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ORIGINAL ARTICLE

# Profiles of Gene Polymorphisms in Cytokines and Toll-Like Receptors with Higher Risk for Gastric Cancer

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**Abstract**
*Background* Chronic inflammation and gastric carcinogenesis show a close association, so gene polymorphisms that modify the intensity of the inflammatory response may contribute to variations in gastric cancer risk.
*Aims* The purpose of this study was to investigate the combined effect of the pro- and anti-inflammatory cytokines and toll-like receptors polymorphisms on the chronic gastritis and gastric cancer risk in a Brazilian population sample.
*Methods* We evaluated 669 DNA samples (200 of gastric cancer [GC], 229 of chronic gastritis [CG], and 240 of healthy individuals [C]). Ten polymorphisms were genotyped: *IL-1RN* and *TLR2* -196 to -174 *del* using the allele-specific PCR method and *TNF-A* (rs1800629; rs1799724), *TNF-B* (rs909253), *IL-8* (rs4073; rs2227532), *IL-10* (rs1800872) and *TLR4* (rs4986790; rs4986791) using PCR–RFLP.
*Results* Polymorphisms *TNF-A-308G/A, IL-8-251A/T, TNF-B + 252A/G* and *TLR4 + 1196C/T* were not associated with risk of any gastric lesion. However, an association with increased risk for GC was observed for polymorphisms *IL-1RNL/2* ($p < 0.001$), *TNF-A-857C/T* ($p = 0.022$), *IL-8-845T/C* ($p < 0.001$), *IL-10-592C/A* ($p < 0.001$), *TLR2ins/del* ($p < 0.001$), and *TLR4 + 896A/G* ($p = 0.033$). In CG, an association was observed only with polymorphisms *IL-1RNL/2* and *IL-10-592A/C* ($p < 0.001$ for both). A combined analysis of these six polymorphisms associated with GC revealed a profile with two to four combined genotypes which confer a higher risk of gastric carcinogenesis, with an OR increased 2.95-fold to 50.4-fold, highlighting the combinations *IL-1RN2/TNF-A-857T/IL-8-845C, IL-1RN2/IL-8-845C/TLR2del, IL-1RN2/IL-10-592A/TLR4 + 896G, IL-10-592A/TLR2del/TLR4 + 896G,* and *IL-1RN2/TNFA-857T/IL8-845C/TLR2del.*
*Conclusions* Our findings evidenced that the combined effect of polymorphisms in genes involved in the inflammatory process may potentiate the risk of gastric cancer, thus emphasizing the importance of evaluating multiple polymorphisms together.

**Keywords** Gene polymorphisms · Cytokines · Toll-like receptors · Gastric cancer · Chronic gastritis

**Abbreviations**

| | |
|---|---|
| C | Control |
| CG | Chronic gastritis |
| CI | Confidence interval |
| dNTPs | Deoxyribonucleotide triphosphates |
| EDTA | Ethylenediamine tetraacetic acid |
| GC | Gastric cancer |

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| | |
|---|---|
| IL-1$\beta$ | Interleukin 1-beta gene |
| IL-1RN | Interleukin 1 receptor antagonist |
| IL-8 | Interleukin 8 gene |
| IL-10 | Interleukin 10 gene |
| MgCl2 | Magnesium chloride |
| N | Number of individuals |
| OR | Odds ratio |
| P | Probability |
| PCR-RFLP | Polymerase chain reaction-restriction fragment length polymorphism |
| SNP | Single nucleotide polymorphism |
| T° | Temperature |
| TLR2 | Toll-like receptor 2 |
| TLR4 | Toll-like receptor 4 |
| TNF-A | Tumor necrosis factor alpha |
| TNF-B | Tumor necrosis factor beta |

## Introduction

Gastric cancer, despite its declining incidence rate, is still the second cause of cancer-related death worldwide, killing 700,000 people each year and remaining the fourth most common type of cancer [1, 2]. In Brazil, it ranks third in incidence and mortality rates, with an estimated incidence of about 20,090 new cases in 2012 [3], which makes it a major problem of public health.

This cancer is a model of multifactorial disease in which genetic and environmental factors are involved [4], such as precancerous gastric lesions and mainly bacterial infection by *Helicobacter pylori*. This type of cancer progresses through a multistep process consisting of chronic gastritis, gastric atrophy, intestinal metaplasia and dysplasia [5].

Chronic inflammation of the gastric mucosa and carcinogenesis show a close association. *H. pylori* is an imminent carcinogenic pathogen, and various studies have shown that infection by this bacterium causes infiltration of inflammatory cells, oxidative damage and gene mutations [6, 7]. The bacterium binds the epithelial cells and activates the host immune response through toll-like receptors (TLRs), which provide the first line of host defense against harmful pathogens [8]. Next, various pro- and anti-inflammatory cytokines are activated in cascade and act as potent inhibitors of gastric acid secretion, consequently leading to hypochlorhydria, gastric atrophy and increased risk for gastric cancer [9, 10].

Single nucleotide polymorphisms (SNP) within regulatory and other functional cytokine regions, such as *IL-1ra*, *TNF-A*, *TNF-B*, *IL-8, IL-10*, and TLRs that markedly influence expression and secretion profiles in response to infectious agents may modify the intensity of the inflammatory response, thereby contributing to variations in gastric cancer risk [11, 12]. Thus, it might be difficult to explain the risk of developing gastric cancer based on only one polymorphism of an inflammatory mediator per se.

