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RESEARCH ARTICLE

Influence of functional polymorphisms in *TNF-α*, *IL-8*, and *IL-10* cytokine genes on mRNA expression levels and risk of gastric cancer

Juliana Garcia de Oliveira[1,2] · Ana Flávia Teixeira Rossi[1] · Daniela Manchini Nizato[3] · Aline Cristina Targa Cadamuro[1] · Yvana Cristina Jorge[1] · Marina Curado Valsechi[4] · Larissa Paola Rodrigues Venâncio[5] · Paula Rahal[4] · Érika Cristina Pavarino[3] · Eny Maria Goloni-Bertollo[3] · Ana Elizabete Silva[1]

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Abstract Functional polymorphisms in promoter regions can produce changes in the affinity of transcription factors, thus altering the messenger ribonucleic acid (mRNA) expression levels of inflammatory cytokines associated with the risk of cancer development. The goal of this study was to evaluate the influence that polymorphisms in the cytokine genes known as *TNF*-α-308 G/A (rs1800629), *TNF*-α-857 C/T (rs1799724), *IL-8*-251 T/A (rs4073), *IL-8*-845 T/C (rs2227532), and *IL-10*-592 C/A (rs1800872) have on changes to mRNA expression levels and on the risks of chronic gastritis (CG) and gastric cancer (GC). A sample of 723 individuals was genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Relative mRNA expression levels were measured using quantitative real-time PCR (qPCR). Polymorphisms *TNF*-α-308 G/A and *IL-8*-251 A/T were not associated with risks of these gastric lesions. However, *TNF*-α-857 C/T, *IL-8*-845 T/C, and *IL-10*-592 C/A were found to be associated with a higher risk of GC, and *IL-10*-592 C/A was found to be associated with a higher risk of CG. The relative mRNA expression levels (RQ) of *TNF-α*, *IL-8*, and *IL-10* were markedly downregulated in the CG group (median RQs=0.128, 0.247, and 0.614, respectively), while the RQ levels of *TNF-α* in the GC group were upregulated (RQ=2.749), but were basal for *IL-8* (RQ=

✉ Juliana Garcia de Oliveira
juliana.usc2012@yahoo.com.br

Ana Flávia Teixeira Rossi
rossi.anaflavia@gmail.com

Daniela Manchini Nizato
danymanchini@hotmail.com

Aline Cristina Targa Cadamuro
ctc.aline@gmail.com

Yvana Cristina Jorge
yvanacj@hotmail.com

Marina Curado Valsechi
mcvalsechi@hotmail.com

Larissa Paola Rodrigues Venâncio
larissa.bluerose@gmail.com

Paula Rahal
prahal@ibilce.unesp.br

Érika Cristina Pavarino
erika@famerp.br

Eny Maria Goloni-Bertollo
eny.goloni@famerp.br

Ana Elizabete Silva
anabete@ibilce.unesp.br

[1] Cytogenetics and Molecular Biology Laboratory, Department of Biology, Sao Paulo State University (UNESP), Rua Cristovão Colombo, 2265, São José do Rio Preto 15054-000, SP, Brazil

[2] Department of Graduate Studies and Research, Sacred Heart University (USC), Rua Irmã Arminda, 10-50, Bauru 17011-970, SP, Brazil

[3] Department of Genetics and Molecular Biology, São José do Rio Preto School of Medicine (FAMERP), Av. Brigadeiro Faria Lima, 5416, São José do Rio Preto 15090-000, SP, Brazil

[4] Genomics Laboratory, Department of Biology, Sao Paulo State University (UNESP), Rua Cristovão Colombo, 2265, São José do Rio Preto 15054-000, SP, Brazil

[5] Laboratory for the Study of Hemoglobin and the Genetics of Hematologic Diseases, Department of Biology, Sao Paulo State University (UNESP), Rua Cristovão Colombo, 2265, São José do Rio Preto 15054-000, SP, Brazil

1.053) and downregulated for *IL-10* (RQ=0.179). When the groups were stratified according to wild-type and polymorphic alleles, only for *IL-8*-845 T/C the polymorphic allele was found to influence the expression levels of this cytokine. *IL-8*-845 C allele carriers were significantly upregulated in both groups (GC and CG; RQ=3.138 and 2.181, respectively) when compared to TT homozygotes (RQ=−0.407 and 0.165, respectively). In silico analysis in the *IL-8* promoter region revealed that the presence of the variant C allele in position -845 is responsible for the presence of the binding sites for two transcription factors (*REL* and *CREB1*), which are involved in increased gene expression. Polymorphic alleles were not shown to have any effect on the expression levels of *TNF-α* and *IL-10*. Taken together, our findings provide evidence for an association of *TNF-α*-857 C/T, *IL-8*-845 T/C, and *IL-10*-592 C/A with a higher risk of gastric cancer and also demonstrate the influence that the polymorphic C allele of *IL-8*-845 has on changes to the gene expression levels of this cytokine.

Keywords Cytokines · Gastric cancer · Chronic gastritis · Gene polymorphisms · Gene expression

Abbreviations

C	Control
CG	Chronic gastritis
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
EDTA	Ethylenediaminetetraacetic acid
GC	Gastric cancer
IL	Interleukin
IL-8	Interleukin-8
IL-10	Interleukin-10
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
OR	Odds ratio
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RQ	Relative quantification
SNP	Single-nucleotide polymorphism
TNF-α	Tumor necrosis factor alpha

Background

Cytokines play central roles in diversifying the inflammatory process, and interleukin (IL)-1*β*, tumor necrosis factor alpha (TNF-α), and interleukin-10 (IL-10) are all known to be the major cytokines involved in inflammation and cancer development [1, 2]. When it comes to gastric cancer, studies have found that infection by *Helicobacter pylori* promotes the production of multiple inflammatory cytokines and disrupts gastric homeostasis within the local mucosa [3].

