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ORIGINAL ARTICLE

Association of tumor necrosis factor alpha gene polymorphisms with *Helicobacter pylori* infection in dyspeptic patients in Sri Lanka

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ABSTRACT

Single nucleotide polymorphisms present on the promoter sequence of the *TNF-α* gene may affect production of TNF-α, a pro-inflammatory cytokine, during immune responses. The presence of *TNF-α* polymorphisms is also reportedly associated with more severe manifestations of *Helicobacter pylori* infection. However, the frequency of *TNF-α* polymorphisms and the associated disease severity vary between different patient groups. In this study, gastric biopsies and blood specimens were collected from 138 patients with dyspepsia undergoing routine upper gastrointestinal endoscopy. Our institution's Ethics Review Committee approved the study and written informed consent was obtained from all participants. The presence of *H. pylori* was confirmed histologically in all patients. The frequency of *TNF-α* polymorphisms in the study cohort was investigated using PCR–restriction fragment length polymorphism and expression of serum TNF-α quantitated using a commercial ELISA assay. The proportions of selected *TNF-α* polymorphisms (*TNF-α -238*, *-308* and *-863*) were similar in *H. pylori*-positive and -negative patients. Homozygous mutations of *TNF-α* polymorphisms were rarely detected in the study group. There was a significant difference in TNF-α concentrations between patients with mild chronic gastritis and *TNF-α -308 GG* genotype and patients with moderate to severe chronic gastritis ($P = 0.008$). It was not possible to identify an association between these genotypes and disease severity because of the low frequency of heterozygous and homozygous mutated genes in Sri Lankan patients with dyspepsia.

Key words *Helicobacter pylori*, Sri Lanka, *TNF-α* polymorphisms.

Tumor necrosis factor-alpha, a pro-inflammatory cytokine, has been implicated in the pathogenesis of *Helicobacter pylori*-associated gastroduodenal disease. *H. pylori* virulence factors promote TNF-α secretion (1), contributing to gastritis (2). Further studies have reported an association between TNF-α secretion and increased cellular apoptosis (3), independent of the vacuolating cytotoxin (4) or cagA status (5) of *H. pylori*. The implications of these findings are that high concentrations of TNF-α may exacerbate pathology. Regulation of TNF-α production occurs at the gene transcriptional level; thus, *TNF-α* polymorphisms are potential determinants of disease susceptibility (6).

The *TNF-α* gene is located on chromosome 6 within the MHC class III region (7, 8). Polymorphisms of the promoter region of the *TNF-α* gene have shown to influence production of TNF-α (9, 10). Several polymorphisms of *TNF-α* have been studied, including *TNF-α −238*,*-308* and *-863*. *TNF-α -238* and *-308* polymorphisms are caused by point mutations in which there is a G to A

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List of Abbreviations: RFLP, restriction fragment length polymorphism.

substitution, whereas *-863* polymorphisms involve C to A substitution at position 863 (11).

The association between *TNF-α -308* and *TNF-α -238* polymorphisms and gastric pathology is still controversial (12). A meta-analysis identified *TNF-α* polymorphisms as a risk factor for gastric cancer in Caucasian, but not in East Asian and other Asian, individuals (12–14). Similarly Gorouhi *et al.*, reported that the association between *TNF-α -308* (G/A) and gastric cancer was limited to western populations (15). Several studies from China (16) and Korea (17) have not found an association between the *TNF-α* genotype and gastric pathology, whereas other studies have reported an association (18, 19).

The role of *TNF-α* polymorphisms in severity of *H. pylori*-associated disease has not been studied in Sri Lanka. Previous histological studies done in Sri Lanka have suggested that most *H. pylori*-infected patients have mild chronic gastritis (20, 21) and that development of gastric atrophy is not a significant problem. Multiple factors can contribute to a shift from mild to atrophic gastritis; however, host genetic factors can play a major role. In this study we investigated polymorphisms of the *TNF-α* gene in Sri Lankan individuals and their correlation with *H. pylori*-associated disease severity. We hypothesized that *TNF-α* may be a useful genotypic marker for identifying groups at higher risk of gastric cancer and peptic ulcer and therefore those for whom preventive *H. pylori* eradication therapy should be considered.

