\Delta\Delta\text{Ct}$ value and the normalized target gene expression were calculated: $\Delta\Delta\text{Ct} =$

$\Delta\text{Ct (IM or GC)} - \Delta\text{Ct (normal adjacent mucosa)}$. Normalized expression in a sample = $2^{-\Delta\Delta\text{Ct}}$.

PCR for H. pylori diagnosis

DNA samples extracted from the adjacent normal gastric mucosa were used for molecular diagnosis of the *H. pylori* bacterium, because it is a more efficient method than histology and urease test [18]. The bacterium genes *urease* and *tsaA* were amplified by Multiplex PCR, using the specific primers F- 5' TTCCTGATGGGACCAAACCTC 3' and R- 5' TTACCGCCAATGTCAATCAA 3', and (F- 5' CCTGCCGTTTTAGGAAACAA 3' and R- 5' TCCGCATTCTACCTAATGG 3') in order to amplify, respectively, segments of 316 pb and 413 pb. The housekeeping human gene *CYP11A1* (F- 5' CTCACCCCTGATGGTGCTAT 3' and R- 5' TTTGGAAGTGCTCACAGCAG 3') that amplifies a 226 pb segment was used as quality control DNA. Positive and negative controls were used in all experiments. The PCR assay solution consisted of 4 mM MgCl_2 , 0.3 mM dNTPs, 0.6 μM of each primer, and 1.75 U Taq DNA polymerase in 1 X buffer. The thermal cycling conditions were: 5 min at 95°C, followed by 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were separated on 2% 1000 agarose gel (Invitrogen) and stained with ethidium bromide. The assay was considered positive when at least one of the bacterial PCR products was present.

Immunohistochemistry

The immunohistochemical analysis was performed in 34 samples with IM, 24 with UG and 11 with GC. Tissue sections of 4 μm were cut from paraffin-embedded tissue blocks and mounted on glass slides pretreated with 3-aminopropyl-triethoxysilane/acetone solution and dried overnight at 60°C. After deparaffinization at

37° overnight, and rehydration step, antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for NOS2, HGF, MET, TFF1 and CLDN18, and in 10mM EDTA-TRIS buffer (pH 9.0) for KRAS for 30 minutes at 95°C in a steam-pan (Exotic, Arno). The endogenous peroxidase activity was blocked with 3% H₂O₂ treatment for 10 minutes. Then, the sections were incubated for 1 hour at room temperature with specific antibody to TFF1 (pS2 mouse monoclonal antibody, clone BC04, Invitrogen, 1:50), HGF (Monoclonal anti hepatocyte growth factor antibody, clone 24612.111, Sigma, 1:50) and MET (Anti hepatocyte growth factor receptor (cMet) antibody, Sigma, 1:50) and overnight at 4°C with specific antibodies for NOS2 (Rabbit polyclonal to iNOS, abcam, 1:200), KRAS (Mouse monoclonal anti-KRAS, clone 9.13, Invitrogen, 1:50) e CLDN18 (Rabbit polyclonal anti-claudin-18 -c-Term, Zymed, Invitrogen, 1:200). After being rinsed with Tris-HCl buffer (pH 7.6), the slides were incubated, for 20 minutes each, with biotinylated secondary antibody and incubated with streptavidin-biotin peroxidase, following the manufacturer's instructions (Histostain Bulk Kit, Zymed). The immunostain was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin. Negative controls were established by replacing the primary antibody with buffer solution. Stomach normal mucosa was used as positive control for TFF1 and CLDN18 antibodies, and liver, breast carcinoma, lung carcinoma and epidermoid lung carcinoma tissues were used as positive controls for HGF, MET, NOS2 and KRAS antibodies, respectively. A single pathologist examined all specimens. All analyses were performed under a light microscope, and the whole tissue extension of all samples was examined. Immunostaining for protein NOS2 (brown cytoplasm staining) was evaluated by staining presence as negative (-) (absent brown staining) or positive (+) (presence brown staining), whereas for KRAS (brown cytoplasm staining), TFF1 (brown cytoplasm staining) and CLDN18 (brown membrane

staining) proteins, the immunostaining was evaluated as negative (-) or positive: (+1) weakly stained, (+2) moderately stained, and (+3) strongly stained, as observed in at least 10% of cells. A significant protein expression was considered only for (+2) and (+3) staining, while (-) and (+1) showed no significant expression. Immunostaining analysis for HGF and MET proteins was not performed because antibodies reaction did not produce satisfactory results, after various tests.

Statistical Analysis

Data about RNAm relative expression were described using the mean $\pm$ SD as a point estimator and the range of values. Nonparametric Mann-Whitney test was used in the comparisons between the groups, and nonparametric Spearman rank correlation was used to evaluate interactions among mRNA expression in the groups. Protein expression was described according to number of positive and negative cases and Fisher's exact test was used to compare the groups. The relationship between mRNA and protein levels and clinicopathological variables was evaluated by Fisher's exact test. All the tests were accomplished using the GraphPad Instat Software. The level of significance was set at $P < 0.05$.

Results

Clinical features

Characteristics about gender, age, smoking and drinking habits, histological type and *H. pylori* infection are shown in Table 2. In the IM group, only five cases (13.5%) were incomplete type of IM and most of the cases were found to be smokers and non-drinkers. To GC group, 57.9% of the cases were intestinal type of GC and 62.5% were smokers and drinkers. *H. pylori* infection was detected by multiplex PCR and we

verified that 37.8% and 52.4% of IM and GC cases, respectively, were positive to the bacterium.

NOS2, HGF, MET, KRAS, TFF1 and CLDN18 mRNA expression in intestinal metaplasia (IM) and gastric cancer (GC)

The results for the gene expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in IM and GC are summarized in Table 3. Relative gene expression was calculated using normal adjacent mucosa as reference. Mean levels of expression around 1,5-fold increased or decreased were considered significant. *NOS2* mRNA expression was increased about 13-fold in both IM and GC in about 60% of the cases. *HGF*, *MET* and *KRAS* mRNA relative expression was increased in IM (48.6%, 37.8% and 26.7% of the cases) and GC (27.3%, 38.1% and 28.6% of the cases), respectively, as follow: 2.6- and 1.8-fold, 2.8- and 3.5- fold and 1.1- and 1.7-fold. mRNA expression for *TFF1* and *CLDN18* was decreased in 54% of IM cases (but the expression mean levels was 1.8- and 1.6-fold higher than normal, respectively) and 68% of GC cases (1.1 and 1.7-fold lower than normal tissue, respectively). Figure 1 shows mean levels of expression for *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in IM and GC groups. The statistical analysis between the groups showed a significant difference between the mRNA expression mean levels only for *TFF1* and *CLDN18* ($P=0.006$, Table 3).

The correlation between mRNA expression levels of genes that participate in the same signal pathway was evaluated. To the IM group, we found that *KRAS* expression was correlated to *HGF* and *MET* expression ($r=0.53$, $P=0.0001$) and it has been verified that such correlation was stronger between *KRAS* and *HGF* ($r=0.70$, $P=0.0001$). A significant correlation between mRNA expression levels of *NOS2* and *HGF* was found in GC ($r=0.80$, $P<0.0001$).

Immunostaining for NOS2, KRAS, TFF1 and CLDN18 proteins in intestinal metaplasia (IM) and gastric cancer (GC).

Immunostaining-positive (ranging from weak to strong) for NOS2, KRAS, TFF1 and CLDN18 proteins were found in 92.6%, 89.3%, 85.2% and 11.5% of the IM cases and 30%, 100%, 50% and 60% of the GC cases (Table 4). To IM, cytoplasmatic staining for NOS2 protein was only found in the metaplastic region, while the normal foveolar epithelium had no staining. In GC, NOS2 showed positive immunostaining in cancer cells and glandular epithelium, but, in general, the foveolar epithelium had no staining for NOS2 (Figure 2. A.1, B.1 and C.1). KRAS protein exhibited a focal staining in the cytoplasm of the normal gastric epithelium of IM and GC cases, with a weak staining in metaplastic areas (89.3% of cases) and moderate or strong staining in cancer areas (70% of cases) (Figure 2. A.2, B.2 and C.2). Regarding TFF1 protein, all the normal areas in IM and GC showed moderate to strong cytoplasmatic immunostaining-positive status while 62.9% and 100% of the areas with IM and GC cells, respectively, showed absent or weak positive staining in relation with normal areas (Figure 2. A.3, B.3 and C.3). The membrane of the normal epithelium showed strong staining for CLDN18 protein in all the cases of IM and GC, but in metaplastic and cancer cells CLDN18 staining was absent or weaker (100% and 90%, respectively), than that observed in the normal adjacent mucosa (Figure 2. A.4, B.4 and C.4). Scores ≤ 1 (absent and weak staining) and ≥ 2 (moderate and strong staining) were used to compare protein expression between the IM and GC groups (Table 4), and a significant difference was found for NOS2, KRAS and TFF1 proteins ($P < 0.05$).

Expression of NOS2, HGF, MET, KRAS, TFF1 and CLDN18 and variables such as smoking, drinking, H. pylori infection and histological type.

In GC group, there was a significant association between *NOS2* and *HGF* expression and *H. pylori* infection, with decreased mRNA mean levels in patients *H. pylori*-positive (2.29 vs. 28.8, $P=0.0203$, and 0.93 vs. 2.96, $P=0.0430$, respectively). While increased *CLDN18* mRNA level were associated with *H. pylori*-positive cases of the GC group (0.90 vs. 0.28, $P=0.0435$). To IM group, *TFF1* expression and smoking was associated with decreased mRNA levels in smokers (1.08 vs. 3.92, $P=0.0453$). *KRAS* and *MET* were not associated with any variable studied. Comparisons between mRNA relative expression of evaluated genes and histological parameters as incomplete and complete metaplasia and gastric cancer type (intestinal vs. diffuse) did not show any association. Likewise, analysis between protein expression and variables did not produce significant data.

Discussion

We analyzed the mRNA and protein expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in IM in comparison to GC and adjacent normal mucosal. Our results indicated increased mRNA relative expression for genes *NOS2*, *HGF*, *MET* and *KRAS*, with mRNA mean levels among 1.15 to 13.62, in both IM and GC groups, and decreased means levels of mRNA for genes *TFF1* and *CLDN18* (0.97 and 0.60, respectively) in GC, but increased in IM (1.84 and 1.64, respectively). In addition, we did not observe precise relationship between mRNA and protein expression for most of the evaluated genes.

This divergence between mRNA and protein expression might be explained by the fact that mRNA changes are more diverse than stable. In proteins, changes are slower,

such as in as post-transcriptional regulation, translational control and protein stability. However, levels of mRNA and protein are not always correlated [19].

NOS2 gene (nitric oxide synthase 2), also known as *iNOS* (inducible oxide nitric synthase), encodes an enzyme responsible for producing nitric oxide (NO), induced by bacterial endotoxins or cytokines mainly in leukocytes and epithelial cells, resulting in high concentrations of NO [20]. NO contributes to mucosal defense through its cytotoxic properties, against ingested bacteria and parasites [21], for example, as part of the host response to the *H. pylori* infection. However, in circumstances in which the mucosa is inflamed or damaged, some studies suggest that NO contributes to tissue injury and it can lead to cell cycle disorders, cell death caused by apoptosis, and DNA damage [22, 23]. In the current study, high levels of *NOS2* mRNA expression (about 13-fold) observed in about 60% of cases of both IM and GC might contribute to genetic changes associated with gastric carcinogenesis. Our findings are similar to other studies that also observed *NOS2* protein expression in 13% cases of IM [24]. To GC, high levels of *NOS2* mRNA expression also was corroborated by some previous studies [25-28], and *NOS2* protein expression was related to tumor angiogenesis, tumor progression, and patient survival [29]. Therefore, *NOS2* expression can participate in the initial events of the gastric carcinogenesis through tissue injuries and genetic changes, as observed in pre-cancerous lesions.

HGF, *MET* and *KRAS* genes share the same signaling pathway resulting in proliferation, angiogenesis, migration and morphogenesis. Both *MET* and *HGF* are often up-regulated in gastric cancer [15], while *KRAS* is activated by mutation [30, 31]. Hepatocyte Growth Factor (HGF) acts as a potent mitogen and pro-motility agent for epithelial cells. HGF is produced for the mesenchymal cells to act on its receptor, MET,

in the epithelial cells in a paracrine fashion. Together, they have key roles in gastrointestinal development, mucosal healing and epithelial cell migration [32].

There is accumulating evidence that the transformation of the normal gastric mucosal cells into malignant cells and gastric cancer may be modulated by transforming growth factors including *HGF* and their receptor, *MET*. We find 2.6-fold up-regulation of *HGF* in IM (48.6% of cases), while in GC tissues (27.3%), there was about 2-fold high expression of *HGF*. *MET* mRNA was up-regulated in about 38% of both IM and GC cases, with 2.8- and 3.5-fold higher than adjacent normal gastric mucosa, respectively. These data could not be verified by immunohistochemistry because the antibody used did not produce satisfactory results.

Our findings are similar to Konturek et al. [33] who observed a 2-fold increase in the mRNA expression of *HGF* (52% of the cases) and *TGF- α* in gastric cancer tissue compared to adjacent mucosa by densitometric analysis. Inoue et al. [34] verified higher expression of MET protein in tissues of gastric adenocarcinoma than in normal mucosa. These data provide evidence that up-regulation of *HGF* and *MET* is a frequently occurring event in gastric carcinogenesis and it was in accordance with previous studies showing an increased expression and higher serum levels of these factors in gastric cancer [35, 36]. However, little is known about HGF and MET expression in intestinal metaplasia. Suzuki et al. [37] observed a slight decrease in HGF content after eradication therapy against *H. pylori* in patients with upper gastrointestinal disease through ELISA method. However, the level of antral HGF was significantly decreased in patients with intestinal metaplasia, but not in those without it. Thus, *HGF* and *MET* up-regulation can be important to early steps of gastric carcinogenesis through promotion of cellular proliferation, as observed in IM, considered a pre-malignant stomach lesion.

On the *KRAS* expression, we did not observe an important increase in mRNA relative expression levels (about 1.15- and 1.70-fold) in IM and GC groups, where only about 28% of cases had up-regulation. However, we found a significant proportion of positive cases for KRAS protein in both groups (89.3% for IM and 100% for GC), with a weak staining in IM and a moderate or strong staining in 70% of GC-positive cases. Once again, evidencing that there is no exact relationship between mRNA and protein expression.

The *KRAS* gene encodes a guanosine triphosphatase protein (p21^{ras}) that functions as a “molecular switch”, transmitting signals from the extracellular to intracellular environmental, mediating signaling events that regulates cell proliferation, cell survival, angiogenesis and cell migration [38]. Our findings suggest that *KRAS* expression is not significantly altered in IM. But, in GC, the high levels of protein expression suggest an important role of the gastric carcinogenesis. KRAS protein can be activated by mutations, leading to increased cell growth. However, in gastric adenocarcinoma, the incidence of *KRAS* mutation seems to vary among populations, with rates of about 10%, possibly related to *H. pylori* infection [30, 31]. In addition, KRAS protein can also be activated by gene amplification in gastric cancer. So, immunohistochemistry has shown that this protein is preferentially expressed in gastric cells with *KRAS* amplification [39]. Moreover, increased expression of KRAS protein has been associated with progression of gastric carcinogenesis, lymph nodes metastases and poor prognosis [40].

Trefoil peptide 1 (*TFF1* or *pS2*) located in gastric mucosa cells interacts directly with mucins to stabilize the mucous layer, protecting the epithelium against injury and maintaining the integrity of the mucosal barrier. Furthermore, *TFF1*, known as tumor suppressor gene has an important role in regulating the balance between gastrointestinal

cell proliferation, death and differentiation [41, 42]. Some studies have reported a loss of TFF1 protein expression in 40 to 60% of gastric cancer cases [11, 41, 43]. Our results are in accordance with these reports because we found a decrease or loss of TFF1 protein expression in 100% of GC areas and 62.9% of IM compared to normal epithelium. Similarly, the *TFF1* mRNA expression levels were reduced in 54% of cases of IM, with an expression mean level of 1.84, but with a significantly lower mean level in GC (68%, mean=0.96) compared to IM.

Fujimoto et al. [44] demonstrated that reduced expression of TFF1 at mRNA and protein levels, by hypermethylation in the promoter region, frequently occurred in adenomas, intestinal metaplasia and gastric adenocarcinomas. Moreover, Kim et al. [45] reported TFF1 protein was expressed in IM, although the immunoreactivity was weaker than in the adjacent normal gastric epithelium, as observed in the present study. Then, progressive loss of TFF1 expression from normal gastric mucosa into intestinal metaplasia may increase susceptibility to carcinogens and finally progression to gastric cancer. In addition, we observed that the *TFF1* mRNA expression levels were lower in smokers than in nonsmokers in IM group, suggesting that substances of the tobacco can decrease TFF1 expression levels in the gastric mucosa, and consequently change their protective role. We did not find literature data about this interaction in gastric tissues, but in breast cancer cells high TFF1 expression was reported to be significant (2-fold), induced by tobacco smoke condensate [46]. This discrepancy might be explained by the fact that, in the stomach, *TFF1* acts as a tumor-suppressor gene, while in breast tissue, it acts as an oncogene. So, it may hypothesized that the carcinogenic properties of the several substances of the tobacco can modulate TFF1 expression, contributing to DNA damage and cell transformation, and increased malignant risk.

