

# Genetic polymorphism of interleukin-8 (IL-8) is associated with *Helicobacter pylori*-induced duodenal ulcer

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**ABSTRACT.** *Background and aims.* *Helicobacter pylori* infection almost invariably causes chronic gastritis, but only a proportion of the infected subjects develop peptic ulcers. The local inflammation associated with *H. pylori* infection is characterized by an increased production of the proinflammatory cytokines IL-1- $\beta$ , IL-6, IL-8 and TNF- $\alpha$ . Since such cytokine production is often determined by the genetic polymorphism of regions regulating cytokine gene expression, we investigated the relationship between TNF- $\alpha$  and IL-8 polymorphisms and the development of duodenal ulcer disease. We also sought a correlation between the promoter polymorphism of the lipopolysaccharide (LPS) receptor CD14 and the formation of peptic ulcer, because CD14 plays a crucial role in the initiation of the cytokine cascade. *Methods.* Genomic DNA extracted from the peripheral blood of 69 patients with *H. pylori*-positive duodenal ulcer disease and 47 *H. pylori*-positive healthy controls was analyzed for TNF- $\alpha$ -308 promoter polymorphism by RFLP, and for IL-8 -251 polymorphism by ARMS. Genetic polymorphism within the promoter of the CD14 gene was identified using the LightCycler instrument via melting point analysis. *Results:* No significant correlation could be revealed between the TNF- $\alpha$  and CD14 promoter polymorphisms and the clinical outcome of *H. pylori* infection. The IL-8 A/T heterozygote mutant variant was detected with a significantly higher frequency (65.22%) among the ulcer patients than among the healthy, *H. pylori*-positive blood donors (36.17%), while the frequency of the normal allelic genotype (TT) was significantly higher in the control group (44.6% vs 15.9%). *Conclusion.* Analysis of the genetic predisposition to enhanced cytokine production revealed a significant association only for the IL-8 polymorphism. This observation draws attention to the possible importance of IL-8 polymorphism as a genetic predisposing factor in the pathomechanism of *H. pylori*-induced duodenal ulcer disease, and to the relative protection from duodenal ulcer disease that is associated with the TT genotype.

**Keywords:** *Helicobacter pylori*, duodenal ulcer, genetic polymorphism, IL-8, TNF- $\alpha$ , CD-14

## INTRODUCTION

*Helicobacter pylori* induces gastric inflammation in virtually all of the hosts colonized, and such gastritis increases the risk of gastric and duodenal ulceration, distal gastric adenocarcinoma and gastric mucosal lymphoproliferative disease. In contrast with infections with other mucosal pathogens however, only a small percentage of the persons carrying *H. pylori* ever develop clinical sequelae. Investigations focused on the pathogenesis of *H. pylori* infections have emphasized that the disease risk involves specific and choreographed interactions between pathogen and host [1], which in turn are dependent upon strain-specific bacterial factors and an induced immune response in the host [2]. Locally, nonspecific immunity and, most importantly, the production of proinflammatory cytokines is stimulated by the bacterium. It is specifically the contact of bacteria

with the gastric epithelial cells that induces the production of proinflammatory cytokines. The cytokines are released from neighboring leukocytes, and further activation of these effector cells occurs in both autocrine and paracrine ways, the chemotactic effect of IL-8 attracting further leukocytes. It is important that IL-8, which plays a key role in the development of the disease, is also secreted by gastric epithelial cells; hence, the role of this cytokine in the initiation, modulation and maintenance of the gastrointestinal inflammatory responses is crucial [3]. In addition to the bacterial virulence, host factors also seem to be important in the outcome of the infection. For instance, the intensity of the local cytokine response can contribute to the development of mucosal destruction [2, 4]. We reported earlier that significantly higher levels of TNF- $\alpha$ , IL-6 and IL-8 were to be found in antral biopsy specimens from duodenal ulcer (DU) patients than in those from *H.*

*pylori*-negative subjects [5]. This prompted us to investigate the role of a genetic predisposition to higher cytokine production in the pathogenesis of DU disease.

