New insight to IL-23/IL-17 axis in Iranian infected adult patients with gastritis : effects of genes polymorphisms on expression of cytokines

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Abstract

Background and Objective : **Chronic inflammation is the hallmark of the pathogenesis of *H. pylori*-induced gastric cancer. IL-17A and IL-17F are inflammatory cytokines expressed by a novel subset of CD4$^+$Th cells and play critical function in inflammation. We evaluated the relationship between IL-17A G197A, IL-17F A7488G and IL23R+2199 A/C polymorphisms with IL-6, IL-17, IL-21, IL-23 and TGF-β1 mRNAs expression in regard to *H. pylori* infection with chronic gastritis.**

Materials and Methods : **Total RNA and genomic DNA were extracted from gastric biopsies of 58 *H. pylori*-infected patient with gastritis. Afterward, mucosal IL-6, IL-17, IL-21, IL-23 and TGF-β1 mRNAs expression and polymorphisms in IL-17A G197A, IL-17F A7488G and IL-23R +2199A/Cin gastric biopsies were determined by real-time PCR and PCR-RFLP.**

Results : **Our results show that IL-17A G197A, IL-17F A7488G andIL23R +2199A/C polymorphisms have no effect on mucosal expression of IL-6, IL-17, IL-21 and TGF-β1 mRNAs expression in *H. pylori*-infected patients with chronic gastritis.**

Conclusion : **These results suggest that IL-17A G197A, IL-17F A7488G and IL23R +2199A/C polymorphisms no alter mucosal cytokine pattern in Iranian patients with *H. pylori*-associated gastritis diseases.** (Acta gastroenterol. belg., **2015**, 78, **212-218**).

Keywords : *H. pylori*, **polymorphism, chronic gastritis.**

Introduction

Helicobacter pylori (*H. pylori*) infection is associated with gastritis (1) and considerable infiltration (2) of neutrophils, monocytes, dendritic cell, lymphocytes, and plasma cells into the gastric mucosa that contribute to maintain and expand the local inflammation (3-5). The risk of different clinical expression of *H. pylori* infection is thought to rely on interactions between the host genetic factors and bacterial factors (6). For instance, polymorphisms of pro-inflammatory cytokine genes such as IL-8, IL-10, IL-17A, IL-1β and TNF-α that enhance inflammatory response of gastric mucosa, have been correlated to an increased risk of gastric cancer and peptic ulcer (7,8). It has been shown that the mucosal levels of several cytokines are significantly higher in *H. pylori* infected patient with virulence factors groups in comparison to patients without virulence factors groups (9,10). Although *H. pylori* activates various mechanisms of the immune system, the inflammatory response is not enough in the majority of patients to eradicate the bacterium from the gastric mucosa. T helper 17 cells (Th17) are a new subset of T helper cells producing IL-17 (11,12). Recent studies revealed a new inflammation pathway of ''IL-23→IL-23R→*STAT3*→Th17→IL-17A/IL-17F'' axis (IL-23/IL-17 axis) was proved to play pivotal role in inflammatory and autoimmune diseases (13), such as psoriasis, lupus nephritis and intestinal inflammation (14-16). In this inflammation pathway, IL-23, the cytokine that is responsible for the expansion of Th17, is comprised by heterodimer of p40/p19 in which p40 is the common subunit shared with IL-12 and p19 is the specific subunit with higher affinity to IL-23R (17). IL-23R, as the key component to IL-23 receptor, was proved to play a significant role in the initiating, maintaining and accelerating of this IL-23/IL-17 inflammatory signal transduction pathway (18). In 2006, Duerr *et al*. found a strong association between Crohn'sdisease and polymorphisms of the *IL23R* gene (19). Since then, *IL23R*gene was proved to be the predisposing gene to many other autoimmune/inflammatory diseases. Among the identified polymorphisms of IL23R, the functional SNP of +2199A/C (rs10889677) located in the 3-untranslated region (UTR) was repeatedly proved to be associated with diverse autoimmune/inflammatory diseases. However, the results are in controversial in different groups with different diseases (19-22). Moreover IL-17F was discovered to share strongest homology to IL-17A. alike to IL-17A, IL-17F was reported to induce the expression in a wide variety of chemokines, cytokines and adhesion molecules by human airway epithelial cells, fibroblasts and vein endothelial cells (23) but IL-17F had significantly weaker activity than IL-17A (24, 25). Several studies have found excess expressing of IL-17A in