Claudins (CLDNs) are a large family of proteins which interact with one another to form a branching network that helps create the tight junctions of epithelial cells [47]. CLDN18 protein is normally detected in the normal gastric mucosa and plays an important role in various biophysiological processes, such as regulation of paracellular permeability and conductance. Disruption of tight junctions can cause loss of cell polarity, resulting in an abnormal influx of growth factors, which could provide auto and paracrine stimulation to tumourigenic epithelial cell [16, 48]. Our results showed down-regulation at *CLDN18* mRNA in 68% of GC cases and 54% of IM cases, but with significantly reduced mRNA mean levels ($P=0.0065$) in GC group (0.605) in comparison to IM group (1.639). Similarly, a decrease or loss of CLDN18 protein expression was observed in 90% of GC cases and 100% of IM cases when compared to adjacent normal mucosa.

Since claudin-18 is expressed only in normal stomach, dysfunction of tight junctions caused by down-regulation of *CLDN18* mRNA may lead to an abnormal influx of stomach-related growth factors, which can promote an increase of cell proliferation and increased risk of gastric cancer. Similarly to our results, Sanada et al. [16] showed that *CLDN18* mRNA expression was down-regulated in 56.5% of GC and no expression of CLDN18 protein was observed in 57% of the cases of gastric cancer associated with poor survival, as in 90% of gastric adenoma and some of the intestinal metaplasia. According to these authors, down-regulation of *CLDN18* may be an early event in gastric carcinogenesis. Moreover, Matsuda et al. [48] found no expression of CLDN18 protein in IM and GC, suggesting a close correlation with carcinoma progression and subsequent metastatic events. However, in other cancer types such as pancreatic, *CLDN18* is frequently up-regulated [47].

Literature data show an intrinsic network that includes NOS2, HGF, MET, and KRAS proteins. In IM, we found a significant correlation between *KRAS* and *HGF/MET* mRNA expression levels, and, yet, a stronger correlation between *KRAS* and *HGF* ($r=0.70$). HGF action can be started through its binding to MET receptor that activates several intracellular messengers. One of them is KRAS that has been shown to mediate the HGF-induced migration of gastric cells and promotion of cell proliferation [49, 50]. In GC, it was observed a correlation between *NOS2* and *HGF* expression. HGF/MET binding activates KRAS expression and, on the other hand, KRAS seems to activate AP-1 (Activator Protein-1), via ERK (extracellular signal-regulated kinase) signaling pathway that induces the activation of NOS2 and COX-2 expression, increasing inflammation, apoptosis and oxidative damage in gastric cells [51]. So, these proteins can interact in order to increase cellular proliferation and damage the gastric cells, leading to key changes in cell homeostasis, contributing to the initial steps of carcinogenesis, as in premalignant lesions.

H. pylori infection is frequently associated with gastric carcinogenesis. So, we investigated the relationship between *H. pylori* infection and mRNA expression levels of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in the IM and GC groups. We found that in the GC group *H. pylori*-positive individuals had lower mRNA expression levels for *NOS2* and *HGF* and higher expression levels for *CLDN18* than that *H. pylori*-negative.

Concerning *NOS2*, the data were discordant with NO role in the mucosal defense through its cytotoxic properties, acting as primary defense against bacterium infection [22]. Wang et al. [27] found higher levels of NO and NOS expression in the *H. pylori*-positive group when compared to negative group. However, Augusto et al. [28] did not find association between *NOS2* expression and virulence factor of *H. pylori*. There is

evidence that generation of polyamines by ornithine decarboxylase (ODC) and also induced by *H. pylori*, results in inhibition of *NOS2* [52, 53]. These biochemical pathways that limit NO production may exist to protect macrophages from potential toxic effects of overproduction of NO in response to other pathogens, but in the case of *H. pylori*, the bacterium appears to be reaping the benefits of these host responses [53]. When it comes to the relationship between HGF expression and *H. pylori* infection, some studies found an increased expression of HGF in gastric mucosa colonized by *H. pylori*, suggesting a possible link between this factor and *H. pylori*-associated gastritis and gastric cancer [37, 54]. Moreover, CagA virulence factor of *H. pylori*, have been shown to interact with *HGF* receptor, *MET*, thereby stimulating cell growth, motility and invasiveness [55]. However, the results are controversial, because Konturek et al. [33] did not find significant correlation between CagA status and genetic alterations concerning gene and protein expression for growth factors. Moreover, similar gene changes were also observed in patients without *H. pylori* infection, suggesting that overexpression of *HGF* is not necessarily affected by the *H. pylori* status. In addition, a recent study has shown that *H. pylori* induces AGS gastric adenocarcinoma cells motility independently of *MET* expression [56]. Thus, to clarify these divergent results, more studies of *H. pylori* interaction with growth factors are necessary. In the present study, we should also consider the small number of cases evaluated in GC group.

To our knowledge, there are no reports about *H. pylori* infection and CLDN18 expression. However, it is known that *H. pylori* recruits and modifies the distribution of zonula occludens-1 (ZO-1) and junctional adhesion molecule (JAM), which are normally located at cell tight junctions. CagA alters the structure and function of the apical-junctional complex may result in loss of control over cytoskeletal architecture, cell polarity, proliferation and differentiation, which are characteristics of oncogenic

transformation [55]. As *CLDN18* participate of the tight junctions network, the higher levels of mRNA expression in *H. pylori*- positive observed in this study, can suggest an interaction between bacterium infection and *CLDN18* in GC group. However, further studies about role of *H. pylori* infection on *CLDN18* expression are required to explain this question.

Our data did not show any association regarding other comparisons between mRNA and protein of genes evaluated and parameters as age, gender, smoking, alcohol drinking, histological type of metaplasia (complete vs. incomplete) and histological type of gastric cancer (intestinal vs. diffuse).

Conclusion

In conclusion, our findings on deregulated expression in important genes related to cycle cellular and maintenance of gastric mucosa integrity in intestinal metaplasia, mainly *NOS2*, *HGF* and *MET*, suggest that this preneoplastic lesion share changes of gene expression similar to the GC, which may have a key role both to the development of this lesion as to the initial steps of gastric carcinogenesis. So, these genetic changes along with others may bring greater risk of malignancy. However, further studies are needed in premalignant gastric lesions to assess deregulation of different genes that lead to progression of gastric cancer.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MCD implemented the experimental design, carried out the assays and drafted the manuscript. MTMF performed the pathological assessment of gastric specimens and

helps in the immunohistochemical analysis. KM was responsible for endoscope exam and collection of the gastric biopsies. AES was responsible for study conception, design and revision of manuscript draft for intellectual content. All authors have read and approved the final version of the manuscript.

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Table 1. Primers sequences for Real Time PCR.

Gene	Primers Sequences	Size (pb)
<i>NOS2</i>	F 5' CTTCAACCATAAGGCCAAAGG 3' R 5' AGCTCATCTGGAGGGGTAGG 3'	119
<i>HGF</i>	F 5' TGGCCATGAATTTGACCTC 3' R 5' CCAGGGCTGACATTTGATG 3'	121
<i>MET</i>	F 5' GCAAGCAAAAAGTTTGTCCAC 3' R 5' TGGCAAGACCAAAATCAGC 3'	94
<i>KRAS</i>	F 5' AGAGTGCCTTGACGATACAGC 3' R 5' TCCCTCATTGCACTGTACTCC 3'	160
<i>TFF1</i>	F 5' GGCCAGACAGAGACGTG 3' R 5' ACAGCAGCCCTTATTTGCAC 3'	97
<i>CLDN18</i>	F 5' GATCGTAGGCATCGTCCTGG 3' R 5' GGATGCATTTTCAGGGCAAAG 3'	65
<i>β-Actin</i>	F 5' TGCCCTGAGGCACTCTTC 3' R 5' CGGATGTCCACGTCACAC 3'	101

Table 2. Clinicopathologic characteristics of intestinal metaplasia (IM) and gastric cancer (GC) groups.

Variables		IM (n=37)	GC (n=22)
Gender	Male	18 (48.6 %)	17 (77.3 %)
	Female	19 (51.4 %)	5 (22.7 %)
Age (years)	<60	16 (43.2%)	9 (40.9%)
	≥ 60	21 (56.8%)	13 (59.1%)
	Mean±SD	61.05±12.65	62.45±14.07
	Range	38 to 80	38 to 86
Smoking	Yes ¹	26 (72.2 %)	10 (62.5 %)
	No	10 (27.8 %)	6 (37.5 %)
Drinking	Yes ²	10 (27.8 %)	10 (62.5 %)
	No	26 (72.2 %)	6 (37.5 %)
Histology		CIM = 32 (86.5%) IIM= 5 (13.5%)	I = 11 (57.9%) D = 8 (42.1%)
<i>H. pylori</i> infection³	Positive	14 (37.8 %)	11 (52.4 %)
	Negative	23 (62.2%)	10 (47.6%)

¹Smokers and past smokers. ²Drinkers and past drinkers. ³Molecular diagnosis. CIM= complete IM. IIM= incomplete IM. I = Intestinal gastric cancer type. D= Diffuse gastric cancer type (Lauren classification).

Table 3. Mean level of mRNA expression of the *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in intestinal metaplasia (IM) and gastric cancer (GC) groups.

Gene		IM	GC	P value IM x GC
<i>NOS2</i>	Up regulated cases	22/36 (61.1%)	13/22 (59.1%)	0.3657
	mean±SD	13.621±21.886	12.792±29.780	
	Range	0.0008 to 78.339	0.005 to 127.410	
<i>HGF</i>	Up regulated cases	18/37 (48.6%)	6/22 (27.3%)	0.0806
	mean±SD	2.602±3.243	1.824±3.075	
	Range	0.001 to 12.042	0.055 to 13.485	
<i>MET</i>	Up regulated cases	14/37 (37.8%)	8/21 (38.1%)	0.3109
	mean±SD	2.832±6.760	3.504±7.573	
	Range	0.000 to 38.187	0.001 to 30.626	
<i>KRAS</i>	Up regulated cases	10/37 (26.7%)	6/21 (28.6%)	0.9047
	mean±SD	1.146±0.996	1.692±2.804	
	Range	0.0007 to 4.223	0.120 to 12.641	
<i>TFF1</i>	Down regulated cases	20/37 (54%)	15/22 (68.2%)	0.0060*
	mean±SD	1.842±3.350	0.968±2.047	
	Range	0.0003 to 16.223	0.0001 to 8.378	
<i>CLDN18</i>	Down regulated cases	20/37 (54%)	15/22 (68.2%)	0.0065*
	mean±SD	1.639±2.053	0.605±0.867	
	Range	0.002 to 8.861	0.001 to 2.627	

Mann-Whitney *U*-test for comparing mRNA expression levels between IM and CG groups. * statistically significant difference ($P<0.05$).

Table 4. NOS2, KRAS, TFF1 and CLDN18 protein expression in intestinal metaplasia (IM) and gastric cancer (GC) groups.

Protein	Staining	IM		GC		P value	
		N (%)	Score ≤ 1 ≥ 2	N (%)	Score ≤ 1 ≥ 2	IM x GC	
NOS2	-	2 (7.4)		7 (70)			
	+	25 (92.6)	NA NA	3 (30)	NA NA	0.0003*	
KRAS	-	3 (10.7)		0 (0)			
	+	25 (89.3)	28 0	3 (30)	3 7	0.0001*	
	++/+++	0 (0)		7 (70)			
TFF1	-	4 (14.8)		5 (50)			
	+	13 (48.1)	17 10	5 (50)	10 0	0.0359*	
	++/+++	10 (37.1)		0 (0)			
CLDN18	-	23 (88.5)		4(40)			
	+	3 (11.5)	26 0	5 (50)	10 1	0.2778	
	++/+++	0 (0)		1 (10)			

Fisher's exact test for comparing protein expression between the groups. (-) absent; (+) weak; (++)/ (++++) moderate/strong staining; NA: no applicable. Statistical Analysis: * = statistically significant difference ($P<0.05$).

Legend to figures.

Figure 1. The mRNA expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in intestinal metaplasia (IM) and gastric cancer (GC) by Real-time PCR. Data are expressed as mean $\pm$ SD. Mann-Whitney *U*-test for comparing mRNA expression levels between IM and CG groups. * statistically significant difference ($P < 0.05$).

Figure 2. Immunohistochemistry analysis. A: normal gastric mucosa (N); B: intestinal metaplasia (IM); C: gastric cancer (GC). 1- NOS-2 brown cytoplasmatic staining. A.1: absent staining in the foveolar epithelium (white arrow), weak in the glandular epithelium (black arrow); B.1: strong staining; C.1: strong staining. 2- k-RAS brown cytoplasmatic staining. A.2: absent staining in the foveolar epithelium (white arrow), weak in the glandular epithelium (black arrow); B.2: absent staining; C: strong staining. 3- TFF1 brown cytoplasmatic staining. A.3: strong staining; B.3: weak staining in IM (black arrow), strong in N (white arrow); C.3: absent staining in GC (black arrow), strong in N (white arrow). 4- CLDN18 brown membrane staining. A.4: strong staining; B: weak staining in IM (black arrow), strong in N (white arrow); C: absent staining in GC (black arrow), strong in N (white arrow). Magnification: 100x.

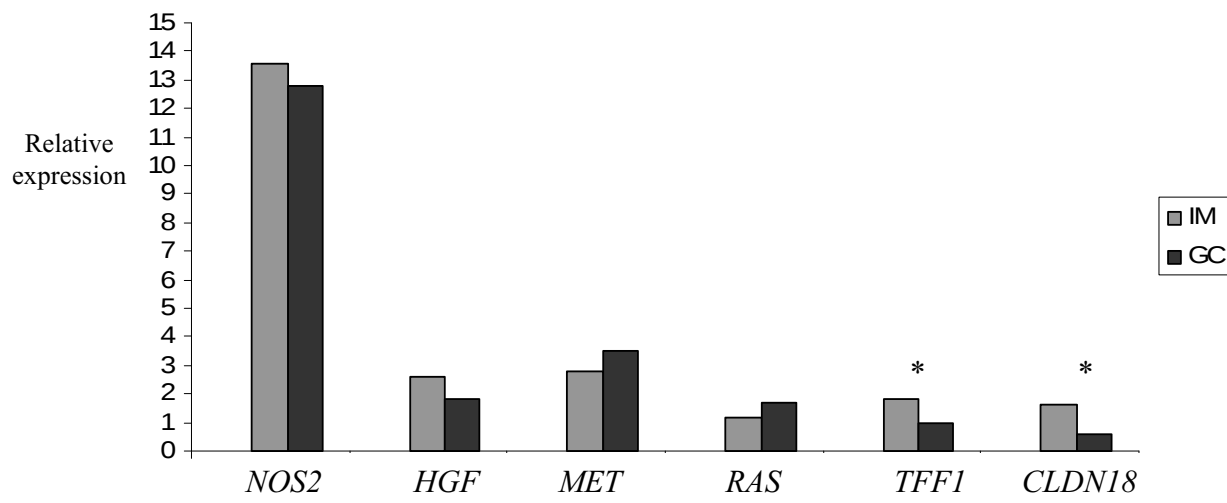


Figure 1.

