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Association between virulence factors of helicobacter pylori and gastric mucosal interleukin-18 mRNA expression in dyspeptic patients

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ABSTRACT

Background: Helicobacter pylori (Hp) infection is associated with gastritis and marked infiltration of the gastric mucosa by several cytokines secreting inflammatory cells that contribute to sustain and expand the local inflammation. Different clinical expressions of the infection may reflect distinctive patterns of cytokine expression. IL-1β, TNF-α, IL-17 and IL-23 have been reported to be involved in Hp-induced gastric mucosal inflammation, but the details and association to different patterns of inflammation and virulence factors remain unclear.
Methods: Total RNA was extracted from gastric biopsies of 51 Hp-infected patients and 44 Hp-negative patients. Mucosal IL-18 mRNA expression in gastric biopsies was determined by Real-Time PCR. Presence of virulence factors was evaluated using PCR.
Results: IL-18 mRNA expression was significantly increased in biopsies of Hp-infected patients compared to Hp-uninfected individuals. There was no association between virulence factors and IL-18 mRNA expression. Also severity of mononuclear infiltration was significantly higher in gastritis patients with vacA (m1)-positive compare patients with vacA (m2)-positive.
Conclusions: IL-18 may play an important role in the inflammatory response and promote the chronic and persistent inflammatory changes in the stomach. This may ultimately influence the outcome of Hp-associated diseases that arise within the context of gastritis.

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1. Introduction

H. pylori (Hp) is a spiral-shaped Gram negative flagellate bacterium that colonizes the gastric mucosa of approximately 50% of the world's population [1]. Hp infection is associated with gastritis and considerable infiltration of neutrophils, monocytes, lymphocytes, and plasma cells into the gastric mucosa that contribute to maintain and expand the local inflammation. Activation and migration of these inflammatory cells into the gastric mucosa is related to increased production of pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-1β, IL-18, IL-17, IL-8 and IL-6 which are believed to contribute to maintaining the gastric inflammation and

causing epithelial cell damage [2]. The risk of different clinical expression of Hp infection is thought to rely on interactions between the host genetic factors and bacterial factors. For instance, polymorphisms of pro-inflammatory cytokine genes such as IL-8, IL-10, IL-17, 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 [3–12]. It has been shown that the mucosal levels of several cytokines are significantly higher in Hp infected patient with virulence factors groups in comparison to patients without virulence factors groups [13–15]. The immune response to Hp infection is thought to consist predominantly with the Th1 type responses [16,17]. This concept is supported by the fact that Hp infected IFN-γ-knockout mice developed the least amount of possible pathological changes [18,19]. The pro-inflammatory cytokine interleukin-18, previously known as an IFN-γ-inducing factor, is a Th1 cytokine in the IL-1 superfamily [20]. IL-18 boosts the production of IFN-γ from Th1 and NK cells in

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Table 1
PCR primers for amplification of virulence factors.

References	Size of PCR product (bp)	Primer sequence	Primer designation
[27]	567 bp (m1)	vacAmF: 5-CAATCTGTCC AATCAAGCGAG-3	vacA m1/m2
	642 bp (m2)	vacAmR: 5-GCGTCTAAAT AATTCCAAGG-3	
[28]	259 bp (s1)	VA1-F: 5-ATGGAAATACA ACAAACACAC-3	vacA s1/s2
	286 bp (s2)	VA1-R: 5-CTGCTTGAAT GCGCCAAAC-3	
[29]	430 bp	oipA-F:CAAGCGCTTAA CAGATAGGC-3 oipA-R:AAGGCGTTTCT GCTGAAG-3	oipA
[30]	247 bp	iceA1F- 5-GTGTTTTTAA CCAAAGTATC-3 iceA1R-5-CTATAGCCAS TYTCTTTGCA-3	iceA1
[30]	229 or 334 bp	iceA2F: 5-GTTGGGTATA TCACAATTTAT-3 iceA2R: 5-TTRCCCTATTTT CTAGTAGGT-3	iceA2
[31]	271 bp	bab7-F: 5-CCAAACGAAAC AAAAAGCGT-3 bab7-R: 5-GCTTGTGTAAA AGCCGTCGT-3	babA2
[32]	232 bp	cag1: 5-ATGACTAACGAA ACTATTGATC-3 cag2 5-CAGGATTTTTGA TCGCTTTATT-3	cagA