Among the TLRs, it has been reported that TLR2, the bacterial lipoprotein receptor, and TLR4, the lipopolysaccharide (LPS) receptor, are involved in the response to infection by *H. pylori* in gastric epithelial cells, thereby triggering a network of mediators of inflammation such as cytokines IL-1$\beta$, TNF-$\alpha$ and TNF-$\beta$, and chemokines such as IL8 [13–15].

IL-1ra, encoded by the *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 [16]. The *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). The short allele is associated with chronic inflammation, autoimmune conditions and increased secretion of IL-1$\beta$ [17]. Another anti-inflammatory cytokine is IL-10, involved in down-regulating cell-edited and cytotoxic inflammatory response that inhibits the formation of pro-inflammatory cytokines such as TNF-A and IL-1B [18].

In contrast, genetic polymorphisms in pro-inflammatory cytokines such as TNF-A, TNF-B and the chemokine IL-8 are known to be related to several autoimmune and infectious diseases and cancer; therefore the increased level of expression of these cytokines has been investigated as a potential factor of susceptibility to gastric cancer [19–25]. TNF-A is mainly produced by macrophages and is involved in the regulation of diverse biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation [26, 27]. TNF-B, produced mainly by stimulated T lymphocytes, plays an important role in regulating immune response [28]. Another group, the IL-8 chemokine, is secreted by monocytes, macrophages, neutrophils, fibroblasts, keratinocytes and other cell types [29]. This chemokine comprises mediators which can stimulate cells that release proteolytic enzymes, allowing the digestion of the extracellular matrix and providing a path for the migration of inflammatory cells and tumor growth metastasis [30].

Considering the many mediators involved in inflammation, and the imbalance between pro- and anti-inflammatory components triggered by *H. pylori* which can lead to chronic inflammation and gastric tumorigenesis, it would be difficult to explain the increased risk for the development of gastric cancer associated with a SNP involving only one type of cytokine. Thus, the aim of this study was to elucidate the significance of polymorphisms occurring in key cytokines and inflammatory receptors involved in the development of chronic gastritis and gastric cancer and

moreover, to evaluate the combined effect of those polymorphisms which were associated with risk of this neoplasia in order to establish the profiles of combined polymorphisms that confer a higher risk of gastric carcinogenesis. To this effect, we genotyped ten polymorphisms in pro-inflammatory cytokines [*TNF-B* 252A/G (rs909253), *TNF-A* (rs1800629 and rs1799724) and *IL-8* (rs4073 and rs2227532)], anti-inflammatory cytokines [*IL-1RN* VNTR and *IL-10* (rs1800872)], and receptors [*TLR2* -196 to -174 del and *TLR4* (rs4986790 and rs4986791)] in a Brazilian population sample.

## Materials and Methods

### Population

The Research Ethics Committee of the University (CEP/IBILCE/UNESP) approved this work (Number 117/08), and written informed consent was obtained from all participating individuals.

This case–control study on chronic gastritis and gastric cancer patients in comparison to healthy individuals was performed using 669 DNA samples from peripheral blood leukocytes stored in our laboratory from previous studies [31, 32]. All subjects of the case groups were recruited from the Service of Endoscopy, Base Hospital, Medical School, São José do Rio Preto, SP, Brazil.

The chronic gastritis (CG) group comprised 229 individuals (113 men and 116 women) with a mean age of 52.3 ± 14.5 years (range, 19–86 years). All of them had a histopathologically confirmed diagnosis of chronic gastritis, mainly non-atrophic, but cases with atrophic gastritis and with a metaplastic area (Sydney System) [33] were also included. Exclusion criteria were specific types of gastritis, such as autoimmune and ischemic. The noncardia gastric cancer (GC) group comprised 200 individuals (156 men and 44 women) with a mean age of 61.7 ± 12.5 years (range, 28–93 years), and with a histopathologically confirmed diagnosis of intestinal or diffuse gastric cancer (Lauren's classification) [34]. Information on status of *H. pylori* infection was obtained from the patients' medical records of 95 cases of gastric cancer and 177 of chronic gastritis, for which the diagnosis was established using the Giemsa staining technique or the urease test, performed at the Pathology Service of Hospital de Base. However, due the reduced number of cases with available diagnosis for bacterium infection, this parameter was not included in the statistical analysis for the polymorphisms evaluated.

The cancer-free control (C) group was composed of 240 healthy individuals (121 men and 119 women) with a mean age of 55.5 ± 17.7 years (range, 20–93 years), recruited among blood donors of the Blood Bank of Base Hospital, Medical School, São José do Rio Preto, SP, Brazil. The inclusion criteria were absence of a cancer diagnosis according to government guidelines for donated blood, tested for 20 related diseases (http://www.hemonline.com.br/portarias/rdc153/indexframe.htm), and no previous history of gastric disease.

Epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, containing questions about current and past occupation, smoking habits, alcohol intake, and family history of cancer. Smokers were defined as individuals who had consumed at least 100 cigarettes during their lifetime, and alcohol consumers were those who used to have more than four drinks per week [35].

### Genotyping

DNA was extracted according to Miller et al. [36] and stored at −20 °C until use for genotyping. Polymorphisms *IL-1RN* and *TLR2* -196 to -174*del* were investigated using the allele-specific polymerase chain reaction (PCR) method [17, 37], and the PCR–RFLP (polymerase chain reaction-restriction fragment length polymorphism) technique was employed to identify the genotypes *TNF-A* (rs1800629 and rs1799724) [18, 38], *TNF-B* (rs909253) [19], *IL-8* (rs4073 and rs2227532) [24, 29], *IL-10* (rs1800872) [39] and *TLR4* (rs4986790 and rs4986791) [40, 41], in both case and control groups. Table 1 summarizes the PCR conditions, the sets of primers and the enzymes used for each analysis. In brief, the assay was carried out 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 from New England Biolabs, Massachusetts, USA) and 7.0 μL $dH_2O$. The products were then electrophoresed on a 1.5–3.0 % agarose 1,000 gel (Invitrogen, Carlsbad, CA, USA), to allow detection by ethidium bromide staining. Random samples were selected and duplicates were performed by other researchers of the laboratory, in an attempt to reduce errors, but there were not significant discrepancies.