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various tumor tissues, including colorectal cancer, prostate cancer, breast cancer and gastric cancer (26,27). Moreover, increasing evidences suggested the role of IL-17A in *H. pylori*-related gastric diseases (28,29). Polymorphisms of IL-17A G197A (rs2275913) and IL-17FA7488G (p.His161Arg rs763780) have recently been identified to be associated with the susceptibility to ulcerative colitis and, rheumatoid arthritis respectively (30, 31). Genetic variations in inflammation-related genes, particularly cytokines and their receptors are thought to play a role in the outcome of infection and development of gastritis. Considering that chronic gastritis and genetic predisposition are important parts of a complex interaction to initiate gastric carcinogenesis, and these polymorphisms has been associated with gastric cancer without role of *H. pylori* in these patients. As a result, our study suggests that these polymorphisms may alter mucosal cytokine pattern by regulating IL-6, IL-21, IL-23, IL-17 and TGF-β1 production and, probably, the consequent release of these cytokines, the development of severe histological changes in the gastric mucosa patients with gastritis.

Subjects and methods

A total of 58 *H. pylori*-infected gastritis patients 23 men (43.43 ± 16.93) and 35 women (40.57 ± 14.47) with gastritis who were undergoing upper gastrointestinal endoscopy were tested for *H. pylori* infection. *H. pylori* infected patients were determined by the RUT, PCR (16srRNA, glmM) and histological examination of biopsies taken from the corpus. Patients were classified as *H. pylori*-infected only if the three tests were positive. 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 (NSAIDs), proton pump inhibitors, antibiotics, or bismuth salts. Informed consents for participation were signed by all the study subjects. The study was approved by the human research ethics committee and informed consent was obtained from each volunteer before participation.

Histological examination

Gastric biopsy specimens were embedded in 10 % buffered formalin and stained with Hematoxylin and Eosin (H&E) to examine gastritis and with giemsa to detect *H. pylori*. The histological detection of gastritis was blindly performed according to the Updated Sydney System (32).

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 UltraPureRNAse/DNAse-Free Distilled water.

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

Genotyping analysis 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*. (33,34). The PCR amplification 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 5 min, followed by 33 cycles of 95°C for 60 s, 65°C for 60 s, and 72°C for 50 s. A final extension was carried out at 72°C for 7 min for IL-17A ; denaturation at 95°C for 6 min, followed by 33 cycles of 95°C for 60 s, 65°C for 55 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 10 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. Gel analysis was performed after staining with ethidium bromide. PCR products were shown to

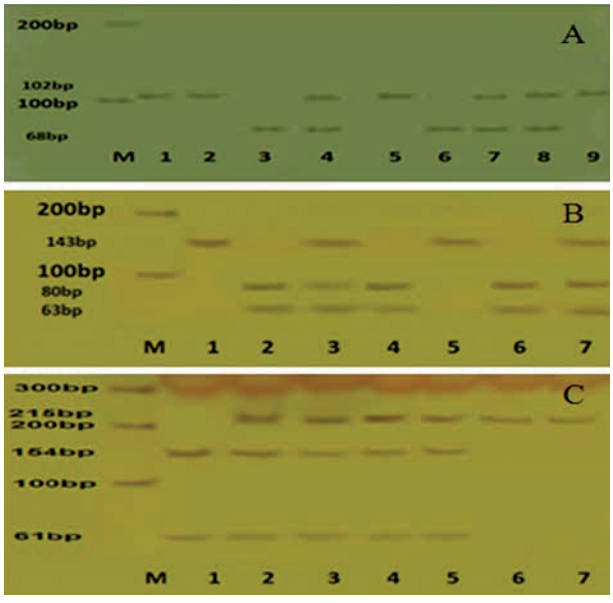

Fig. 1. — PCR-RFLP polyacrylamide gel electrophoresis of the IL-17A G197A and IL-17F A7488G and IL23R +2199A/C (rs10889677) polymorphins indicating : (A) IL-17A G197A, No. 1, 2, 5, 9 (AA = 102 bp) 4, 7, 8 (AC = 102, 68, 34 bp) 3, 6 (GG = 68, 34 bp) genotypes and (B) IL-17F A7488G, No. 2, 4, 6 (AA = 80,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

Table 1. — **Primer and probe sequences employed in this study**

Gene	Primer and probe sequence
β-actin	Forward 5-AGCCTCGCCTTTGCCGA-3
	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

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. No significant difference was found after genotyping the randomly selected samples.