Table 2
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-CCGCCGCCCGTCCACACCGCC-TAMRA
IL-18	Forward 5-GACCAAGGAAATCGGCCTCTA -3 Reverse 5-CCATACCTCTAGGCTGGCTATCTT-3 Probe FAM-ATTCTGACTGTAGAGATAATGCACCCCGGAC-TAMRA

cooperation with IL-12 [21]. Because the Th1 type immune response is thought to be predominant in Hp infected gastric mucosa, we hypothesized that IL-18 should play an important role in this process. However, the effect of Hp infection on IL-18 production remains unclear, because one report suggested that antral, but not corporal, IL-18 mRNA levels were up-regulated during Hp infection [22] and another indicated that mucosal IL-18 mRNA levels were independent of Hp infection [23]. It has been shown that the mucosal levels of pro-inflammatory cytokine in the site of infection with H. pylori are related to the HP strains expressing some virulence factors. Moreover, the risks of Hp-induced gastric inflammation, atrophy, metaplasia, and malignancy have been associated with the presence of Hp related virulence factors [24]. IL-18 has recently been associated with Hp related gastric mucosal inflammation and gastro-duodenal disease risks, but the time course and relationship with bacterial virulence factors remain unknown [25]. The present study investigated IL-18 mRNA expression levels and its relation with bacterial virulence factors in Hp positive patients with gastritis compared to Hp-negative individuals with gastritis.

2. Materials and methods

2.1. Patients and sampling

A total of 51 Hp-infected gastritis patients, 19 men (43.58 ± 17.66) and 32 women (42.41 ± 13.69) and 44 uninfected gastritis patients, 20 men (38.80 ± 13.97) and 24 women (37.58 ± 17.27), were participated in this study. Hp infection was determined by the rapid urease test, PCR test (for detection of 16srRNA and ureA genes) and histological examination of biopsies taken from the corpus. Patients were classified as Hp-infected only if all three tests were positive. Detection of bacterial virulence factors was done using PCR test and one biopsy from each patient was used for assessment of IL18 mRNA levels using Real-Time-PCR.

2.2. Histological examination

Gastric biopsy specimens were embedded in 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 (on a four-point scale: 0, no; 1, mild; 2, moderate; and 3, severe changes) based on the degree of polymorphonuclear leukocyte (PMN) and mononuclear cell (MNC) infiltration, and atrophy according to the Updated Sydney System [26].

2.3. PCR amplification

DNA for polymerase chain reaction (PCR) was extracted using the Biospin Tissue Genomic DNA Extraction Kit (BioFlux, Japan). Specific primers for PCR amplification of different genes are shown in Table 1. For vacA, cagA, iceA and babA2 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). For oipA, amplification was performed with 35 cycles of denaturation (at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s), and one final extension (at 72 °C for 5 min).

2.4. Quantitative analysis for IL-18 mRNA in the gastric mucosa using Real-Time-PCR

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 2. The quantification of IL-18 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 94 °C), 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 [33].

2.5. Statistical analysis

In statistics, normality tests are used to determine whether a data set is well-modeled by a normal distribution or not, cytokine expression is presented as means and differences between infected and uninfected groups 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.

Table 3
Frequency distribution of the vacA allele variants s and m and the cagA status for the total of study patients analyzed.

Genotype	Total	
	N	%
vacA s-region		
s1	37	72.5
s2	8	15.7
s1 and s2	6	11.8
vacA m-region		
m1	13	25.5
m2	33	64.7
m1 and m2	5	9.8
cagA		
Positive	31	60.8
Negative	20	39.2
Total	51	100

3. Results

3.1. Genotype

Table 3 gives an overview of the frequency distribution of the vacA s and m alleles and the cagA status. The vacA s1 and m2 alleles as well as cagA were predominant. Mixed vacA s1/s2 genotypes were found in six patients (11.8%) and mixed vacA m1/m2 genotypes were found in five patients (9.8%). Concerning genotype combinations, vacA s1/m2/cagA+ were most frequent, followed by vacA s1/m1/cagA+. The vacA s2/m1 genotype was not observed (Table 4). The oipA, babA2, iceA1 and iceA2 genes respectively were found in 98%, 86.1%, 62.6% and 37.2% of the Hp isolates (Table 5).

