From genes polymorphisms to mucosal expression of cytokines: evaluating IL-23/IL-17 axis in adult patients with gastritis

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Abstract

Background and Objective: Chronic inflammation is the typical sign of gastritis that may shift into gastric cancer. IL-17A and IL-17F as a novel inflammatory cytokines subset of CD4+Th play the main role in inflammation. A key cytokine receptor in the inflammatory IL-17/IL-23 axis, the interleukin 23 receptor (IL23R), may be related to gastritis. We evaluated the correspondence between IL-17A G197A, IL-17F A7488G and IL23R+2199 A/C polymorphisms with TGF-β1, IL-6, IL-17, IL-21 and IL-23 mucosal mRNAs expression in uninfected *H. Pylori* (HP) chronic gastritis patients.

Materials and Methods: Total RNA and genomic DNA were separated from gastric biopsies of 44 patients with gastritis. Subsequently, mucosal mRNAs expression of TGF-β1, IL-6, IL-17, IL-21 and IL-23 were assessed by real-time PCR. To polymorphisms determination of IL-17A G197A, IL-17F A7488G and IL-23R +2199A/C the PCR-RFLP was used in gastric biopsies.

Results: Results point that IL-17A G197A, IL-17F A7488G and IL23R +2199A/C polymorphisms did not influence the mucosal expression of TGF-β1, IL-6, IL-17 and IL-21 ($p > 0.05$). In an opposite result, we don't find a correspondence between IL-17A G197A, IL-17F A7488G polymorphisms and mucosal expression of IL-23 ($p > 0.05$). In a contrary, we found a correlation between IL23R +2199A/C polymorphism and mucosal expression of IL-23 in patients with chronic gastritis ($p < 0.05$).

Conclusion: These findings propose that IL23R +2199A/C polymorphism may change the mucosal expression of IL-23 pattern in patients with gastritis disease in the absence of HP, but to support the conclusion, more research may be required.

Keywords: Cytokines; polymorphism; gastritis; IL-23, IL-17.

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Introduction

There are a connection between inflammation in gastric mucosa, gastritis [1, 2] and infiltration immune cells, such as mononuclear and polynuclear cells, in the gastric mucosa that keeps up and expands the local inflammation [3, 4]. Polymorphisms in a few cytokines and their receptors are thought to play a part in outcome of gastric inflammation[2, 5-7]. It is proposed that polymorphisms of inflammatory cytokine, causes inflammatory response of gastric mucosa, like TNF-α, IL-1β, IL-8, IL-10 and IL-17A, have been related to an increased risk of gastric cancer and peptic ulcer in the digestive system [8-10]. T helper 17 cells (Th17) are IL-17 producing Th cells[11,12]. Recent studies revealed an inflammatory pathway of IL-23/IL-17 axis play the pivotal role in inflammatory and autoimmune diseases such as long-lasting autoimmune disease, inflammation of the kidney and intestinal inflammation [13]. The IL-23 is responsible for stimulating of Th17 in this inflammatory pathway[13].

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IL-23R play a significant role in the starting, keeping up and accelerating in the signaling of IL-23/IL-17 inflammatory axis [14]. A strong association was found by Duerr et al between Crohn's disease (CD) and polymorphisms in the IL23R gene in 2006 [15]. The result of previous studies shows the functional SNP of IL23R +2199A/C (rs10889677) located in the 3-untranslated region (UTR) was repeatedly proved to be associated with diverse autoimmune and inflammatory diseases. However, similar studies have different outcomes in various diseases [15-17]. On the other hand, IL-17F has the most homology with IL-17A, and like IL-17A, triggers a wide range of cytokines and adhesion molecules by different types of human cells [18] However, IL-17A has more activities than IL-17F [19]. Various studies on colorectal, prostate, breast and gastric tumor cells show an increased expression of IL-17A [20, 21]. Polymorphisms of IL-17A G197A (rs2275913) and IL-17F A7488G (rs763780) have recently been identified to be associated with the susceptibility to ulcerative colitis and rheumatoid arthritis respectively [22, 23]. Genetic variations in inflammation-related genes, particularly cytokines and their receptors are thought to play a role in the outcome of infection and the development of gastritis. Considering that chronic gastritis and genetic predisposition are important parts of a complex interaction to initiate gastric carcinogenesis, and these polymorphisms have been associated with gastric cancer in these patients. As a result, our study suggests that these polymorphisms may alter the mucosal cytokine IL-23/IL-17 axis pattern by regulating TGF-β1, IL-6, IL-17, IL-21 and IL-23 mucosal mRNAs produced and, probably, the consequent release of these cytokines. Ultimately, these changes can lead to development of severe histological changes in the gastric mucosa patients with gastritis without HP role (Fig.1).