MATERIALS AND METHODS

Study cohort

The study cohort consisted of patients undergoing routine endoscopy at a tertiary care hospital in Sri Lanka for dyspeptic symptoms. Other inclusion criteria were age over 18 years and no antibiotics for a month before endoscopy. Patients aged less than 18 years, currently on antibiotics or with a history of antibiotic intake in the previous 4 weeks, with mental instability or with malignant diseases (e.g., gastric cancer) were excluded. Written informed consent was obtained before enrolling patients in the study. An interviewer-based questionnaire was used to collect patient data.

Ethical approval

Approval for the study was obtained from the Ethics Review Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura (Application no: 14/15) and Colombo South Teaching Hospital, Kalubowila (Application no: 450).

Specimen collection

Four antral gastric biopsy specimens were collected from each patient during endoscopy. One specimen was placed in an in-house rapid urease test solution, two in 10% formalin saline solution, and the remaining one in a sterile Eppendorf tube. The specimens were transported to the Departments of Pathology and Microbiology for processing. A blood specimen (5 mL) was collected from each patient and centrifuged. The serum was aliquoted into cryovials and stored at −80°C.

Histopathological investigations

The biopsy specimens (transported in 10% formalin saline) were dehydrated using an alcohol gradient, placed in xylene and embedded in paraffin wax. Sections were cut using a microtome (4 μm thick) and placed on clean glass slides. The slides were stained with hematoxylin and eosin and Giemsa stains separately. Specimens were graded according to the updated Sydney system (22)by an experienced consultant pathologist.

In-house biopsy urease test

The specimen placed in the in-house rapid urease test solution was placed in an incubator (37°C) for 24 hr. The solution was observed at 1, 2, 12 and 24 hr intervals for color change from yellow-orange to pink-red.

DNA extraction, PCR and RFLP

In accordance with the manufacturer's instructions, DNA was extracted from the remaining biopsy specimen using a commercial DNA extraction kit (QIAamp DNA mini kit; Qiagen, Hilden, Germany). PCR was performed on DNA extracted from each specimen using the specific primers listed in Table 1 to amplify the promoter region of the *TNF-α* gene (11).

All PCR reactions were carried out using Flexigene thermal cycler (version 31.04; Techne, Cole-Parmer, Staffordshire, UK). All PCR reactions were performed in a 25 μL reaction mixture consisting of 1× buffer (Sigma–Aldrich, St Louis, MO, USA) with 0.2 mM each of dATP, dCTP, dGTP and dTTP (Promega, Madison, WI, USA), 0.2 μM of forward and reverse primer, 1.25 U of Taq polymerase (Sigma–Aldrich) and 2 μL (∼100 ng) of nucleic acid (Table 1).

TNF-α polymorphisms were determined by digesting the three PCR products with specific restriction enzymes (11). RFLP reactions were optimized by digesting lambda DNA with each of the restriction enzymes. Optimized RFLP conditions were applied to

Table 1. Specific primers and PCR conditions used in the amplification of TNF-α promoter region

Target gene	Primer	Primer sequence 5′- to -3′	Product size (bp)	PCR reaction conditions
TNF-α -238	Forward Primer	AGA AGA CCC CCC TCG GAA CC	151	94°C for 5 min, 58°C for 1 min, 72°C for 45 s 40 cycles of 94°C for 1 min, 58°C for1 min, 72°C for 45 s 72°C for 10 min
	Reverse Primer	ATC TGG AGG AAG CGG TAG TG		
TNF-α -308	Forward Primer	GCA ATA GGT TTT GAG GGC CAT G	144	93°C for 3 min 35 cycles of 92°C for 1 min, 63°C for 1 min, 72°C for 1 min 72°C for 10 min.
	Reverse Primer	GGG ACA CAC AAG CAT CAA GGA T		
TNF-α -863	Forward Primer	GGC TCT GAG GAA TGG GTT AC	125	94°C for 3 min 35 cycles of 94°C for 30 s, 59°C for 1 min, 72°C for 2 min 72°C for 10 min
	Reverse Primer	CTA CAT GGC CCT GTC TTC GTT ACG		

