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Polimorfismos gênicos de citocinas e receptores envolvidos no processo inflamatório da carcinogênese gástrica e alterações nos níveis de expressão gênica

Tese apresentada para obtenção do Título de Doutor em Genética

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"Penso noventa e nove vezes e nada descubro; deixo de pensar, mergulho em profundo silêncio e eis que a verdade se revela."

Albert Einstein

Lista de abreviaturas e símbolos:

µg	Micro-grama
µl	Micro-litro
µM	Micro-molar
BMP	do inglês *Bone morphogenetic protein*
bp	do inglês base pairs
C	do inglês control
C	Controle
°C	do inglês celsius grade
CG	Câncer gástrico
CG	do inglês chronic gastritis
CI	do inglês confidence interval
COX-2	do inglês cyclooxygenase-2
CXCL	do inglês chemokine
del	do inglês deletion
EDTA	do inglês *Ethylenediamine tetraacetic acid*
EGFR	do inglês *Epidermal growth factor receptor*
EPHX1	do inglês *Epoxide hydrolase 1, microsomal (xenobiotic)*
ERCC2	do inglês *Excision repair cross-complementing rodent repair deficiency, complementation group 2*
F	do inglês female
GC	Gastrite crônica
GC	do inglês gastric cancer
GRO-α	do inglês *Growth-regulated alpha protein*
GSTT1	do inglês *Glutathione S-transferase theta 1*
IL8	do inglês *Interleukin 8*

IL10	do inglês *Interleukin 10*
IL-1ra	do inglês Interleukin – 1 receptor antagonist protein
IL-1RN	do inglês *Interleukin – 1 receptor antagonist*
ins	do inglês insertion
LPS	Lipopolissacarídeos
LTA	do inglês *Lymphotoxin-alpha; tumor necrosis factor beta*
M	do inglês male
MHC	do inglês major histocompatibility complex
ml	Mili-litro
MTHFR	do inglês *Methylenetetrahydrofolate reductase*
MIP-3α	do inglês *Macrophage inflammatory protein 3 alpha*
NAT-2	do inglês *N-acetyltransferase 2*
NF-κB	do inglês nuclear factor-kappa B
OR	do inglês odds ratio
p	do inglês probability
pb	Pares de bases
PMN	Polimorfonucleares
qPCR	do inglês quantitative polymerase chain reaction
SULT 1A1	do inglês *Sulfotransferase 1A1*
SNPs	do inglês Single nucleotide polymorphisms
T°	do inglês Temperature
TNFA	do inglês *Tumor nuclear factor A*
TNFB	do inglês *Tumor nuclear factor B*
TLR	do inglês *Toll like receptor*
XPA	do inglês *Xeroderma pigmentosum A*
XPC	do inglês *Xeroderma pigmentosum C*

Sumário

RESUMO

O câncer gástrico é uma doença caracterizada como multifatorial associada a fatores ambientais e genéticos. A carcinogênese do estômago pode progredir de uma inflamação crônica da mucosa gástrica, resultante da infecção pela bactéria *Helicobacter pylori* que ativa a resposta inflamatória do hospedeiro. Portanto, selecionamos um grupo de polimorfismos presentes em genes de citocinas pró-inflamatórias (*IL8, TNFA* e *TNFB*), anti-inflamatórias (*IL10* e *IL-1RN*) e receptores de reconhecimento de padrões moleculares associados aos microorganismos, denominados *toll like receptor* (TLR). Considerando a escassez e controvérsia de estudos de polimorfismos desses genes em câncer gástrico e seu envolvimento na carcinogênese de estômago, propôs-se avaliar, pelas técnicas de PCR- alelo específico ou PCR-RFLP, a associação dos polimorfismos *TLR2* -196 a –174 *del, TLR4* (+896 A/G rs4986790 e +1196 C/T rs4986791), *IL-1RN* VNTR, *TNFB* 252A/G (rs909253), *TNFA* (-308 G/A rs1800629 e –857 C/T rs1799724), *IL8* (-251 T/A rs4073 e –845 T/C rs2227532) e *IL10* (-592 C/A rs1800872) com risco de câncer gástrico, gastrite crônica e fatores de risco ambientais. Também, procurou-se associar, pela técnica de qPCR em tempo real, os polimorfismos com os níveis de expressão dos genes das citocinas, cujas variantes ocorrem em regiões promotoras (*TNFA*, *IL8* e *IL10*). De modo geral, a genotipagem dos referidos genes foi realizada em amostras de DNA de 723 indivíduos (207 de câncer gástrico-CG; 276 de gastrite crônica-GC e 246 controles saudáveis-C), enquanto que nas análises de expressão gênica foi utilizado o cDNA de tecido gástrico proveniente de 45 pacientes com CG e 47 com GC. Observou-se que, os SNPs *TLR4*+1196C/T, *TNFB*+252A/G, *TNFA*-308G/A e *IL8*-251T/A não foram associados com o risco de gastrite crônica e câncer gástrico. Contudo, as freqüências dos genótipos *TLR2* ins/del +del/del e *TLR4*+896 AG+GG foram significativamente maiores (p<0,01) no grupo CG (33,5% e 13%, respectivamente) do que no grupo C (17,9% e 6,5%, respectivamente). Também foi observado que o haplótipo G-C da combinação *TLR4*+896A/G+1196C/T (p=0.02) e a combinação dos alelos variantes *TLR2del/TLR4*+896G (p=0,02) associaram-se com a suscetibilidade ao câncer gástrico. Para o *IL1-RN* VNTR, suas frequências genotípicas e alélicas foram maiores no grupo CG (58% e 0,37, p <0,01, respectivamente) e GC (48% e 0,30, p <0,01, respectivamente) do que no grupo C (35% e 0,20%, respectivamente). Da mesma forma, diferenças estatisticamente significantes também foram encontradas para as frequências genotípicas e alélicas dos SNPs *TNFA*-857C/T (p<0,01 e p =0,02, respectivamente), *IL8*-845T/C (p<0,01) e *IL10*-592C/A (p<0,01) entre os grupos de CG *versus* C. Além do mais, as combinações dos polimorfismos *TNFA*-308T/A*NFA*-857T/*IL8*-251A/*IL8*-845C; *TNFA*-308T/T*NFA*-857T/*IL8*-845C/*IL10*-592A e *TNFA*-308A/ *IL8*-251A/ *IL8*-845C/ *IL10*-592A demonstraram maior risco relativo para o câncer gástrico, com OR de 9,33, 8,00 e 8,75, respectivamente. A análise de regressão logística múltipla demostrou associação de fatores como sexo masculino, idade avançada e consumo de álcool, mas não se encontrou associação com infecção pela *H. pylori.* No estudo de expressão gênica, não se obteve resultados para o gene *TNFA*, devido falha na etapa de validação dos primers, enquanto os genes *IL8* e *IL10*, de modo geral, mostraram níveis de expressão do RNAm reduzidos em ambos grupos CG e GC. Portanto, comparando os níveis de RNAm, houve diferença estatisticamente significante apenas para o gene *IL10*, entre os grupos CG e GC (p<0.01). Quando as amostras foram agrupadas de acordo com os polimorfismos *IL8*-845T/C e *IL10*-592 A/C, pôde-se observar a influência das variantes polimórficas na expressão do RNAm destas citocinas. Portanto, estes resultados evidenciam que os polimorfismos de genes de mediadores inflamatórios como *TLR2* del, *TLR4*+896A/G, *IL-1RN*, *TNFA*-857C/T , *IL8*-845T/C e *IL10*-592 A/C influenciam na suscetibilidade individual para o desenvolvimento do câncer gástrico na população brasileira avaliada. Também é evidente a relevância funcional das variantes polimórficas nas regiões reguladoras *IL8*-845C e *IL10*-592A na modulação da expressão gênica, reforçando a importância do papel destas citocinas na carcinogênese do estômago.

Palavras-chave: câncer gástrico; gastrite crônica; citocinas; receptores de padrões microbianos; polimorfismos; expressão gênica.

ABSTRACT

Gastric cancer is a multifactorial disease characterized as associated with environmental and genetic factors. The carcinogenesis of the stomach can progress to a chronic inflammation of the gastric mucosa, resulting from infection by the bacterium *Helicobacter pylori* that activates the inflammatory response of the host. Therefore, we selected a group of polymorphisms in genes of pro-inflammatory cytokines (*IL8*, *TNFA* and *TNFB*), anti-inflammatory (*IL10* and *IL-1RN*) and receptor recognition of molecular patterns associated with microorganisms, the toll like receptors (TLR). Considering the scarcity and controversy of these polymorphisms studies in gastric cancer and their involvement in carcinogenesis of the stomach, this study proposed to evaluate, by PCR allele-specific or PCR-RFLP the association of polymorphisms *TLR2* -196 to -174 del , *TLR4* (rs4986790 and rs4986791), *IL-1RN* VNTR, *TNFB* 252A/G (rs909253), *TNFA* (rs1800629 and rs1799724), *IL8* (rs4073 and rs2227532) and *IL10* (rs1800872) with risk of gastric cancer, chronic gastritis and environmental risk factors. Also, we tried to associate, for the technique of real-time qPCR, polymorphisms with levels of expression of cytokine genes whose variations occur in the promoter region (*TNFA*, *IL8* and *IL10*). Overall, the genotyping of these genes was performed on DNA samples from 723 individuals (207 gastric cancer-GC, 276 chronic gastritis-CG, and 246 healthy controls-C), whereas in the analysis of gene expression was used cDNA of gastric tissue from 45 patients with GC and 47 CG. It was observed that *TLR4* SNPs +1196C/T, *TNFB* +252A/G, *TNFA*-308G/A and *IL8*-251 T/A were not associated with risk of chronic gastritis and gastric cancer. In the analysis of polymorphisms of toll like receptor, the frequencies of genotypes *TLR2* ins / del + del / del and *TLR4* +896 AG were significantly higher (p <0.01) in GC group (33.5% and 13% respectively) than in C group (17.9% and 6.5% respectively). We also observed that the G-C haplotype combination of *TLR4* +896 A/G +1196 C/T (p=0.02) was associated with susceptibility to gastric cancer. For the *IL1-RN* VNTR, their genotypes (L/2 and 2/2) and allele frequencies were significantly higher in GC group (58% and 0.37, p <0.01, respectively) and CG (48% and 0.30, p <0.01, respectively) than in C group (35% and 0.20% respectively). Likewise, statistically significant differences were also found for the genotype and allele frequencies of *TNFA*-857C/T (p <0.01 and p = 0.02, respectively), *IL8*-845T/C (p <0.01) and *IL10*-592C/A (p <0.01) SNPs, between groups of GC versus C, whereas the group of chronic gastritis showed genotype and allele frequencies significantly higher than the control group only for the *IL10*-592C/A (p <0.01) SNP. Moreover, the combinations of genotypes *TNFA*-308A/*TNFA*-857T/*IL8*-251A/*IL8*-845C; *TNFA*-308A/*TNFA*-857T/*IL8*-845C/*IL10*-592A and *TNFA*-308A/*IL8*-251A/*IL8*- 845C/*IL10*-592A showed a higher relative risk for gastric cancer, with OR of 9.33, 8.00 and 8.75, respectively. A multiple logistic regression analysis demonstrated association of factors such as male gender, older age and alcohol consumption, but no association with infection by *H. pylori*. In the study of gene expression, the results were not for the *TNFA* gene, due to failure in the validation phase of the primers, whereas *IL8* and *IL10* genes, in general, showed reduced levels of mRNA expression in both groups of GC and CG. Therefore, comparing the expression levels showed a statistically significant only for the *IL10* gene, between the GC and CG groups (p <0.01). When samples were grouped according to the *IL8*-845T/C and *IL10*-592 A/C polymorphisms, one could observe the influence of polymorphic variants in the mRNA expression of these cytokines. Therefore, these results suggest that polymorphisms *TLR2* del, *TLR4* +896 A/G, *IL-1RN*, *TNFA*-857C/T, *IL8*-845T/C and *IL10*-592 A/C inflammatory mediators genes influence the individual susceptibility to the development of gastric cancer in our population studied. It is also evident the functional relevance of polymorphic variants in regulatory regions *IL8* -845C and *IL10*-592A in the modulation of gene expression, reinforcing the importance of the role of cytokines in carcinogenesis of the stomach.

Keywords: gastric cancer, chronic gastritis, cytokines, receptors for microbial standards; polymorphisms, gene expression.

Introdução

I. Introdução

I.1- Câncer gástrico

O câncer gástrico apresenta-se como um dos problemas mais sérios de saúde em vários países, apesar de ser o quarto tipo mais comum de neoplasia mundial representa a segunda principal causa de morte relacionada ao câncer (TAKAISHI; OKUMURA; WANG, 2008). No Brasil, segundo o Instituto Nacional do Câncer, estimaram-se para o ano de 2010, 21.500 casos novos de câncer de estômago, sendo 13.820 entre os homens e 7.680 casos entre as mulheres (INCA, 2011). Esse dado é realmente preocupante uma vez que quando diagnosticado em estágio avançado, a doença é usualmente incurável (ZHANG et al., 2008).

O estômago é um órgão sacular dividido em quatro regiões anatômicas, a cárdia, o fundo, o corpo e o antro. Ele está ligado superiormente ao esôfago pela região da cárdia e, inferiormente ao duodeno pelo esfíncter pilórico. A glândula gástrica típica é composta por três tipos de células: células mucosas; células principais, que secretam grandes quantidades de pepsinogênio e células parietais, que secretam ácido clorídrico e fator intrínseco (Figura 1). A principal característica da fisiologia gástrica é a secreção de ácido clorídrico, além da função do muco na proteção contra lesões (ROBBINS; COTRAN, 2005).

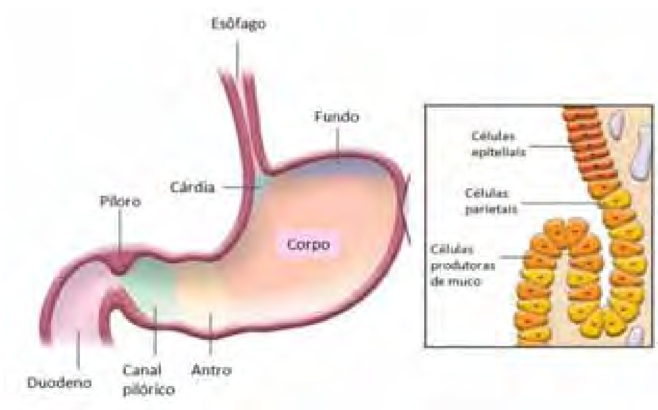

Figura 1. Representação das regiões anatômicas e histológicas do estômago. Modificado de Fox e Wang (2007).

A carcinogênese do estômago apresenta etiologia complexa, na qual fatores genéticos e ambientais estão envolvidos, caracterizando um modelo multifatorial. Entre estes últimos, está o consumo elevado de sal e álcool, fumo, cirurgia gástrica anterior, infecção pela bactéria *Helicobacter pylori* e histórico de lesões gástricas (KONTUREK et al., 2009; WU et al., 2010). O câncer gástrico progride por meio de um processo de múltiplas etapas, com a participação de lesões gástricas com potencial pré-canceroso como gastrite crônica, atrofia gástrica, metaplasia intestinal e displasia (CORREA, 2004).

Na gastrite crônica, as lesões vão desde processo inflamatório superficial até a atrofia do epitélio. Aproximadamente 10% dos pacientes com atrofia gástrica desenvolvem adenocarcinoma em um período de 15 anos (CHELI; GIACOSA, 1983), motivo pelo qual é considerada uma lesão pré-maligna (GENTA, 1998).

O adenocarcinoma representa aproximadamente 95% dos casos de neoplasias malignas gástricas, sendo classificado com base em aspectos histopatológicos e clínicos em dois tipos: intestinal e difuso (LAUREN, 1965). As lesões do tipo intestinal são as mais frequentes, bem diferenciadas, localizadas na região do corpo e antro gástrico, dependentes de fatores ambientais e associadas com a presença de lesões pré-cancerosas. São encontradas predominantemente em homens e indivíduos idosos. O tipo difuso é pouco diferenciado e encontrado principalmente na região da cárdia, tem prognóstico ruim e não está associado a lesões pré-cancerosas. É um pouco mais frequente em mulheres e pacientes jovens e tem alta ocorrência familial, principalmente entre indivíduos com tipo sangüíneo A (TAHARA, 2004; FOX, WANG, 2007; INCA, 2011).

No curso de múltiplas etapas da carcinogênese do estômago, várias alterações genéticas em oncogenes, genes supressores de tumor, genes de reparo do DNA, reguladores do ciclo celular e moléculas de adesão celular são acumuladas acompanhando as alterações morfológicas do tecido. Adicionalmente, também são relatadas instabilidade genética e alterações em fatores de crescimento, bem como em citocinas e receptores celulares, que podem contribuir para a carcinogênese do estômago. Muitas vias oncogênicas estão bem estabelecidas, como aquelas mediadas por reguladores do ciclo celular (ciclina E, ciclinas D1 e D2), fator nuclear- κB, cicloxigenase-2 (COX-2) e receptor de fator de crescimento epidermal (EGFR). Entretanto têm surgido evidências da importância de outras vias, incluindo o fator transformante de crescimento (TGF-β) e proteínas morfogenéticas do osso (BMP), ligados a indução de metástase, como também as vias de sinalização da Wnt/ β-catenina, *Hedgehog e Notch* relacionadas à diferenciação intestinal das células epiteliais gástricas e motilidade e agressividade tumoral, respectivamente (SMITH et al., 2006; WU et al., 2010 para revisão).

I.2- Helicobacter pylori

A colonização do estômago pela *H. pylori* representa um fator etiológico importante da carcinogênese gástrica e, desde 1994, esta bactéria é considerada pela Organização Mundial de Saúde um carcinógeno classe I (IARC, 1994). Este patógeno encontra-se envolvido com o processo inflamatório da mucosa do estômago, e portanto, associado com a gastrite crônica, as úlceras pépticas, a metaplasia intestinal e com os adenocarcinomas do estômago (DAWN; PEEK, 2006). O principal evento patofisiológico da infecção pela *H. pylori* na mucosa gástrica é a indução do processo inflamatório, seguido de infiltrado de neutrófilos, células mononucleares e seus produtos. Este ambiente estimula a transcrição e síntese de diversas citocinas pró- e anti-inflamatórias e caso não ocorra um controle apropriado desta resposta imune pode-se desencadear a carcinogênese gástrica (SUGIMOTO; YAMAOKA; FURUTA, 2010).

Esta bactéria é o principal agente etiológico em 95% dos casos de gastrite crônica e em 20% das úlceras gástricas e tem sido relacionada com o aumento do risco de câncer em até nove vezes (KUIPERS, 1999; SCHOLTE et al., 2002). Cerca de 60-80% dos casos de câncer gástrico e 90% das metaplasias intestinais têm um diagnóstico molecular positivo para *H. pylori*, entretanto, a infecção por este agente não parece ser um fator intrínseco para o desenvolvimento do câncer, cujo risco é de apenas 1-2% para os indivíduos infectados (SCHOLTE et al., 2002). Sugerindo que fatores adicionais, como fatores genéticos do hospedeiro, fatores ambientais, bem como a diversidade da virulência dos genes da *H. pylori* possam estar envolvidos na tumorigênese do câncer gástrico (ANDO et al., 2009).

O conhecimento sobre o papel da *H. pylori* na tumorigênese gástrica é ainda incompleto. Sabe-se que esta bactéria possui uma enzima, a urease, que permite a hidrólise da uréia gástrica em amônia e dióxido de carbono. Isto permite que a *H. pylori* mantenha um pH alcalino na superfície da mucosa gástrica (STOICOV et al., 2004; SCOTT et al., 2007). A vasta maioria destes bacilos que colonizam os hospedeiros é de vida livre, porém 20% se ligam fortemente às células epiteliais gástricas, podendo induzir mudanças ultra-estruturais nestas (SMITH et al., 2006).

Também existem evidências substanciais indicando que diferenças genéticas do parasita têm um papel no resultado clínico da infecção pela *H. pylori*, particularmente de genes relacionados à virulência como, *cag*A, *vac*A, *ice*A, *bab*A e o *dup*A (SMITH et al., 2006; HUSSEIN, 2010).

O gene *cag*A faz parte da chamada ilha de patogenicidade e as linhagens positivas para este gene estão associadas com úlceras pépticas, gastrites atróficas e câncer gástrico, além da expressão desta proteína estar associada com a redução do processo apoptótico (STOICOV et al., 2004). Outro gene relacionado à virulência, o *vac*A, é responsável pela codificação de uma citotoxina vacuolizante, que induz a formação de vacúolos em células eucarióticas e estimula a apoptose celular.

O gene *bab*A codifica uma outra proteína de membrana, BabA, que se liga ao receptor Lewis B das células gástricas e há evidências significantes indicando que o acúmulo da expressão desta proteína pode influenciar na gravidade da doença (SMITH et al., 2006). O gene *ice*A também tem sido estudado em cepas de *H. pylori* sendo suas principais variantes alélicas a *ice*A1 e a *ice*A2. A expressão é aumentada pelo contato da *H. pylori* com células epiteliais gástricas. Cepas *H. pylori* positivas para *ice*A1 mostram associação com desenvolvimento de úlcera péptica e secreção aumentada de IL8 pela mucosa gástrica, enquanto uma maior prevalência de cepas *ice*A2 positivas são observadas em pacientes com dispepsia não-ulcerativa (HUSSEIN, 2010). O gene recentemente descoberto como novo fator de virulência, o *dup*A (gene promotor de úlcera duodenal), têm sido associado ao aumento da secreção da IL8 pelas células epiteliais e alguns estudos o relacionam ao desenvolvimento de câncer gástrico (HUSSEIN, 2010). Contudo, os mecanismos moleculares pelos quais a *H. pylori* participa provocando desregulação nos processos de proliferação celular, apoptose e resposta imune pelo hospedeiro precisam ser melhores compreendidos.

I.3- Fatores genéticos do hospedeiro

Também é conhecido que fatores genéticos do hospedeiro podem aumentar o risco de câncer gástrico (AL-MONDHRI et al., 2006), portanto, é possível estabelecer uma relação entre câncer gástrico e os polimorfismos de uma variedade de genes humanos, os quais podem modificar os efeitos decorrentes da exposição natural ao ambiente, diminuindo ou aumentando a suscetibilidade dos indivíduos a tumorigênese (NARDONE; MORGNER, 2003).

Os polimorfismos genéticos são comuns e apresentam baixo risco absoluto de ocasionar o câncer, porém, por modularem a suscetibilidade individual ao desenvolvimento de neoplasias e dependerem fortemente de fatores ambientais, possuem um risco populacional elevado (KIBERSTIS; ROBERTS, 2002). Grande parte dos polimorfismos gênicos apontados na epidemiologia do câncer possui relação com a codificação de enzimas envolvidas no metabolismo químico, no mecanismo de reparo do DNA, resposta inflamatória, regulação de proliferação celular, adesão celular e crescimento vascular (WÜNSCH FILHO; ZAGO, 2005).

Em pacientes com câncer gástrico, alguns polimorfismos têm sido relatados associados com risco aumentado para a doença e estudados como possíveis biomarcadores para predição desta neoplasia (GAO; NIETERS; BRENNER, 2009). Estes polimorfismos estão relacionados a diversas vias moleculares que participam da carcinogênese, como o *MTHFR* C677T envolvido no metabolismo do folato (DONG et al, 2007), genes de reparo do DNA (*XPA, XPC, ERCC2*) que possuem um importante papel no reparo ao dano no DNA causado pelo processo inflamatório desencadeado pela *H. pylori* (DONG et al, 2008). Pode-se ainda citar alguns polimorfismos em genes como os *GSTT1, SULT1A1, NAT2* e *EPHX1* importantes na ativação e detoxificação de hidrocarbonetos policíclicos aromáticos (BOCCIA et al., 2007), *TERT* importantes na manutenção dos telômeros (DUARTE et al., 2011), genes envolvidos no processo inflamatório como *NOS2* (JORGE et al., 2010) e também genes de citocinas pró-inflamatórias (*IL-1B* e *TNFA*) e anti-inflamatórias (*IL-1RN*), quimiocinas (*IL8*) (EL-OMAR et al., 2000; ZHANG et al., 2008) e receptores do sistema imune (*TLRs,* receptores *"toll like"*) (HOLD et al., 2007), importantes no padrão de resposta inflamatória desencadeada pela *H. pylori.* Esses polimorfismos demonstram a importância dos carcinógenos ambientais junto à suscetibilidade para certos perfis genéticos individuais (Figura 2) (MILNE et al., 2009; LAO-SIRIEIX; CALDAS; FITZGERALD, 2010 para revisão).

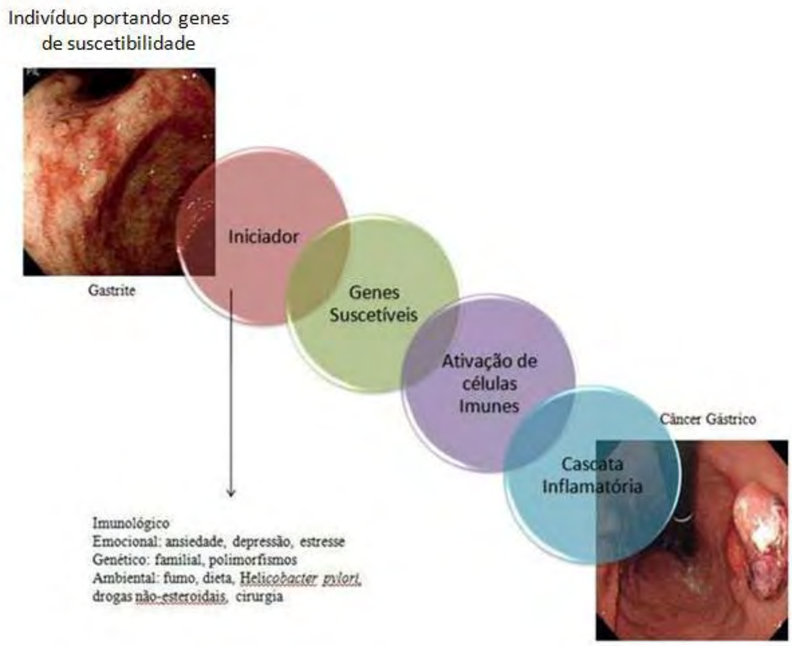

Figura 2. Inflamação e câncer. Diversas causas têm sido sugeridas na iniciação tumoral gástrica, entre elas a infecção pela *H. pylori*, drogas (fumo e álcool), alterações genéticas e ambientais. Indivíduos portadores de genes de suscetibilidade ao câncer gástrico são expostos a fatores como os descritos acima, a ativação do sistema imune desencadeia um processo inflamatório (gastrite) na mucosa gástrica. A persistência desta inflamação (gastrite crônica) pode levar ao câncer gástrico. Modificado de Hong et al. (2010).