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

In the current study we selected biopsies from all 58 *H. pylori*-infected biopsies 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 shown in Table 1. The quantification of mRNA levels was performed using a Rotor-Gene 3000 (Corbett). Real-time-PCR reactions were performed 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 94ºC), 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 (35).

Statistical analysis

Data were analyzed using SPSS 16.0 (SPSS Inc, Chicago, IL). Cytokine expression is presented as means and differences between infected patients 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 IL-6, IL-21, IL-23, IL-17A and TGF-β1 in biopsies of H. pylori-infected with SNPs in IL-17A G197A gene

IL-6, IL-21, IL-23, IL-17A and TGF-β1 mRNAs were detectable in all biopsies was taken from *H. pylori*-infected. Since polymorphisms in IL-17 may affect its function, we studied the expression of inflammation-related cytokines in the gastric mucosa of 17 patients with polymorphisms IL-17A A/G, 4 patients with A/A and 21 patients with G/G (wild type). As shown in Fig. 2, the expression of IL-6 ($p = 0.726$), IL-17 ($p = 0.842$), IL-21 ($p = 0.734$), IL-23 ($p = 0.994$) and TGF-β1 ($p = 0.732$) were not significant higher in patients with SNPs in IL-17A gene.

Expression of IL-6, IL-21, IL-23, IL-17 and TGF-β1 in biopsies of H. pylori-infected with SNPs in IL-17F A7488G gene

IL-6, IL-21, IL-23, IL-17 and TGF-β1mRNAs were detectable in all biopsies was taken from *H. pylori-*

