Evaluation of H.pylori Infection and IL23R Gene Polymorphism in Dyspeptic Subjects

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Abstract: CagA strains of *H.pylori* (Hp) are known to be associated with gastroduodenal diseases. Polymorphisms in inflammation related genes, such as cytokines and their receptors, were thought to partly determine the outcome of Hp infection and the progression of gastritis. It is supposed that interleukin 23 receptor (IL23R), a basic cytokine receptor in the inflammatory IL-17/IL-23 axis, may be related to gastritis. In the present study, we evaluated the association of IL23R +2199 rs10889677 polymorphism and cagA positivity with chronic gastritis. In addition, we studied the infiltration of polymorphonuclear (PMN) and mononuclear (MN) Leukocytes into surrounding tissues of corpus. Biopsies taken from the corpus of the patients were classified as two groups: Hp-infected and Hp-uninfected. The severity of gastritis was graded from normal to severe, chronic gastritis and chronic active gastritis. Virulence factor, cagA, was evaluated using PCR and the polymorphism in IL23R was investigated by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). AA and AC carriers of IL23R +2199 polymorphism, but not CC genotype in Hp-uninfected patients, were not associated with cellular infiltration and gastritis in both groups ($p > 0.05$). CagA positivity was significantly associated with increased risk of PMN ($P= 0.013$), but not with MN infiltration ($P= 0.069$). Also gastritis was found to be associated with cagA positivity ($P= 0.044$). Our results show decreased Hp infection probability in patients with CC genotype of 2199 +IL23R. According to the clinical and pathological features in Hp-infected group, IL23R polymorphism doesn't influence chronic gastritis and chronic active gastritis.

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Introduction

Hp is a spiral-shaped Gram negative flagellate bacterium that colonizes the gastric mucosa of approximately 50 % of the world's population (1, 2). Hp infection induces inflammation in gastric mucosa that involved in chronic gastritis (3, 4). Hp-associated inflammatory response is defined by an immense mucosal infiltration of macrophages, PMNs, T cells, and plasma cells (5, 6).Gastritis may progress to other steps Such as, gastric atrophy, intestinal metaplasia, and gastric cancer (7-9). It may also lead to precancerous lesions looking like monoclonal lymphocytic proliferation, lymphoid follicle (LF) development and later primary gastric lymphoma (PGL) which develop only in a portion of individual with gastritis because of multifactorial effects of host virulence and bacterial factors that vary among different racial and social groups (10). Among bacterial factors, studies in developed countries mention that cagA-Positive strains of Hp are more often associated to gastroduodenal diseases than cagA-negative strains (11-14); however this has not been proven in developing countries with a high widespread of Hp infection. Among host factors several inflammatory proteins including cytokines, growth factors, and chemokines have been known to control adaptive immune response in contrast to Hp infection (15, 16). Firstly, El-Omar was reported an association between gastric cancer risk and interleukin 1 gene cluster polymorphisms (17). Studies from the western world show roles of anti- and pro- inflammatory cytokine genes such as Interleukin-1 beta (IL-1β), Interleukin 1 receptor antagonist (*IL-1RN),* Interleukin-10 *(IL-10),* and tumor necrosis factor alpha (*TNF*-α) gene polymorphisms affect risk in gastritis (18) and

