Association between TNF-α promoter polymorphism and *Helicobacter pylori* cagA subtype infection

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Abstract

***Aims*—To assess the importance of tumour necrosis factor α (TNF-α) promoter polymorphism in relation to infection with the cytotoxin associated gene A (cagA) subtype of *Helicobacter pylori* within a dyspeptic Korean population.**

***Methods*—Eighty three patients with gastric disease and 113 healthy controls were studied. The DNA from gastric biopsy specimens was analysed by *H pylori* specific and cagA specific polymerase chain reaction (PCR). To characterise TNF-α polymorphism at positions −308 and −238, PCR based restriction fragment length polymorphism analysis was performed.**

***Results*—*Helicobacter pylori* infection was closely correlated with G to A transition at position −308 of the TNF-α promoter when compared with healthy controls (odds ratio (OR), 2.912; 95% confidence interval (CI), 1.082 to 7.836; p = 0.034). Although TNF-α −308 polymorphism in patients with *H pylori* was not significantly different from that in patients without *H pylori*, the −308A polymorphism was strongly associated with *H pylori* cagA subtype infection when compared with the polymorphism in cagA negative *H pylori* infection (OR, 8.757; 95% CI, 1.413 to 54.262; p = 0.019) and healthy controls (OR, 3.683; 95% CI, 1.343 to 10.101; p = 0.011). G to A genetic change at position −238 of the TNF-α gene was not significantly associated with *H pylori* cagA subtype infection. In addition, genetic polymorphisms at both sites of the TNF-α promoter in patients with *H pylori* infection did not correlate with the severity of disease.**

***Conclusion*—TNF-α −308A polymorphism was significantly related to infection with the *H pylori* cagA subtype in Korean patients with gastric disease.**

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Keywords: *Helicobacter pylori*; cagA; tumour necrosis factor α; polymorphism

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Helicobacter pylori is a Gram negative, urease positive bacterium active as a human gastric pathogen.[1] It is well known to be strongly associated with chronic gastritis as well as gastric and duodenal ulcers in humans.[2] *Helicobacter pylori* infection is also considered to be a risk factor for gastric cancer,[3] and the International Agency for Research on Cancer has categorised *H pylori* infection as a group I carcinogen.[4] However, few patients with *H pylori* infection develop gastric cancer,[5] and recent studies have focused on whether specific *H pylori* subtypes are associated with gastric carcinogenesis. It has been reported that the potential pathogenic differences between the subtypes of *H pylori* result from the cytotoxin associated gene A (cagA).[6 7] Although the function of cagA is not clear, it is recognised as a marker of enhanced virulence, and several studies have noted a correlation between cagA subtype and the severity of gastric mucosal inflammation.[8–10]

Helicobacter pylori induces the production of tumour necrosis factor α (TNF-α), which is closely related to epithelial injury.[11 12] TNF-α plays a crucial role in host defence against infection, but a high concentration of TNF-α may cause severe pathology.[13] Because TNF-α production is regulated, in part, at the transcriptional level,[14] many studies have implicated TNF-α promoter polymorphisms as potential determinants of disease susceptibility. The gene for TNF-α is located within the class III region of the major histocompatibility complex, which is a highly polymorphic region. The most common exchanges are G to A transitions in the TNF-α promoter at positions −308 (−308A) and −238 (−238A), and these genetic changes have been reported to influence TNF-α concentrations.[15 16]

In our study, we investigated the association of genetic polymorphisms at positions −308 and −238 of the TNF-α promoter with infection with *H pylori* and its cagA subtype in a dyspeptic Korean population using polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) analysis.

Methods

SUBJECTS AND PREPARATION OF SAMPLES

Eighty three patients with gastric disease and 113 unrelated healthy controls from the department of internal medicine, Inje University Paik Hospital, Pusan, Korea were recruited. Informed consent was obtained from each patient and the study was approved by the local ethics committee. The diagnosis of gastric disease was established by endoscopic examination and confirmed histologically. Biopsy specimens were taken from the gastric antrum and corpus and genomic DNA was isolated by the standard method using proteinase K and phenol/chloroform extraction.[17]

DETECTION OF *H PYLORI* AND cagA SUBTYPE

Helicobacter pylori infection was determined by PCR for the *H pylori* specific urease A (ureA) gene.[18 19] Nested PCR amplified a 204 bp ureA

