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Frequency of virulence factors in *Helicobacter pylori*-infected patients with gastritis

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ABSTRACT

The outcome of *Helicobacter pylori* infection has been related to specific virulence-associated bacterial genotypes. The vacuolating cytotoxin (vacA), cagA gene, oipA and babA2 gene are important virulence factor involving gastric diseases. The objective of this study was to assess the relationship between virulence factors of *H. pylori* and histopathological findings.

Material and methods: Gastroduodenoscopy was performed in 436 dyspeptic patients. Antrum biopsy was obtained for detection of *H. pylori*, virulence factors and for histopathological assessment. The polymerase chain reaction was used to detect virulence factors of *H. pylori* using specific primers.

Results: vacA genotypes in patients infected with *H. pylori* were associated with cagA, iceA1 and iceA2. In the patients with *H. pylori* infection there was a significant relationship between cagA positivity and neutrophil activity ($P = 0.004$) and chronic inflammation ($P = 0.013$) and with *H. pylori* density ($P = 0.034$). Neutrophil infiltration was found to be more severe in the s1 group than in the s2 group ($P = 0.042$). Also was a significant relationship between oipA positivity and neutrophil activity ($P = 0.004$) and with *H. pylori* density ($P = 0.018$). No significant relationships were observed between other vacA genotypes and histopathological parameters.

Conclusion: H. pylori strains showing cagA, vacA s1 and oipA positivity are associated with more severe gastritis in some histological features but virulence factors of *H. pylori* do not appear to determine the overall pattern of gastritis.

1. Introduction

Helicobacter pylori (*H. pylori*) is an important human pathogen that colonize the stomach. This bacterium induces gastritis, peptic ulcer and is associated with gastric carcinoma [1,2]. The clinical outcome of *H. pylori* infection has been associated with bacterial virulence factors, host gastric mucosal factors, and the environment [3]. But relationship between *H. pylori* genotype and its association with clinical outcome is not fully understood. Several possible disease-specific virulence factors have been suggested to be associated with *H. pylori* infection [4–7]. The main bacterial virulence factors include adhesins (BabA, SabA), the vacuolating cytotoxin VacA, and the products of the cag pathogenicity island (cag PAI). CagA was the most examined putative virulence factor and it is encoded by the cagA gene. A number of studies have confirmed that infection with cagA-positive strains is associated with more severe gastritis and higher prevalence of peptic ulcer and gastric cancer in western countries [8,9]. Conversely, relationship between cagA-positive status and its association with clinical outcome are not fully understood in Asian countries, where the majority of the *H. pylori* strains are cagA-positive [10–12]. The vacuolating cytotoxin A gene, which is another important virulence factor of

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H. pylori, the vacA is present in all *H. pylori* strains and induces vacuolation of epithelial cells [8]. The vacA gene includes two variable parts. The *H. pylori* strains have one of two types of vacA signal sequence (s1 and s2) and two types of mid region (m1 and m2). Cytotoxin production and virulence are higher in the s1/m1 subtypes than in the s1/m2 subtype, and lower still in the s2/m2 subtype [13]. Similar cagA status, there are geographic differences between vacA status and the *H. pylori*-related diseases. In western countries infection with vacA s1 strain is more common in patients with peptic ulcer than in those with chronic gastritis. However in Asian countries, the association between vacA diversity and clinical outcome is not established [14]. BabA is an adhesion molecule that mediates the attachment of *H. pylori* to Lewis b blood group antigens on human gastric epithelial cells [15]. Three bab alleles have been identified: babA1, babA2, and babB and only the babA2 gene product is necessary for Lewis b binding activity [15]. Studies in western countries have shown that about 70% of *H. pylori* strains in Western countries were typed as babA2, which was associated with increased virulence [16]. Moreover, the triple-positive phenotype (babA2, cagA, and vacA s1) was detected at a higher frequency in isolates from patients with ulcers and adenocarcinomas, which might serve as useful markers of high-risk patients in western countries [16]. The iceA gene is induced by contact with epithelium and has two main allelic variants, iceA1 and iceA2. The presence of iceA1 allele is associated with peptic ulcer disease in western countries [17]. OipA is a proinflammatory response-inducing protein associated with high *H. pylori* density and more severe neutrophil infiltration. OipA mediates adherence of *H. pylori* to gastric epithelial cells and contributes to the pathogenesis of gastroduodenal diseases [18]. Therefore, the aim of this study was to analyze the frequency of virulence factors and to correlate the presence of babA2 with cagA, oipA and vacA, iceA genotypes of *H. pylori* strains in Iran patients and to study its association with the histologic severity of gastritis.

2. Materials and methods

2.1. Study population

The subjects included in this study were 436 patients with non-ulcer dyspepsia (NUD) (195 patients with *H. pylori* infection and 241 *H. pylori* uninfection), having recurrent abdominal pain from endoscopy unit of the Hajar Hospital in Shahrekord, Iran. From each patient, written consent was obtained and 3 biopsies were collected from gastric antrum. Two specimens were used for rapid urease test and DNA extraction, and one specimen was used for histopathology study. *H. pylori*-infection was determined by the rapid urease test, PCR (16srRNA and glmM) and histological examination of biopsies taken from the corpus. Patients were classified as *H. pylori*-infected only if the four tests were positive, respectively. This study was performed by the Ethics Committee approval No: 1025 of Shahrekord University of Medical Sciences, Shahrekord, Iran.