I.4- Inflamação e câncer

Já há alguns anos, estudos epidemiológicos têm revelado que inflamações crônicas predispõem para diferentes tipos de cânceres (BALKWILL; MANTOVANI, 2001; COUSSENS; WERB, 2002, HONG et al, 2010). É conhecido que as células neoplásicas são capazes de atrair diferentes tipos celulares para o microambiente tumoral pela secreção de proteases extracelulares, fatores pró-angiogênicos e citocinas (MACARTHUR; HOLD; EL-OMAR; 2004). Condições inflamatórias afetando órgãos como o fígado, pâncreas, estômago, cólon e próstata são associadas com risco aumentado de câncer e pesquisas genéticas têm demonstrado o papel dos componentes chaves da inflamação na carcinogênese. Estes incluem citocinas inflamatórias primárias (IL-1, TNF), IL6, fatores de crescimento hematopoiéticos (M-CSF) e fator de transcrição NF-κB (GRETEN et al., 2004; PIKARSKY et al., 2004; KARIN, 2006; LEE; HAHN, 2008).

Polimorfismos em regiões reguladoras e funcionais de genes das citocinas e receptores do sistema imune influenciam a sua expressão e o perfil de resposta aos agentes infecciosos podendo contribuir para variações no risco de câncer gástrico (CRUSIUS et al., 2008). Um excelente exemplo disto é o papel destes polimorfismos na patogenia do câncer gástrico induzido pela *H. pylori*. A infecção por esta bactéria causa inflamação da mucosa gástrica com infiltração de células inflamatórias, cuja distribuição e agressividade variam dependendo do perfil genético do hospedeiro, podendo assim afetar o resultado clínico e progressão para doenças mais graves (MILNE et al., 2009).

Os receptores conhecidos como *Toll like* (TLR), que reconhecem padrões moleculares associados aos microorganismos, são importantes membros da resposta imune inata, e seus genes têm sido descritos como polimórficos. Esta variação genética pode ser vantajosa em nível populacional, mas pode tornar alguns indivíduos com genótipos associados à ativação excessiva da resposta imune e inflamação, propícios a desenvolver outras doenças, incluindo o câncer (EL-OMAR; NG; HOLD, 2008; SABROE et al., 2008 para revisão).

Entre eles, têm sido demonstrados que os TLR2 (receptor de lipoproteínas bacterianas) e o TLR4 (receptor de lipopolissacarídeo-LPS) estão envolvidos na resposta a infecção pela *H. pylori* (Figura 3). Ambos, TLR2 e TLR4, medeiam a ativação de fatores de transcrição, principalmente via NF-κB, resultando na inflamação. Estes receptores são proteínas de membrana tipo I e um de seus componentes inclui uma estrutura com repetição rica em leucina (RRL), que está envolvida na ligação protéica. Por meio dessa estrutura os TLRs reconhecem os padrões moleculares associados aos patógenos. Seus componentes intracelulares são associados com o domínio Toll/IL-1R, que se assemelham aos receptores da interleucina 1. Atualmente, dez tipos de TLRs têm sido encontrados em humanos (SEYA et al., 2010).

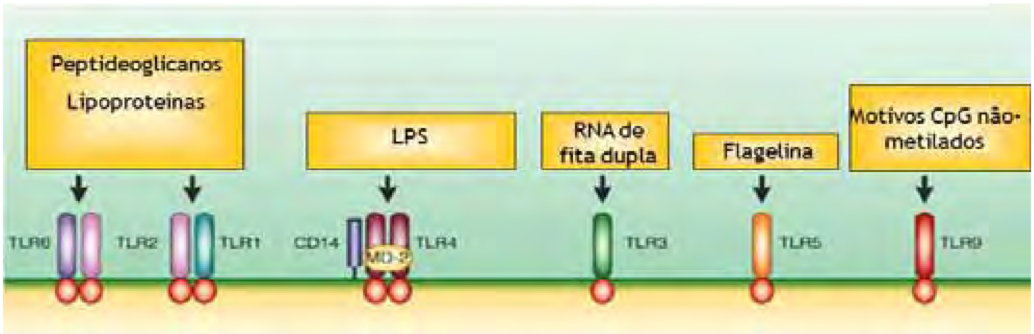

Figura 3. Alguns tipos de TLRs humanos e os padrões microbianos, os quais estes receptores reconhecem. Modificado de Medzhitov (2001).

Há evidências que TLR2 é expresso em células epiteliais gástricas infectadas pela *H. pylori*. Após transfecção com TLR2, células infectadas com a bactéria *H. pylori,* tiveram o fator de transcrição NF-κB ativado e um conseqüente aumento da expressão de IL8. Estes resultados demonstraram que o TLR2 pode ter um papel significante na imunidade da mucosa gástrica após infecção pela *H. pylori* (SMITH et al., 2003).

Polimorfismos de nucleotídeo único (SNPs, Single-Nucleotide Polymorphisms) em *TLR2* têm sido associados à suscetibilidade para várias doenças inflamatórias e infecciosas, como lepra (KANG; CHAE, 2001), aumento do risco de sepse Gram-negativa (SCHRODER et al., 2003) e infecções bacterianas recorrentes em crianças (KUTUKCULER et al., 2007). Em adição, outros estudos recentes têm relacionado polimorfismos neste gene com câncer colorretal e cervical (BORASKA- JELAVIC et al., 2006; PANDEY et al., 2009). O polimorfismo correspondente a uma deleção de 22 pares de bases na posição -196 a -174 da região promotora do *TLR2* tem sido relatado por alterar a atividade promotora, diminuindo os promotores responsivos (NOGUCHI et al., 2004). Na população japonesa, tal polimorfismo foi associado com maior suscetibilidade ao câncer gástrico não-cardia (TAHARA et al., 2007) e metaplasia intestinal (TAHARA et al., 2008)

Da mesma forma, o TLR4, responsável pela imunidade inata contra bactérias Gram-negativas, é mais importante no contexto de macrófagos/monócitos ou células dendríticas. Este receptor também pode ser ativado por substâncias endógenas produzidas durante estresse ou dano celular, como exógenos (bacterianos). Portanto, polimorfismos genéticos deste receptor podem ter um importante impacto no processo inflamatório. Em condições infecciosas, estas variações podem levar a uma baixa resposta ao LPS, redução da densidade epitelial do TLR4 e resposta de citocinas inflamatórias (DREXLER; FOXWELL, 2010).

Alguns polimorfismos de *TLR4* têm sido implicados com maior suscetibilidade a diversas doenças, como aterosclerose (KIECHL et al., 2003), asma (HOLD et al., 2007), malária (MOCKENHAUPT et al., 2006) e também infecção pela bactéria *H. pylori* (LORENZ; FREES; SCHWARTS, 2001). Assim, dois SNPs no gene *TLR4*+896 A/G e

+1196 C/T, têm recebido especial atenção em alguns estudos, embora os dados sejam controversos (EL-OMAR; NG; HOLD, 2008).

A potente resposta inflamatória induzida pelos *tolls* muitas vezes são protetoras e erradicam o microorganismo. Entretanto se a inflamação persistir pode resultar em doenças autoimunes, inflamatórias e câncer. Os TLR2 e TLR4 implicados no reconhecimento da bactéria *H. pylori* no estômago, como já destacado, são os iniciadores da resposta inflamatória contra esta bactéria, assim desencadeando uma rede de mediadores da inflamação, como as citocinas inflamatórias IL-1β, TNF-α e TNF-β, quimiocinas como IL8 e de citocinas anti-inflamatórias como IL10. O desbalanço entre estes componentes, promovido pela *H. pylori,* pode levar a uma inflamação crônica e tumorigênese gástrica (Figura 4) (SABROE et al., 2008; DREXLER et al., 2010).

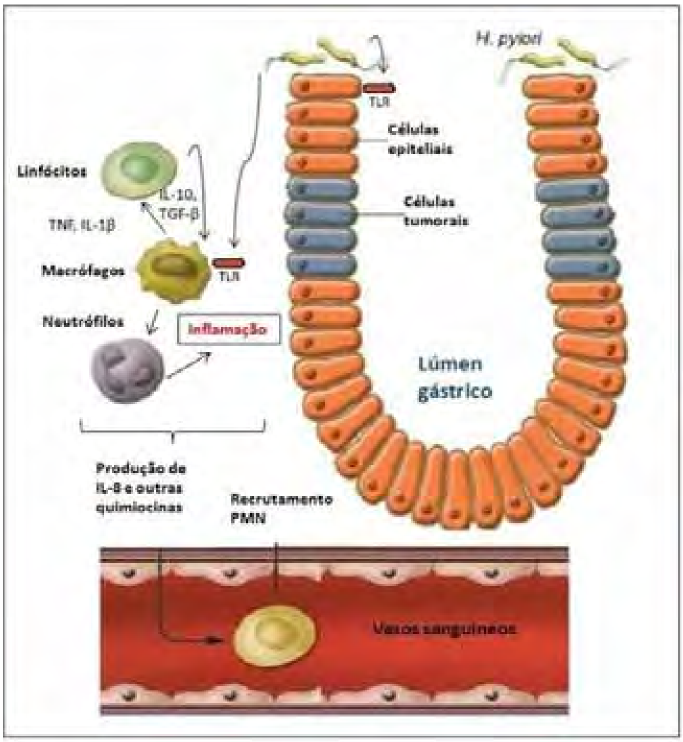

Figura 4. Perfil da resposta imune desencadeada pela *H. pylori* e iniciação tumoral. Reconhecimento de padrões moleculares (LPS, lipopolissacarídeo; lipoproteínas) da bactéria pelos receptores do tipo *toll like* (TLR), posterior recrutamento de células inflamatórias, liberação de citocinas pró-inflamatórias (TNF-α, TNF-β, IL-1β e IL8) e hipoexpressão de citocinas anti-inflamatórias. PMN, polimorfonucleares. Modificado de Fox e Wang (2007).

Alguns pesquisadores têm focado seus estudos na interleucina 1β (IL-1β) como um fator chave do hospedeiro envolvido na tumorigênese associada à infecção pela *H. pylori*, e têm examinado polimorfismos isolados do gene da *IL-1β* e do antagonista do seu receptor *IL-1RN* (MACARTHUR; HOLD; EL-OMAR, 2004; ALLAVENA et al., 2008 para revisão).

No *cluster* da interleucina-1 localizado no cromossomo 2q, além do gene *IL-1β* que codifica a citocina pró-inflamatória IL-1β há mais dois genes relacionados, *IL-1A* e *IL-1RN* que codificam as citocinas IL-1α e o antagonista do receptor endógeno da interleucina 1β (IL-1ra), respectivamente. O gene *IL-1RN* tem uma repetição penta alélica em tandem (VNTR, Variable Number of Tandem Repeats) de 86 pb, resultando em um alelo curto (IL-1RN*2, com duas repetições) ou alelos longos (IL-1RN*L, com três a seis repetições). IL-1ra é uma citocina anti-inflamatória que competitivamente se liga aos receptores de IL-1β, mas não induz uma resposta, entretanto modulam os efeitos danosos potenciais desta interleucina (EL-OMAR et al., 2000; FENG et al., 2008; XUE et al., 2010). O alelo 2 (IL-1RN*2) está associado com inflamações crônicas, condições auto-imunes e aumento da secreção da IL-1β (PEREZ-PEREZ et al., 2005). Alguns estudos têm demonstrado uma associação significante do alelo IL-1RN*2 com risco aumentado de câncer gástrico, embora os dados sejam controversos (EL-OMAR et al., 2000; MACHADO et al., 2001; VANVAKOPOULOS et al., 2002; GARZA-GONZALEZ et al., 2005; PEREZ-PEREZ et al., 2005; MELO-BARBOSA et al., 2009).

El-Omar et al. (2000) foram os primeiros a demonstrar a associação dos polimorfismos gênicos da IL-1β e IL-1ra com maior risco de câncer gástrico e suas lesões precursoras. Posteriormente, Perez-Perez et al. (2005) evidenciaram associação do genótipo homozigoto para o alelo curto *IL-1RN**2 com risco aumentado para este tipo de câncer, assim como do polimorfismo *IL-1β* -511T. Estes autores também demonstraram que a combinação de genótipos de risco do hospedeiro e da bactéria pode ser útil para definir um perfil genético específico de suscetibilidade ao câncer gástrico.

Além da associação com câncer gástrico, o alelo curto *IL-1RN**2 tem sido associado com vários resultados clínicos em diversas doenças inflamatórias, incluindo lúpus eritematoso sistêmico (BLAKEMORE et al., 1994), colite ulcerativa (MANSFIELD; HOLDEN; TARLOW, 1994), alopecia (CORK et al., 1995, BARAHAMANI et al., 2002) e câncer de próstata (LINDMARK et al., 2005). Há também relatos de uma associação do alelo *IL-1RN**2 e produção aumentada de IL-1β *in vitro,* assim podendo ter um papel importante no agravamento do processo inflamatório (SENO et al., 2007).

Em adição, muitas outras citocinas estão envolvidas no processo inflamatório em resposta a infecção pela *H. pylori* e têm importante papel no desenvolvimento da gastrite ou câncer gástrico (SENO et al., 2007). SNPs em diversos outros genes como o fator de necrose tumoral (TNF), as interleucinas IL8 e IL12 e genes de citocinas anti-inflamatórias IL10 e IL4 têm sido associadas com maior suscetibilidade ao câncer de estômago (CRUSIUS et al., 2008).

Os TNF-α e TNF-β ou linfotoxina-A (LT-A), citocinas pro-inflamatórias, pertencem à superfamília TNF e são codificados na mesma região cromossômica do complexo principal de histocompatibilidade (MHC) em 6p21.3 (PEREZ-PEREZ et al., 2005). TNF-α é secretada principalmente por macrófagos e está envolvida na regulação de diversos processos biológicos incluindo proliferação celular, diferenciação, apoptose, metabolismo lipídico e coagulação (SUZUKI et al., 2009; GHOSH et al., 2010). Enquanto TNF-β, produzida principalmente por linfócitos T estimulados, tem um papel importante na regulação da resposta imune (GOEDDEL et al., 1986). Estas citocinas têm sido implicadas em uma variedade de doenças, incluindo as autoimunes (HAVEMOSE-POULSEN et al., 2005; SORENSEN et al., 2009; LIANG et al., 2010) e o câncer (ZHANG et al., 2008; KOHAAR et al., 2009; KUMAR et al., 2010; GUO et al., 2010).

O nível de expressão do gene *TNF-α* parece ser fortemente influenciado por polimorfismos em sua região promotora, como *TNFA* -238 G/A, -308 G/A, -857 C/T, -863 C/A e -1031 T/C (SUGIMOTO et al.; 2007a; ZHANG et al.; 2008). Dentre eles, o alelo A do polimorfismo *TNFA* -308 e o alelo T do polimorfismo *TNFA*-857 estão associados com aumento da atividade transcricional e consequente aumento da produção de citocina. Esta expressão aumentada também tem sido demonstrada *in vitro* para os polimosfismos *TNFA* -308 G/A e *TNFB* +252 A/G (BOUMA et al., 1996). Também há estudos que mostram que o alelo *TNFB* +252 G está associado a uma maior atividade biológica da linfotoxina-α (OZAKI et al., 2002) e uma maior concentração de proteína C-reativa (PCR), um biomarcador de inflamação (SUZUKI et al., 2004; CLARKE et al., 2006). Portanto estes polimorfismos têm sido pesquisados como um potencial fator de suscetibilidade ao câncer de estômago (ZHANG et al., 2008).

Outro grupo de citocinas importante na carcinogênese são as quimiocinas, que compreendem mediadores com habilidade de migração e ativação leucocitária, podendo estimular células que liberam enzimas proteolíticas, permitindo a digestão da matriz extracelular e providenciando uma via para a migração de células inflamatórias, crescimento tumoral e metástase. Uma quimiocina que tem sido implicada na carcinogênese é a IL8 que apresenta um papel crítico na angiogênese e progressão tumoral, cujo gene esta mapeado em 4q13-q21 (SPARMANN; BAR-SAGI, 2004). Esta quimiocina é secretada por monócitos, macrófagos, neutrófilos, fibroblastos, queratinócitos e outros tipos de células (DAN et al., 2010). Estudos prévios têm revelado que polimorfismos no gene *IL8* estão associados com diversas doenças, como bronquiolite viral (HULL; THONSON; KWIATKOWSKI, 2000), síndrome respiratória aguda (HILDEBRAND et al., 2007) e câncer gástrico (TAGUCHI et al., 2005), atrofia gástrica e metaplasia intestinal (LI et al., 2010).

Da mesma forma, muitas citocinas anti-inflamatórias, como IL10 e IL4 também têm sido associadas com o câncer. Por exemplo, a IL10, cujo gene está localizado na região cromossômica 1q31-q32, é secretada por células tumorais e macrófagos e entre outros efeitos inibe células T citotóxicas, suprimindo assim a resposta imune anti-tumoral, demonstrando sua importância para a iniciação e progressão do câncer (COUSSENS; WERB, 2002). Por exemplo, camundongos deficientes de IL10 desenvolvem gastrite atrófica grave e enterocolite crônica (KUHN et al., 1993; SUTTON et al., 2000).

Considerando-se os vários mediadores envolvidos no processo inflamatório decorrente da infecção pela *H. pylori* seria difícil explicar o risco elevado para o desenvolvimento do câncer gástrico associado com um SNP envolvendo apenas um tipo de citocina.

Assim, são poucos os estudos que têm investigado a ocorrência de associação de polimorfismos em genes de citocinas no desenvolvimento do câncer gástrico, sendo que, os resultados ainda não são conclusivos (SENO et al., 2007; CRUSIUS et al., 2008). Por exemplo, Seno et al. (2007) analisaram em uma população japonesa 207 SNPs em 11 genes de citocinas, incluindo *IL-1A, IL-1B, IL1-RN, IL4, IL4R, IL8, IL10, IL12, TNFA, TNFB e INF-γ.* Este estudo evidenciou uma associação negativa com o desenvolvimento do câncer gástrico não-cárdia para os polimorfismos dos genes *IL4* (984 e 2983 AA/GA) e *IL1-RN*. Nenhuma outra associação foi encontrada envolvendo os outros polimorfismos avaliados (SENO et al., 2007). De modo contrário, Crusius et al. (2008) que também investigaram vários polimorfismos em genes de citocinas (*IL-1A, IL-1B, IL1-RN, IL4, IL4R, IL8, IL10, IL12, TNFA)* em uma população européia, encontraram associação positiva entre o genótipo *IL1-RN**2/2 e câncer gástrico não-cárdia e associação negativa deste câncer com o polimorfismo *IL8* -251A/A. Portanto, evidenciando a necessidade de novas investigações envolvendo polimorfismos em grupos de citocinas, principalmente na população brasileira, composta por indivíduos de diferentes ancestralidades, em que ainda não há estudos tão amplos envolvendo estes genes.

Por exemplo, Gatti et al. (2004) avaliaram polimorfismos no gene *IL-1β* (-511C/T e -31T/C) e *IL1-RN* não encontrando associação com adenocarcinoma gástrico. O mesmo grupo investigou a associação de polimorfismos na região promotora do gene *IL6*, encontrando associação do genótipo *IL6* -174G/G com o câncer gástrico (GATTI et a., 2007). Posteriormente, Rocha et al. (2005) avaliaram os polimorfismos *IL-1β* (-511C/T e -31T/C), *IL1-RN* e *TNFA* -308G/A e relataram que apenas o alelo polimórfico *IL-1RN*2* estava associado ao câncer gástrico de não-cárdia. Mais recentemente, outro estudo também demonstrou em uma amostra da população brasileira associação do alelo IL-1RN*2 e o desenvolvimento de úlceras gástricas e câncer gástrico (MELO-BARBOSA et al., 2009).

Também se deve considerar que muitos polimorfismos ocorrem em regiões reguladoras de genes de citocinas. Acredita-se que alguns destes estão relacionados à alteração na afinidade de fatores de transcrição, resultando assim em expressão diferencial da respectiva citocina (HAUKIM et al., 2002). SNPs como *IL-1β* -511 T/C, *IL6* -174 G/C, *IL8* -251T/A, *IL10* -1082 A/G e *TNFA* -308 G/A têm sido associados com a expressão diferencial de suas respectivas citocinas *in vitro* (DI GIOVINE et al., 1992; WILSON et al., 1993; TURNER et al., 1997; FISHMAN et al., 1998; GALBRAITH et al., 1998; OLOMOLAIYE; WOOD; BIDWELL, 1998; BROGAN et al., 1999; HULL; THONSON; KWIATKOWSKI, 2000; SHAHBAZI et al., 2002).

Ainda são escassos os estudos que têm avaliado a relação entre as variantes polimórficas na região promotora desses genes e os níveis de expressão das citocinas com o risco para a carcinogênese. Rad et al. (2004) encontraram associação entre os portadores dos polimorfismos *IL-1β* -511T, -31C, IL-1RN*2 e *IL10* -1082G, -819C e -592C com maior expressão das respectivas citocinas em pacientes com gastrite crônica. Da mesma forma, portadores do alelo T do polimorfismo *IL4* -592C/T com menor atividade transcricional do seu promotor apresentaram uma associação com o risco para câncer de próstata (TINDALL et al., 2010). Portanto, são necessários estudos que procurem correlacionar os polimorfismos e níveis de expressão gênica destas citocinas com o risco na carcinogênese gástrica também na população brasileira.

Objetivos

II. Objetivos

Considerando-se a escassez de estudos na literatura com polimorfismos de grupos de citocinas e receptores envolvidos na infecção gástrica pela *H. pylori* na população brasileira, bem como a relação de polimorfismos em região reguladora e expressão gênica. Este projeto teve por objetivos:

1) Determinar as freqüências alélicas e genotípicas de polimorfismos nos genes dos receptores *toll like* – TLR (*TLR2* deleção -196 a -174, *TLR4* +896A/G e +1196C/T), genes de citocinas pró-inflamatórias (*TNFA* -308G/A e -857C/T, *TNFB* +252A/G *e IL8* -251T/A e -845T/C) e anti-inflamatórias (*IL-1RN* VNTR e *IL10* -592A/C) em três grupos da população brasileira (câncer gástrico, gastrite crônica e controle) e avaliar a ocorrência de associação desses polimorfismos com risco aumentado para essas lesões gástricas;

2) Estimar a combinação entre polimorfismos e haplótipos que resultam em um aumentado risco para as lesões gástricas;

3) Avaliar a associação desses polimorfismos nos diferentes grupos ajustados com fatores de risco como idade, sexo, tabagismo, etilismo e infecção pela *H. pylori*, e

4) Avaliar a expressão relativa do RNAm pela técnica de qPCR em tempo real dos genes *TNFA, IL8* e *IL10,* que apresentam polimorfismos em suas regiões reguladoras, em amostras da mucosa de pacientes com as lesões gástricas, e possíveis associações entre os níveis de expressão gênica e os fatores de risco e as variantes polimórficas.

Artigos

III. 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 I

Título: Polymorphisms of the *TLR2* and *TLR4* genes are associated with risk of gastric cancer in a Brazilian population.

Autores: Juliana Garcia de Oliveira e Ana Elizabete Silva.

Periódico: *World Journal of Gastroenterology*, submetido em 11/2010

Artigo II

Título: Polymorphism of IL-1ra anti-inflammatory cytokine is associated with risk of gastric cancer and chronic gastritis, but not the TNF-β pro-inflammatory cytokine.

Autores: Juliana Garcia de Oliveira e Ana Elizabete Silva.

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

Artigo III

Título: Polymorphisms in promoter regions of TNF-*α,* IL8 and IL10 cytokines and gene expression in chronic gastritis and gastric cancer.

Autores: Juliana Garcia de Oliveira, Daniela Manchini Nizato, Ana Flávia Teixeira, Marina Curado Valsechi, Paula Rahal e Ana Elizabete Silva.

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

Artigo I

Ms. Wjg/2010/027904 BRIEF ARTICLE

Polymorphisms of the *TLR2* and *TLR4* genes are associated with risk of gastric cancer in a Brazilian population

Running title: *TLR2* and *TLR4* polymorphisms in gastric cancer

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Abstract

AIM: To investigate *TLR2* -196 to –174 *del*, and *TLR4* (+896A/G rs4986790 and +1196C/T rs4986791) polymorphisms at risk of chronic gastritis and gastric cancer in a Brazilian population and association of gastric lesions with risk factors such as smoking, alcohol intake and *H. pylori* infection.

METHODS: In this case-control study, polymorphism at *TLR2* -196 to -174 *del* was investigated by using the allele-specific polymerase chain reaction (PCR) method, while the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique was carried out to identify the *TLR4* (rs4986790 and rs4986791) genotypes in 675 Brazilian individuals (229 with chronic gastritis-CG, 200 with gastric cancer-GC and 246 controls -C).