Table 1 Tumour necrosis factor α (TNF-α) promoter polymorphisms at positions −308 and −238 in patients with gastric disease and healthy controls

Locus	Genotype	Patients (n = 83)		Healthy controls (n = 113)
		ureA$^+$ (n = 62)	ureA$^-$ (n = 21)	
−308	G/G	52 (83.9%)	21 (100%)	106 (93.8%)
	G/A	9 (14.5%)	0	7 (6.2%)
	A/A	1 (1.6%)	0	0
	Allele frequencies for −308A	0.0887	0	0.0309
−238	G/G	58 (93.5%)	20 (95.2%)	100 (88.5%)
	G/A	4 (6.5%)	1 (4.8%)	12 (10.6%)
	A/A	0	0	1 (0.9%)
	Allele frequencies for −238A	0.0323	0.0238	0.0619

In a 2 × 2 analysis, possession of TNF-308A was associated with an odds ratio of 2.912 (95% confidence interval, 1.082 to 7.836; $\chi^2 = 4.505$; p = 0.034) for ureA$^+$ versus healthy controls.

fragment from the template genomic DNA. Forward primer (5'-ATATTATGGAAGAAG CGAGAGC-3', position 2783 to 2804) and reverse primer (5'-ATGGAAGTGTGAGCC GATTTG-3', position 3096 to 3076) were used for the first round PCR. The second round nested PCR was performed using forward primer (5'-CATGAAGTGGGTATT GAAGC-3', position 2893 to 2912) and reverse primer (5'-ATGGAAGTGTGAGCC GATTTG-3', position 3096 to 3076). To determine the presence of the cagA subtype, a cagA specific primer set (5'-TAATGCTA AATTAGACAACTTGAGCGA-3', position 1218 to 1244; 5'-TAGAATAATCAACAAA CATCACGCCAT-3', position 1513 to 1487), which yields a 296 bp fragment on amplification, was used. For amplification of ureA and cagA gene fragments, cycles comprising a one minute denaturing step at 94°C, one minute annealing step at 55°C, and one minute elongation step at 72°C were used. After 35 cycles, a final elongation step was performed for five minutes at 72°C. A 10 μl aliquot of each PCR mixture was subjected to 1% agarose gel electrophoresis and ethidium bromide staining for the detection of amplified DNA products, which were confirmed by DNA sequencing.

TNF-α GENOTYPING BY PCR-RFLP

PCR based RFLP was performed to analyse TNF-α promoter polymorphism at positions −308 and −238. Nested PCR amplified a 118 bp fragment including the positions −308 and −238 of the TNF-α promoter from the template genomic DNA. For the first round PCR, two primers (5'-GAAGGAAACAGACC ACAGAC-3', position −372 to −353; 5'-ATCT GGAGGAAGCGGTAGTG-3', position −106 to −128) were used, and then a different primer set (5'-AGGCAATAGGTTTTCAGGTCCA TG-3', position −332 to −309; 5'-CACACT CCCCATCCTCCCAGATC-3', position −215 to −237) was used for the second round PCR. The underlined nucleotides of the primers indicate the mismatches that were introduced to create restriction sites or minimise the duplex formation of primers. The same reaction conditions described above were used. The nested PCR products were digested with NcoI to detect the −308 polymorphism.[20] The PCR products that also had a BglII restriction site (owing to the uniquely designed primer: position −215 to −237) were digested with BglII to detect the −238 polymorphism. The restricted products were analysed on a 15% polyacrylamide gel.

STATISTICAL ANALYSIS

Distributions of TNF-α promoter polymorphisms were compared by the χ^2 test. Odds ratios (OR) and 95% confidence intervals (CI) were also calculated. p Values smaller than 0.05 were regarded as significant. Statistical analysis was performed using the SAS system, version 6.12 (SAS Institute Inc, Cary, North Carolina, USA).