Genetic polymorphisms within the promoter of the inflammation-related cytokine genes are thought to influence the expression of these cytokines, and there are numerous infectious and noninfectious diseases in which these polymorphic changes have been shown to correlate with disease susceptibility or outcome of the disease [6]. The present study was conducted to examine any association between TNF- $\alpha$  and IL-8 polymorphisms and the development of DU disease in *H. pylori*-infected patients. The clinical importance of the TNF- $\alpha$  gene -308 promoter/enhancer polymorphism is supported by the fact that the rare allele TNF2 has direct effects on the transcription of TNF- $\alpha$  [7-10]. A common variant of the IL-8 gene promoter region (-251A) also tends to be associated with increased IL-8 production [11, 12]. CD14, expressed on the surface of monocytes and hepatic Kupffer cells, is the receptor for lipopolysaccharide (LPS) [13], a cell wall component of Gram-negative bacteria. Since the LPS receptor CD14 plays a crucial role in the initiation of the cytokine cascade [13], we also sought a possible correlation between the -159 C $\rightarrow$ T promoter polymorphism of CD14 and the development of peptic ulcer. A single nucleotide polymorphism (SNP) in the promoter region of the CD14 gene (C/T at position -159) has been described [14]. TT homozygotes have significantly higher serum levels of sCD14 [15]. TT genotype frequencies have been found to be increased in Crohn's disease [16] and in myocardial infarction [17]. An association between a genomic polymorphism within the CD14 locus and septic shock was recently reported [18]. Karhukorpi *et al.* [19] observed a tendency to a higher frequency of the CD14 TT genotype in DU patients as compared with subjects without DU. It therefore appeared logical to supplement our study with an investigation of CD14 polymorphism.

## PATIENTS AND METHODS

### Patients

Sixty-nine *H. pylori*-positive patients with DU were studied. The project was approved by the Clinical Ethical Committee of the Medical Faculty of the University of Szeged (Szeged, Hungary), and informed consent was obtained from all of the patients. Multiple biopsy specimens were taken during upper gastrointestinal endoscopy from adjacent sites of the gastric antrum and corpus for histology. In addition, the  $^{13}\text{C}$ -urea breath test (UBT) was carried out. Only patients with *H. pylori* infection documented by histology and with a positive  $^{13}\text{C}$ -UBT result were considered eligible for the study.

Forty-seven *H. pylori*-positive blood donors without gastric or duodenal disease served as controls. The status of infection with *H. pylori* in these controls was determined by serology with a commercial ELISA kit (HP IgG ELISA (Dia.Pro, Milan, Italy), and by  $^{13}\text{C}$ -UBT positivity.

The patient population comprised 30 men and 39 women, with a mean age of  $54.46 \pm 1.19$  years (28-77), whereas in the control group there were 22 men and 25 women, with a mean age of  $49.85 \pm 1.82$  years (24-73). The two groups matched with regard to age (Mann-Whitney U  $p=0.0971$ ) and sex (Fisher's exact test  $p=0.8494$ ). All case subjects

and controls were of Hungarian ethnic origin and resided in Hungary.

### Genotyping procedures

DNA was extracted from the peripheral blood of the patients and the controls using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

The analysis of the polymorphisms was based on polymerase chain reaction (PCR) techniques performed either in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA, USA) or in a light cycler (Roche Laboratories, Basel, Switzerland).

**TNF- $\alpha$ .** The G $\rightarrow$ A transition at position -308 in the promoter region defines the rare allele 2 associated with an elevated expression of TNF- $\alpha$  [10]. A single base change at the 3' end of primer A1 (underlined) was required for the formation of an NcoI recognition sequence [20]. The PCR primers were:

A1: 5' AGGCAATAGGTTTTGAGGGCCCAT 3' and

A2: 5' TCCTCCCTGCTCCGATTCCG 3'

100 ng of genomic DNA was amplified using Taq DNA polymerase (Fermentas, Vilnius, Lithuania) with 1.5 mM MgCl<sub>2</sub> under the following conditions: 94 °C for 3 min, followed by 36 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with an extension at 72 °C for 5 min. The amplified product was digested with NcoI and analyzed on a 12% polyacrylamide gel. Digestion confirmed two alleles. Allele 1 gave two fragments, of 87 bp and 20 bp, while allele 2 gave a single, 107 bp fragment.

