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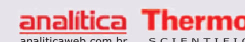

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Relationship of *IL-1* and *TNF-α* polymorphisms with *Helicobacter pylori* in gastric diseases in a Brazilian population

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Abstract

It is well known that the risk of development of gastric cancer (GC) in *Helicobacter pylori*-infected patients depends on several factors. Thus, the aim of this study was to investigate the effect of proinflammatory cytokine gene polymorphisms for *IL-1β*, *IL-1RN* and *TNF-α* on the development of GC in a Brazilian population. A total of 202 biopsies obtained from Brazilian patients with chronic gastritis and GC were included in the study. Infection with *H. pylori cag*A$^+$ was determined by the polymerase chain reaction (PCR) as previously described. *IL-1β*, *IL-1RN* and *TNF-α* polymorphism genotyping was performed by restriction fragment length polymorphism PCR. Associations between gene polymorphisms, clinical diseases and virulence markers were evaluated using either the χ^2 test or the Fisher exact test. Our results demonstrated that the *IL-1β* -511 C/C and *IL-1β* -511 C/T alleles were associated with chronic gastritis in *H. pylori*-positive patients (P = 0.04 and P = 0.05, respectively) and the *IL-1β* -511 C/C genotype was associated with GC (P = 0.03). The frequency of *IL-1RN* alleles from patients with chronic gastritis and GC indicated that there was no difference between the genotypes of the groups studied. Similar results were found for *TNF-α* -308 gene polymorphisms. Our results indicate that the *IL-1β* -511 C/C and C/T gene polymorphisms are associated with chronic gastritis and GC development in *H. pylori*-infected individuals.

Key words: Chronic gastritis; Gastric cancer; Polymorphisms; *Helicobacter pylori*; Interleukins; *IL-1β*; *IL-1RN*; *TNF-α*

Introduction

Infection with *Helicobacter pylori* is a major risk factor for gastric cancer (GC), which is the second leading cause of cancer death worldwide (WHO). This Gram-negative bacterium is a well-established etiologic factor and has been classified as a type 1 carcinogen because of its causative role in the development of GC (1). Gastric carcinogenesis is a complex process resulting from the interaction between genetic and environmental factors (2). It is known that diet, smoking, alcohol consumption, and low intake of fruits and vegetables can be implicated in disease development (3). The presence of *H. pylori* is responsible for triggering a pathological progression in the gastric mucosa that begins with chronic gastritis and progresses to atrophic gastritis, intestinal metaplasia, dysplasia, and eventually GC (4).

Some bacterial factors are correlated with the severity of disease. However, bacterial factors alone are not sufficient to explain the diverse outcomes of *H. pylori*-related diseases (5-7). The cytotoxin-associated gene (*cag*A) was the first strain-specific gene identified in *H. pylori* that was considered to be a marker for the presence of a pathogenicity island (cag-PAI) (8). Infection with cag-PAI-bearing *H. pylori* has been recognized as increasing risk for the development of several gastric disorders, such as peptic ulceration, GC and mucosa-associated lymphoid tissue lymphoma (9).

The immune response of the host is considered to be a key event in the development of GC. This reaction is induced by the contact of *H. pylori* with gastric cells and is followed by the stimulation of proinflammatory cytokine production (10). Additionally, several studies have reported a relationship between *H. pylori* infection and an increase in the inflammatory gene response characterized by the up-regulation of several genes such as *IL-1β*, *IL-1RN*, *IL-8*, *IL-10*, and tumor necrosis factor-alpha (*TNF-α*). These cytokines are considered to be important mediators of gastric pathophysiology and could play

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a critical role in the etiology of GC (1,11).

IL-1β is a proinflammatory cytokine induced by *H. pylori* infection and is a powerful inhibitor of gastric acid secretion. Its effects promote hypochlorhydria, favoring further colonization of *H. pylori* and more severe gastritis (12). It has been shown that *IL-1β* -31 and *IL-1β* -511 polymorphisms are associated with increased production of *IL-1β* and are therefore related to the development of GC (13-15). Thus, persons harboring *IL-1β* polymorphisms who are also colonized by an *H. pylori* toxigenic strain have an increased risk of developing GC (15).

Another cytokine that has an important influence on IL-1β levels is IL-1ra, the gene (*IL-1RN*) of which is also known to be polymorphic. The *IL-1RN* gene has a penta-allelic 86-bp tandem repeat polymorphism in intron 2. The rare variant *IL-1RN**2 has been associated with GC (13). Furthermore, it is known that gastrin is up-regulated by *H. pylori* infection. However, acid secretion from parietal cells is inhibited mainly due to the proinflammatory cytokines IL-1β and TNF-α (16).