RESULTS: The SNP *TLR4*+1196C/T was not associated with risk of chronic gastritis or gastric cancer and the homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population. However, the frequency of *TLR2* -196 to -174 ins/del + del/del and *TLR4*+896AG genotypes was significantly higher (*P*<0.01) in the cancer group (33.5% and 13%, respectively) than in the control group (17.9% and 6.5%, respectively). It was also observed that the G-C haplotype of the *TLR4*+896A/G+1196C/T (*P*=0.02) and the combination of variant alleles of the *TLR2del/TLR4*+896G (*P*=0.02) are associated with susceptibility to gastric cancer. In addition, the multiple logistic regression showed that male gender (OR=2.51; 95% CI=1.58-4.00; *P*<0.01), age above 60 years (OR=1.66; 95% CI=1.08-2.54; *P*=0.02), alcohol intake (OR=2.93; 95% CI=1.81-4.77; *P*<0.01), *TLR2* -196 to -174 *del* (OR=2.21; 95% CI= 1.36-3.60; *P*<0.01) and *TLR4*+896G (OR=2.39; 95% CI=1.14- 4.99; *P*=0.02) polymorphisms were associated with a higher susceptibility to developing this neoplasm.

CONCLUSION: Our data indicate that *TLR2* -196 to -174 *del* and *TLR4*+896G may increase the risk of gastric cancer in a Brazilian population.

Key words: polymorphisms, *TLR2, TLR4,* gastric cancer, gastritis.

INTRODUCTION

Gastric cancer is one of the most serious health problems in many countries, including Brazil, which ranks third in incidence and mortality, and with an estimated incidence in 2010 of about 21,500 new cases, with an incidence of 14.25 per 100.000 males and 7.70 per 100.000 females[1]. The *H. pylori* is the major etiological risk factor for this malignancy, which progresses through a multi-step process, developing from gastritis, to gastric atrophy, intestinal metaplasia, dysplasia, and finally to carcinoma [2]. It is widely accepted that chronic *H. pylori* infection induces a gastric atrophy and hypochlorhydria, which are precursors of all pathophysiological changes of gastric carcinogenesis [3]. However, colonization with *H. pylori* can lead to various outcomes. Nearly all *H. pylori* positive subjects have chronic gastritis, and only 1-2% development of stomach cancer among infected[4]. Hence, other factors are likely to be involved in gastric tumorigenesis such as host genetic factors, as well as the diversity of *H. pylori* virulence genes.

Host genetic factors, as polymorphisms in inflammatory and immune response genes, are mainly related to the recognition of the bacteria by the immune system and the variation in the level of cytokine response [5]. Among host factors, several inflammatory proteins including cytokines, growth factors, and chemokines have been known to control immune response against *H. pylori* infection [6, 7]. Therefore, many studies have focused on the analyses of polymorphisms in genes associated with the inflammatory response in the gastric mucosa and risk for malignancy [8-11].

Other mediators also have polymorphic variants that modulate the innate immune response pattern, as the toll-like receptors (TLR), which provide first line of host defense against harmful pathogens [12].

Among the TLRs, it has been reported that the TLR2, lipoproteins bacterial receptor and the TLR4, the lipopolysaccharide (LPS) receptor, are involved in the response to infection by *H. pylori* on gastric epithelial cells [3, 13-15]. Both the TLR2 and the TLR4 promote transcription of genes involved in immune activation including *NF-κB* and also mintage-activated protein (MAP) kinase pathways [16]. *TLR4* is up regulated in gastric epithelial cell lines infected with *H. pylori* and in macrophages and expression of TLR4 protein has been demonstrated in chronic active gastritis, in precancerous lesions, and also in gastric tumor cells. TLR2 activates NF-κB in epithelial cells, in response to *H. pylori* infection, causing the expression of IL8, MIP-3α and GRO-α [7]. Thus, it is conceivable that functionally relevant polymorphisms in TLR genes can alter the host immune response to pathogens as infection induced by *H. pylori*.

Single Nucleotide Polymorphisms (SNPs) in *TLR2* have been associated with susceptibility to various infectious and inflammatory diseases such as leprosy [17], increased risk of Gram-negative sepsis [18], asthma [19], recurrent bacterial infections [20] and sporadic colorectal cancer susceptibility [21]. The specific polymorphism *TLR2* -196 to -174 *del/del* genotype has been reported to show decreased transactivation of responsive promoters [22]. Such fact aroused the interest for this polymorphism, and previous studies in the Japanese population demonstrated its association with increased susceptibility to non-cardia gastric cancer [23] and intestinal metaplasia [24].

Likewise, the *TLR4* presents some polymorphisms implicated in increased susceptibility to various diseases such as atherosclerosis [25], asthma [3], malaria [26], and also infection with the *H. pylori* associated with gastric cancer and its precursors [27]. Two SNPs in TLR4+896A/G (rs4986790) and +1196C/T (rs4986791) have received special attention in some studies, although the results are still controversial [6, 28, 29].

Therefore, the aim of this study was to evaluate the influence of the 22-bp nucleotide deletion -196 to –174 *del*, in the promoter region of the gene *TLR2* and +896A/G and +1196C/T polymorphisms (Asp299Gly and Thr399Ile) respectively in *TLR4* gene on the risk of chronic gastritis and gastric cancer in a Brazilian population and whether there is an association of gastric lesions with risk factors such as smoking, alcohol intake and *H. pylori* infection.

MATERIALS AND METHODS

Subjects

This was a case-control study on chronic gastritis and gastric cancer, in which a total of 675 DNA samples from peripheral blood leukocytes were genotyped. The case groups comprised 229 individuals (113 men and 116 women) with a histopathologically confirmed diagnosis of chronic gastritis – CG (Sidney System) [30], with a mean age of 52,3+14,5 years (range 19 to 86 years), and 200 individuals (156 men and 44 women) with a histopathologically confirmed diagnosis of gastric cancer – GC (Lauren's classification) [31], with a mean age of 61,7±12,5 years (range 28 to 93 years). All subjects were recruited from the Hospital de Base in São José do Rio Preto, SP, and from the Pio XII Foundation in Barretos, SP, Brazil. *H. pylori* infection was histologically established either by the Giemsa staining technique or by the urease test, performed at the Pathology Services of the Hospital de Base and the Pio XII Foundation. Results of *H. pylori* infection were obtained for the available cases. The cancer-free control group (C) with no previous history of gastric disease was composed of 246 healthy individuals (126 men and 120 women), mainly blood donors, with a mean age of 55,5+17,7 years (range 20 to 93 years).

Epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, with questions about current and past occupation, smoking habits, alcohol intake and family history of cancer. Considering the admixture ethnic of Brazilian population, the individuals were ethnically classified on their visual appearance as Caucasians (~90%) and Negroids (~10%) in the three groups evaluated.

The National Research Ethics Committee approved this work, and written informed consent was obtained from all individuals.

Genotyping

About 5 mL of whole blood were collected from all study participants in sterile EDTA-coated vacutainers. DNA was extracted according to a previous report [32], and stored at -20ºC until use for genotyping.

Polymorphism at *TLR2* -196 to –174 *del* was investigated using the allele-specific polymerase chain reaction (PCR) method [24], and the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique was carried out in order to identify the *TLR4* (rs4986790 and rs4986791) genotype [33, 34] in cases and control groups. In brief, the procedure was carried in a total reaction volume of 25 µL, containing 2.5 µL 10×PCR buffer, 2 µL dNTPs (1.25 µmol/L), 0.5 µL $MgCl_2$ (25 mmol/L), 1.25 µL of each primer (25 mmol/L, Sigma-Aldrich, USA), 15.3 µL dH_2O, 2 µL DNA (100 ng/ µL), and 0.2 µL Taq DNA polymerase (5 U/µL, Invitrogen, USA). PCR for *TLR2* -196 to -174 *del* was as follows: initial denaturation step at 95ºC for 5 min, amplification was carried out by 35 cycles at 95ºC for 30 s, at 60ºC for 40 s, and at 72ºC for 40 s, followed by a final elongation cycle at 72ºC for 7 min (Table 1). PCR for both *TLR4* polymorphisms were as follows: after an initial denaturation step at 94ºC for 3 min, amplification was carried out by 30 cycles at 94ºC for 30 s, at 62ºC for 30 s, and at 72ºC for 30 s, followed by a final elongation cycle at

72°C for 7 min. Then, 10 µL of PCR products were digested with 0.5 µL of the specific enzyme (5 U/µL) in a 10 µL volume including 2.5 µL 10×buffer 1 (New England Biolabs, USA) and 7.0 µL dH_2O (Table 1). The products were then electrophoresed on a 3% agarose 1000 (Invitrogen, USA) gel, to allow detection by ethidium bromide staining.

In order to confirm the veracity of the results, one confirmed polymorphic case was used as positive control for every RFLP procedure, to attest the good functioning of the restriction enzyme. For the *TLR4*+896A/G (rs4986790) and +1196C/T (rs4986791) polymorphisms, we also used other fragments of the *TLR2* and *IL-1β* gene that was known to have the enzyme recognition site to verify the correct functioning of the enzymes *Bst*XI and *Hinf*I (Table 1, Figure 1), considering we did not detect any polymorphic homozigous subjects.

Statistical analysis

Fisher's exact test was used to compare the groups regarding genotype and allele frequencies, and the chi-square test for determining Hardy-Weinberg equilibrium. Multiple logistic regression models were used to determine the effects of the variables in gastric cancer and chronic gastritis. The models included age (reference: <60 and 53 years old – median of the groups), gender (reference: female), smoking habits (reference: nonsmokers), drinking habits (reference: nondrinkers), and *H. pylori* infection (reference: *H. pylori*-negative). The results are shown as odds ratios (OR), showing 95% confidence intervals (95% CI). OR's were calculated using a dominant model due to low frequency of polymorphic homozygous (i.e., combining heterozygous and homozygous for the minor allele *vs.* homozygous for the major allele) for all SNPs. The haplotype frequencies of *TLR4* were inferred by Haploview program (4.0 version). Statistical analyses were performed using the GraphPad Instat, and SPSS (11.5 version) computer software programs. A probability level (*P*) of less that 0.05 was adopted as a significance criterion.

RESULTS

The samples of the 675 subjects were genotyped for the *TLR2* -196 to –174 *del* and *TLR4* (+896A/G rs4986790 and +1196C/T rs4986791). The genotype and allele frequencies for these polymorphisms are presented in Table 2. The genotype and allele frequency distribution of the three polymorphisms complied with Hardy-Weinberg equilibrium in both cases and control groups (data not shown). The banding patterns of these polymorphisms are represented in Figure 1.

For *TLR2* -196 to –174 *del*, the genotype (ins/del and del/del) and allele (del) frequencies were increased statistically (P<0.01) in gastric cancer group (33.5% and 0.19 respectively) than in control group (17.9% and 0.10 respectively).

Similarly, *TLR4*+896A/G (rs4986790) showed genotype and allele frequencies statistically significant (P=0.02) between gastric cancer (13% and 0.07%, respectively) and control groups (6.5% and 0.03%, respectively), due to the higher frequency of polymorphic genotype (+896AG) in the gastric cancer group. In contrast, for *TLR4*+1196C/T (rs4986791), no significant difference was found between gastric cancer and control group (Table 2). Among the groups of gastritis and control, no statistically significant difference was observed for all three polymorphisms (Table 2). Homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population. We also compared the genotype and allele frequencies between gastric cancer and gastritis groups and no significant difference was found for the polymorphisms studied (data not shown).

The *TLR4* haplotype analysis (Table 3) demonstrated higher frequency of both wild alleles (haplotype A-C) in control subjects compared with gastric cancer (0.957 and 0.914, respectively; P<0.01). However, the opposite was observed for frequency of variant haplotype G-C, which was higher in the gastric cancer group compared with control group (0.063 and 0.031, respectively; *P*=0.02).

In another statistical analysis, which evaluated the combined effect between the three polymorphisms (*TLR2* -196 to -174 *del*, *TLR4*+896A/G and +1196C/T), the combination of variant alleles of the polymorphisms *TLR2 ins/del* and *del/del* with *TLR4* +896 AG showed a higher risk of gastric cancer compared to healthy individuals (OR=4.13; 95% CI=1.26-13.50; *P*=0.02). The other combinations of variant alleles did not show any significant difference (Table 4). The combination of the three variant alleles was found in only one individual of the group of chronic gastritis (data not shown).

The potential associations between the distributions of *TLR2* -196 to –174 *del* and *TLR4* (+896A/G and +1196C/T) genotypes and exposure to risk factors for gastric cancer and chronic gastritis in comparison with control group are presented in Table 5.

In the gastric cancer group, the multiple logistic regression shows that male gender (OR=2.5; 95% CI=1.58-4.00; *P*<0.01), age above 60 years (OR=1.66 ; 95% CI=1.08-2.54; *P*=0.02), alcohol intake (OR=2.93; 95% CI=1.81-4.77; *P*<0.01), *TLR2* -196 to -174 ins/del+del/del (OR=2.21; 95% CI= 1.36-3.60; *P*<0.01) and *TLR4* +896AG (OR=2.39; 95% CI=1.14- 4.99; *P*=0.02) were associated with a higher susceptibility to developing this neoplasm. The comparison between gastritis and control group showed that none of the polymorphisms were associated with risk of chronic gastritis, and that age above 53 years (OR=0.64; 95% CI=0.44-0.93; *P*=0.02) and smoking (OR=0.63; 95% CI=0.42-0.94; *P*=0.02) were negatively associated with the development of gastritis, while alcohol intake (OR=1.65; 95% CI=1.06-2.57; *P*=0.02) increased the risk for gastritis. In another multiple logistic regression analysis considering also the individuals tested for *H. pylori* infection (95 with gastric cancer and 177 with gastritis), there was no statistically significant association (OR = 0.73, 95% CI = 0.29-1.78, *P* = 0.49, data not shown in Table 5). There was also no association of three polymorphisms with *H. pylori* when evaluated negative and positive individuals within a group of gastric cancer and gastritis (data not shown).

DISCUSSION

TLRs participate in *H. pylori* bacterium recognition in gastric mucosa, and SNPs in TLRs are associated with impaired immune response, inducing a potent inflammatory response. Therefore, it is relevant to carry out studies on host genetic factors that can be associated with susceptibility of gastric diseases. Hence, we investigated whether *TLR2* -196 to –174 *del* and *TLR4* (+896A/G rs4986790 and +1196C/T rs4986791) polymorphisms affect the risk of developing gastric cancer and chronic gastritis in a Brazilian population. Our results have demonstrated for the first time in this population, an association of *TLR2* -196 to -174 *del* and of *TLR4*+896AG polymorphisms with susceptibility to gastric cancer. The polymorphism *TLR4*+1196 C/T was not associated with risk to the gastric lesions evaluated, and the homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population.

Some studies that investigated the association of *TLR2* -196 to -174 *del* polymorphisms at risk of developing diseases related to an inflammatory process have shown conflicting results. For instance, *del* allele or del/del genotype of TLR2 -196 to-174 polymorphism was significantly associated with cervical cancer susceptibility[35] and risk of non-gastric cancer in a Japanese population, but not for gastritis, gastric ulcer and duodenal ulcer[23], while *TLR2* –196 to –174 *ins* allele was associated with more severe intestinal metaplasia in older patients [24]. However, Wang et al. [36] failed to show association of *TLR2* -196 to -174 *del/del* and *ins/del* carriers with ulcerative colitis.

The -196 to -174 *del* polymorphisms in *TLR2* gene located on chromosome 4, causes a 22-bp nucleotide deletion that alters the promoter activity of gene. The *TLR2 del/del* genotype is reported to show decreased transactivation of responsive promoters [22]. In our study, we observed significantly higher frequencies of genotypes *TLR2 ins/del* and *del/del* in the gastric cancer group compared to the healthy individuals, emphasizing its role in the gastric carcinogenesis.

The *TLR4* gene is mapped on chromosome 9 and consists of three exons. In exon 3, two non-synonymous SNPs *TLR4*+896A/G and +1196C/T allows the substitution of amino acids Asp299Gly and Thr399Ile, respectively. In the analysis by Haploview, the frequencies of *TLR4* G-C (299Gly-399Thr) and G allele were higher in patients with gastric cancer indicating an association of this haplotype with increased risk of gastric cancer to its carriers. The substitution of Asp299Gly amino acids disrupt the normal structure of the extracellular region of the TLR4 and may cause decreased ligand recognition or protein interaction, and decreased responsiveness to lipopolysaccharide, disrupting transport of TLR4 to the cell membrane [13, 37]. This change leads to an exaggerated inflammatory response with severe tissue destruction, likely due to a failure in stimulating regulatory cells and production of IL10 cytokine [38]. Arbour et al. [13] were the first to report that individuals having either the Asp299Gly and/or Thr399Ile polymorphisms had a blunted response towards inhaled LPS. Thus, during the cascade of progression of gastric carcinogenesis, the subjects with this polymorphism can have an increased risk of severe inflammation followed by development of hypochlorhydria and gastric atrophy, which are regarded as important precursor alterations of gastric cancer [12].

Both SNPs in *TLR4* are presented in about 10% of Caucasian and African populations and are reported to have a positive correlation with susceptibility to infectious diseases, whereas studies in Asian populations have shown the absence of these polymorphisms [26, 39]. However, in our study with a Southeastern Brazilian population, we found that both SNPs *TLR4* (+896A/G and +1196C/T) were present in heterozygous in about 6.5% to 13% and 2.5% to 4.5% respectively in the evaluated groups. Other studies in the Brazilian population found similar frequencies of heterozygous TLR4+896A/G polymorphism in Chagas disease (5.6%), ulcerative colitis (7,1%) and Crohn`s disease (7%) [40, 41]. But, to the best of our knowledge, there are not studies on *TLR2* and *TLR4* polymorphisms in gastric cancer of Brazilian population.

With regards to *TLR4*+896A/G and +1196C/T polymorphisms, Garza-Gonzalez et al. [29] showed no association with the risk of gastric cancer in the Mexican population, while Trejo de la O et al. [7] observed that both SNPs in *TLR4* had an association with duodenal ulcer and gastric cancer also in Mexican patients. Yet, other studies have demonstrated association with only one of these polymorphisms and risk of gastric cancer and precancerous lesions, either *TLR4*+1196C/T (Thr399Ile)[6,42] or *TLR4*+896A/G (Asp299Gly) polymorphisms[3] are associated with susceptibility to gastric carcinogenesis. In addition, in this study in Caucasian population[3], homozygous polymorphic TLR4+896 GG was not found, corroborating our results.

The Brazilian population displays great genetic heterogeneity as a consequence of high miscegenation during colonization, resulting mainly from the mating between native Amerindians, African and Europeans [43]. This demonstrates the importance of this study and also explains the differences from other studied populations with different ethnic origins.

Concerning *H. pylori* infection, we evaluated the association between *TLR2* and TLR4 polymorphisms in the case groups with the available information in their medical records (95 with gastric cancer and 177 with gastritis) and no association was found. The reduced number of samples available for statistical analysis may have harmed these results. RAD et al.

[8] studied the role of various toll-like receptors (TLRs 2/4/7 and 9) in response to *H. pylori* using mice mutants lacking these receptors. The results demonstrated the importance of the TLR2 in response to this bacterium, unlike the TLR4. TLR4-receptor lacking mice had little or no change in response to *H. pylori* compared with controls. Although the role that the effect of polymorphism in *TLR2* in the activity of this receptor in gastric cells is not fully understood, this deletion is likely to alter their activity. Since *TLR2* has an important role in immune response against the *H. pylori* bacterium, the change of its function becomes relevant in carcinogenesis of the stomach [8].

Besides the influence of the *H. pylori*, other risk factors as smoking and alcohol intake were analyzed. It should be emphasized the statistically significant results for alcohol intake in the gastric cancer and chronic gastritis groups. The relationship of alcohol consumption with upper gastrointestinal tract cancer has been reported previously [44- 46]. Really, ethanol oxidation generates acetaldehyde, which presents carcinogenic effects, since it interferes with many DNA synthesis and repair sites, leading to tumor development [47].

In conclusion, our findings indicate a significant role of both *TLR2* -196 to -174 *del* and *TLR4*+896A/G (Asp299Gly) polymorphisms with susceptibility to gastric cancer in the Southeastern Brazilian population evaluated, whereas no association was observed for *TLR4* +1196C/T polymorphism. Thus, it is feasible to highlight that host genetic factors as the interaction of polymorphisms in genes of toll-like receptors can play an important role in gastric carcinogenesis.

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Table 1. Primer sequences, restriction enzymes and fragment sizes for *TLR2* and *TLR4* gene polymorphisms and gene *IL-1β*.

Genes	Primers	Enzyme T°/Time	Fragment (pb)	Ref
TLR2 *del -196* to *-174*	F: 5`-CACGGAGGCAGCGAGAAA-3` R: 5`-CTGGGCCGTGCAAAGAAG-3`	-	286 ins/ins: 286 ins/del: 286, 264 del/del: 264	[29]
TLR4 +896A/G rs4986790	F:5′-AGCATACTTAGACTACCACCTCGATG 3′ R:5′-GTTGCCATCCGAAATTATAAGAAAAG 3′	*Bst*XI 37° C, 1 h	131 A/A: 131 A/G: 131, 108 G/G: 108	[36]
TLR4+1196C/T rs4986791	F:5`-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3` R:5`-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3`	*Hinf*I 37° C, 1 h	407 C/C: 407 C/T: 407, 378 T/T: 378	[35]
IL1-β	F: CATGTGACCTGCTCGTCAGT R: CCCTAGGGATTGAGTCCACA	*Hinf*I 37° C, 1h	370 195, 175	[50]
TLR2	F: 5`-CACGGAGGCAGCGAGAAA-3` R: 5`-CTGGGCCGTGCAAAGAAG-3`	*Bst*XI 37° C, 1 h	286 188, 98	[29]

T°, temperature.

Table 2. Genotype and allele frequencies of *TLR2* (-196 to –174 *del*) and *TLR4* (rs4986790 and rs4986791) polymorphisms in gastric cancer (GC), chronic gastritis (CG), and control (C) groups.

Genotypes/ Alleles	GC N=200(%)		C N=246(%)		CG N=229(%)
TLR2 -196 to -174					
ins/ins	133 (66.5)		202 (82.1)		175 (76.4)
del/ins	58 (29.0)		40 (16.2)		47 (20.6)
del/del	9 (4.5)		4 (1.7)		7 (3.0)
OR(95% CI)		2.31 (1.49-3.58)		1.41 (0.90-2.21)	
P		<0.01		0.14	
Alleles					
ins	0.81		0.90		0.87
del	0.19		0.10		0.13
OR(95% CI)		2.17 (1.47-3.20)		1.42 (0.95-2.12)	
P		<0.01		0.10	
TLR4 +896A/G (rs4986790)					
AA	174 (87.0)		230 (93.5)		205 (89.5)
AG	26 (13.0)		16 (6.5)		24 (10.5)
GG	0		0		0
OR(95% CI)		2.14 (1.11-4.12)		1.68 (0.86-3.25)	
P		0.02		0.13	
Alleles					
A	0.93		0.97		0.95
G	0.07		0.03		0.05
OR(95% CI)		2.06 (1.09-3.91)		1.64 (0.86-3.13)	
P		0.02		0.14	
TLR4 +1196C/T (rs4986791)					
CC	191 (95.5)		240 (97.5)		223(97.4)
CT	9 (4.5)		6 (2.5)		6 (2.6)
TT	0		0		0
OR(95% CI)		1.86 (0.65-5.38)		1.07 (0.34-3.38)	
P		0.29		1.00	
Alleles					
C	0.98		0.99		0.99
T	0.02		0.01		0.01
OR(95% CI)		1.86 (0.65-5.28)		1.07 (0.34-3.35)	
P		0.29		1.00	

CI, confidence interval; OR, odds ratio; *P*, probability.

Table 3. *TLR4* haplotype frequency distribution between gastric cancer (GC), chronic gastritis (CG) and control (C) groups.

Haplotypes *TLR4* +896/+1196	GC	C	X^2	*P*	CG	C	X^2	*P*
A-C	0.914	0.957	6.802	<0.01	0.940	0.957	1.361	0.24
G-C	0.063	0.031	5.247	0.02	0.046	0.031	1.598	0.20
A-T	0.021	0.011	1.461	0.22	NF	NF	NF	NF

X^2, chi-square test; NF= not found; *P*, probability; haplotype G-T not found

Table 4. Combined effect of *TLR2* (-196 to -174 *del*) and *TLR4* (rs4986790 and rs4986791) polymorphisms on risk of gastric cancer (GC) and chronic gastritis (CG).

Risk genotype	Groups								
	GC (n=200)	C (n=246)	OR (95% CI) P	CG (n=229)	C (n=246)	OR (95% CI) P	GC (n=200)	CG (n=229)	OR (95% CI) P
Neither	113	187	1.00 (reference)	160	187	1.00 (reference)	113	160	1.00 (reference)
***TLR2* ins/del or del/del/ *TLR4*+896 AG**	10	4	4.13 (1.26-13.50)	11	4	3.21 (1.00-10.29)	10	11	1.28 (0.52-3.13)
			0.02			0.06			0.64
***TLR2* ins/del or del/del / *TLR4*+1196 CT**	4	1	6.61 (0.73-59.99)	1	1	1.16 (0.07-18.84)	4	1	5.66 (0.62-51.37)
			0.07			1.00			0.16
***TLR4*+896 AG/ +1196 CT**	1	1	1.65 (0.10-26.73)	3	1	3.50 (0.36-34.05)	1	3	0.47 (0.04-4.59)
			1.00			0.34			0.64

C, control group; CI, confidence interval; OR, odds ratio; *P*, probability.

Table 5. Distribution of risk factors, genotypes of *TLR2* (-196 to –174 *del*) and *TLR4* (rs4986790 and rs4986791), and odds ratios (OR) for gastric cancer (GC), chronic gastritis (CG), and control (C) groups.