Single-nucleotide polymorphisms (SNPs) in pro- and anti-inflammatory cytokine genes modify the intensity of the inflammatory response. Examples of these polymorphisms include *IL-1β*, *IL-1RN*, *IL-8*, *TNF-α*, and *IL-10*, which may contribute to variations in the risk of developing gastric cancer [1, 4–6]. Some SNPs that occur within regulatory regions of cytokine genes are believed to be involved in changes to the affinity of transcription factors, and they therefore affect the cytokine expression and secretion profile in response to agents of infection [7].

TNF-α is known as a gastric acid-suppressive cytokine that can thus change the site of *H. pylori* infection in the stomach. It is also important to note that the presence of *H. pylori* increases TNF-α expression, a process which reflects the interaction between this inflammatory mediator and gastric carcinogenesis [8]. Polymorphisms that increase *TNF-α* expression have now been associated with autoimmune diseases and cancer [9–11].

Interleukin-8 (IL-8) is the most potent known chemokine, and it is responsible for inducing chemotaxis, which consists of directed migration of cells to an inflammation site. Many stimuli can induce IL-8 secretion, including lipopolysaccharides (LPS), live bacteria, and early pro-inflammatory cytokines such as TNF and IL-1 [12]. A well-characterized SNP at the -251 T/A (rs4073) position of the *IL-8* gene has been studied to determine its involvement in several pathologies, including gastric cancer [13–16]. Studies have shown that the polymorphic allele A of the -251 T/A polymorphism upregulates IL-8 levels after LPS stimulation in vitro [17, 18]. The *IL-8*-845 T/C polymorphism (rs2227532) has received attention from researchers, who have attempted to associate it with inflammatory diseases and cancer [19, 20].

In contrast, the anti-inflammatory cytokine IL-10 is involved in cell downregulation and cytotoxic inflammatory response. It inhibits the formation of pro-inflammatory cytokines such as TNF-α and IL-8 [3, 21]. Polymorphisms in the IL-10 promoter region gene have been described as modulating transcriptional activation, which itself may alter IL-10 protein production in vitro. One of these polymorphisms is *IL-10*-592 C/A (rs1800872), the allele A of which is associated with gastric cancer development [2] and decreased messenger ribonucleic acid (mRNA) expression of this cytokine [22].

Few studies have evaluated the relationship that polymorphic variants within promoter regions of cytokine genes and changes to both mRNA expression levels share with carcinogenesis risk [23–25]. Thus, the goal of this study was to assess the mRNA expression levels of *TNF-α*, *IL-8*, and *IL-10* cytokine genes in tissue samples from patients with gastric cancer and chronic gastritis in order to then assess the association that the functional polymorphisms *TNF-α*-308 G/A (rs1800629), *TNF-α*-857 C/T (rs1799724), *IL-8*-251 T/A (rs4073), *IL-8*-

845 T/C (rs2227532), and *IL-10*-592 C/A (rs1800872) share with the increased risk of developing these lesions. The findings provide evidence that the *IL-8*-845 C allele polymorphism influences the modulation of mRNA expression levels and can therefore be associated with gastric carcinogenesis.

Materials and methods

Ethics declaration

Written informed consent for the collection of biological material (peripheral blood and gastric biopsies) and for access to medical records for research purposes were both obtained from the all individuals included in the study. The National Research Ethics Committee of IBILCE/UNESP approved this study (number 28/2009).

Subjects and samples

This study was performed in two steps. The first step was a case-control study on chronic gastritis (CG) and gastric cancer (GC), in which a total of 723 peripheral blood DNA samples from a previous study [26], which had been stored in our laboratory and genotyped for cytokine polymorphisms, were assessed. The first case group comprised 276 patients (142 men and 134 women) with a histopathologically confirmed diagnosis of chronic gastritis according to the Sidney System [27] and a mean age of 53.7±14.41 years (range 19–86 years). The second case group comprised 207 patients (161 men and 46 women) with a histopathologically confirmed diagnosis of gastric cancer according to Lauren's classification [28] and a mean age of 62.0±12.6 years (range 28–93 years). The control group (C) consisted of 240 healthy individuals (121 men and 119 women) with no previous history of gastric disease or cancer, most of whom were blood donors, with a mean age of 56.10±17.70 years (range 20–93 years). They were selected according to criteria described in a previous study [26].

The second step consisted of an evaluation of the mRNA relative expression levels of the *TNF-α*, *IL-8*, and *IL-10* genes in complementary deoxyribonucleic acid (cDNA) gastric tissue samples. Biopsies from the lesion areas were collected during endoscopic evaluations of 47 patients with CG (29 males and 18 females) with a mean age of 53.10±9.41 years (range 41–84 years), and 45 patients with GC (38 males and 7 females) with a mean age of 62.77±14.20 years (range 33–88 years). The biopsies were collected mainly from the gastric antral region and the corpus region. Peripheral blood was also collected from these patients for polymorphism genotyping.

All subjects were recruited from the Hospital de Base in São José do Rio Preto, São Paulo state, Brazil. Epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, which included questions on the patients' current and past occupations, smoking habits, alcohol intake, and family history of cancer. The details of this questionnaire are reported in a previous study [26].

Genotyping analyses

Approximately 5 mL of whole blood was collected from each of the study participants in sterile ethylenediaminetetraacetic acid-coated vacutainers. DNA was extracted according to Miller et al. [29] and stored at −20 °C until the genotyping was performed. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to identify the gene polymorphisms in both the case and control groups, as described in previous reports [19, 30–33]. Table 1 summarizes the PCR conditions, the sets of primers, and the enzymes used in each assay.