precursors (17, 19). However Asian studies did not find any such association (20-22). IL-23 is a heterodimeric cytokine composed of the p40 and p19 in which p40 is the common subunit shared with Interleukin-12 (IL-12) and p19 is the special subunit with a higher affinity to IL-23R (23).The locus of IL23R gene is on chromosome 1p31 and encodes a subunit of the IL-23 R. The heterodimeric IL-23R compound composed of IL-23R subunit associate with the IL-12Rβ1 subunit shared with the IL-12R complex. One of the most studied polymorphism in IL23R gene is R381Q (rs11209026) is located in the coding sequence of the IL23R and interchange arginine to glutamine in codon 381 (24). Recently, an inflammation pathway of IL-23/IL-17 axis reported to play fundamental role in inflammatory and autoimmune diseases(25), such as psoriasis (26), lupus nephritis(27), and intestinal inflammation (28). The new delineated pathway IL-23/IL-17 has proved to be included in the etiology of several chronic inflammatory diseases such as inflammatory bowel disease (IBD) (29), and ankylosing spondylitis. In addition, there is high level experssion of IL-23 in Hp-infected gastric mucosa (30). IL-23R, as the key component to IL-23R, was shown to play an influential role in the launching, supporting and accelerating of IL-23/IL-17 inflammatory signal transduction pathway (30). In 2006, Duerr et al. indicated a strong relation between Crohn's disease and polymorphisms of the IL23R gene (29). Different genotypes of IL23R gene have been evaluated for association with chronic inflammatory disorders (31). From then, IL23R gene was shown to be an influential gene in many other autoimmune/inflammatory diseases. Among the recognized polymorphisms of IL23R, the functional SNP of +2199A/C (rs10889677) located in the 3´-untranslated region (UTR) has been repeatedly shown to be related to different autoimmune/inflammatory diseases. However, the results are in debate in different diseases. In a study from Hungary, the AA genotype of rs10889677 reported as a risk factor for rheumatoid arthritis (32) However, researchers concluded A allele has a protective role for ankylosing spondylitis (33). Contradictory, some studies indicated that wild type C allele increases the risk to Graves' ophthalmopathy (34) and idiopathic dilated cardiomayopathy (35). Unfortunately, there is no study on this issue from Iran. In the present study we therefore aimed to evaluate an association of IL23R +2199A/C polymorphism with gastritis, using a case-control approach. We also evaluated the association of cagA positivity, IL23R +2199A/C polymorphism, and their interactions with cellular infiltrations and precancerous stages using a case-only approach in a population from central area of Iran.

Material and Methods

A total of 435 patients with nonulcer dyspepsia (NUD) who were undergoing upper gastrointestinal endoscopy were tested for Hp infection using in-house RUT. Hp infected and uninfected patients were determined by the rapid urease test, PCR 16srRNA, urea and histological examination of biopsies taken from the corpus. Patients were classified as Hp-infected only if the three tests were positive and Hp-uninfected if the three tests were negative, respectively. Demographic and clinical data were obtained through interview using a standard clinical pro forma. Exclusion criteria included history of gastric neoplasm or surgery, liver disease, and previous treatment with non-steroidal anti-inflammatory drugs, proton pump inhibitors, antibiotics, or bismuth salts. Informed consents for participation were signed by all the study subjects. The study protocol was approved by the Clinical Research Ethics Committee of the Shahrekord University of Medical Sciences.

Histological examination

Sections of biopsy specimens were embedded 10 % buffered formalin and stained with Hematoxylin and Eosin to examine gastritis and with Giemsa to detect Hp. The histological severity of gastritis was blindly graded from normal to severe based on the grade of PMN and MN infiltration, chronic gastritis and chronic active gastritis according to the Updated Sydney System (36) on a four-point scale: 0, no; 1, mild; 2, moderate; and 3, severe changes.

DNA isolation

Genomic DNA was extracted from biopsies taken from the corpus using Biospin Tissue Genomic DNA Extraction Kit (Bio Flux, Japan). All extracted DNA was resuspended in UltraPure RNAse/DNAse-Free Distilled water.

Genotyping for IL23R +2199A/C (rs10889677) polymorphism

Genotyping analysis IL23R genotyping was performed by PCR-RFLP as reported by Chen *et al* (24). Primer sequences for +2199A/C variation of IL23R gene are as follows: sense 5´-AGGGGATTGCTGGGCCATAT-3´, anti-sense 5´-TGTGCCTGTATGTGTGACCA-3´. The PCR amplification was performed in a total volume of 25 uL mixture containing: 100 ng genomic DNA, 1.0 mM of each primer, 200 mM of each dNTP, 2.0 mM of $MgCl_2$ and 1.0 U Taq DNA polymerase and 10 X Taq buffer (Fermentas) using the Biometra Tgradient 96 (Biometra, Germany). PCR conditions were as follows: denaturation at 95 °C for 5 min, followed by

38 cycles of 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 60 s. A final extension was carried out at 72 °C for 10 min and cooling down to 4 °C. The PCR products were digested by restriction endonuclease MnLI (Fermentas), according to the manufacturer's instructions, at 37°C overnight and then separated by 10% polyacrylamide gel electrophoresis. Gel analysis was performed after staining with ethidium bromide. PCR products were shown to be digested into three types of fragments (Fig. 1). To confirm the genotyping results, selected PCR samples in both groups including samples of each genotype were re-genotyped by other laboratory personnel. There was no difference after re-genotyped the randomly selected samples.