**IL-8.** A single nucleotide T $\rightarrow$ A polymorphism at -251 nt relative to the transcription start site, accompanied by increased IL-8 production, was typed by an amplification refractory mutation system (ARMS) [11]. The allele-specific primers were:

5' CCACAATTTGGTGAATTATCAAT 3' (-251A) and

5' CCACAATTTGGTGAATTATCAAA 3' (-251T)

The consensus primer was 5' TGCCCCTTCACTCTGTTAAC 3', giving a PCR product of 336 bp. In each reaction, a second set of primers for exon 3 of the HLA-DRB1 gene (forward: 5' TGCCAAGTGGAGCACCCAA 3', reverse: 5' GCATCTTGCTCTGTGCAGAT 3', product size 796 bp) was used as a control for PCR efficiency. Reactions were carried out using the Advantage-GC cDNA polymerase mix and buffer (Clontech, Palo Alto, CA, USA) under the following conditions: 96 °C for 120 s; four cycles of 96 °C for 35 s, 68 °C for 45 s, and 72 °C for 35 s; four cycles of 96 °C for 35 s, 65 °C for 45 s, and 72 °C for 45 s; four cycles of 96 °C for 35 s, 62 °C for 45 s, and 72 °C for 55 s; ten cycles of 96 °C for 35 s, 58 °C for 45 s, and 72 °C for 65 s; ten cycles of 96 °C for 35 s, 55 °C for 45 s, and 72 °C for 75 s; four cycles of 96 °C for 35 s, 52 °C for 45 s, and 72 °C for 85 s; four cycles of 96 °C for 35 s, 50 °C for 45 s, and 72 °C for 90 s; and 72 °C for 5 min.

**CD14.** A genetic polymorphism within the promoter region of the CD14 LPS receptor gene was identified using a rapid-cycle PCR with fluorescent-labeled oligonucleotide hybridization probes on the LightCycler instrument and subsequent fluorescent probe melting point analysis [15]. This polymorphism consists of a single base exchange (C $\rightarrow$ T) at position -159, resulting in an elevated expres-

**Table 1**  
Allele and genotype frequencies of the TNF- $\alpha$  -308 gene polymorphism

	<i>H. pylori</i> -positive DU patients		<i>H. pylori</i> -positive controls	
	n	%	n	%
Allele <sup>a</sup>				
1	121	87.68	74	78.72
2	17	12.32	20	21.28
Genotype <sup>b</sup>				
1/1	54	78.26	29	61.70
1/2	13	18.84	16	34.04
2/2	2	2.90	2	4.26

<sup>a</sup> Fisher's exact test:  $p=0.071$ .

<sup>b</sup> Chi-square test:  $\chi^2=3.805$ ,  $p=0.149$ .

sion of the CD14 receptor molecule. PCR was performed in disposable capillaries (Roche Diagnostics) in a reaction volume of 10  $\mu$ L containing 20-80 ng of DNA, 0.5  $\mu$ M of each of the primers (forward: 5' GGTGCCAACAGATGAGGTTTCAC 3'; reverse: 5' CTTCGGCTGCCTCTGACAGTT 3'), 1  $\mu$ L of reaction buffer (Roche Diagnostics), and 0.2  $\mu$ M of each of the probes. The detection probe specific for the T allele (5' TTCCTGTTACGGCCCCCT 3') was labeled at the 3' end with fluorescein. The anchor probe (5' GGAGACACAGAACCCTAGATGCCCTGCA 3') was labeled with LightCycler Red 640 at the 5' end and modified at the 3' end by phosphorylation to block extension. The PCR conditions were as follows: initial denaturation at 95 °C for 120 s, followed by 60 cycles of denaturation (95 °C for 0 s), annealing (55 °C for 10 s), and extension (72 °C for 10 s). The melting curve consisted of one cycle at 95 °C for 0 s, 45 °C for 10 s, and then a temperature increase to 95 °C at 0.2 °C/s. The fluorescence signal was monitored continuously during the temperature ramp and then plotted against the temperature.