Variables	F (GC/C) %	OR (95%CI)	p	F (CG/C) %	OR (95% CI)	p
Gender						
F	22/48.8	reference	<0.01	50.7/48.8	reference	0.79
M	78/51.2	2.5 (1.58-4.00)		49.3/51.2	0.95 (0.64 -1.39)	
Age (years)						
≤60	39.5/56.9	reference	0.02	54.5/45.5	≤ 53 reference	0.02
>60	60.5/43.1	1.66 (1.08-2.54)		45.5/54.5	> 53 0.64 (0.44-0.93)	
Ethinicity						
Negroids	13.0/8.6	reference	0.56	9.2/8.6	reference	0.80
Caucasian	87.0/91.4	0.81 (0.41-1.62)		90.8/91.4	1.08 (0.56-2.09)	
Smoking						
Nonsmokers	29.5/36.6	reference	0.35	44.5/36.6	reference	0.02
Smokers	70.5/63.4	0.79 (0.48-1.29)		55.5/63.4	0.63 (0.42-0.94)	
Alcohol						
Nondrinkers	43.5/74,4	reference	<0.01	68.1/74.4	reference	0.02
Drinkers	56.5/25.6	2.93 (1.81-4.77)		31.9/25.6	1.65 (1.06-2.57)	
TLR2						
ins/ins	66.5/82.1	reference	<0.01	76.4/82.1	reference	0.37
ins/del del/del	33.5/17.9	2.21 (1.36-3.60)		23.6/17.9	1.23 (0.77 -1.95)	
***TLR4* +896A/G (rs4986790)**						
AA	87.0/93.5	reference	0.02	89.5/93.5	reference	0.18
AG	13.0/6.5	2.39 (1.14- 4.99)		10.5/6.5	1.60 (0.80-3.22)	
***TLR4* +1196C/T (rs4986791)**						
CC	95.5/97.5	reference	0.73	97.4/97.5	reference	0.92
CT	4.5/2.5	1.22 (0.37-3.96)		4.6/2.5	0.94 (0.28-3.10)	

F, frequency of individual (%);OR, odds ratio; CI, confidence interval; *P*, probability.

Figure Legend:

Figure 1. Electrophoretic pattern of fragments generated by PCR-allele-specific (A) *TLR2* -196 to -174 del: ins/ins=286 bp; ins/del=286 +264 bp; del/del=264 bp and PCR-RFLP for the polymorphisms (B) *TLR4* +896A/G: A/A= 131 bp; A/G=131 +108 bp and positive control of enzyme *Bst*XI, TLR2 gene fragment of 286 bp with the enzyme cutting site: 188+98 bp (last lane) (C) *TLR4* +1196 C/T: C/C= 407 bp; C / T: 407 +378 bp and positive control of enzyme *Hinf*I, *IL-1β* gene fragment of 370 bp with the enzyme cutting site: 195+175 bp (last lane). Lane 1 (M): molecular weight marker of 100 bp.

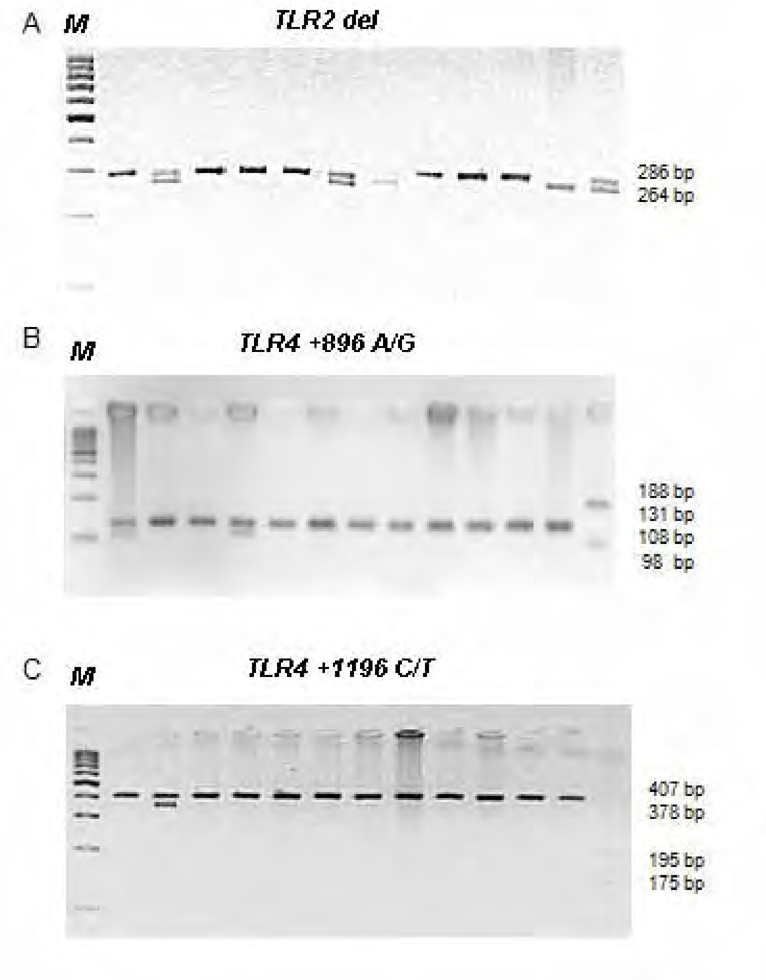
A M
TLR2 del
286 bp
264 bp
B M
TLR4 +896 A/G
188 bp
131 bp
108 bp
98 bp
C M
TLR4 +1196 C/T
407 bp
378 bp
195 bp
175 bp

Figure 1.

Artigo II

Polymorphism of IL-1ra anti-inflammatory cytokine is associated with risk of gastric cancer and chronic gastritis, but not the TNF-β pro-inflammatory cytokine.

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Article Type: Research article

Abstract

Backgroud: Genetic polymorphisms in genes that codify inflammatory cytokines have been associated with gastric carcinogenesis. This study evaluated *IL-1RN* VNTR and *TNFB* +252A/G polymorphisms on the risk of chronic gastritis and gastric cancer in a Brazilian Southeast population and whether there is an association of gastric lesions with risk factors such as smoking, alcohol intake and *H. pylori* infection.

Methods: In this case-control study, polymorphism at *IL-1RN* was investigated using the allele-specific polymerase chain reaction (PCR) method, while the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique was carried out to identify the *TNFB* +252A/G genotype in 675 Brazilian individuals (229 with chronic gastritis-CG, 200 with gastric cancer-GC and 246 healthy individuals-C).

Results: The SNP *TNFB* +252A/G was not associated with risk of chronic gastritis or gastric cancer. However, for *IL-1RN* VNTR, the genotype (L/2 and 2/2) and 2 allele frequencies were increased statistically in GC group (58% and 0.37; p<0.01, respectively) and CG (48% and 0.30; p<0.01, respectively) than in control group (35% and 0.20%, respectively). Also, there was a statistically significant difference among the groups of GC and CG (p=0.04 and p<0.01, respectively). In addition, the multiple logistic regression showed the association of factors such as male gender, older age, alcohol consumption and *IL-1RN* *L/2 and 2/2 genotypes for GC and CG in comparison of control group to the gastric carcinogenesis process.

Conclusions: Our data indicated that *IL-1RN**2 allele may increase the risk of gastric cancer and precancerous lesion in Brazilian population, so reinforcing the importance of host genetic factors in the susceptibility to gastric cancer and the participation of cytokines in both inflammation and carcinogenic process.

Key words: polymorphisms, *IL-1RN, TNF-β,* gastric cancer, chronic gastritis.

Background

The relationship between inflammation and cancer is well established, mainly in gastrointestinal tract [1]. Many of the factors involved in chronic inflammation and immune response play a role in the proliferation cell, stimulation of angiogenesis and inhibition of apoptosis [2]. Gastric cancer is a model of multifactorial disease resulting from environmental and genetic factors, which may progress from a chronic inflammation of gastric mucosa induced by *Helicobacter pylori* infection [3].

H. pylori is the most important risk factor for gastric cancer, triggering chronic inflammation of the stomach and leading to stepwise development of the malignancy [4]. It is widely accepted that chronic *H. pylori* infection induces a gastric atrophy and hypochlorhydria, which are precursors of all pathophysiological changes of gastric carcinogenesis [5].

Although the *H. pylori* infection is widespread throughout the world human population, only about 1-2% of exposed individuals progress for gastric cancer [6]. Thus, host genetic patterns beyond virulence factors of bacterium, mainly those that regulate immunological and inflammatory response may also contribute to a neoplasic progression [7]. Genetic polymorphisms in genes that codify inflammatory cytokines have been associated with an increase in synthesis of those interleukins and have emerged as a hypochloridria determining factor for cancer susceptibility [8, 9].

The interleukin-1 (*IL-1*) gene cluster on chromosome 2q contains three related genes, *IL-1A*, *IL-1B* and *IL-1RN*, which encode the proinflammatory cytokines IL-1α and IL-1β, as well as their endogenous receptor antagonist (IL-1ra), respectively [10]. IL-1ra encoded by *IL-1RN* gene is an anti-inflammatory cytokine that competitively binds to IL-1 receptors but does not elicit a response, thereby modulating the potentially damaging effects of IL-1 [11, 12]. *IL-1RN* gene has penta-allelic 86 bp variable number tandem repeats in the second intron, resulting in a short allele (IL-1RN*2, with two repeats) or long alleles (IL-1RN*L, with three to six repeats) [13].

The short allele is associated with chronic inflammation, autoimmune conditions and increase secretion of IL-1β [9]. Initially, El-Omar et al. (2000) reported increased risk for developing gastric cancer for individuals having the *IL-1RN**2/*2 genotypes. This finding was subsequently confirmed in others populations studies [7, 8, 11, 14, 15], althought data are controversial [16, 17].

In addition, genetic polymorphisms in pro-inflammatory cytokines as TNF-β are known to be related to several autoimmune, infectious, and neoplasic diseases [18-24]. TNF-β (tumor necrosis factor) or lymphotoxin-α (LT-α) is encoded by the same gene cluster for TNFA, located in tandem within the MHC complex on chromosome 6p21.3 [25]. TNF-β is a lymphokine which also plays an important role in the regulation of the immune response as part of the cytokine network. It is mainly produced by stimulated T cells, B and natural killer cell lymphocytes [26].

The increased level of expression *in vitro* of TNF-β has been shown for polymorphism *TNFB* +252A/G [27]. Also, there are studies showing that the *TNFB* +252G-carriage is associated with a high serum concentration of C-reative protein (CRP), a biomarker of inflammation [28, 29]. Therefore, this polymorphism has been investigated as a potencial susceptibility to gastric cancer [19, 30].

Therefore, the aim of this study was to determine the occurrence of association of the VTNR (Variable Number of Tandem Repeats) in *IL-1RN* and SNP (Single Nucleotide Polymorphism) of *TNFB* +252A/G polymorphisms on the risk of chronic gastritis and gastric cancer in a Brazilian Southeast population and whether there is an association of gastric lesions with risk factors such as smoking, alcohol intake and *H. pylori* infection.

Methods

Population

The National Research Ethics Committee approved this work, and written informed consent was obtained from all individuals.

This was a case-control study on chronic gastritis and gastric cancer, in which a total of 675 DNA samples from peripheral blood leukocytes were genotyped.

The case groups comprised 229 individuals (113 men and 116 women) with a histopathologically confirmed diagnosis of chronic gastritis – CG (Sidney System) [31] with a mean age of 52,3±14,5 years (range 19 to 86 years), and 200 individuals (156 men and 44 women) with a histopathologically confirmed diagnosis of gastric cancer – GC (Lauren's classification) [32] with a mean age of 61,7±12,5 years (range 28 to 93 years). All subjects were recruited from the Hospital de Base in São José do Rio Preto, SP, and from the Pio XII Foundation in Barretos, SP, Brazil. Results of *H. pylori* infection were obtained for the available cases in the medical records (95 with gastric cancer and 177 with chronic gastritis). The diagnosis was established by the Giemsa staining technique or by the urease test, performed at the Pathology Services of both Hospitals. The cancer-free control group (C) with no previous history of gastric disease was composed of 246 healthy individuals (126 men and 120 women), mainly blood donors, with a mean age of 55,5± 17,7 years (range 20 to 93 years). Epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, with questions about current and past occupation, smoking habits, alcohol intake and family history of cancer. Considering the admixture ethnic of Brazilian population evaluated, the individuals were ethnically classified on their visual appearance as Caucasians (90%) and Negroids (10%).

***IL-1RN* and *TNFB*+252 A/G genotyping**

About 5 mL of whole blood were collected from all study participants in sterile EDTA-coated vacutainers. DNA was extracted according to a previous report [33] and stored at -20ºC until use for genotyping.

Polymorphisms at *IL-1RN* were investigated using the allele-specific polymerase chain reaction (PCR) method [10], and the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique was carried out to identify the *TNFB* genotype [34] in cases and control groups. In brief, it was carried in a total reaction volume of 25 μL, containing 2.5 μL 10×PCR buffer, 2 μL dNTPs (1.25 μmol/L, Invitrogen, Carlsbad, CA, USA), 0.5 μL $MgCl_2$ (25 mmol/L), 1.25 μL of each primer (25 mmol/L, Sigma-Aldrich, St. Louis, MO, USA), 15.3 μL dH_2O, 2 μL DNA (100 ng/ μL), and 0.2 μL Taq DNA polymerase (5 U/µL, Invitrogen, Carlsbad, CA, USA). PCR for IL-1RN was as follows: initial denaturation step at 94ºC for 3 min, amplification was carried out by 35 cycles at 94ºC for 45 s, at 61ºC for 45 s, and at 72ºC for 1,5 min, followed by a final elongation cycle at 72ºC for 7 min. The size of amplified fragments is shown in Table 1. The alleles 1, 3, 4 and 5 were grouped and called longs alleles (L). PCR for *TNF-β* polymorphism was as follows: after an initial denaturation step at 94ºC for 5 min, amplification was carried out by 35 cycles at 94ºC for 1 min, at 65ºC for 1 min, and at 72ºC for 1 min, followed by a final elongation cycle at 72ºC for 7 min. Then, 10 µL of PCR products were digested with 0.5 µL of the specific enzyme *Nco*I (5 U/µL) in a 10 µL volume including 2.5 µL 10×buffer 1 (New England Biolabs, Massachusetts, USA) and 7.0 µL dH_2O (Table 1). The products were then electrophoresed on a 1,5% agarose (Invitrogen, Carlsbad, CA, USA) gel, to allow detection by ethidium bromide staining.

Statistical analysis

Fisher's exact test was utilized to compare the groups with regard to genotype and allele frequencies, and Chi-square test for determining Hardy-Weinberg equilibrium. Odds ratios (OR) and 95% confidence intervals (CIs) for polymorphisms evaluated were computed using multiple logistic regression models adjusting for age, gender, smoking, drinking and *H pylori* infection in gastric cancer and chronic gastritis groups. OR were calculated using a dominant model (i.e., combining heterozygous and homozygous for the minor allele) for two polymorphisms. Statistical analyses were performed using the GraphPad Instat; and SPSS (11.5 version) computer software programs. A probability level (p) of less than 0.05 was adopted as significance criterion.

Results

The samples of the 675 subjects were genotyped for the *IL-1RN* VNTR and *TNFB* +252 A/G in gastric cancer, chronic gastritis and control groups. The genotype and allele frequency distribution of the two polymorphisms were in agreement with Hardy-Weinberg equilibrium in both cases and control groups (data not shown).

The genotype and allele frequencies for these polymorphisms are presented in Table 2. For *IL-1RN* VNTR, the genotypes (L/2 and 2/2) and allele-2 frequencies were increased statistically in GC group (58% and 0.37, p<0.01, respectively) and CG (48% and 0.30; p<0.01, respectively) than in control group (35% and 0.20, respectively). Also, there was a statistically significant difference for genotype and allele frequencies among the both case groups (GC *vs*. CG, p=0.04 and p=0.02, respectively). Contrary, for *TNFB* +252A/G, no significant difference was found between the three groups evaluated. Although we found a higher frequency of genotypes A/G and G/G in the groups of GC (57%) and CG (55%) compared to control (48.8%) this was not statistically significant (Table 2).

When we evaluated the combined effect between the two polymorphisms studied the combination of variant alleles *IL1-RN**2 and *TNFB* +252*G showed a higher risk of GC (OR=2.01; 95%CI=1.49-2.71; p<0.01) and CG (OR= 1.48; 95%CI= 1.07-2.05; p<0.01) compared to healthy individuals. The same was observed when comparing the two case groups, that showed an increased risk of gastric cancer (OR= 1.35; 95% CI= 1.06-1.71; p<0.01) when compared to gastritis.

The potential associations between the distributions of *IL-1RN* VNTR and *TNFB* +252 A/G genotypes adjusting for risk factors for gastric cancer and chronic gastritis in comparison of control group are presented in Table 4.

In the GC group, the multiple logistic regression show that male gender (OR=2.27; 95% CI=1.42-3.62; p<0.01), age above 60 years (OR=1.70 ; 95% CI=1.10-2.61; p<0.01), alcohol intake (OR=3.09; 95% CI=1.91-5.02; p=<0.01), *IL-1RN**L/2 and 2/2 (OR=2.53; 95% CI= 1.66-3.87; p<0.01) were associated with a higher susceptibility to developing this cancer. The comparison between gastritis and control groups showed that *IL1-RN**L/2 and 2/2 (OR=1.79; 95% CI=1.22-2.62; p<0.01) and alcohol intake (OR=1.81; 95% CI=1.16-2.83; p<0.01) were associated with risk of chronic gastritis, while age over 53 years (OR=0.66; 95% CI=0.45-0.96; p=0.03) and smoking (OR=0.58; 95% CI=0.38-0.87; p<0.01) had a protective role in the development of gastritis (Table 4). In another multiple logistic regression analysis considering only the individuals of GC and CG groups tested for *H. pylori* infection (95 and 177, respectively) also adjusted for the other risk factor, was not observed statistically significant association (data not shown).

Discussion

Cytokine gene polymorphisms have generated further evidence to support the role of inflammation in gastrointestinal carcinogenesis, mediated by an array of pro- and anti-inflammatory cytokines. Polymorphisms of these genes are associated with differences in gastric mucosal cytokine mRNA level, which result in an individual final clinical outcome [35]. Considering this scenario, we investigated the role of polymorphisms of two genes cytokines, an anti-inflammatory (*IL-1RN* VNTR) and other pro-inflammatory (*TNFB* +252 A/G) on the risk of developing gastric cancer and chronic gastritis in a Southeast Brazilian population.

Our results demonstrated in this population, an association of *IL-1RN**2 allele variant and of the combined effect of polymorphisms *IL-1RN** L/2 or 2/2 / *TNFB* +252 A/G or G/G with susceptibility to gastric cancer and chronic gastric. The polymorphism *TNFB* +252A/G alone was not associated with risk of gastric lesions studied.

The results of our study add to the current controversy about the role of *IL-1RN* VNTR polymorphism in carcinogenesis gastric risk, recently reported in three meta-analyses [12, 36, 37]. The first, Kamangar et al. (2006), observed that the overall associations between *IL-1RN* pro-inflammatory polymorphisms and gastric cancer were null, even in studies conducted in Western countries. A second meta-analysis, Peleteiro et al. (2010), showed that the allele *IL-1RN**2 is associated with increase of the risk of gastric precancerous lesions, supporting a role for this polymorphism in the early stages of gastric carcinogenesis. The last meta-analysis mentioned, Xue et al. (2010) observed that the allele *IL-1RN**2 is significantly associated with an increased risk of developing gastric carcinoma and even more significantly with noncardia gastric carcinoma or with intestinal-type gastric carcinoma. However, this association is evident among Caucasians, but not among Asians or Hispanics. Probably these discrepancies may be due to low frequency of the risk allele in some populations, such as in Asian.

Rocha et al. (2005) conducted an assessment study of cytokine polymorphisms in an admixture population of Southeastern Brazil, with background, approximately 33% of Portuguese, 33% of Amerindians and 33% of African ancestry. They also demonstrated an association between the polymorphic allele *IL-1RN**2 and the development of gastric cancer, and association of cagA-positive *H. pylori* status. However, they found the 2/2 homozygous frequency was low (3,6%) compared to our study (15%) and in study in the European Caucasian population (28%) and Asian population (6%) [38, 39]. More recently, other study in a population of northern region of Brazil, with strong genetic contribution of Portuguese (50%) and Amerindians (40%) and low African (10%) ancestry, also showed a positive association between allele *IL-1RN**2 and gastric ulcer and gastric adenocarcinoma and infection by *H. pylori* CagA+, with 2/2 homozygous frequency about 6% [7]. These results indicate that the genotype frequencies of *IL-1RN* are related to the ethnicity of the population studied. In the present study, in another population of Southeastern Brazil, the African ancestry is estimated to be more than 10% [40]. Based on the broad geographic extension of Brazil there is variation in population composition among different regions that occurred during its colonization by Europeans and African matching with native Amerindians [41], and consequently differences in their genotype distribution.

This polymorphic allele *IL1-RN* * 2 has been linked to increased production of pro-inflammatory cytokine IL1-β, which causes a chronic inflammatory process and reduced production of gastric acid (hypochlorhydria) [42]. The reactive oxygen species and nitric oxide-derived inflammatory process are known as mutagens, whereas hypochlorhydria can allow an infection marked by bacteria that consequently increase the production of carcinogens. The DNA damage caused by this cascade of factors may be amplified by increased cell division inherent in the inflammatory process. Thus, genotypes that enhance the production of inflammatory cytokines may be related to the carcinogenic process of the stomach [43].

In addition, about the importance of pro-inflammatory cytokines, that can also modify the immune/inflammatory responses upon *H. pylori* infection, and influence gastric carcinogenesis, assessed the *TNFB* +252 A/G polymorphism.

Although we found a higher frequency of genotypes A/G and G/G in the groups of gastric cancer and gastritis compared to healthy individuals this was not statistically significant. Similarly, Lee et al. (2004) and Garza-Gonzalez et al. (2007) found no association in Korean and Spanish populations, respectively, as in the present study [44, 45]. Likewise, Guo et al. (2005) also found no relationship between the polymorphic allele and the groups of gastric adenocarcinoma and squamous cell carcinoma of the esophagus [34]. However, a recent study in the Japanese population showed association of genotypes G/G and G/A with increased risk of non-cardia gastric cancer of diffuse type and attributed this association 252G allele ability to increase production of pro-inflammatory cytokines such as TNF-β [30]. These discrepant results may also be linked to ethnicity among others factors.

When analysing the influence of the *H. pylori* infection on risk for the gastric lesions evaluated, no association has been observed. However, several studies have observed this association between *IL-1RN* polymorphisms and gastric lesions risk in the presence of *H. pylori* bacterium [11, 46, 47,]. Furthermore, Rocha et al. (2005) and Melo-Barbosa et al. (2009) found a strong association between this polymorphism and infection by positive *H. pylori* CagA strains and the development of gastric lesions and adenocarcinoma [7, 38]. This influence of infection by *H. pylori* is well defined in the progression of gastric cancer in some populations. Individuals with pro-inflammatory genotypes overexpress gastric IL-1β in response to *H. pylori* infection, leading to increased inflammation, gastric atrophy, hypochlorhydria and the development of gastric carcinoma [8]. This discrepancy might be possible due to the reduced number of cases with diagnosis for *H. pylori* in both gastric cancer and chronic gastritis groups in our study, did not find this association.

Other risk factors as male gender, age above 60 years and alcohol intake were analyzed and observed statistically associated with risk of gastric cancer. According to the National Cancer Institute the highest incidence of gastric cancer occurs in men around age 70, and about 65% of patients diagnosed with this type of cancer were over 50 years [48]. In Brazil, gastric cancer is third in incidence among men and fifth in women, so our results are consistent with epidemiological data reported in literature. Another risk factor well established in the literature in relation to gastric carcinogenesis is the excessive consumption of alcohol. The oxidation of ethanol generates acetaldehyde, which primarily has direct carcinogenic and mutagenic effects. This metabolite interferes with many sites of synthesis and DNA repair, which can lead to tumor development [49].

Conclusions

In conclusion, our findings do not evidence any association of *TNFB* +252A/G polymorphism alone with susceptibility to gastric lesions, but indicate a significant role of *IL-1RN**2 alelle for both gastric cancer and chronic gastritis in Southeastern Brazilian population evaluated. Thus we have shown the importance of polymorphisms in genes of anti-inflammatory factors in stomach carcinogenesis, reinforcing the contribution of the host genetic factors in the development of gastric cancer.

Abbreviations List:

C: Control;

CG: Chronic Gastritis;

CI: Confidence Interval;

dH_2O: Distilled Water;

dNTPs: Deoxyribonucleotide Triphosphates;

EDTA: Ethylenediamine Tetraacetic Acid;

GC: Gastric cancer

IL-1β: Interleukin 1-beta gene;

IL-1ra: Interleukin-1 receptor antagonist

IL-1RN: Interleukin-1 receptor antagonist gene

$MgCl_2$: Magnesium Chloride;

MHC: Major Histocompatibility Complex

N: Individual numbers;

OR: Odds Ratio;

P: Probability;

Bp: Base pairs

PCR-RFLP: Polymerase Chain Reaction – Restriction Fragment Length Polymorphism;

SNP: Single Nucleotide Polymorphism;

TNF-α: Tumoral necrose factor α

TNF-β: Tumoral necrose factor β

T^o: Temperature;

Competing interests

The authors declare that they have no competing interests.

Author's contributions

JGO carried out the molecular genetic studies, acquisition of data, performed the statistical analysis and drafted the manuscript. AES participated in the conception and design of the study, data interpretation, and contributed to the preparation and revision of the manuscript. All authors have read and approved the final manuscript.

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Table 1. Primer sequences, restriction enzyme and fragment sizes for *IL1RN* and *TNFB* gene polymorphisms.

Genes	Primers	Enzyme T°/Time	Fragment (bp)
IL1RN	F: 5`- CCCCTCAGCAACACTCC -3` R: 5`- GGTCAGAAGGGCAGAGA -3`	–	allele 1=4 repetitions (410) allele 2=2 repetitions (210) allele 3=5 repetitions (500) allele 4=3 repetitions (325) allele 5=6 repetitions (595)
TNFB+252 A/G	F:5'- CTCCTGCACCTGCTGCCTGGATC-3' R:5'- GAAGAGACGTTCAGGTGGTGTCAT -3'	*NcoI* 37°C, 1 h	G/G: 368 A/G: 368+235+133 A/A: 235+133

bp, base pairs; T°, temperature.