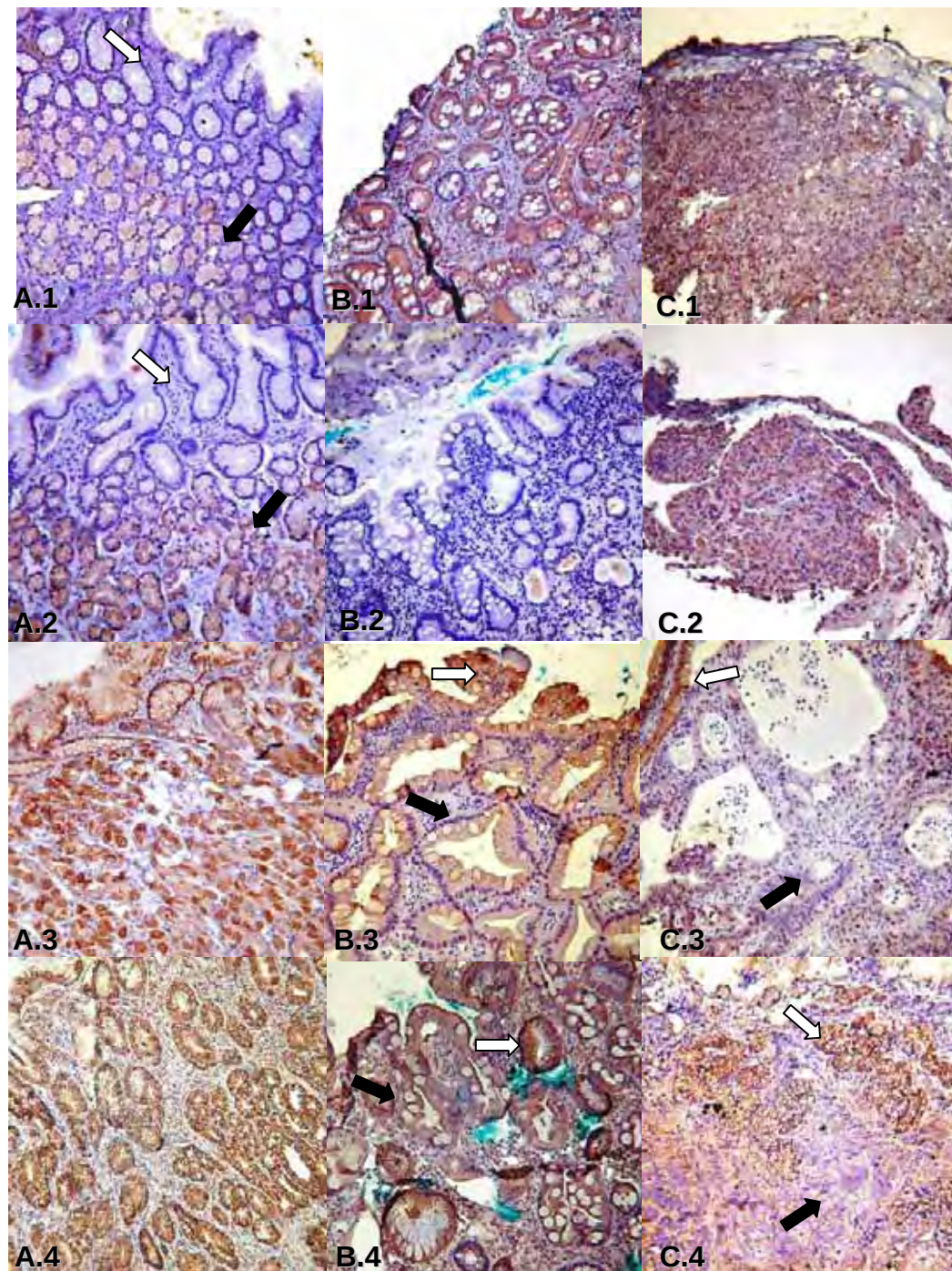
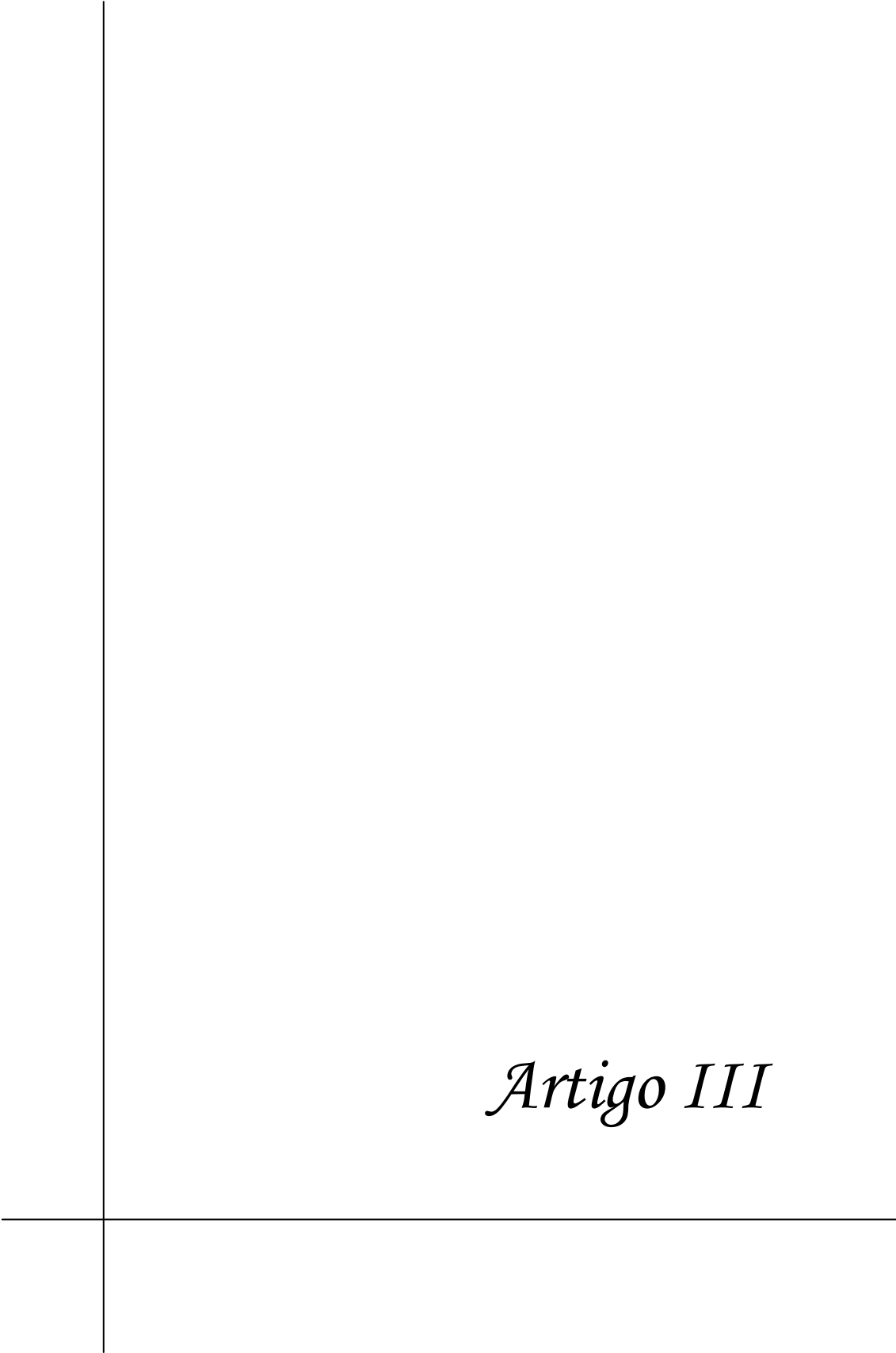


Figure 2.



Artigo III

Artigo III: *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* expression in gastric ulcer and gastric cancer.

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Abstract

Background: Gastric ulcer (GU) has been pointed as a pre-neoplastic condition of the stomach cancer. However, there are few studies regarding gene expression changes in this lesion, as well as its relationship to gastric carcinogenesis. We examined mRNA and protein expressions of important genes involved in cell mechanisms and gastric mucosa protection in GU and gastric cancer (GC).

Methods: Gastric mucosal biopsies specimens were taken from 30 patients with GU and 22 with GC and their respective normal mucosa. mRNA was extracted and used in Real Time PCR assay to evaluate mRNA expression for *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18*. Immunohistochemistry was performed in order to evaluate the staining of these proteins in the respective tissues.

Results: Up-regulation was found respectively in *NOS2*, *HGF*, *MET* and *KRAS* in 43.3%, 26.7%, 17.2% and 26.7% of GU cases (4.4-, 1.05-, 2.8- and 2.6-fold, respectively). Concerning GC, the same genes were up-regulated about 12.8-, 1.8-, 3.5- and 1.7-fold in 59.1%, 27.3%, 38.1% and 28.6% of the cases, respectively. *TFF1* and *CLDN18* were down-regulated in 73.3% and 58.6% of GU (mRNA mean levels of 0.53 and 0.93, respectively) and 68.2% of GC cases (mRNA mean levels of 0.97 and 0.60, respectively). Despite the absence of an exact relationship between mRNA and protein expression, in general, immunohistochemistry in *NOS2*, *KRAS*, *TFF1* and *CLDN18* proteins evidenced positive staining (weak to strong) in GU and GC specimens.

Conclusions: These results suggest that ulcer and gastric cancer share changes in the expression of key genes that participate in important cell mechanisms, which can both be involved in the process of ulcer healing and also act in the initiation and progression of gastric carcinogenesis.

Introduction

Gastric cancer development is a multistep process that involves *H. pylori* infection, chronic gastritis, atrophic gastritis, intestinal metaplasia and dysplasia¹. However, the theory that gastric carcinoma arises within the site of a benign peptic ulcer has been increasing in the past decades². Studies have verified a coexisting gastric cancer in two percent of patients with a diagnosis of gastric ulcers³. Subsequently, it was estimated that gastric ulcer patients had a 1.8 times higher risk of developing gastric carcinoma, which was attributed to presence of atrophic gastritis due to *H. pylori* as a common primary factor to both gastric cancer and gastric ulcer patients⁴. Moreover, the surgery performed to reduce the gastric acid secretion in patients with gastric ulcer history, did not decrease the gastric cancer risk⁵.

Gastroduodenal peptic ulcer is characterized by circumscribed loss of tissue that occurs in portions of the digestive tract exposed to chlorohydro-peptic secretion, occurring in 5 to 10% of population. Among the etiological factors, those who break the balance between aggressive factors (acid and pepsin) and protective factors from gastric mucosa barrier, stand out acetylsalicylic acid, biliary acids, nonsteroidal anti-inflammatories, corticosteroids, alcohol and tobacco⁶. Nevertheless, the most important environmental factor is *H. pylori* infection, affecting over 90% adults with duodenal ulcer and 70% to 90% of those with gastric ulcer⁷.

To date, very little is known about genetic alterations and gene expression changes in gastric ulcer. In a recent report of our group, trisomies of the chromosomes 7 and 17 in patients with gastric ulcer have been detected, mainly in the positive *H. pylori* cases, besides immunostaining to p53⁸. Furthermore, it is well known that, during the process of ulcer healing, complex biological responses occur, including cell proliferation, migration, differentiation, regeneration, active angiogenesis, and

extracellular matrix deposition^{9,10}, many of them also participating in the carcinogenic process. In addition, complex interaction of many proteins at the injury site such as growth factors (EGF, TGF- α , TGF- β , FGF and HGF/MET), peptides trefoil (TFF), COX-2, among others are also required^{9,10}. Thus, several studies have reported increased expression of the proteins CGRP¹¹, TFF1 and TFF2^{12,13}, and of the genes *HGF*¹⁴, *TGF- β* and *TGF- β R2*¹⁵ and *COX-2*¹⁶ in the mucosa of patients with gastric ulcer in healing.

Taking into account the potential of gastric ulcer as a precancerous lesion of the stomach, and that gene expression deregulation occurs in initial steps of carcinogenesis, genetic studies about important genes involved in stomach carcinogenesis in patients with gastric ulcer can be useful to understand if these lesions have genetic changes in common. In the presence of these facts, we analyzed the expression levels of the mRNA and protein of key genes deregulated in the gastric cancer, such as *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18*, in patients with gastric ulcer in comparison with normal gastric mucosa and patients with gastric cancer and their potential relation with clinicopathological variables such as *H. pylori* infection.

Material and Methods

Samples

We analyzed samples for 30 patients with gastric ulcer and 22 with gastric adenocarcinoma, who had undergone upper endoscopy from March 2006 to March 2008 at the Base Hospital, São José do Rio Preto, SP, Brazil. Three biopsies of lesion region and other three biopsies of apparently normal region were collected of each patient, mainly in the antrum and corpus of the stomach. Immediately after this procedure, they were put in RNA-later reagent (Ambion) and stored at 4°C for one day,

and then, at -20°C until RNA and DNA extraction. Archival paraffin-embedded, formalin-fixed tissues were used for immunohistochemical staining of the proteins. Information about age, gender, smoking and drinking habits and the written informed consent was obtained from all patients, and the study was approved by the National Research Ethics Committee (CONEP).

The gastric ulcer group (GU) was composed of 22 men and 8 women, with a mean age of 54.90 ± 12.37 years (range 30 to 75 years), mainly smokers (64.3%) and non drinking (60.7%), while the gastric cancer group (GC) was composed of 17 men and 5 women, with a mean age of 62.45 ± 42.07 years (range 38 to 86 years), which the majority (62.5%) was smokers and alcoholics. All specimens were histopathologically diagnosed. Gastric adenocarcinoma was classified as intestinal (57.9%) and diffuse (42.1%), according to the Laurén classification¹⁷.

RNA and DNA extraction and reverse transcription

Total cellular RNA and DNA were extracted from gastric biopsies using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA and DNA concentration was determined in a NanoDrop[®] ND1000 spectrophotometer, and RNA integrity was visualized on 1% agarose gel with 2 fragments of 18S and 28S rRNA. DNA samples were maintained in freezers at -20°C and used for molecular diagnosis of *H. pylori*. RNA samples were stored in freezers at -80°C and used for reverse transcription. cDNA was synthesized from 5 μg of total RNA using random primers and High Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. The integrity of all cDNA preparations was tested by PCR assay of the *β -actin* gene. Briefly, for total volume of 20 μL , have been used 1 μL of cDNA, 1.5mM MgCl_2 , 0.2mM dNTPs, 0.5 μM each *primer* (F-5'GAGGCACTCTTCCAGCCTTC3' and R-5'GTTGGCGTACAGGTCTTGC3') and

1.5 U of Taq DNA polymerase in buffer 1 X. The cycling thermal conditions were as follow: a initial step at 94°C for 3 minutes, follow by 35 amplification cycles at 94 °C for 45 seconds, 61°C for 30 seconds and 72 °C for 1 minute and a final extension at 72°C for 10 minutes. A 114 bp band was visualized on 2% agarose gel stained with ethidium bromide.

Quantitative Real Time PCR

The expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* mRNA was measured by real time PCR, based on the SYBR Green methodology, using an ABI Prism[®] 7300 (Applied Biosystems). *β-actin* gene was used as reference. The primer sequences used in the study were obtained using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with exception of *CLDN18* sequence, obtained of Sanada et al¹⁸. The primer sequences were: *NOS2* F-5'CTTCACCATAAGGCCAAAGG3' and R-5'AGCTCATCTGGAGGGGTAGG3', *HGF* F-5'TGGCCATGAATTTGACCTC3' and R 5'CCAGGGCTGACATTTGATG3', *MET* F-5'GCAAGCAAAAAGTTTGTCCAC3' and R-5'TGGCAAGACCAAAA TCAGC3', *KRAS* F-5'AGAGTGCCTTGACGATACAGC3' and R-5'TCCCTCATTGCACTGTACTCC3', *TFF1* F-5'GGCCCAGACAGAGACGTG3' and R-5'ACAGCAGCCCTTATTTGCAC3', *CLDN18* F-5'GATCGTAGGCATCGTC CTGG3' and R-5'GGATGCATTTTCAGGGCAAAG 3'and *β-actin* F-5'TGCCCT GAGGCACTCTTC3' and R-5'CGGATGTCCACGTCACAC3'.

An initial validation step with 10-fold dilution series was performed to obtain the amplification efficiencies of the primers (*E*), calculated according to the equation $E = 10^{[-1/\text{slope}]}$. The efficiencies of amplification ranged between 90% and 97%. After the validation step, the real time PCR assays were performed in 10 µL of SYBR[™] Green Master Mix (Applied Biosystems), 25 ng of cDNA and 0.6 µM of *β-actin*, *TFF1* and

CLDN18 primers, 0.7 μ M of *KRAS* primer and 0.8 μ M of *NOS2*, *HGF* and *MET* primers. Thermal cycling conditions for all the genes were: 2 min at 50°C and 10 min at 95°C for initial denaturation, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, and a dissociation step at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Triplicates of the samples were assayed in each run. β -actin, α -tubulin and β 2-microglobulin housekeeping genes were evaluated to case and control samples, to verify which contained the lowest variation of amplification between the samples. After this, β -actin gene was used as reference because it showed the lowest variation. The relative expressions of the genes were analyzed using the $2^{-\Delta\Delta C_t}$ method, according to the description by Livak and Schmittgen¹⁹. First, the mean of cycle number at the threshold level of fluorescence (C_t) for each sample was determined. Next, the ΔC_t value was calculated as being the difference between the C_t value of target gene and the C_t value of β -actin: $\Delta C_t = C_t$ (target gene) - C_t (β -actin). Finally, the $\Delta\Delta C_t$ value and the normalized target gene expression were calculated: $\Delta\Delta C_t = \Delta C_t$ (ulcer or cancer tissues) - ΔC_t (normal adjacent mucosa). Normalized expression in a sample = $2^{-\Delta\Delta C_t}$.