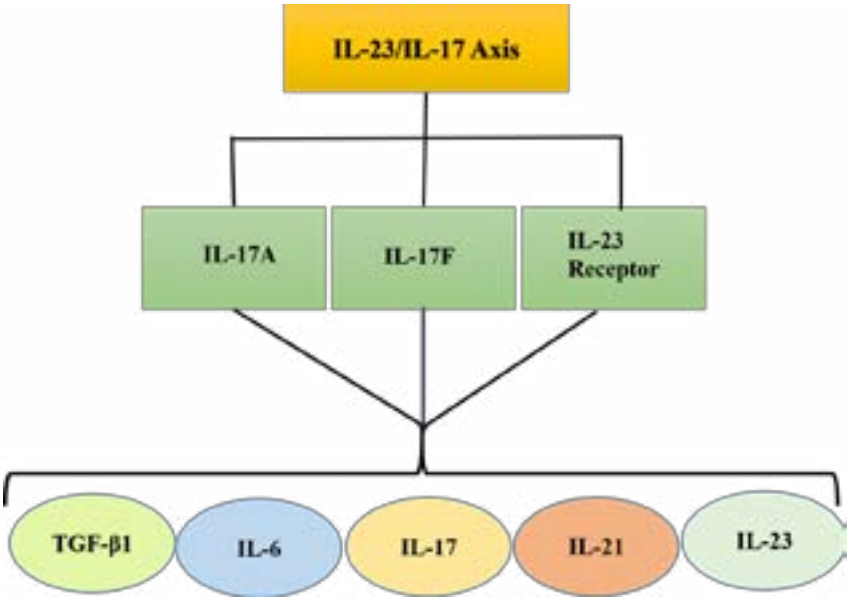

Fig.1. Schematic figure showing possible mechanism of polymorphism in IL-17A, IL-17F and IL-23 receptor that may affect some of the IL-17/IL-23 axis. IL-17/IL-23 axis is the key inflammatory pathway including main cytokines as TGF-β, IL-6, IL-17, IL-21 and IL-23. Polymorphisms in IL-17A, IL-17F and IL-23R were shown as pivotal site of IL-17/IL-23 that may affect expression of TGF-β, IL-6, IL-17, IL-21 and IL-23 separately.

Subjects and methods

Among patients undergoing upper gastrointestinal endoscopy a total of 44 gastritis patients without HP infection, 20 men (45.5 percent) and 24 women (54.5 percent), were selected and histological examination of biopsies taken from the corpus. The process of obtaining data, including demographic and clinical data was done through interviews using a standard clinical pro forma. Exclusion criteria for the study was include the history of gastric neoplasm, surgery, Enterohepatic-related problems, and previous treatment with non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors, antibiotics, or bismuth salts. Informed consents for participation were signed with full knowledge by all the study patients. The demographic data of all subjects were demonstrated in Table 1. There was no significant difference through the subjects with respect to the age and gender distribution, so that patients don't have the same age range.

Table 1. Demographic data of study subjects

Variable	Frequency	Age Mean ± SD (year)
Overall	44 (100%)	-
Gender		
Male	20 (45. 5%)	38.8 ±13.97
Female	24 (54.5%)	37.58 ± 17.27

Ethic approval

The study was approved by the human research ethics committee at the Shahrekord University of Medical Sciences and informed consent was obtained from each volunteer before participation.

Histological examination

Gastric biopsy specimens were embedded in 10 % buffered formalin to preparation of paraffin block, then stained with Hematoxylin and Eosin (H&E) to examine gastritis. The histological examination of gastritis was blindly performed according to the Updated Sydney System.

DNA isolation

Genomic DNA was purified from biopsies taken the corpus using Biospin Tissue Genomic DNA Extraction Kit (Bio Flux, Japan). All extracted DNA was resuspended in Ultra-Pure RNase/DNase-Free Distilled water.

Genotype Determination for IL-17A G197A, IL-17F A7488G and IL23R +2199A/C polymorphisms

Genotyping analysis of IL-17A, IL-17F and IL23R genotyping were performed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) as reported by Wu et al and Chen B et al 24, 25. The PCR cycles was performed in a total volume of 25 μL mixture containing: 100 ng genomic DNA, 1.0 μM of each primer, 200 μM of each dNTP, 2.0 mM of MgCl2 and 1.0 U Taq DNA polymerase and 10X Taq buffer (Fermentas) using the BiometraTgradient 96 (Biometra, Germany). PCR conditions were as follows: Denaturation at 96 °C for 6 min, followed by 34 cycles of 95 °C for 60 s, 65 °C for 60 s, and 72 °C for 52 s. A final extension was carried out at 72 °C for 6 min for IL-17A; denaturation at 95 °C for 6 min, followed by 34 cycles of 95 °C for 63 s, 65 °C for 56 s, and 72 °C for 60 s. A final extension was carried out at 72 °C for 6 min for IL-17F; 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 11 min for IL23R then products cooling down to 4 °C. The PCR products were digested by restriction endonuclease XagI (Fermentas) for IL-17A G197A, NlaIII (Fermentas) for IL-17F A7488G and MnLI (Fermentas) for IL23R +2199A/C according to the manufacturer's instructions, at 37°C overnight and then separated by 10% polyacrylamide gel electrophoresis. Gene analysis was performed on gel stained with Ethidium Bromide (EtBr). PCR products were shown to be digested into three types of fragments. To confirm the genotyping results, selected PCR samples in patient, including samples of each genotype were re-genotyped by other laboratory personnel. No significant difference was found after genotyping the randomly selected samples (Fig.2).