Table 2. Genotype and allele frequencies of *IL-1RN* VNTR and *TNFB*+252A/G polymorphisms in gastric cancer (GC), chronic gastritis (CG), and control (C) groups.

Genotypes/ Alleles	GC N=200 (%)		C N=246 (%)		CG N=229 (%)
***IL-1RN* VNTR**					
L/L	84 (42.0)		160 (65.0)		119 (52.0)
L/2	86 (43.0)		76 (31.0)		86 (37.5)
2/2	30 (15.0)		10 (4.0)		24 (10.5)
OR (95%IC)		2.56 (1.75-3.77)		1.72 (1.18-2.48)	
p		<0.01		<0.01	
Alleles					
L	0.63		0.80		0.70
2	0.37		0.20		0.30
OR (95%IC)		2.37 (1.75-3.20)		1.70 (1.26-2.30)	
p		<0.01		<0.01	
***TNFB*+252 A/G**					
A/A	86 (43.0)		126 (51.2)		103 (45.0)
A/G	97 (48.5)		93 (37.8)		95 (41.5)
G/G	17 (8.5)		27 (11.0)		31 (13.5)
OR (95%IC)		1.32 (0.95-2.02)		1.28 (0.89-1.84)	
p		0.08		0.19	
Alleles					
A	0.67		0.70		0.65
G	0.33		0.30		0.35
OR (95%IC)		1.14 (0.86-1.51)		1.22 (0.93-1.60)	
p		0.38		0.16	

CI, confidence interval; L, alleles 1, 3, 4 e 5; N, numbers; OR, odds ratio; p, probability.

Table 3. Combined effect of *IL-1RN* (L/2 or 2/2 genotypes) and *TNFB*+252 A/G (A/G or G/G genotypes) polymorphisms on risk of gastric cancer (GC) and chronic gastritis (CG).

Risk genotype	Groups								
	GC (n=200)	C (n=246)	OR (95% CI) *P*	CG (n=229)	C (n=246)	OR (95% CI) *P*	GC (n=200)	CG (n=229)	OR (95% CI) *P*
Neither	34	79	1.00 (reference)	59	79	1.00 (reference)	34	79	1.00 (reference)
IL-1RN L/2 or 2/2/	64	38	2.01 (1.49-2.71)	55	38	1.48 (1.07-2.05)	64	38	1.35 (1.06-1.71)
TNFB+252 A/G or G/G			<0.01			0.01			0.01

p, probability; OR, odds ratio.

Table 4. OR and 95% confidence intervals (CIs) for *IL-1RN* VNTR and *TNFB*+252 A/G adjusting for risk factors for gastric cancer (GC), chronic gastritis (CG), and control (C) groups.

Variables	F (GC/C) %	OR (95%CI)	p	F (CG/C) %	OR (95% CI)	p
Gender						
F	22.0/48.8	reference	<0.01	50.7/48.8	reference	0.79
M	78.0/51.2	2.27 (1.42-3.62)		49.3/51.2	0.95 (0.64-1.39)	
Age (years)						
≤60	39.5/56.9	reference	0.01	54.5/45.5	≤53 reference	0.03
>60	60.5/43.1	1.70 (1.10-2.61)		45.5/54.5	>53 0.66(0.45-0.96)	
Smoking						
Nonsmokers	29.5/36.6	reference	0.20	44.5/36.6	reference	<0.01
Smokers	70.5/63.4	0.72 (0.44-1.19)		55.5/63.4	0.58 (0.38-0.87)	
Alcohol						
Nondrinkers	43.5/74.4	reference	<0.01	68.1/74.4	reference	<0.01
Drinkers	56.5/25.6	3.09 (1.91-5.02)		31.9/25.6	1.81(1.16-2.83)	
IL1-RN						
L/L	42.0/65.0	reference	<0.01	52.0/65.0	reference	<0.01
L/2	58.0/35.0	2.53 (1.66-3.87)		48.0/35.0	1.79 (1.22-2.62)	
2/2						
***TNFB*+252 A/G**						
A/A	43.0/51.2	reference	0.13	45.0/51.2	reference	0.20
A/G						
G/G	57.0/48.8	1.38 (0.90-2.09)		55.0/48.8	1.27 (0.87-1.84)	

CI, confidence interval; F, frequency of individual (%); p, probability; OR, odds ratio.

Artigo III

Polymorphisms in promoter regions of TNF-*α*, IL8 and IL10 cytokines and gene expression in chronic gastritis and gastric cancer.

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Abstract

Background: Inflammation is a primary defense process against various extracellular stimuli, such as *H. pylori* infection in the gastric mucosa. Here we evaluated the association of the pro and anti-inflammatory cytokines polymorphisms, on the risk of chronic gastritis and gastric cancer. Besides, we tried to correlate the polymorphisms in promoter region of *TNF-α, IL8* and *IL10* with mRNA expression levels of these cytokines.

Methods: In this case-control study, polymorphism at *TNFA* (rs1800629 and rs1799724)*, IL8* (rs4073 and rs2227532) and *IL10* (rs1800872) genes were investigated using the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique in 723 Brazilian individuals. For gene expression levels analysis, mRNA of 47 patients with CG and 45 with GC were used in Real-time qPCR assay.

Results: The SNPs *TNFA*-308 G/A and *IL8*-251T/A were not associated with risk of gastric lesions. However, *TNFA*-857C/T, *IL8*-845T/C and *IL10*-592 C/A showed genotype or allele frequencies statistically significant between GC and C groups and the combined genotypes *TNFA*-308A/*TNFA*-857T/*IL8*-251A/*IL8*-845C, *TNFA*-308A/*TNFA*-857T/*IL8*-845C/*IL10*-592A, and *TNFA*-308A/ *IL8*-251A/ *IL8*-845C/ *IL10*-592A led to more potent risk of GC. SNPs *IL8*-845T/C and *IL10*-592C/A also presented genotype or allele frequencies statistically significant between the groups CG and C. Risk factors such as male gender, age and alcohol intake were associated with a higher susceptibility on gastric carcinogenesis. In general, *IL8* and *IL10* relative gene expression levels were down-regulated in the case groups. For *TNFA* was not obtained results due to failure to validate the primers. Comparing mRNA expression mean levels between GC and CG groups, there was a significant difference only for *IL10* gene ($p<0.01$) with means levels of -2.66 ± 2.19 and 0.37 ± 3.47, respectively. When grouped the samples according to *IL8*-845 T/C and *IL10*-592 A/C polymorphism, the polymorphic variants influenced the expression of these cytokines.

Conclusions: We found that promoter region polymorphisms of *TNFA*-857 C/T, *IL8*-845 T/C and *IL10*-592 C/A were significantly associated with increased risk for gastric carcinogenesis. Also demonstrated the functional relevance of variants SNPs *IL8*-845C and *IL10*-592A, reinforcing the important role of these cytokines in gastric carcinogenesis.

Background

Inflammation is a primary defense process against various extracellular stimuli, such as viruses, pathogens, and foods [1]. *H. pylori* is an imminent carcinogenic pathogen [2], and various studies have shown that infection by this bacterium causes infiltration of inflammatory cells, oxidative damage and gene mutations, which forms a critical cross-link between chronic inflammation and gastric carcinogenesis [3].

Single-nucleotide polymorphisms (SNPs) in pro- and anti-inflammatory cytokine as *IL-1B*, *IL-1RN*, *TNFA*, and *IL10* that modify the intensity of the inflammatory response may contribute to variations on risk of gastric cancer [4-7]. In particular, it is believed that some SNPs occurring within regulatory regions of cytokine genes are related to the change in the affinity of transcription factors, thus affecting the cytokine expression and secretion profile in response to infection agents [8].

TNF-α is a potent gastric acid inhibitor, which consequently causes considerable change in *H. pylori* infection and its distribution on the corpus region of stomach [9]. Therefore, it seems likely that the expression level of TNF-α may be involved in cancer pathogenesis and progression. The gene expression level of TNF-α is proven to be greatly influenced by polymorphisms in its promoter region, that have been studied as a putative determinant factor of susceptibility toward various autoimmune diseases [10-12], and cancer [13,14]. The A allele of TNFA-308 G/A (rs1800629) and the T allele of TNFA-857 C/T (rs1799724) polymorphisms show higher transcriptional activity and increased production of TNF-α cytokine [15].

IL8, the most potent known chemokine, is responsible for inducing chemotaxis, which consists of directed migration of cells to a site of inflammation. This chemokine also mediates the activation and migration of neutrophils into tissue from peripheral blood. Multiple stimuli will induce the secretion of IL8, including lipopolysaccharide (LPS), live bacteria, early pro-inflammatory cytokines such as TNF and IL1 [16]. A well-characterized SNP at position -251T/A (rs4073) of *IL8* gene was associated with several diseases as bronchiolitis, macular degeneration, breast cancer, oral squamous cell carcinoma, prostate cancer, and gastric cancer [17-20]. Studies have shown that the A polymorphic allele of -251T/A polymorphism up-regulated IL8 levels after stimulation with LPS [21]. The polymorphism *IL8*-845T/C (rs22277532) has received attention from researchers, who have attempted to establish an association with inflammatory diseases [22, 23].

In contrast, anti-inflammatory cytokine IL10 is involved in down regulating cell-actived and cytotoxic inflammatory response. It inhibits the formation of pro-inflammatory cytokines such as TNF-α and IL1B [24]. Several studies have reported functional effect of IL10 promoter region polymorphisms on transcriptional activation which may alter IL10 protein production *in vitro*. Among them, the IL10-592 C/A (rs1800872), whose allele A is associated with risks of development of gastric cancer [25], and decreased mRNA expression of this cytokine [26].

Few studies have evaluated the relationship between polymorphic variants within promoter region of cytokine genes and the expression levels on the risk for carcinogenesis [27, 28]. Thus, the objective of this study was to investigate the association of the TNFA-308 G/A (rs1800629), TNFA-857C/T (rs1799724), IL8-251T/A (rs4073), IL8-845T/C (rs22277532) and IL10-592C/A (rs1800872) polymorphisms on the risk of chronic gastritis and gastric cancer in a Brazilian Southeastern population. Besides, try to correlate the polymorphisms and mRNA expression levels of these cytokines with the risk of gastric carcinogenesis in this population.

Methods

Population

This study was conducted prospectively in two steps. First, was performed a case-control study on chronic gastritis and gastric cancer, in which a total of 723 DNA samples from peripheral blood were genotyped for cytokine polymorphisms. The case groups comprised 276 individuals (142 men and 134 women) with a histopathologically confirmed diagnosis of chronic gastritis – CG (Sidney System) [29] with a mean age of 53,7±14,41 years (range 19 to 86 years), and 207 individuals (161 men and 46 women) with a histopathologically confirmed diagnosis of gastric cancer – GC (Lauren's classification) [30] with a mean age of 62,0±12,6 years (range 28 to 93 years). Results of *H. pylori* infection were obtained for the available cases in the medical records (101 with gastric cancer and 224 with chronic gastritis). The diagnosis was established by the Giemsa staining technique or by the urease test, performed at the Pathology Services of the Hospital. The cancer-free control group (C) with no previous history of gastric disease was composed of 240 healthy individuals (121 men and 119 women), mainly blood donors, with a mean age of 56.10± 17.70 years (range 20 to 93 years).

The second step was to evaluate the mRNA expression levels of genes *TNF-A*, *IL8* and *IL10* in cDNA samples of gastric tissues. Biopsies from the lesion area were collected during endoscope evaluation of 47 individuals with CG (29 men and 18 women) with a mean age of 53.10±9.41 years (range 41 to 84 years), and 45 individuals with GC (38 men and 7 women) with a mean age of 62.77±14.20 years (range 33 to 88 years). The biopsies were collected mainly from the antrum and corpus regions of the stomach.

All subjects were recruited from the Hospital de Base in São José do Rio Preto, SP, and from the Pio XII Foundation in Barretos, SP, Brazil. Epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, with questions about current and past occupation, smoking habits, alcohol intake and family history of cancer. Considering the admixture ethnic of Brazilian population evaluated, the individuals were ethnically classified on their visual appearance as Caucasians (90%) and Negroids (10%). The National Research Ethics Committee approved this work, and written informed consent was obtained from all individuals.

Genotyping of cytokine gene polymorphisms

About 5 mL of whole blood were collected from all study participants in sterile EDTA-coated vacutainers. DNA was extracted according to a previous report [31] and stored at -20ºC until use for genotyping.

The PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique was carried out to identify the cytokine gene polymorphisms in cases and control groups [23, 32-35]. The Table 1 summarizes the PCR conditions, the sets of primers and the enzymes used for each analysis. In brief, PCR was carried in a total reaction volume of 25 μL, containing 2.5 μL 10×PCR buffer, 2 μL dNTPs (1.25 μmol/L, Invitrogen, Carlsbad, CA, USA), 0.5 μL $MgCl_2$ (25 mmol/L), 1.25 μL of each primer (25 mmol/L, Sigma-Aldrich, St. Louis, MO, USA), 15.3 μL dH_2O, 2 μL DNA (100 ng/ μL), and 0.2 μL Taq DNA polymerase (5 U/μL, Invitrogen, Carlsbad, CA, USA). Then, 10 μL of PCR products were digested with 0.5 μL of the specific enzyme (5 U/μL) in a 10 μL volume including 2.5 μL 10×buffer 1 (all New England Biolabs, Massachusetts, USA) and 7.0 μL dH_2O. The products were then electrophoresed on a

1.5 – 3.0% agarose 1000 (Invitrogen, Carlsbad, CA, USA) gel, to allow detection by ethidium bromide staining.

RNA extraction and reverse transcription

Total cellular RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) 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 -80ºC and used for reverse transcription. cDNA was synthesized from 2,5 μg of total RNA using random primers and a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, *USA*), according to the manufacturer's instructions. The integrity of all cDNA preparations was tested by PCR assay of the β-actin gene (F: 3`-GGCATCGTGATGGACTCCG-5` and R: 3`- GCTGGAAGGTGGACAGCG-5`) and visualized on 1.5% agarose gel.

Quantitative Real Time PCR- qPCR

The relative expression levels of *TNFA*, *IL8* and *IL10* mRNA was measured by real time PCR (qPCR), based on the SYBR Green methodology, using an ABI Prism® 7300 (Applied Biosystems, Foster City, CA, ***USA***). *B-actin* gene (F-5' TGCCCTGAGGCACTCTTC 3' and R-5' CGGATGTCCACGTCACAC) was used as reference because it showed the lowest variation in comparison with *α-tubulin* and β2-*microglobulin* in previous study [36]. The primer sequences used to *IL8* and *TNFA* genes were obtained using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), as follow: *IL8* F-5' GATGCCAGTGAAACTTCAAGC 3' and R-5'ATTGCATCTGGCAACCCTAC 3'. For *TNFA* gene was designed and tested two primer sequences, both between exons 3 and 4, primer-1: *TNFA* F-5'AGCCCATGTTGTAGCAAACC 3' and R-5' GAGGTACAGGCCCTCTGATG 3', primer-2: *TNFA* F-5'AGCCCATGTTGTAGCAAACC 3'and R-5'GAGGTACAGGCCCTCTGATG3'. The primer sequence to *IL10* gene was obtained according to Sorensen et al. [11] *IL10* F-5' ACGGCGCTGTCATCGATT 3' and R-5' GGCATTCTTCACCTGCTCCA 3'.

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 $E=10^{[-1/slope]}$. Both primer sequences for *TNFA* (primer-1 and primer-2) presented efficiency below 90%, so were not processed. After the validation step for *IL8* and *IL10* genes, the qPCR assays were performed in 10 µL of SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, *USA*), 25 ng of cDNA and 0.4 µM of *β-actin* and 0.5µM of *IL8* and *IL10* primers. Samples of normal gastric mucosa were mixed to form a pool that was used as a calibrator (standard sample). Thermal cycling conditions for all the genes were: 2 min at 50ºC and 10 min at 95ºC for initial denaturation, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, and a dissociation step at 95ºC for 15 s, 60ºC for 30 s and 95ºC for 15 s. Triplicates of the samples were assayed in each run. Gene relative quantification (RQ) of both genes was calculated according to the description by Pfaffl [37] and normalized with the *β-actin* control reference gene and corresponding normal gastric mucosa. The transcript levels around 1.5-fold were considered significant, whether increased or decreased.

Statistical Analysis

Fisher's exact test was utilized to compare the groups with regard to genotype and allele frequencies, and chi-square test for determining Hardy-Weinberg equilibrium. Odds ratios (OR) and 95% confidence intervals (CIs) for polymorphisms evaluated were computed using multiple logistic regression models adjusting for age, gender, smoking, drinking and *H pylori* infection in gastric cancer and chronic gastritis groups. OR were calculated using a dominant model (i.e., combining heterozygous and homozygous for the minor allele) for all polymorphisms. Data about RNAm relative expression were described using mean ±SD as a point estimator and the range of values. To determine the possible association between relative gene expression difference between groups was used *t* test with *Welch's* correction and within groups to analyze the influence of factors such as polymorphisms, sex, age, ethnicity, smoking, alcohol consumption and the presence of *H. pylori* was used Man-Whitney *U*-tes*t*. Statistical analyses were performed using the GraphPad Instat; and SPSS (11.5 version) computer software programs. A probability level (p) of less that 0.05 was adopted as significance criterion.

Results

Case-control study

The samples of the 723 subjects were genotyped for the *TNFA*-308G/A and -857C/T, *IL8*-251T/A and -845T/C and *IL10*-592 C/A in gastric cancer (GC), chronic gastritis (CG) and control (C) groups. The genotype and allele frequency distribution of all polymorphisms were in agreement with Hardy-Weinberg equilibrium in both cases and control groups (data not shown).

The genotype and allele frequencies for these polymorphisms are presented in Table 2. The polymorphisms *TNFA*-857 C/T, *IL8*-845 T/C and *IL10*-592 C/A showed genotype frequencies statistically significant ($p<0.01$) between GC (50.7%, 23.7% and 49.8%, respectively) and C (34.6%, 6.3% and 29.6%, respectively), as well as allele frequencies ($p=0.02$; $p<0.01$ and $p<0.01$, respectively). In addition, for the *IL10*-592 C/A polymorphism there was a statistically significant difference for genotype ($p<0.01$) and allele ($p<0.01$) frequencies among CG (53.3 and 0.30, respectively) and C (29.6 and 0.18, respectively) groups. However, for the *IL8*-845 T/C polymorphism was observed only difference in allele frequencies ($p=0.03$) among the CG (0.06) and C (0.03) groups. Contrary, for *TNFA*-308G/A and *IL8*-251T/A, no significant difference was found between the three groups evaluated.

The potential associations between the distributions of *TNFA*-308 G/A, *TNFA*-857 C/T, *IL8-251T/A*, *IL8*-845 T/C and *IL10* -592 C/A genotypes adjusting for risk factors for gastric cancer and chronic gastritis in comparison with the control group are presented in Table 3. In the GC group, the multiple logistic regression shows that male gender (OR= 2.09; 95% CI=1.28-3.40; $p<0.01$), age above 56 years (OR=1.98; 95%CI-1.26-3.10; $p<0.01$), alcohol intake (OR=3.14 95% CI=1.91-5.17; p=<0.01), *TNFA*-857 C/T (OR=1.81; 95% CI= 1.17-2.81; $p<0.01$), *IL8*-845 T/C (OR=3.97; 95%CI= 2.00-7.88; $p<0.01$) and *IL10*-592 C/A (OR= 2.36; 95%CI=1.51-3.67; $p<0.01$) were associated with a higher risk of developing of this cancer. The comparison between gastritis and control groups showed that alcohol intake (OR=1.95; 95% CI=1.26-3.03; $p<0.01$) and *IL10*-592 CA+AA genotypes (OR=2.78; 95% CI=1.91-4.05; $p<0.01$) was associated with the risk of chronic gastritis, while smoking (OR=0.57; 95% CI=0.38-0.85; $p<0.01$) had a protective role in the development of gastritis. For individuals tested for *H. pylori* infection (101 with gastric cancer and 224 with gastritis) there was no statistically significant association (data not shown).

The combined analysis of the five polymorphisms evaluated (*TNFA*-308G/A, *TNFA*-857C/T, *IL8*-251T/A, *IL8*-845T/C and *IL10*-592C/A) revealed statistically significant differences considering the various combinations according to Table 4, in GC group compared to control group. Among the different combinations it should be highlighted that the combined genotypes with four SNPs *TNFA*-308A/ *TNFA*-857T/*IL8*-251A/*IL8*-845C, *TNFA*-308A/*TNFA*-857T/*IL8*-845C/*IL10*-592A, and *TNFA*-308A/ *IL8*-251A/ *IL8*-845C/ *IL10*-592A lead to a greater risk of gastric cancer with OR (95%CI) respectively of 9.33 (1.33-65.26); 8.00 (1.08-58.82) and 8.75 (1.22-62.36).

mRNA expression in chronic gastritis and gastric cancer

For *TNFA* gene, data of gene expression was not obtained due to the fact that both two primers sequences tested have not been approved in the validation step. Thus, the Table 5 shows only the results for the mean levels of mRNA relative expression of *IL8* and *IL10* in the GC and CG groups. Considering all individuals of GC and CG groups regardless of their genotype for *IL8* and *IL10* genes, it can be observed that, in general, both genes presented down regulation, with relative expression mean levels respectively of -0.45+2.63 and -2.66+2.19 for GC group and -0.71+3.40 and 0.37+3.47 for CG group. Thus, significant difference was observed only for *IL10* ($p<0.01$) when compared expression mean levels between GC and CG groups.

For *IL8*, considering all cases, the mRNA expression level was up-regulated in 22.0% and 30.0% of GC and CG cases with an maximum increase of 4.16 and 10.76-fold after normalization with the *β-actin* reference gene and comparison with normal mucosa, respectively (Table 5). When the samples were grouped according to polymorphic variants (Table 6) for the *IL8-251T/A* no significant difference was found in the expression mean levels of *IL8* gene between the individuals with wild genotype (-251TT) and polymorphic allele-carriers (-251TA/AA), in both groups of GC and CG. However, at *IL8*-845 T/C, whose C allele was related to increased risk of gastritis and gastric cancer in our results, it was observed that among C allele-carriers individuals, the *IL8* mRNA relative expression was up-regulated in 52.6% of cases of GC and 72.2% of cases of CG. Thus, statistically significant difference in the gene expression levels between TT homozygous and C allele-carriers (TC heterozygous and CC homozygous) was found in the groups of GC ($p<0.01$) and CG ($p<0.01$) (Table 6, Figure 1).

In addition, at *IL10* gene, the mRNA expression level was down-regulated in 78.0% and 28.0% of GC and CG cases with a maximum reduction of -10.57 and -5.99-fold in comparison with normal mucosa and after normalization with the *β-actin* reference gene, respectively. Moreover, when they were grouped according to the polymorphism of *IL10*-592 C/A (Table 6) was evident a statistically significant difference in CG group ($p<0.01$) between CC homozygous individuals (mRNA mean level 1.7+4.21) and A allele-carriers (heterozygous CA and homozygous AA, mRNA mean level -0.9+1.79). For the GC group there is no statistically significant difference ($p=0.65$) was observed (Figure 1)

There was no statistically significant difference in relative gene expression of IL8 and IL10 cytokines evaluated within the group of chronic gastritis and gastric cancer when stratified according to risk factors (gender, age, smoking, alcohol intake (data not shown).

Discussion

Chronic or recurrent inflammation is known to play a causative role in the promotion and progression of many human tumors and the association of different cytokines with tumor development is well documented. However studies investigating the role of genetic polymorphisms on cytokine secretion are controversial and the experimental approaches used, mainly *in vitro*, have several disadvantages not reflecting the real situation [27]. To overcome these difficulties, we investigated the influences of SNPs in the promoter region at *TNFA* (rs1800629 and rs1799724), *IL8* (rs4073 and rs2227532) and *IL10* (rs1800872) genes at risk of developing gastric cancer and chronic gastritis and determined cytokines mRNA levels in the mucosa of stomach lesions.

Among the SNPs *TNFA*-308 G/A and -857 C/T evaluated in this study was found association with chronic gastritis and gastric cancer only at *TNFA*-857 C/T. The influence these two polymorphisms in the promoter region of *TNFA* gene on risk of gastric cancer in different populations is not well established. For example, Zhang et al.

[13] in a meta-analysis concluded that *TNFA*-308 locus would be a risk factor for gastric cancer, especially in Caucasian populations. Besides, *TNFA*-857 locus may be related to gastric cancer risk, although most studies are concentrated in Asia. However, other recent meta-analysis that examined Asian and non-Asians studies showed no association of the polymorphism *TNFA*-308 G/A with gastric cancer, evidencing a great controversy [38].

TNFA has been recognized as an important player in both inflammation and cancer development. Several polymorphisms have been associated with a higher production of this cytokine [39]. Studies realized mainly in European populations reported a significantly greater risk of GC in *TNFA*-308 A carriers [6, 25]. Contrary, these findings have not been able to be replicated, mainly in Asian countries [27, 33, 39, 40]. Although the frequency of the A allele in our study in a sample of Southeastern Brazilian population (18%) is comparable to Caucasians (19-20%), but much higher than the Asian - 1,7% [15], there was clearly no association with risk of gastric cancer for this polymorphism in the present study. Similarly another study in Southeastern Brazilian population also found no association of *TNFA*-308 SNP with non-cardia gastric carcinoma [41].

In contrast, the SNP *TNFA*-857 C/T was associated with gastric cancer risk in the current study, as also in another studies with gastric and duodenal ulcer [42], rugal hyperplastic gastritis by *H. pylori* infection and gastric cancer [43]. Despite all studies being conducted in Asian populations, the T allele frequency (0.28) in the present study is similar to that reported in Japanese population (0.22) [44].