DNA extracted from patient specimens: the RFLP reactions were carried out in 15 μL reaction volumes consisting of 1× reaction buffer, restriction enzyme and PCR products. For *TNF-α -238* polymorphism, 5 μL of PCR products and 5 U of Msp I enzyme (Promega) was used with 2 μg of BSA (Promega) and digested for 4 hr at 37°C. PCR products (6 μL) for the *TNF-α -308* polymorphism were digested for 16 hr at 37°C with 1 U of Nco I enzyme. The restriction enzymes Msp I and Nco I were inactivated by keeping the reaction mixture at 65°C for 20 min. For the *TNF-α -863* polymorphism, 5 μL of PCR products were mixed with 5 U of Tai I enzyme and incubated at 65°C for 3 hr. EDTA (20 mM) was added to inactivate the enzyme.

PCR products were visualized by running on a 1.5% agarose gel while the RFLP products were visualized in a 3% agarose gel under a UV transilluminator (Quantum ST4; Vilber Lourmat, Marne-la-Vallée, France).

When the PCR product of *TNF-α -238* (151 bp) is digested by Msp I, the wild type (*TNF-α -238* GG) will yield one band at 132 bp (a 19 bp band is not visible) and the heterozygous genotype (*TNF-α -238* GA) two separate bands (132 bp and 151 bp), whereas specimens containing the mutated genotype (*TNF-α -238* AA) will result in undigested PCR product. For *TNF-α -308* polymorphism, wild type (*TNF-α -308* GG) specimens yield one band at 126 bp (a 18 bp band is not visible) and heterozygous specimens (*TNF-α -308* GA) yield two separate bands at 144 bp and 126 bp, whereas the mutated genotype (*TNF-α -308* AA) remains undigested. The wild type genotype of *TNF-α -863* (*TNF-α -863* CC) remains undigested whereas the heterozygous genotype (*TNF-α -863* CA) yields two bands at 125 bp and 104 bp and the mutated genotype (*TNF-α -863* AA) one band at 104 bp.

Expression of TNF-α in serum specimens of *H. pylori*-positive patients

TNF-α concentrations in serum specimens were measured using an ELISA assay (Mabtech AB, Nacka Strand, Sweden) according to the manufacturer's instructions. Two wells (blank) were kept empty while an ELISA plate was coated with mAb TNF3/4 and incubated overnight at 4°–8°C. The serum specimens and plate were allowed to reach room temperature (20°–25°C) and the coated plate washed with PBS solution. Blocking solution (PBS with 0.05% Tween 20 with 0.1% BSA) was added to each well (except blanks) and incubated at room temperature for 1 hr. The plate was washed with washing buffer (PBS with 0.05% Tween) and standards and specimens were added in duplicate. The plate was then incubated for 2 hr at room temperature. After washing the plate with wash buffer, mAb TNF5–biotin solution was added to each well (except blanks) and incubated at room temperature for 1 hr. The plate was again washed and Streptavidin-HRP solution added before incubating for another hour at room temperature. After washing, substrate solution was added and allowed to incubate for 45 min before adding the stop solution. The absorbance at 450 nm was measured using an MPSCREEN MR-96A ELISA reader (MP Biomedicals, Santa Ana, CA, USA). The amount of TNF-α in each specimen was calculated using Graphpad Prism version 7 (Graphpad Software).

Data analysis

The frequency of the TNF-α polymorphisms was calculated as a percentage of the study cohort. The χ^2 test was used to determine the association between *TNF-α* polymorphisms and *H. pylori* infection. Using the Mann–Whitney U test, serum TNF-α concentrations were compared between *H. pylori*-positive patients with mild chronic gastritis and *H. pylori*-positive patients with moderate to severe gastritis.