PCR for H. pylori diagnosis

DNA samples extracted from the adjacent normal gastric mucosa were used for molecular diagnosis of the *H. pylori* bacterium, because it is a more efficient method than histology and urease test²⁰. The bacterium genes *urease* and *tsaA* were amplified by Multiplex PCR, using the specific primers F- 5' TTCCTGATGGGACCAAACCTC 3' and R- 5' TTACCGCCAATGTCAATCAA 3', and (F- 5' CCTGCCGTTTTAGGAAACAA 3' and R- 5' TCCGCATTCCTACCTAATGG 3') in order to amplify, respectively, segments of 316 pb and 413 pb. The housekeeping human gene *CYP11A1* (F- 5' CTCACCCCTGATGGTGCTAT 3' and R- 5' TTTGGAAGTGCTCACAGCAG 3') that amplifies a 226 pb segment was used as

quality control DNA. Positive and negative controls were used in all experiments. The PCR assay solution consisted of 4 mM MgCl₂, 0.3 mM dNTPs, 0.6 μM of each primer, and 1.75 U Taq DNA polymerase in 1 X buffer. The thermal cycling conditions were: 5 min at 95°C, followed by 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were separated on 2% 1000 agarose gel (Invitrogen) and stained with ethidium bromide. The assay was considered positive when at least one of the bacterial PCR products was present.

Immunohistochemical Analysis

Four-micron-thick tissue sections were heat-fixed on glass slides at 37°C overnight. Slides were then rehydrated through sequential solutions of xylene for 5 min (4X), four baths in 100% ethanol (3X), 95 % ethanol (1X), 80% ethanol (1X), 50% ethanol (1X) and distilled water (2X). Freshly prepared 10mM EDTA-TRIS buffer (pH 9.0) for KRAS and 10mM Citrate buffer (pH 6.0) for NOS2, HGF, MET, TFF1 and CLDN18 were pre-heated for 40 min in a stem-pan (Exotic, Arno) until 95°C and then, slides were put in the buffer for 30 min for antigen retrieval. After cooled for 20 min at ambient temperature, the endogenous peroxidase activity was blocked with 3% H₂O₂ treatment for 10 minutes. Then, the sections were incubated for 1 hour at room temperature with specific antibody for TFF1 (mouse monoclonal anti-pS2, clone BC04, Invitrogen, 1:50), HGF (Monoclonal anti hepatocyte growth factor antibody, clone 24612.111, Sigma, 1:50) and MET (Anti hepatocyte growth factor receptor -cMet antibody, Sigma, 1:50) and overnight at 4°C with specific antibodies for NOS2 (Rabbit polyclonal to iNOS, Abcam, 1:200), KRAS (Mouse monoclonal anti-KRAS, clone 9.13, Invitrogen, 1:50) e CLDN18 (Rabbit polyclonal anti-claudin-18 -c-Term, Zymed, Invitrogen, 1:200). After being rinsed with Tris-HCl buffer (pH 7.6), the slides were incubated, for 20 minute each, with biotinylated secondary antibody and incubated with

streptavidin-biotin peroxidase, following the manufacturer's instructions (Histostain Bulk Kit, Zymed). The immunostain was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin. Negative controls were established by replacing the primary antibody with buffer solution.

Normal stomach was used as positive control for TFF1 and CLDN18 antibodies, and liver, breast carcinoma, lung carcinoma and epidermoid lung carcinoma tissues were used as positive controls for HGF, MET, NOS2 and KRAS antibodies, respectively. A single pathologist examined all specimens. All analyses were performed under a light microscope, and the whole tissue extension of all samples was examined. Immunostain for protein NOS2 (brown cytoplasm staining) was evaluated by staining presence as negative (-, absent brown staining) or positive (+, presence brown staining), whereas for KRAS (brown cytoplasm staining), TFF1 (brown cytoplasm staining) and CLDN18 (brown membrane staining) proteins, the immunostain was evaluated as negative (-) or positive: +1 (weakly stained), +2 (moderately stained), and +3 (strongly stained), as observed in at least 10% of cells. A significant protein expression was considered only for (+2) and (+3) staining, while (-) and (+1) showed no significant expression. Immunostaining analysis for HGF and MET proteins was not performed because antibodies reaction did not produce satisfactory results, after various tests.

Statistical Analysis

Data about RNAm relative expression were described using mean $\pm$ SD as a point estimator and the range of values. Nonparametric Mann-Whitney test was used in the comparisons between the groups, and nonparametric Spearman rank correlation was used to evaluate interactions among mRNA expression in the groups. Protein expression was described according to number of positive and negative cases and Fisher's exact test was used to compare the groups. The relationship between mRNA and protein

levels and clinicopathological variables was evaluated by Fisher's exact test. All the tests were accomplished using the GraphPad Instat Software. The level of significance was set at $P < 0.05$.

Results

mRNA expression in gastric ulcer and gastric cancer

Table 1 shows the results for the gene expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in gastric ulcer (GU) and gastric cancer (GC). Relative gene expression was calculated using normal adjacent gastric mucosa (NM) as reference, and a mean level of expression around 1.5-fold was considered significant, whether increased or decreased. Comparisons between mRNA expression levels in GU and GC were illustrated in Figure 1. *NOS2* expression was up-regulated in 43.3% and 59.1% of GU and GC cases with an increase of 4.5- and 12.8-fold in comparison with normal mucosa, respectively. *HGF* and *MET* mRNA expressions were up-regulated only for 26.7% and 17.2% of GU cases and 27.3% and 38.1% of GC cases, respectively. *HGF* mRNA mean level was not observed increased in UG group (1.05-fold), but both *HGF* and *MET* were increased 1.8- and 3.5-fold, in GC group, and *MET* was increased 2.8-fold in GU group. Regarding *KRAS*, 26.7% and 28.6% of cases of GU and GC, respectively, showed up-regulated, with respective mRNA relative mean levels increased in 2.6- and 1.7-fold. While for both *TFF1* and *CLDN18*, most of GU (73.3% and 58.6%, respectively) and GC (68.2% for both genes) cases were down-regulated, with decreased expression mean levels in both groups, as follows: 0.531 and 0.934 for GU and 0.968 and 0.605 for GC groups, respectively.

When we compared relative expression mean levels between GU and GC groups, there was a significant difference only for *MET* ($P = 0.0001$, Figure 1).

Correlation analysis to compare mRNA expression interaction among genes that participate in the same signaling pathway, showed in GU group that *NOS2* mRNA expression is related to *KRAS* mRNA expression ($r=0.57$, $P=0.0009$), and with *HGF* expression ($r=0.64$, $P=0.0001$). In GC group, a correlation between mRNA expression levels of *NOS2* and *HGF* ($r=0.80$, $P=0.0001$) has been observed.

Immunohistochemical analysis for NOS2, KRAS, TFF1 and CLDN18.

Immunohistochemical analyses results in gastric ulcer and gastric cancer specimens were listed in Table 2. Cytoplasmatic brown-staining for *NOS2* was detected in 61.5% and 30% of ulcer and cancer area, respectively. While *NOS2* expression was visualized in the normal glandular epithelium in 100% and 75% of GU and GC cases (Figure 2. A.1, B.1 and C.1). In the normal foveolar epithelium, we observed negative immunostaining in 57 % of UG and 100% of GC cases. Regarding *KRAS* protein, ulcer and cancer areas were positive in 76.9% and 100%, respectively, with *KRAS* showing moderate (++) and strong (+++) cytoplasmatic staining in 38.5% of GU cases and 70% of GC specimens. But, most of the normal epithelium of both GU and GC specimens showed a weak focal staining (+) (Figure 2. A.2, B.2 and C.2). With regards to *TFF1* protein, in the UG group, 100% of the specimens had similar patterns with moderate/strong cytoplasmatic staining (++) between normal epithelium and ulcer area (Figure 2. A.3 and B.3). But in the GC group only 50% of specimens showed a weak staining in the cancer cells and the remaining 50% did not express *TFF1* protein in the cancerous areas, despite the fact that the normal epithelium showed moderate/strong staining for *TFF1* (Figure 2. C.3). Positive brown-staining for *CLDN18* protein was visualized in the membrane of gastric normal epithelium in both UG and GC groups. The ulcer area was positive for *CLDN18* in 88.2% of the specimens with a pattern of weak staining. However, *CLDN18* was not expressed in 40% of cancer cells and

showed a weak membrane staining (+) in the other 50% of cases, in comparison with normal epithelium (+++) (Figure 2. A.4, B.4 and C.4). Scores ≤ 1 (absent and weak staining) and ≥ 2 (moderate and strong staining) were used to compare protein expression between the GU and GC groups, and a significant difference was found only for TFF1 protein ($P=0.0001$).

mRNA and protein expression and clinicopathological variables

Association between *NOS2*, *KRAS*, *HGF*, *MET*, *TFF1* and *CLDN18* expression and variables as age, gender, tobacco and alcohol habits and *H. pylori* infection was measured in gastric ulcer and gastric cancer groups. Molecular diagnosis of *H. pylori* bacterium showed that 15 of the 26 (57.7%) cases of GU and 11 of the 21 (52.4%) cases of GC were *H. pylori*-positive (data not shown). Lower mRNA levels of *NOS2* and *HGF* and higher levels of *CLDN18* expression were significantly associated with *H. pylori* infection in GC group (2.29 vs. 28.82, 0.93 vs. 2.96 and 0.90 vs. 0.28, respectively). For the remaining genes, the comparisons between mRNA and protein expression and variables studied did not result in any significant association (data not shown).

Discussion

In this study, we performed the analysis of mRNA and protein expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in gastric ulcer and gastric cancer biopsies. Our current results showed similar mRNA expression pattern in GU and GC groups with increased mRNA mean levels for the genes *NOS2*, *HGF*, and *KRAS*, and reduced for the genes *TFF1* and *CLDN18*, but significantly different only for *MET*, due to higher mRNA expression in GC group. Immunohistochemical analysis of *NOS2*, *KRAS* and *CLDN18* protein expressions did not show significant differences between

groups, except for the TFF1 protein, where 100% of GC cases showed negative/weak staining, whereas 100% of GU cases the staining was moderate/strong, in both normal epithelium and ulcer area.

Although these genes are known to be deregulated in gastric cancer, very little is known about its expression pattern in gastric ulcer. For example, various studies have found elevated levels of *NOS2* in about 70 to 80% of cases of gastric cancer²¹⁻²⁵. *NOS2* or iNOS (inducible nitric oxide synthase) is transcriptionally induced in inflammatory cells by proinflammatory agents and inflammatory cytokines for the production of nitric oxide (NO)²⁶. High concentration and sustained release of NO by *NOS2* during inflammation can result in the formation of highly toxic oxidizing species such as peroxynitrites and tissue aggravation and DNA damages^{26,27}.

We found that *NOS2* mRNA was 4.5-fold increased in GU and about 13-fold increased in GC. Immunohistochemistry for *NOS2* protein showed that 61.5% of GU cases were positive, but staining-positive was observed only in 30% of cases of GC. Previous studies have also shown little correlation between *NOS2* mRNA and protein, indicating a considerable degree of post-transcriptional regulation^{28,29}. Also, our results are in accordance with the report of Guo et al.¹⁶, who found high levels of *NOS2* activity during ulcer healing. This study verified a beneficial role of selective *NOS2* inhibitor in gastric ulcer healing, reinforcing the suggestion that NO can aggravate tissue damage. However, there are also observations that NO may benefit ulcer healing through the promotion of apoptosis in inflammatory cells³⁰. With regards to *NOS2* protein expression, Rajnakove et al.³¹ found that the expression of all isoforms of NOS in human stomach tumor tissue was significantly lower than normal mucosa, which is similar to our results, where we found positive staining for *NOS2* protein in normal glandular epithelium of most GU and GC specimens.

HGF (Hepatocyte Growth Factor) and its specific receptor, MET, are frequently found up-regulated in various types of cancer, as esophagus, salivary gland, breast, thyroid and bladder cancers³²⁻³⁶. HGF is a cytokine that induces many biological functions as promoting cell migration, cell proliferation, angiogenesis, apoptosis inhibition, and morphogenesis in hepatocytes and many epithelial cells type³³. Similarly, its receptor MET is expressed in the normal epithelium of the majority of tissues, where it is primarily located at the intercellular junctions together with cell adhesions molecules. HGF has also been shown to have an important role in repairing the kidney, gastrointestinal, lung, liver, myocardial tissue, and retinal epithelium³⁷. Our data showed that about 27% of both GU and GC cases were up-regulated to *HGF*, with normal mRNA expression mean levels in GU (1.05) and increased in GC (1.82), but without significant differences between of the groups. However, for *MET*, we observed a significant increase in the mRNA mean level in GC group in comparison with GU group (3.50 vs. 2.77, respectively), where only 17.2% of GU cases were up-regulated in relation to 38.1% of GC cases.

Immunohistochemistry analysis was not performed because antibodies for HGF and MET proteins did not yield satisfactory results. Previous reports associated with high expression of *HGF* and *MET* in gastric ulcer healing^{9,14}. Similarly, in leg ulcers, it was observed an increased expression of MET protein in chronic ulcer compared with controls, however HGF protein expression did not reach statistical significance between patients with chronic ulcers and controls³⁸.

Growth factors activate epithelial cell migration and proliferation and accelerate wound/ulcer healing *in vivo* and *in vitro* by binding to their specific receptors on the cell surface, a process that triggers a number of intracellular signaling events that culminate in the cell migration and proliferation⁹. The lower *HGF* mRNA relative expression

observed in our study, can be due to technical variation because mRNA was extracted from biopsies of gastric ulcer containing areas with normal epithelium, so it can mask the results. Also, it is known that expression of *HGF* transcripts in normal skin is greater than in leg ulcers areas³⁷.

Aberrant expression of *KRAS*, such as overexpression of wild type or point mutations, is frequent in a variety of human cancer such as pancreatic adenocarcinoma, lung carcinoma, gastric cancer and pancreatic carcinosarcoma³⁹⁻⁴². These mutations are capable of transducing a strong mitogenic signal to stimulate cell proliferation, which is directly involved in the initiation and development of human neoplasia⁴³. We found an up-regulation of *KRAS* of 2.6- and 1.7-fold in GU and GC, respectively and a moderate/strong protein expression in 38.5% of GU and 70% of GC cases. The overexpression of *KRAS* in UG may be a result of its role in the cellular proliferation to repair the injured area of gastric mucosa, while in gastric cancer, it might be related to an increase in the proliferation of gastric cancer cells.

It is noteworthy that target genes of KRAS/ERK signaling pathway have been overexpressed in gastric cancer, indicating that *KRAS* overexpression and activation of RAS/ERK are key events in gastric cancer development, as well as acute myelogenous leukemia and acute lymphocytic leukemia, breast and prostate cancers^{44,45}. Additionally, in gastric cancer, the increased expression of KRAS protein was correlated with lymph nodes metastases and poor prognosis⁴¹. To the best of our knowledge, there are only studies about KRAS protein expression in experimental gastric healing, suggesting a role in the restoration of the epithelial components during gastric ulcer healing⁹. Thus, our data can be the first to show *KRAS* mRNA and protein expression in human gastric ulcer, and supporting the experimental data, suggest a role in the ulceration sites through the cell proliferation promotion.

Literature data show an intrinsic network which includes HGF, MET, KRAS and NOS2 proteins. High NO concentration is observed in injured gastric mucosa, probably due to a protective role that NO exerts on epithelial cells, mediated by increased gastric mucosal blood flow, increased mucus and bicarbonate secretion, stabilization of mucosal mast cells and facilitation of the formation of mucoïd cap over the injured mucosa⁴⁶. Despite this protective role, NO retards cell migration and induces apoptosis, presumably by generating reactive metabolites such as peroxynitrites and superoxide radicals. This destructive role of NO seems to be inhibited by the expression of HGF and its up-regulation can be used by gastric cells to cancel the destructive effects of NO. HGF action can be started through its binding to MET receptor that activates several intracellular messengers, one of them is KRAS that has been shown to mediate the HGF-induced migration of gastric cells^{46,47}. In GU group, our data showed high expression of both *NOS2* mRNA and protein, and although *HGF* has shown mRNA normal mean levels, a high expression of *HGF*-receptor (*MET*) and *KRAS* were observed. These results reinforce that all these genes are overexpressed in gastric ulcer as part of ulcer healing process, which involves epithelial cell proliferation, migration, re-epithelialization, and reconstruction of gastric glands⁹. In GC, this pathway eventually leads to accelerated proliferation, cell morphological and actin filament changes, and other processes that are important to gastric cancer progression⁴⁸.