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 using a NanoDrop® ND1000 spectrophotometer, and its integrity was visualized in 1 % agarose gel. The RNA samples were stored at −80 °C and were later 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 each of the cDNA preparations was tested using a PCR assay of the *β-actin gene* (F: 5′-GGCATCGTGATG GACTCCG-3′ and R: 5′-GCTGGAAGGTGGACAGCG-3′) with visualization in 1.5 % agarose gel.

Real-time quantitative PCR

The relative expression levels of *TNF-α*, *IL-8*, and *IL-10* mRNA was measured by quantitative real-time PCR (qPCR), based on the SYBR Green methodology for *IL-8* and *IL-10* and the TaqMan methodology for *TNF-α*, using an ABI Prism® 7300 system (Applied Biosystems, Foster City, CA, USA). In both methodologies, the *β-actin* gene was used as a reference because it had presented lower variation than *α-tubulin* and *β2-microglobulin* in a previous study [34]. The primer sequences used for the *IL-8* and *β-actin* genes were obtained using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), as follows: *IL-8* F-5′ GATGCCAGTGAAACTTCAAGC 3′ and R-5′ATTGCATCTGGCAACCCTAC 3′; *B-actin* F-5′ TGCCCTGAGGCACTCTTC 3′ and R-5′ CGGATGTCCA CGTCACAC. The primer sequence for the *IL-10* gene was obtained according to Sorensen et al. [35]: *IL-10* F-5′ ACGG CGCTGTCATCGATT 3′ and R-5′ GGCATTCTTCACCTGC

Table 1 PCR-RFLP conditions: primer sequences, restriction enzyme, and fragment sizes

Gene	Primer (5′-3′)	PCR cycles	Melting T°	Enzyme T°/ time	Agarose gel (%)	Allele (bp)	Reference
TNF-α-308 G/A (rs1800629)	F: GAGGCAATAGGTTTTGAGGGCCAT R: GGGACACACAAGCATCAAG	36	65 °C	*Nco*I, 37 °C/1 h	3.0	G: 126+21 A: 147	[30]
TNF-α-857 C/T (rs1799724)	F: AAGTCGAGTATGGGGACCCCCCGTTAA R: CCCCAGTGTGTGGCCATATCTTCTT	36	65 °C	*Hinc*II, 37 °C/1 h	3.0	C: 107+24 T: 131	[31]
IL-8-251 T/A (rs4073)	F: TTCTAACACCTGCCACTCTAG R: CTGAAGCTCCACAATTTGGTG	35	60 °C	*Mfe*I, 37 °C/12 h	3.0	A: 76+32 T: 108	[32]
IL-8-845 T/C (rs2227532)	F: AACCCAGCAGCTCCAGTG R: AGATAAGC CAGCCAATCATT	35	61 °C	*Vsp*I, 37 °C/1 h	1.5	T: 341+193 C: 534	[19]
IL-10-592 C/A (rs1800872)	F: GGTGAGCACTACCTGACTAGC R: CCTAGGTCACAGTGACGTGG	30	60 °C	*Rsa*I, 37 °C/12 h	1.5	A: 412 C: 236+176	[33]

bp base pairs, *T°* temperature

TCCA 3′. For TaqMan gene expression analysis, the probes (Hs00357333_g1 in the case of *β-actin* and Hs 00174128_m1 in the case of *TNF-α*) were obtained from the website https://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/ PCR/ real-time-pcr/real-time-pcr-assays/taqman-gene-expression.html.

An initial validation step with a tenfold dilution series was performed to obtain the amplification efficiencies of the primers (*E*), which were calculated according to the equation $E=10^{[-1/\text{slope}]}$. After the validation step for the *IL-8* and *IL-10* genes, the qPCR assays were performed in 10 μL of SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, USA), 25 ng of cDNA, 0.4 μM of *β-actin* primer and 0.5 μM of *IL-8* and *IL-10* primer. The qPCR reaction for *TNF-α* analysis was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in a 20-mL reaction volume containing 50 ng of cDNA. Thermal cycling conditions for all of the genes were 2 min at 50 °C and 10 min at 95 °C for initial denaturation, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and a dissociation step at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s.

Relative quantification (RQ) of all genes was obtained according to the model described by Livak [36] and normalized with the *β-actin* reference gene and a pool of adjacent gastric mucosa. All reactions were performed in triplicate and included a negative control, and the data were expressed as medians.

In silico transcription factor detection

The dataset evaluated in this study comprised a promoter sequence (1500 bp upstream to 200 bp downstream of an annotated transcription start site) from the human *IL-8* gene (NCBI: NC_000004.12, GRCh38.p2). The set of vertebrate transcription factors (TFs) with their binding sites was obtained from the non-redundant, curated, and publically available JASPAR database, Version 5.0_ALPHA (JASPAR_Core) (http://jaspar.genereg.net) [37, 38]. The transcription factor binding site (TFBS) analysis was performed in the SNP rs2227532 region in order to identify TFs associated with this SNP. The evaluation was performed in two different situations—a TFBS search when the sequence possesses the ancestral allele (T) and a TFBS search in a sequence in which the polymorphism (C) was present—in order to identify possible changes to TFBS due to the presence of the polymorphism.

Statistical analysis

Odds ratios (ORs) and 95 % confidence intervals (CIs) were calculated for the polymorphisms under study using multiple logistic regression models that were adjusted for age, gender, smoking habits, and drinking habits in both the gastric cancer group and the chronic gastritis group. ORs were calculated using the SNPStat program, which considers the log-additive model (major allele homozygotes vs heterozygotes vs minor allele homozygotes), the dominant model (major allele homozygotes vs heterozygotes+minor allele homozygotes), and the recessive model (major allele homozygotes+heterozygotes vs minor allele homozygotes) for all polymorphisms. Data on relative mRNA expression were expressed as medians in order to determine the possible association between relative gene expressions in each group. The data were analyzed using the graphical method for outlier detection [39], and the outliers were removed from the subsequent analyses. The distribution of continuous data was determined using the D'Agostino & Pearson omnibus normality test. The Mann-Whitney test was used to analyze the influence of relative expression and of factors such as polymorphisms, gender, age, smoking habits, and alcohol consumption. Statistical analyses were performed using the GraphPad Prism 5 version 5.01 and the SPSS (11.5 version) computer software programs. Probability level (p) <0.05 was adopted as a significance criterion.

