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Department of Surgery, The University of Hong Kong, Hong Kong, China

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## Case Control Study

# Characterization and strong risk association of *TLR2* del -196 to -174 polymorphism and *Helicobacter pylori* and their influence on mRNA expression in gastric cancer

Caroline de Matos Lourenço, Manoela Dias Susi, Mariah Cristina Antunes do Nascimento, Vilson Serafim Junior, Ana Paula Simedan Vila, Gabriela Helena Rodrigues-Flemming, Eny Maria Goloni-Bertollo, Ana Elizabete Silva, Juliana Garcia de Oliveira-Cucolo

**ORCID number:** Caroline de Matos Lourenço (0000-0003-3629-1447); Manoela Dias Susi (0000-0001-7913-2564); Mariah Cristina Antunes do Nascimento (0000-0002-6422-4068); Vilson Serafim Junior (0000-0002-3349-0531); Ana Paula Simedan Vila (0000-0003-4353-0696); Gabriela Helena Rodrigues-Flemming (0000-0002-6714-6931); Eny Maria Goloni-Bertollo (0000-0002-2622-4673); Ana Elizabete Silva (0000-0003-1491-8878); Juliana Garcia de Oliveira-Cucolo (0000-0002-2125-9930).

**Author contributions:** Oliveira-Cucolo JG and Silva AE conceived and designed the experiments; Lourenço CM, Susi MD, Serafim Junior V and Vila APS performed the experiments; Nascimento MCA provided technical support; Oliveira-Cucolo JG, Serafim Junior V and Rodrigues-Flemming GH analyzed and interpreted the data; Silva AE and Goloni-Bertollo EM contributed to the collection of samples/reagents/materials and analysis tools; Oliveira-Cucolo JG, Rodrigues-Flemming, Goloni-Bertollo EM and Silva AE drafted and revised the manuscript; All of the authors approved the final version of the manuscript for publication.

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**Caroline de Matos Lourenço, Manoela Dias Susi,** Department of Graduate-Level Research, Sacred Heart University, Bauru, São Paulo 17011-970, Brazil

**Mariah Cristina Antunes do Nascimento, Vilson Serafim Junior, Ana Paula Simedan Vila, Gabriela Helena Rodrigues-Flemming, Eny Maria Goloni-Bertollo, Juliana Garcia de Oliveira-Cucolo,** Department of Molecular Biology, São José do Rio Preto School of Medicine, São José do Rio Preto, São Paulo 15090-000, Brazil

**Ana Elizabete Silva,** Department of Biology, São Paulo State University, São José do Rio Preto, São Paulo 15054-000, Brazil

**Corresponding author:** Ana Elizabete Silva, PhD, Adjunct Professor, Department of Biology, São Paulo State University, Rua Cristóvão Colombo, 2265, São José do Rio Preto, São Paulo 15054-000, Brazil. [ae.silva@unesp.br](mailto:ae.silva@unesp.br)

## Abstract

### BACKGROUND

Toll-like receptor-2 (*TLR2*) is responsible for recognizing *Helicobacter pylori* (*H. pylori*) and activating the immune response. Polymorphisms in *TLR2* may modulate gastric carcinogenesis.

### AIM

To evaluate whether the *TLR2* 19216T/C (rs3804099) and *TLR2* -196 to -174 ins/del (rs111200466) polymorphisms contribute to gastric carcinogenesis in the Brazilian population, and to determine the influence of both polymorphisms and *H. pylori* infection on *TLR2* mRNA expression.

### METHODS

DNA was extracted from 854 peripheral blood leukocyte or gastric tissue samples [202 gastric cancer (GC), 269 chronic gastritis (CG), and 383 control/healthy (C)] and genotyped by allele-specific PCR or restriction fragment length polymorphism (RFLP)-PCR. Quantitative polymerase chain reaction by *TaqMan*® assay was used to quantify *TLR2* mRNA levels in fresh gastric tissues (48 GC, 36 CG, and 14 C).

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## RESULTS

Regarding the TLR2 -196 to -174 polymorphism, the *ins/del* and *del/del* genotypes were associated with a higher risk of GC by comparison with the C in all of the analyzed inheritance models (codominant, dominant, recessive, overdominant and log-additive;  $P < 0.0001$ ). Similarly, an increased risk was observed when comparing the GC and CG groups [codominant ( $P < 0.0001$ ), dominant ( $P < 0.0001$ ), recessive ( $P = 0.0260$ ), overdominant ( $P < 0.0001$ ) and log-additive ( $P < 0.0001$ )]. In contrast, TLR2 19216T/C was associated with a protective effect in the GC group compared to the C group [dominant ( $P = 0.0420$ ) and log-additive ( $P = 0.0300$ )]. Regarding the association of polymorphisms with *H. pylori* infection, individuals infected with *H. pylori* and harboring the TLR2 -196 to -174 *ins/del* polymorphism had an increased risk of gastric carcinogenesis [codominant ( $P = 0.0120$ ), dominant ( $P = 0.0051$ ), overdominant ( $P = 0.0240$ ) and log-additive ( $P = 0.0030$ )], while TLR2 19216T/C was associated with a protective effect [codominant ( $P = 0.0039$ ), dominant ( $P < 0.0001$ ), overdominant ( $P = 0.0097$ ) and log-additive ( $P = 0.0021$ )]. TLR2 mRNA levels were significantly increased in the GC group (median RQ = 6.95) compared to the CG group (RQ = 0.84,  $P < 0.0001$ ) and to the normal mucosa group (RQ = 1.0). In addition, both *H. pylori* infection ( $P < 0.0001$ ) and the presence of the polymorphic TLR2 -196 to -174del ( $P = 0.0010$ ) and TLR2 19216 C ( $P = 0.0004$ ) alleles influenced TLR2 mRNA expression.

## CONCLUSION

The TLR2 -196 to -174 *ins/del* and TLR2 19216 T/C polymorphisms are strongly associated with GC. TLR2 mRNA expression levels are upregulated in neoplastic tissues and influenced by both the presence of *H. pylori* and variant genotypes.

**Key words:** Toll-like receptor 2; *Helicobacter pylori*; Gastric cancer; Chronic gastritis; Polymorphisms; Gene expression

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**Core tip:** This study showed that two polymorphisms in toll-like receptor-2 (TLR2) are strongly associated with gastric cancer and *Helicobacter pylori* (*H. pylori*) infection in the Brazilian population. TLR2 mRNA expression was increased in gastric cancer tissue compared with chronic gastritis and normal tissues. Moreover, TLR2 mRNA expression levels were upregulated in gastric cancer in the presence of the TLR2 -196 to -174 del variant allele or the wild-type TLR2 19216 T allele and in the presence of *H. pylori*. Thus, gene polymorphisms that change expression levels, such as those in TLR2, may alter the immune response and, consequently, the development and clinical manifestations of cancer.