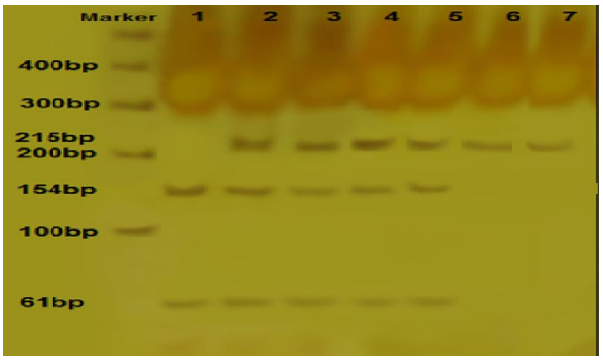

Fig 1: PCR-RFLP polyacrylamide gel electrophoresis of the IL23R +2199A/C (rs10889677) polymorphism indicating the No.1 (CC = 154, 61 bp), 2,3,4,5 (AC = 215, 154, 61 bp), 6,7(AA= 215 bp) genotypes.

Determination of cagA (+/-) in Hp-infected subjects

Amplification for cagA was performed by polymerase chain reaction as reported by Bagheri *et al* (1).Primer sequences for cagA gene are as follows: sense 5′ ATGACTAACGAAACTATTGATC-3′, anti-sense 5′ CAGGATTTTTGATCGCTTTATT-3′. For cagA evaluation, the PCR program comprised 35 cycles of denaturation (at 94 °C for 30 s), annealing (at 56 °C for 30 s, extension at 72 °C for 30 s), and one final extension (at 72 °C for 5 min).

Statistical analysis

Data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL). Hardy–Weinberg equilibrium in all subjects was analyzed with the x^2 goodness-of-fit test before the ensuing analyses. The confounding effects of age and gender were adjusted using conditional logistic regression. Logistic regression analyses were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for gastritis in association with genotypes. Also Statistical analysis was performed by Mann-Whitney Rank Sum test or non paired t test depending on the data set. Values of p <0.05 were considered as significant.

Results

Demographic and clinical characteristics

Genomic DNA was obtained among the193 (44.4%) Hp-infected and 242(55.6%) Hp-uninfected gastritits then the DNA all subjects were genotyped. The demographic data of all subjects were demonstrated in Table 1. There was no significant difference between the two groups with respect to the age and gender distribution ($p > 0.05$)

Table 1: Demographic data of study subjects.

Variable	Hp-infected (%)	Hp-Uninfected (%)	*P* value
Overall	193(44.4%)	242(55.6%)	
Gender Male Female	78(41.9%) 115(46.2%)	108(58.1%) 134(53.8%)	0.377
Age Mean±SD (year)	47.24 ±17.28	48.29 ±19.49	0.556

IL23R +2199CC genotype and Hp infection susceptibility

The frequencies of the polymorphism in cases and controls are shown in Table 2. Frequencies of IL23R +2199 genotypes in Hp-infected (CC, 16.8%; CA, 37.4% and AA, 45.8%) were different from those in Hp-uninfected (CC, 24.8%; CA, 29.2% and AA, 46.0%). Compared with AA genotype and CA genotype, the CC genotype significantly decreased Hp infection risk with ORs of 1.634 (95% CI: 0.985-2.710).

IL23R +2199A/C polymorphisms and gastritis

In our study population, IL23R +2199A/C (rs10889677) variants (AA, AC, CC) evaluated in Hp-infected and Hp-uninfected population ($p > 0.05$). Carriers of IL23R +2199A/C were not associated with chronic active gastritis and chronic gastritis in both groups. Also allele C (AC/CC) was not associated with risk for gastritis development in both group (Table 3 and 4).

IL23R +2199A/C polymorphisms and cellular infiltration

IL23R+2199A/C genotypes evaluated with grade of mononuclear and polymorphonuclear infiltration on Hp-infected and Hp-uninfected groups. The results are compared in Table 5 and Table 6. There was no significant association in both groups ($p > 0.05$).