Results

Association of *TNF-α*, *IL-8*, and *IL-10* polymorphisms with gastric cancer and chronic gastritis

The results of the logistic regression analysis, as well as the genotype and allele frequencies of all of the polymorphisms in both case groups and the control group (GC vs C, CG vs C, and GC vs CG), are shown in Table 2.

When it came to the *TNF-α*-308 G/A and *IL-8*-251 T/A polymorphisms, no significant difference was found among the three groups evaluated (Table 2). However, in the case of *TNFA*-857 C/T, the CT+TT genotypes were associated with gastric cancer in the dominant model (OR=1.88, 95 % CI=1.24–2.85, p=0.002) when compared to the control group and in the dominant and log-additive models (OR=1.46, 95 % CI=1.04–2.05, p=0.027 and OR=1.63, 95 % CI=1.10–2.41, p=0.014) when compared to the CG group.

In the case of *IL-8-845* T/C, the CC polymorphic genotype was found to be associated with gastric cancer in all three of the models evaluated. Higher OR values were found in the recessive model (OR=15.54, 95 % CI=1.87–129.10, p<0.001) when the GC group was compared to the C group. When both case groups were compared to each other, there was a statistically significant difference in genotype frequencies only in the log-additive and dominant models (OR=1.67, 95 % CI=1.12–2.51, p=0.010 and OR=2.19, 95 % CI=1.27–3.77, p=0.004, respectively).

In the case of *IL-10-592* C/A, the AA polymorphic genotype was found to be associated with both gastric cancer and chronic gastritis lesions when it was compared to values in the control group when all three models were applied. Higher OR values were found in the recessive model when the GC group was considered (OR=3.24, 95 % CI=1.28–8.20, p=0.008) and in the dominant model when the CG group was considered (OR=2.84, 95 % CI=1.96–4.14, p<0.001) (Table 2). However, when the two case groups were compared to each other, there was no statistically significant difference in genotype frequencies.

Gene expression of *TNF-α*, *IL-8*, and *IL-10* in gastric cancer and chronic gastritis

The relative expression of *TNF-α*, *IL-8*, and *IL-10* in the GC and CG groups is shown in Table 3 and in Fig. 1. *TNF-α* and *IL-8* mRNA were upregulated in the GC group (RQ=2.749 and RQ=1.053, respectively) when compared to the CG group, which was downregulated (RQ=0.128 and RQ=0.247, p<0.001 and 0.036, respectively). In contrast, *IL-10* mRNA was downregulated in both the GC group (RQ=0.179) and the CG group (RQ=0.614), though with a statistically significant difference between the two groups (p<0.001). In addition, there was no statistically significant difference in the relative gene expression of *TNF-α*, *IL-8*, and *IL-10* within the GC and CG groups when the groups were stratified according to risk factors: gender, age, smoking habits, and alcohol intake (data not shown).

Influence of *TNF-α*, *IL-8*, and *IL-10* polymorphic alleles on mRNA expression levels

In another analysis, the GC and CG samples were grouped according to the polymorphic *TNF-α*-857 C/T, *IL-8*-845 T/C, and *IL-10*-592 C/A genotypes. This grouping showed these genotypes to be associated with gastric cancer and chronic gastritis when the influence of these polymorphisms on respective mRNA expression levels was determined (Table 4, Fig. 2). *TNF-α*-857 C/T was also evaluated more closely. In the GC group, individuals carrying the CC wild-type genotype were found to present increased mRNA expression levels (RQ=3.079) when compared to CT/TT polymorphic genotype carriers (RQ=1.703), though no significant difference was found (p=0.793). Similarly, in the CG group, higher mRNA expression levels were found in CC wild-type carriers (RQ=0.257) than in the CT/TT polymorphic genotype carriers (RQ=0.103, p=0.050).

When the *IL-8*-845 T/C samples were grouped according to polymorphic allele, it was found that, among the C allele carriers (TC/CC), relative *IL-8* mRNA expression levels were significantly more upregulated in both the GC group (RQ=3.138) and the CG group (RQ=2.181) than in the case of TT wild-homozygous individuals in the GC group (median RQ=0.407, p<0.001) and in the CG group (median RQ=0.165, p<0.001). When the individuals from the GC and CG groups were grouped according to the *IL-10*-592 C/A polymorphism, the mRNA expression levels were more downregulated in the carriers of the polymorphic A allele (RQ=0.133 and RQ=0.614, respectively) than those in the CC homozygous individuals (RQ=0.184 and RQ=0.833, respectively), though the differences were not significant (p=0.979 and 0.149, respectively).

In silico transcription factor detection in the *IL-8-845* T/C promoter region

The JASPAR Core analysis identified nine TFBSs for the *En1*, *HOXA5*, *ARID3A*, *Sox17*, *Pdx1*, *Sox6*, *Prrx2*, *Stat5a* and *Stat5b*, *and RFX5* transcription factors in the *IL-8* promoter sequence when the ancestral T allele was evaluated. With the presence of polymorphic C allele, seven TBFS were identified. It was observed that, with the change of base T>C, the binding sites for *Sox17*, *Pdx1*, *Sox6*, and *Prrx2* were lost; on the other hand, the binding sites to *REL* and *CREB1* were recognized.