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## INTRODUCTION

The cascade of gastric carcinogenesis described by Correa *et al*<sup>[1]</sup> describes the role of *Helicobacter pylori* (*H. pylori*) infection in the development of chronic gastritis (CG), considered the initial stage of tumor progression, and that ends with the development of gastric cancer (GC). Thus, *H. pylori* infection represents the main cause of CG, and infected patients have a 10-fold higher chance of developing GC<sup>[2]</sup>. Thus, this bacterium is widely known as a class I carcinogen in gastric diseases<sup>[3]</sup>.

Pattern recognition receptors (PRRs), including toll-like receptors (TLRs) 2 and 4, recognize different pathogen-associated molecular patterns (PAMPs) shared by most microorganisms, including *H. pylori*<sup>[4]</sup>. TLR2 is involved in the recognition of bacterial



lipopolysaccharide (LPS); TLR2 activation results in the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and functions as an innate immune response, but a Th1 adaptive immune response is also triggered by binding to *H. pylori* neutrophil-activating protein<sup>[5]</sup>.

The presence of *H. pylori* disrupts gastric mucosa homeostasis and initiates an inflammatory response, stimulating the production and secretion of proinflammatory mediators, such as interleukin (IL)-1 $\beta$ , IL-2, IL-6, IL-8, IL-12, and reactive oxygen and nitrogen species that cause DNA damage<sup>[6]</sup>. This proinflammatory microenvironment may promote the development of precancerous lesions, such as chronic gastritis, gastric atrophy, intestinal metaplasia, and dysplasia, that eventually progress to gastric cancer<sup>[7]</sup>.

TLR2 polymorphisms are associated with the pathogenesis of GC, vary among different populations and ethnic groups, and can modulate the immune response to *H. pylori* infection and its persistence. For example, *TLR2* -196 to -174 ins/del, a 22-bp deletion in the promoter region, is associated with protection against GC in the Chinese population<sup>[8,9]</sup> but has no association with gastric diseases in the Japanese population<sup>[10,11]</sup>. However, studies in the Caucasian population demonstrated an increased risk for gastric cancer<sup>[12]</sup> and other types of cancer, such as breast<sup>[13]</sup>, colorectal<sup>[14]</sup>, and head and neck cancer<sup>[15]</sup>.

For *TLR2* 19216T/C (rs3804099), a synonymous variant located on chromosome 4, it is not yet clear which allele (C or T) is associated with the susceptibility to disease. Some studies in the Asian population have indicated a protective association between the TC or CC genotype and different types of cancer, such as colorectal<sup>[16]</sup>, breast<sup>[17]</sup>, and hepatocellular carcinoma<sup>[18]</sup>. Conversely, in the Russian population, the CC genotype was closely associated with a risk of severe coronary atherosclerosis<sup>[19]</sup>.

Thus, given the contradictory results and genetic heterogeneity of the Brazilian population, it is important to evaluate the role of these polymorphisms in the susceptibility to gastric carcinogenesis. In addition, we evaluated the influence of *TLR2* polymorphisms and *H. pylori* infection on *TLR2* mRNA expression. Our findings showed that the *TLR2* -196 to -174 ins/del and *TLR2* 19216T/C polymorphisms are associated with an increased risk of and protection against gastric cancer development, respectively. *TLR2* mRNA expression levels were upregulated in gastric cancer tissues and were influenced by both the presence of *H. pylori* and variant genotypes.

## MATERIALS AND METHODS

### Ethics statement

The Research Ethics Committee of Universidade do Sagrado Coração (USC) in Bauru, São Paulo, Brazil approved this study (Registration Number 382.514), and written informed consent for the collection of biological material (peripheral blood and gastric tissues) was obtained from all individuals.

### Subjects and samples

This was a case-control study on CG and GC patients and healthy individuals. DNA was obtained from a total of 852 peripheral blood leukocyte or gastric tissue samples and genotyped for *TLR2* polymorphisms [*TLR2* del -196 to -174 (rs111200466) and *TLR2* 19216T/C (rs3804099)]. All samples included in this study were obtained from patients with gastric complaints who underwent an upper digestive endoscopy between January 2010 and March 2016 in the Gastroenterology Department, State Hospital of Bauru, São Paulo, Southeastern Region, Brazil. Patients treated with antibiotics, anti-inflammatory agents, chemotherapy drugs, radiotherapy or proton pump inhibitors within 30 days before endoscopy were not included in the study.

The case groups included 269 patients (123 men and 146 women; 147 *H. pylori*-positive and 122 *H. pylori*-negative; mean age, 50.89  $\pm$  23.03 years) with a confirmed histopathological diagnosis of CG per the Sydney System<sup>[20]</sup> and 202 patients (152 men and 50 women; 86 *H. pylori*-positive and 116 *H. pylori*-negative; mean age, 66.26  $\pm$  16.32 years) with a confirmed histopathological diagnosis of GC per Lauren's classification<sup>[21]</sup>. The gastric disease-free control group (C) consisted of 381 patients (176 men and 205 women; mean age, 51.26  $\pm$  16.77 years) who underwent endoscopy by medical indication, gave up gastric biopsy exclusively for this study, and were histopathologically confirmed to be negative for any gastric disease and for *H. pylori* infection by a trained professional of Sacred Heart University -Bauru-SP following the hospital standard (Table 1).

*H. pylori* infection was histologically established by Giemsa staining or the urease test performed by the Pathology Services of the State Hospital of Bauru, and the results were subsequently confirmed using PCR, as described in a previous study<sup>[22]</sup>.

**Table 1** Epidemiological data of individuals with normal gastric mucosa (control group, chronic gastritis, and gastric cancer patients), *n* (%)

Variables	Control, <i>n</i> = 381	Chronic gastritis, <i>n</i> = 269	Gastric cancer, <i>n</i> = 202
Gender			
Female	205 (53.8)	146 (54.3)	50 (24.8)
Male	176 (46.2)	123 (45.7)	152 (75.2)
Age in yr			
mean ± SD	51.26 ± 16.77	50.89 ± 23.03	66.26 ± 16.32
Smoking			
Yes	79 (20.7)	84 (31.2)	142 (70.3)
No	302 (79.3)	185 (68.8)	60 (29.7)
Drinking			
Yes	98 (25.7)	115 (42.7)	128 (63.3)
No	281 (74.3)	154 (57.3)	74 (36.7)
<i>Helicobacter pylori</i>			
Positive	0 (0)	122 (45.3)	86 (42.6)
Negative	381 (100)	147 (54.7)	116 (57.4)

In addition, to quantify *TLR2* mRNA levels, biopsies were collected during the endoscopic evaluation (gastric antrum and corpus regions) from 48 patients (29 men and 19 women; mean age, 53.10 ± 9.41 years; 16 *H. pylori*-positive and 32 *H. pylori*-negative) in the CG group, 36 patients (25 men and 11 women; mean age, 62.32 ± 14.66 years; 21 *H. pylori*-positive and 15 *H. pylori*-negative) in the GC group, and 14 individuals in the C group who were free of gastric cancer and *H. pylori* infection (9 men and 5 women; mean age, 49.58 ± 21.01 years).

### Polymorphism genotyping

DNA was extracted from peripheral blood following a previously published protocol<sup>[23]</sup> with modifications (using Ficoll-Paque™ PLUS to separate blood components), while total RNA and DNA were simultaneously extracted from tissue samples using the QIAamp® tissue kit (Qiagen, Germany) according to the reagent protocol and stored at -20 °C.