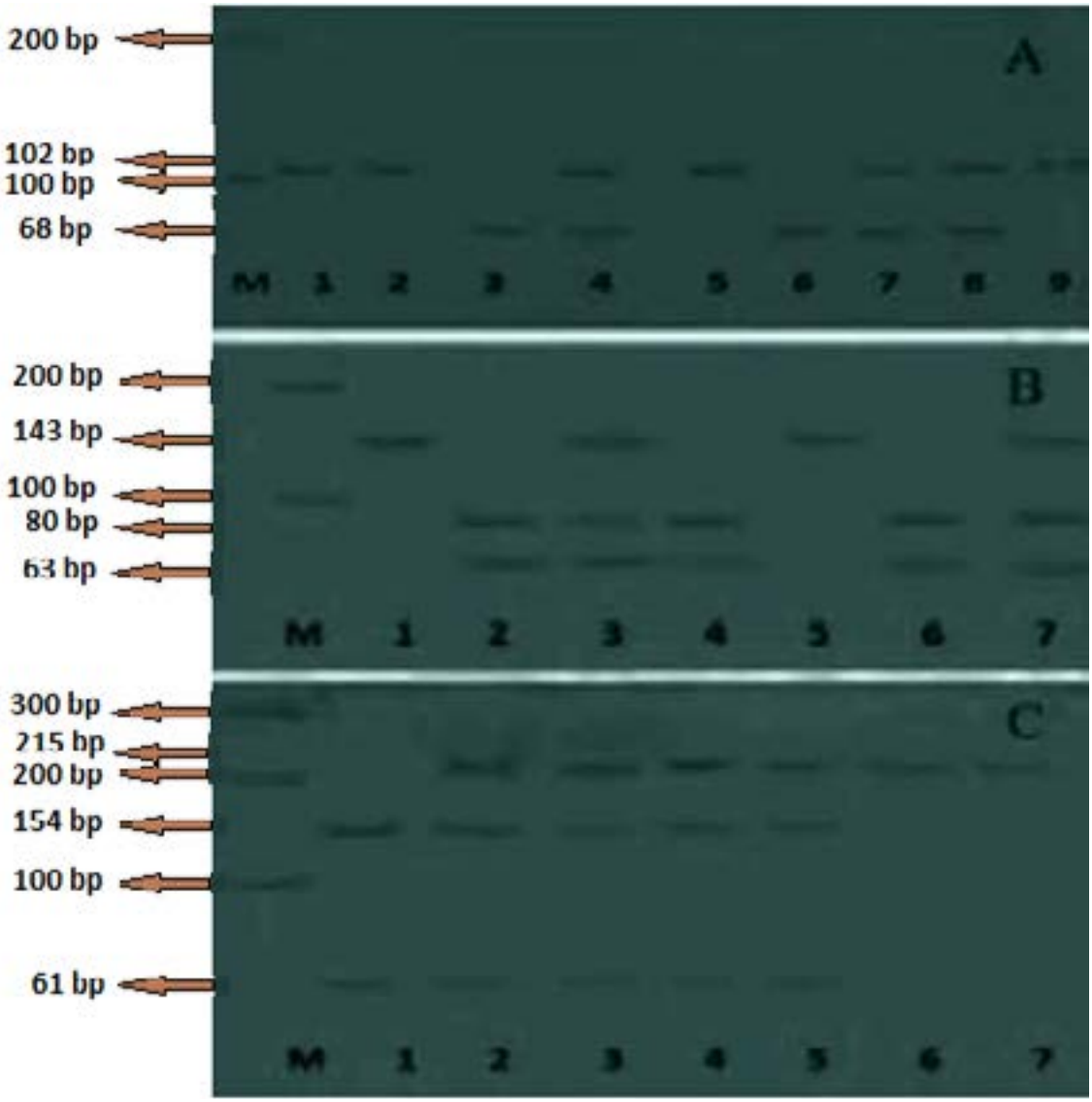

Fig. 2. PCR-RFLP polyacrylamide gel electrophoresis of the IL-17A G197A and IL-17F A7488G and IL23R +2199A/C (rs10889677) polymorphism indicating: (A) IL-17A G197A, No. 1, 2, 5, 9 (AA = 102 bp) 4, 7, 8 (AC = 102, 68, 34 bp) 3, 6 (GG = 68, 34 bp) genotypes and (B) IL-17F A7488G, No. 2, 4, 6 (AA = 80, 63 bp) 3, 7 (GA = 143, 80, 63 bp) 1, 5 (GG = 143 bp) genotypes and (C) IL23R +21199A/C (re10889677), No 1 (CC = 154, 61 bp), 2, 3, 4, 5 (AC = 215, 154, 61 bp), 6, 7 (AA = 215 bp) genotypes