TNF-α has an important role in host defense against *H. pylori* infection, but a high concentration of this cytokine may cause severe pathology. TNF-α production is regulated in part at the transcriptional level, and many studies have implicated *TNF-α* polymorphisms as potential determinants of disease susceptibility (17).

The risk of development of GC in *H. pylori*-infected patients depends on several factors. Genotyping of bacterium and host gene polymorphisms could be important in the early identification of individuals at high risk to develop severe gastric diseases. Therefore, the aim of the present study was to evaluate the effect of proinflammatory cytokine gene polymorphisms in *IL-1β*, *IL-1RN*, and *TNF-α* in the Brazilian population.

Patients and Methods

Patients

The study was approved by the Ethics Committees of Faculdade de Medicina, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil, and of Universidade São Francisco, Braganca Paulista, SP, Brazil, as well as by the National Committee of Ethics in Research, Brasília, DF, Brazil. Informed consent to participate was obtained from all patients or their guardians.

We studied 202 volunteer patients of both genders with gastric disease. Mean patient age was 59.2 ± 11.36 years (range: 19-87 years). Exclusion criteria were as follows: previous gastroduodenal surgery, prior treatment with antimicrobial therapy to eradicate the microorganism, use of NSAIDs and/or proton pump inhibitors in the last three months, and being a smoker, alcoholic, drug user, or user of therapeutic drugs in the last 30 days.

Biopsy collections

Biopsies of patients with gastric complaints were obtained during endoscopy from the lesser curvature of the antrum (the distal region of the stomach) within 2 cm of the pyloric ring. Biopsies from GC were obtained during gastric surgery to remove gastric carcinoma. One biopsy from each site was used for a rapid urease test. Two biopsies were used for histopathological evaluation. One sample was used for bacterial genotyping by the polymerase chain reaction (PCR). *H. pylori* infection was confirmed when positive results were obtained in at least two of the following tests: rapid urease test, histological analysis and gastric biopsy PCR.

Histopathology

Tissue from the gastric mucosa was fixed in 10% formalin for 24 h, dehydrated in alcohol and xylene and embedded in paraffin. Sequential 3-5-µm sections were cut and stained with hematoxylin-eosin for routine histology. Gastritis was classified according to Sydney's system (18), and the presence of *H. pylori* was confirmed in carbolfuchsin-stained sections.

DNA extraction and *H. pylori* genotyping

To determine the presence of *H. pylori*, we used the rapid urease test (Probac, Brazil), histology, and PCR. The bacterium was considered to be present if two or more methods were positive.

A biopsy of the antrum of each patient was used for extraction of genomic DNA. DNA isolation was performed according to the phenol-chloroform protocol adapted by Fox et al. (19).

All primers (Invitrogen™ Life Technologies, USA) used in this study are presented in Table 1. PCR assays are

Table 1. Specific primers used for polymorphism analyses.

Gene	Primer	Sequence (5'→3')	PCR (bp)	References
IL-1β -511	F	TCCTCAGAGGCTCCTGCAAT	304	14
	R	TGTGGGTCTCTACCTTGGGTG		
TNF-α -308	F	CCCCAAAAGAAATGGAGGC	107	14
	R	TCTTCTGGGCCACTGACTGAT		
IL-1RN	F	CCCCTCAGCAACACTCC	240-595	13
	R	GGTCAGAAGGGCAGAGA		
SSA	F	TGGCGTGTCTATTGACAGCGAGC	300	20
	R	CCTGCTGGGCATACTTCACCATG		
*glm*M	F	AAGCTTTTAGGGGTGTTAGGGGTTT	294	21
	R	AAGCTTACTTTCTAACACTAACGC		
*cag*A	D008	TTAGAATAATCAACAAACATCACGCCAT	297	22
	R008	TTAGAATAATCAACAAACATCACGCCAT		

F = forward; R = reverse.

performed in a total volume of 50 µL, containing 50 pmol primer, 100 ng genomic DNA, 1.0 mM of each dNTP (Invitrogen™ Life Technologies) and 2.5 U Taq DNA polymerase (Invitrogen™ Life Technologies). The reaction mixtures were cycled in an automated GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems, USA). PCR was performed using the following conditions: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing ranging from 45°C to 60°C for 1 min and extension at 72°C for 1 min. The final cycle included a 7-min extension step to ensure full extension of the PCR products. The presence of *H. pylori* was confirmed by PCR of the *SSA* (20) and *glm*M (21) genes. The *cag*A gene was analyzed using the primers D008 and R008 described by Covacci et al. (22).