The *TLR2* -196 to -174 *del* polymorphism (rs111200466) was detected by allele-specific PCR, and restriction fragment length polymorphism (RFLP)-PCR was used to assess the *TLR2* 19216 T/C (rs3804099) polymorphism. For both PCR techniques, the reaction solution contained the following: 1 × buffer, 15.3 µL of ultrapure H<sub>2</sub>O, 2.0 µL (0.10 µmol/L) of dNTPs, 0.5 µL (25 mmol/L) of MgCl<sub>2</sub>, 1.25 µL of each primer (25 mmol/L), 0.2 µL (1 U) of *Taq* DNA polymerase, and 200 ng of genomic DNA. The amplification products of the *TLR2* -196 to -174 *del* analysis and the digestion products of the *TLR2* 19216 T/C analysis were visualized on a 3% agarose-1000 gel (Invitrogen®) with ethidium bromide in the presence of a 100 bp molecular marker. To ensure greater genotyping reliability, a positive control (a heterozygous sample for the evaluated polymorphism) was included in all reactions. Approximately 10% of the samples were processed in duplicate for quality control purposes. Table 2 summarizes the location of both polymorphisms, minor allele frequency (MAF), PCR conditions, primer sets, and enzymes used in each assay.

### RNA extraction, reverse transcription, and real-time quantitative PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. RNA concentration and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, United States) and a Bioanalyzer (Agilent, United States). A reverse transcription reaction was performed using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA, United States) according to the protocol instructions.

Quantitative PCR was performed with a *TaqMan*® System (Life Technologies, United States) and the StepOne Plus Real-Time PCR system 2.2.3 (Applied Biosystems, United States) using a *TaqMan* probe specific for the *TLR2* gene (Hs00610101\_m1) and two reference genes, GUSB (Hs00187320\_m1) and TBP (Hs00187332\_m1). The reactions were performed in triplicate and included a negative control. Relative quantification (RQ) was performed using the 2<sup>-ΔΔC<sub>t</sub></sup> method<sup>[24]</sup> after normalization to both reference genes, and 14 normal *H. pylori*-negative gastric tissue samples (C group) were used as a calibrator (RQ = 1.0). RQ was also performed for

**Table 2** Nucleotide primer sequences, PCR conditions, and minor allele frequency for polymorphisms *TLR2* -196 to -174 ins/del (rs111200466) and *TLR2* 19216T/C (rs3804099)

Genes	Location	MAF	Primers, 5'-3'	Cycles	T° melting	Enzyme	Genotypes, bp
<i>TLR2</i> -196 to -174 ins/del (rs111200466)	Chromosome 4:153684312-153684338 (intron variant)	0.1952 (del)	F: CACGGAGGCA GCGAGAAA	35	60 °C	-	ins/ins: 286 bp
			R: CTGGGCCGTGC AAAGAAG				ins/del: 286 + 264 bp
							del/del: 264 bp
<i>TLR2</i> 19216T/C (rs3804099)	Chromosome 4:153703504 (synonymous variant)	0.4048(C)	F: TCCCTGGGCAG TCTTGAACATT TAG	30	65 °C	Taq	TT: 415 bp
			R: TGTCCAAATCA GTATCTCGCAG TTCC				TC: 415 + 109 + 306 bp
							CC: 306 + 109 bp

MAF: Minor allele frequency - extracted from 1000 Genomes Project Phase 3; bp: Base pairs R: Reverse; F: Forward.

the samples stratified by polymorphism genotype (at least one polymorphic allele vs wild-type homozygote) and *H. pylori* infection (negative vs positive). The data were expressed as median values.

### Statistical analysis

SNPStats software was used to calculate the odds ratios (ORs) and 95% confidence intervals (CIs) for the risk associations between polymorphisms and gastric diseases. The multiple logistic regression models were adjusted for age, gender, and *H. pylori* infection. The effect of the polymorphisms was evaluated in the models as (1) codominant (heterozygous vs wild-type homozygous, and polymorphic homozygous vs wild-type homozygous); (2) dominant (heterozygous + polymorphic homozygous vs wild-type homozygous); (3) recessive (polymorphic homozygous vs wild-type homozygous + heterozygous); (4) overdominant (heterozygous vs wild-type homozygous + polymorphic homozygous); or (5) log-additive (polymorphic homozygous with 2 + heterozygous vs wild-type homozygous).

The RQ values for *TLR2* mRNA were statistically analyzed. The continuous data distribution was evaluated using the D'Agostino-Pearson omnibus test for normality. The Mann-Whitney test and Wilcoxon's signed rank test were used for comparisons between groups (GC, CG and C) to analyze the influence of *H. pylori* infection and polymorphisms on *TLR2* mRNA expression. Statistical analyses were performed using GraphPad Prism 5 software and SNPstats online tool (<https://www.snpstats.net/start.htm>). A probability level (*P*) of < 0.05 was considered to indicate statistical significance. MAF and Hardy-Weinberg equilibrium were evaluated using OEGE software<sup>[25]</sup>.

## RESULTS

### *TLR2* -196 to -174 ins/del and *TLR2* 19216T/C polymorphisms, and risk of gastric lesions and *H. pylori* infection

The genotype and allele frequency distributions of the two polymorphisms complied with Hardy-Weinberg equilibrium in both the case and control groups (data not shown). The genotype frequencies of the *TLR2* -196 to -174 ins/del and *TLR2* 19216 T/C polymorphisms in all three groups (GC vs C, CG vs C, and GC vs CG) are shown in Table 3.

*TLR2* -196 to -174 del was associated with a higher risk of GC by comparison with the C group in the codominant [odds ratio (OR) = 3.70, 95%CI: 2.41-5.70 for *TLR2* -196 to -174 ins/del; OR = 5.73, 95%CI: 1.80-18.21 for *TLR2* -196 to -174 del/del; *P* < 0.0001], dominant (OR = 3.87, 95%CI: 2.55-5.86; *P* < 0.0001), recessive (OR = 4.00, 95%CI: 1.27-12.62; *P* = 0.0130), overdominant (OR = 3.44, 95%CI: 2.24-5.27; *P* < 0.0001), and log-additive models (OR = 3.23, 95%CI: 2.23-4.69; *P* < 0.0001). Similarly, this polymorphism was associated with an increased risk when comparing the GC and CG groups in the codominant (OR = 2.68, 95%CI: 1.71-4.20 for *TLR2* -196 to -174 ins/del; OR = 5.06, 95%CI: 1.45-17.70 for *TLR2* -196 to -174 del/del; *P* < 0.0001), dominant (OR



**Table 3** Genotype frequencies of TLR2 -196 to -174 ins/del and TLR2 19216 T/C polymorphisms in both case and control groups (GC vs C, CG vs C and GC vs CG), *n* (%)