Quantitative analysis for TGF-β1, IL-6, IL-17, IL-21 and IL-23 in the gastric mucosa using real-time PCR

In the current study, we selected biopsies from all 44 biopsies from under study population, patients with either G197A, A7488G and +2199A/C polymorphisms in IL-17A, IL-17F and IL23R matched by sex, age and pathology. Total RNA was isolated from whole gastric biopsy specimens using total RNA extraction biozol (bioflux, Japan). An aliquot containing 0.2 µg of total RNA was used for the reverse transcription reaction, which was conducted using the superscript first-strand cDNA synthesis system (Fermentas, Finland) according to the manufacturer's instructions. The sequences of oligonucleotide primer and probe are specified in Table 2. The relative amount of TGF-β1, IL-6, IL-17, IL-21and IL-23mRNAs levels were performed using a Rotor-Gene 3000 (Corbett). Real-time-PCR reactions were conducted in a total volume of 25 µl containing 3 µl of synthesized cDNA solution, 12.5 µl of 2x Rotor-Gene Probe PCR Master Mix (Qiagen, Germany), 500 nM of each primer and 250 nM of the TaqMan probe. Amplification program included a pre warming step (10 min at 94 ºC), denaturation step (94 °C for 15 s) and an annealing/extension step (60 °C for 60 s). Relative quantification of cytokine to β-actin (cytokine mRNA/β-actin mRNA) was determined using the $2^{-\Delta C_T}$ method [26].

Table 2. Primer and probe sequences employed in this study

Gene	Primer and probe sequence
β-actin 3	Forward 5-AGCCTCGCCTTTGCCGA- Reverse 5-CTGGTGCCTGGGGCG-3 Probe FAM-CCGCCGCCCGTCCACACCCGCC-TAMRA
TGF-β1	Forward 5-CAGCAACAATTCCTGGCGATA-3 Reverse 5-AAGGCGAAAGCCCTCAATTT-3 Probe FAM-CTGCTGGCACCCAGCGACTCG-TAMRA
IL-6	Forward 5-GGTACATCCTCGACGGCATCT-3 Reverse 5-GTGCCTCTTTGCTGCTTTCAC-3 Probe FAM-TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT-TAMRA
IL-17	Forward 5-AATCTCCACCGCAATGAGGA-3 Reverse 5-ACGTTCCCATCAGCGTTGA-3 Probe FAM-CGGCACTTTGCCTCCCAGATCACA-TAMRA
IL-21	Forward 5-TGTGAATGACTTGGTCCCTGAA-3 Reverse 5-AACAGGAAAAAGCTGACCACTCA-3 Probe FAM-TCTGCCAGCTCCAGAAGATGTAGAGACAAAC-TAMRA
IL-23	Forward 5-TCAGTGCCAGCAGCTTTCAC-3 Reverse 5-TCTCTTAGATCCATGTGTCCCAC-3 Probe FAM-CTCTGCACACTGGCCTGGAGTGCA-TAMRA

Statistical analysis

Data were analyzed using SPSS 16.0 (SPSS Inc, Chicago, IL). Cytokine expression is presented as means and differences between patients with gastritis were analyzed using the Mann-Whitney test and for comparison of more than two groups Kruskal-Wallis tests were used. P-values of less than 0.05 were considered significant.

Results

Expression of mucosal mRNAs TGF-β1, IL-6, IL-17, IL-21 and IL-23 in biopsies of gastritis patients with SNPs in IL-17A G197A gene

TGF-β1, IL-6, IL-17, IL-21and IL-23 mRNAs were detectable in all biopsies were taken from gastritis patients. Since polymorphisms in IL-17A may affect mucosal cytokine IL-23/IL-17 axis function, we studied the expression of inflammation related cytokines in the gastric mucosa of patients with polymorphisms IL-17A (A/A, A/G and G/G). As shown in Fig. 3, the expression of TGF-β1 (p=0.059), IL-6 (p=0.834), IL-17(p=0.501), IL-21(p=0.999) and IL-23 (p=0.330) were not significant higher in patients with SNPs in IL-17A gene than in wild type cases.