In order to confirm the reliability of the electrophoretic pattern one confirmed case of polymorphism was used as positive control for every RFLP procedure to attest the functioning of the restriction enzyme. For polymorphisms *TLR4* + 896A/G (rs4986790) and +1196C/T (rs4986791), we also used other fragments of the *TLR2* and *IL-1β* genes, known to have the enzyme recognition site, to verify the

**Table 1** Primers sequences and PCR conditions for genotyping allele-specific PCR and PCR–RFLP

| Genes | Primers (5′–3′) | Cycles | Temp. melting | Enzyme, temp/time | Agarose gel (%) | Alleles or fragments (bp) |
|---|---|---|---|---|---|---|
| *IL-1RN VNTR* | F: CCCCTCAGCAACACTCC | 35 | 61 °C | – | 1.5 | Allele 1 = 4 repetitions (410) |
| | R: GGTCAGAAGGGCAGAGA | | | | | allele 2 = 2 repetitions (210) |
| | | | | | | allele 3 = 5 repetitions (500) |
| | | | | | | allele 4 = 3 repetitions (325) |
| | | | | | | allele 5 = 6 repetitions (595) |
| *TNF-A-308 G/A* (rs1800629) | F: GAGGCAATAGGTTTTGAGGGCCAT | 36 | 65 °C | *Nco*I, 37 °C/1 h | 3.0 | G: 126 + 21 |
| | R: GGGACACACAAGCATCAAG | | | | | A: 147 |
| *TNF-A-857 C/T* (rs1799724) | F: AAGTCGAGTATGGGGACCCCCCGTTAA | 36 | 65 °C | *Hinc*II, 37 °C/1 h | 3.0 | C: 107 +24 |
| | R: CCCCAGTGTGTGGCCATATCTTCTT | | | | | T: 131 |
| *TNF-B + 252 A/G* (rs909253) | F: CTCCTGCACCTGCTGCCTGGATC | 35 | 65 °C | *NcoI,* 37 °C/1 h | 1.5 | G: 368 |
| | R: GAAGAGACGTTCAGGTGGTGTCAT | | | | | A: 235 + 133 |
| *IL-8-251 T/A* (rs4073) | F: TTCTAACACCTGCCACTCTAG | 35 | 60 °C | *Mfe*I, 37 °C/12 h | 3.0 | A: 76 + 32 |
| | R:CTGAAGCTCCACAATTTGGTG | | | | | T: 108 |
| *IL-8-845 T/C* (rs2227532) | F: AACCCAGCAGCTCCAGTG | 35 | 61 °C | *Vsp*I, 37 °C/1 h | 1.5 | T: 341 + 193 |
| | R: AGATAAGC CAGCCAATCATT | | | | | C: 534 |
| *IL-10-592 C/A* (rs1800872) | F: GGTGAGCACTACCTGACTAGC | 30 | 60 °C | *Rsa*I, 37 °C/12 h | 1.5 | A: 412 |
| | R: CCTAGGTCACAGTGACGTGG | | | | | C: 236 + 176 |
| *TLR2 del -196 to -174* | F: CACGGAGGCAGCGAGAAA | 35 | 60 °C | – | 3.0 | ins: 286 |
| | R: CTGGGCCGTGCAAAGAAG | | | | | del: 264 |
| *TLR4 +896A/G* (rs4986790) | F: AGCATACTTAGACTACCACCTCGATG | 30 | 62 °C | *Bst*XI, | 3.0 | A: 131 |
| | R: GTTGCCATCCGAAATTATAAGAAAAG | | | 37 °C/1 h | | G: 108 +23 |
| *TLR4 + 1196C/T* (rs4986791) | F: GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA | 30 | 62 °C | *Hinf*I, | 3.0 | C: 407 |
| | R: ACCTGAAGACTGGAGAGTGAGTTAAATGCT | | | 37 °C/1 h | | T: 378 +29 |
| *IL1-β*[a] | F: CATGTGACCTGCTCGTCAGT | 35 | 56 °C | *Hinf*I, | 1.5 | 370 |
| | R: CCCTAGGGATTGAGTCCACA | | | 37 °C/1 h | | 195, 175 |
| *TLR2*[a] | F: CACGGAGGCAGCGAGAAA | 35 | 60 °C | *Bst*XI, | 3.0 | 286 |
| | R: CTGGGCCGTGCAAAGAAG | | | 37 °C/1 h | | 188, 98 |

[a] Fragments used to verify the correct functioning of the enzymes *Bst*XI and *Hinf*I

correct functioning of the enzymes *Bst*XI and *Hinf*I, respectively (Table 1), once no homozygous polymorphic subjects were detected.

### Statistical Analysis

The chi-square test was used for determining Hardy–Weinberg equilibrium. Genotype and allele frequencies were analyzed using multiple logistic regression models adjusted for age, gender, smoking and drinking. ORs were calculated using a dominant model (i.e., combining heterozygous and homozygous for the minor allele) for all polymorphisms. Statistical analyses were performed using the GraphPad Instat and SPSS (version11.5) computer programs. A probability level ($P$) of less than 0.05 was adopted as significance criterion.