Analysis of polymorphisms

The analysis of the polymorphisms *IL-1β* (-511 C/T - rs16944), *TNF-α* (-308 G/A - rs1800629) and intron 2 VNTR of the *IL-1RN* were analyzed by PCR in a 50-µL total reaction volume. The reaction mixture contained the following reagents: 2.5 µL genomic DNA, 20 µmol of each primer, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1.5% $MgCl_2$, 2.5 U Platinum® Taq DNA polymerase and enzyme reaction buffer.

Genotyping of the *IL-1β* -511 T>C SNP was performed by restriction fragment length polymorphism PCR (RFLP-PCR) as previously described. PCR products were digested with AvaI (New England Biolabs, USA) at 37°C for 2 h and then analyzed on 2% agarose gels. Individuals with the genotype *IL-1β* -511 C/C produced digested DNA bands of 190/104 bp. Individuals with the *IL-1β* -511 T/T genotype had no amplicon digestion and generated only one band of 304 bp. A heterozygous genotype of *IL-1β* -511 C/T produced 304/190/104-bp bands.

The *TNF-α* polymorphism (-308 G/A) creates a restriction site for *Nco*I (C ↓ CATGG). The amplicon of 107 bp was digested with the restriction enzyme *Nco*I (New England Biolabs) at 37°C for 2 h and analyzed on 2% agarose 1000 (Invitrogen) gels. The *TNF-α* -308 G/G genotype produced 87/20-bp bands after amplicon digestion. Individuals with the *TNF-α* -308 A/A genotype did not have amplicon digestion and generated only one band of 107 bp. The heterozygous *TNF*-α -308 G/A genotype produced three bands 107/87/20 bp in length.

The products generated by PCR of *IL-1RN* were subjected to electrophoresis on 2% agarose gels and classified as follows: allele 1-4 repeats (410 bp); allele 2-2 repeats (240 bp); allele 3-5 repeats (500 bp); allele 4-3 repeats (235 bp), and allele 5-6 repeats (595 bp) (13). All analyses were made in duplicate.

Statistical analysis

Associations between gene polymorphisms, clinical diseases and virulence markers were evaluated using the χ^2 test with Yates continuity correction. The statistical significance was assessed by ANOVA multiple comparisons using a *t*-test with post-test correction. Hardy-Weinberg equilibrium was tested among the groups. A P value of <0.05 was considered to be statistically significant. The SPSS software (version 11.5; SPSS Inc., USA) was used for data analysis.

Results

Prevalence of *H. pylori* infection and genotyping

There were no differences in gender or age distribution between the study groups. *H. pylori* infection was analyzed in biopsy specimens from 202 patients who underwent endoscopy and was detected in 87% (176/202). Histopathological analysis showed that 13% (26/202) had normal mucosa and were uninfected, 55% (112/202) had chronic gastritis, and 32% (64/202) had GC. All patients with GC were infected

Table 2. Frequency of the *IL-1β*, *IL-1RN* and *TNF-α* polymorphisms.

Genotypes	Chronic gastritis (N = 138)		Cancer (N = 64)
	H. pylori ⁻ (N = 26)	*H. pylori* ⁺ (N = 112)	*H. pylori* ⁺ (N = 64)
IL-1β -511			
C/C	-	28 (25%)*	13 (20%)*
C/T	20 (77%)	52 (46%)*	35 (55%)
T/T	6 (23%)	32 (29%)	16 (25%)
*T	0.61	0.51	0.52
‡P values	-	<0.001	0.03
IL-1RN			
1/1	7 (27%)	45 (40%)	20 (31%)
1/2	17 (65%)	57 (51%)	42 (65%)
1/3,4	-	2 (2%)	-
2/2	2 (8%)	6 (5%)	2 (4%)
*2	0.40	0.31	0.36
‡P values	-	NS	NS
TNF-α -308			
G/G	22 (85%)	78 (70%)	44 (68%)
G/A	4 (15%)	30 (27%)	20 (32%)
A/A	-	3 (3%)	-
*A	0.07	0.16	0.16
‡P values	-	NS	NS

NS = not significant. *P < 0.05 for *H. pylori* ⁻ versus *H. pylori* ⁺ chronic gastritis and *H. pylori* ⁺ gastric cancer (χ^2 test). ‡P values for ANOVA multiple comparisons using a *t*-test with post-test correction.

with *H. pylori*. Of the 112 *H. pylori*-positive patients, 84 (75%) were *cag*A-positive. There was a high prevalence of infection with *cag*A-positive strains among GC patients.