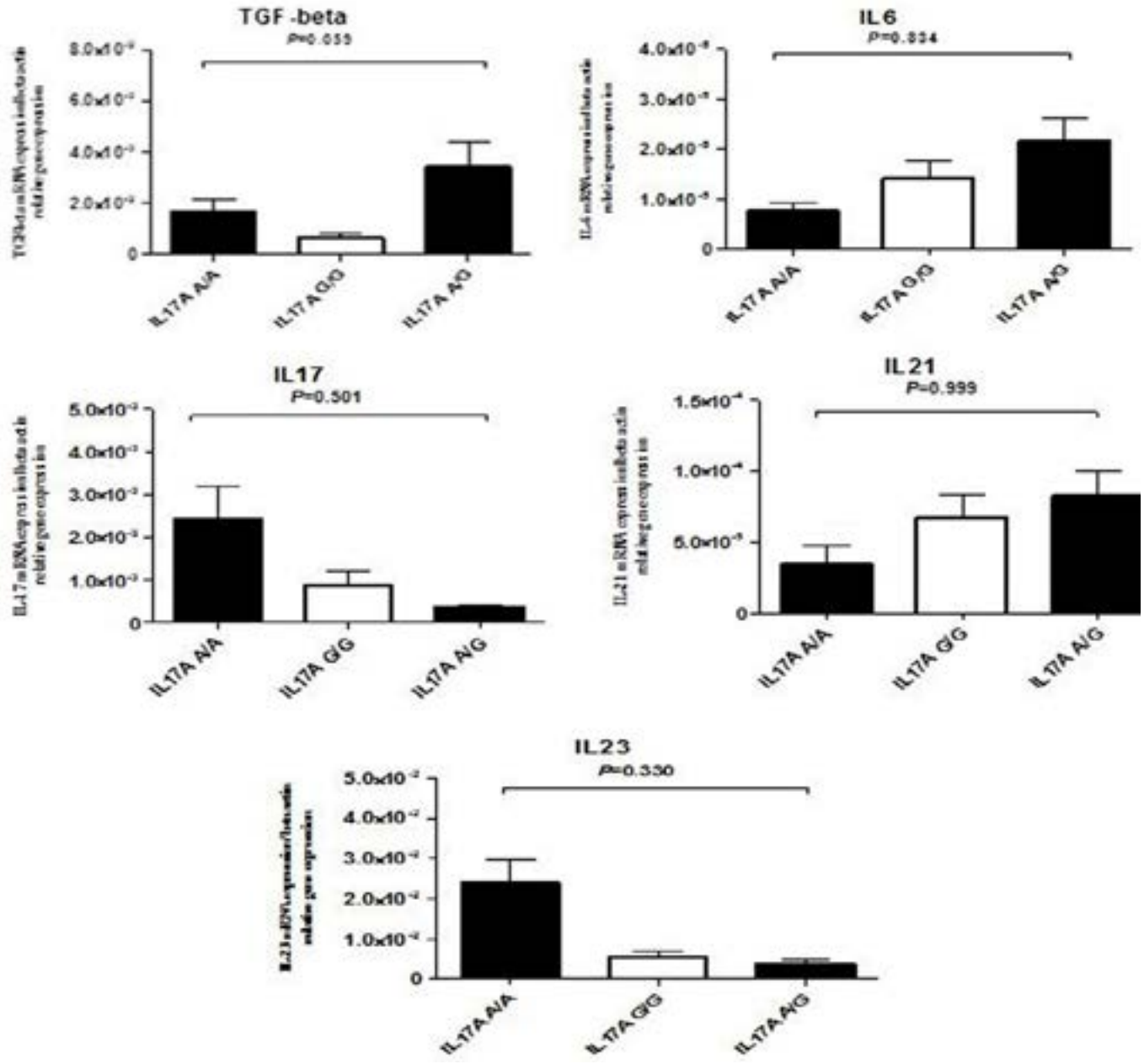

Figure 3. Expression of cytokines in biopsies gastritis patients with SNPs in IL-17A G197Agene. Reverse transcription and quantitative PCR were performed to estimate the relative expression levels of each cytokine. Expression of TGF-β IL-6 (B), IL-17 (C), IL-21 (D) and IL-23 (E)were normalized with expression values of human β-actin. Difference in mRNA expression between patients with SNPs in IL-17A G197Awere not significant in gastritis patients. 0.05 > p was considered statistically significant using the Mann–Whitney test.

Expression of mucosal mRNAs TGF-β1, IL-6, IL-17, IL-21and IL-23 in biopsies of gastritis patients with SNPs in IL-17F A7488G gene

TGF-β1, IL-6, IL-17, IL-21and IL-23 mRNAs were detectable in all biopsies were taken from gastritis patients. Since polymorphisms in IL-17A may affect mucosal cytokine IL-23/IL-17 axis function, we studied the expression of inflammation related cytokines in the gastric mucosa of patients with polymorphisms IL-17A (A/A, A/G and G/G). As shown in Fig. 4, the expression of TGF-β1 (p=0. 059), IL-6 (p=0. 834), IL-17 (p=0. 501), IL-21 (p=0. 999) and IL-23 (p=0. 330) were not significantly higher in patients with SNPs in IL-17A gene than in wild type cases.