Table 2. Adjusted Odds Ratios (ORs) and 95% confidence intervals (CIs) for Hp-infected in relation to IL23R +2199 genotypes

Genotype	Hp-infected (%)	Hp-Uninfected (%)	*P* value	*OR*[#] *(95% CI)*
IL23R +2199				
AA	82(45.8%)	93(46.0%)	0.523	1.009(0.674-1.511)
AC	67(37.4%)	59(29.2%)	0.056	0.690(0.449-1.059)
CC	30(16.8%)	50(24.8%)	0.037	1.634(0.985-2.710)

[#] Adjusted for age and gender

Table 3: Odds Ratios (ORs) and 95% Confidence Intervals (CIs) for gastritis in relation to IL23R +2199 genotypes in Hp-infected subjects

IL23R +I2199	Hp-infected (%)	Chronic active Gastritis(%)	Chronic Gastritis(%)	*P* value	*OR*[#] *(95% CI)*
AA	61(48.8%)	28(42.4%)	33(55.9%)	0.131	0.581(0.286-1.179)
AC	43(34.4%)	25(37.9%)	18(30.5%)	0.387	1.389(0.660-2.925)
CC	21(16.8%)	13(19.7%)	8(13.6%)	0.360	1.564(0.598-4.088)
AC/CC**	64(51.2%)	38(57.6%)	26(44.1%)	0.131	1.723(0.848-3.500)

[#] Adjusted for age and gender.
**C allele, common between homozygote (CC) and heterozygote (AC) situation of IL23R +I2199 polymorphism

Table 4: Odds Ratios (ORs) and 95% Confidence Intervals (CIs) for gastritis in relation to IL23R +2199 genotypes in Hp-uninfected subjects

L23R +I2199	Hp-Uninfected (%)	Chronic active Gastritis(%)	Chronic Gastritis(%)	*P* value	OR[#] (95% CI)
AA	28(40.6%)	10(50.0%)	18(36.7%)	0.309	1.722(0.602-4.929)
AC	24(34.8%)	6(30.0%)	18(36.7%)	0.594	0.738(0.241-2.260)
CC	17(24.6%)	4(20.0%)	13(26.5%)	0.571	0.692(0.195-2.455)
AC/CC**	41(59.4%)	10(50.0%)	31(63.3%)	0.309	0.581(0.203-1.662)

[#] Adjusted for age and gender.
**C allele, common between homozygote (CC) and heterozygote (AC) situation of IL23R +I2199 polymorphism

Table 5: Association of IL23R +2199 genotypes with cellular infiltrations in Hp-infected subjects

Genotype	No.	Mononuclear infiltration*	Polymorphonuclear infiltration*
IL-23R		1.33 ±0.664(0-3)	
		1.38 ±0.733(0-3)	
AA	66	1.32 ±0.646 (0-3)	0.47 ±0.561(0-3)
AC	46		0.57 ±0.544(0-3)
CC	22		0.55 ±0.510 (0-3)
P value		0.949	0.556

*The histopathological parameters were scored as: 0, none; 1, mild; 2, moderate; 3, severe

Table 6: Association of IL23R +2199 genotypes with cellular infiltrations in Hp-uninfected subjects

Genotype	No.	Mononuclearin filtration*	Polymorphonuclear infiltration*
IL-23R		1.02 ±0.832 (0-3)	
AA	40	1.27 ±0.667 (0-3)	0.28 ±0.506 (0-3)
AC	26		0.23 ±0.430 (0-3)
CC	22	1.18 ±0.853 (0-3)	0.23 ±0.528 (0-3)
P value		0.388	0.858

*The histopathological parameters were scored as: 0, none; 1, mild; 2, moderate; 3, severe

Table 7: Association of cagA (+/-) with cellular infiltration in Hp-infected subjects

Genotype	No	Mononuclear infiltration*	Polymorphonuclear infiltration*
cagA (+)	96	1.43 ± 0.645 (0-3)	0.60 ± 0.535 (0-3)
cagA (-)	51	1.22 ±0.757 (0-3)	0.37 ±0.528 (0-3)
P value		0.069	0.013

*The histopathological parameters were scored as: 0, none; 1, mild; 2, moderate; 3, severe

HP cagA positivity, cellular infiltration and gastritis

CagA positivity was comparable among Hp-infected subjects with PMN and MN infiltration (Table 7). Patients with PMN infiltration were associated with cagA positivity than were those with cagA negativity (p=0.013). However, MN infiltration was not associated with cagA positivity among Hp-infected subjects (p=0.069). Gastritis was significantly higher in patients with cagA-positive compared to those observed in cagA-negative patients (Table 8), then cagA positivity imparted risk for gastritis (p = 0.044, OR = 2.128, 95% CI = 1.014–4.470).

