IL-1β (+3953 C/T) and IL-8 (-251 A/T) Gene Polymorphisms in *H. pylori* Mediated Gastric Disorders

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ABSTRACT

Background: Previous studies imply that IL-1 and IL-8 gene variations may play a crucial role in the genetic predisposition to different gastric disorders upon *H. pylori* infection. **Objective:** The aim of this study was to determine the potential association between the prevalence of certain polymorphic sites and the risk of gastric disorders in Iranian population. **Methods:** One hundred and forty three unrelated individuals with different gastric disorders and 374 normal individuals with no gastric disorders and with a negative serology test for *H. pylori* (control group) were studied for the association between IL-1β (+3953 C/T) and IL-8 (-251 A/T) gene polymorphisms and *H. pylori*-mediated gastritis and gastric ulcer. An analysis of genotype frequency for these genes was performed using RFLP-PCR. **Results:** Based on the data obtained from culture and pathologic findings, the patients were classified into three subpopulations: *H pylori*$^+$ non-ulcerative gastritis$^+$, *H. pylori*$^+$ ulcerative gastritis$^+$ and *H. pylori*$^-$ non-ulcerative gastritis$^+$. A significantly higher frequency of TT genotype (p=0.02) in IL-1β +3953 in *H. pylori*$^+$ ulcerative gastritis$^+$ was revealed compared to the control group. There were no significant differences among other subpopulations. No significant differences in allele and genotype frequencies of IL-8 (-251A/T) were found among the patients. **Conclusion:** The data suggest that TT genotype in IL-1β +3953 may be a major contributing genetic risk factor for *H. pylori* induced gastric ulcer. Moreover, the role of other bacterial and host response factors, such as bacterial adherence peptides, host chemokines, and genes involved in gastric acid secretion, must be further investigated in different ethnic populations.

Keywords: Gastric Diseases, Helicobacter pylori, IL-1β, IL-8, Polymorphism

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INTRODUCTION

It has been proposed that the complex interactions between the bacterial and host genetic factors, along with environmental conditions, play a significant role in determining the differential clinical outcomes among various individuals infected by *Helicobacter pylori* (1,2).The immune system plays an important role in the pathogenesis of gastroduodenal diseases by regulating the nature and intensity of the inflammatory response to *H. pylori* infection (3). IL-1β, a potent inhibitor of gastric acid, is speculated to be responsible for the changes in the gastric environment upon *H. pylori* infection (4,5). Not only, it has important biologic effects, but it also does regulate the inflammatory and the immune response upon promoting the expression of other cytokines, such as IL-6 and IL-12. Polymorphic gene sequences of some cytokines could be potential markers of susceptibility and clinical outcome in different human infectious diseases (6). The biallelic polymorphisms reported in IL-1β , all representing C>T base transition at positions -511(NT-022135.14:g.2392610 C>T), -31(NT-022135.14:g.2302130 C>T), and +3953(NT-022135.14:g.2306115 C>T) from the transcriptional initiation site (7,8). These polymorphisms have been shown to affect gastric mucosal IL-1β production in response to *H. pylori* infection, significantly (9). The effect of these polymorphisms is therefore most likely mediated through the regulation of IL-1β expression (3,10,11). Interleukine-8 (IL-8), a member of neutrophils and chemokine family, is a chemoattractant of neutrophils and lymphocytes (12). It regulates humoral and immune response through its inflammatory and procoagulant properties (13,14). *H. pylori* adheres to gastric epithelial cells, activates nuclear factor-κB, and stimulates IL-8 production (15,16). Gastric mucosal levels of IL-8 are in parallel with the histological severity of gastritis. The (-251 A/T) polymorphism, which is found in the promoter region of IL-8 gene, is the only one known to influence its expression (17). To determine the potential association between the prevalence of certain polymorphic sites and the risk of gastric disorders in different geographic regions, this study aimed to find an association between IL-1β (+3953 C/T) and IL-8 (-251 A/T) gene polymorphism and *H. pylori*-mediated gastritis and gastric ulcer in an Iranian population using fragment length polymorphism polymerase chain-reaction (RFLP- PCR) method.