## Results

The DNA samples of the 669 subjects with gastric cancer, chronic gastritis and controls were genotyped for ten polymorphisms in pro- and anti-inflammatory cytokines and receptors as follows: *IL-1RN, TNF-A* (-308G/A rs1800629 and -857C/T rs1799724), *TNF-B* (+252A/G rs909253), *IL-8* (-251T/A rs4073 and -845T/C rs2227532), *IL-10* (-592C/A rs1800872), *TLR2* -196 to -174 *del* and *TLR4* (+896A/G rs4986790 and +1196C/T rs4986791). The genotype and allele frequency distributions of the ten polymorphisms were found to be in Hardy–Weinberg equilibrium in both case and control groups (data not shown).

The genotype and allele frequencies for these polymorphisms and OR values between case and control groups, adjusted for age, gender, smoking and drinking, are presented in Table 2. The *TNF-A-308G/A, IL-8-251A/T, TNF-B + 252A/G* and *TLR4 + 1196C/T* SNPs were not associated with risk of any of the gastric lesions. However, an association was observed between increased risk of gastric cancer and polymorphisms *IL-1RNL/2* (OR = 2.60, 95 %CI = 1.65–4.10, $p < 0.001$), *TNF-A-857C/T* (OR = 1.70, 95 %CI = 1.08–2.67, $p = 0.022$), *IL-8-845T/C* (OR = 3.46, 95 %CI = 1.69–7.07, $p < 0.001$), *IL-10-592C/A* (OR = 2.34, 95 %CI = 1.47–3.70, $p < 0.001$), *TLR2ins/del* (OR = 2.20, 95 %CI = 1.28–3.78, $p < 0.001$) and *TLR4 + 896A/G* (OR = 2.09, 95 %CI = 1.08–4.02, $p = 0.033$), while in chronic gastritis an association was observed only with polymorphisms *IL-1RN L/2* (OR = 1.88, 95 %CI = 1.25–2.83, $p < 0.001$) and *IL-10-592A/C* (OR = 3.00, 95 %CI = 1.99–4.50, $p < 0.001$).

The six polymorphisms associated with risk of gastric cancer were submitted to combined analysis, so as to establish the polymorphism profiles associated with higher risk of this neoplasm (Table 3). Analyzing the various combinations between the gastric cancer group (maximum five polymorphisms) and the control group (maximum four polymorphisms), it became evident that the combined effect of two to four polymorphisms increased the risk of gastric cancer, with an odds ratio from 2.95 (95 %CI 2.15–4.06, $p < 0.001$) for the combined genotype *TNF-A*-857T/*IL-10*-592A to 50.40 (95 %CI 7.04–360.62, $p < 0.001$) for the combination *IL-1RN2/TNF-A*-857T/*IL-8*-845C. Worth considering are also other combinations of two genotypes such as *IL-1RN2/IL-8*-845C (OR = 30.24, 95 %CI = 7.64–119.66, $p < 0.001$) and *IL-8*-845C/TLR4 + 896G (OR = 36.00, 95 %CI = 4.74–273.09, $p < 0.001$), three genotypes such as *IL-1RN2/TLR2del/TLR4 + 896G* (OR = 36.00, 95 %CI = 4.74–273.09, $p < 0.001$), *IL-10-592A/IL-8-845C/TLR2del* (OR = 36.00, 95 %CI = 4.74–273.09, $p < 0.001$), *IL-1RN2/IL-10-592A/TLR4 + 896G* (OR = 38.76, 95 %CI = 5.19–289.0, $p < 0.001$), *IL-10-592A/TLR2del/TLR4 + 896G* (OR = 38.76 95 %CI = 5.19–289.0, $p < 0.001$) and *IL-1RN2/IL-8-845C/TLR2del* (OR = 49.26, 95 %CI = 6.86–353.47, $p < 0.001$), and four genotypes such as *IL-1RN2/TNF-A-857T/IL-10-592A/TLR2del* (OR = 21.90, 95 %CI = 5.25–91.35, $p < 0.001$) and *IL-1RN2/TNF-A-857T/IL-8-845C/TLR2del* (OR = 38.76, 95 %CI = 5.19–289.58, $p < 0.001$).

In another analysis, aimed to assess the effect of the number of risk genotypes on the risk of gastric cancer, regardless of which gene was polymorphic, we compared the risk genotype frequencies in gastric cancer patients with those of the controls (Table 4). Individuals were classified according to the number of risk genotypes (*IL-1RN2, TNF-A-857T, IL-8-845C, IL-10-592A, TLR2del* and *TLR4 + 896G*) as follows: 0, reference group with no risk genotype; 1, individuals with one of the risk genotypes; 2, individuals with two of the risk genotypes; 3, individuals with three of the risk genotypes; and 4, individuals with four of the risk genotypes. Patients with five risk genotypes were also found in the GC group, but not in the C group, so it was not possible to perform statistical analysis for this category. We found that the OR of developing GC increased progressively with the increasing number of risk genotypes 1 to 4 (Table 4), with a 9.28 times higher OR value (95 %CI = 3.83–22.49, $p < 0.001$) in patients of the GC group with four risk genotypes.