#### Statistical analysis

All statistical calculations were performed with the GraphPad Prism statistical program. To compare the genotype frequencies,  $\chi^2$  tests were performed and Yates' correction was applied, resulting in corrected P values. Allelic frequencies were compared by Fisher's exact test. For comparison of age and sex between the patients and the controls, the Mann-Whitney U test and Fisher's exact test was used. Statistical significance was taken at the  $p=0.05$  level. The relationship between the presence of individual alleles and disease is presented as the odds ratio with a 95% confidence interval (OR 95% CI). The genotype frequen-

cies for each polymorphism were tested for deviation from the Hardy-Weinberg equilibrium by the  $\chi^2$  test, with one degree of freedom used.

## RESULTS

The genotype frequencies of the TNF- $\alpha$ , CD14 and IL-8 polymorphisms in the control group did not deviate significantly from those expected for the Hardy-Weinberg equilibrium, ( $\chi^2=0.0123$ ,  $p=0.911$ ;  $\chi^2=0.0212$ ,  $p=0.884$ ;  $\chi^2=2.4040$ ,  $p=0.121$ , respectively). Among the DU patients, only the IL-8 genotype frequency deviated significantly from that for the Hardy-Weinberg equilibrium ( $\chi^2=6.4375$ ,  $p=0.011$ ).

#### TNF- $\alpha$ polymorphism

The frequency distribution of the genotypes for the TNF- $\alpha$  gene polymorphism studied is shown in table 1. There was no significant difference in the distribution of the TNF- $\alpha$  -308 G $\rightarrow$ A gene polymorphism between the *H. pylori*-positive DU patients and the *H. pylori*-positive healthy controls ( $\chi^2=3.805$ ,  $p=0.149$ ). Likewise, no significant difference in the rate of carriage of the high-secreting allele was seen between the two populations ( $p=0.071$ ).

#### CD14 polymorphism

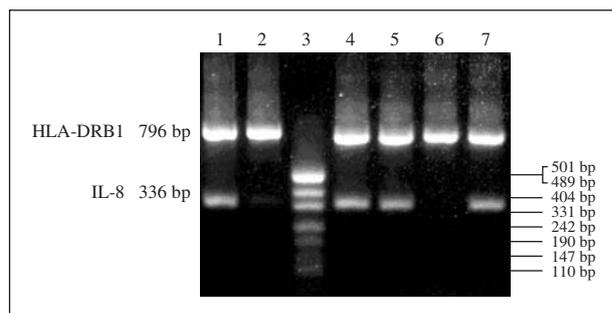
The frequency distribution of the genotypes for the CD14 gene polymorphism is presented in table 2. A significant correlation between the presence of the CD14 -159 C $\rightarrow$ T promoter polymorphism and the development of DU disease was not observed in the *H. pylori*-positive populations studied. No significant differences were found in either the

**Table 2**  
Allele and genotype frequencies of the CD14 -159 gene polymorphism

	<i>H. pylori</i> -positive DU patients		<i>H. pylori</i> -positive controls	
	N	%	n	%
Allele <sup>a</sup>				
C	73	52.90	47	50.00
T	65	47.10	47	50.00
Genotype <sup>b</sup>				
C/C	20	28.98	12	25.53
C/T	33	47.83	23	48.94
T/T	16	23.19	12	25.53

<sup>a</sup> Fisher's exact test:  $p=0.689$ .

<sup>b</sup> Chi-square test:  $\chi^2=0.1916$ ,  $p=0.098$ .



**Figure 1**

PCR/ARMS analysis of the IL-8 -251 polymorphism. Lanes 1 and 2 correspond to genotype AA, lanes 4 and 5 to the heterozygote genotype AT, and lanes 6 and 7 to genotype TT (336 bp). Lane 3 shows a molecular weight marker with band sizes of 501 - 110 bp. The upper band corresponds to a HLA-DRB1 gene product (796 bp) as control.

genotype frequency distributions ( $\chi^2=0.1916$ ,  $p=0.908$ ), or the rate of carriage of the allele linked to high expression of CD14 [15]. The T allele was found in 47.1% of the patients as compared with 50.0% of the controls ( $p=0.689$ ).