MATERIALS AND METHODS

Patients and Controls. Of the patients attending the endoscopy ward of Motahhary Clinic of Shiraz University of Medical Sciences during the period of October 2006 to March 2007, 125 were enrolled in this study. Exclusion criteria for patients' recruitment to the study were previous attempts to eradicate *H. pylori*, use of antibiotics or proton pump inhibitors within the last 2 weeks prior to endoscopy, and previous gastric surgery. The diagnosis of non-ulcerative gastritis and/or gastric ulcer was established on the basis of endoscopic findings. The diagnosis of *H. pylori* infection and the histological confirmation of the gastric disease were established by a central study pathologist. Corpus and antral biopsies taken from each patient were transferred to the lab in an appropriate transfer medium (Brain Heart Infusion Broth supplemented with 20% glucose) for *H. pylori* isolation and identification. From each patient 2 blood samples were taken; one in an EDTA containing tube that was stored at 4°C until subsequent preparation for

PCR and another in tubes without EDTA to prepare serum. The serum samples were stored at -70°C until subsequent preparation for serology tests. The control subjects were 374 unrelated individuals who attended Shiraz blood transfusion organization for blood donation. They had been confirmed as not having any gastric disorders by clinical presentation and the absence of *H. pylori* by serology tests. Written informed consent was obtained from each individual. 10 ml blood collected in EDTA and stored at 4 °C until preparation for PCR.

Serology Test. To perform *H. pylori* serology test, Enzyme Linked Immunosorbent Assay (ELISA) was used to study the presence of IgG antibodies to the whole cell in patient's serum according to the manufacturer's instruction (*H. pylori* IgG, MP Biomedicals- Italy).

Isolation of *H. pylori.* Biopsy samples from patients were gently homogenized and cultured on rapid urease test media and Brucella agar base (Merck, Germany) supplemented with 10% lysed horse blood and amphotericin B (2 µg/L), trimethoprim (5 µg/L) and nalidixic acid (10 µg/L). The cultures were kept in a microaerophilic atmosphere (6% O2, 7.1% CO_2, 7.1% H_2, and 79.8% N_2) at 37 °C for 5-10 days. Then the isolates were confirmed as *H. pylori* by positive oxides, catalase and rapid urease tests. The samples were also evaluated for the presence of *H. pylori* by Gram staining and rapid urease tests. If both tests were positive simultaneously, the sample was considered *H. pylori* positive.

DNA Extraction. Blood samples containing EDTA were poured in a 50 ml screw capped plastic tube and DNA was extracted from blood lymphocytes using a 'salting out' method (18).

Typing of IL-1 and IL-8 Gene Polymorphism Using RFLP-PCR. The polymorphic region containing the *Taq*I restriction site at position +3953 of the IL-1β gene was amplified using the following primers: forward, 5′-GTTGTCATCAGACTTTGACC-3′; reverse, 5′-TTCAGTTCATATGGACCCAGA-3′ (19) The polymorphic region containing the *Mfe*I restriction site at position +251 of the IL-8 gene was amplified using the following primers: forward, 5′-CCCAAGCTTGTGTGCTCTGCTGT-3′; reverse, 5′-GATTCTGCTCTTATGCCTCA-3′. All PCR were performed in a 10 µl mixture containing: 1x PCR buffer, 0.2 mM dNTP (Cinnagene, Iran), 0.5U Taq polymerase, 2 mM $MgCl_2$, 5 pM of each primer and 1 µl of the DNA sample. The PCR amplification included an initial denaturation step at 95°C for 2 min followed by: 1 cycle at 95°C for 2 minutes, 55°C for 2 minutes, and 72°C for 1 minute, then 32 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and a final extension at 72°C for 10 minutes for IL-1ß, and 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute and a final extension at 72°C for 10 minutes for IL-8. The presence of the 249-bp and 816-bp fragments for IL-1β and IL-8, respectively, was confirmed by electrophoresis on agarose gel and staining with ethidium bromide. The PCR amplified products of IL-1β and IL-8 were digested overnight with 8 units of *Taq*I (MBI, Fermentas, Lithuania) at 65°C and 4 units of *Mfe*I at 37°C, respectively, in appropriate buffers recommended by the supplier (MBI, Fermentas, Lithuania). The digests were analyzed by electrophoresis in a 2% agarose gel with 1x Tris-Acetate-EDTD buffer followed by ethidium bromide staining.

In position +3953 within exon 5 of the IL-1β gene there is a single base pair polymorphism, therefore *Taq*I digestion of the 249-bp fragments results in products that either remain intact (allele 2) or will be cut into two fragments of 135 bp and 114 bp (allele 1)

(Figure 1). The amplified fragment of the IL-8 promoter possesses an *Mfe*I site at the 520th base. The -251 A/T alleles were differentiated by *Mfe*I digestion, producing two fragments of 296 and 520 bp in the -251 A allele (Figure 2). The PCR products from the AA homozygotes were fully digested by *Mfe*I, while those from AT heterozygotes were half digested, and those from TT homozygotes were not digested at all.