Results

ASSOCIATION BETWEEN *H PYLORI* INFECTION AND TNF-α POLYMORPHISM

Gastric disease related to *H pylori* infection was determined by the presence of the *H pylori* specific ureA gene. Of the 83 patients with gastric disease, 62 were classified as *H pylori* positive (ureA$^+$) and 21 as *H pylori* negative (ureA$^-$). Table 1 shows the genotype changes at positions −308 and −238 of the TNF-α promoter in the patients with gastric disease and in healthy controls. Genetic changes at position −308 were detected in 10 of 62 ureA$^+$ patients, whereas no genetic variation was seen in the 21 ureA$^-$ patients. A change from genotype G/G to G/A was seen in nine of the 10 patients; the remaining patient showed a G/G to A/A change (allele frequency for −308A = 0.0887). The statistical analysis for ureA$^+$ patients versus healthy controls showed a significant association between the presence of ureA and the −308A polymorphism (p = 0.034). This result indicated that the −308A polymorphism was related to an increased risk of developing *H pylori* infection (OR, 2.912; 95% CI, 1.082 to 7.836). However, for ureA$^+$ versus ureA$^-$, no significant correlation was found.

At the −238 position of the TNF-α gene, changes were detected in four of the 62 ureA$^+$ patients and one of the 21 ureA$^-$ patients, all of which were G/G to G/A genotype changes. However, *H pylori* infection was not significantly associated with the −238A polymorphism when compared with healthy controls (p = 0.280) or ureA$^-$ patients (p = 0.779).

ASSOCIATION BETWEEN *H PYLORI* cagA SUBTYPE INFECTION AND TNF-α POLYMORPHISM

To detect the cagA subtype of *H pylori*, cagA specific PCR was performed on biopsy specimens from the 83 patients with gastric disease. Amplified cagA DNA fragments were detected in 46 patients. Sixteen patients were cagA$^-$, although they were ureA$^+$. Table 2 shows that nine of 46 cagA$^+$ patients had genetic changes at position −308 of the TNF-α promoter; eight were heterozygotes (G/A) and one was a homozygote (A/A) (allele frequency for −308A = 0.1087). In cagA$^-$ patients, 36 of 37 showed no genetic variation, but one G/G to G/A genotype change was seen. Statistical analysis demonstrated that the −308A polymorphism was strongly associated with *H pylori* cagA subtype infection for cagA$^+$ versus cagA$^-$ (p = 0.019), and for cagA$^+$ versus healthy controls (p = 0.011). These results

Table 2 Association of Helicobacter pylori cagA subtype with tumour necrosis factor α (TNF-α) promoter polymorphisms at positions −308 and −238 in patients with gastric disease

		Patients	
Locus	*Genotype*	*cagA⁺ (n = 46)*	*cagA⁻ (n = 37)*
−308	G/G	37 (80.4%)	36 (97.3%)
	G/A	8 (17.4%)	1 (2.7%)
	A/A	1 (2.2%)	0
	Allele frequencies for −308A	0.1087	0.0135
−238	G/G	43 (93.5%)	35 (94.6%)
	G/A	3 (6.5%)	2 (5.4%)
	A/A	0	0
	Allele frequencies for −238A	0.0326	0.0270

In a 2 × 2 analysis for cagA⁺ versus healthy controls, possession of TNF-308A was associated with an odds ratio (OR) of 3.683 (95% confidence interval (CI), 1.343 to 10.101; $\chi^2 = 6.458$; p = 0.011). For cagA⁺ versus cagA⁻, TNF-308A was associated with an OR of 8.757 (95% CI, 1.413 to 54.262; $\chi^2 = 5.502$; p = 0.019).

Table 3 Genotype distribution for tumour necrosis factor α (TNF-α) promoter polymorphisms at positions −308 and −238 in Helicobacter pylori infected patients with carcinoma and benign disease

		ureA⁺	
Locus	*Genotype*	*Carcinoma (n = 37)*	*Benign disease (n = 25)*
−308	G/G	32 (86.5%)	20 (80%)
	G/A	4 (10.8%)	5 (20%)
	A/A	1 (2.7%)	0
	Allele frequencies for −308A	0.0811	0.1
−238	G/G	35 (94.6%)	23 (92%)
	G/A	2 (5.4%)	2 (8%)
	A/A	0	0
	Allele frequencies for −238A	0.027	0.04

indicated that the –308A polymorphism was associated with infection with *H pylori* harbouring the cagA gene compared with cagA⁻ *H pylori* infection (OR, 8.757; 95% CI, 1.413 to 54.262) and with healthy controls (OR, 3.683; 95% CI, 1.343 to 10.101). Furthermore, in 2 × 3 analysis for ureA⁻/cagA⁻, ureA⁺/cagA⁻, and ureA⁺/cagA⁺, the Mantel-Haenszel method for the χ^2 test, which represents variation tendency, showed that there was a significant trend to the –308A polymorphism ($\chi^2 = 5.621$; p = 0.018), although the original χ^2 test indicated no significant correlation with each other ($\chi^2 = 5.837$; p = 0.054).