The gastric ulcer healing involves a great number of factors that have stimulated expression at lesions sites, such as *EGF*, *TNF- α* , *TNF- β* and trefoil peptides (TFFs). TFF, a group of small molecule polypeptide mainly secreted by gastrointestinal mucous cells is also a factor that has stimulated expression during gastric ulcer healing^{9,10}. *TFF1* (*pS2*) is stomach specific and has the function of mucosal protection, epithelial restitution after injury, carcinoma suppression, and apoptosis adjustment¹⁰. We found

that 100% of gastric cancer cases showed absent or weak staining for TFF1 protein by immunohistochemistry, inversely, in GU 100% of cases showed moderate/strong staining. Conversely, *TFF1* mRNA mean levels were more reduced in GU (0.53) than in GC (0.97). Despite the decreased levels of *TFF1* mRNA in GU group, the immunohistochemistry analysis of protein showed increased levels of TFF1 at the ulcer area, reinforcing its role in the repair and healing of the damaged gastric mucosal. This finding is in accordance with other studies^{10,13,49}, for example, Shi et al.¹³ found decreased levels of TFF1 protein expression from UG (mean score = 73.7%) to GC (mean score = 57%).

This apparent discordance between mRNA and protein expression in the present study can be related to higher levels of *TFF1* expression in normal adjacent mucosa than in the ulcer area, which suggests a cytoprotective role in non-ulcerated gastric mucosa. Similarly, Saitoh et al.⁴⁹ found that *TFF1* mRNA at the non-ulcerated sites in live human stomach with gastric ulcer was increased during the healing stage and, consequently, these sites might escape ulceration. In addition, similar to our results in gastric cancer, several studies have shown a decreased expression of TFF1 protein in this cancer type^{13,50-52}, and reinforced the findings that deregulation of the *TFF1* leading to decreased protein levels is a critical and common event in human gastric tumorigenesis.

The other gene evaluated in our study was *CLDN18*, which belongs to a class of proteins named claudins that interact with one another to form the tight junctions (TJs) of epithelial cells⁵³. It is known to function in cell-cell adhesion, membrane polarity, and regulation of ion and uncharged (aqueous) molecule passage through the paracellular pathway. Disruption of TJs can cause loss of cell polarity, resulting in an abnormal influx of growth factors, which could provide auto and paracrine stimulation

to tumourigenic epithelial cell^{18,54}. In the present study, *CLDN18* mRNA was down-regulated in 68.2% of GC cases and the protein showed absent or weak staining in 90% of the specimens. No significant changes of the mRNA expression levels were observed in GU, despite that, about 59% of GU cases showed reduced mRNA levels in relation to adjacent normal mucosa. Immunohistochemistry analysis confirmed these findings, since CLDN18 protein staining was weak in 88% of GU cases and absent/weak in 90% of GC cases. In gastric cancer, absence or loss of expression of CLDN18 has suggested a close correlation with carcinoma progression and subsequent metastatic events^{18,54}. However, in other cancer types such as pancreatic, CLDN18 is frequently up-regulated⁵³.

To our knowledge, there are no reports about CLDN18 expression in gastric ulcer. So, our study showed for the first time, weak expression of CLDN18 protein in ulcer tissues in comparison to normal gastric epithelium, which showed strong membrane staining in normal areas of both ulcer and gastric cancer specimens. Thus, this result suggests that a decrease or a loss of CLDN18 expression can also occur in precancerous lesions such as gastric ulcer and may have a key role in the progression and maintenance of gastric cancer.

In the present study, we did not observe associations between status of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* expression with clinicopathological features, such as age, gender, tobacco and alcohol habits, except that individuals *H. pylori*-positive of gastric cancer group had lower expression levels for *NOS2* and *HGF* and higher expression levels for *CLDN18*. Conversely, Wang et al.²³ found higher levels of NO and NOS expression in *H. pylori*-positive group, while Augusto et al.²⁵ did not find association between *NOS2* expression and virulence factor of *H. pylori*. The relationship between HGF expression and *H. pylori* infection is contradictory. Some

previous studies found an increased expression of HGF in gastric mucosa colonized by *H. pylori*⁵⁵. Similarly, CagA virulence factor of *H. pylori* has been shown to interact with HGF receptor, *MET*, thereby stimulating cell growth, motility and invasiveness⁵⁶, suggesting a possible link between this growth factor and *H. pylori*-associated gastritis and gastric cancer. However, Konturek et al.⁵⁷ did not find significant correlation between CagA status and gene and protein expression of growth factors. They also observed similar gene changes in patients without *H. pylori* infection. So, to clarify these divergent results, further studies of *H. pylori* interaction with growth factors are necessary.

To our knowledge, there are no reports about interactions between *H. pylori* infection and *CLDN18* expression. However, it is known that *H. pylori* recruits and modifies the distribution of zonula occludens-1 (ZO-1) and junctional adhesion molecule (JAM), which are normally located at cell tight junctions altering the structure and function of the apical-junctional complex. This dysfunction may result in loss of control over cytoskeletal architecture, cell polarity, proliferation and differentiation, which is characteristic of oncogenic transformation⁵⁶. Thus, virulence factors of *H. pylori* are likely to interact with *CLDN18*, leading to changes in TJs structure and function, suggesting that *H. pylori* infection may change *CLDN18* expression.

In conclusion, we verified that gastric ulcer and gastric cancer share changes in the expression of key genes that participate in important mechanisms as cell proliferation, apoptosis, maintenance and integrity of gastric mucosa, namely *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18*. Despite that, in ulcer, these expression changes appear to be mainly related to the response of the tissue in the healing process. In gastric cancer, it must act in initiation and progression of gastric carcinogenesis. However, we cannot rule out the possibility that these alterations in the gene/protein expressions

together with other genetic alterations may provide a greater risk of malignant progression in a percentage of gastric ulcer in the progress to gastric cancer. To clarify these aspects, further studies should be conducted involving different gene classes implicated in initial steps and progression of gastric cancer.

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Table 1. Mean level of mRNA expression for *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in gastric ulcer (GU) and gastric cancer (GC) in comparison to normal adjacent gastric mucosa.

Genes	N(%)		mean±SD		Range		P value GU x GC
	GU	GC	GU	GC	GU	GC	
Up-regulated cases							
<i>NOS2</i>	13/30 (43.3)	13/22 (59.1)	4.479±6.941	12.792±29.780	0.0005 to 25.428	0.005 to 127.410	0.4217
<i>HGF</i>	8/30 (26.7)	6/22 (27.3)	1.051±1.336	1.824±3.075	0.0002 to 6.596	0.055 to 13.485	0.5643
<i>MET</i>	5/29 (17.2)	8/21 (38.1)	2.771±11.249	3.504±7.573	0.000 to 61.181	0.001 to 30.626	0.0001*
<i>KRAS</i>	8/30 (26.7)	6/21 (28.6)	2.642±7.390	1.692±2.804	0.000 to 31.341	0.120 to 12.641	0.0500
Down-regulated cases							
<i>TFF1</i>	22/30 (73.3)	15/22 (68.2)	0.531±0.486	0.968±2.047	0.0001 to 2.334	0.0001 to 8.378	0.2438
<i>CLDN18</i>	17/29 (58.6)	15/22 (68.2)	0.934±1.382	0.605±0.867	0.001 to 5.959	0.001 to 2.627	0.1349
Mann-Whitney <i>U</i> -test for comparing mRNA expression levels. * statistically significant difference (<i>P</i> <0.05).							

Mann-Whitney *U*-test for comparing mRNA expression levels. * statistically significant difference ($P<0.05$).

Table 2. Expression of NOS2, KRAS, TFF1 and CLDN18 proteins in gastric ulcer (GU) and gastric cancer (GC) specimens.

Protein	Staining	GU	GC	P value
		N (%)	N (%)	GU x GC
NOS2	-	5 (38.5)	7 (70)	0.2138
	+	8 (61.5)	3 (30)	
KRAS	-	3 (23)	0 (0)	0.2138
	+	5 (38.5)	3 (30)	
	++/+++	5 (38.5)	7 (70)	
TFF1	-	0 (0)	5 (50)	0.0001*
	+	0 (0)	5 (50)	
	++/+++	16 (100)	0 (0)	
CLDN18	-	2 (11.8)	4(40)	0.3704
	+	15 (88.2)	5 (50)	
	++/+++	0 (0)	1 (10)	

Fisher's exact test for comparing protein expression between the groups. N= number of samples; Statistical Analysis: - and + were grouped for comparison between GU and GC. * = statistically significant difference ($P<0.05$).

Legend to figures

Figure 1. mRNA expression of *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* and *CLDN18* in gastric ulcer (GU) and gastric cancer (GC) by Real-time PCR. Data are expressed as mean $\pm$ SD. Mann-Whitney *U*-test was used to compare mRNA expression levels between GU and CG groups. * statistically significant difference ($P < 0.05$).

Figure 2. Immunohistochemistry analysis in normal gastric mucosa (A), gastric ulcer (B) and gastric cancer (C) tissues. 1- NOS2 brown cytoplasmatic staining. A.1: absent in the foveolar epithelium (black arrow) and weak in glandular epithelium (white arrow); B.1: moderate staining; C.1: strong staining. 2- KRAS brown cytoplasmatic staining. A.2: absent in the foveolar epithelium (black arrow) and weak in glandular epithelium (white arrow); B.2: strong staining; C.2: strong staining. 3- TFF1 brown cytoplasmatic staining. A.3: strong staining; B.3: strong staining C.3: absent staining. 4- CLDN18 brown membrane staining. A.4: strong staining; B.4: strong staining in normal epithelium (white arrow) and absent in the ulcer (black arrow); C.4: strong staining in normal epithelium (white arrow) and absent in tumor cells (black arrow). Magnification 100x.

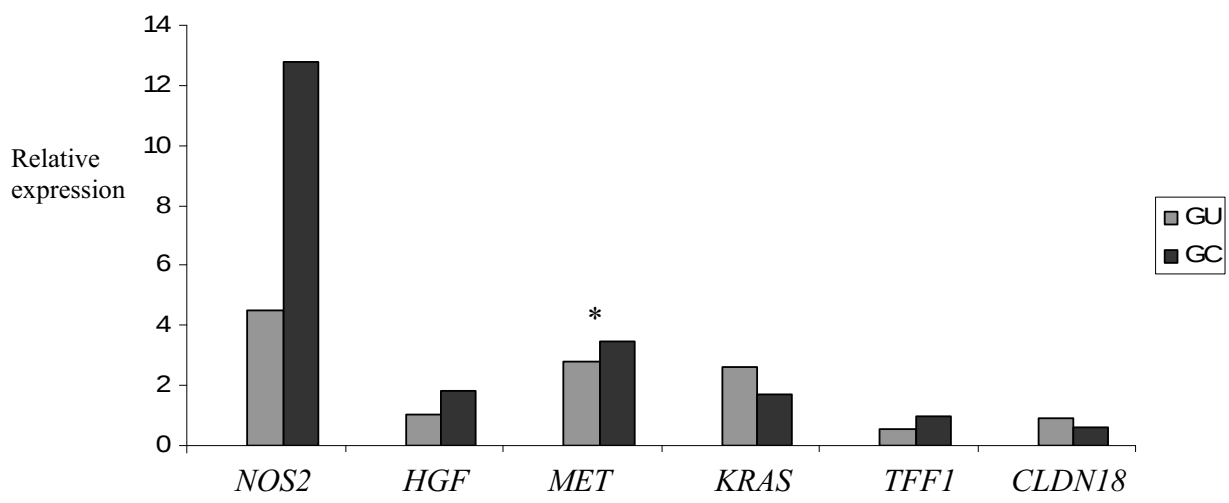


Figure 1.

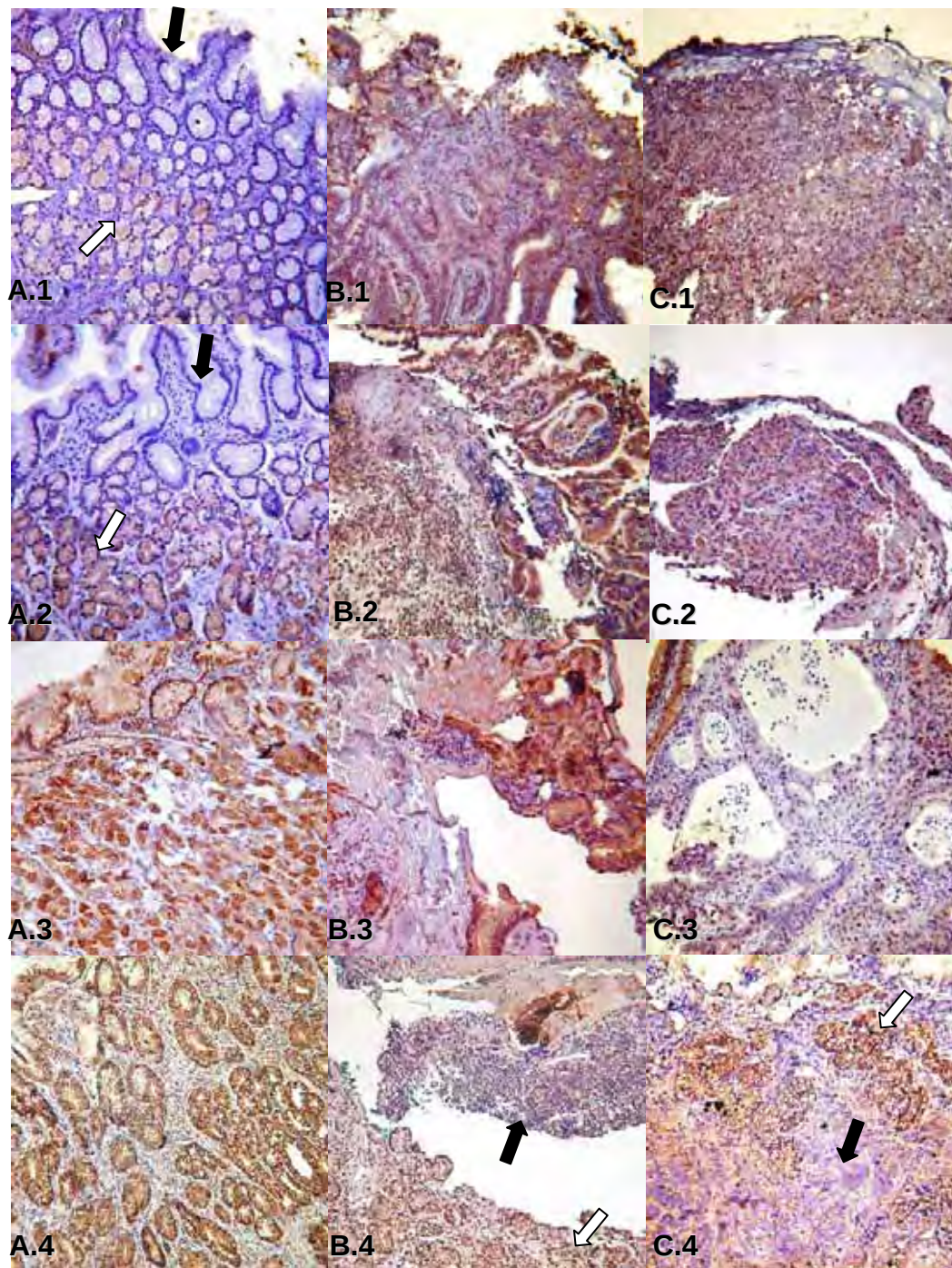


Figure 2.

Discussão

IV - Discussão

O presente estudo investigou a expressão de RNAm e proteína dos genes *TERT*, *COX-2*, *NOS2*, *HGF*, *MET*, *KRAS*, *TFF1* e *CLDN18*, com funções relevantes em processos do ciclo celular e proteção da mucosa gástrica em biópsias de lesões pré-cancerosas como metaplasia intestinal (MI) e úlcera gástrica (UG) em comparação com suas respectivas mucosas normais e câncer gástrico (CG). Destaca-se a importância do estudo de lesões pré-neoplásicas para melhor compreensão das etapas iniciais do processo de carcinogênese do estômago e possível indicação de alvos para estratégias de diagnóstico precoce e prevenção futuras.

TERT codifica a enzima transcriptase reversa da telomerase e atua na elongação dos telômeros durante a divisão celular, sendo fundamental para a senescência e imortalização celular (BELGIOVONE; CHIODI, MONDELLO, 2008). *COX-2* e *NOS2* codificam enzimas induzíveis durante o processo inflamatório, exercendo papéis tanto na defesa da mucosa gástrica quanto na progressão carcinogênica, por meio de suas atividades proliferativa e angiogênica (STACK; DUBOIS, 2001; SAUKKONEN et al., 2003; KEKLIKOGU et al., 2008). *HGF*, *MET* e *KRAS* participam das vias de sinalização celular, atuando como fatores de promoção da proliferação, migração e angiogênese (FRIDEY; ADJEI, 2005; TOSCHI; JÄNNE, 2008). *TFF1* e *CLDN18* são fatores específicos que atuam na proteção da mucosa gástrica, aumentando as barreiras existentes contra substâncias nocivas ao organismo (TOMASETTO; RIO, 2005; SEMBA et al., 2006).