IL8-251T/A is commonly referred to with increased risk and poor prognosis of several diseases, including gastric cancer [17, 34, 45]. Contrary to our expectation, the results of this study showed an approximate frequency of A variant allele in cases (47%-GC and 50%-CG) and healthy controls (47%), so not demonstrating increased susceptibility to these gastric lesions. Although it has been reported that IL8 -251 A allele is the high producer allele [34], it is still controversial. Lee et al. [40] demonstrated that IL8-251 T allele possessed transcriptional activity two to five-fold stronger than the A counterpart. On the other hand, Hacking et al. [46] found no difference in promoter activity of the two alleles by performing analysis on the -251 T/ A promoter SNP. It is also possible that SNPs at other positions of the *IL8* gene may also have an effect on production of this cytokine.

When we investigated another polymorphism in the promoter region of *IL8* gene at position -845, our results demonstrated for the first time, an association of the *IL8*-845 C polymorphic variant with susceptibility to chronic gastritis and gastric cancer and the influence of this polymorphism on the gene expression level of this cytokine. The scarcity of studies with this polymorphism may be explained by the low frequency of C allele, which is absent in European and Asian populations [23]. In contrast, this C variant allele is common in African Americans (13%) [47], with similar frequency to that found in our study for gastric cancer patients (15%), but different for chronic gastritis patients (6%) and healthy individuals (3%), indicating the probable influence of this polymorphism in gastric carcinogenesis.

It is known that IL8 expression is greatly affected by the activation of nuclear factor-kappa B (NF-κB). It can be induced by the activators of NF-κB, such as TNF-α and CXCL12 in an inflammatory process [48]. We found in our samples of both gastric cancer and chronic gastritis a heterogeneity of relative gene expression to this cytokine, with samples showing down-and up-regulated expression, However, when grouped those individuals carrying the C variant allele was observed increased expression mean levels of 1.6 and 2.3-fold respectively, in the groups of gastric cancer and chronic gastritis, while the TT wild homozygous in both groups showed down regulated expression. These results evidence the genetic tendency for C polymorphic allele to increase transcriptional activity and overexpression of this cytokine with increased active immune response and possibly more susceptibility for carcinogenesis.

In the current study we observed an association of the polymorphism *IL10*-592 C/A with both gastric lesions evaluated. The frequency of the -592A allele in our analysis among the healthy controls was 18%, much lower than those of Asian, Latinos and European Caucasians (65%, 50% and 30%, respectively) [40, 49, 50]. However among our patients of case groups this frequency was increased around 30%, so evidencing the susceptibility to both gastric cancer and chronic gastritis in our population. These results are corroborated by other studies in that the *IL10*-1082 A/A, *IL10*-819 T/T and *IL10*-592 A/A genotypes or the ATA haplotype of *IL10*-1082/-819/-592 polymorphisms were associated to an increased risk of development of *H. pylori* infection-related gastric cancer [25, 42].

Contrary, a recent meta-analysis showed that *IL10*-592 C/A polymorphism was not a risk factor to GC on the basis of all studies examined. According to this meta-analysis when stratifying for the race, it was found that *IL10*-592 AA genotype was a protective factor on development of GC in Asians. But, there were no significantly differences in genotype distribution between GC case and control among Caucasians and Latinos. The authors indicate the difference of genetic background among Asians and other races [51].

In addition, we also observed a low gene expression level of this cytokine in gastric cancer compared with gastritis group, as well as in the groups of gastric cancer and chronic gastritis stratified according with the polymorphic variant (CC and CA + AA), where A allele-carriers showed more reduced expression of IL10 cytokine, that was statistically significant within gastritis group. Rad *et al*. [27] evidenced that GCC haplotype carriers (IL10 -1082G; -819C; -592C) were associated with high and ATA carriers with low IL10 gene expression levels. More recently, Faupel-Badger *et al*.[52] demonstrated that genotypes correlated with low IL10 production was associated with increased risk of prostate cancer and with high-grade disease. These results confirmed the relevance of IL10 polymorphisms in the down-regulation of the inflammatory response. The association with the low levels of IL10 may be related to their role as an anti-inflammatory cytokine that down-regulated IL-1β, TNF-α, interferon gamma, and other proinflammatory cytokines. Relative deficiency of IL10 may result in a Th1 driven hyperinflammatory response to *H. pylori* with greater damage to the gastric mucosa [25]. Imbalances among pro-inflammatory and anti-inflammatory mediators and acid gastric production may favor the development of gastric atrophy and its progression to cancer.

The combination of multiple pro- and anti-inflammatory polymorphisms as shown in our study, which the combination of four polymorphisms (*TNFA*-308A/ *TNFA*-857T/*IL8*-251A/*IL8*-845C, *TNFA*-308A/*TNFA*-857T/*IL8*-845C/*IL10*-592A, and *TNFA*-308A/ *IL8*-251A/ *IL8*-845C/ *IL10*-592A) increased the values of OR at risk of gastric cancer, has also been reported by other researchers. Perez-Perez et al. [26] analyzed gene polymorphisms of *IL-1B*, *IL-1RN*, *TNFA* and *IL10* and showed that the OR for non-cardia gastric cancer increased progressively with increasing number of pro-inflammatory genotypes. Other groups also had similar results [25, 53], probably by an additive effect of pro-inflammatory profile of these SNPs, resulting in an exacerbated immune response and increased risk for gastric cancer.

Despite the influence of infection by *H. pylori* is well defined in the progression of gastric cancer in some populations, in the present study, we observed no association between *H. pylori* infection and risk of gastric cancer, probably due to the limited number of individuals with diagnosis for this bacterium in the samples evaluated. While *H. pylori* infection and host genetic factors interact to initiate a hypochlorhydric and atrophic phenotype, other factors may contribute to subsequent neoplasic transformation, which does not depend on continued presence of the infection. Among environmental risk factors, in the present study, male gender, age and alcohol intake were associated with risk of gastric cancer. According to the National Cancer Institute the highest incidence of gastric cancer occurs in men around age 70, and about 65% of patients diagnosed with this type of cancer were over 50 years. In Brazil, gastric cancer is third in incidence among men and fifth in women, so our results are consistent with epidemiological data reported in literature. The excessive consumption of alcohol is also another risk factor well established in relation to gastric carcinogenesis [54]. The oxidation of ethanol generates acetaldehyde, which primarily has direct carcinogenic and mutagenic effects. This metabolite interferes with many sites of synthesis and DNA repair, which can lead to tumor development [55]. Thus, cytokine gene polymorphisms represent one component of a complex interplay among the host, pathogen, and environmental factors involved in gastric carcinogenesis.

Conclusions

In summary, we found that the promoter region polymorphisms of *TNFA*-857 C/T, *IL8*-845 T/C and *IL10*-592 C/A are significantly associated with increased risk for gastric carcinogenesis and the genotypic profile composed of *TNFA*-308A/ *TNFA*-857T/*IL8*-251A/*IL8*-845C; *TNFA*-308A/*TNFA*-857T/*IL8*-845C/*IL10*-592A, and *TNFA*-308A/ *IL8*-251A/ *IL8*-845C/ *IL10*-592A led to a higher risk of GC. The relative gene expression levels of IL8 and IL10 cytokines are down-regulated in most samples of gastric cancer and chronic gastritis. However, we observed the influence of C allele-carriers of the *IL8*-845T/C SNP that showed increased expression in comparison to TT wild homozygous, and also of A allele-carriers of the polymorphism *IL10*-592 A/C, that, on the contrary, showed greater reduction in the expression of this cytokine. These results show the functional relevance of these promoter region polymorphisms *in vivo* and their importance to clarify the role of pro- and anti-inflammatory cytokine in gastric carcinogenesis.

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Table 1. Nucleotide primer sequences and PCR conditions for genotyping by PCR-RFLP.

Genes	Primer (5`-3`)	PCR Cycles	T°*melting*	Enzyme, T°/Time	Agarose Gel	Alleles	Reference
TNFA-308 G/A (rs1800629)	F: GAGGCAATAGGTTTTGAGGGCCAT R: GGGACACACAAGCATCAAG	36	65°C	*Nco*I, 37°C/1h	3%	G: 126+21 bp A: 147 bp	[32]
TNFA-857 C/T (rs1799724)	F: AAGTCGAGTATGGGGACCCCCCGTTAA R: CCCCAGTGTGTGGCCATATCTTCTT	36	65°C	*Hinc*II, 37°C/1h	3%	C: 107 +24 bp T: 131 bp	[33]
IL8-251 T/A (rs4073)	F: TTCTAACACCTGCCACTCTAG R:CTGAAGCTCCACAATTTGGTG	35	60°C	*Mfe*I, 37°C/12h	3%	A: 76+32 bp T: 108 bp	[34]
IL8-845 T/C (rs2227532)	F: AACCCAGCAGCTCCAGTG R: AGATAAGC CAGCCAATCATT	35	61°C	*Vsp*I, 37°C/1h	1.5%	T: 341+193 bp C: 534 bp	[23]
IL10-592 C/A (rs1800872)	F: GGTGAGCACTACCTGACTAGC R: CCTAGGTCACAGTGACGTGG	30	60°C	*Rsa*I, 37°C/12h	1.5%	A: 412 bp C: 236+176 bp	[35]

bp, bases pairs; T°, temperature.

Table 2. Genotype and allele frequencies of *TNFA*, *IL8* and *IL10* polymorphisms in case and control groups.

Genes	Genotypes/Alleles	GC N=207 (%)	C N=240 (%)	OR(95%CI)	p	CG N=276 (%)	C N=240 (%)	OR(95%CI)	p
	GG	138 (66.7)	167 (69.6)	1.14(0.76-1.70)	0.54	194 (70.3)	167 (69.6)	0.96(0.66-1.41)	0.92
TNFA-308 G/A	GA + AA	69 (33.3)	73 (30.4)			82 (29.7)	73 (30.4)		
(rs1800629)	G	0.82	0.84	1.14(0.80-1.62)	0.47	0.84	0.84	0.96(0.69-1.35)	0.86
	A	0.18	0.16			0.16	0.16		
	CC	102 (49.3)	157 (65.4)	1.94(1.33-2.85)	<0.01	167 (60.5)	157 (65.4)	1.23(0.86-1.76)	0.27
TNFA-857 C/T	CT + TT	105 (50.7)	83 (34.6)			109 (39.5)	83 (34.6)		
(rs1799724)	C	0.72	0.79	1.42(1.04-1.93)	0.02	0.78	0.79	1.01(0.75-1.37)	0.93
	T	0.28	0.21			0.22	0.21		
	TT	62 (30.0)	61 (25.4)	0.79(0.52-1.20)	0.29	58 (21.0)	61 (25.4)	1.28(0.84-1.93)	0.25
IL8-251 T/A	AT + AA	145 (70.0)	179 (74.6)			218 (79.0)	179 (74.6)		
(rs4073)	T	0.53	0.53	0.98(0.75-1.28)	0.94	0.50	0.53	1.11(0.87-1.42)	0.38
	A	0.47	0.47			0.50	0.47		
	TT	158 (76.3)	225 (93.7)	4.65(2.51-8.58)	<0.01	249 (90.2)	225 (93.7)	1.62(0.84-3.13)	0.15
IL8-845 T/C	TC + CC	49 (23.7)	15 (6.3)			27 (9.8)	15 (6.3)		
(rs2227532)	T	0.85	0.97	5.20(2.95-9.16)	<0.01	0.94	0.97	1.96(1.07-3.59)	0.03
	C	0.15	0.03			0.06	0.03		
	CC	104 (50.2)	169 (70.4)	2.35(1.59-3.47)	<0.01	129 (46.7)	169 (70.4)	2.71(1.88-3.90)	<0.01
IL10-592 C/A	CA + AA	103 (49.8)	71 (29.6)			147 (53.3)	71 (29.6)		
(rs1800872)	C	0.70	0.82	2.20(1.59-3.03)	<0.01	0.70	0.82	2.17(1.60-2.95)	<0.01
	A	0.30	0.18			0.30	0.18		

GC, gastric cancer; CG, chronic gastritis; C, health individuals; P, probability; OR, odds ratio.

Table 3. OR and 95% confidence intervals (CIs) for *TNFA, IL-8* and *IL-10* polymorphisms adjusting for risk factors for gastric cancer (GC), chronic gastritis (CG), and control (C) groups.

Variables		GC x C			CG x C			
		F(GC/C) %	OR(95%CI)	p		F(CG/C) %	OR(95%CI)	p
Gender	F	22.0/50.6	reference	<0.01		48.5/50.6	reference	0.83
	M	78.0/49.4	2.09 (1.28-3.40)			51.5/49.4	1.04 (0.71-1.52)	
Age	≤56 years	31.8/52.0	reference	<0.01	≤53 years	52.0/45.0	reference	0.05
	>56 years	68.2/48.0	1.98 (1.26-3.10)		>53 years	48.0/55.0	0.70 (0.48-1.0)	
Smoking	Nonsmokers	28.9/35.4	reference	0.50		44.9/35.4	reference	<0.01
	Smokers	71.1/64.6	0.83 (0.50-1.40)			55.1/64.6	0.57 (0.38-0.85)	
Alcohol	Nondrinkers	43.0/74.1	reference	<0.01		66.0/74.0	reference	<0.01
	Drinkers	57.0/25.9	3.14 (1.91-5.17)			34.0/26.0	1.95 (1.26-3.03)	
TNFA-308	GG	66.6/69.5	reference	0.89		70.3/69.6	reference	0.88
G/A	GA+AA	33.4/30.5	1.03 (0.64-1.65)			29.7/30.4	1.03 (0.69-1.53)	
(rs1800629)								
TNFA-857	CC	49.3/65.5	reference	<0.01		60.50/65.4	reference	0.24
C/T	CT+TT	50.7/34.5	1.81 (1.17-2.81)			39.5/34.6	1.25 (0.85-1.83)	
(rs1799724)								
IL8-251 T/A	TT	30.0/25.5	reference	0.14		21.5/25.4	reference	0.19
(rs4073)	AT+AA	70.0/74.5	0.69(0.43-1.13)			78.5/74.6	1.33 (0.86-2.0)	
IL8-845 T/C	TT	76.3/93.7	reference	<0.01		90.2/93.7	reference	0.41
(rs2227532)	TC+CC	23.7/6.3	3.97 (2.00-7.88)			9.8/6.3	1.33 (0.66-2.69)	
IL10-592 C/A	CC	50.2/70.5	reference	<0.01		46.7/70.4	reference	<0.01
(rs1800872)	CA+AA	49.8/29.5	2.36 (1.51-3.67)			53.3/29.6	2.78 (1.91-4.05)	

GC, gastric cancer; CG, chronic gastritis; C, health individuals; P, probability; OR, odds ratio.

Table 4. Combined effect of *TNFA* (rs1800629 and rs1799724), *IL8* (rs4073 and rs2227532) and *IL10* (rs1800872) polymorphisms on risk of gastric cancer (GC).

Risk genotype	Groups		
	GC (n=207)	C (n=240)	OR (95% CI) p
Neither	3	13	1.00 (reference)
TNFA-308A / *TNFA*-857T	40	21	1.50 (1.14-1.98) <0.01
TNFA-308A/ IL8-251A	46	52	1.17 (0.99-1.35) 0.05
TNFA-308A/ IL8-845C	16	5	3.03 (1.40-6.52) <0.01
TNFA-308A/ IL10-592A	27	19	1.15 (1.11-2.06) <0.01
TNFA-857T/ IL8-251A	59	61	1.15 (1.02-1.30) 0.03
TNFA-857T/ IL8-845C	25	7	2.55 (1.38-4.70) <0.01
TNFA-857T/ IL10-592A	49	31	1.33 (1.09-1.63) <0.01
IL8-251A/ IL8-845C	30	11	1.98 (1.26-3.10) <0.01
IL8-251A/IL10-592A	74	51	1.20 (1.05-1.37) <0.01
IL8-845C / IL10-592A	21	7	2.50 (1.35-4.63) <0.01
TNFA-308A/ TNFA-857T/ IL8-251A	20	12	1.81 (1.16-2.80) <0.01
TNFA-308A/ IL8-251A/ IL8-845C	11	3	4.19 (1.45-12.05) <0.01
TNFA-308A/ IL8-845C/ IL10-592A	14	5	2.96 (1.36-6.44) <0.01
TNFA-857T/ IL8-251A/ IL8-845C	10	5	2.76 (1.24-6.17) 0.01
TNFA-857T /IL8-845C/ IL10-592A	9	3	4.00 (1.37-11.67) <0.01
IL8-251A/ IL8-845C/ IL10-592A	17	6	2.69 (1.35-5.35) <0.01
TNFA-308A/ TNF-857T/ IL8-251A/ IL8-845C	6	1	9.33 (1.33-65.26) <0.01
TNFA-308A/TNFA-857T/ IL8-845C/ Il10-592A	4	1	8.00 (1.08-58.82) 0.02
TNFA-308A/ IL8-251A/ IL8-845C/ IL10-592A	5	1	8.75 (1.22-62.36) 0.01
TNFA-857T/ IL8-251A/ IL8-845C/ IL10-592A	5	3	3.33 (0.97-10.55) 0.06

GC, gastric cancer; C, health individuals; P, probability; OR, odds ratio.

Table 5. Down and up-regulated samples and mean level of mRNA expression of the *IL8* and *IL10* in gastric cancer (GC) and chronic gastric (CG).

Variable	*IL8*		*IL10*	
	GC (N=45)	CG (N=47)	GC (N=45)	CG (N=47)
N (%) down-regulated	11 (24.0)	23 (49.0)	35 (78.0)	13 (28.0)
N (%) up-regulated	10 (22.0)	14 (30.0)	1 (2.0)	10 (21.0)
Relative expression (mean±SD)	-0.45±2.63	-0.71±3.40	-2.66±2.19	0.37±3.47
Relative expression				
Minimum	- 7.68	- 7.72	-10.57	-5.99
Maximum	4.16	10.76	3.85	15.03
p	0.68		<0.01	

t test with Welch correction for comparing mRNA expression levels;

Table 6. Ccomparisons of gene expression levels of mRNA within groups (gastric cancer-GC and chronic gastritis-CG) to assess the influence of polymorphic variants.

M

Polymorphic genes		IL8-251 T/A (rs4073)		p	IL8-845 T/C (rs2227532)		p	IL10-592 C/A (rs1800872)		p
		TT	TA / AA		TT	TC / CC		CC	CA/AA	
		Up-regulated cases			Up-regulated cases			Down-regulated cases		
GC (N=45)	N(%)	5/28 (17.8)	4/17 (23.5)		0/26 (0.0)	10/19 (52.6)		20/26 (77.9)	15/19 (78.9)	
	Mean±SD	-0.3±2.35	-0.6±3.09	0.86	-1.9±2.46	1.6±0.82	<0.01	-2.3±2.16	-3.12±2.21	0.65
	Range	-7.07 to 4.16	-7.68 to 2.85		-7.68 to 1.37	0.18 to 4.16		-5.70 to 3.85	10.57 to -0.61	
CG (N=47)	N(%)	6/20 (30.0)	6/27 (22.2)		1/29 (3.4)	13/18 (72.2)		4/23 (17.3)	10/24 (41.6)	
	Mean±SD	0.1±4.07	-1.3±2.73	0.15	-2.6±2.09	2.3±2.80	<0.01	1.7±4.21	-0.9±1.79	<0.01
	Range	-7.72 to 10.76	-5.46 to 4.10		-7.77 to 1.64	-3.07 to 10.77		-2.66 to 15.03	-5.99 to 3.31	

an-Whitney *U*-test for comparing mRNA expression levels.

Figure Legend:

Figure 1. mRNA expression of *IL8* and *IL10* genes and agrouped according to the *IL8*-845 T/C and *IL10* -592 C/A polymorphisms in gastric cancer (GC) and chronic gastritis (CG) by qPCR. Data are expressed as mean. *t* test with Welch correction was used to compare mRNA expression levels between GC and CG groups; Mann-Whitney U-test was used to compare mRNA expression levels within groups. Statistically significant difference ($p<0.05$).

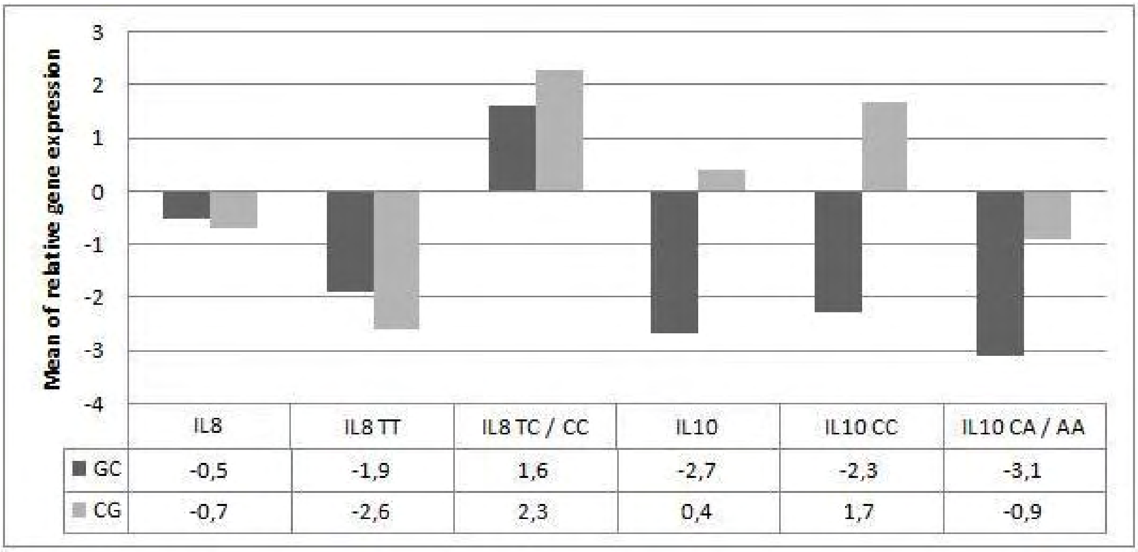
Mean of relative gene expression
3
2
1
0
-1
-2
-3
-4
IL8
IL8 TT
IL8 TC / CC
IL10
IL10 CC
IL10 CA / AA
GC
-0,5
-1,9
1,6
-2,7
-2,3
-3,1
CG
-0,7
-2,6
2,3
0,4
1,7
-0,9

Figure 1.

Discussão

VI. Discussão

Polimorfismos em genes de citocinas e receptores do sistema immune influenciam o perfil de resposta aos agentes infecciosos, como a *H. pylori* podendo contribuir para variações no risco da carcinogênese gástrica (GERMANO; ALLAVENA; MANTOVANI, 2008; HUSSEIN et al, 2010). No presente estudo a investigação de associação dos polimorfismos em genes dos receptores *toll like* – TLR (*TLR2* deleção -196 a -174, *TLR4* +896A/G e +1196C/T), genes de citocinas pró-inflamatórias (*TNFA* -308G/A e -857C/T, *TNFB* +252A/G *e IL8* -251T/A e +845T/C) e anti-inflamatórias (*IL-1RN* VNTR e *IL10* -592A/C) com o risco de desenvolvimento do câncer gástrico (CG) e gastrite crônica (GC) evidenciou associação dos polimorfismos *TLR2* ins/del, *TLR4*+896A/G, *TNFA*-857 C/T, *IL8-845* T/C, *IL-1RN* e *IL10*-592 C/A com o risco aumentado para a carcinogênese gástrica. Quanto aos fatores de risco relacionados ao câncer gástrico e gastrite crônica observou-se associação principalmente com idade avançada, sexo masculino e consumo de álcool. Os níveis de expressão gênica das citocinas IL8 e IL10, cujos polimorfismos estão presentes em regiões promotoras, evidenciaram de forma geral uma baixa expressão nos grupos casos. Porém, quando as amostras foram agrupadas de acordo com os polimorfismos *IL8*-845 T/C e *IL10*-592 A/C evidenciou-se que as variantes polimórficas afetam os níveis de expressão destas citocinas, demonstrando a relevância funcional destes SNPs *in vivo.*

Os TLRs participam do reconhecimento inicial da *H. pylori* na mucosa gástrica, e diferentes SNPs presentes nestes receptores estão associados com uma resposta inflamatória mais potente (DREXLER; FOXWELL et al., 2010). Os resultados deste estudo, demonstraram pela primeira vez na população brasileira uma associação dos alelos *TLR2* del e *TLR4* +896 G com suscetibilidade ao câncer gástrico. O polimorfismo *TLR4*+1196 C/T não foi associado com risco para as lesões gástricas avaliadas, e os genótipos homozigotos *TLR4* +896G/G e *TLR4* +1196T/T estavam ausentes nessa população.

Alguns estudos têm investigado a associação da deleção *TLR2* -196 a -174 del no desenvolvimento de doenças relacionadas ao processo inflamatório, mas com resultados conflitantes. Pandey et al. (2009) encontraram que o alelo *TLR2* del está associado com suscetibilidade para o câncer cervical. Assim como, Tahara et al. (2007) investigaram a influência deste polimorfismo na ocorrência de câncer gástrico não-cárdia e lesões como gastrite e úlcera duodenal e sugeriram uma associação do genótipo *TLR2* -196 a -174 del/del apenas para este tumor na população japonesa. De modo contrário, este mesmo grupo mostrou que o alelo *TLR2* –196 a –174 ins estava associado com maior gravidade da metaplasia intestinal em pacientes idosos (TAHARA et al., 2008). Contudo, Wang et al. (2007) na população japonesa falharam em mostrar associação dos genótipos *TLR2* -196 a -174 del/del e ins/del em pacientes com colite ulcerative.

O polimorfismo *TLR2* -196 a -174 del localizado no cromossomo 4, causa uma deleção de 22 nucleotídeos que altera a atividade promotora deste gene. O genótipo *TLR2* del/del tem sido relacionado como responsável pela diminuição da atividade promotora (NOGUCHI et al., 2004).