## Discussion

Polymorphisms in cytokine and receptor genes of the immune system influence the response to infectious agents, such as *H. pylori*, and may contribute to variations in the risk of gastric carcinogenesis [42, 43]. Therefore, it is relevant to conduct studies on host genetic factors that may

**Table 2** Genotype and allele frequencies of *IL1-RN, TNF-A, TNF-B, IL-8, IL-10, TLR2* and *TLR4* polymorphisms adjusted for age, gender, smoking and drinking in gastric cancer (GC), chronic gastritis (CG), and control (C) groups

| Polymorphisms genotypes/alleles | | Controls, $N = 240$ *n* (%) | Patients | | | |
|---|---|---|---|---|---|---|
| | | | Gastric cancer, $N = 200$ | | Chronic gastritis, $N = 229$ | |
| | | | *n* (%) | OR (95 %CI), *p* | *n* (%) | OR (95 %CI), *p* |
| *IL-1RN* VNTR | L/L | 158 (66.0) | 84 (42.0) | 2.60 (1.65–4.10), <0.001 | 119 (52.0) | 1.88 (1.25–2.83), 0.002 |
| | L/2 + 2/2 | 82 (34.0) | 84 + 32 (58.0) | | 86 + 24 (48.0) | |
| | L | 0.81 | 0.63 | 2.51 (1.85–3.40), <0.001 | 0.71 | 1.76 (1.30–2.39), 0.002 |
| | 2 | 0.19 | 0.37 | | 0.29 | |
| *TNFA-308 G/A* (rs1800629) | G/G | 167 (69.5) | 136 (68.0) | 0.93 (0.56–1.54), 0.782 | 160 (70.0) | 1.03 (0.66–1.61), 0.870 |
| | G/A + A/A | 73 (30.5) | 61 + 3 (32.0) | | 66 + 3 (30.0) | |
| | G | 0.84 | 0.85 | 0.95 (0.66–1.38), 0.852 | 0.84 | 0.97 (0.68–1.38), 0.928 |
| | A | 0.16 | 0.15 | | 0.16 | |
| *TNFA-857 C/T* (rs1799724) | C/C | 157 (65.5) | 99 (49.5) | 1.70 (1.08–2.67), 0.022 | 147 (64.0) | 1.03 (0.68–1.57), 0.868 |
| | C/T + T/T | 83 (34.5) | 101 (50.5) | | 82 (36.0) | |
| | C | 0.79 | 0.72 | 1.42 (1.04–1.94), 0.027 | 0.80 | 0.91 (0.66–1.26), 0.628 |
| | T | 0.21 | 0.28 | | 0.20 | |
| *TNFB + 252 A/G* (rs909253) | A/A | 124 (51.6) | 86 (43.0) | 1.33 (0.84–2.10), 0.224 | 102 (44.5) | 1.21 (0.80–1.84), 0.351 |
| | A/G + G/G | 116 (48.4) | 114 (57.0) | | 127 (55.5) | |
| | A | 0.70 | 0.67 | 1.15 (0.87–1.54), 0.341 | 0.65 | 1.26 (0.96–1.66), 0.093 |
| | G | 0.30 | 0.33 | | 0.35 | |
| *IL8-251 T/A* (rs4073) | T/T | 61 (25.4) | 60 (30.0) | 0.88 (0.53–1.46), 0.638 | 38 (16.5) | 1.28 (0.95–2.23), 0.730 |
| | T/A + A/A | 179 (74.6) | 140 (70.0) | | 191 (83.5) | |
| | T | 0.53 | 0.53 | 1.00 (0.76–1.30), 1.000 | 0.46 | 1.29 (0.99–1.67), 0.058 |
| | A | 0.47 | 0.47 | | 0.54 | |
| *IL8-845 T/C* (rs2227532) | T/T | 225 (93.7) | 154 (77.0) | 3.46 (1.69–7.07), <0.001 | 220 (96.0) | 0.61 (0.24–1.56), 0.310 |
| | T/C + C/C | 15 (6.3) | 46 (23.0) | | 9 (4.0) | |
| | T | 0.96 | 0.85 | 4.91 (2.77–8.70), <0.001 | 0.97 | 0.71 (0.32–1.55), 0.438 |
| | C | 0.04 | 0.15 | | 0.03 | |
| *IL10-592 C/A* (rs1800872) | C/C | 169 (70.5) | 100 (50.0) | 2.34 (1.47–3.70), <0.001 | 106 (46.0) | 3.00 (1.99–4.50), <0.001 |
| | C/A + A/A | 71 (29.5) | 100 (50.0) | | 123 (54.0) | |
| | C | 0.84 | 0.74 | 1.84 (1.31–2.56), <0.001 | 0.70 | 2.26 (1.65–3.10), <0.001 |
| | A | 0.16 | 0.26 | | 0.30 | |
| *TLR2 del -196 to -174* | ins/ins | 200 (83.4) | 133 (66.5) | 2.20 (1.28–3.78), <0.001 | 175 (76.4) | 1.49 (0.91–2.46), 0.112 |
| | ins/del + del/del | 36 + 4 (16.6) | 58 + 9 (33.5) | | 47 + 7 (23.6) | |
| | ins | 0.91 | 0.81 | 2.32 (1.56–3.46), <0.001 | 0.87 | 1.52 (1.01–2.29), 0.049 |
| | del | 0.09 | 0.19 | | 0.13 | |