RESULTS

Characteristics of the study cohort

The study cohort consisted of 138 patients with dyspepsia aged from 18–87 years. Seventy-seven were male and 61 female. The cohort comprised study 22 *H. pylori*-positive and 116 *H. pylori*-negative patients (*H. pylori* infection confirmed by histology). Of the 22 *H. pylori*-positive patients, 13 had mild chronic gastritis and nine moderate to severe chronic gastritis, whereas most patients in the *H. pylori*-negative group (114/116) had mild chronic gastritis and two had moderate to severe chronic gastritis.

Association between *TNF-α* polymorphisms and *H. pylori* infection

The frequency of *TNF-α* polymorphisms among 22 *H. pylori*-positive and 116 *H. pylori*-negative patients is presented in Table 2. Most patients in both the *H. pylori*-positive and negative groups had the wild type genotype. Among the 22 *H. pylori* positive patients, one patient was found to have each of the *TNF-α -238* AA genotype (4.5%), *TNF-α -308* AA genotype (4.5%) and *TNF-α -863* AA (4.5%) genotype, whereas wild type genotypes were identified in 18 (81.8%) (*TNF-α -238* GG), 20 (90.9%) (*TNF-α -308* GG) and 15 (68.2%) (*TNF-α -863* CC) of these patients. There was no significant difference in genotype expression between the *H. pylori*-positive and -negative groups.

When serum TNF-α expression in *H. pylori*-positive patients with dyspepsia ($n = 22$) was compared with that in healthy *H. pylori*-negative individuals ($n = 44$), the mean TNF-α concentration was found to be 224.6 pg/mL for *H. pylori*-positive patients whereas it was 276.4 pg/mL in the *H. pylori*-negative group. The difference in serum TNF-α expression between these two groups is not significant ($P = 0.4414$).

Association of *TNF-α* polymorphisms and TNF-α cytokine expression in patients with *H. pylori* infection

Among the *H. pylori*-positive patients with the *TNF-α -238* GG genotype, the mean TNF-α concentration was 139.89 pg/mL, whereas patients with *TNF-α -238* GA genotype had a comparatively higher mean TNF-α concentration of 507.86 pg/mL and a patient with *TNF-α -238 AA* genotype had the highest TNF-α concentration of 984.69 pg/mL. Among patients with the *TNF-α -308* GG genotype, the mean TNF-α concentration was 158.65 pg/mL. Two patients, one with *TNF-α -308* GA genotype and another with *TNF-α -308* AA genotype, had 27.79 pg/mL TNF-α in serum. Among patients with *TNF-α -863* CC genotype, the mean concentration of TNF-α was 158.65 pg/mL, whereas among patients with *TNF-α -863* CA genotype, the mean concentration was 124.89 pg/mL (Table 3).

Frequency of *TNF-α* polymorphisms, TNF-α concentrations and their association with severity of chronic gastritis in *H. pylori*-positive patients

Among the 22 patients with *H. pylori* infection and *TNF-α -238* GG genotype, the 11 with mild chronic gastritis had a mean TNF-α concentration of 218.14 pg/mL, whereas those with moderate to severe chronic gastritis had a mean concentration of

Table 2. Association of *TNF-α* polymorphisms with *H. pylori* infection in the study cohort

	H. pylori infection		
TNF-α polymorphism	Positive ($n = 22$) N (%)†	Negative ($n = 116$) N (%)‡	*P* value
TNF-α -238 polymorphism			
GG (Wild type)	18 (81.8)	93 (80.2)	
GA (Heterozygous)	3 (13.6)	16 (13.8)	0.962
AA (Mutated)	1 (4.5)	7 (6.0)	
TNF-α -308 polymorphism			
GG (Wild type)	20 (90.9)	103 (88.8)	
GA (Heterozygous)	1 (4.5)	12 (10.3)	0.301
AA (Mutated)	1 (4.5)	1 (0.8)	
TNF-α -863 polymorphism			
CC (Wild type)	15 (68.2)	76 (65.5)	
CA (Heterozygous)	6 (27.3)	35 (30.2)	0.963
AA (Mutated)	1 (4.5)	5 (4.3)	

†Percentage calculated for H. pylori-positive patients; ‡Percentage calculated for *H. pylori*-negative patients.