Table 2 Genotype and allele frequencies and logistic regression analysis of *TNF-α*, *IL-8*, and *IL-10* polymorphisms between gastric cancer and chronic gastritis groups

Polymorphism	Genotype/ allele	Control vs *n* (%)	Case group		Gastric cancer vs chronic gastritis
			Gastric cancer—*n* (%)	Chronic gastritis—n (%)	
TNF-α-308 G/A	GG	167 (69.6)	138 (66.7)	193 (70.2)	
(rs1800629)	GA	69 (28.8)	66 (31.9)	78 (28.4)	
	AA	4 (1.7)	3 (1.4)	4 (1.4)	
Log-additive—OR (95 % CI), *p*			1.02 (0.68–1.52), 0.940	0.98 (0.69–1.39), 0.900	1.19 (0.80–1.75), 0.390
Dominant—OR (95 % CI), *p*			1.04 (0.67–1.61), 0.870	0.99 (0.67–1.45), 0.940	1.21 (0.79–1.84), 0.380
Recessive—OR (95 % CI), *p*			0.78 (0.15–4.00), 0.770	0.85 (0.21–3.51), 0.820	1.16 (0.23–5.99), 0.860
	G	0.84	0.82	0.84	
	A	0.16	0.18	0.16	
OR (95 % CI), *p*			1.14 (0.80–1.62), 0.470	0.96 (0.69–1.35), 0.860	1.18 (0.84–1.66), 0.338
TNF-α-857 C/T (rs1799724)	CC		157 (65.4)	102 (49.3)	166 (60.4)
	CT	64 (26.7)	95 (45.9)	99 (36)	
	TT	19 (7.9)	10 (4.8)	10 (3.6)	
Log-additive—OR (95 % CI), *p*			1.34 (0.96–1.87), 0.082	1.02 (0.76-1.37), 0.880	1.46 (1.04–2.05), 0.027
Dominant—OR (95 % CI), *p*			1.88 (1.24–2.85), 0.002	1.23 (0.85–1.77), 0.270	1.63 (1.10–2.41), 0.014
Recessive—OR (95 % CI), *p*			0.47 (0.20–1.13), 0.085	0.46 (0.21–1.02), 0.050	1.17 (0.45–3.02), 0.750
	C	0.79	0.72	0.78	
	T	0.21	0.28	0.22	
OR (95 % CI), *p*			1.42 (1.04–1.93), 0.020	1.01 (0.75–1.37), 0.930	1.39 (1.04–1.88), 0.027
IL-8-251 T/A (rs4073)	TT		61 (25.4)	62 (29.9)	58 (21.1)
	TA		134 (55.8)	98 (47.3)	162 (58.9)
	AA		45 (18.8)	47 (22.7)	55 (20)
Log-additive—OR (95 % CI), *p*			0.91 (0.68–1.22), 0.540	1.14 (0.87–1.50), 0.340	0.84 (0.63–1.12), 0.250
Dominant—OR (95 % CI), *p*			0.78 (0.49–1.22), 0.270	1.28 (0.84–1.94), 0.250	0.75 (0.42–1.32), 0.050
Recessive—OR (95 % CI), *p*			1.04 (0.63–1.72), 0.870	1.09 (0.69–1.70), 0.720	1.12 (0.70–1.79), 0.650
	T	0.53	0.53	0.50	
	A	0.47	0.47	0.50	
OR (95 % CI), *p*			0.98 (0.75–1.28), 0.940	1.11 (0.87–1.42), 0.380	0.88 (0.68–1.14), 0.363
IL-8-845 T/C (rs2227532)	TT	225 (93.8)	158 (76.3)	248 (90.2)	
	TC	14 (5.8)	35 (16.9)	19 (6.9)	
	CC	1 (0.4)	14 (6.8)	8 (2.9)	
Log-additive—OR (95 % CI), *p*			3.28 (1.87–5.77), <0.001	1.14 (0.87–1.50), 0.340	1.67 (1.12–2.51), 0.010
Dominant—OR (95 % CI), *p*			3.80 (1.97–7.31), <0.001	1.28 (0.84–1.94), 0.250	2.19 (1.27–3.77), 0.004
Recessive—OR (95 % CI), *p*			15.54 (1.87–129.10), <0.001	1.09 (0.69–1.70), 0.720	1.72 (0.67–4.45), 0.250
	T	0.97	0.85	0.94	
	C	0.03	0.15	0.06	
OR (95 % CI), *p*			5.20 (2.95–9.16), <0.001	1.96 (1.07–3.59), 0.030	2.65 (1.71–4.09), <0.001
*IL-10-592*C/A (rs1800872)	CC	169 (70.4)	104 (50.2)	128 (46.5)	
	CA	64 (26.7)	82 (39.6)	130 (47.3)	
	AA	7 (2.9)	21 (10.1)	17 (6.2)	
Log-additive—OR (95 % CI), *p*			2.17 (1.52–3.09), <0.001	2.42 (1.75–3.36), <0.001	1.03 (0.76–1.40), 0.840
Dominant—OR (95 % CI), *p*			2.49 (1.63–3.82), <0.001	2.84 (1.96–4.14), <0.001	0.91 (0.61–1.34), 0.620
Recessive—OR (95 % CI), *p*			3.24 (1.28–8.20), 0.008	2.69 (1.07–6.74), 0.028	1.66 (0.81–3.40), 0.160
	C	0.82	0.70	0.70	
	A	0.18	0.30	0.30	
OR (95 % CI), *p*			2.20 (1.59–3.03), <0.001	2.20 (1.59–3.03), <0.001	1.02 (0.76–1.22), 0.943

Frequencies adjusted for age, gender, smoking habits, and drinking habits

Table 3 Relative gene expression levels of the *TNF-α*, *IL-8*, and *IL-10* in the gastric cancer and chronic gastritis groups