**Table 2** continued

| Polymorphisms genotypes/alleles | | Controls, $N = 240$ $n$ (%) | Patients | | | |
|---|---|---|---|---|---|---|
| | | | Gastric cancer, $N = 200$ | | Chronic gastritis, $N = 229$ | |
| | | | $n$ (%) | OR (95 %CI), $p$ | $n$ (%) | OR (95 %CI), $p$ |
| *TLR4 + 896 A/G* (rs4986790) | A/A | 224 (93.4) | 174 (87.0) | 2.09 (1.08–4.02), 0.033 | 205 (89.5) | 1.68 (0.81–3.47), 0.159 |
| | A/G | 16 (6.6) | 26 (13.0) | | 24 (10.5) | |
| | A | 0.97 | 0.93 | 2.01 (1.06–3.81), 0.037 | 0.95 | 1.60 (0.84–3.06), 0.195 |
| | G | 0.03 | 0.07 | | 0.05 | |
| *TLR4 + 1196 C/T* (rs4986791) | C/C | 234 (97.5) | 191 (95.5) | 0.84 (0.22–3.23), 0.804 | 223 (97.0) | 1.33 (0.38–4.59), 0.645 |
| | C/T | 6 (2.5) | 9 (4.5) | | 6 (3.0) | |
| | C | 0.99 | 0.98 | 1.81 (0.64–5.15), 0.300 | 0.99 | 1.04 (0.33–3.27), 1.000 |
| | T | 0.01 | 0.02 | | 0.01 | |

be associated with susceptibility to gastric diseases. This is a large epidemiological study focusing on the significance of ten polymorphisms in seven cytokine genes and TLRs regarding the susceptibility to gastric carcinogenesis in a Southeast Brazilian population.

Our results demonstrated the existence of an association of the anti-inflammatory cytokine variant alleles *IL-1RN*2 and *IL-10*-592A, of the pro-inflammatory variant alleles *TNF-A-857T* and *IL-8-845C* and of the toll-like receptors *TLR2 del* and *TLR4 + 896G* with a higher risk of developing gastric cancer and, for some of them, also of chronic gastritis. Despite the relevance of the study, some limitations should be considered that did not enable the analysis of association with *H. pylori* infection and histological type of gastric cancer.

Several previous studies have found a significant association of the polymorphic allele *IL-1RN2* with an increased risk of gastric diseases [16, 44, 45], although the data are controversial [46]. The allele *IL1-RN*2 has been linked to increased in vitro production of pro-inflammatory cytokine IL1-$\beta$ that causes a chronic inflammatory process and hypochlorhydria [47].

IL-10, another anti-inflammatory cytokine, has also been reported to be capable of inhibiting the production of pro-inflammatory cytokines such as IL-1$\beta$ and TNF-A [48, 49]. Polymorphisms in the 5′-flanking region of IL-10 at positions -1082 A/G, -819T/C and -592A/C were shown to be related with high transcriptional promoter activity [48]. All of them and the combined haplotypes have been suggested to be associated with gastric cancer risk in different populations [50, 51]. However, in the present study performed in a Brazilian population sample, we observed an association only of polymorphism *IL-10-592A* with risk of gastric cancer. Thus, genotypes that enhance the production of inflammatory cytokines may be related to the carcinogenic process of the stomach [52]. In contrast, a recent meta-analysis based on several previous studies concluded that polymorphism *IL-10*-592 C/A was not a risk factor for gastric cancer. However, when stratifying the data by ethnicity, genotype *IL-10*-592 AA was found to be a factor of protection against the development of this neoplasm in Asians, but not among Caucasians and Latinos, indicating differences in the genetic background of Asians and other ethnicities [53].

In addition, regarding the importance of pro-inflammatory cytokines, which can also modify the immune/inflammatory responses upon *H. pylori* infection and influence gastric carcinogenesis, we detected association with polymorphisms *TNF-A*-857 C/T and *IL-8*-845 C/T. So far, the influence of the *TNF-A*-857 C/T polymorphism in the promoter region on the risk of gastric cancer in different populations is not well established. For example, Zhang et al. [54] concluded in their meta-analysis that the *TNF-A*-857 C/T locus may be related to gastric cancer risk,

**Table 3** Combined effect of *IL1-RN, TNF-A-857C/T, IL-8-845T/C, IL-10-592A/C, TLR2 del-196 to -174* and *TLR4 + 896A/G* polymorphisms on risk of gastric cancer (GC)