Table 3. Concentrations of TNF-α according to *TNF-α* polymorphisms among patients with *H. pylori* infection

			Serum TNF-α (pg/mL)	
Polymorphism	Genotype	N	Mean ± SD	Range
TNF-α -238	GG (Wild type)	18	139.89 ± 318.90	12.90–821.21
	GA (Heterozygous)	3	570.86 ± 333.68	108.915–757.00
	AA (Mutated)	1	-	984.69
TNF-α -308	GG (Wild type)	20	158.65 ± 359.06	12.90–984.69
	GA (Heterozygous)	1	-	27.79
	AA (Mutated)	1	-	27.79
TNF-α -863	CC (Wild type)	14	158.65 ± 382.79	12.90–984.69
	CA (Heterozygous)	7	124.89 ± 277.79	35.23–737.99
	AA (Mutated)	1	-	280.23

423.08 pg/mL. Among the patients with *TNF-α -308* GG genotype, there was a significant difference in TNF-α concentrations between patients with mild chronic gastritis ($n = 13$; mean concentration 251.19 pg/mL) and those with moderate to severe chronic gastritis ($n = 7$; mean concentration 637.36 pg/mL). Among the patients with the *TNF-α -863* CC genotype, there was no significant difference in TNF-α concentrations between patients with mild chronic gastritis (276.84 pg/mL) and those with moderate to severe chronic gastritis (566.63 pg/mL) (Table 4).

Association of *TNF-α* polymorphisms with severity of chronic gastritis in *H. pylori*-negative patients

Only two of 116 *H. pylori*-negative patients had moderate to severe gastritis, whereas the other 114 had mild chronic gastritis. Mild chronic gastritis was diagnosed in 91 patients (78.4%) with the *TNF-α -238* GG genotype, 101 (87.1%) with *TNF-α -308* GG genotype and 75 (64.7%) with *TNF-α 863* CC genotype. Twelve patients with *TNF-α -308* GA genotype and one with *TNF-α -308* AA genotype were diagnosed as having mild chronic gastritis. None of the patients with moderate to severe chronic gastritis carried the *TNF-α -238* GA or AA genotypes. Both patients with moderate to severe chronic gastritis had the wild type genotype for *TNF-α -238* and *-308* polymorphisms (Table 5).

DISCUSSION

In the current study, we investigated three *TNF-α* polymorphisms in a Sri Lankan cohort: *TNF-α -308*, *TNF-α -238* and *TNF-α -863*. None of the three polymorphisms was significantly associated with *H. pylori* infection or disease severity in our patient cohort. Additionally, none of our Sri Lankan cohort was diagnosed with gastric atrophy, indicating a low risk of developing gastric cancer, which is a favorable finding for Sri Lankans. For all three gene polymorphisms, the wild type genotype was predominant among *H. pylori*-positive patients with mild gastritis and those with moderate to severe gastritis.

However, TNF-α concentrations were significantly higher in patients with moderate to severe gastritis regardless of their *TNF-α* polymorphisms, suggesting that gene polymorphism alone is not responsible for the enhanced expression of TNF-α seen in patients with moderate to severe gastritis. *TNF-α -308* heterozygous (GA) and mutant (AA) genotypes were found to be associated with an increased risk of gastric cancer in a Chinese study (23). Further, Jang *et al*. reported that patients with gastric cancer tended to carry the *TNF-α -308* A allele and that they found the homozygous A/A genotype in these patients from South Korea (24). Among the 22 *H. pylori* positive patients in our study, the mutant *TNF-α -308* AA genotype was not found in patients with mild gastritis and only one patient with this genotype had moderate to severe gastritis. The absence of gastric atrophy in our study cohort may be partly attributable to the predominance of the wild type genotype of *TNF-α -308* and the rare occurrence of the AA genotype in Sri Lanka. Studies by Samaranayake *et al*. and Fernando *et al*. have reported a low proportion of AA genotype in Sri Lanka (25, 26), strengthening our findings. Thus, we hypothesized that *TNF-α -308* polymorphism may be useful as a genotypic marker for identifying groups at higher risk of gastric cancer and peptic ulcer, and thus for whom prevention of these diseases by *H. pylori* eradication therapy is indicated.