Variable	*TNF-α*		*IL-8*		*IL-10*	
	GC (*n*=45)	CG (*n*=47)	GC (*n*=45)	CG (*n*=47)	GC (*n*=45)	CG (*n*=47)
Median	2.749	0.128	1.053	0.247	0.179	0.614
Range	0.080–20.34	0.005–0.832	0.007–4.532	0.004–4.840	0.001–0.732	0.017–3.535
p	<0.001		0.036		<0.001	

Mann-Whitney test was used to compare mRNA expression levels

GC gastric cancer, *CG* chronic gastritis

Discussion

Our findings show that the *TNF-α*-857 C/T, *IL-8*-845 T/C, and *IL-10*-592 C/A functional polymorphisms are associated with increased risk of developing gastric cancer and/or chronic gastritis. In addition, we observed significantly higher *IL-8* gene expression levels in the polymorphic C allele carriers.

TNF-α has been recognized as an important player in the development of both inflammation and cancer, including gastric cancer [9]. Among the *TNF-α*-308 G/A and -857 C/T SNPs evaluated in this study, only *TNF-α*-857 C/T was found to be associated with gastric cancer. Several studies have reported the same association [9, 20, 40–43], but studies attempting to associate cytokine expression with the presence of SNPs are uncommon [7].

A recent meta-analysis found a modest association between the *TNF-α*-308 A variant and gastric cancer in a Caucasian population, though no association between the polymorphism and gastritis or peptic ulcers was detected [9]. Allele A frequency in our study (0.18) is comparable to the frequency found in Caucasians (0.19) [2], and it is much higher than the frequency found in Asian patients (0.07) [44]. However, the same association with gastric cancer was not found in our population. Similarly, another study conducted in a southeastern Brazilian population also failed to find an association between the *TNF-α*-308 SNP and non-cardia gastric carcinoma [45]. In the present study, however, the *TNF-α*-857 C/T SNP was associated with gastric cancer, similar to other studies on gastric diseases in European [46] and Asian [47] populations.

In patients with gastric cancer, we found *TNF-α* mRNA to be overexpressed when compared to patients with chronic gastritis. This result indicates the involvement of this cytokine in carcinogenesis. Models with TNF-α-deficient mice have revealed that it is one of the essential cytokines for gastric cancer promotion, along with IL-1 and IL-6 [48]. High *TNF-α* mRNA expression has been reported in gastric cancer cases [49], as well as in gastric lesion cases such as peptic ulcers [50], chronic gastritis, intestinal metaplasia, and dysplasia [51], whether associated with *H. pylori* infection or not. Thus, these data corroborate our results. However, when we analyzed the influence of the *TNF-α*-857 C/T polymorphism on gene expression, we did not find a relationship between the polymorphic T allele carriers and those with the wild-type allele, a finding which was probably due to the reduced number of samples analyzed. In contrast, Higuchi et al. reported that the T allele of *TNF-α*-857 C/T (rs1799724) and the A allele of *TNF-α*-308 G/A (rs1800629) presented higher transcriptional activity and an increase in TNF-α cytokine production [7].

Among the SNPs of the *IL-8* gene evaluated, *IL-8*-251 A/T is the one that is most commonly related to increased risk for and poor prognosis of several diseases, including gastric cancer [52–54]. However, our results did not show increased susceptibility to the gastric diseases evaluated for their

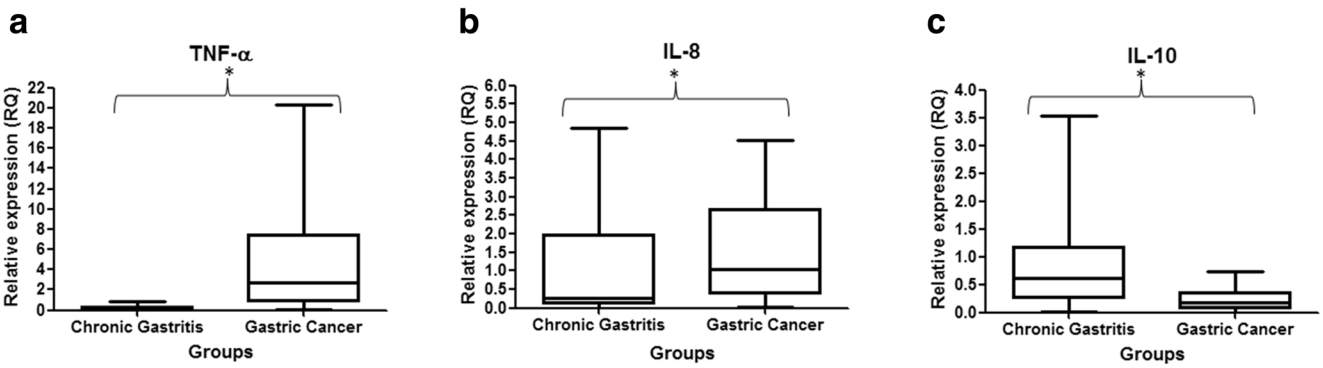

Fig. 1 Relative gene expression levels of **a** *TNF-α*, **b** *IL-8*, and **c** *IL-10* in gastric cancer and chronic gastritis groups. *Significant difference ($p<0.05$). *RQ* relative quantification

Table 4 Comparisons of gene expression levels of *TNF-α*, *IL-8*, and *IL-10* within gastric cancer and chronic gastritis groups, which were stratified according to wild and polymorphic genotypes