3.2. Mucosal IL-18 mRNA levels in gastric mucosa

IL-18 mRNA level was detectable in all samples regardless of whether biopsies were taken from Hp-infected or uninfected patients. IL-18 expression was significantly increased in biopsy specimens of Hp-infected patients compared with uninfected patients ($p < 0.04$) (Fig. 1). If the mean ± SD (HP positive group) is 0.002 and the mean ± SD. (HP-negative group) is 0.0007 then the difference in the IL-18 mRNA expression in the Hp-positive compared with the Hp-negative patients is 0.002/0.0007 or 2.6-fold.

3.3. Effect of virulence factors in Hp-infected on the mucosal IL-18 mRNA level in gastric mucosa

A mucosal IL-18 mRNA level was independent of virulence factors status. If the mean ± SD (HP positive group) is 0.0016 and the mean ± SD (HP-negative group) is 0.0026 then the difference in the

Table 4
Frequency distribution of the different genotype combinations for the total of patients analyzed.

Genotype	Frequency	Percentage
vacA s1 m1 cagA+	10	19.6
vacA s1 m1 cagA−	5	9.8
vacA s1 m2 cagA−	5	9.8
vacA s1 m2 cagA+	16	31.4
vacA s2 m2 cagA−	7	13.7
vacA s2 m2 cagA+	3	5.9
vacA s1+2 m2 cagA−	2	3.9
vacA s1+2 m2 cagA+	3	5.9
Total	51	100

Table 5
Frequency of virulence factors in Hp isolates.

Genotype				Number (%)
oipA	iceA1	iceA2	babA	
+	+	+	+	4 (7.8)
+	+	−	+	24 (47)
+	+	−	−	4 (7.8)
+	−	+	+	14 (27.4)
+	−	+	−	1 (2)
+	−	−	+	2 (3.9)
+	−	−	−	1 (2)
−	−	−	−	1 (2)
				51 (100%)

IL-18 mRNA expression in the cagA-positive HP strains compared with the cagA-negative HP strains is 0.0016/0.0026 or 0.59-fold, the difference in the IL-18 mRNA expression in the babA2-positive HP strains compared with the babA2-negative HP strains is 0.0018/0.0032 or 0.56-fold, the difference in the IL-18 mRNA expression in the iceA1-positive HP strains compared with the iceA1-negative HP strains is 0.0017/0.0024 or 0.74-fold and the difference in the IL-18 mRNA expression in the iceA2-positive HP strains compared with the iceA2-negative HP strains is 0.0024/0.0018 or 1.3-fold (Fig. 2).

3.4. Effect of vacA allele variants in H. pylori-infected on the mucosal IL-18 mRNA level in gastric mucosa

Our results showed that in H. pylori-infected patients; mucosal IL-18 mRNA level was independent on the vacA status. The difference in the IL-18 mRNA expression in the vacA s1-positive HP strains compared with the vacA s2-positive HP strains is 0.0019/0.0018 or 1.05-fold. Mucosal IL-18 mRNA expression in gastritis patients with vacA m1-positive was not significantly higher than those observed in gastritis patients with vacA m2-positive (0.0019/0.002 or 0.96-fold). Mucosal IL-18 mRNA expression in gastritis patients with vacA s1m1-positive was not significantly higher than those observed in gastritis patients with vacA s1m2-positive (0.0019/0.0021 or 0.9-fold) and vacA s2m2-positive (0.0019/0.0018 or 1.03-fold) (Fig. 3).