Polymorphisms	Models	Genotypes	C, <i>n</i> = 381	Case							
				GC		CG			GC x CG		
				<i>n</i> = 202	OR (95%CI)	<i>P</i> value	<i>n</i> = 269	OR (95%CI)	<i>P</i> value	OR (95%CI)	<i>P</i> value
TLR2 -196 to -174 ins/del (rs111200466)	Codominant	ins/ins	316 (82.9)	112 (55.5)	1.00	< 0.0001	212 (78.8)	1.00	0.4100	1.00	< 0.0001
		ins/del	60 (15.8)	79 (39.1)	3.70 (2.41-5.70)		53 (19.7)	1.32 (0.88-1.98)		2.68 (1.71-4.20)	
		del/del	5 (1.3)	11 (5.5)	5.73 (1.80-18.21)		4 (1.5)	1.19 (0.32-4.49)		5.06 (1.45-17.70)	
	Dominant	ins/ins	316 (82.9)	112 (55.5)	1.00	< 0.0001	212 (78.8)	1.00	0.1900	1.00	< 0.0001
		ins/del	65 (17.1)	90 (44.5)	3.87 (2.55-5.86)		57 (21.2)	1.31 (0.88-1.94)		2.84 (1.84-4.39)	
		del/del									
	Recessive	ins/ins	376 (98.7)	191 (94.5)	1.00	0.0130	265 (98.5)	1.00	0.8500	1.00	0.0260
		ins/del									
		del/del	5 (1.3)	11 (5.5)	4.00 (1.27-12.62)		4 (1.5)	1.13 (0.30-4.26)		3.77 (1.08-13.12)	
	Overdominant	ins/ins	321 (84.2)	123 (60.9)	1.00	< 0.0001	216 (80.3)	1.00	0.1900	1.00	< 0.0001
		del/del									
		ins/del	60 (15.8)	79 (39.1)	3.44 (2.24-5.27)		53 (19.7)	1.31 (0.87-1.97)		2.49 (1.59-3.88)	
TLR2 19216T/C (rs3804099)	Log-additive	---	---	---	3.23 (2.23-4.69)	< 0.0001	---	1.25 (0.88-1.79)	0.2200	2.54 (1.72-3.74)	< 0.0001
	Codominant	T/T	157 (41.2)	101 (50.0)	1.00	0.0920	131 (48.7)	1.00	0.1600	1.00	0.8900
		T/C	175 (45.9)	83 (41.1)	0.72 (0.49-1.06)		106 (39.4)	0.73 (0.52-1.01)		0.95 (0.63-1.44)	
		C/C	49 (12.9)	18 (8.9)	0.56 (0.30-1.05)		32 (11.9)	0.78 (0.47-1.29)		0.85 (0.43-1.69)	
	Dominant	T/T	157 (41.2)	101 (50.0)	1.00	0.0420	131 (48.7)	1.00	0.0580	1.00	0.7100
		T/T	224 (58.8)	101 (50.0)	0.68 (0.45-0.99)		138 (51.3)	0.74 (0.54-1.01)		0.93 (0.63-1.38)	
		T/C									
	Recessive	T/T	332 (87.1)	184 (91.1)	1.00	0.1600	237 (88.1)	1.00	0.7100	1.00	0.6800
		T/C									
		C/C	49 (12.9)	18 (8.9)	0.65 (0.36-1.19)		32 (11.9)	0.91 (0.57-1.47)		0.87 (0.45-1.68)	
	Overdominant	T/T	206 (54.1)	119 (58.9)	1.00	0.2500	163 (60.6)	1.00	0.0970	1.00	0.9000
		C/C									
		T/C	175 (45.9)	83 (41.1)	0.81 (0.56-1.17)		106 (39.4)	0.77 (0.56-1.05)		0.98 (0.65-1.46)	
Log-additive	---	---	---	---	0.74 (0.56-0.97)	0.0300	---	0.83 (0.66-1.05)	0.1200	0.93 (0.69-1.26)	0.6400

C: Control; CG: Chronic gastritis; GC: Gastric cancer.

= 2.84, 95%CI: 1.84-4.39;  $P < 0.0001$ ), overdominant (OR = 2.49, 95%CI: 1.59-3.88;  $P < 0.0001$ ), recessive (OR = 3.77, 95%CI: 1.08-13.12;  $P = 0.0260$ ), and log-additive models (OR = 2.54, 95%CI: 1.72-3.74;  $P < 0.0001$ ). However, no association was found with this polymorphism in the comparison of the CG and C groups (Table 3).

TLR2 19216T/C was associated with a protective effect against GC development compared to the C group by the dominant (OR = 0.68, 95%CI: 0.47-0.99;  $P = 0.0420$ ) and log-additive models (OR = 0.74, 95%CI: 0.56-0.97;  $P = 0.0300$ ). However, no association was found for the CG vs C and CG vs GC comparisons (Table 3).

Both polymorphisms were also investigated to evaluate their association with *H. pylori* infection. Thus, all the samples, including those in the case and control groups, were divided into *H. pylori*-negative cases ( $n = 619$ , 73%) and *H. pylori*-positive cases ( $n = 233$ , 27%). For the TLR2 -196 to -174 ins/del polymorphism, an association was observed with *H. pylori*-positive individuals in the codominant (OR = 1.55, 95%CI: 1.09-2.19 for TLR2 -196 to -174 ins/del; OR = 2.48, 95%CI: 1.01-6.08 for TLR2 -196 to -

174 del/del;  $P = 0.0120$ ), dominant (OR = 1.62, 95%CI: 1.16-2.27;  $P = 0.0051$ ), overdominant (OR = 1.50, 95%CI: 1.06-2.12;  $P = 0.0240$ ), and log-additive models (OR = 1.56, 95%CI: 1.17-2.08;  $P = 0.0030$ ). In contrast, the TLR2 19216T/C polymorphism was associated with protection against *H. pylori* infection in the codominant (OR = 0.60, 95%CI: 0.44-0.83 for TLR2 19216T/C; OR = 0.58, 95%CI: 0.35-0.98 for TLR2 19216C/C;  $P = 0.0039$ ), dominant (OR = 0.60, 95%CI: 0.44-0.81;  $P < 0.0001$ ); overdominant (OR = 0.67, 95%CI: 0.49-0.91;  $P = 0.0097$ ), and log-additive models (OR = 0.70, 95%CI: 0.55-0.88;  $P = 0.0021$ ) (Table 4).

### TLR2 mRNA expression in gastric lesions is influenced by *H. pylori* infection

The relative expression of TLR2 mRNA in the GC and CG groups is shown in Figure 1. We observed significantly increased TLR2 mRNA expression in the GC group (median RQ = 6.95) compared to CG group (median RQ = 0.84,  $P < 0.0001$ ) and C group (RQ = 1;  $P < 0.0001$ ). However, we did not find a statistically significant difference between the CG and C groups ( $P = 0.9387$ ).

Another analysis was performed to determine whether *H. pylori* infection influences TLR2 mRNA levels. Samples from the GC and CG groups were separated according to the presence or absence of *H. pylori* infection and compared. The results showed significantly higher TLR2 mRNA expression in *H. pylori*-positive cases (median RQ = 6.38) than in *H. pylori*-negative cases (median RQ = 0.79;  $P < 0.0001$ ; Figure 2). We observed significantly increased TLR2 mRNA expression in the *H. pylori*-positive cases group compared to C group (median RQ = 1,  $P < 0.0001$ ), but not between *H. pylori*-negative cases vs C group (RQ = 1;  $P = 0.4305$ ).