IL-1β, *IL-1RN* and *TNF-α* polymorphisms

The genotype distribution in each group was tested using Hardy-Weinberg equilibrium (HWE). Regarding the *IL-1β* -511 C/T polymorphism, P values of 0.56 and 0.44 were found for chronic gastritis and cancer, respectively. For *TNF-α* -308, P values of 0.95 and 0.13 were found for chronic gastritis and cancer, respectively. *IL-1RN* was not in HWE and was not evaluated.

The genotype frequencies of the polymorphisms studied are shown in Table 2. The frequency of the *IL-1β* -511 C/T polymorphism in patients with chronic gastritis differed significantly between the *H. pylori*-positive group (52/112) and the uninfected group (20/26) (P = 0.05). Similarly, a significant difference was found in the frequency of the *IL-1β* -511 C/C genotype in chronic gastritis and GC patients (P = 0.04 and P = 0.03, respectively).

With respect to the frequency of the *IL-1RN* alleles from patients with chronic gastritis and GC, our data indicated that there was no difference in the genotypes from the groups studied. Similar results were found for the *TNF-α* -308 gene polymorphisms.

Table 3. Frequency of the polymorphisms of *IL-1β*, *IL-1RN* and *TNF-α* related to the presence or absence of virulence factor *cag*A.

Genotypes	Chronic gastritis (N = 112)		Cancer (N = 64)
	*cag*A$^-$ (N = 28)	*cag*A$^+$(N = 84)	*cag*A$^+$ (N = 64)
IL-1β -511			
C/C	2 (7%)	22 (27%)	13 (20%)
C/T	20 (71%)	39 (46%)	35 (55%)
T/T	6 (22%)	23 (27%)	16 (25%)
*T	0.57	0.50	0.52
‡P values	-	NS	NS
IL-1RN			
1/1	7 (25%)	36 (43%)	20 (31%)
1/2	18 (65%)	43 (51%)	42 (65%)
1/3,4	1 (3%)	1 (1%)	-
2/2	2 (7%)	4 (5%)	2 (4%)
*2	0.39	0.30	0.36
‡P values	-	NS	NS
TNF-α -308			
G/G	25 (90%)	60 (72%)	44 (68%)
G/A	3 (10%)	20 (24%)	20 (32%)
A/A	-	3 (4%)	-
*A	0.05	0.15	0.16
‡P values	-	NS	NS

‡P values for ANOVA multiple comparisons using a *t*-test with post-test correction. NS = not significant.

Correlation between polymorphisms and *H. pylori cag*A

Table 3 shows the frequency of each polymorphism and the distribution of *cag*A$^+$ and *cag*A$^-$ samples from patients with chronic gastritis and GC. Regarding the *IL-1β* -511 polymorphism, we found an association between heterozygosity and *cag*A$^-$ in chronic gastritis patients (P = 0.02). An individual analysis of the genotype frequencies of *IL-1RN**2 and *TNF-α* -308 and the presence of *cag*A revealed a similar distribution among all groups.

Discussion

Distinct host cytokine responses to *H. pylori*-induced gastric mucosal inflammation appear to play a significant role in clinical outcome including the development of gastric diseases and GC. However, the relationship between gene host cytokine polymorphisms and the nature and severity of the clinical outcome has not been well characterized. Furthermore, the ethnic diversity of the Brazilian population is known to be an important factor that has been associated with variations in the frequency of several markers. In view of the need to characterize the allelic frequencies of gene host cytokine polymorphisms in mixed populations, the present study evaluated the effect of proinflammatory cytokine gene polymorphisms in *IL-1β*, *IL-1RN*, and *TNF-α* in patients with chronic gastritis and GC.

Infection with this chronic and active bacterium will inevitably cause an inflammatory response in the host. Histologically, the host response to this bacterium is characterized by infiltration of lymphocytes, macrophages and neutrophils in the gastric mucosa. This reaction is most likely induced by the contact of *H. pylori* with the gastric cells and is followed by the stimulation of proinflammatory cytokine production (10).

In addition to their role in *H. pylori* infections, gene polymorphisms play an important role in GC development (16,23). Currently, there are approximately 1400 publications in PubMed covering more than 100 different genes with polymorphisms linked to GC. The most comprehensive knowledge exists for cytokine-encoding genes, which account for roughly one quarter of these publications. Within this subset of publications, the *IL-1β* gene locus is the focus of more than 200 studies and represents a major target for research on gastric carcinogenesis (24).