Corroborando dados prévios da literatura, nossos resultados mostraram um aumento de expressão dos níveis de RNAm para os genes *TERT*, *COX-2*, *NOS2*, *HGF*, *MET* e *KRAS* nas amostras de CG. A expressão das respectivas proteínas, em geral, mostrou-se elevada em comparação com a mucosa normal, com exceção das proteínas

HGF e MET, para as quais a análise imunohistoquímica não produziu resultados satisfatórios e, portanto, não foram avaliadas. De modo geral, *TFF1* e *CLDN18* apresentaram níveis reduzidos de expressão para ambos mRNA e proteínas.

Em câncer gástrico, a reativação da telomerase tem sido descrita em 61 a 90% dos casos (HU et al., 2004; SABAH et al., 2004; GULMANN et al., 2005). A expressão aumentada da proteína COX-2 foi relacionada à proliferação celular, angiogênese e resistência a apoptose (YAMACH et al., 2008). Níveis elevados de expressão gênica e protéica para *NOS2* foram associados com angiogênese, sobrevida e progressão tumoral (WANG et al., 2005; CHEN et al., 2006; AUGUSTO et al., 2007). *HGF* e *MET* são freqüentemente encontrados com expressão aumentada em câncer gástrico (KONTUREK et al., 2001; INOUE et al., 2004; STOCK; OTTO, 2005), enquanto *KRAS* é preferencialmente ativado por mutação (WATARI et al., 2007), e a expressão aumentada de sua proteína já foi correlacionada com progressão da carcinogênese gástrica e metástases em linfonodos (LI et al., 2006).

No presente estudo, a proteína TFF1 estava ausente ou com marcação fraca nas amostras de CG, embora o nível médio de expressão relativa do mRNA não mostrasse uma redução significativa, enquanto *CLDN18* apresentou expressão gênica e protéica reduzida em CG comparado com a mucosa normal. Alguns estudos têm descrito uma perda de expressão da proteína TFF1 em 40 a 60% dos casos de câncer gástrico (MACHADO et al., 2000; LEUNG et al., 2002; SHI; CAI; YANG, 2006) e da proteína CLDN18 em 57,5% dos casos, correlacionada com baixa sobrevida (SANADA et al.; 2006).

No grupo de metaplasia intestinal, nossos resultados mostraram uma expressão elevada dos níveis médios de mRNA para *TERT*, *COX-2*, *NOS2*, *HGF*, *MET*, *TFF1* e *CLDN18*, apesar que para *TFF1* e *CLDN18* 54% dos casos de MI tinham baixa

expressão em comparação com a mucosa normal. Esses dados foram concordantes com a expressão das proteínas TFF1 e CLDN18 que apresentaram coloração fraca ou perda de expressão na maioria dos casos de metaplasia. Para as demais proteínas, a expressão protéica mostrou-se frequentemente concordante com os resultados de expressão gênica. Quanto à expressão gênica de *KRAS*, não foi observada variação significativa em relação à mucosa normal e a proteína mostrou-se fracamente marcada na maioria das amostras.

Estudos genéticos sobre expressão gênica e protéica em metaplasia intestinal são bastante restritos, mas nossos resultados são concordantes com alguns deles. Por exemplo, Gulmann et al. (2006) e Sun et al. (2006) verificaram a expressão das proteínas TERT e COX-2, respectivamente, em 38% e 23% dos casos de metaplasia. A expressão reduzida de *TFF1*, tanto RNAm quanto proteína, foi demonstrada por Fujimoto et al. (2000) em adenomas, MI e CG, enquanto, Kim et al. (2004) observaram positividade imuno-histoquímica mais fraca em MI que na mucosa normal adjacente. Alguns autores também demonstraram redução na expressão da proteína CLDN18 em MI e CG, e sugeriram que esta mudança no nível de expressão é um evento precoce na carcinogênese gástrica, podendo estar relacionada à progressão e metástase (SANADA et al., 2006; MATSUDA et al., 2007). Entretanto muito pouco se conhece sobre os padrões de expressão gênica e protéica de *HGF*, *MET* e *KRAS* em metaplasia intestinal. Para *HGF*, Suzuki et al. (2004) verificaram uma redução dos níveis da proteína em indivíduos com metaplasia intestinal após o tratamento de erradicação da bactéria *H. pylori*.

Diante dos resultados obtidos no presente estudo há indicações de que *HGF* e *MET*, associados com aumento da proliferação celular, podem apresentar expressão desregulada em metaplasia intestinal, assim podendo também participar das etapas iniciais da carcinogênese gástrica. Ainda neste estudo, foi constatado que *KRAS* não

apresenta mudanças importantes nos níveis de mRNA e proteína em metaplasia intestinal.

No grupo de úlcera gástrica foi verificado um aumento de expressão relativa do mRNA para os genes *TERT*, *COX-2*, *NOS2*, *MET* e *KRAS*, e expressão diminuída para *TFF1* em comparação com a mucosa normal. *HGF* e *CLDN18* não apresentaram alterações significantes em seus níveis de expressão gênica, embora cerca de 27% dos casos de UG tenham apresentado níveis aumentados do mRNA de *HGF* e 59% dos casos mostraram baixa expressão gênica de *CLDN18*. De modo geral, as proteínas *TERT*, *COX-2*, *NOS2*, *KRAS* e *TFF1* apresentaram marcação positiva em UG, enquanto *CLDN18* apresentou expressão fraca ou ausente em comparação com o epitélio normal.

Em úlcera gástrica alguns estudos têm avaliado mudanças na expressão gênica e protéica associadas com o processo de cicatrização. Portanto, nossos resultados estão de acordo com outros quanto às expressões de *COX-2* (GUO et al., 2006; HATAZAWA et al., 2007), *NOS2* (AKIBA et al., 1998; GUO et al., 2006) e *TFF1* (SAITOH et al., 2000; SHI et al., 2006; OH et al., 2008). Os dados da literatura também mostram uma expressão aumentada de *HGF*, *MET* e também *COX-2* nos sítios de úlcera gástrica em cicatrização (HORI et al., 2000; OH et al., 2008; TARNAWSKI, 2005).

No presente estudo, 26,7%, 17,2% e 51,7% dos casos de UG respectivamente, apresentaram aumento de expressão para *HGF*, *MET* e *COX-2*, apesar do nível médio de RNAm de *HGF* ter-se apresentado normal. Portanto, nossos resultados também corroboram que esses genes devem atuar como fatores importantes no processo de cicatrização da úlcera, por meio da promoção da proliferação e migração celular e reconstrução das glândulas gástricas (TARNAWSKI, 2005). Do mesmo modo, *COX-2*, *NOS2* e *TFF1* também estão envolvidos no processo de reparação da mucosa gástrica

pela indução de fatores angiogênicos, aumento de fluxo sanguíneo e formação da capa de muco sobre a lesão (GUO et al., 2006; NYLANDER-KOSKI et al., 2007).

Em úlceras gástricas experimentais, *KRAS* geralmente apresenta-se com expressão aumentada, sugerindo seu papel como ativador da proliferação e restauração dos componentes epiteliais danificados. No entanto, até o presente, não há relatos sobre o padrão de expressão deste gene em úlceras gástricas humanas. A expressão aumentada do RNAm e da proteína de *KRAS* verificada neste estudo, pode corroborar os dados encontrados para as úlceras experimentais. Da mesma forma, não conhecemos relatos sobre expressão gênica e protéica de *TERT* e *CLDN18* em úlcera gástrica. Deste modo, nossos dados indicam uma expressão aumentada de *TERT* e redução de expressão da proteína CLDN18 em úlcera gástrica, ambas mudanças que participam do processo carcinogênico do estômago.

As proteínas avaliadas neste estudo como HGF, MET, KRAS, NOS2, COX-2 e TFF1 participam de vias de sinalização em comum, responsáveis pela ativação de fatores importantes associadas com processos de proliferação e migração celular, apoptose, inflamação, danos oxidativos e reparação da mucosa gástrica (HOFFMANN, 2005; NYLANDER-KOSKI, 2007; CHO et al., 2009). Dessa forma, foi investigada a ocorrência de correlação entre a expressão desses genes nos grupos de metaplasia, úlcera e câncer gástrico. Os dados evidenciaram uma correlação direta entre a expressão gênica de *KRAS*, *HGF* e *MET* em metaplasia intestinal, entre *NOS2*, *RAS* e *HGF* em úlcera gástrica, e entre *NOS2* e *HGF* em câncer gástrico. Esta via de sinalização pode iniciar-se com a ligação da proteína HGF ao seu receptor MET, que ativa KRAS e promove a proliferação e migração celular. KRAS por sua vez, pode induzir a ativação de *NOS2* e *COX-2*, aumentando a inflamação, apoptose e dano oxidativo nas células

gástricas, que pode alterar a homeostase celular e contribuir para o início e progressão da carcinogênese gástrica (NYLANDER-KOSKI, 2007; CHO et al., 2009).

Em ulcera, contudo, *HGF* e *MET* parecem ser induzidos em resposta aos danos produzidos pelo acúmulo de óxido nítrico nas células epiteliais gástricas, e através de *KRAS*, podem promover a reestruturação do tecido ulcerado pela proliferação e migração de células epiteliais para as áreas danificadas (TARNAWSKY, 2005; NYLANDER-KOSKI, 2007).

Portanto, os resultados do presente estudo sugerem que mudanças nos padrões de expressão dos genes ora avaliados podem estar associadas com alterações importantes envolvidas tanto nas etapas iniciais como na progressão maligna, como observado em metaplasia e câncer gástrico. Dessa forma, evidenciando que metaplasia intestinal apresenta mudanças de expressão gênica semelhante ao câncer gástrico que podem conferir maior risco para esta neoplasia. Porém, em úlcera tais alterações devem estar associadas principalmente com o processo de reparo do epitélio gástrico. Contudo, ainda deve ser considerado que essas mudanças de expressão envolvendo genes que também participam do processo carcinogênico, juntamente com outras alterações genéticas e epigenéticas podem conferir maior risco para progressão da úlcera a malignização.

Neste trabalho procurou-se verificar a existência de associações entre os níveis de expressão gênica e protéica com variáveis ambientais e clinicopatológicas, como sexo, idade, tabagismo, etilismo, infecção por *H. pylori* e tipo histológico de MI e CG. Foi observada associação significativa entre baixa expressão de *TFF1* e tabagismo no grupo de MI. Este dado sugere que a expressão de *TFF1* pode ser modulada por carcinógenos presentes no tabaco, que enfraquece as defesas da mucosa gástrica,

proporcionadas pela expressão de *TFF1*, e possibilita maior risco de danos ao DNA e progressão para o fenótipo maligno (TOMASETTO; RIO, 2005; MARTIN et al., 2007).

Também foi verificada uma associação entre indivíduos *H. pylori* positivos e expressão reduzida de *NOS2* e *HGF* e expressão aumentada de *CLDN18* no grupo de CG. Há indicações de que a infecção pela *H. pylori* e fatores de virulência da bactéria podem alterar o padrão de expressão de alguns genes como o da gastrina, COX-2, ANXA2, IL-1 e NOS2 (STOICOV et al., 2004; STOCK; OTTO, 2005; WANG et al., 2005), contudo os resultados são contraditórios. Por exemplo, há relatos de aumento de expressão de *NOS2* em indivíduos infectados por *H. pylori* comparados com os não infectados (WANG et al., 2005), como também da falta de associação entre esses fatores (AUGUSTO et al., 2007). Da mesma forma, para *HGF*, alguns estudos têm verificado tanto expressão aumentada em indivíduos *H. pylori* positivos (SUZUKI et al., 2004; NGUYEN et al., 2008), como falta de associação (KONTUREK et al., 2004).

Quanto à associação entre expressão de *CLDN18* e infecção por *H. pylori* não foi encontrado nenhum estudo na literatura, mas com base em outros relatos sobre a interação de proteínas derivadas de *H. pylori* com a ZO-1 (Zonula Occludens-1), que participa das junções “tight” (NGUYEN et al., 2008), pode-se sugerir que a infecção por *H. pylori* pode participar na modulação da expressão de *CLDN18* em câncer gástrico. Deve-se ainda se considerar, no presente estudo, o número relativamente reduzido de amostras das lesões avaliadas para este tipo de associação, deste modo mais estudos com número maior de casos são necessários para esclarecer estes dados.

Conclusões

V - Conclusões

No presente estudo, considerando-se as lesões avaliadas e as técnicas empregadas, é possível obter-se as seguintes conclusões:

- a) Em geral, metaplasia intestinal exibe padrões similares de expressão gênica ao grupo de câncer gástrico, referente aos genes *TERT*, *NOS2*, *HGF* e *MET*, mas menor para o gene *COX-2* e mais elevada para *TFF1* e *CLDN18*. *KRAS* não apresenta mudanças de expressão significantes nas amostras de metaplasia intestinal;
- b) Úlcera gástrica apresenta mudanças de expressão dos genes *TERT*, *NOS2*, *KRAS*, *TFF1* e *CLDN18* similares as do câncer gástrico, porém *MET* é mais expresso no câncer em relação à úlcera. Tais alterações devem estar relacionadas ao processo de reparo da mucosa gástrica, como também podem conferir maior risco de malignização;
- c) Em câncer gástrico a expressão de *TERT*, *COX-2*, *NOS2*, *HGF*, *MET* e *KRAS* é aumentada em comparação com a mucosa normal adjacente, enquanto *TFF1* e *CLDN18* exibem perda de expressão;
- d) Não há uma relação precisa entre os níveis de mRNA e expressão das proteínas nos grupos avaliados. Porém, em geral, a análise imuno-histoquímica confirma expressão aumentada para as proteínas *TERT*, *COX-2* e *NOS2* e diminuída para *CLDN18* nos três grupos avaliados. *KRAS* está expressa em úlcera e câncer gástrico, enquanto *TFF1* exibe perda de expressão em metaplasia intestinal e câncer gástrico;

e) Há evidências de correlação entre os níveis de expressão gênica para *NOS2*, *HGF*, *MET* e *KRAS* nos três grupos avaliados;

f) De modo geral, não há associação evidente entre os níveis de expressão gênica e protéica dos genes avaliados com variáveis clinicopatológicas, porém o hábito tabagista pode estar associado com expressão diminuída de *TFF1* no grupo de metaplasia intestinal e a infecção por *H. pylori*, pode modular a expressão de *NOS2*, *HGF* e *CLDN18*, como observado no grupo de câncer gástrico.

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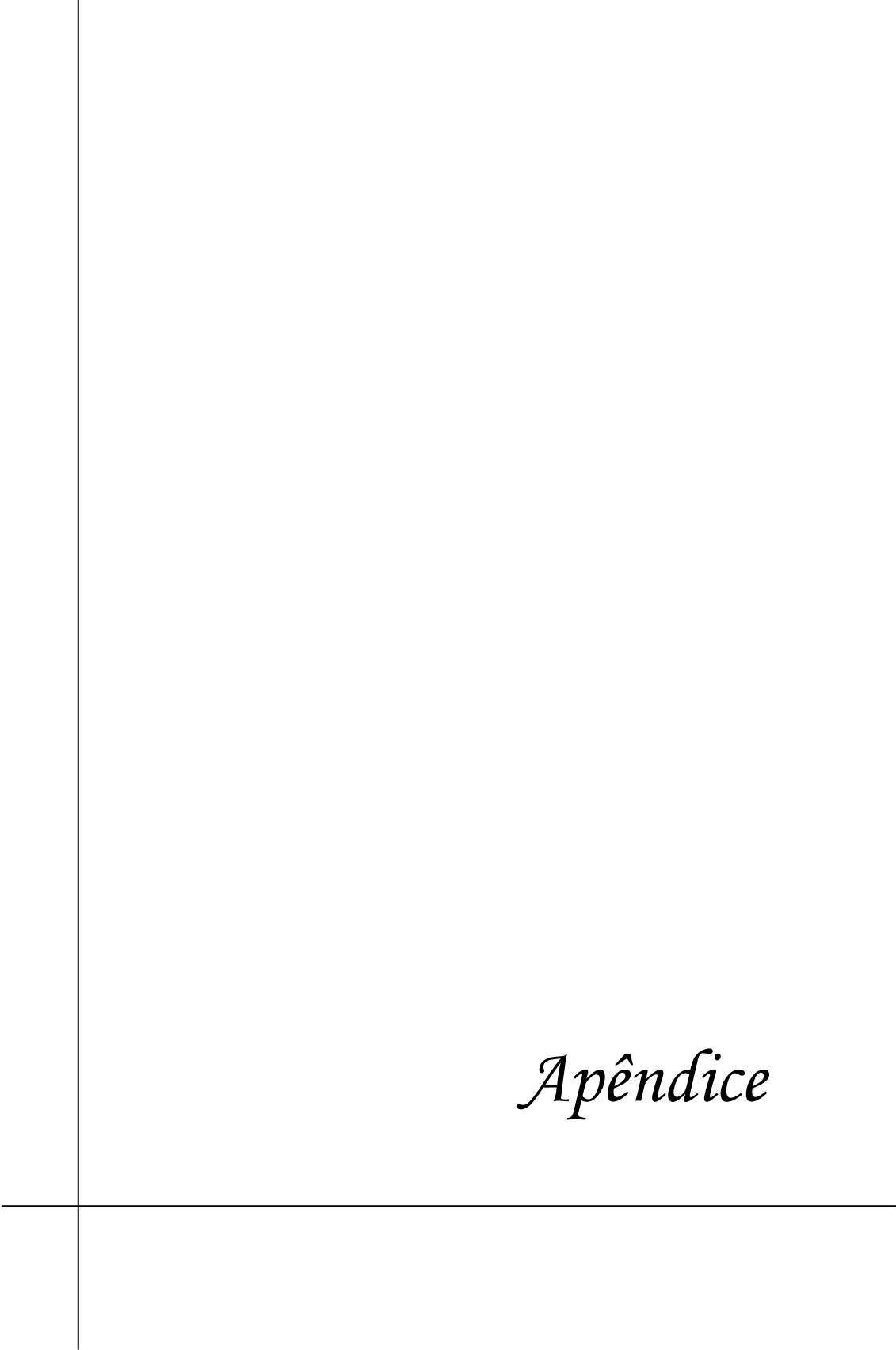
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Apêndice

Apêndice 1 – Questionário

Questionário do projeto: Expressão transcricional e protéica de genes relacionados à atividade da telomerase e ciclo celular em metaplasia intestinal e úlcera gástrica, e associação com *Helicobacter pylori*.