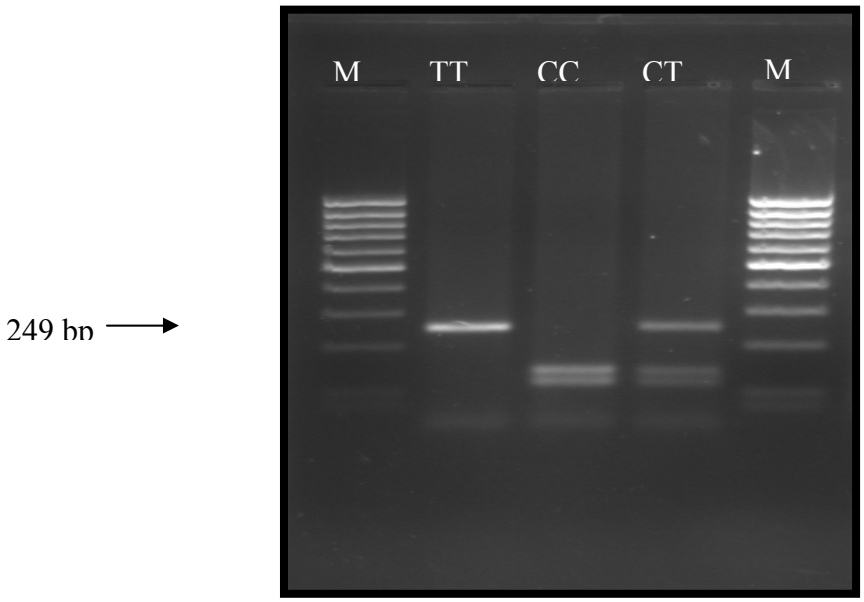

Figure 1. Differentiation of the IL-1+3953C/T promoters by *Taq*I digestion; M: ladder, TT: Thymine Thymine, CC: Cytosine Cytosine, CT: Cytosine Thymine

Statistical Analysis. Allele and genotype frequencies were calculated by direct gene counting. Statistical analysis of the differences between groups was determined by χ^2 test using EPI 2000 and SPSS software version 13. P values less than 0.05 were considered significant. The study power was calculated for each allele and genotype. The Hardy-Weinberg equilibrium was assessed statistically for both genes.

RESULTS

The Patients' Subpopulations. The study group consisting of unrelated individuals with clinical gastric problems was classified into 3 categories on the basis of endoscopic findings and bacterial culture as follows: *H. pylori*$^+$ non-ulcerative gastritis$^+$ (n=54), *H. pylori*$^+$ulcerative gastritis$^+$ (n=36) and *H. pylori*$^-$ non-ulcerative gastritis$^+$ (n=35). The subjects selected for this study were all from the same geographical location, i.e. the southern part of Iran.