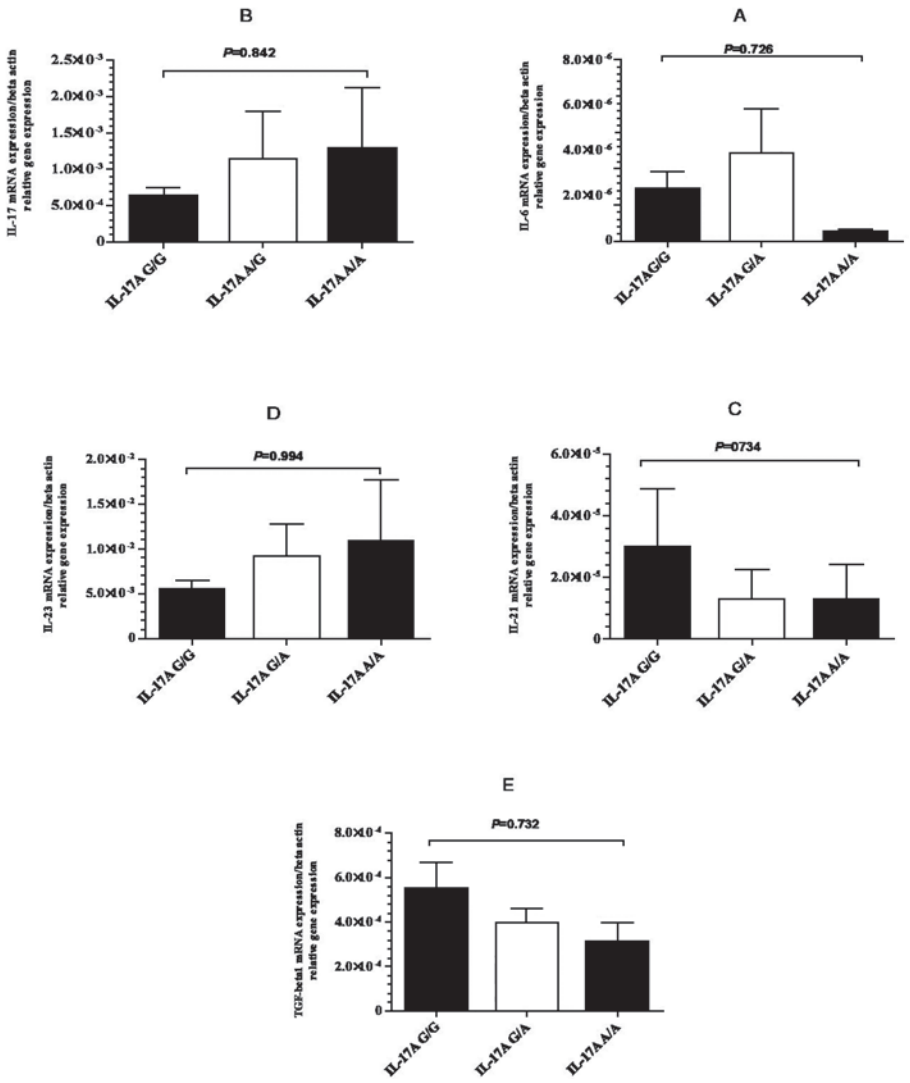

Fig. 2. — Expression of cytokines in biopsies of *H. pylori* infected patients with SNPs in IL-17A gene and patients with the wild type IL-17A gene. Quantitative PCR was performed to estimate the relative expression levels of each cytokine. Expression of IL-6 (A), IL-17(B), IL-21 (C), IL-23 (D) and TGF-β1 (E) were normalized with expression values of human β-actin. Difference in mRNAs expression between patients with SNPs in IL-17A G197A were not significant in *H. pylori*-infected patients. *P* value < 0.05 was considered statistically significant using the Kruskal-Wallis test.

infected. Since polymorphisms in IL-17F may affect its function, we studied the expression of inflammation-related cytokines in the gastric mucosa of 10 patients with polymorphisms IL-17F A/G and 25 patients with A/A (wild type). As shown in Fig. 3, the expression of IL-6 ($p = 0.826$), IL-17 ($p = 0.378$), IL-21 ($p = 0.434$), IL-23 ($p = 0.534$) and TGF-β1 ($p = 0.194$) was not significant higher in patients with SNPs in IL-17A gene.

Expression of IL-6, IL-21, IL-23, IL-17 and TGF-β1 in biopsies of H. pylori-infected with SNPs in IL23R +2199A/C gene

IL-6, IL-21, IL-23, IL-17 and TGF-β1 mRNAs were detectable in all biopsies was taken from *H. pylori*-infected. Since polymorphisms in IL23R may affect its function, we studied the expression of inflammation-related cytokines in the gastric mucosa of 7 patients with polymorphisms IL23R C/C, 10 patients with A/C and 17 patients with A/A (wild type). As shown in Fig. 4, the expression of IL-6 ($p = 0.422$), IL-17 ($p = 0.120$), IL-21 ($p = 0.646$), IL-23 ($p = 0.093$) and TGF-β1 ($p = 0.483$) were not significant higher in patients with SNPs in IL-IL23R +2199A/C gene.

Discussion

The correlation of genetic host factors in gastroduodenal diseases associated with *H. pylori* infection has been demonstrated in previous reports with genes participating in inflammation (36-38). Regulation of the immune response seems to be critical in determining the severity of damage to the host (38-40). In this study, we suspect