Studies investigating the association between *TNF-α−238* polymorphism and risk of gastric diseases have yielded contradictory and inconclusive results.

Table 4. Severity of chronic gastritis according to *TNF-α* polymorphisms and TNF-α serum concentrations in *H. pylori*-positive patients

	Mild chronic gastritis		Moderate to severe chronic gastritis		
TNF-α polymorphism	N	Mean ± SD (pg/mL)	N	Mean ± SD (pg/mL)	*P* value
TNF-α -238					
GG (Wild type)	1	218.14 ± 282.44	7	423.08 ± 353.91	0.276
GA (Heterozygous)	1	432.96 ± 458.26	1	-	-
AA (Mutated)	2	-	1	-	-
	0				
TNF-α -308					
GG (Wild type)	1	251.19 ± 300.81	7	637.36 ± 275.33	0.008
GA (Heterozygous)	3	-	1	-	-
AA (Mutated)	0	-	1	-	-
	0				
TNF-α -863					
CC (Wild type)	8	276.84 ± 320.17	7	566.63 ± 387.48	0.296
CA (Heterozygous)	5	210.14 ± 297.73	1	-	-
AA (Mutated)	0	-	1	-	-

No significant association was found between the *TNF-α -238* GA genotype and gastric cancer in a Chinese cohort (23), whereas a meta-analysis concluded that the *TNF-α -238* GA genotype is significantly associated with increased gastric cancer risk among Asians (27). In the present study, the *TNF-α -238* AA and GA genotypes were rare, making it impossible to identify an association between the presence of *TNF-α -238* A allele and severity of *H. pylori*-associated disease in Sri Lankan individuals.

TNF-α -863 polymorphisms have been studied in a Japanese cohort and found to be associated with increased risk of development of gastric ulcer and gastric cancer in patients with *TNF-α 863* A allele (18). Among patients with dyspepsia in Taiwan, those with the *TNF-α -863* A allele reportedly have significantly more numerous neutrophils and a higher risk of ulcer development (28). However, in our Sri Lankan cohort, *TNF-α -863* polymorphism was not significantly associated with *H. pylori* infection or severity of gastritis. The low frequency or absence of CA and AA genotypes in our cohort made it impossible to draw conclusions about the roles of these genotypes in development of complications such as gastric cancer.

Table 5. Association of TNF-α polymorphisms with severity of chronic gastritis in *H. pylori*-negative patients

	Severity of chronic gastritis	
TNF-α polymorphism	Mild N (%)†	Moderate to severe N (%)†
TNF-α 238 polymorphism		
GG (Wild type)	91 (78.4)	2 (1.7)
GA (Heterozygous)	16 (13.8)	0 (0.0)
AA (Mutated)	7 (6.0)	0 (0.0)
TNF-α 308 polymorphism		
GG (Wild type)	101 (87.1)	2 (1.7)
GA (Heterozygous)	12 (10.3)	0 (0.0)
AA (Mutated)	1 (0.9)	0 (0.0)
TNF-α 863 polymorphism		
CC (Wild type)	75 (64.7)	1 (0.9)
CA (Heterozygous)	35 (30.2)	0 (0.0)
AA	4 (3.4)	1 (0.9)

†Percentage was calculated from the *H. pylori*-negative patient population.

The low frequency of mutated and heterozygous *TNF-α* polymorphisms among patients with dyspepsia in our Sri Lankan study cohort emphasizes the importance of ensuring a larger sample size to improve the statistical power, its small size being a limitation of the present study.

In conclusion, the wild type genotype of *TNF-α* was predominant in our Sri Lankan study cohort, preventing us from finding and no clear association between *H. pylori* infection and gene polymorphism. Our findings on the frequency of *TNF-α* polymorphisms indicate that larger studies are needed to investigate the association of these gene polymorphisms with *H. pylori* infection and associated disease severity.

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DISCLOSURE

There are no conflicts of interest to declare.

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