### IL-8 polymorphism

Figure 1 depicts representative results relating to the IL-8 genotyping. To detect the nucleotide swap, ARMS was used. By means of the two, allele-specific primers, the homozygote mutant (AA), and the heterozygote (AT) and the homozygote TT variants (336 bp product) were easily distinguishable. To monitor the effectiveness of the PCR reaction, the HLA-DRB1 exon 3 gene was also demonstrated in each case (product size: 796 bp).

The genotypic frequencies were significantly different in the *H. pylori*-positive patient and healthy control groups (table 3). There was a significantly higher frequency of the IL-8 -251 T/A heterozygote genotype in the diseased group (45/69, 65.22% versus 17/47, 36.17%;  $\chi^2=11.26$ ,  $p=0.0008$ ). Similarly, the rate of carriage of the high-secreting allele (IL-8 A) was significantly different in the two populations (51.45% among the DU patients versus 37.23% in the healthy blood donor group;  $p=0.043$ , OR=1.7863, CI 95%: 0.23-0.95), although the numbers of homozygotes were low and not dissimilar. Conversely, the prevalence of the IL-8 TT, wild-type genotype was significantly lower in the DU patients.

## DISCUSSION

Although there is evidence that *H. pylori* infection plays a crucial role in the pathogenesis of DU, there is a striking difference between the number of infected individuals and the number that go on to develop ulcer. Among *H. pylori*-positive subjects, the incidence of ulcer formation is 15-20% [21]. Hence, the progression toward the disease appears to depend on the combined effects of the bacterial pathogenicity and host factors. Environmental factors such as diet or the use of NSAID may also interfere in the progression toward the various diseases due to infection.

We demonstrated previously that in *H. pylori*-positive patients with DU there was considerable local TNF- $\alpha$ , IL-6 and IL-8 production in gastric biopsy samples, but only IL-8 was produced in a significantly higher amount by the peripheral white blood cells as compared with non-DU patients [5]. This prompted us to investigate whether SNP in the IL-8 promoter region could confer a higher risk of ulcer development as compared with the polymorphisms of the TNF- $\alpha$  gene.

Polymorphisms in the TNF- $\alpha$  gene have tentatively been associated with an increased risk of gastric carcinoma [22]. In our study, no significant connection was found between the TNF- $\alpha$ -308 polymorphism and the development of DU in the *H. pylori*-positive subjects. There are two possible explanations. First, the regulation of cytokine protein expression is complex and multifactorial. This means that, following induction, both transcriptional and translational regulation and posttranslational protein processing are major steps involved in protein expression. The TNF-308 polymorphism affects TNF transcription in both a cell-type and a stimulus-specific manner and this effect may even be dependent on the differentiation states of the cell and the inducers [23]. Brinkman *et al.* [24] found that there was no difference between the level of transcription of the -308 A and -308 G alleles in LPS-stimulated peripheral monocytes. Similar observations were reported by Stuber *et al.* [25]. The TNF- $\alpha$  gene utilizes different sets of transcriptional elements, and TNF- $\alpha$  protein expression is probably not regulated exclusively at the transcriptional level determined by the -308 site on the promoter. Secondly, even with a potentially high TNF-producing ability, the -308 polymorphism of the TNF- $\alpha$  gene may not pose a risk of ulcer development. This is in good accordance with the finding in our previous study that there was no increased TNF- $\alpha$ -producing ability among DU patients in general, when whole blood cell cultures were investigated [5]. Kunstmann *et al.* reported that a genotype change at

**Table 3**  
Allele and genotype frequencies of the IL-8 -251 gene polymorphism

	<i>H. pylori</i> -positive DU patients		<i>H. pylori</i> -positive controls	
	N	%	n	%
Allele				
T	67 <sup>a</sup>	48.55 <sup>a</sup>	59 <sup>a</sup>	62.77 <sup>a</sup>
A	71 <sup>a</sup>	51.45 <sup>a</sup>	35 <sup>a</sup>	37.23 <sup>a</sup>
Genotype				
T/T	11 <sup>b</sup>	15.94 <sup>b</sup>	21 <sup>b</sup>	44.68 <sup>b</sup>
T/A	45 <sup>b</sup>	65.22 <sup>b</sup>	17 <sup>b</sup>	36.17 <sup>b</sup>
A/A	13	18.84	9	19.15

<sup>a</sup> Fisher's exact test:  $p=0.043$ , OR=1.7863, CI 95%: 0.23-0.95.