| Risk genotypes | Groups | | OR (95 %CI), *p* |
|---|---|---|---|
| | GC ($N = 200$) | C ($N = 240$) | |
| Neither | 6 | 71 | 1.00 (reference) |
| Two risk polymorphisms | | | |
| *TNF-A-857T/IL-10-592A* | 43 | 30 | 2.95 (2.15–4.06), <0.001 |
| *IL-1RN2/TNF-A-857T* | 60 | 28 | 3.21 (2.32–4.43), <0.001 |
| *TNF-A-857T/TLR2del* | 31 | 17 | 4.33 (2.76–6.80), <0.001 |
| *IL-1RN2/IL-10-592A* | 56 | 18 | 4.46 (2.93–6.80), <0.001 |
| *IL-1RN2/TLR2del* | 39 | 14 | 5.26 (3.21–8.61), <0.001 |
| *IL-10-592A/TLR2del* | 31 | 12 | 5.79 (3.36–9.96), <0.001 |
| *TNF-A-857T/TLR4 + 896G* | 11 | 6 | 8.30 (3.56–19.33), <0.001 |
| *IL-8-845C/IL-10-592A* | 20 | 7 | 8.57 (4.09–17.92), <0.001 |
| *TNF-A-857T/IL-8-845C* | 22 | 7 | 8.75 (4.20–18.22), <0.001 |
| *IL-1RN2/TLR4 + 896G* | 15 | 6 | 9.16 (4.05–20.70), <0.001 |
| *TLR2del/TLR4 + 896G* | 10 | 4 | 11.71 (4.19–32.71), <0.001 |
| *IL-10-592A/TLR4 + 896G* | 16 | 4 | 13.63 (5.08–36.60), <0.001 |
| *IL-8-845C/TLR2del* | 14 | 3 | 17.26 (5.49–54.27), <0.001 |
| *IL-1RN2/IL-8-845C* | 29 | 2 | 30.24 (7.64–119.66), <0.001 |
| *IL-8-845C/TLR4 + 896G* | 6 | 1 | 36.00 (4.74–273.09), <0.001 |
| Three risk polymorphisms | | | |
| *IL-1RN2/IL-10-592A/IL-8-845C* | 21 | 8 | 7.68 (3.86–15.27), <0.001 |
| *IL-1RN2/TNF-A-857T/TLR2del* | 18 | 7 | 8.35 (3.97–17.58), <0.001 |
| *TNF-A-857T/IL-10-592A/TLR2del* | 17 | 6 | 9.48 (4.23–21.23), <0.001 |
| *IL-1RN2/TNF-A-857T/IL-10-592A* | 26 | 6 | 10.42 (4.75–22.89), <0.001 |
| *IL-1RN2/IL-10-592A/TLR2del* | 20 | 4 | 14.42 (5.43–38.30), <0.001 |
| *TNF-A-857T/TLR2del/TLR4 + 896G* | 4 | 2 | 14.60 (3.05–69.74), <0.001 |
| *IL-8-845C/TLR2del/TLR4 + 896G* | 2 | 1 | 18.00 (1.82–177.25), 0.024 |
| Three risk polymorphisms | | | |
| *TNF-A-857T/IL-10-592A/TLR4 + 896G* | 6 | 2 | 18.25 (4.15–80.14), <0.001 |
| *TNF-A-857T/IL-8-845C/TLR2del* | 8 | 2 | 20.85 (4.93–88.07), <0.001 |
| *TNF-A-857T/IL-10-592A/IL-8-845C* | 11 | 2 | 23.61 (5.75–96.83), <0.001 |
| *IL-10-592A/IL-8-845C/TLR4 + 896G* | 3 | 1 | 24.00 (2.78–207.70), 0.000 |
| *TNF-A-857T/IL-8-845C/TLR4 + 896G* | 4 | 1 | 28.80 (3.56–232.76), <0.001 |
| *IL-1RN2/TLR2del/TLR4 + 896G* | 6 | 1 | 36.00 (4.74–273.09), <0.001 |
| *IL-10-592A/IL-8-845C/TLR2del* | 6 | 1 | 36.00 (4.74–273.09), <0.001 |
| *IL-10-592A/TLR2del/TLR4 + 896G* | 7 | 1 | 38.76 (5.19–289.0), <0.001 |
| *IL-1RN2/IL-10-592A/TLR4 + 896G* | 7 | 1 | 38.76 (5.19–289.0), <0.001 |
| *IL-1RN2/IL-8-845C/TLR2del* | 13 | 1 | 49.26 (6.86–353.47), <0.001 |
| *IL-1RN2/TNF-A-857T/IL-8-845C* | 14 | 1 | 50.40 (7.04–360.62), <0.001 |
| Four risk polymorphisms | | | |
| *TNFA-857T/IL8-845C/TLR2del/TLR4 + 896G* | 1 | 1 | 10.28 (0.71–147.29), 0.173 |
| *IL-1RN2/TNFA-857T/IL-10-592A/TLR2del* | 9 | 2 | 21.90 (5.25–91.35), <0.001 |
| *IL-1RN2/TNFA-857T/IL8-845C/TLR2del* | 7 | 1 | 38.76 (5.19–289.0), <0.001 |

*GC* gastric cancer group, *C* cancer-free control group, *OR* odds ration, *CI* confidence interval

although most studies were concentrated on Asian populations [55]. Similarly, the SNP *TNF-A*-857 C/T was associated with gastric and duodenal ulcer [51], besides rugal hyperplastic gastritis by *H. pylori* infection and gastric cancer [56].

When we investigated the polymorphism in the promoter region of the *IL-8* gene at position -845, our results demonstrated for the first time an association of the *IL-8*-845C variant with susceptibility to chronic gastritis and gastric cancer. The scarcity of studies with this polymorphism may

**Table 4** ORs for the association with gastric cancer according to the number of risk genotypes for *IL1-RN, TNF-A-857C/T, IL-8-845T/C, IL-10-592A/C, TLR2 del-196 to -174* and *TLR4 + 896A/G* polymorphisms

| Number of risk genotypes | Controls, $N = 240$ *n* (%) | Gastric cancer, $N = 200$ *n* (%) | OR (95 %CI), *p* |
|---|---|---|---|
| 0 | 60 (25.0) | 6 (3.0) | 1 (reference) |
| 1 | 65 (27.0) | 33 (16.5) | 1.63 (1.32–2.02), <0.001 |
| 2 | 61 (25.5) | 80 (40.0) | 1.85 (1.53–2.22), <0.001 |
| 3 | 49 (20.5) | 62 (31.0) | 2.08 (1.62–2.52), <0.001 |
| 4 | 5 (2.0) | 15 (7.5) | 9.28 (3.83–22.49), <0.001 |

2 % of the gastric cancer group were with 5 risk genotypes. In the control group there were no individuals found with 5 risk genotypes

be explained by the low frequency of allele C, which is absent in European and Asian populations [29].