Table 8: Association of cagA (+/-) with gastritis in Hp-infected subjects

Genotype	No. (%)	Chronic active Gastritis(%)	Chronic Gastritis(%)
cagA (+)	93(68.9%)	55 (76.4%)	38 (60.3%)
cagA (-)	42(31.1%)	17 (23.6%)	25 (39.7%)
P value		0.044	
OR[#] (95% CI)		2.128 (1.014-4.470)	

[#] Adjusted for age and gender.

Discussion

In the present study we found that frequencies of IL23R+2199A/C genotypes and alleles, except for genotype CC, were not comparable in infected patients and uninfected Hp subjects. IL23R+2199CC genotype decreases susceptibility to Hp that may indicate Hp infection is associated with pathway of IL-23/IL-17 axis. Also, we found that variants of IL23R gene, IL23R +2199 CA, IL23R +2199 AA, IL23R +2199 CC, were not associated with gastritis in Hp-uninfected and Hp-infected patients. These findings suggest that IL23R polymorphisms may independent of the presence or absence of Hp has no effect on chronic active gastritis and chronic gastritis. Whereas, one study suggest IL23R +2199CC, genotype significantly decreased gastric cancer risk and some of IL23R+2199A/C genotypes associated with increased risk of certain subtypes of gastric cancer, but not with all of them (24). This may indicate that the effect of IL23R polymorphism on inflammatory processes varied with inflammatory Steps. This result is consistent with the different mechanisms of inflammation so that in precancerous and gastritis stages some of cytokines are dominant and have specific role in start of inflammation process but as stage Progress, another cytokines participate, therefore, we observe many cytokines affection in the latter stages. As there is no enough biological report that revealed the function of IL23R +2199 polymorphism, especially in precancerous, it is difficult to fully elucidate this phenomenon about our study. IL23R+2199A/C allele carrier genotypes were not found to be associated with neutrophil and lymphocyte infiltration in HP-infected and HP-uninfected which is consistent with the above. Our results may corroborate those of Horvath et al (37)., who studies indicate that IL-23 makes a contribution to both Th1 and Th17 responses during Hp infection especially during the chronic stage of infection (37). We suggest that may be an induced inflammatory response by Hp that caused by Th1 but not with Th17, So IL23R polymorphisms does not affect the inflammatory response in our study. Another study suggests the presence of CagA contributes to regulation of cytokine production in DCsco-cultured with Hp in the mouse model of infection, this data suggests that IL-23 makes a minor contribution to the development of chronic gastritis in this model of Hp infection (37). Contradictory, a study from Iran reported a higher levels of IL-23 in Hp-infected patients (including DU and AS groups) than in the Hp negative control group (38). Also studies revealed that the inflamed gastric mucosa of Hp-positive patients could secrete IL-23 (39). However another study reported no significant difference in mucosal IL-17 and IL-23 mRNA expression between Hp-infected and uninfected patients (1) that accordance with our study. Gastric mucosa of patients with both duodenal and gastric ulcers was equally potent for secretion of IL-23 compared with patients with chronic active gastritis with no signs of peptic ulcer disease. The release of IL-23 was greater by Hp-infected gastric mucosa than by gastric mucosa not infected by Hp mainly for patients with chronic gastritis and only after stimulation with LPS. Similar findings have been published elsewhere (30, 40). Nevertheless, LPS of Hp has also been described to behave in a different manner (41). CagA positivity was more frequently associated with neutrophil infiltration but we don't found association between cagA positivity and lymphocyte infiltration. Also cagA positivity correlated with gastritis that shows cagA virulence factor may have initiated role in precancerous stages especially in chronic active gastritis and chronic gastritis. The results of one study also showed that the serum levels of IL-23 were not influenced by the cagA status of Hp. These results indicate that bacterial factors other than cagA may act as inducers of IL-23 (38).

In this study in particular, we have demonstrated that polymorphism of IL 23R doesn't play a role in control of Hp-induced gastritis. Whether IL23R is an independent mediator in the pathogenesis of gastritis or not cannot be excluded with safety from the presented findings. Further investigation is necessary to elucidate fully the exact role of IL23R in the pathogenesis of gastritis. Our results highlight the importance of cagA virulence factors in explaining differential outcomes after infection with Hp. However, the importance of host genetic factors rather than Hp virulence in explaining variations in outcomes after infection in different Asian countries has been reported (42).

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