Group		*TNF-α*-857 C/T (rs1799724)		*p* value	*IL-8*-845 T/C (rs2227532)		*p* value	*IL-10*-592 C/A (rs1800872)		*p* value
		CC	CT/TT		TT	TC/CC		CC	CA/AA	
GC (*N*=45)	Median	3.079	1.703	0.793	0.407	3.138	<0.001	0.184	0.133	0.979
	Range	2.749–4.205	0.08–20.34		0.007–3.138	0.867–4.532		0.026–0.732	0.001–0.565	
CG (*N*=47)	Median	0.257	0.103	0.050	0.165	2.181	<0.001	0.833	0.614	0.149
	Range	0.005–0.832	0.008–0.788		0.004–2.819	0.111–4.840		0.168–3.535	0.017–1.526	

Mann-Whitney test used to compare mRNA expression levels

GC gastric cancer, *CG* chronic gastritis

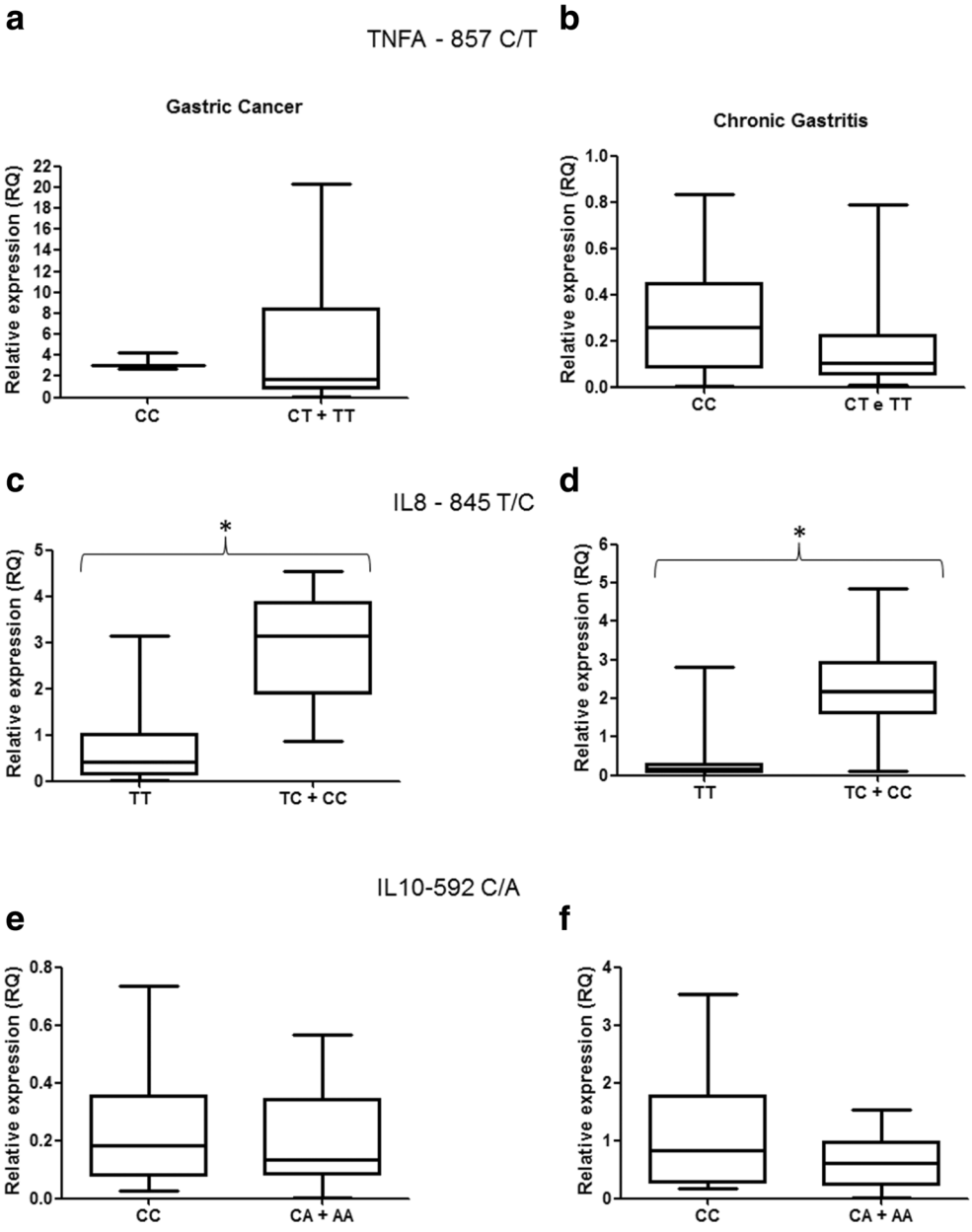

Fig. 2 Relative gene expression levels between wild-genotype carriers compared to those with at least one polymorphic allele in gastric cancer and chronic gastritis groups: **a** and **b** *TNF-α*-857 CC (wild-type) and CT+TT (polymorphic); **c** and **d** *IL-8*-845 TT (wild-type) and TC+CC (polymorphic); and **e** and **f** *IL-10*-592 CC (wild-type) and CA+AA (polymorphic). *Significant difference ($p<0.05$). *RQ* relative quantification

association with this SNP, nor did we observe any influence of the A allele on gene expression levels. Though it has been reported that the *IL-8*-251A allele is associated with higher levels of IL-8 [32], this finding is still controversial. Lee et al. [55] demonstrated that the *IL-8*-251 T allele has a two to five times stronger transcriptional activity than the A allele. On the other hand, Hacking et al. [56] found no difference in the promoter activity of the two alleles in an analysis of the -251 T/A promoter SNP. It is also possible that SNPs at other positions on the *IL-8* gene, such as *IL-8*-845 T/C, may also have an effect on the production of this cytokine.