Other key components in controlling the inflammatory response are the TLRs that participate in *H. pylori* bacterium recognition in the gastric mucosa, and SNPs in TLRs are associated with impaired immune response, inducing a potent inflammatory response [57]. Our results showed an association of polymorphisms *TLR2* -196 to -174 *del* and *TLR4* + 896AG with susceptibility to gastric cancer. The association of polymorphism *TLR2* -196 to -174 *del* with risk of developing diseases related to an inflammatory process has shown conflicting results. In a Japanese population, this polymorphism was significantly associated with risk of gastric cancer but not for gastritis, gastric ulcer and duodenal ulcer [58], while allele *TLR2* –196 to –174 *ins* was associated with more severe intestinal metaplasia in older patients [37].

The *TLR4* + 896A/G polymorphism allows the substitution of amino acid Asp299Gly that disrupts the normal structure of the extracellular region of TLR4 and may cause decreased ligand recognition or protein interaction and decreased responsiveness to lipopolysaccharide, thereby disrupting the transport of TLR4 to the cell membrane [13, 59]. This change leads to an exaggerated inflammatory response with severe tissue destruction, likely due to a failure in stimulating regulatory cells and production of IL-10 cytokine [59].

In general, as reported above, the results of both our study and the literature indicate the involvement of the allelic variants *IL-1RN* 2, *IL-10*-592 A, *TNF-A-857T*, *IL-8-845C*, *TLR2 del* and *TLR4 + 896G* in gastric carcinogenesis. However, considering the various mediators involved in the cascade of inflammatory process, is not possible to explain the association of gastric cancer with only one SNP. A combined analysis involving various pro- and anti-inflammatory gene polymorphisms is therefore important, because it allows evaluation of gene–gene interaction, thus determining the genetic profiles of associations with a higher risk of gastric cancer in various populations with different genetic backgrounds.

In the present study, the combined analysis of the six polymorphisms associated with gastric cancer (*IL-1RN*2, *IL-10*-592A, *TNF-A-857T*, *IL-8-845C*, *TLR2 del* and *TLR4 + 896G)* showed that the combination of two (*IL-1RN2*/*IL-8*-845C and *IL-8*-845C/TLR4 + 896G), three (*IL-1RN2/TNF-A-857T/IL-8-845C, IL-1RN2/IL-8-845C/TLR2del, IL-1RN2/IL-10-592A/TLR4 + 896G, IL-10-592A/TLR2del/TLR4 + 896G, IL-1RN2/TLR2del/TLR4 + 896G, IL-10-592A/IL-8-845C/TLR2de*), and four of them (*IL-1RN2/TNF-A-857T/IL-8-845C/TLR2del* and *IL-1RN2/TNF-A-857T/IL-10-592A/TLR2 del*) increased the OR values significantly, ranging from 2.95 to 50.40 for risk of gastric cancer. Assessing the risk of GC by number of risk genotypes, ranging from 1 to 4 according to the polymorphic alleles (*IL-1RN2, TNF-A-857T, IL-8-845C, IL-10-592A, TLR2del* and *TLR4 + 896G*), the analysis showed that the OR values increased progressively with the increasing number of risk genotypes. Individuals carrying four risk genotypes, regardless of gene polymorphism, had a 9.28-fold increased risk of developing gastric cancer, as compared to those with only one risk genotype, whose risk was increased only 1.63-fold. However, this risk may increase up to 50.40-fold according to the gene polymorphisms involved, as shown above. Moreover, our results show mainly that it is possible to establish genetic profiles associated with highest risk for the development of gastric cancer associated with pro- and anti-inflammatory gene polymorphisms.

Few studies have done combined analysis of different polymorphisms in gastric cancer. El-Omar et al. [50] analyzed 11 polymorphisms of the *IL-1B*, *IL-1RN*, *IL-4, 1L-6, 1L-10* and *TNF-A* cytokine genes and showed that the OR for noncardia gastric cancer increased progressively with the number of pro-inflammatory genotypes to 27.3 for three or four polymorphisms. Similarly, Hold et al. [50, 60] also observed that the combined genotypes *IL-1B*-511T and/or homozygous *IL-1RN2* and *TLR4* + 896G increased the risk of gastric cancer. In addition, Machado et al. [61] analyzed the combined effect of pro-inflammatory genetic polymorphisms *IL-1B*, *IL-1RN*, and *TNF-A* and reported that individuals carrying high-risk genotypes at the three loci have an increased risk for chronic gastritis and gastric cancer. These findings are probably due to an additive effect of the pro-inflammatory profiles of these gene polymorphisms, resulting in an exacerbated immune response.

Although Seno et al. [62] evaluated a large number of polymorphisms (207 SNPs in 11 cytokine genes), they did not perform a combined analysis of the polymorphisms. They, however, made a haplotype or diplotype analysis, which revealed that only the *IL-4* gene diplotypes (984 and 2983 AA/GA) were negatively associated with the development of gastric cancer. Crusius et al. [11] investigated

various polymorphisms in cytokine genes (*IL-1A, IL-1B, IL1-RN, IL-4, IL-4R, IL-8, IL-10, IL-12, TNF* and *LTA*) in the European population and found a positive association of noncardia gastric cancer with *IL1-RN*2 and a negative association with *IL-8-251A/A*. They, however, observed no increase in OR with the progressive increase in number of genotypes.

## Conclusion

In conclusion, our study demonstrated that individuals with a combination of polymorphisms in cytokine and inflammatory receptor genes such as *IL-1RN2, IL-8-845C, TLR2del, IL-10-592A, TLR4 + 896G*, and *TNF-A-857T* may be at higher risk of developing gastric cancer. Therefore, further studies are needed to assess the combined effects of several polymorphisms and thereby possibly identify the profiles of polymorphisms associated with higher risk of developing gastric cancer in different populations.

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**Conflict of interest** None.

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