O gene *TLR4*, mapeado no cromossomo 9, consiste de 3 éxons. No terceiro éxon, dois SNPs não-sinônimos, *TLR4*+896 A/G e +1196 C/T promovem a substituição dos aminoácidos Asp299Gly e Thr399Ile, respectivamente. No presente estudo, as frequências do haplótipo *TLR4* G-C (299Gly-399Thr) e do alelo G foram maiores em pacientes com câncer gástrico indicando uma associação com risco aumentado para esta neoplasia. A substituição dos aminoácidos Asp299Gly quebra a estrutura normal da região extracelular do receptor TLR4 e pode causar uma diminuição do reconhecimento de seus ligantes, assim reduzindo a responsividade a lipopolissacarídeos (LPS) bacterianos e interrompendo o transporte deste receptor para a membrana celular (ARBOUR et al., 2000; SCRODER et al., 2005). Estas mudanças permitem uma resposta inflamatória exacerbada com grande destruição tecidual, provavelmente devido a uma falha no estímulo de células regulatórias e producão de citocina IL10 (HIGGINS et al., 2003; EL-OMAR, NG; HOLD et al., 2008).

Arbour et al. (2000) foram os primeiros a relatar que indivíduos com as substituições Asp299Gly e/ou Thr399Ile na região promotora do gene *TLR4* apresentavam uma redução da resposta a LPS. Portanto, durante a cascata da carcinogênese gástrica, os indivíduos com estes polimorfismos podem ter maior risco de uma inflamação grave, seguida pelo desenvolvimento de hipocloridria e atrofia gástrica, que são alterações precursoras importantes do câncer de estômago (EL-OMAR; NG; HOLD, 2008).

Ambos polimorfismos de *TLR4* estão presentes em 10% das populacões caucasianas e africanas e estão relacionados com a suscetibilidade para doenças infecciosas, embora estudos na população asiática não tenham detectado estas variantes (MOCKENHAUPT et al., 2006; CHENG et al., 2007). Já, no presente em uma amostra da população do sudeste brasileiro encontrou-se que ambos os SNPs do gene *TLR4* (+896A/G e +1196C/T) estão presentes em heterozigose em aproximadamente 6,5% e 13% e 2,5 e 4,5% dos pacientes com câncer gástrico e gastrite crônica, respectivamente. Outros estudos na população brasileira encontraram frequências similares de heterozigotos do polimorfismo

TLR4+896A/G em pacientes com doença de Chagas (5,6%), colite ulcerative (7,1%) e doença de Crohn (7,0%) (QUEIROZ et al., 2009; RAMASAWMY et al., 2009). Porém, este é o primeiro estudo que evidenciou associação do SNP TLR4 +896A/G em câncer gástrico na população brasileira.

Apesar da indicação do importante papel dos polimorfismos em região promotora, do gene *TLR4* na suscetibilidade ao câncer gástrico e lesões pré-cancerosas não há um consenso na literatura, mesmo em estudos em populações da mesma etnia. Por exemplo, na população mexicana, Garza-Gonzalez et al. (2007) não mostraram associação dos polimorfismos *TLR4* +896A/G e +1196C/T com o risco de câncer gástrico, enquanto Trejo de la O et al. (2008) observaram que ambos os SNPs de TLR4 tinham uma associação com úlcera duodenal e câncer gástrico. Na população indiana, Achyut et al. (2007) avaliaram estes dois polimorfismos em pacientes com gastrite e lesões pré-cancerosas como metaplasia intestinal e atrofia gástrica e concluíram que a substituição *TLR4* Thr399Ile pode ser um fator de risco para estas lesões gástricas. De modo contrário, na população caucasiana o polimorfismo *TLR4* +896 A/G foi associado com o desenvolvimento de lesões pré-malignas e também com risco para o carcinoma gástrico não-cárdia (HOLD et al., 2007). Neste estudo, também não foram encontrados homozigotos polimórficos *TLR4* +896 GG, corroborando os resultados do presente estudo. Porém, Santini et al. (2008) demonstraram que *TLR4* +1196C/T (Thr399Ile) estava associado com maior suscetibilidade ao câncer gástrico, mas não demonstraram associação do polimorfismo *TLR4* +896A/G (Asp299Gly), assim evidenciando uma variação quanto a esses dois polimorfismos e risco na carcinogênese do estômago.

Os TLRs estão implicados no reconhecimento de bactérias como *H. pylori* no estômago, assim são os iniciadores da resposta inflamatória, consequentemente desencadeando uma rede de mediadores da inflamação, como as citocinas.

Na avaliação das citocinas anti-inflamatória *IL-1RN* e pró-inflamatória *TNFB*, os resultados demonstraram uma associação do alelo variante *IL-1RN**2 e do efeito combinado dos polimorfismos *IL-1RN** L/2 ou 2/2 / *TNFB* +252 A/G ou G/G com suscetibilidade ao câncer gástrico e gastrite crônica. Enquanto o polimorfismo *TNFB* +252A/G isolado não foi associado com o risco para estas lesões gástricas analisadas.

Os resultados deste estudo auxiliam a esclarecer a controvérsia sobre o papel do polimorfismo *IL-1RN* VNTR na carcinogênese gástrica, recentemente discutida em três meta-análises. Kamangar et al. (2006) observaram que as associações entre o polimorfismo *IL-1RN* e câncer gástrico eram nulas, mesmo em populacões ocidentais. Peleteiro et al. (2010) encontraram associação do alelo *IL-1RN**2 com risco aumentado de lesões pré-cancerosas, suportando o papel deste polimorfismo nos estágios iniciais da carcinogênese gástrica. Enquanto, Xue et al. (2010) observaram que o alelo *IL-1RN**2 encontra-se significantemente associado com aumento do risco de desenvolvimento de carcinoma gástrico, especialmente na região não-cárdia e do tipo intestinal. Contudo, os autores verificaram que esta associação era evidente entre caucasianos, mas não em asiáticos e hispânicos. Provavelmente devido à baixa frequência do alelo de risco nestas populacões, como na Ásia.

No Brasil, dois estudos em diferentes regiões, o primeiro em uma população miscigenada do estado de Minas Gerais com contribuição étnica de aproximadamente 33% de portugueses, 33% de ameríndios e 33% de africanos, e o outro na população norte do Brasil que possui forte composição genética de portugueses (50%) e ameríndios (40%) e pouca descendência africana (10%) demonstraram uma associação entre o alelo *IL-1RN**2 e o desenvolvimento do câncer gástrico, assim como uma associação com cepas *H. pylori* cag-A positivas (ROCHA et al. 2005; MELO-BARBOSA et al., 2009). Melo-Barbosa et al. (2009), também encontraram associação com úlcera gástrica. Contudo, as freqüências de homozigotos 2/2 relatadas por estes autores, ou seja, respectivamente 3,6% e 6% são menores quando comparadas ao presente estudo (15%) e estudos na população caucasóide européia (28%) (FURUTA et al., 2002).

Estes resultados indicam que a frequência genotípica de *IL-1RN* esta relacionada a etnicidade da população estudada. No presente estudo, na população do sudeste brasileiro, a contribuição genética de africanos é estimada em torno de 10% (PENA et al., 2004). Considerando-se a ampla extensão geográfica do Brasil, há uma variação na composição da população entre as diferentes regiões do país, isso devido a diferenças que ocorreram na colonização por europeus e africanos e a mistura com nativos ameríndios (SALZANO; FREIRE-MAIA, 1970), portanto contribuindo para diferenças na sua distribuição genotípica.

O alelo polimórfico *IL1-RN* * 2 tem sido relacionado a uma produção aumentada de citocinas pró-inflamatórias, como IL-1β, que ocasiona um processo inflamatório crônico e redução na produção de ácido gástrico (hipocloridria) (KUMAR-KUMAR; DIXIT et al., 2009). Ainda como conseqüências do processo inflamátorio são produzidos alguns mutágenos, conhecidos como espécies reativas do oxigênio e óxido nítrico, além da hipocloridria já citada que pode permitir a resistência da infecção pela bactéria *H. pylori* e consequente aumento da produção de carcinógenos. O dano ao DNA causado por esta cascata de fatores pode ser amplificado por um aumento da divisão celular inerente à inflamação. Portanto, genótipos que aumentam a produção de citocinas inflamatórias podem estar associadas à carcinogênese do estômago (HONG et al., 2010).

Devido à importância de citocinas pró-inflamatórias que também podem modificar a resposta immune contra a infecção pela *H. pylori* e assim influenciar no desenvolvimento tumoral, analisou-se o polimorfismo *TNFB* +252 A/G. Embora tenha-se encontrado uma maior frequência dos genótipos A/G e G/G no grupo de câncer gástrico e gastrite crônica comparando aos indivíduos saudáveis, esta diferença não foi estatisticamente significante. Similarmente, outros estudos não encontraram associação em populações coreanas e espanholas, respectivamente (LEE et al., 2004; GARÇA-GONZALEZ et al., 2007), assim como, entre o alelo polimórfico e os grupos de adenocarcinoma gástrico e carcinoma de esôfago (GUO et al., 2005). Entretanto, um estudo recente na população japonesa mostrou uma associação dos genótipos G/G e G/A com risco aumentado de câncer gástrico não-cárdia do tipo difuso e atribuíram esta ligação à habilidade do alelo 252G de aumentar a produção de citocinas pró-inflamatórias como TNF-β (SUZUKI et al., 2009).

No presente estudo, também se avaliou as influências de SNPs nas regiões promotoras das citocinas pró-inflamatórias *TNFA* (-857 C/T e -308 G/A) e *IL8* (-251 T/A e -845 T/C) e da citocina anti-inflamatória *IL10* (-592 C/A) no risco do desenvolvimento de câncer gástrico e gastrite crônica e alterações nos níveis de expressão de RNAm destas citocinas na mucosa das lesões gástricas.

Dos polimorfismos investigados no gene *TNFA* foi encontrada associação com câncer gástrico e gastrite crônica apenas para o SNP *TNFA*-857 C/T, mas não para o -308 G/A. Em uma meta-análise com os polimorfismos *TNFA*-308 G/A and -857 C/T na influência de câncer gástrico em diversas populações, Zhang et al. (2008) concluíram que o locus *TNFA*-308 pode ser um fator de risco para o câncer gástrico, especialmente em populações caucasóides. Além disso, o locus *TNFA*-857 pode também estar associado ao risco para este câncer, embora a maioria destes estudos esteja concentrada na Ásia. Contudo, em outra recente meta-análise em populações asiáticas e não-asiáticas os autores não demonstraram a ocorrência de associação entre *TNFA*-308 G/A e câncer gástrico (PERSSON et al., 2011).

A citocina pró-inflamatória *TNF-α* tem sido reconhecida por apresentar um papel relevante na inflamação e no desenvolvimento do câncer. Vários polimorfismos têm sido associados com a maior produção desta citocina (GARZA-GONZALEZ et al., 2005). Estudos conduzidos principalmente em populações européias indicam um significante aumento do risco para câncer gástrico em portadores do alelo *TNFA*-308 A (EL-OMAR et al, 2003; MACHADO et al., 2003). Ao contrário, estes resultados não têm sido confirmados, principalmente em países asiáticos (RAD et al., 2004; LEE et al., 2005; GARZA-GONZALEZ et al., 2005; SUGIMOTO et al., 2007). Embora a frequência do alelo A no presente estudo (18%) seja comparável a frequência relatada em populações caucasianas (~20%), e superior à asiática (1,7%) (HIGUCHI et al., 1998), não houve uma associação com o risco para este tipo de câncer. Este dado corrobora outro estudo, também realizado na população do sudeste brasileiro, que não encontrou associação do *TNFA*-308 A com carcinoma gástrico não-cárdia (ROCHA et al., 2005).

De modo contrário, o SNP *TNFA*-857 C/T foi associado com o risco para o câncer gástrico no presente estudo, como também em outros trabalhos com úlceras gástrica e duodenal (ZANBOM et al. 2005), gastrite hiperplásica causada por infecção pela *H. pylori* e câncer gástrico (OHYAMA et al., 2004). Apesar de todos estas análises terem sido realizadas na Ásia, a frequência do alelo T no presente estudo (0.28) é similar à encontrada na população japonesa (0.22) (SOGA et al., 2003).

O polimorfismo *IL8*-251 T/A é comumente relacionado ao risco aumentado e pobre prognóstico de várias doenças, entre elas o câncer gástrico (HULL et al., 2000; TAGUCHI et al., 2005; HILDEBRAND et al., 2007). Porém, os resultados do presente estudo mostraram frequências similares do alelo variante A nos grupos casos (0,47 no grupo CG e 0,50 no grupo GC) e controle (0,47), não demonstrando, portanto, um aumento da suscetibilidade para estas lesões gástricas.

Embora haja relatos que o alelo *IL8* -251 A esteja associado com maior produção desta citocina (TAGUCHI et al., 2005), estes dados são bastante controversos. Lee et al. (2005) demonstraram que o alelo *IL8*-251 T possui uma atividade transcricional de duas a cinco vezes maior que o alelo A. Entretanto, Hacking et al. (2004) não encontraram diferença na atividade promotora entre os dois alelos nas análises com o SNP -251 T/A. Portanto, é possível que SNPs em outras posições do gene IL8 possam afetar a produção desta citocina como o *IL8*-845 T/C.

Nossos resultados, demostraram positivamente associação do outro polimorfismo na região promotora *IL8*-845 T/C com a suscetibilidade para câncer gástrico e gastrite crônica. Além do mais apresentou resultados inéditos da influência deste polimorfismo nos níveis de expressão gênica desta citocina.

A escassez de estudos com este SNP em câncer pode ser explicada pela baixa frequência do alelo variante C, que se encontra ausente nas populacões da Europa e Ásia (ROVIN; LU; ZHANG, 2002; DAN et al., 2010). Em contraste, este alelo é presente em negros americanos (0,13) (ROVIN; LU; ZHANG, 2002), com freqüência similar a encontrada em nosso estudo no grupo CG (0,15), mas diferente para os pacientes com GC (0,06) e indivíduos saudáveis (0,03), indicando uma forte influência deste polimorfismo na carcinogênese gástrica, e reforçando a contribuição genética africana em nossa população.

A expressão da citocina IL8 é afetada pela ativação do fator nuclear NF-κB. Ela pode ser induzida por ativadores do NF-κB, como TNF-α e CXCL12 durante o processo inflamátorio (REHMANN; WANG, 2009). No estudo aqui realizado encontramos ampla heterogeneidade da expressão gênica relativa desta citocina nas amostras de CG e GC, com indivíduos apresentando expressão reduzida, enquanto outros apresentaram expressão aumentada. Porém, quando os indivíduos portadores do alelo polimófico C foram agrupados, observou-se uma média do nível de expressão relativa aumentada de 1,6 e 2,3 vezes respectivamente, nos grupos de câncer gástrico e gastrite crônica. Enquanto que homozigotos TT de ambos os grupos mostraram expressão reduzida. Estes resultados evidenciam a influência do alelo polimórfico C em aumentar a atividade transcricional e a super-expressão desta citocina, consequentemente com resposta imune mais vigorosa e possivelmente suscetibilidade para a carcinogênese.

Ainda neste estudo, observou-se uma associação da citocina anti-inflamatória referente ao polimorfismo *IL10*-592 C/A com ambas as lesões gástricas avaliadas. A frequência do alelo polimórfico -592A no grupo controle (0,18) foi muito menor que a relatada nas populações asiáticas (0,65), latinas (0,50) e caucasianas européias (0,30) (SCASSELATI et al., 2004; ALONSO et al., 2005; LEE et al., 2005). Contudo, nos pacientes dos grupos casos esta frequência foi elevada (0,30), assim evidenciando a suscetibilidade para a carcinogênese gástrica na nossa população. Estes resultados são corroborados por outros estudos em que os genótipos *IL10*-1082 A/A, *IL10*-819 T/T e *IL10*-592 A/A ou o haplótipo ATA dos polimorfismos *IL10*-1082/-819/-592 foram relatados aumentar o risco para o desenvolvimento de câncer gástrico conjuntamente à infecção pela *H. pylori* (EL-OMAR et al., 2003; ZAMBON et al., 2005).

De modo contrário, uma recente meta-análise mostrou que o polimorfismo *IL10*-592 C/A não é um fator de risco para o câncer gástrico com base em todos os estudos avaliados. Porém, quando estratificados por raça, foi observado que o genótipo *IL10*-592 AA apresentava fator protetor no desenvolvimento desta neoplasia em asiátcos, mas não houve diferenças de distribuição genotípica entre os grupos de câncer gástrico e controle nas populações caucasianas e latinas. Os autores ressaltaram para explicar seus resultados a diferença de composição genética entre os asiáticos e as outras etnias (ZHU; WANG; ZHANG, 2011).

No presente estudo, a análise combinada de quatro polimorfismos (*TNFA*-308A/ *TNFA*-857T/*IL8*-251A/*IL8*-845C, *TNFA*-308A/T*NFA*-857T/*IL8*-

845C/*IL10*-592A e *TNFA*-308A/ *IL8*-251A/ *IL8*-845C/ *IL10*-592A) aumentou os valores de ORs em cerca de 8 vezes em relação ao risco de câncer gástrico. Outros estudos também têm demostrado uma relação entre o número de alelos polimórficos e genótipos combinados no risco de câncer. Por exemplo, Perez-Perez et al. (2005) analizaram alguns polimorfismos dos genes *IL-1β, IL-1RN, TNF-α* e *IL10* e mostraram que os valores de ORs para câncer gástrico não-cárdia aumentava progressivamente com o número crescente de genótipos pró-inflamatórios. Outros grupos também têm observado resultados similares (EL-OMAR et al., 2003; HOLD et al., 2007), provavelmente devido ao efeito de interação desses SNPs de citocinas pró-inflamatórias, assim resultando em uma resposta imune mais exacerbada e conseqüentemente maior risco para o desenvolvimento de câncer gástrico.

De modo geral, a análise da expressão gênica relativa da citocina IL10, no presente estudo, mostrou um baixo nível de expressão desta citocina no grupo de câncer gástrico (-2.66±2.19) comparado com o grupo de gastrite crônica (0.37±3.47). Da mesma forma, quando as amostras dos grupos CG e GC foram estratificadas conforme as variantes polimórficas (CC e CA + AA), os portadores da variante A mostraram expressão mais reduzida da citocina IL10, que foi estatisticamente significante dentro do grupo de gastrite crônica.

Rad et al. (2004) evidenciaram que o haplótipo GCC (*IL10* -1082G; -819C; -592C) foi associado com expressão elevada, enquanto o haplótipo ATA com baixa expressão de IL10. Em estudo recente, Faupel-Badger et al. (2008) demonstraram que os genótipos correlacionados com baixa produção de IL10 estavam associados com risco aumentado de câncer de próstata e com maior gravidade desta doença. Estes resultados confirmam a relevância do polimorfismo de IL10 na regulação da resposta inflamátoria. A associação com os níveis reduzidos de IL10 pode estar relacionada ao seu papel como uma citocina antiinflamátoria que regula a expressão de citocinas pró-inflamatórias como IL-1β, TNF-α, interferon gama. A deficiência de IL10 pode ativar a via de diferenciação Th1 da resposta imune, frente à infecção pela *H. pylori*, caracterizada por uma hiper-inflamação com grandes danos a mucosa gástrica. Portanto, desbalanços entre mediadores pró e anti-inflamátorios e a produção de ácido gástrico podem favorecer o desenvolvimento de atrofia gástrica e sua progressão para o câncer (EL-OMAR et al., 2003).

Quando analisada a influência da infecção pela *H. pylori* combinada aos polimorfismos avaliados no presente estudo, nenhuma associação foi observada, provavelmente devido ao número reduzido de amostras dos grupos casos com o diagnóstico disponível para esta bactéria. Entretanto, diversos estudos têm observado associação entre polimorfismos em genes que atuam na resposta inflamatória e infecção por esta bactéria. Por exemplo, Rad et al. (2009) estudaram o papel de vários receptores *toll-like* (TLRs 2/4/7 e 9) na resposta imune induzida pela *H. pylori* em camundongos mutantes que não apresentavam estes receptores. Os resultados demonstraram a importância do TLR2 na resposta a esta bacteria. Porém camundongos nulos para o receptor TLR4 tiveram apenas uma pequena ou nenhuma alteração na resposta à *H. pylori* quando comparado a camundongos controles. Portanto, evidenciando que o TLR2 tem um importante papel na resposta immune contra a *H. pylori*, e mudanças na sua função são relevantes na carcinogênese do estômago (RAD et al., 2009).

Do mesmo modo, polimorfismos em *IL-1RN* têm sido associados a lesões gástricas na presença desta bactéria (FENG et al., 2008; CRUSIUS et al., 2008; AL-MOUNDHRI et al., 2009), assim como com cepas CagA positivas (ROCHA et al., 2005; MELO-BARBOSA et al., 2009). Esta influência da infecção pela *H. pylori* está bem definida na progressão do câncer gástrico em algumas populações. Indivíduos com genótipos pró-inflamatórios apresentam uma expressão aumentada da citocina IL-1β em resposta a *H. pylori*, permitindo um aumento da resposta inflamátoria, atrofia gástrica, hipocloridria e o desenvolvimento do carcinoma (PEREZ-PEREZ et al., 2005).

Enquanto a *H. pylori* e fatores genéticos do hospedeiro interagem para iniciar a hipocloridria e fenótipo atrófico, outros fatores podem contribuir para uma subsequente transformação, que não dependem da presença contínua da infecção (El-OMAR et al., 2001). Entre estes fatores, no presente estudo, foram encontrados como risco para o câncer gástrico, a idade avançada, o gênero masculino e o consumo de álcool. De acordo com o Instituto Nacional do Câncer (2011), no Brasil o câncer gástrico é mais comum em homens, ocupando o terceiro em incidência entre os homens e o quinto entre as mulheres, sendo então os resultados ora apresentados consistentes com os dados epidemiológicos na literatura. Da mesma forma, está bem defindo que o câncer gástrico está relacionado com idade avançada, em que aproximadamente 65% dos indivíduos diagnósticados com esta neoplasia têm idade acima dos 50 anos, e predominante em homens acima dos 70 anos de idade. O consumo excessivo de álcool também é outro fator de risco importante na carcinogênese do estômago (INCA, 2011). A oxidação do etanol gera acetaldeído, que primariamente têm efeitos carcinogênicos e mutagênicos. Estes metabólitos intereferem em muitos sítios de síntese e reparo do DNA, que podem levar ao desenvolvimento tumoral (JELSKI; SZMITKOWSKI, 2008).

Portanto, polimorfismos gênicos em mediadores inflamátorios (receptores e citocinas) são componentes de um complexo intrincado entre fatores do hospedeiro, da *H. pylori* e de fatores ambientais envolvidos na carcinogênese gástrica.

Conclusões

V. Conclusões

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

1. Não há evidências de associação entre os polimorfismos *TLR4* +1196C/T, *TNFA* -308G/A, *TNFB* +252A/G *e* *IL8* -251T/A com as lesões avaliadas, o que pode estar relacionado a variações nas frequências destes SNPs conforme a origem étnica das populações. Já, os polimorfismos nos genes dos receptores *TLR2* deleção -196 a -174 e *TLR4* +896A/G, das citocinas pró-inflamatórias *TNFA* -857C/T e *IL8* -845T/C e anti-inflamatórias *IL-1RN* VNTR e *IL10* -592A/C encontram-se associados com risco de câncer gástrico e/ou gastrite crônica, assim enfatizando a contribuição de fatores genéticos envolvidos com a resposta inflamatória na carcinogênese do estômago;

2. As combinações dos polimorfismos envolvendo receptores e citocinas pró e anti-inflamatórias como *TLR2del/TLR4*+896G; *IL-1RN* 2/*TNFB* +252G e *TNFA*-308A/*TNFA*-857T/*IL8*-251A/*IL8*-845C; *TNFA*-308A/*TNFA*-857T/*IL8*-845C/*IL10*-592A e *TNFA*-308A/ *IL8*-251A/*IL8*-845C/ *IL10*-592A demonstraram maior risco para a carcinogênse gástrica, assim como o haplótipo G-C dos polimorfismos *TLR4*+896A/G+1196C/T; evidenciando um perfil genético que pode conferir maior risco aos seus portadores;

3. Entre os fatores de risco ambientais, o sexo masculino, a idade avançada e o consumo de álcool destacam-se na carcinogênese do estômago, para a população analisada e

4. Os polimorfismos nas regiões promotoras *IL8* -845 T/C e *IL10* -592 C/A influenciam a expressão gênicas destas citocinas, sendo que os portadores do alelo polimórfico *IL8*-845C tem expressão relativa do mRNA aumentada em relação aos portadores homozigotos *IL8*-845 T/T. Ao contrário, os portadores da variante *IL10*-592 A tem expressão reduzida desta citocina anti-inflamatória em relação aos homozigotos *IL10*-592 C/C. Esses resultados mostram a relevância funcional destes polimorfismos e auxiliam a demonstrar a importância das variações destes mediadores inflamatórios na carcinogênese gástrica.

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Apêndice

Apêndice 1. Questionário

Questionário do projeto: Avaliação do perfil de polimorfismos de genes de citocinas e receptores envolvidos no processo inflamatório da *H. pylori* e suscetibilidade genética ao câncer gástrico e alterações nos níveis de expressão gênica

Responsáveis: Doutoranda Juliana Garcia de Oliveira e Profa. Dra. Ana Elizabete Silva (Departamento de Biologia IBILCE-UNESP, 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

( ) gastrite ( ) leve ( ) moderada ( ) severa

( ) câncer gástrico: ( ) intestinal ( ) difuso

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

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

Apêndice 2. Características demográficas e fatores de risco dos 729 indivíduos dos grupos de gastrite crônica (N=276), câncer gástrico (N=207) e controle (N=246).