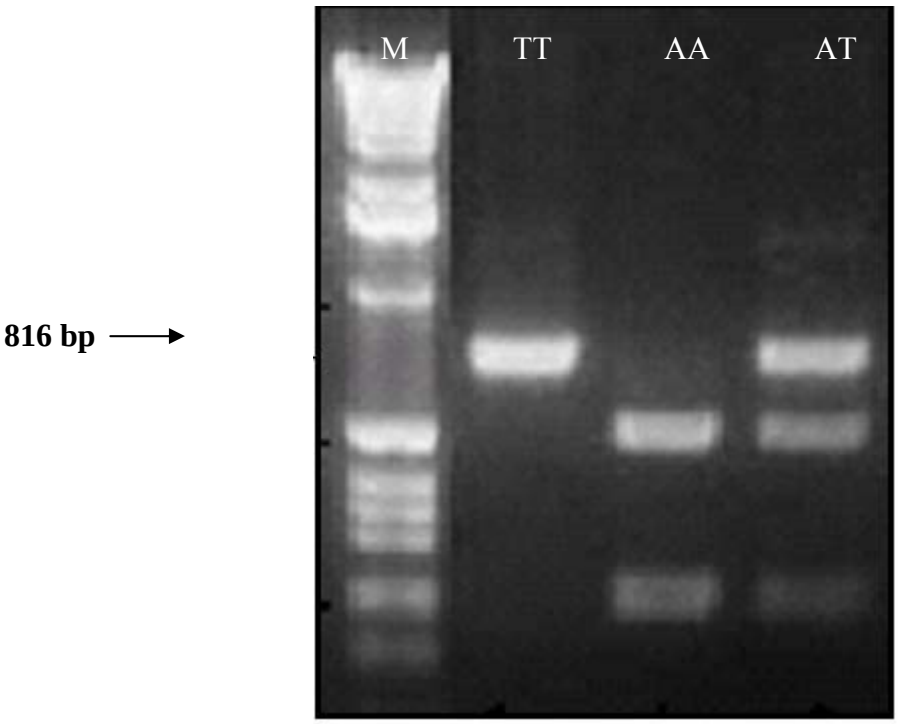

Figure 2. Differentiation of the IL-8-251A/T promoters by *Mfe*I digestion; M: ladder, TT: Thymine Thymine, CC: Cytosine Cytosine, CT: Cytosine Thymine

Distributions of Genotypes and Haplotypes in the IL-1ß and IL-8. Table 1 shows the genotype and allelic distributions of two polymorphic loci in IL-8 and IL-1β in the subpopulations of *H. pylori*$^{+}$ulcerative gastritis^{+} and *H. pylori*$^{+}$ non-ulcerative gastritis^{+}. No significant differences in the allele or genotype frequencies could be demonstrated between these two groups of patients.

Table 1. The frequencies of IL-1β and IL-8 genotypes and alleles in *H. pylori*$^+$ ulcerative gastritis$^+$ (group A) and *H. pylori*$^+$ non-ulcerative gastritis$^+$ patients (group B).

Cytokine genotypes	group A patients n (%)	group B patients n (%)	P value[a]
IL-1ß(+3953)			
Genotype			
CC	18 (50%)	28 (51.9%)	0.86
CT	13 (36.1%)	22 (40.7%)	0.65
TT	5 (13.9%)	4 (7.4%)	0.31
Total	36	54	
Allele			
C	49 (68.1%)	78 (72 %)	0.55
T	23 (31.9%)	30 (28%)	
IL-8(-251)			
Genotype			
AA	8 (22.2%)	11 (20.4%)	0.83
AT	16 (44.5%)	25 (46.3%)	0.86
TT	12 (33.3%)	18 (33.3%)	1.00
Total	36	54	
Allele			
A	32 (30.8%)	47 (43.5%)	0.90
T	40 (69.2%)	61 (56.5%)	

[*]P values were determined by χ^2 test

As it has been shown in Table 2, the frequencies of the TT genotype for IL-1β were significantly increased in *H. pylori* $^+$ ulcerative gastritis$^+$ group compared to the control group ($p<0.05$). There were no significant differences in any observed allele distribution of IL-1β +3953 polymorphisms between the two study groups. There were also no significant differences in any allele or genotype distribution of IL-8 polymorphism between the two study groups.

Table 2. The frequencies of IL-1β and IL-8 genotypes and alleles in *H. pylori*$^{+}$ ulcerative gastritis^{+} patients (group A) and controls.

	group A patients	controls	P value[a]
Cytokine genotypes	n (%)	n (%)	
IL-1ß(+3953)			
Genotype			
CC	18 (50%)	214 (57.2%)	0.40
CT	13 (36.1%)	142 (38%)	0.83
TT	5 (13.9%)	18 (4.8%)	0.02
Total	36	374	
Allele			
C	49 (68.1%)	570 (76.2%)	0.20
T	23 (31.9%)	178 (23.8%)	
IL-8(-251)			
Genotype			
AA	8 (22.2%)	79 (21.3%)	0.89
AT	16 (44.5%)	190 (51.2%)	0.44
TT	12 (33.3%)	102 (27.5%)	0.89
Total	36	371	
Allele			
A	32 (30.8%)	348 (46.9%)	0.69
T	40 (69.2%)	394 (53.1%)	

*P values were determined by χ^2 test

According to Table 3, no significant differences in the allele or genotype frequencies for IL-8 and IL-1β were seen between the two subpopulations of *H. pylori* $^{+}$ non-ulcerative gastritis^{+} and *H. pylori* $^{-}$ non-ulcerative gastritis^{+} patients.