Genetic changes at position −238 of the TNF-α promoter were detected in three of the 46 cagA⁺ patients and two of the 37 cagA⁻ patients, all of whom were G/A heterozygotes. When cagA⁺ was compared with cagA⁻, there was no correlation between *H pylori* cagA subtype infection and TNF-α –238 polymorphism (p = 0.832).

ASSOCIATION BETWEEN DISEASE SEVERITY AND TNF-α POLYMORPHISM IN *H PYLORI* INFECTION

Based on histopathological analysis, the *H pylori* associated gastric diseases tested were subdivided into two groups: carcinoma and benign disease (gastritis and peptic ulcer). Of 62 ureA⁺ patients, 37 patients were classified as having carcinoma and 25 patients as having benign lesions (19 with gastritis, three with gastric ulcer, and three with duodenal ulcer). Table 3 shows the distribution of the TNF-α genotype in ureA⁺ patients with carcinoma or benign disease. Genetic changes at position −308 of the TNF-α promoter were detected in five of the 37 ureA⁺ patients with carcinoma and five of the 25 ureA⁺ patients with benign disease (p = 0.496). At position −238 of the TNF-α promoter, genetic changes were detected in two of the 37 ureA⁺ patients with carcinoma and two of the 25 ureA⁺ patients with benign disease (p = 0.683). The five patients with the GA genotype at position −308 all had chronic gastritis. Of the two patients with the GA genotype at position −238, one had gastritis and the other had gastric ulcer. Thus, there was no statistical correlation between the severity of disease and TNF-α polymorphisms at both regions.

Discussion

In our study, TNF-α promoter polymorphisms at positions −308 and −238 in an *H pylori* associated dyspeptic Korean population were investigated. The analysis for ureA⁺ versus ureA⁻ showed that the −308 polymorphism was not significantly associated with *H pylori* infection, although the result was significant when compared with healthy controls. Although Kunstmann *et al* reported recently that genotype change at position −308 of the TNF-α promoter was significantly more frequent in *H pylori* positive patients than in *H pylori* negative patients,[21] this relation was significant for duodenal ulcers only, not for gastric ulcers. Thus, the conflicting results might be caused by the differences of the sample group, although possible differences in the characteristics of the Korean population should not be ruled out. The gastric diseases investigated here included carcinoma, gastritis, and gastric ulcers, in addition to duodenal ulcers. The heterogeneity of diseases studied might have contributed to the lack of a significant relation between the TNF-α –308 polymorphism and *H pylori* infection. In addition, no significant association between the TNF-α –308 polymorphism and the severity of gastric disease was found. In contrast, *H pylori* cagA subtype infection was associated strongly with the –308A polymorphism when compared with the cagA⁻ *H pylori* infected and healthy control groups. These results suggest that the TNF-α –308A genotype plays a crucial role in the genetic predisposition for infection with the *H pylori* cagA subtype in the Korean population with gastric disease.

Genetic variations in regulatory regions of cytokine genes are associated with susceptibility to several complex disorders. G to A transition at position −308 in the TNF-α promoter has been shown to increase TNF-α concentrations and disease susceptibilities in human subjects.[22 23] Although the molecular mechanism by which genetic polymorphism influences cytokine gene expression is not clear, several studies suggested that the −308 polymorphism affected transcription factor binding. The binding characteristics of activator protein 2 (AP-2) to the region around −308 was found to be altered by the −308A allele.[24] Based on functional analysis showing the repressive effect of AP-2 binding on TNF-α promoter activity,[25] it is possible that the −308A polymorphism leads to an increase of TNF-α gene expression. Transfection studies also indicated that TNF-α expression was

higher in the presence of the −308A allele compared with the −308G allele.[26] These findings confirmed the importance of this site in the transcriptional regulation of the TNF-α gene. In addition, recent functional studies showed that mice deficient in TNF-α were resistant to the development of benign and malignant skin tumours,[27 28] suggesting that there is a direct relation between TNF-α polymorphism and the high incidence of disease. Thus, it is possible that increased TNF-α concentrations, as a result of –308A polymorphism, alter the immune response, which confers susceptibility to gastric disease with *H pylori* cagA subtype infection.

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