### Stratification of TLR2 polymorphisms and their influence on mRNA expression in gastric cancer and chronic gastritis

To evaluate the influence of the TLR2 -196 to -174 ins/del and TLR2 19216T/C polymorphisms on mRNA expression, the samples were grouped based on the presence of at least one polymorphic allele or a wild-type genotype (Table 5 and Figure 3).

In the GC group, individuals with at least one polymorphic TLR2 -196 to -174 del allele had greater than four-fold higher TLR2 mRNA expression in tumor tissue (median RQ = 8.74) than those with the wild-type genotype (median RQ = 2.58,  $P = 0.0010$ ). In contrast, carriers of TLR2-19216 TC + CC polymorphic variants showed reduced expression (median RQ = 3.63) compared to those harboring the wild-type TLR2 -19216 TT allele (median RQ = 18.54,  $P = 0.0004$ ). In the CG group, when the individuals were grouped as TLR2 -196 to -174 ins/del + del/del or TLR2-19216 TC + CC polymorphic allele carriers and homozygous wild-type allele carriers, the differences between the groups were not statistically significant ( $P = 0.5334$  and  $P = 0.8827$ , respectively).

## DISCUSSION

In our analyses, we demonstrated an association between the TLR2 -196 to -174 ins/del and TLR2 19216 T/C polymorphisms and gastric cancer (an increased risk and a protective effect, respectively), as well as greater susceptibility to *H. pylori* presence. Infection of the gastric mucosa by *H. pylori* leads to increased inflammation that contributes to cancer development, and factors that promote an exacerbated immune response may interfere with this process<sup>[26]</sup>.

Regarding the TLR2 -196 to -174 ins/del (rs111200466) polymorphism, two meta-analyses have shown no association with gastric cancer risk, but in the Chinese population, an increased risk for gastric carcinogenesis was reported in *H. pylori*-infected individuals, reinforcing the importance of this microorganism in disease pathogenesis<sup>[27,28]</sup>. In contrast, a recent study in a southern Chinese population showed a risk association for gastric cancer; however, no association with *H. pylori* infection was observed<sup>[8]</sup>.

Other studies have reported a risk association between the TLR2 -196 to -174 del variant and head and neck cancer<sup>[15,29]</sup>, cervical cancer in the Tunisian population<sup>[30]</sup>, breast cancer in the Greek population<sup>[13]</sup>, and prostate<sup>[31]</sup> and bladder cancer in the Indian population<sup>[32]</sup>. In the Brazilian population, previous studies demonstrated a risk association for gastric and colorectal cancer<sup>[14,33]</sup>.

For TLR2 19216T/C (rs3804099), our results demonstrated a protective association between the TC and CC genotypes and gastric cancer. This protective association has been previously reported for some types of cancer, such as colorectal, breast, gastric, and hepatocellular carcinoma, but all these studies were in the Asian population<sup>[16-18,34]</sup>. Two recent studies evaluated this polymorphism in the Thai population but failed to

**Table 4** Genotype frequencies of *TLR2* -196 to -174 ins/del and *TLR2* 19216T/C polymorphisms in *Helicobacter pylori*-positive and *Helicobacter pylori*-negative groups, n (%)

Polymorphisms	Models	Genotypes/ alleles	Case		OR (95%CI)	P value
			<i>H. pylori</i> -positive, n = 619	<i>H. pylori</i> -negative, n = 233		
<i>TLR2</i> -196 to -174 ins/del (rs111200466)	Codominant	ins/ins	481 (77.7)	159 (68.2)	1.00	0.0120
		ins/del	127 (20.5)	65 (27.9)	1.55 (1.09-2.19)	
		del/del	11 (1.8)	9 (3.9)	2.48 (1.01-6.08)	
	Dominant	ins/ins	481 (77.7)	159 (68.2)	1.00	0.0051
		ins/del	138 (22.3)	74 (31.8)	1.62 (1.16-2.27)	
		del/del				
	Recessive	ins/ins	608 (98.2)	224 (96.1)	1.00	0.0880
		ins/del				
		del/del	11 (1.8)	9 (3.9)	2.22 (0.91-5.43)	
	Overdo- minant	ins/ins	492 (79.5)	168 (72.1)	1.00	0.0240
		del/del				
		ins/del	127 (20.5)	65 (27.9)	1.50 (1.06-2.12)	
<i>TLR2</i> 19216T/C (rs3804099)	Log- additive	---	---	---	1.56 (1.17-2.08)	0.0030
	Codominant	T/T	261 (42.2)	128 (54.9)	1.00	0.0039
		T/C	281 (45.4)	83 (35.6)	0.60 (0.44-0.83)	
		C/C	77 (12.4)	22 (9.4)	0.58 (0.35-0.98)	
	Dominant	T/T	261 (42.2)	128 (54.9)	1.00	< 0.0001
		T/C - C/C	358 (57.8)	105 (45.1)	0.60 (0.44-0.81)	
	Recessive	T/T - T/C	542 (87.6)	211 (90.6)	1.00	0.2200
		C/C	77 (12.4)	22 (9.4)	0.73 (0.45-1.21)	
	Overdo- minant	T/T - C/C	338 (54.6)	150 (64.4)	1.00	0.0097
		T/C	281 (45.4)	83 (35.6)	0.67 (0.49-0.91)	
	Log- additive	---	---	---	0.70 (0.55-0.88)	0.0021

*H. pylori*: *Helicobacter pylori*.