3.5. Correlation between Mucosal IL-18 mRNA level and types of disease in Hp-infected patients

In those infected with Hp, IL-18 mRNA expression in mucosa did not correlate with types of disease, the difference in the IL-18
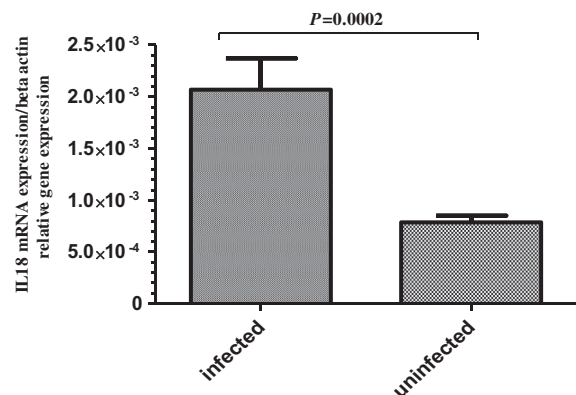

Fig. 1. Mucosal IL-18 mRNA expression in gastritis patients. RNA was extracted from gastric biopsies of 51 Hp-infected patients, 44 Hp non-infected patients with gastritis and analyzed for IL-18 by real-time-PCR. Levels are normalized to β-actin. *P* values <0.05 was considered statistically significant using the Mann–Whitney test.
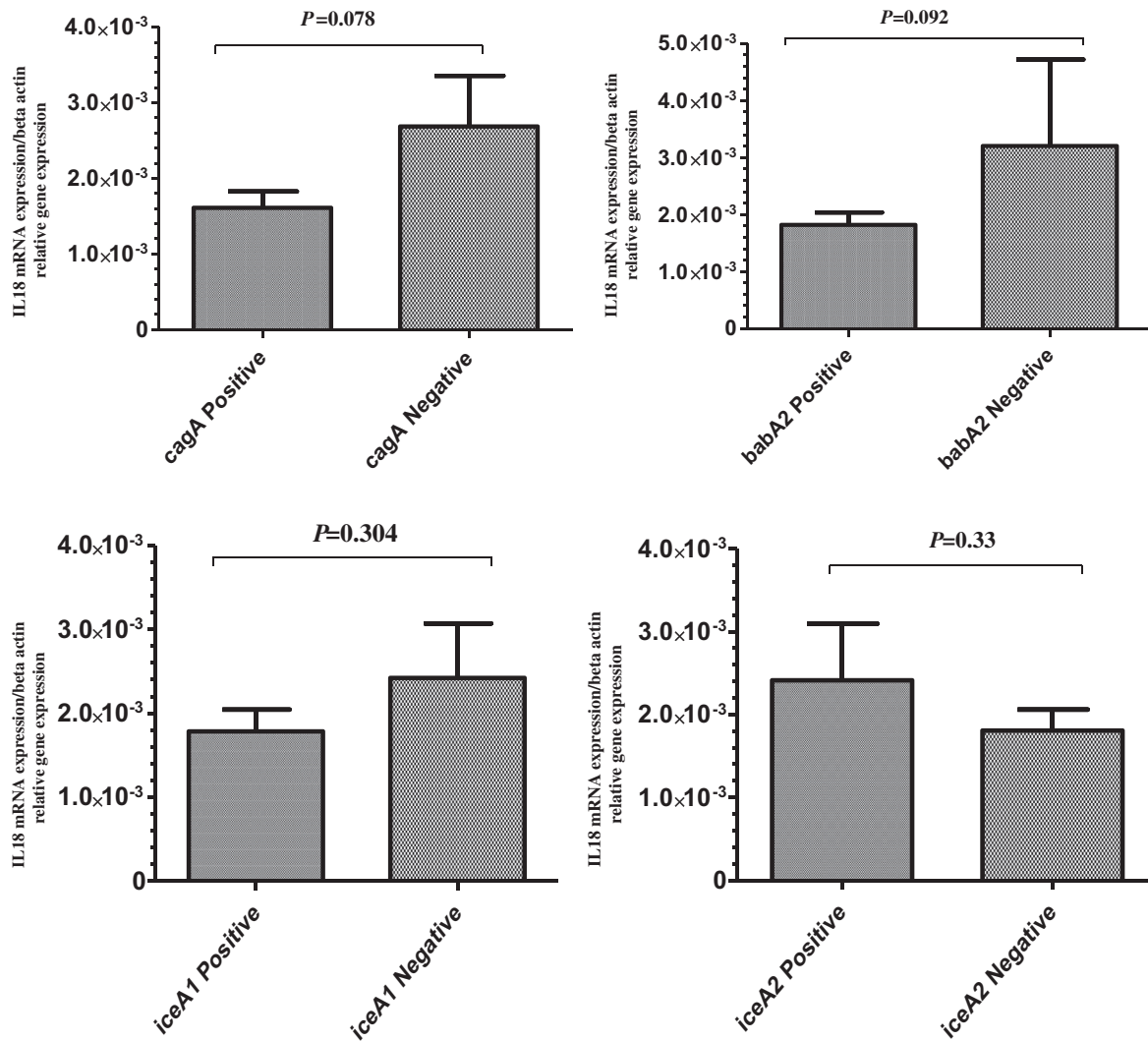
Fig. 2. Mucosal IL-18 mRNA expression in Hp infected patients and virulence factors. P values <0.05 was considered statistically significant using the Mann–Whitney test.