IL-1β is an important factor for initiating and amplifying the inflammatory response. It acts as a potent inhibitor of acid secretion and its expression levels are increased in *H. pylori*-infected patients (25,26). Some investigators have

shown that *IL-1β* -511 polymorphisms are associated with increased *IL-1β* production and are therefore related to the development of GC (15).

In the present study, we found an association between the *IL-1β* -511 CT/CC genotypes and the presence of chronic gastritis and GC in patients infected with *H. pylori*. These results are similar to those of previous studies conducted on Mexican patients (27). It has also been reported that the *IL-1β* -511 CC/CT genotypes potentiate cytokine production and are significantly associated with the clinical development of *H. pylori* infection (27,28). Additionally, a greater risk of chronic gastritis and GC development has also been reported in the Japanese population harboring the *IL-1β* -511 CC genotype and in the Chinese population with the CT genotype. Furthermore, within the Thai population, the *IL-1β* -511 CC genotype is considered to be a risk factor for GC development (29). Conversely, some studies have yielded conflicting results. A recent meta-analysis showed a positive association between the T allele at the *IL-1β* -511 position and GC development in Caucasians but not in Asians (12). Similar results indicate that there is no association in Italian (30), Jamaican (31), or Brazilian populations (32,33).

The *IL-1RN* polymorphisms are closely associated with the regulation of IL-1β activity and are related to GC development (12,13). It has been proposed that individuals with the *IL-1RN**2 genotype have elevated levels of circulating IL-1β. The increased IL-1β levels result in a prolonged inflammatory response and increase the risk of disease development. However, our data indicate that there was no association between IL-1RN genotypes and the subgroups studied. Similar results were reported in a meta-analysis by Camargo et al. (12). Although the results presented here and those observed by other investigators (33) did not indicate an association between the *IL-1RN**2 allele and the development of GC, other studies conducted in Brazil have shown that this allele is associated with the development of GC in different Brazilian populations (32,34). Such results could be attributed to the miscegenation of the Brazilian population because some studies indicate that the frequencies of these markers usually show large ethnic variations (35). Additionally, in a meta-analysis, Kamangar et al. (36) reported no association between the genotypes of *IL-1β* -511 or *IL-1RN* and GC development.

In addition to the previously described polymorphisms, several investigators have reported that changes in the promoter region of *TNF-α* are related to an increased risk of GC development (37). Regarding the *TNF-α* -308 G/A polymorphisms, our data indicate that there was no difference between subgroups. The data presented here are similar to those reported by Melo Barbosa et al. (32) in a Brazilian population and by Gorouhi et al. (37) in a meta-analysis and are equivalent to the frequencies detected in Caucasians, Asians and Hispanics. Additionally, these authors did not detect any association between *TNF-α* -308 GG or GA genotypes and the development of GC. Moreover, these authors found that genotype A/A was associated with a moderately increased risk of developing GC. The data presented here revealed no association between the genotypes of *TNF-α* -308 and the development of GC. This discrepancy could easily be attributed to the heterogeneity of the Brazilian population.

Infection by *cag*A-positive *H. pylori* strains has been associated with several clinical outcomes. Epidemiological studies have associated the presence of the *cag*A gene with the development of several gastric diseases and GC (38) and with cellular proliferation (39). Our study showed that patients with chronic gastritis infected by *cag*A-positive *H. pylori* had the highest cytokine mRNA and protein levels (40). However, our data did not reveal an association between the presence of any polymorphism and infection with *cag*A-positive strains. Other studies have reported an association between *H. pylori cag*A-positive strains and the polymorphisms in patients with gastritis and GC in Brazil (32) and in Portugal (15). Our data indicate that there was no preferential colonization of specific hosts by specific bacterial strains. The association of polymorphisms with certain bacterial strain types indicated that host-specific colonization or adaptation occurs, which may explain the heterogeneity of *H. pylori* strains in different populations. The Brazilian population is composed of a genetic mix of various ethnic groups, including Portuguese, Africans and Amerindians. Thus, the heterogeneity between the allele frequencies of the polymorphisms studied in our population compared with those of other ethnic groups is a product of the miscegenation of the Brazilian population.

Our results indicated that in the Brazilian population the *IL-1β* -511 CC and CT gene polymorphisms were associated with chronic gastritis and GC development in *H. pylori*-infected individuals. No correlation between *IL-1RN* and *TNF-α* gene polymorphisms was observed in any gastric disease. More extensive studies investigating a broader range of polymorphisms, as well as other pro- and anti-inflammatory genes, are required to conclusively assess the impact of gene polymorphisms on gastric mucosal cytokine production.

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