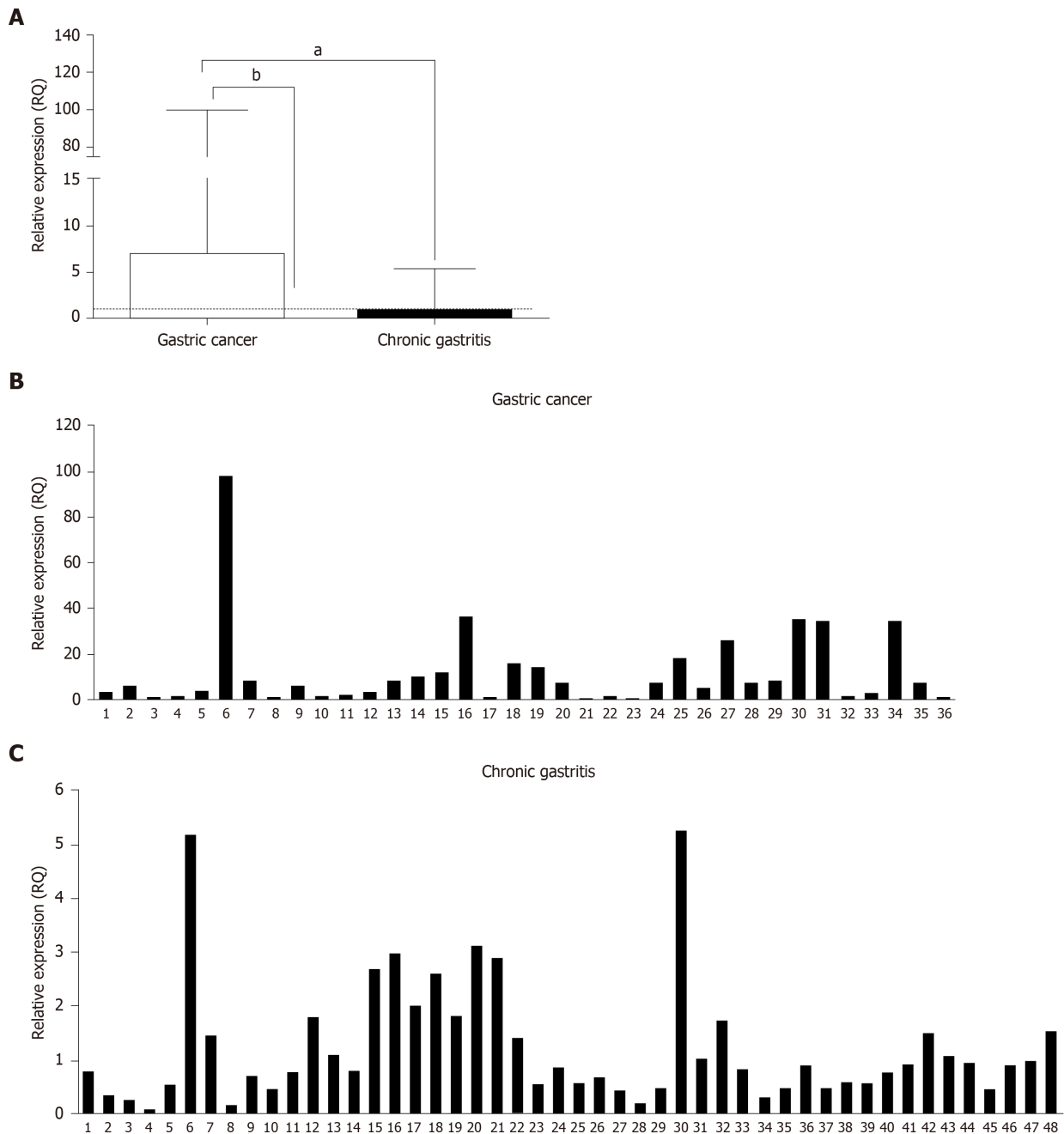
demonstrate any association with the development of gastric lesions or with *H. pylori* infection<sup>[35,36]</sup>. Thus, our study shows this protective association of *TLR2* 19216T/C with gastric cancer in the Brazilian population.

When we analyzed *TLR2* mRNA expression, we found significantly higher levels of *TLR2* mRNA in gastric cancer tissues than in chronic gastritis tissues. In the literature, a study in gastric cancer indicates that the *TLR2* mRNA expression levels were significantly increased in tumor tissues compared to either adjacent non-tumor tissues or normal tissues from GC-free individuals, regardless of risk factors or *H. pylori* infection<sup>[37]</sup>. Similarly, a previous study from our research group also evaluated *TLR2* receptor expression in different premalignant lesions (chronic gastritis, gastric atrophy, metaplasia) and observed a slight increase in *TLR2* gene and protein expression in relation to normal gastric tissues<sup>[38]</sup>.

Therefore, these studies showed that *TLR2* mRNA expression levels are mainly increased in tumor tissue, and not in the chronic gastritis group, as this is a benign lesion that likely does not yet present significant alterations in genes associated with carcinogenesis.

Similar results were observed in colorectal cancer in the Brazilian population, where the relative mRNA expression in tumor tissues was 2.36-fold higher than that in adjacent normal tissues, and a strong immunostaining pattern was observed in the epithelium of tumor tissues<sup>[14]</sup>. In 2018, Semlali *et al*<sup>[16]</sup> analyzed *TLR2* mRNA expression in the Saudi Arabian population and showed a decrease in *TLR2* mRNA in colon cancer tissues compared to normal colon tissues, and this result was confirmed by immunohistochemistry.

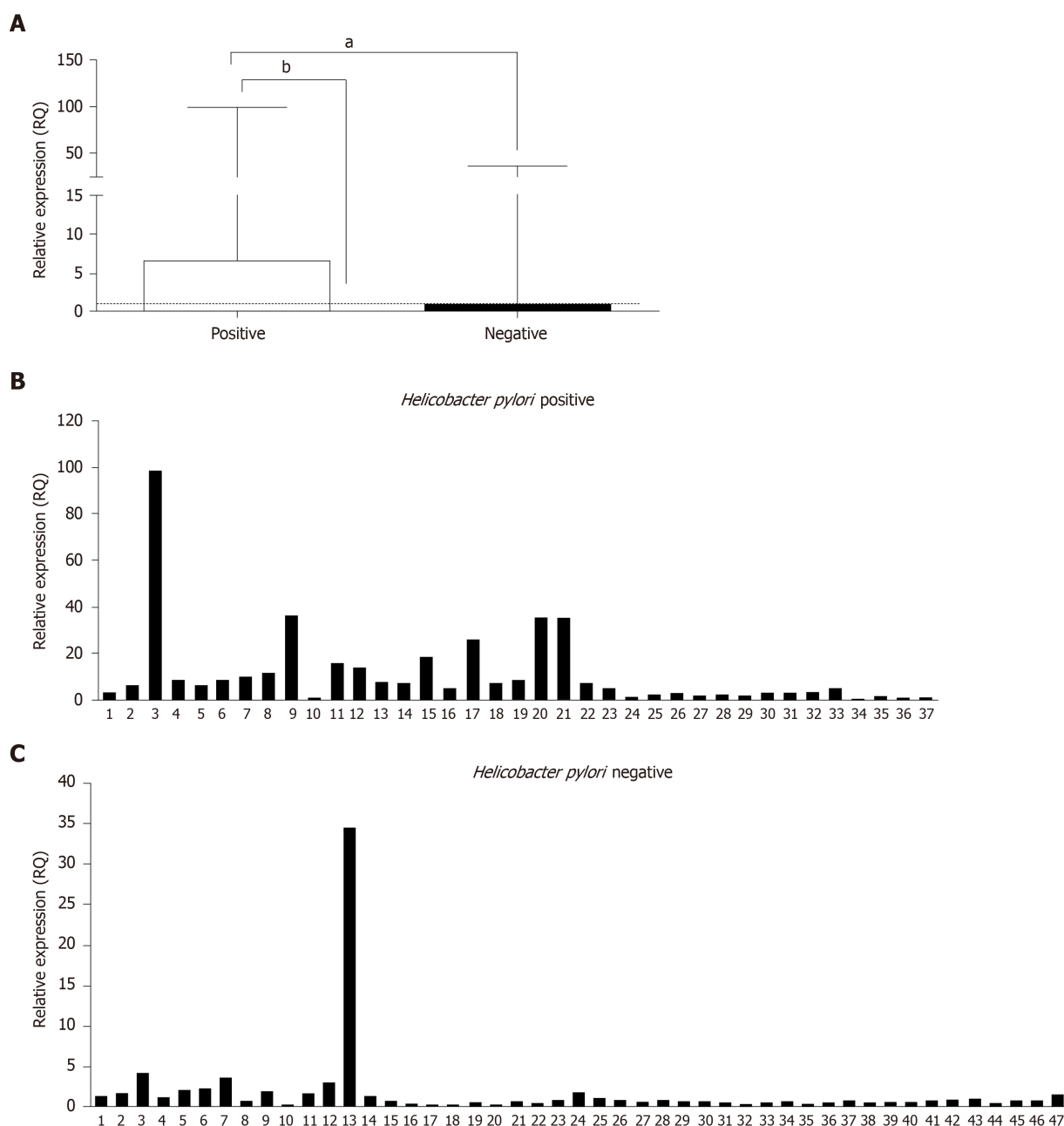
This is the first study in gastric lesions to demonstrate the regulation of gene expression in the presence of variant genotypes (*TLR2* -196 to -174 ins/del + del/del and *TLR2* 19216 T/C+C/C) and *H. pylori* infection, regardless of the type of lesion (cancer or