mRNA expression in the chronic gastritis compared with the chronic active gastritis in HP-positive Patients is 0.0027/0.0014 or 1.8-fold (Fig. 4).

3.6. Correlation between virulence factors and gastric mucosal inflammation

Severity of mononuclear infiltration was significantly higher in gastritis patients with vacA (m1)-positive compared to those observed in gastritis patients with vacA (m2)-positive (Table 6).

4. Discussion

IL-18 is a cytokine with pleiotrophic immunomodulatory functions that increase in a variety of human inflammatory conditions, such as Crohn's disease [34]. This cytokine could directly enhance IL-1, IL-6, and TNF-α cytokine production from macrophages that would progress gastritis [20,35]. This study was designed to essay the hypothesis that Nod-like receptors (NLRs) are activated by the presence of microbial products like, peptidoglycan, cagA protein and vacA toxin that induce the transcription factor NF-kB to activate innate and adaptive immune response genes. Inflammasomes are protein compounds that state in cytoplasm. These compounds influence innate immune responses and inflammation by responding to molecules drawn from pathogen and also products drawn from host that released in response to a range of tissue disturbance [36]. Depend on identification of an inflammatory stimulus, inflammasomes put together and activated caspase-1 processes the cytoplasmic precursors of IL-1β and IL-18 to generate the mature, biologically active cytokines, which are consequently released to initiate inflammation and defense mechanisms [36]. It appears as though that IL-18 