Table 3. The frequencies of IL-1β and IL-8 genotypes and alleles in *H. pylori*$^+$ non-ulcerative gastritis$^+$ (group B) and *H. pylori*- non-ulcerative gastritis$^+$ patients (group C).

	group B patients	group C patients	P value[a]
Cytokine genotypes	n (%)	n (%)	
IL-1ß(+3953)			
Genotype			
CC	28 (51.9%)	18 (51.4%)	0.76
CT	22 (40.7%)	15 (42.8%)	0.65
TT	4 (7.4%)	2 (5.8%)	0.29
Total	54	35	
Allele			
C	78 (72%)	47 (67.1%)	0.46
T	30 (28%)	23 (32.9%)	
IL-8(-251)			
Genotype			
AA	11 (20.4%)	3 (9.1%)	0.17
AT	25 (46.3%)	18 (54.6%)	0.46
TT	18 (33.3%)	12 (36.4%)	0.77
Total	54	33	
Allele			
A	47 (43.5%)	24 (36.4%)	0.35
T	61 (56.5%)	42 (63.6%)	

[a]P values were determined by χ^2 test

No significant differences in the allele or genotype frequencies for IL-8 and IL-1β in *H. pylori*$^+$non-ulcerative gastritis$^+$ patients compared to the normal population were demonstrated (Table 4). The distribution of each of the genotypic variants satisfied Hardy-Weinberg equilibrium.

Table 4. The frequencies of IL-1β and IL-8 genotypes and alleles in *H. pylori*+ non-ulcerative gastritis+ patients (group B) and controls.

	group B patients	controls	P value[a]
Cytokine genotypes	n (%)	n (%)	
IL-1ß(+3953)			
Genotype			
CC	28 (51.9%)	214 (57.2%)	0.46
CT	22 (40.7%)	142 (38.0%)	0.70
TT	4 (7.3%)	18 (4.8%)	0.42
Total	54	374	
Allele			
C	78 (72.2%)	570 (76.2%)	0.37
T	30 (27.7%)	178 (23.8%)	
IL-8(-251)			
Genotype			
AA	11 (20.4%)	79 (21.3%)	0.88
AT	25 (46.3%)	190 (51.2%)	0.50
TT	18 (33.3%)	102 (27.5%)	0.37
Total	54	371	
Allele			
A	47 (43.5%)	348 (46.9%)	0.51
T	61 (56.5%)	394 (53.1%)	

*P values were determined by χ^2 test

DISCUSSION

Although more than half of the world's population is infected with *H. pylori*, most people remain asymptomatic. It has been shown that allelic variants in cytokine genes influence gene expression and subsequently the susceptibility to and severity of various infectious diseases (20). Since gastric mucosal inflammation underlies the pathophysiology of *H. pylori* associated gastroduodenal diseases, it seems that production and release of cytokines have a key role in the inflammatory cascade. However, current re-