Responsáveis: Doutoranda Márcia Cristina Duarte e Profa. Dra. Ana Elizabete Silva (Departamento de Biologia IBILCE-UNESP, São José do Rio Preto-SP), Dr. Kenji Miyazaki e Dr. Aldenis Albanese Borim (Hospital de Base de São José do Rio Preto-SP).

I. IDENTIFICAÇÃO

Nome:.....Prontuário:.....

Data de nascimento:...../...../..... Sexo: () F () M

Grupo étnico: () caucasóide () negróide () asiático

Endereço:.....Fone:.....

Cidade:.....Estado:.....

Profissão atual:.....tempo de atuação:.....

Profissão anterior:.....tempo de atuação:.....

II. DADOS PESSOAIS E FAMILIAIS

- Consumo de bebida alcoólica: () sim () não () ex-etilista

Há quantos anos:.....Tipo de bebida:.....dose/dia:.....

- Consumo de cigarro: () sim () não () ex-fumante

Há quanto anos:.....Quantidade (un/dia):.....

- Doenças anteriores:

() úlcera () gastrite () câncer (tipo:.....)

() outras (tipo:.....)

- Tratamentos anteriores: () sim () não

Tipo:.....

- Cirurgias anteriores: () sim () não

Tipo:.....

- Uso de medicamentos: () sim () não

Tipo:.....

História de câncer ou outras doenças na família (grau de parentesco)

() câncer (tipo:.....) () úlcera () gastrite

() outras (tipo:.....)

III- DIAGNÓSTICO PATOLÓGICO

() Metaplasia intestinal: () completa () incompleta

() úlcera gástrica

() câncer gástrico: () intestinal () difuso

() *Helicobacter pylori*: () positivo () negativo

Data:...../...../..... Responsável pelo procedimento:.....

Apêndice 2 - Caracterização das amostras dos indivíduos com metaplasia intestinal quanto a sexo, idade, tabagismo, etilismo, classificação histológica e diagnóstico molecular de infecção por *H. pylori*.

Código	Sexo	Idade (anos)	Tabagismo	Etilismo	Histologia	<i>H. pylori</i>
MI01	M	66	S	N	MIC	-
MI02	F	77	N	N	MIC	-
MI03	F	57	N	N	MIC	-
MI04	M	50	S	N	MIC	-
MI05	M	87	S	N	MIC	-
MI06	M	55	N	N	MIC	-
MI07	F	55	S	N	MIC	-
MI08	M	78	S	S	MIC	-
MI09	F	65	N	N	MIC	-
MI10	F	42	S	N	MIC	-
MI11	M	55	S	S	MIC	-
MI12	M	78	N	N	MII	-
MI13	F	43	S	N	MIC	+
MI14	F	73	S	S	MII	-
MI15	M	51	S	N	MII	-
MI16	F	51	N	N	MIC	+
MI17	F	60	S	N	MIC	-
MI18	M	59	N	N	MII	-
MI19	M	66	N	N	MIC	-
MI20	M	65	S	S	MIC	-
MI21	F	47	S	N	MIC	+
MI22	M	75	S	S	MIC	-
MI23	F	51	S	S	MIC	+
MI24	F	64	N	N	MIC	-
MI25	M	67	S	S	MIC	-
MI26	M	46	S	N	MIC	+
MI27	F	61	N	S	MIC	+
MI28	F	60	S	N	MIC	+
MI29	F	58	S	N	MIC	+
MI30	M	63	S	S	MIC	+
MI31	F	38	S	N	MIC	+
MI32	F	65	S	N	MIC	+
MI33	M	60	S	N	MIC	+
MI34	M	80	*	*	MII	-
MI35	M	80	S	S	MIC	-
MI36	F	38	S	N	MIC	+
MI637	M	73	S	N	MIC	+

MI = metaplasia intestinal; Sexo: M= masculino e F = feminino; Tabagismo e Etilismo: S= sim e N= não; Histologia: MIC = metaplasia intestinal completa, MII = metaplasia intestinal incompleta e MICI = metaplasia intestinal completa e incompleta; *H. pylori*: + = positivo e - = negativo; *= dado não disponível.

Apêndice 3 - Caracterização das amostras dos indivíduos com úlcera gástrica quanto a sexo, idade, tabagismo, etilismo e diagnóstico molecular de infecção por *H. pylori*.

Código	Sexo	Idade (anos)	Tabagismo	Etilismo	H. pylori
UG01	M	40	S	N	+
UG02	M	43	N	N	+
UG03	F	30	S	N	+
UG04	M	75	S	N	-
UG05	F	48	S	N	-
UG06	M	49	S	S	-
UG07	M	49	S	S	-
UG08	M	62	S	N	+
UG09	M	45	S	N	+
UG10	F	55	N	N	+
UG11	M	55	S	S	-
UG12	M	55	S	S	+
UG13	F	48	S	N	+
UG14	F	31	N	N	+
UG15	M	42	S	S	+
UG16	M	53	N	S	-
UG17	M	70	N	N	-
UG18	M	81	S	S	*
UG19	M	50	N	S	-
UG20	M	62	N	S	-
UG21	F	54	S	N	+
UG22	M	60	S	N	-
UG23	F	58	S	S	+
UG24	M	51	S	N	*
UG25	F	74	N	N	-
UG26	M	51	N	N	+
UG27	M	60	N	N	+
UG28	M	60	*	*	*
UG29	M	75	S	S	+
UG30	M	59	*	*	*

UG = úlcera gástrica; Sexo: M= masculino e F = feminino; Tabagismo e Etilismo: S= sim e N= não; *H. pylori*: + = positivo e - = negativo; *= dado não disponível

Apêndice 4 - Caracterização das amostras dos indivíduos com câncer gástrico quanto a sexo, idade, tabagismo, etilismo, classificação histológica e diagnóstico molecular de infecção por *H. pylori*.

Código	Sexo	Idade (anos)	Tabagismo	Etilismo	Histologia	<i>H. pylori</i>
CG01	M	60	S	S	CGI	+
CG02	M	74	S	S	CGI	-
CG03	M	41	N	N	CGD	-
CG04	F	38	N	N	CGD	-
CG05	M	45	S	S	CGD	+
CG06	M	68	*	*	CGD	-
CG07	M	70	*	*	CGD	-
CG08	M	58	*	*	CGD	-
CG09	F	75	*	*	CGD	-
CG10	M	60	*	*	CGI	-
CG11	F	80	*	*	CGI	-
CG12	M	48	S	N	CGI	+
CG13	F	78	S	N	CGD	+
CG14	M	71	S	S	CGI	+
CG15	M	77	N	S	CGI	+
CG16	M	48	N	S	CGI	+
CG17	F	86	N	N	CGI	+
CG18	M	61	N	N	CGI	+
CG19	M	47	S	S	CGI	+
CG20	M	78	S	S	*	-
CG21	M	56	S	S	*	+
CG22	M	55	S	S	*	*

CG = câncer gástrico; Sexo: M= masculino e F = feminino; Tabagismo e Etilismo; S= sim e N= não; Histologia: CGI = câncer gástrico intestinal e CGD = câncer gástrico difuso; *H. pylori*: + = positivo e - = negativo; *= dado não disponível.

Apêndice 5 - Material e Métodos

1. Caracterização das amostras

Todas as amostras foram coletadas por profissional qualificado no Centro de Endoscopia do Hospital de Base de São José do Rio Preto e tiveram seu diagnóstico patológico confirmado pelo Serviço de Patologia do mesmo hospital. De cada paciente com diagnóstico endoscópico de úlcera gástrica, metaplasia intestinal e câncer gástrico foram coletadas três biópsias da lesão e três de uma região aparentemente normal do estômago, número este de acordo com as normas do Serviço de Patologia. As biópsias foram acondicionadas em uma solução para preservação do RNA (RNA later, Ambion) e estocadas em freezer a -20 °C para a posterior extração de RNA e DNA. Todos os pacientes foram devidamente informados e consultados sobre sua participação no projeto e de todos foi obtido o Termo de Consentimento Livre e Esclarecido, além de um questionário com informações sobre o estilo de vida e história prévia de problemas de saúde (Apêndice 1), sendo todo o procedimento realizado pelo próprio pesquisador.

2. Extração de RNA e DNA

Para todas as amostras com diagnóstico histopatológico confirmado foi extraído o RNA e o DNA total utilizando o protocolo do reagente Trizol. Inicialmente, duas biópsias de cada amostra foram fragmentadas mecanicamente e colocadas em microtubos contendo 1mL do reagente Trizol para a quebra das membranas celulares, desagregação das proteínas e liberação do material genético (a biópsia remanescente foi estocada em freezer -20°C para extrações posteriores caso a primeira não tenha obtido quantidade e/ou qualidade de RNA suficiente). Após 5 minutos em temperatura ambiente, adicionou-se 200µL de clorofórmio e o material foi mantido à temperatura ambiente por 3 minutos e depois centrifugado por 15 minutos a 2°C. Após a

centrifugação, ocorre a separação do material em três fases: uma vermelha inferior, contendo proteínas e DNA, uma intermediária, contendo restos celulares e DNA e uma superior, contendo RNA. A fase superior foi transferida para outro microtubo no qual adicionou-se 500µL de álcool isopropílico. O material foi mantido à temperatura ambiente por 10 minutos e, posteriormente, centrifugado por 10 minutos a 2°C para a precipitação do RNA total. Após a centrifugação, o sobrenadante foi descartado e o *pellet* de RNA foi ressuspendido e lavado com etanol 75%, centrifugado novamente por 5 minutos a 2°C e seco por cerca de 10 minutos à temperatura ambiente. Finalmente, foi adicionado 30µL de água DEPEC para a diluição do RNA em banho-maria a 60°C por 10 minutos. Logo depois, foi realizada a medida da concentração do RNA em espectrofotômetro (NanoDrop) e este foi rapidamente estocado em freezer -80 °C para evitar a sua degradação.

As fases inferior e intermediária foram reservadas para a posterior extração de DNA, às quais adicionou-se 300µL de etanol absoluto gelado que foi incubado por 3 minutos à temperatura ambiente e, posteriormente, centrifugado a 2 °C por 5 minutos para a precipitação do DNA. O *pellet* de DNA foi lavado duas vezes com solução de citrato de sódio 0,1 M em etanol 10%. Para cada lavagem, o material foi mantido sob agitação por 30 minutos à temperatura ambiente, e depois centrifugado à 2°C por 5 minutos. Após a última lavagem, o *pellet* de DNA foi ressuspendido em etanol 75%, mantido à temperatura ambiente por 15 minutos e centrifugado por 5 minutos a 2°C. O sobrenadante foi descartado, o *pellet* foi seco à temperatura ambiente por 10 minutos e foi adicionado 50µL de água ultra-pura autoclavada para a diluição do DNA em banho-maria à 37°C por aproximadamente três dias. Foi realizada a leitura da concentração do DNA de todas as amostras no espectrofotômetro (NanoDrop) e estas foram estocadas em freezer -20°C, para a posterior avaliação molecular da bactéria *H. pylori*.

3. Eletroforese do RNA

Todas as amostras de RNA foram submetidas à eletroforese em gel de agarose 1% para verificação da integridade do RNA. De cada amostra foi utilizado 1µg de RNA. As amostras de boa qualidade apresentaram duas bandas correspondentes às duas subunidades do RNA ribossômico e foram utilizadas para a síntese de cDNA total. Aquelas que não apresentaram RNA de boa qualidade foram submetidas à nova extração de RNA da biópsia remanescente.

4. RT-PCR

Cerca de 5 µg de RNA foi utilizado para a síntese de c-DNA com o kit High Capacity (Applied Biosystems). Para uma reação de 50µL, foram adicionados 5 µL de random primer, 2,5 µL de mix dNTP 10 mM, 5 µL de tampão 5 X, 80U de RNAOut (Invitrogen), 5µL de Transcriptase Reversa e água DEPEC para completar o volume final. No termociclador, as condições da reação foram 25°C por 10 minutos e, posteriormente, 37°C por 120 minutos. As amostras de cDNA obtidas foram armazenadas em freezer -20 °C.

5. PCR do gene β -actina

Todas as amostras de cDNA foram testadas através de PCR para amplificar o gene da β -actina, com o intuito de confirmar o sucesso da síntese do cDNA. Para uma reação de 20µL, foi utilizado 1µL de cDNA, 1,5mM de MgCl₂, 0,2mM de dNTPs, 0,5µM de cada *primer* (F-5'GAGGCACTCTTCCAGCCTTC3' and R-5'GTTGGCGTACAGGTCTTGC3') e 1,5U de Taq DNA polimerase em tampão 1X. As condições da reação foram as seguintes: 94°C por 3 minutos, seguidos de 35 ciclos de amplificação a 94°C por 45 segundos, 61°C por 30 segundos e 72°C por 1 minuto e extensão final a 72°C por 10 minutos. A banda amplificada de 114 pb foi visualizada em gel de agarose 2% corado com brometo de etídeo.

6. Desenho das seqüências de primers

As seqüências de RNAm dos genes *TERT1*, *COX-2*, *NOS2*, *HGF*, *MET*, *RAS* e *TFF1* bem como dos genes endógenos β -actina, β 2-microglobulina e α -tubulina foram obtidas do banco de dados do NCBI e utilizadas para a confecção dos *primers* por meio do programa Primer3 (disponível em http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Todos apresentaram temperatura de anelamento de 60°C e amplificam fragmentos que variam de 83 a 160 pb (Tabela 1) pertencendo a dois ou mais éxons consecutivos, para evitar a amplificação de fragmentos de DNA contaminante. A seqüência do gene *CLDN18* foi obtida conforme descrito por Sanada et al., 2006.

7. Amplificação por PCR em tempo real

A reação de PCR em tempo real foi realizada utilizando o kit comercial SYBR Green (Applied Biosystems), com *primers* específicos para os genes *TERT*, *COX-2*, *NOS2*, *HGF*, *MET*, *RAS*, *TFF1* e *CLDN18*. Os genes *housekeeping* β -actina, β 2-microglobulina e α -tubulina foram testados como controles internos da reação. Resumidamente, a reação de 20 μ L foi composta de 10 μ L de SYBR Green, concentração específica de cada *primer* obtida na etapa de otimização e 25 ng de cDNA. As condições no equipamento de PCR em tempo real ABI Prism® 7300 (Applied Biosystems) compreenderam uma etapa inicial de 2 min a 50°C, seguida de uma etapa a 95°C por 10 min e 40 ciclos de amplificação a 95°C por 15 seg e 60°C por 1 min. No final, foi acrescentado um passo de dissociação que compreende duas etapas de 15 seg a 95°C, intercaladas por uma etapa de 30 seg a 60°C. Esta etapa tem por objetivo a construção de uma curva padrão para verificar a ocorrência de *primer-dimer*, produtos inespecíficos e contaminações. Na etapa de otimização, para cada gene proposto para este estudo, foi realizada uma reação contendo *primers* com diferentes concentrações,

variando de 0,25 a 1,0 μ M. De acordo com as curvas de amplificação geradas pelo equipamento de PCR em tempo real, foi determinada a melhor concentração a ser utilizada para cada par de *primer* nas reações futuras. A melhor concentração foi estipulada como aquela que primeiro amplifica a amostra em determinado ciclo de amplificação (C_t – *Threshold Cycle*). Este ciclo não deve mudar conforme aumenta a concentração dos *primers*. Desde modo, foram obtidas concentrações de *primer* que amplificam otimamente a amostra, sem o risco de formação de produtos inespecíficos e *primer-dimer*.