is crucial for Hp-specific IFN-γ output. It has been shown that the levels of IL-18 cytokine in IL-18$^{-/-}$ mice have decreased levels, although extra-expression of IL-18 in transgenic mice demonstrate strongly up-regulated IFN-γ levels, obviously indicating intense association between IL-18 and the ability to produce IFN-γ [37]. Recent research has also highlighted, the function of T cells in the pathophysiology of gastric epithelial injury has gained attention, in which Th1-dominant infiltration of CD4$^+$ T cells in the gastric mucosa infected with H. pylori. Fan et al. [38] clearly demonstrate that H. pylori infection induces apoptosis in the gastric epithelial cells, increased IFN-γ production is associated with cell death. Moreover, there is accumulating evidence that IFN-γ can enhances the expression of Fas on the epithelial cells, and mucosal T cells positive for FasL play a key role in the apoptosis of gastrointestinal
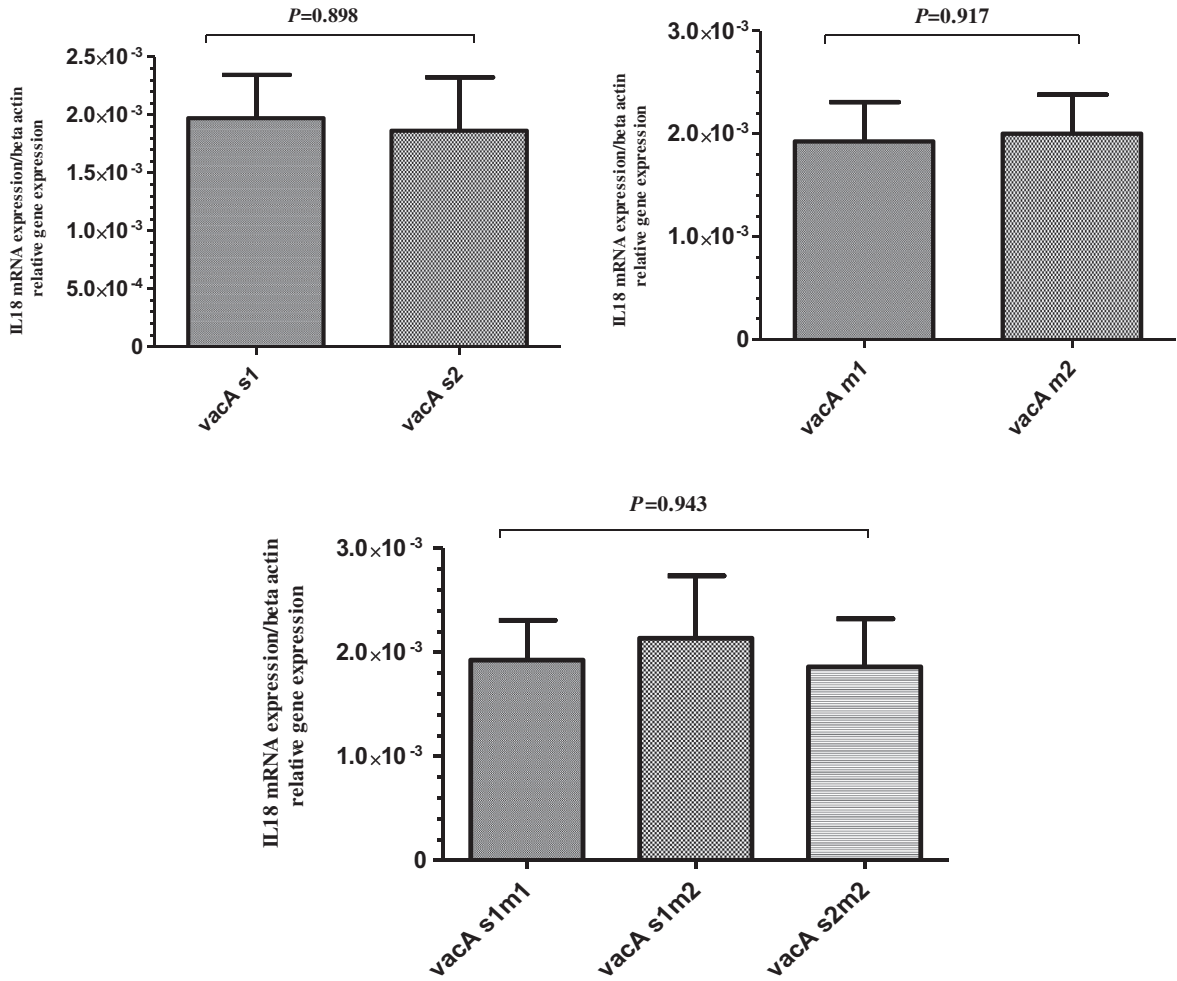
Fig. 3. Mucosal IL-18 mRNA expression in Hp infected patients with vacA allele variants. P values <0.05 was considered statistically significant using the Mann–Whitney and Kruskal–Wallis tests.

epithelial cells. It has been indicated that in Hp-infected subjects, IL-18 mRNA expression and IL-18 production were increased in the gastric mucosa [39]. Our results showed that IL-18 transcripts were increased in the antral mucosa of Hp-infected with gastritis compare to Hp-uninfected subjects with gastritis. This finding

Fig. 4. Correlation between Mucosal IL-18 mRNA level and types of disease in Hp-infected patients.