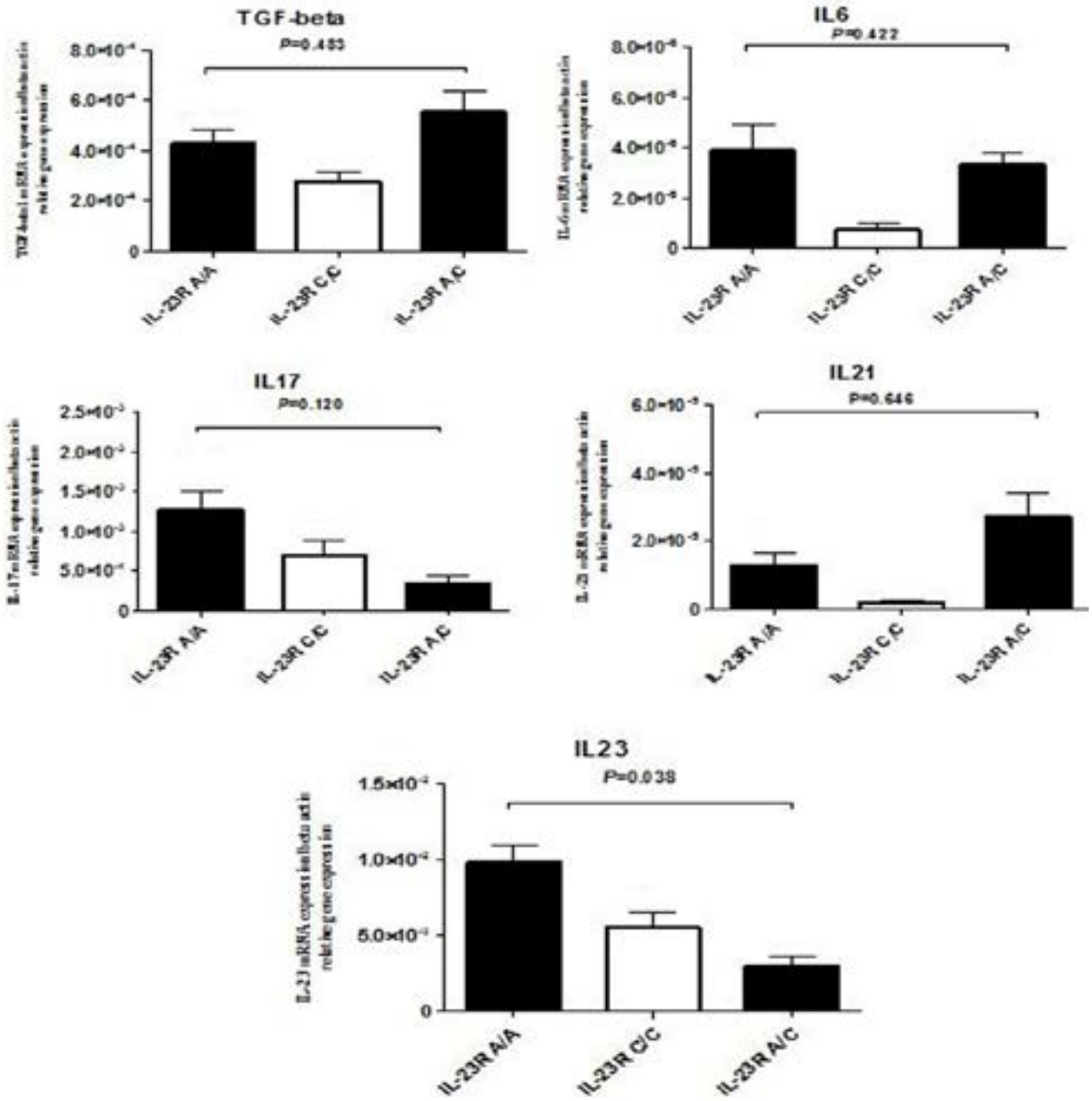

Figure 4. Expression of cytokines in biopsies gastritis patients with SNPs in IL-17F A7488Ggene. Reverse transcription and quantitative PCR were performed to estimate the relative expression levels of each cytokine. Expression of TGF-β1 (A), IL-6 (B), IL-17 (C), IL-21 (D) and IL-23 (E)were normalized with expression values of human β-actin. Difference in mRNA expression between patients with SNPs in IL-17F A7488Gwere not significant in gastritis patients. 0.05 > p was considered statistically significant using the Mann–Whitney test.