Nesta etapa também observou-se a qualidade dos *primers*, sendo que aqueles com curvas de amplificação ruins foram novamente desenhados. Após a etapa de otimização, os *primers* foram validados para um gradiente de concentrações de amostras. O objetivo foi verificar se determinado *primer* é capaz de amplificar com a mesma eficiência amostras com diferentes concentrações. Esta eficiência é medida pela fórmula $E = 10^{(1/-s)} - 1$, onde s é o *slope* da curva padrão, que corresponde à inclinação da reta obtida quando se analisa a variação do C_t dos genes estudados em função do Log de diferentes quantidades de cDNA. A eficiência dos *primers* ficou entre 90 e 100 %. Para os genes *housekeeping* e os genes supressores de tumor *TFF1* e *CLDN18* foram utilizadas amostras com concentrações de 100, 10, 1, 0,1 e 0,01 ng de cDNA, e para os demais genes as concentrações foram de 50, 25, 12,5, 6,25 e 3,12 ng. Para as etapas de otimização e validação foi utilizado um pool de amostras da mucosa normal dos indivíduos deste estudo e para cada *primer* e/ou amostra a reação foi realizada em triplicata.

Após a análise dos genes *housekeeping*, β -actina foi escolhido como referência por apresentar menor variação de C_t nas diferentes amostras e grupos estudados. A expressão relativa do RNAm foi calculada de acordo com o método $2^{-\Delta\Delta C_t}$ descrita por

Livak e Schmittgen (2001). Primeiro, a média dos Ct para cada amostra foi determinada. Depois, o valor de ΔCt foi calculado como sendo a diferença entre o valor de Ct do gene alvo e o valor de Ct da β -actina: $\Delta Ct = Ct (\text{gene alvo}) - Ct (\beta\text{-actina})$. Finalmente, o valor de $\Delta\Delta Ct$ foi calculado: $\Delta\Delta Ct = \Delta Ct (\text{metaplasia, úlcera ou câncer}) - \Delta Ct (\text{mucosa normal adjacente})$.

8. Diagnóstico molecular da *H. pylori*

As amostras de DNA obtidas foram utilizadas para o diagnóstico molecular da bactéria *H. pylori* e, nesta etapa, apenas as amostras referentes às mucosas normais dos pacientes foram avaliadas. Foram utilizados os *primers* específicos para a *H. pylori* referentes aos genes urease A (F- 5' TTCCTGATGGGACCAAACCTC 3' e R- 5' TTACCGCCAATGTCAATCAA 3') e *tsaA* - alquil hidroperóxido redutase (F- 5' CCTGCCGTTTTAGGAAACAA 3' e R- 5' TCCGCATTCCTACCTAATGG 3'), além do gene constitutivo humano *CYP1A1* (F- 5' CTCACCCCTGATGGTGCTAT 3' e R- 5' TTTGGAAGTGCTCACAGCAG 3') para verificar a integridade do DNA e eficiência da reação de PCR, que amplificam fragmentos de 316, 413 e 226, respectivamente. A reação multiplex foi realizada em volume final de 25 μ L contendo 4 mM de $MgCl_2$, 0,3 mM de dNTPs, 0,6 μ M de cada par de *primer*, 1,75 U de Taq DNA polimerase em tampão 1 X. Os produtos da reação foram submetidos à eletroforese em gel de agarose 1000 (Invitrogen) a 2 % e visualizados com coloração de brometo de etídeo.

Tabela 1. Sequência dos *primers* utilizados, desenhados com auxílio do programa *Primer3*.

Gene	Sequência do <i>primer</i>	Tamanho do fragmento
β -actina	TGCCCTGAGGCACTCTTC CGGATGTCCACGTCACAC	101 pb
β 2M	GGGATCGAGACATGTAAGCAG GCAAGCAAGCAGAATTTGG	83 pb
α -tubulina	TCAACACCTTCTTCAGTGAAACG AGTGCCAGTGCGAACTTCATC	150 pb
<i>TERT</i>	CTGGAGCAAGTTGCAAAGC TGCCTGACCTCTGCTTCC	84 pb
<i>COX-2</i>	CATCCTGAATGGGGTGATG GATAGCCACTCAAGTGTTGCAC	109 pb
<i>NOS2</i>	CTTCACCATAAGGCCAAAGG AGCTCATCTGGAGGGGTAGG	119 pb
<i>HGF</i>	TGGCCATGAATTTGACCTC CCAGGGCTGACATTTGATG	121 pb
<i>c-MET</i>	GCAAGCAAAAAGTTTGTCCAC TGGCAAGACCAAAATCAGC	94 pb
<i>k-RAS</i>	AGAGTGCCTTGACGATACAGC TCCCTCATTGCACTGTACTCC	160 pb
<i>TTF1</i>	GGCCCAGACAGAGACGTG ACAGCAGCCCTTATTTGCAC	97 pb

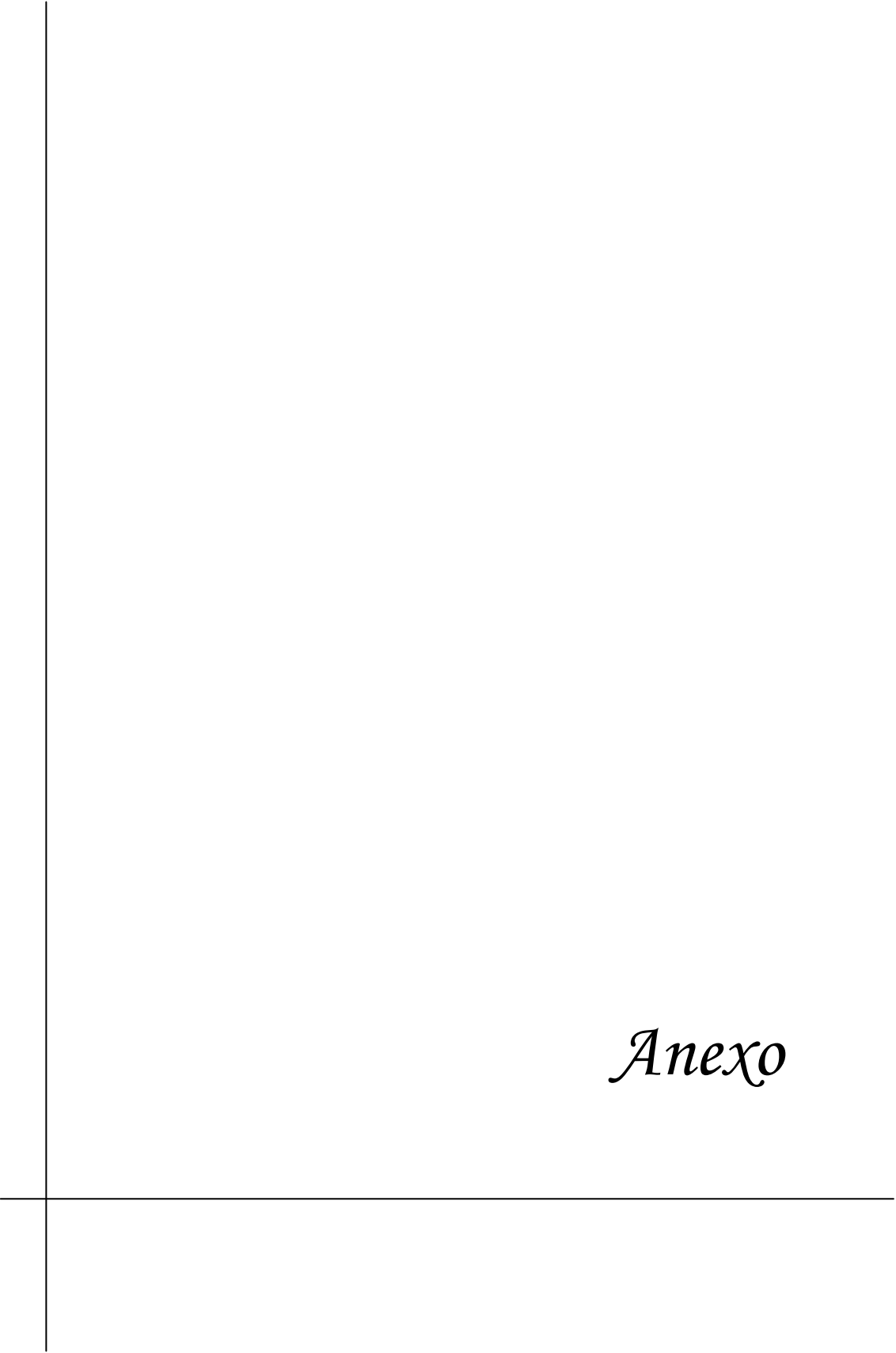
9. Imunohistoquímica

A partir do material emblocado em parafina, correspondente as amostras utilizadas no estudo de expressão gênica, procedeu-se a avaliação imunohistoquímica com a aplicação de anticorpos específicos. Foram feitos cortes histológicos de aproximadamente 4 μ m, montados em lâminas silanizadas. Resumidamente, para silanização de cerca de 300 lâminas foram utilizados 5 mL de organosilano em 250 mL de acetona por cinco minutos, seguidos de dois banhos em água destilada por cinco minutos cada. Posteriormente, as lâminas com os cortes histológicos foram fixados à 37°C *overnight*. Os cortes foram após hidratados utilizando soluções sequenciais de xileno por 5 min (4X), quatro banhos em etanol 100% (3X), etanol 95 % (1X), etanol 80% (1X), etanol 50% (1X) e água destilada (2X). Para a recuperação antigênica, foi utilizado tampão EDTA-TRIS 10mM (pH 9.0) para KRAS e tampão citrato 10mM (pH

6.0) para TERT, COX-2, NOS2, HGF, MET, TFF1 e CLDN18. O tampão foi pré-aquecido por 40 min em uma panela de vapor (Exotic, Arno) até 95°C e então as lâminas foram colocadas por 30 min, com exceção de TERT e COX-2. Para estes anticorpos, a recuperação antigênica foi realizada em autoclave à 120°C por 15 min. Após esfriar em temperatura ambiente por cerca de 20 min, a atividade da peroxidase endógena foi bloqueada com 3% H₂O₂ por 20 min.

Em seguida as lâminas foram incubadas por 1 hora em temperatura ambiente com anticorpos específicos para TERT (mouse monoclonal antibody, clone 2D8, ABR - Affinity BioReagents, 1:100), COX-2 (mouse monoclonal antibody, clone 4H12, Novocastra, 1:100) TFF1 (mouse monoclonal anti-pS2, clone BC04, Invitrogen, 1:50), HGF (Monoclonal anti hepatocyte growth factor antibody, clone 24612.111, Sigma, 1:50) e MET (Anti hepatocyte growth factor receptor -cMet antibody, Sigma, 1:50) e *overnight* a 4°C com anticorpos específicos para NOS2 (Rabbit polyclonal to iNOS, Abcam, 1:200), KRAS (Mouse monoclonal anti-KRAS, clone 9.13, Invitrogen, 1:50) e CLDN18 (Rabbit polyclonal anti-claudin-18 -c-Term, Zymed, Invitrogen, 1:200). Após a lavagem com tampão Tris-HCl (pH 7.6), as laminae foram incubadas, por 20 min cada, com anticorpo secundário biotinilado e com peroxidase estreptoavidina-biotina, seguindo as especificações do fabricante (Histostain Bulk Kit, Zymed).

A marcação foi visualizada com 3,3'-diaminobenzidina (DAB) e contra-corado com hematoxilina de Mayer. Os controles negativos foram estabelecidos substituindo-se o anticorpo primário por solução tampão. Estômago normal foi utilizado como controle positivo para TFF1 e CLDN18, e amígdala, câncer de cólon, fígado, carcinoma de mama, carcinoma de pulmão e carcinoma epidermóide de pulmão foram utilizados como controles positivos para TERT, COX-2, HGF, MET, NOS2 e KRAS, respectivamente.



Anexo

Anexo 1: Parecer consubstanciado do Comitê de Ética em Pesquisa Institucional



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

COMITÊ DE ÉTICA EM PESQUISA - CEP PARECER DO PROJETO DE PESQUISA

IDENTIFICAÇÃO

Nome do pesquisador: Márcia Cristina Duarte
Departamento: Depto de Biologia
Instituição: IBILCE/UNESP – São José do Rio Preto
Projeto: Expressão transcricional e protéica de genes relacionados à atividade da telomerase e ciclo celular em metaplasia intestinal e úlcera gástrica, e associação com *Helicobacter pylori*.

PARECER CONSUBSTANCIADO

O projeto trata-se de um estudo para a investigação da expressão de diferentes genes em lesões gástricas neoplásicas e pré-neoplásicas e também da possível associação entre esses e a presença da bactéria *Helicobacter pylori*. Foi solicitada bolsa de Doutorado e auxílio a pesquisa para a FAPESP. O período de execução será de 48 meses. O estudo será executado no Laboratório de Citogenética e Biologia Molecular e no Laboratório de Estudos Genômicos, ambos do IBILCE/UNESP. O principal objetivo deste estudo é avaliar a expressão dos genes *hTERT*, *WT*, *E2F1*, *E2F3*, *HGF*, *c-MET*, *ANXA1* e *ANXA5* ao nível transcricional e protéico e sua associação com a infecção pela *H. pylori* em amostras de pacientes com metaplasia intestinal (30), úlcera gástrica (30) e adenocarcinoma gástrico (10). Tais amostras serão obtidas a partir de biópsia durante endoscopia (Ambulatório do Hospital de Base) ou de fragmentos cirúrgicos pelo médico responsável (Hospital de Base). As mucosas adjacentes normais também serão coletadas. As amostras correspondentes emblocadas em para fina serão utilizadas (Serviço de Patologia do Hospital de Base) também com a finalidade de verificar a expressão protéica por imuno-histoquímica. O RNA extraído será submetido à técnica de PCR-real time para quantificação dos níveis de RNAm. O DNA de *H. pylori* será também investigado. Após a assinatura do termo de consentimento informado, os pacientes serão entrevistados no momento da inclusão no estudo. Os dados obtidos serão analisados por programas específicos para a análise estatística. As referências citadas são pertinentes, atuais e provenientes de periódicos de circulação internacional. Os nomes dos pacientes serão protegidos por códigos registrados nos questionários e nas fichas clínicas. As informações obtidas serão analisadas em conjunto. Não haverá análise ou relato individual de casos e as amostras serão obtidas em procedimentos diagnósticos ou cirúrgicos previamente indicados, não havendo, portanto, risco em participar do projeto. O projeto apresenta benefícios que se referem à contribuição para o entendimento da carcinogênese gástrica e, portanto, com o futuro levantamento de medidas de prevenção e tratamento do câncer gástrico. A metodologia proposta permitirá a obtenção de dados para o cumprimento dos objetivos propostos. O termo de consentimento e a forma de obtê-lo se encontram adequados. O pesquisador e sua orientadora apresentam produção científica publicada em veículos especializados.

O projeto é relevante, e apresenta-se bem elaborado,

1.

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Datas previstas para apresentação dos relatórios

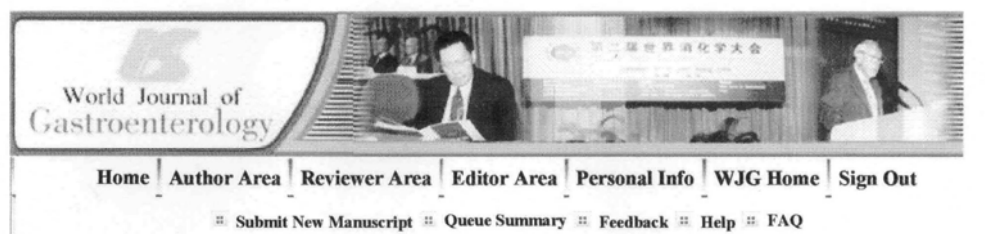
1º relatório: 17/11/2006

2º relatório: 17/11/2007

São José do Rio Preto, 17 de novembro de 2005.

Profa. Dra. Cláudia Regina Bonini Domingos
Coordenadora do CEP

Anexo 2: Comprovante de submissão do artigo “Expression of *TERT* and *COX-2* in precancerous gastric lesions compared to gastric cancer” ao periódico World Journal of Gastroenterology .

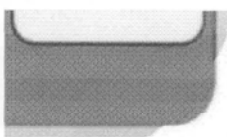


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Expression of hTERT and COX-2 in

Title: precancerous gastric lesions compared to gastric cancer

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