<sup>b</sup> Chi-square test with Yates' correction:  $\chi^2=11.26$ ,  $p=0.0008$ .

position -308 of the TNF- $\alpha$  promoter was significantly more frequent in *H. pylori*-positive patients than in *H. pylori*-negative patients [26]; this relationship was significant only for DUs, and not for gastric ulcers. Thus, the conflicting results might be caused by the differences between the sample groups, as we investigated only *H. pylori*-positive subjects, although possible differences in the characteristics of the Korean and Hungarian populations should not be ruled out.

CD14 is one of the key molecules that mediate the effects of LPS [13]. The promoter region polymorphism (-159C/T) in the CD14 gene is functionally important for regulating CD14 levels [15]. Our working hypothesis was that TT genotypes associated with an increased CD14 expression, might confer susceptibility to DU in *H. pylori*-infected patients, potentiating the effect of LPS to shift the cytokine response toward TH1 [27].

*H. pylori* LPS is reportedly much less potent in eliciting cytokines or chemokines than LPS from *Salmonella enterica* or *Escherichia coli* [28]. However, it has recently been stated that *H. pylori* LPS binds to CD14 on macrophages and stimulates the release of IL-8 [29, 30]. No association between ulcer and CD14 polymorphism was evident in our study, however. The interaction of bacterial components with different Toll receptors [31] may promote cytokine signals and may also demand consideration. It is noteworthy however, that in an *in vitro* study, the gastric mucosal recognition of *H. pylori* was independent of Toll-like receptor 4 [32]. In contrast, another study suggested that TLR4 may play a crucial role in the initiation of inflammatory responses to *H. pylori* infection [33]. A further study with a larger sample size is warranted to explore the role of TLR receptor polymorphisms in DU.

A significantly higher frequency of the IL-8 AT genotype was observed among the *H. pylori*-positive DU patients than among the *H. pylori*-positive healthy subjects without gastrointestinal problems. This genotype reflects a higher IL-8-producing ability [11, 12]. Conversely, the frequency of the TT genotype (with a relatively low IL-8-producing potential) was significantly higher among the *H. pylori*-positive, healthy non-DU subjects. This suggests the possibility that a relative protection from DU disease is observed in association with the TT genotype. This observation is consistent with the results of Hamajima *et al.* [34], who concluded that *H. pylori*-positive healthy individuals with the IL-8 -251 TT genotype might display a milder inflammatory reaction. Among our patients, there were only a few individuals who carried the AA genotype; it is very likely that this reflects the relatively small number of patients investigated to date. In our study, the association with IL-8 was only explored at the level of a single SNP (-251 A/T) and not at the level of the haplotype [12]. The higher incidence of the -251 AT genotype with a concomitant higher IL-8-producing potential [11, 12] highlights the importance of the genetic determination of IL-8 production in *H. pylori*-induced DU. This is in good accordance with our previously published finding that inducible IL-8 was higher in patients with DU than in *H. pylori*-positive healthy subjects [5]. IL-8 is a crucial cytokine in the pathogenesis of DU, where not only the inflammatory cells, but also the gastric epithelial cells themselves can be a source of IL-8 production [2, 3]. *H. pylori*-induced gastric inflammatory diseases are associated with

the massive recruitment of phagocytes, and particularly neutrophils, to the gastric mucosa.

It is therefore tempting to speculate that a predisposition to a higher IL-8 response to the same bacterial stimulus (i.e. *H. pylori*) might also be a factor predisposing to ulcerative processes. There was no such connection between the polymorphisms of the TNF- $\alpha$  and CD14 genotypes. It appears highly likely that just one polymorphism cannot determine the final outcome of *H. pylori* infection. The use of genome-wide SNPs, to detect realistic effect sizes will typically require thousands of individuals [35]. Our pilot study involved 67 cases and 47 controls, and the conclusions must therefore be considered only preliminary. However, determination of the frequencies of IL-8 polymorphism in *H. pylori*-induced diseases could be informative and provide further evidence concerning the role of IL-8 in DU formation, thereby suggesting the clinical value of this genotype assessment.

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