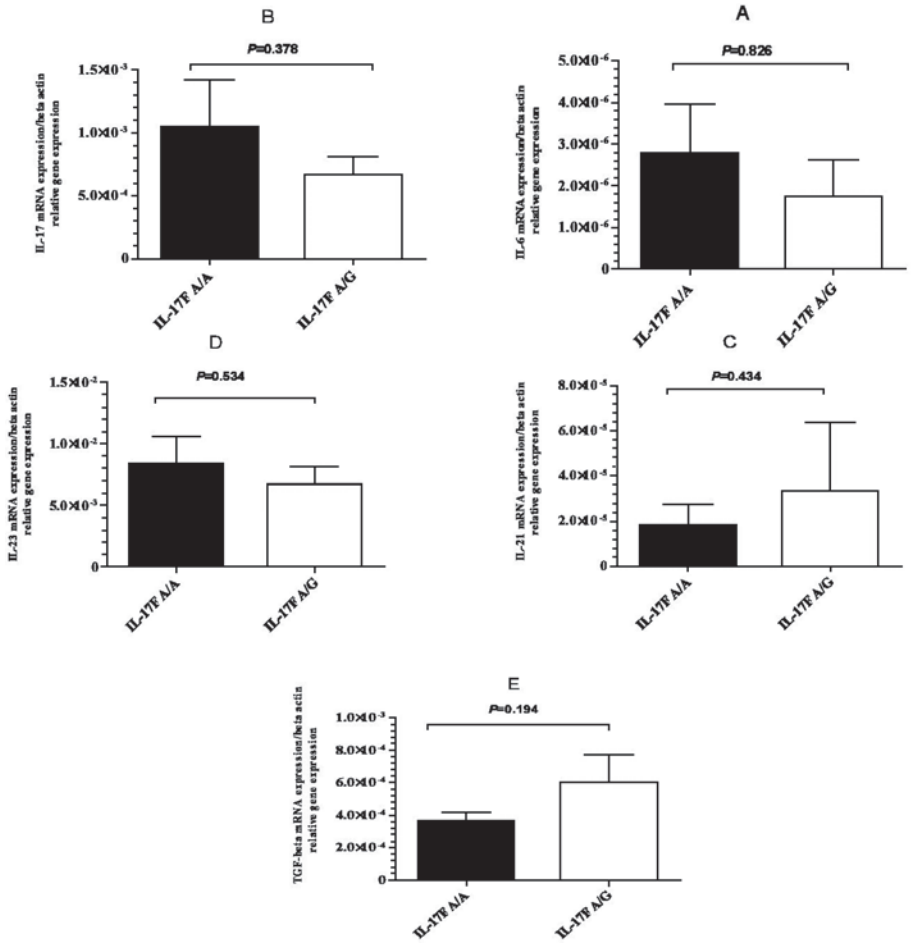

Fig. 3. — Expression of cytokines in biopsies of *H. pylori* infected patients with SNPs in IL-17F gene and patients with the wild type IL-17F gene. Quantitative PCR was performed to estimate the relative expression levels of each cytokine. Expression of IL-6 (A), IL-17(B), IL-21 (C), IL-23 (D) and TGF-β1 (E) were normalized with expression values of human β-actin. Difference in mRNAs expression between patients with SNPs in IL-17F A7488G were not significant in *H. pylori*-infected patients. *P* value < 0.05 was considered statistically significant using the Mann-Whitney test.

that 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 expression of cytokines such as IL-6, IL-21, IL-23, IL-17A and TGF-β1 in chronic gastritis stage. We found that IL-17A, IL-17F and IL23R genotypes were not related to mucosal expression of IL-6, IL-21, IL-23, IL-17A and TGF-β1 mRNAs in *H. pylori*-infected patients. These findings suggest that IL-17A, IL-17F and IL23R polymorphisms have no effect on mucosal expression of IL-6, IL-21, IL-23, IL-17A and TGF-β1 mRNAs involved in IL-23/IL-17 axis. Recent studies have suggested that IL-17 plays a main role in the inflammatory response to the *H. pylori* infection and finally influences the outcome of the *H. pylori*-related diseases and regulates immunity towards this bacterium (28, 29, 41). Arisawa *et al*. reported that IL-17F 7488 polymorphism was reported to interact with *H. pylori* infection to increase the inflammation and activity scores (42). It is well known that IL-17F can induce a diverse set of pro-inflammatory cytokines and chemokines as well as powerful inhibitor of gastric acid secretion IL-1β (43, 44) which potentiate tumor growth in noncardia site. 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 (33). Horvath *et al*. studies indicate that IL-23 makes a contribution to both Th1 and Th17 responses during *H. pylori* infection particularly during the chronic stage of infection (45). Another study indicated that polymorphisms of IL23R gene were associated with gastric cancer in a low risk Chinese population and they found that the IL23R +2199CC genotype significantly decreased gastric cancer risk (34). Together with their previous result that IL-17F 7488GG genotype markedly elevated the risk of gastric cancer. The possible explanation for their result is, by modifying transcription factor binding sites or affecting the structure of messenger RNA (mRNA), the IL23R