**Figure 1** Relative gene expression levels of *TLR2*. A: Comparison between gastric cancer (GC) and chronic gastritis groups (CG),  $^aP < 0.0001$ ; and GC or CG groups with samples of normal mucosa (RQ = 1 represented for dashed line),  $^bP < 0.0001$  and  $P = 0.9387$ , respectively. B: Individual representation of RQ for each sample of GC group; C: Individual representation of relative quantification (RQ) for each sample of CG group. Significant difference,  $^a, ^bP < 0.05$ . RQ represented by median with interquartile range. Mann-Whitney and Wilcoxon Signed statistical test.

gastritis). Thus, when we stratified gastric cancer tissue samples according to wild-type or variant genotypes, carriers of the *TLR2* -196 to -174 *ins/del* + *del/del* genotypes had higher *TLR2* mRNA expression levels than carriers of the *ins/ins* genotype. This finding supports the hypothesis that gene expression may be affected by allele variants in the promoter region and that *H. pylori* infection can influence the inflammatory profile mediated by TLRs. Proença *et al*<sup>[14]</sup> observed similar results in colorectal cancer, where *TLR2* mRNA expression was 2.19-fold higher in *TLR2* -196 to -174 *del* variant carriers than in wild-type genotype carriers<sup>[14]</sup>.

Conversely, the opposite was observed for *TLR2* 19216 T/C+C/C variant carriers (this polymorphism has a protective effect in gastric cancer), who had reduced *TLR2* expression levels with respect to TT wild-type genotype carriers. Thus, these data corroborate the results regarding the protective effect of the *TLR2* 19216 T/C+C/C genotype, which leads to the negative regulation of gene expression. An *in silico* analysis of the *TLR2* nucleotide substitution in rs3804099 predicted a 70% probability that this SNP affects *TLR2* mRNA splicing due to the creation of an additional splice



**Figure 2** Relative gene expression levels of *TLR2* according to *Helicobacter pylori* infection. A: Comparison between *Helicobacter pylori* (*H. pylori*)-positive and *H. pylori*-negative groups, <sup>a</sup> $P < 0.0001$ ; *H. pylori*-positive or *H. pylori*-negative groups with samples of normal mucosa (relative quantification (RQ) = 1 represented for dashed line), <sup>b</sup> $P < 0.0001$  and  $P = 0.4305$ , respectively. B: Individual representation of RQ for each sample in *H. pylori*-positive group; C: Individual representation of RQ for each sample in *H. pylori*-negative group. <sup>a, b</sup> $P < 0.05$ . RQ represented by median with interquartile range. Mann-Whitney and Wilcoxon Signed statistical test.

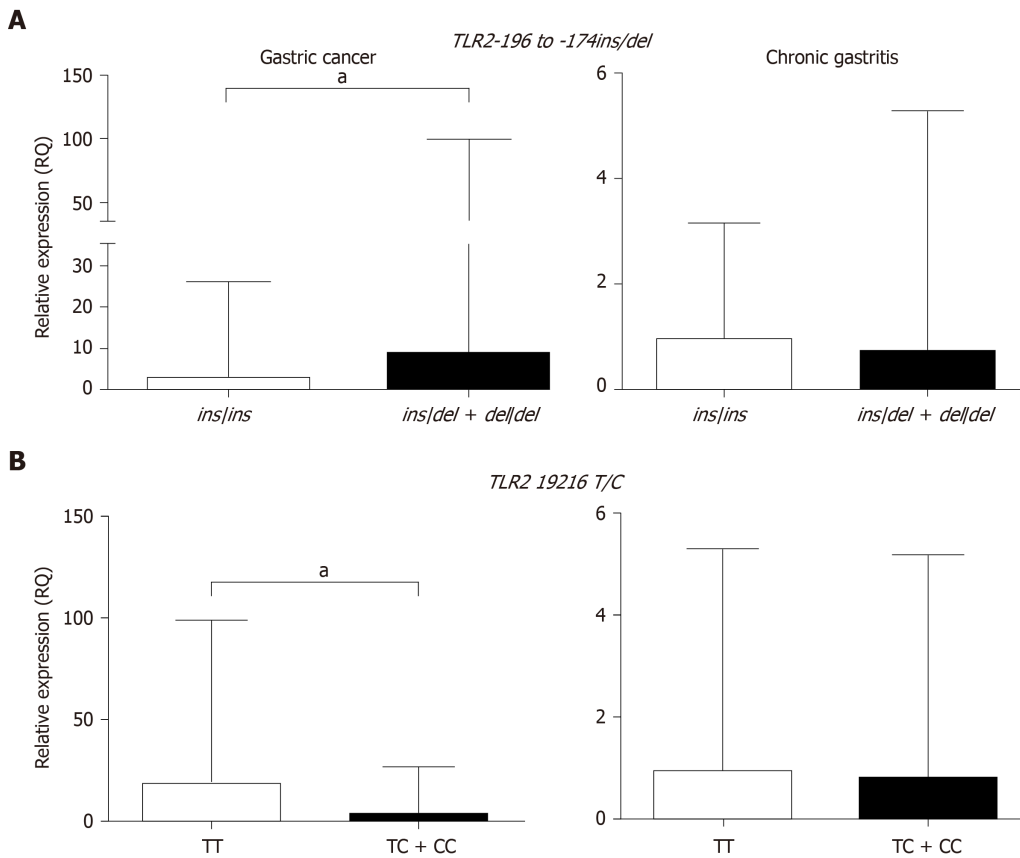
site. Thus, this variant may alter protein expression, receptor conformation, and function<sup>[16]</sup>.

Although the *TLR2* 19216T/C polymorphism results in a synonymous alteration (Asn199Asn), some genetic studies have shown a contribution of synonymous mutations to the risk of human diseases, including cancer<sup>[39]</sup>. A polymorphism in the epidermal growth factor receptor (*EGFR*) gene (rs2293347) has been identified as a potential predictor of the clinical outcome of treatment for advanced non-small-cell lung carcinoma<sup>[40]</sup>. Similarly, nonsynonymous SNPs in the Nijmegen breakage syndrome 1 (*NBS1*) gene (rs709816 and rs1061302) were associated with smoking-related cancers<sup>[41]</sup>, and the tumor suppressor p53 (*TP53*) gene (rs111287251) was associated with overall tumor susceptibility<sup>[42]</sup>.