suggests that Hp pathogen-derived molecules my cause IL-18 mRNA expression. Studies also suggest that some bacterial factors may influence cytokine production. For example cagA and vacA are the most extensively studied virulence factors of Hp and have been related to cytokine expression [40]. Hp strains that have the cag PaI are associated with high amounts in vivo expression of IL-8 in epithelial cell [41]. It has been shown that vacA has multiple effects on epithelial cells, albeit these result in cellular damage rather than pro-inflammatory cytokine release [42]. According to a study, cagA seems to represent a bacterial virulence factor that influence the release of IL-18 and this cytokine is necessary for the initiation of the inflammatory and the Th1 responses [43]. In accord with the role of Hp virulence factors in expression of different cytokine and as there is no study about association between IL-18 mRNA and Hp virulence factors, we design this study. We did not find correlation between IL-18 mRNA expression and virulence factors that suggest two reasons: (i) vacA as exist in all of the Hp-infected biopsies may solitarily suffice to induce expression of IL-18 mRNA then we were unable to observe probable effect of another virulence factor such as oipA, iceA, babA, cagA. (ii) There is no association between virulence factors and expression of IL-18 mRNA. Also a study suggests some factors may have a role in the occurrence of other factors. For example cagA modulates vacA induced vacuolation [44], Indicating that vacA and cagA modulate each other's effects, though do not show the level at which this
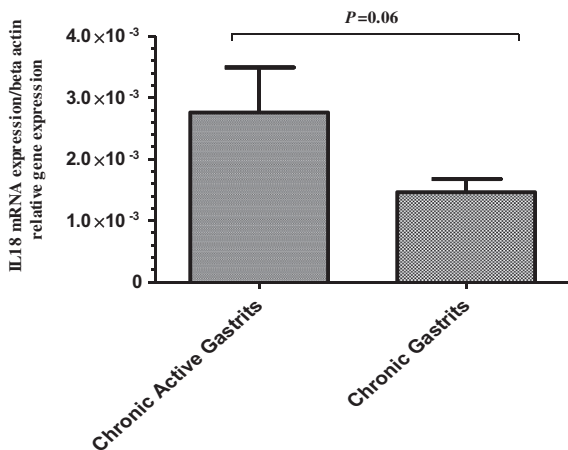
Table 6
Relationship between histological parameters in gastric biopsy specimens and virulence factors.

Genotype	No.	Mononuclear infiltration[b]	Polymorphonuclear infiltration
cagA (+)	28	1.61 (1−3)	0.82 (1−3)
cagA (−)	15	1.87 (1−3)	0.47 (1−3)
P[a]		0.256	0.109
vacA	11	2.09 (1−3)	0.64 (1−3)
m1	30	1.53 (1−3)	0.73 (1−3)
m2			
P[a]		0.019	0.817
s1	32	1.75 (1−3)	0.75 (1−3)
s2	6	1.33 (1−3)	0.50 (1−3)
P[a]		0.248	0.098
s1m1	11	2.09 (1−3)	0.64 (1−3)
s1m2	19	1.53 (1−3)	0.84 (1−3)
s2m1	0		
s2m2	6	1.33 (1−3)	0.50 (1−3)
P[c]		0.055	0.176

[a] Median scores were compared with the Mann−Whitney test.
[b] The histopathological parameters were scored as: 0, none; 1, mild; 2, moderate; 3, severe.
[c] Median scores were compared with the Kruskal−Wallis test.

occurs [44]. As there is no enough biological report to reveal the function of Hp virulence factors, especially in relation to IL-18 mRNA expression, it is difficult to fully elucidate this phenomenon about our study. Also we did not observe relation between vacA alleles and genotypes, s1, s2, m1, m2, s1m1, s1m2, s2m2, and IL-18 mRNA expression. This may show that all of genotypes probably have same effects on the level of IL-18 mRNA expression. Our work indicated severe mononuclear infiltration and chronic gastritis were significantly higher in gastritis patients with vacA (m1)-positive compared to those observed in gastritis patients with vacA (m2)-positive. This may suggest that some vacA genotype affect the production of lymphocyte pathway. In the present study we did not find the association between antral IL-18 mRNA expressions in chronic and chronic acute inflammatory scores in Hp infected patients which suggest that IL-18 may have the same physiologic, pro-IL-18 or IL-18 forms in both chronic and chronic acute steps. The functional role of IL-18 in Helicobacter-induced gastric inflammation and the contribution of different Hp virulence factors to produce IL-18 require further investigation.

Acknowledgments

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