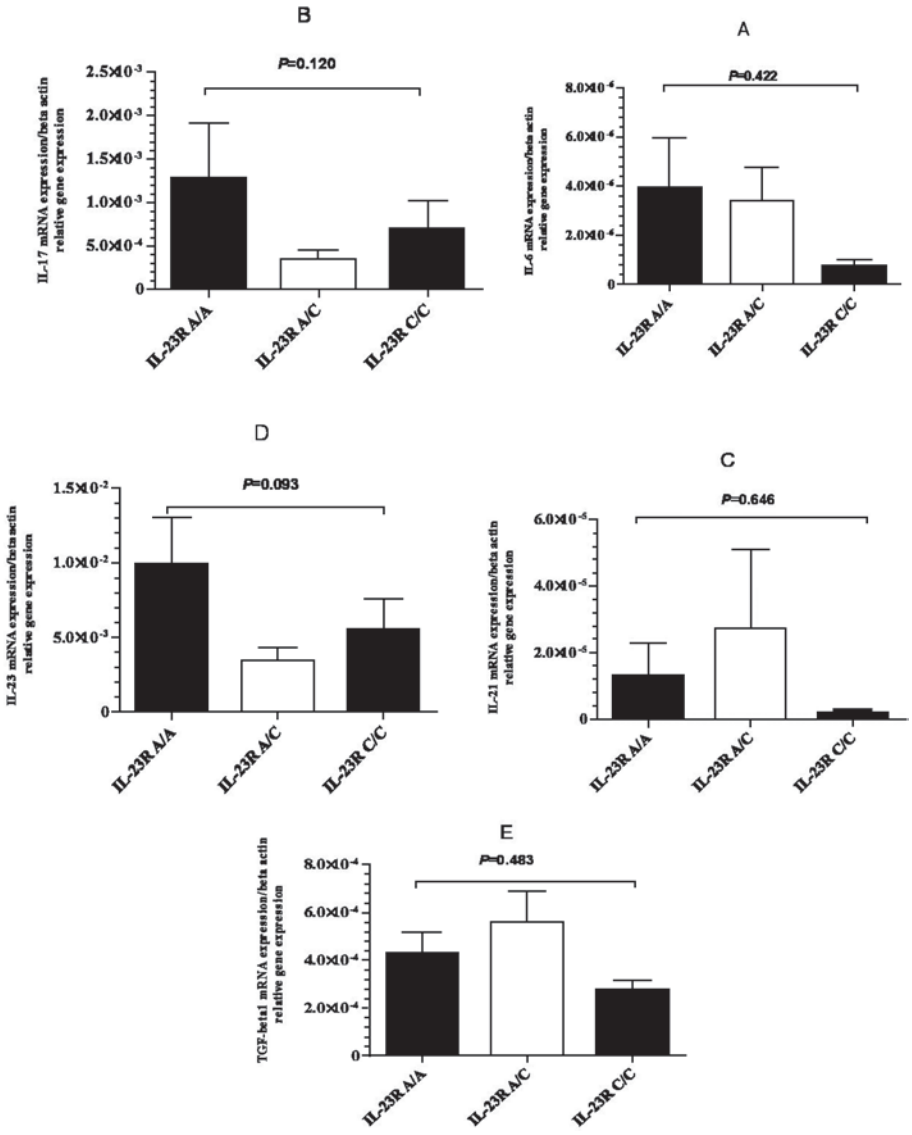

Fig. 4. — Expression of cytokines in biopsies of *H. pylori* infected patients with SNPs in IL23R gene and patients with the wild type IL23R gene. Quantitative PCR was performed to estimate the relative expression levels of each cytokine. Expression of IL-6 (A), IL-17(B), IL-21 (C), IL-23 (D) and TGF-β1 (E) were normalized with expression values of human β-actin. Difference in mRNAs expression between patients with SNPs in IL23R +2199A/C were not significant in *H. pylori*-infected patients. *P* value < 0.05 was considered statistically significant using the Kruskal-Wallis test.

+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 (28). IL23R is an important modulator in the inflammatory process. And patients with precancerous lesions in younger age are more likely to have early stages lesions and may have stronger immunity to prevent against the carcinogenic agents. Therefore, the inverse association based on the immunity activation is easier to be implemented in younger population. According to the previous literature ; IL-23/IL-17 axis has two different roles in tumorigenesis. On one hand, it promotes a pro-inflammatory environment by dominating innate and inflammatory cells which is associated with inflammatory correlated carcinoma. By contrast, this IL-23/IL-17 pathway might also purpose tumor immune surveillance of elimination, equilibrium and escape (46). The former role could partially involve in the pathogenesis gastric cancer, while ovary cancer prone to be associated with the later mechanism. However, our study suggests that these polymorphisms may alter mucosal cytokine pattern by regulating IL-6, IL-21, IL-23, IL-17A and TGF-β1 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 *H. pylori* and mucosal expression

cytokines in explaining differential outcomes in infected patients rather polymorphism because many studies do not consider the role *H. pylori* in their study. Further studies are needed to analyze these polymorphisms with different clinical expression in *H. pylori*-infected patients.

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