Expression of mucosal mRNAs TGF-β1, IL-6, IL-17, IL-21and IL-23 in biopsies of gastritis patients with SNPs in IL23R +2199A/C gene

TGF-β1, IL-6, IL-17, IL-21 and IL-23mRNAs were detectable in all biopsies were taken from gastritis patients. Since polymorphisms in IL23R may affect IL-23/IL-17 axis function, we studied the expression of inflammation-related cytokines in the gastric mucosa of patients with polymorphisms in IL23R (A/A, A/C and C/C genotypes). As shown in Fig. 5, the expression of TGF-β1 (p=0.483), IL-6 (p=0.422), IL-17(p=0.120) and IL-21(p=0.646) were not significantly higher in patients with SNPs in IL23R +2199A/C gene, but IL-23 (p=0.038) was significantly higher in patients with SNPs in IL23R (A/A genotype (gene.

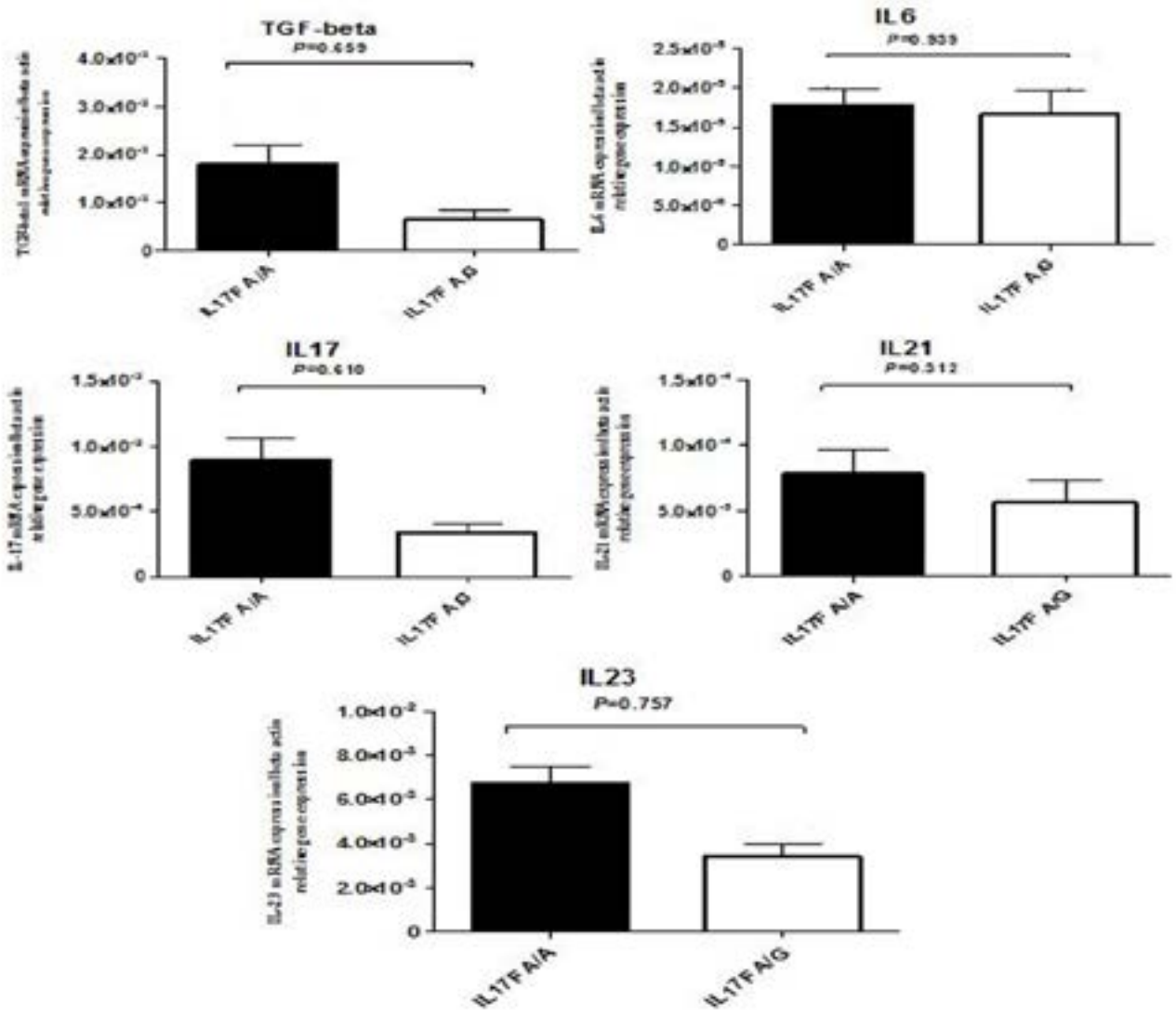

Figure 5. Expression of cytokines in biopsies gastritis patients with SNPs in IL23R +2199A/C gene. Reverse transcription and quantitative PCR were performed to estimate the relative expression levels of each cytokine. Expression of TGF-β1 (A), IL-6 (B), IL-17 (C), IL-21 (D) and IL-23 (E)were normalized with expression values of human β-actin. Difference in mRNA expression between patients with SNPs in IL23R +2199A/A was significant in IL23 expression but don't observed significant between patients with other SNPs in gastritis patients. 0.05 > p was considered statistically significant using the Mann–Whitney test.