When we investigated the *IL-8*-845 T/C polymorphism, our results revealed an association of *IL-8*-845 C with susceptibility to gastric cancer and the influence of the C allele on the *IL-8* mRNA expression level. The scarcity of studies on this polymorphism may be explained by the low frequency of the C allele, which is absent in European and Asian populations [19, 57]. In contrast, this allele is common in individuals of African descent (19 %) [58], with a frequency similar to that of gastric cancer patients in our study (15 %). We found heterogeneity in relative mRNA gene expression in our *IL-8* samples from both gastric cancer and chronic gastritis, with samples showing down- and upregulated expression. However, when the individuals carrying the C variant allele were grouped together, gene expression level increased significantly. This increase occurred in both the cancer group and the gastritis group, while the TT wild-type homozygous genotype presented downregulated expression in both groups. These results suggest the tendency of the C polymorphic allele to increase transcriptional activity and promote overexpression of this cytokine changes which can produce a more potent inflammatory response and possibly greater susceptibility to carcinogenesis.

In silico analysis in the *IL-8* promoter region showed that the presence of the variant C allele in position -845 is responsible for the appearance of REL and CREB1 transcription factor binding sites. The c-Rel protein is a member of the NF-kB family of transcription factors. The vertebrate Rel/NF-kB transcription factors include RelA, RelB, c-Rel, p50/p105, and p52/p100. The Rel/NF-kB proteins participate in cell growth control and neoplasia [59]. The RAS-RAF signaling pathway activates the NF-kB transcription factor, which, in turn, leads to the production of numerous cytokines [60], including pro-inflammatory *IL-8*, the continued production of which supports cell transformation and invasiveness [61, 62]. It is also known that, once *IL-8* expression has been induced, this chemokine may also feed forward to activate NF-kB and to exacerbate the inflammatory response [63]. CREB is a transcription factor that is known for its role in cell proliferation, differentiation, and survival. The CREB family encompasses three members, referred to as CREB1, CREM (cAMP response element modulator), and ATF-1 (activating transcription factor-1) [64]. CREB proteins can be phosphorylated in their kinase-inducible domain. This allows for increased transactivation potential. It remains unclear how this transcription factor may affect cytokine production; however, it has recently been reported that CREB1 is involved in the production of inflammatory mediators such as TNF-α, IL-1, and IL-8 in tumor cell lines [65]. CREB has also been found to be involved in *IL-8* production in human neutrophils induced by stimulation with *Trichomonas vaginalis* infection [66]. Therefore, it can be inferred that the *IL-8*-845 C variant is associated with the appearance of transcription factor binding sites for two TFs (REL and CREB) that are capable of increasing gene expression.

Our study also showed an association of the *IL-10*-592 C/A polymorphism with both gastric diseases evaluated. The frequency of the *IL-10*-592A allele in the healthy controls of our study was 18 %, much lower than those reported for Asian, Latino, and European Caucasian populations (65, 50, and 30 %, respectively) [67–69]. However, in the patients in our case groups, this frequency increased by approximately 30 %. This finding represents our population's susceptibility to both gastric cancer and chronic gastritis. These results are corroborated by other studies, in which the *IL-10*-1082 A/A, *IL-10*-819 T/T, and *IL-10*-592 A/A genotypes or the ATA haplotype of the *IL-10*-1082/-819/-592 polymorphisms were associated with *H. pylori* infection-related gastric cancer [2, 46, 70]. Meanwhile, Zhu et al. [70] performed a meta-analysis study and found that the *IL-10*-592 C/A polymorphism was not a risk factor for gastric cancer; they also found that the *IL-10*-592 AA genotype was a protective factor against the development of this neoplasia in Asians.

When we analyzed the mRNA expression level of the IL-10 anti-inflammatory cytokine, we observed less frequent expression in the gastric cancer group (RQ=0.179) than in the chronic gastritis group (RQ=0.614). We also observed a slight but not significant reduction in both the gastric cancer group and the chronic gastritis group when they were stratified according to the polymorphic A allele (CA/AA). Rad et al. [71] analyzed the *IL-10*-1082 G/A, -819 C/T, and -592 C/A polymorphisms and found that GCC haplotype carriers were associated with high *IL-10* gene expression levels, unlike the ATA haplotype. Later, Wang et al. [72] analyzed the same polymorphisms and reported that the *IL-10* mRNA levels were significantly higher in patients with non-small cell lung cancer with the non-ATA haplotype, a finding which shows the association of cytokine IL-10 expression levels with tumor progression. Reduced *IL-10* levels can lead to a hyper-inflammatory response with greater damage to the gastric mucosa infected by *H. pylori* [2]. Therefore, tumor progression may be linked to pro- and anti-inflammatory imbalance control and consequent gastric acidity, since a hypochlorhydric environment in the presence of bacteria encourages the development of both atrophic gastritis and gastric cancer [73].

The carcinogenic process is very complex. In addition to polymorphism phenomena, several events are involved in the regulatory mechanisms of cytokine gene expression, including factors such as transmute chemokine receptors, mRNA instability, and posttranslational modification [74].

Conclusions

Our findings show that promoter region polymorphisms in pro- and anti-inflammatory cytokines such as *TNF*-α-857 C/T, *IL-8*-845 T/C, and *IL-10*-592 C/A play an important role in the increased risk of gastric carcinogenesis. Taken together, they also suggest the influence of the *IL-8*-845 C allele in the elevation of the mRNA expression levels of this pro-inflammatory cytokine.

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Conflicts of interest None

Authors' contributions AES and JGO conceived and designed the experiments. JGO, AFT, DMN, and ACTC performed the experiments. JGO, AES, and ACTC analyzed and interpreted the data. YCJ, MCV, PR, EMGB, ECP, and AES contributed the set of samples, reagents, materials, and analysis tools. JGO and AES drafted the manuscript and revised it carefully. All authors have approved the final version of manuscript to be published.

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