The high expression of *TLR2* results in the recruitment of MyD88 to the TLR/TIR domain and in the production of inflammatory response cytokines by a classic signaling pathway, the *NF-κB* pathway, that exacerbates inflammation and thus facilitates tumor progression<sup>[5]</sup>.

In conclusion, our findings show that the *TLR2* -196 to -174 *ins/del* and *TLR2* 19216





**Figure 3** Relative gene expression levels of *TLR2* according to polymorphic genotypes. A: Comparison between *TLR2*-196 to -174 ins/ins (wild-type) and ins/del + del/del (polymorphic) in gastric cancer (GC),  $P = 0.0010$  and chronic gastritis (CG),  $P = 0.5334$  groups. B: *TLR2*-19216 TT (wild type) and TC + CC (polymorphic) in GC,  $P = 0.0004$  and CG,  $P = 0.8827$  groups. Significant difference,  $^aP < 0.05$ . Relative quantification (RQ) represented by median with interquartile range. Mann-Whitney  $U$  statistical test.

*T/C* polymorphisms are associated with gastric cancer (an increased risk and a protective effect, respectively) and *H. pylori* infection in the Brazilian population. Additionally, our results indicate that *TLR2* mRNA levels are upregulated in gastric cancer tissue, mainly in the presence of the *TLR2* -196 to -174 del variant allele, the *TLR2* 19216 T wild-type allele, and *H. pylori* infection. Thus, genetic polymorphisms may change gene expression, such as *TLR2* polymorphisms, alter the immune response profile, and consequently increase the risk and clinical manifestations of gastric cancer.

**Table 5** TLR2 gene expression level groups to assess influence of polymorphic genotypes in gastric cancer and chronic gastritis

Groups		TLR2-196 to -174ins/del		P value	TLR2-19216 T/C		P value
		ins/ins	ins/del + del/del		TT	TC/CC	
GC (n = 36)	Median	2.58	8.74	0.0010 <sup>1</sup>	18.54	3.63	0.0004 <sup>1</sup>
	Interquartile range	1.45-7.26	7.50-34.66		11.82-35.55	1.62-7.76	
CG (n = 48)	Median	0.92	0.79	0.5334	0.92	0.71	0.8827
	Interquartile range	0.56-1.09	0.48-1.92		0.53-1.53	0.49-1.44	

<sup>1</sup>Mann Whitney U Test. Data are presented as the relative quantification (RQ) median with interquartile range. Significant difference ( $P < 0.05$ ).

## ARTICLE HIGHLIGHTS

### Research background

*Helicobacter pylori* (*H. pylori*) infection is a carcinogen for gastric cancer (GC), and Toll-like receptors are involved in recognition and activation of the inflammatory response for this bacterium. The presence of single nucleotide polymorphism (SNP) genes responsible for activating the innate immunity may influence the risk of precancerous lesions and GC, among them of which include TLR2 polymorphisms. GC is the fifth most common cancer worldwide, mortality rates are still high. In Brazil, GC is the fourth most frequent type of cancer in men, and the sixth in women, with an estimated incidence of 21230 new cases in 2020.

### Research motivation

Considering the inconsistent results in the literature, as well as the importance of these receptors in immune response and for the susceptibility to inflammatory diseases and cancer, new studies are needed. The Brazilian population is highly mixed; thus it becomes important to confirm the real role among the factors that influence changes in the recognition of *H. pylori* and gastric carcinogenesis.

### Research objectives

The aim of this study was to evaluate whether the TLR2 19216T/C (rs3804099) and TLR2 -196 to -174 ins/del (rs111200466) polymorphisms contribute to gastric carcinogenesis in the Brazilian population. In addition, we also evaluate the influence of both polymorphisms and *H. pylori* infection on TLR2 mRNA expression. The results may highlight important polymorphisms that act on gastric carcinogenesis.

### Research methods

A case-control study was conducted to evaluate two TLR2 SNPs (TLR2 19216T/C -rs3804099 and TLR2 -196 to -174 ins/del - rs111200466) in CG and GC patients. A total of 854 DNA samples of peripheral blood [269 CG, 202 GC, and 383 samples from healthy individuals (C)] were genotyped by allele-specific PCR or restriction fragment length polymorphism (RFLP)-PCR. Quantitative polymerase chain reaction by TaqMan<sup>®</sup> assay was used to quantify TLR2 mRNA from fresh gastric tissues (48 GC, 26 CG, and 14 C).

### Research results

The data showed that for the TLR2 -196 to -174 polymorphism, the ins/del and del/del genotypes were associated with a higher risk of GC compared with the C and CG groups. In contrast, TLR2 19216T/C was associated with a protective effect in the GC group compared to the C group. Regarding the association of polymorphisms with *H. pylori* infection, for the TLR2 -196 to -174 ins/del polymorphism, an association was observed with *H. pylori*-positive, while TLR2 19216T/C was associated with protection against *H. pylori* infection. TLR2 mRNA levels were significantly higher in the GC group compared to the CG group and normal mucosa. In addition, when the samples were grouped according to polymorphic genotypes and the presence of *H. pylori*, the two SNPs (TLR2 -196 to -174del and TLR2 19216 C alleles) and *H. pylori* infection influenced TLR2 mRNA expression.

### Research conclusions

Our findings highlight that the polymorphisms of the TLR2 -196 to -174 ins/del and TLR2 19216 T/C receptors are associated with gastric cancer (an increased risk and a protective effect, respectively) and *H. pylori* infection, and therefore may act as a potential factor in the progression of gastric carcinogenesis. TLR2 mRNA expression levels are upregulated in gastric cancer tissues and are influenced by the TLR2 -196 to -174 del variant allele, the TLR2 19216 T wild-type allele and *H. pylori* infection. Considering that most cases of GC have a good prognosis and are treatable when diagnosed at an early stage, it is of the utmost importance to establish molecular markers capable of identifying risk groups and providing early diagnosis in individuals with increased risk of developing this neoplasm. Thus, polymorphisms in genes that affect its expression, such as TLR2, could have an effect on the development and clinical manifestation of disease.

## Research perspectives

The pattern of the host's immune response associated with genetic and environmental factors are essential for understanding the pathology of gastric cancer. Overall, our results indicate that the *TLR2* gene plays an important role in gastric carcinogenesis, highlighting the importance of the *TLR2* -196 to -174 del and *TLR2* 19216 T polymorphisms in increasing gene expression and *H. pylori* infection, possibly triggering a stronger inflammatory response, which in turn enhances the risk of tumor progression. In the future, it would be important to increase the biopsies collected during the endoscopic evaluation to quantify *TLR2* mRNA levels, and investigate another polymorphism in the *TLR2* gene (rs3804100, rs7696323, and rs10116253), described in the literature as associated with cancer, but not yet analyzed in our Brazilian population.

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