search suggests that several bacterial virulence genes such as *CagA* and *VacA,* as well as the individual host's genetic predisposition, are factors that influence the progression of the disease (21). IL-1β, a potent inhibitor of gastric acid, is speculated to be responsible for the changes in the gastric environment upon *H. pylori* infection (5). In the present study, the association between IL-1β +3953 gene polymorphism and *H. pylori* induced non-ulcerative gastritis was investigated and no association was found between chronic active gastritis, and the frequencies of IL-1β +3953 alleles or a specific genotype in this position (p=0.367) (Table 4). The frequencies of CC, CT and TT genotypes and T and C alleles were further investigated in *H. pylori*$^{+}$ non-ulcerative gastritis^{+} patients and *H. pylori*$^{-}$non-ulcerative gastritis^{+} patients (Table 3) and no significant association was observed. Although these findings might suggest that the importance of the IL-1β +3953 alleles is not significant in Iranian population compared with their counterparts from other parts of the world, it is possible that polymorphisms in other positions (-511 and -31) in case of *H. pylori* induced chronic gastritis or other undefined markers within the IL-1β gene are more related to gastritis among Iranians. Watanabe et al. have shown that IL-1 plays a major role in *H. pylori* infection by simultaneously promoting the inflammatory response and suppressing acid secretion (22). In some studies, IL-1β has been described as a factor for gastric cancer development (23-25). However, Zambon et al. showed that neither IL-1β nor IL-1RN genotypes were involved in any aspect of mucosal inflammation, i.e. polymorphonuclear or mononuclear cell infiltrate. This was in contrast to the significant association of inflammation with *H. pylori* virulence factors, *cagA* and *vacA* in their study. They suggested that the mechanism by which IL-1RN and IL-1β gene polymorphisms might influence gastric mucosal inflammation may be related to a different IL-1 synthesis, secretion and activity (26). No significant association was found between the frequencies of IL-1β +3953 alleles and duodenal ulcer disease when *H. pylori* positive ulcer positive patients were compared to the normal population in our study. However, investigation in a larger population and on other polymorphic loci of IL-1β could be recommended. Besides, TT genotype was found as a risk factor for duodenal ulcer disease when *H. pylori* positive ulcer positive patients were compared to the normal population (Table 2) (p=0.02). This result seems to be logical and could be explained by the fact that +3953 T alleles enhance IL-1β production in man (27). The results of the present study suggest that the +3953 TT which is the high producing genotype of IL-1β is an important host biological factor influencing the clinical outcome which results in duodenal ulcer in southern Iran. The frequencies of IL-1β genotypes and alleles in *H. pylori* positive ulcer positive patients under study were also compared to *H. pylori* positive patients with non-ulcerative gastritis (Table 1) and no significant associations between alleles and genotypes were observed. As level of IL-1β is higher in duodenal ulcer patients compared to the non-ulcerative gastritis patients in the antrum area (28), the results could not be explained by a difference in IL-1β expression. This study also focused on determining an association between IL-8 along with IL-1 and *H. pylori* induced duodenal ulcer and non-ulcerative gastritis in an Iranian population in comparison with the normal group. No significant association was found between the frequencies of IL-8 -251, and A allele which is a high producing allele of IL-8 (17) and *H. pylori* induced duodenal ulcer compared to normal population (Table 2). In a study of Hungarians, an AT heterozygote mutant variant was detected with a significantly higher frequency among the ulcerative patients (29). This could be explained by the fact that there are population specific differences in this case. Although the p values were so high that even improving the sample size does not seem to be effective in

changing the results, this study could be carried out with a larger population size. No significant association was observed when *H. pylori* positive patients with non-ulcerative gastritis were compared to the normal population (Table 4). A study performed in 2005 on a group of Thai patients showed that gastric mucosal IL-8 levels were significantly higher in *H. pylori*-positive cases than in the negative ones (30). In 2004 it was reported by a group of Hungarian scientists that an IL-8 A/T heterozygote mutant variant had been detected with a significantly higher frequency among the ulcerative patients (65.22%) in comparison with 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 than in the patients (44.6% vs. 15.9%). Analysis of the genetic predisposition to increased cytokine production showed a significant association for IL-8 polymorphism. This observation draws attention to the possible importance of IL-8 polymorphism as a genetic predisposing factor in the patho-mechanism of *H. pylori*-induced duodenal ulcer disease, and the relative protection from duodenal ulcer disease that is associated with the TT genotype (29). When *H. pylori* positive patients with duodenal ulcer were compared to *H. pylori* positive patients with non-ulcerative gastritis, no association was observed (Table 1), also there was no association when *H. pylori* positive patients with non-ulcerative gastritis were compared to *H. pylori* negative patients with non-ulcerative gastritis (Table 3). As IL-8 production is higher in patients with duodenal ulcer than in non-ulcerative gastritis ones (28), it seems to be other functional sites in IL-8 or some pathogenic properties of the organism that interferes with the production of this interleukin. Therefore, studies on other IL-8 polymorphic loci and identifying other functional sites and studying the pathogenic properties of the isolated *H. pylori* strains are recommended. However, the fact that we did not observe an association and failed to confirm some previous studies might have been due to a Type II error (false-negative). This is, however, unlikely because our sample had more than 80% power to detect the relative risks similar to other studies at 5% significant level. The failure to replicate reported associations is a common event in the search for genetic determinants of complex diseases, either due to genuine population heterogeneity or to a different sort of bias (31). The lack of replication in our population may alternatively be explained by different racial composition of our population, or the presence of environmental factors to which other populations, but not the Iranian population, are exposed to. In addition, genetic differences are known to exist between different ethnic groups, such as African Americans and Caucasians.

In conclusion, the crucial role of the host immune response to *H. pylori* infection was underlined and the importance of polymorphisms in IL-1β +3953 as a factor which gives rise to ulcerative gastritis was confirmed in the present study. Moreover, the role of other bacterial and host response factors, such as bacterial adherence peptides, host chemokines, and genes involved in gastric acid secretion, must be further elucidated in the light of differences in the population structure and the genetic diversity among populations.

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