Discussion

Of the beginning, the discovery of HP was ground breaking news to shift in gastroenterology research worldwide. The bacterial diversity and unclear association with certain digestive problem were on the top list of queries about this mysterious organism [27].

The correlation of genetic host factors in gastroduodenal diseases has been demonstrated in previous reports with genes participating in the inflammatory response[28,29]. Regulation of the immune response seems to be critical in determining the severity of damage to the host cells [29, 30]. Previously, we have shown that IL-17A G197A, IL-17F A7488G and IL23R +2199A/C polymorphisms no alter the mucosal cytokine pattern in Iranian patients with HP-associated gastritis diseases[31]. In this study, we eliminate the role of HP as a side factor, it may lead to sharp result in our study, then we suspect IL-17A, IL-17F and IL23R genotypes confers an increased risk for development of gastritis and the rapid progression of gastric mucosal atrophy via affect the mucosal expression mRNA IL-17 and IL-23 in chronic gastritis stage. In the present study, we found that IL-17A and IL-17F genotypes were not related to the mRNA mucosal expression of IL-17 and IL-23. As a same way, we don't observed relationship between IL23R genotypes and mucosal expression of IL-17 in gastritis subjects. In the other hand, we found that A/A genotype of IL23R polymorphism can increase the mucosal expression of mRNA in IL-23 in under study subject. These findings suggest that IL-17A, IL-17F and IL23R (A/C and C/C genotypes) polymorphisms have no effect on mucosal expression of IL-17 and IL-23, but IL23R polymor-

phism affects the mucosal expression of IL-23 involved in IL-23/IL-17 axis. Recent studies have suggested that IL-17 plays a main role in the inflammatory response and finally influences the gastric diseases and regulates immunity response [32-34]. Arisawa et al reported that IL-17F 7488 polymorphism was related to increases in the gastric inflammation [35]. It is well known that IL-17F can induce a diverse set of pro-inflammatory cytokines and chemokines as well as powerful inhibitors of gastric acid secretion[36, 37]. Xiaoqin et al suggested that IL-17F might promote tumor progression by facilitating tumor proliferation and invasion into neighboring tissues and lymph node and the IL-17A 197 polymorphism is associated with increased risk of certain subtypes of gastric cancer, but not with total gastric cancer risk [24]. Horvath et al. studies indicate that IL-23 makes a contribution to both Th1 and Th17 responses during the chronic stage of gastritis [38]. Another study indicated that polymorphisms of IL23R gene were associated with gastric cancer in a low risk Chinese population, they found the IL23R +2199CC genotype significantly decreased gastric cancer risk [25]. Together with their previous result that IL-17F 7488GG genotype markedly elevated the risk of gastric cancer [34]. The possible explanation for their result is, by modifying transcription factor binding sites or affecting the structure of mRNA, the IL23R +2199A/C polymorphism located in 3-UTR might lower the level of IL-23R expression and modulate the function of IL-23/IL-17 inflammatory axis, which was reported to get actively involved in the chronic gastritis step of intestinal type of gastric cancer formation [34].

IL23R is an important modulator in the inflammatory process. Patients with precancerous lesions in younger age are more likely to have early stage lesions and may have stronger immunity to prevent against the carcinogenic agents [39]. Therefore, the inverse association based on the immunity activation is easier to be implemented in younger population [40]. On one hand, it promotes a pro-inflammatory environment by dominating innate and inflammatory cells which are associated with inflammatory correlated carcinoma according to the previous literature; IL-23/IL-17 axis has two different roles in tumorigenesis [40]. By contrast, this IL-23/IL-17 pathway might also purpose tumor immune surveillance of elimination, equilibrium and escape [41]. The former role could partially involve in the pathogenesis gastric cancer, while ovary cancer prone to be associated with the latter mechanism. However, our study suggests that these polymorphisms may alter the mucosal cytokine pattern by regulating IL-17 and IL-23 production and, probably the consequent release of these cytokines, causes the development of severe histological changes in the gastric mucosa patients with gastritis. Our results highlighted the role of IL23R A/A genotype in mucosal expression IL-23 in patients with gastritis without the role of HP as interfering factor. We suggest same study in other population to more evaluate the role of these polymorphisms in IL-23/IL-17 pathway.

Conflicts of interest

All authors have no conflicts of interest to declare.

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