Variáveis		Gastrite Crônica N=276(%)	Câncer Gástrico N=207(%)	Controle N=246(%)
Sexo	F	134 (48,5)	46 (22,0)	120 (48,8)
	M	142 (51,5)	161 (78,0)	126 (51,2)
Idade (anos)	Média/Desvio Padrão	53,6±14,4	62,0±12,6	55,9±17,7
	Variação	19-86	29-93	20-93
Etnia	Caucasóides	250 (90,5)	178 (89,5)	225 (91,4)
	Negróides	26 (9,5)	29 (10,5)	21 (8,6)
Tabagismo	Fumantes	152 (55,0)	147 (71,0)	155 (63,0)
	Não Fumantes	124 (45,0)	60 (29,0)	91 (37,0)
Etilismo	Etilistas	95 (35,0)	118 (57,0)	62 (25,2)
	Não Etilistas	181 (65,0)	89 (43,0)	184 (74,8)
Infecção por Hp[1]	Positivo	46 (45,0)	114 (51,1)	-
	Negativo	56 (55,0)	109 (48,9)	-

[1] Números relativos a 102 e 223 indivíduos dos grupos de gastrite crônica e câncer gástrico, respectivamente.

Apêndice 3. Padrão eletroforético das análises polimórficas dos receptores *toll-like* (TLR), Artigo 1:

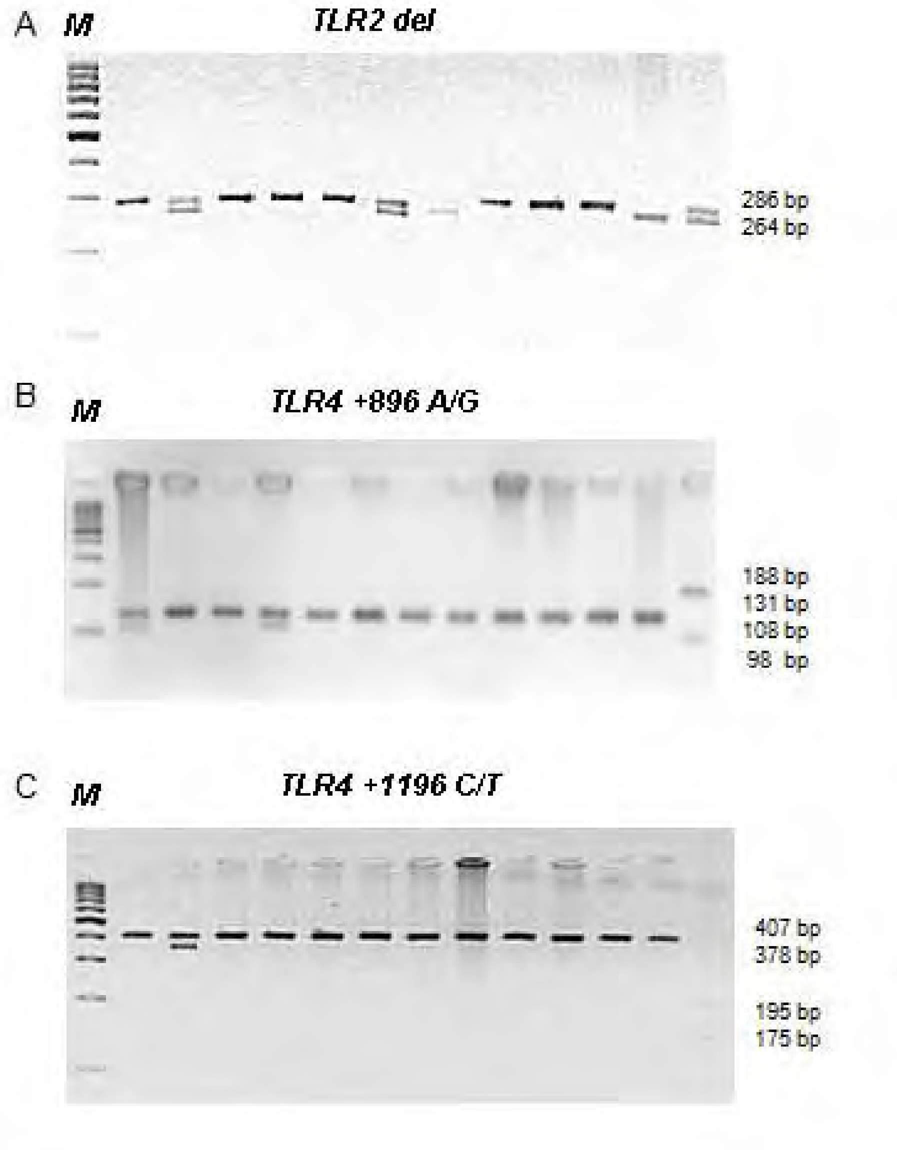


Figura 1. Padrão eletroforético dos fragmentos gerados pela técnica de PCR-alelo específico para: (A) *TLR2 del* -196 a -174: ins/ins=286 pb; ins/del: 286+264 pb; del/del: 264 pb e PCR-RFLP para os polimorfismos (B) *TLR4*+896 A/G: A/A: 131 pb; A/G 131+108 pb e controle positivo da enzima *Bst*XI, fragmento do gene *TLR2* de 286 bp com sítio de corte da enzima: 188+98 bp (última linha) e (C) *TLR4*+1196 C/T: C/C: 407 pb; C/T: 407+378 pb e controle positivo da enzima *Hinf*I, fragmento do gene *IL-1β* de 370 bp com sítio de corte da enzima: 195+175 bp (última linha). M, marcador molecular de 100pb.

Apêndice 4. Padrão eletroforético dos polimorfismos das citocinas TNF-β e IL1-RN, referente ao artigo 2.

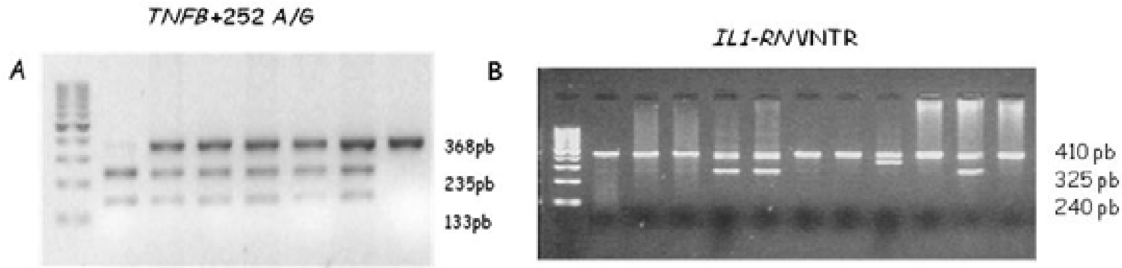


Figura 2. Padrão eletroforético dos fragmentos gerados pela técnica de PCR-RFLP: (A) *TNFB*+252 A/G: A/A= 368pb; A/G = 368 + 235 + 133 pb e GG = 235 +133 pb e PCR-alelo específico (B) das principais repetições em tandem de *IL1-RN*: alelo 1= 410pb; alelo 2 = 240 pb e alelo 3 = 325 pb.

Apêndice 5. Padrão eletroforético dos polimorfismos das citocinas TNF-α, IL8 e IL10, referente ao artigo 3.

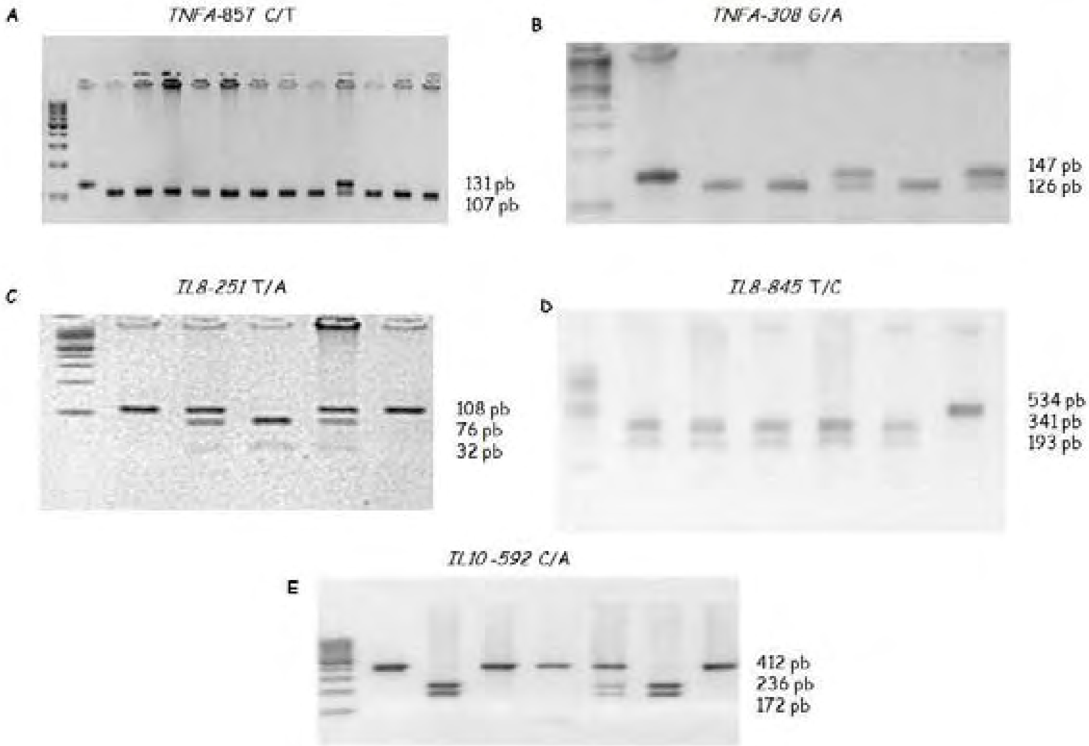
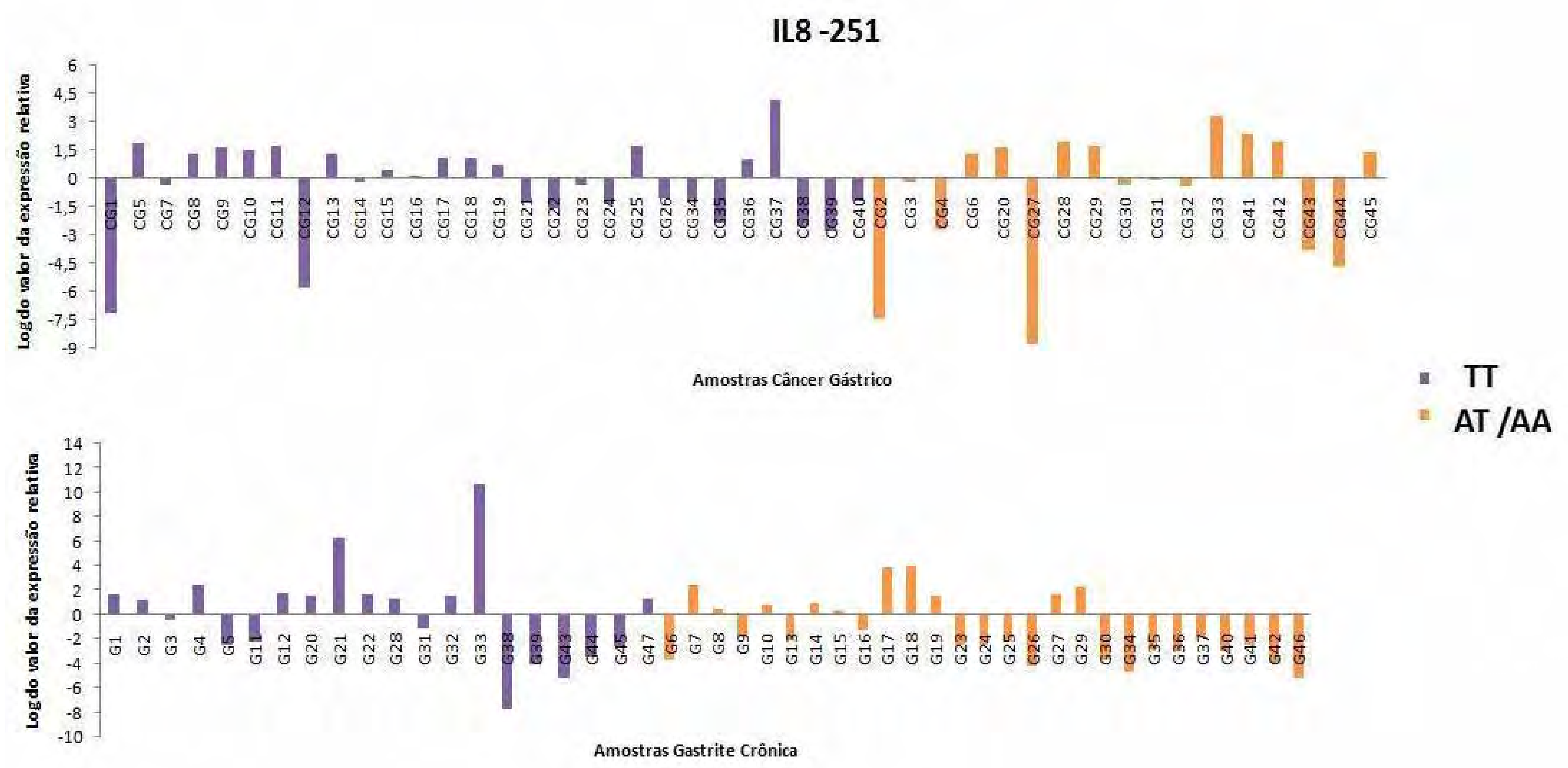
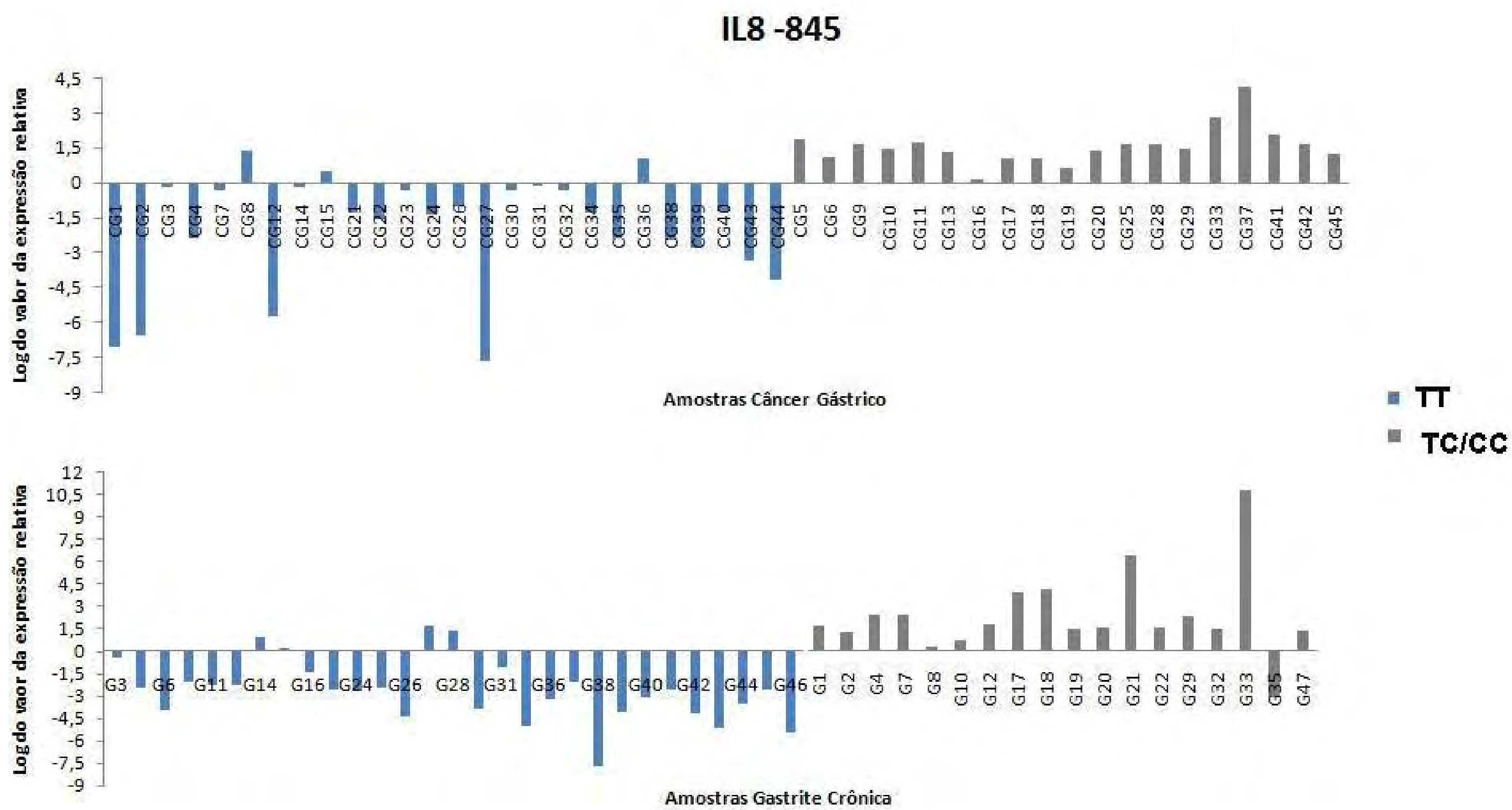


Figura 3. Padrão eletroforético dos fragmentos gerados pela técnica de PCR-RFLP: (A) *TNFA*-857C/T: CC= 107+24pb; CT=131+107+24pb e TT= 131pb; (B) *TNFA*-308 G/A: G/G= 126+31 pb; G/A= 147+126+31pb e A/A= 147pb; (C) *IL8*-251 A/T: A/A=76+32pb; A/T= 108+76+32pb e T/T= 108pb; (D) *IL8*-845T/C: T/T= 341+193pb; T/C= 534+341+193pb e C/C= 534pb; (E) *IL10*-592 C/A: C/C=412 pb; C/A= 412+236+172pb e A/A= 236+172pb.

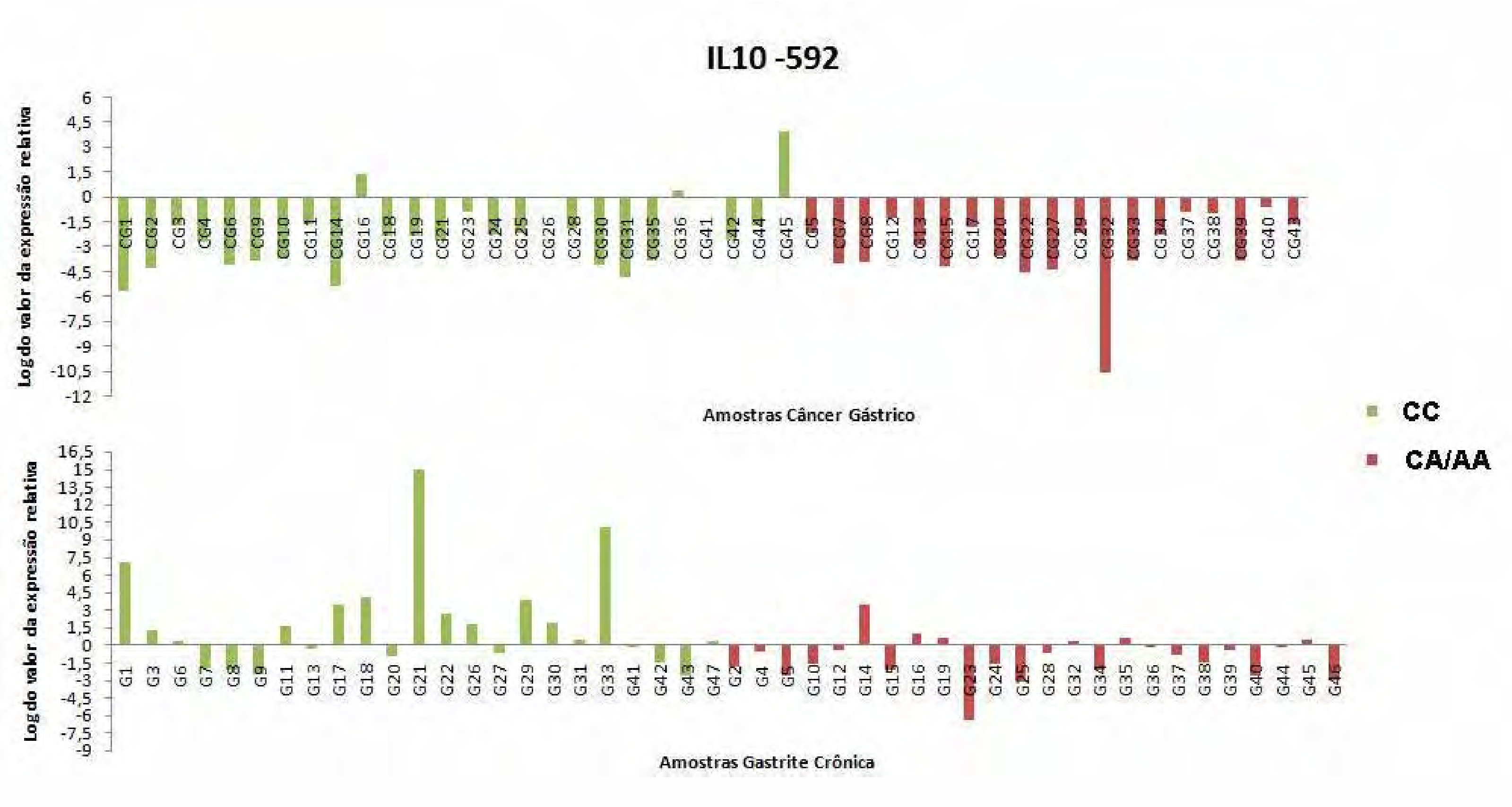
Apêndice 6. Valor da expressão gênica das amostras de câncer gástrico (CG1-45) e gastrite crônica (G1-47) separadas de acordo com as variantes polimórficas para *IL8*- 251 T/A .



Apêndice 7. Valor da expressão gênica das amostras de câncer gástrico (CG1-45) e gastrite crônica (G1-47) separadas de acordo com as variantes polimórficas para IL8- 845 T/C.



Apêndice 8. Valor da expressão gênica das amostras de câncer gástrico (CG1-45) e gastrite crônica (G1-47) separadas de acordo com as variantes polimórficas para *IL10*-592 C/A.



Anexos

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

unesp

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

Comitê de Ética em Pesquisa
IBILCE-UNESP

PARECER CONSUBSTANCIADO
PROJETO DE PESQUISA

IDENTIFICAÇÃO

Nome do pesquisador: Juliana Garcia de Oliveira
Departamento: Depto. Biologia
Instituição: IBILCE/UNESP
Projeto: "Avaliação do perfil de polimorfismos de genes de citocinas e receptores envolvidos no processo inflamatório de *H.Pylori* e suscetibilidade genética ao câncer gástrico e alterações nos níveis de expressão gênica".

PARECER Nº 117/08

O projeto é relevante por estar voltado ao estudo de epidemiologia molecular e suscetibilidade a doenças, e que vem avançando rapidamente.

Os objetivos são claros. A metodologia é adequada á proposta. Existe infra-estrutura adequada para execução da pesquisa.

A pesquisadora atendeu as solicitações feitas pelo CEP conseqüentemente atendeu as normas.

Assim o CEP aprova o projeto em questão.

(X) APROVADO, aguardar apreciação final da CONEP
(   ) APROVADO COM RECOMENDAÇÃO
(   ) PENDENTE
(   ) NÃO APROVADO

Datas previstas para apresentação dos relatórios
1º relatório: dezembro/2008 9

São José do Rio Preto, 03 de dezembro de 2008.

Dr. Raul Aragão Martins
Coordenadora do CEP

Anexo 2. Parecer consubstanciado do Comitê de Ética Ambiental.

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

Parecer

OFÍCIO: 28/2009
INTERESSADOS: Profa. Dra. Ana Elizabete Silva
Juliana Garcia de Oliveira
ASSUNTO: Parecer

São José do Rio Preto, 03 de novembro de 2009.

PARECER DA COMISSÃO DE ÉTICA AMBIENTAL

Trata o presente da análise do potencial de risco dos resíduos químicos a serem gerados do projeto de pesquisa intitulado, "**Avaliação de polimorfismos de genes envolvidos no processo inflamatório induzido pela *Helicobacter pylori* na carcinogênese do estômago**". Este projeto de pesquisa será enviado à FAPESP para solicitação de auxílio à pesquisa para ser desenvolvido pela doutoranda Juliana Garcia de Oliveira, pós-graduanda do Programa de Pós-Graduação em Genética. O projeto será executado no Laboratório de Citogenética e Biologia Molecular Humana, do Depto. de Biologia do Instituto de Biociências, Letras e Ciências Exatas da UNESP – São José do Rio Preto, sob coordenação da Profa. Dra. Ana Elizabete Silva, também deste Departamento.

Os resíduos químicos gerados em função de procedimentos de laboratório serão devidamente armazenados para posterior encaminhamento ao entreposto de coleta, ou ainda serão tratados e descartados na pia, ou serão incinerados, de acordo com sua natureza. A metodologia para descarte dos resíduos deverá ser realizada de acordo com pesquisa realizada pelo solicitante e encaminhada a Comissão de Ética Ambiental, que avaliou e aprovou o tratamento proposto.

Sendo assim, esta Comissão julga que os procedimentos estão de acordo com as normas estabelecidas pela CEA/IBILCE.

Atenciosamente,

mCBisinoti

Prof^a^. Dr^a^. Márcia Cristina Bisinoti
Comissão de Ética Ambiental
Presidente
IBILCE/UNESP

Instituto de Biociências, Letras e Ciências Exatas – Departamento de Química e Ciências Ambientais
Rua Cristovão Colombo, 2265 – Jardim Nazareth CEP 15054-000 – São José do Rio Preto SP Brasil
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Anexo 3. Comprovante da submissão do artigo "Polymorphisms of the *TLR2* and *TLR4* genes are associated with risk of gastric cancer in a Brazilian population" ao periódico World Journal of Gastroenterology.

World Journal of Gastroenterology

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Authors: J[illegible] Garcia Oliveira ,Ana Elizabete Silva

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São José do Rio Preto, ______/______/______

____

Juliana Garcia de Oliveira