2.2. Histological examination

Sections of biopsy specimens were embedded in 10% buffered formalin and stained with hematoxylin and eosin to examine gastritis and with giemsa to detect *H. pylori* (Fig. 1). The histological severity of gastritis was blindly graded from normal to severe based on the degree of mononuclear cell (MNC) and polymorphonuclear leukocyte (PMN) infiltration, and atrophy according to the Updated Sydney system [19] on a four-point scale: 0, no; 1, mild; 2, moderate; and 3, severe changes.

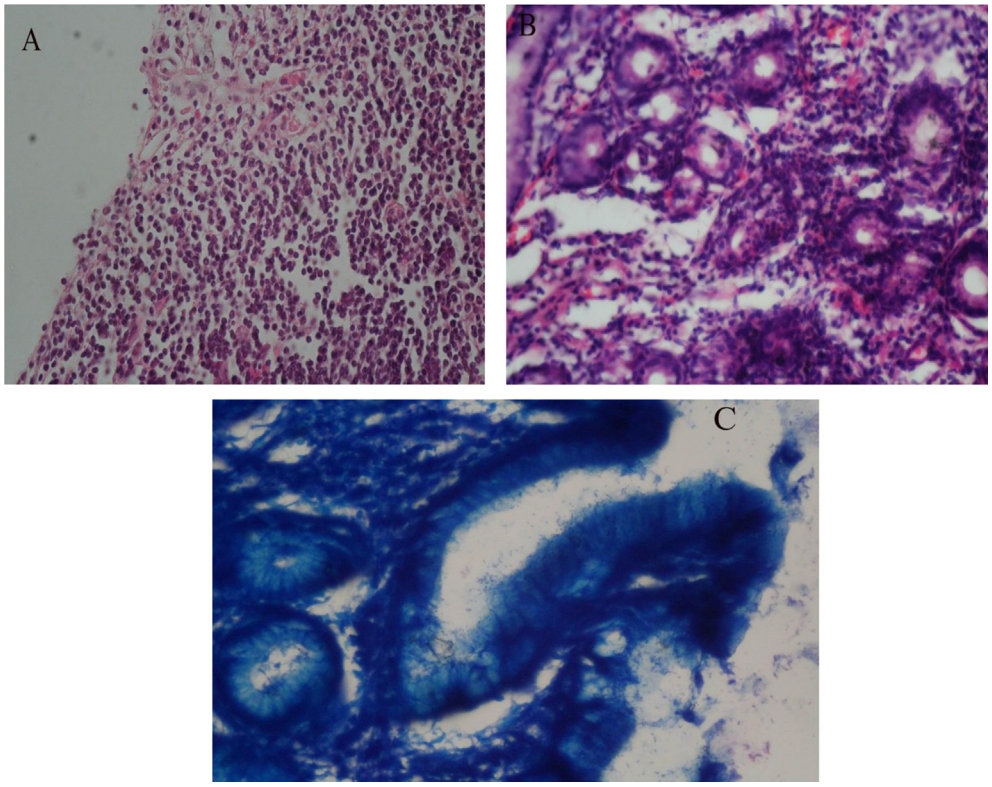

Fig. 1. Histological examination. (A) Mild chronic superficial gastritis with chronic inflammatory cells present in the superficial lamina propria in excess of normal. This is a borderline biopsy sample and illustrates the least number of cells acceptable for a diagnosis of gastritis. (B) Gastric pits infiltrated by neutrophils in a case of *Helicobacter pylori* gastritis. (C) *H. pylori* organisms present in the mucous layer on the gastric mucosal surface (400×).

Table 1
Demographic data of study subjects.

Variable	*H. pylori* infected (%)	*H. pylori* uninfected (%)	*P* Value
Total	195 (44.4%)	241 (55.6%)	0.56
Gender			
Male	80 (41.9%)	106 (58.1%)	
Female	115 (46.2%)	136 (53.8%)	
Mean ± SD (year)	46.74 ± 16.79	48.56 ± 19.82	0.298

2.3. DNA extraction and detection of virulence factors by PCR

DNA for polymerase chain reaction (PCR) was extracted using the Biospin Tissue Genomic DNA Extraction Kit (BioFlux, Japan). Detection of virulence factors was performed by PCR and reported by bagheri et al. [4,20]. 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. Statistical analysis

All data are expressed as mean ± SEM. Age was analyzed by unpaired Student's T-Test. The Chi-squared (χ^2) and Fisher's exact test were used for analyzing categorical variables data and to compare differences in the prevalence of *H. pylori* genotypes. Statistical analysis of the data was carried out by SPSS, version 16, statistical software program and ANOVA was used for continuing data. A *P* values <0.05 were considered to be statistically significant.

3. Results

The characteristics of the population studied are listed in Table 1. There was no significant difference between *H. pylori* infected and *H. pylori* uninfected subjects with respect to the age and gender distribution. Also Fig. 2 shows the results of electrophoresis of PCR products.

3.1. Genotypes

Results of PCR product indicating the presence of the cagA gene was obtained with 139 patients with *H. pylori* infection (71.2%) and

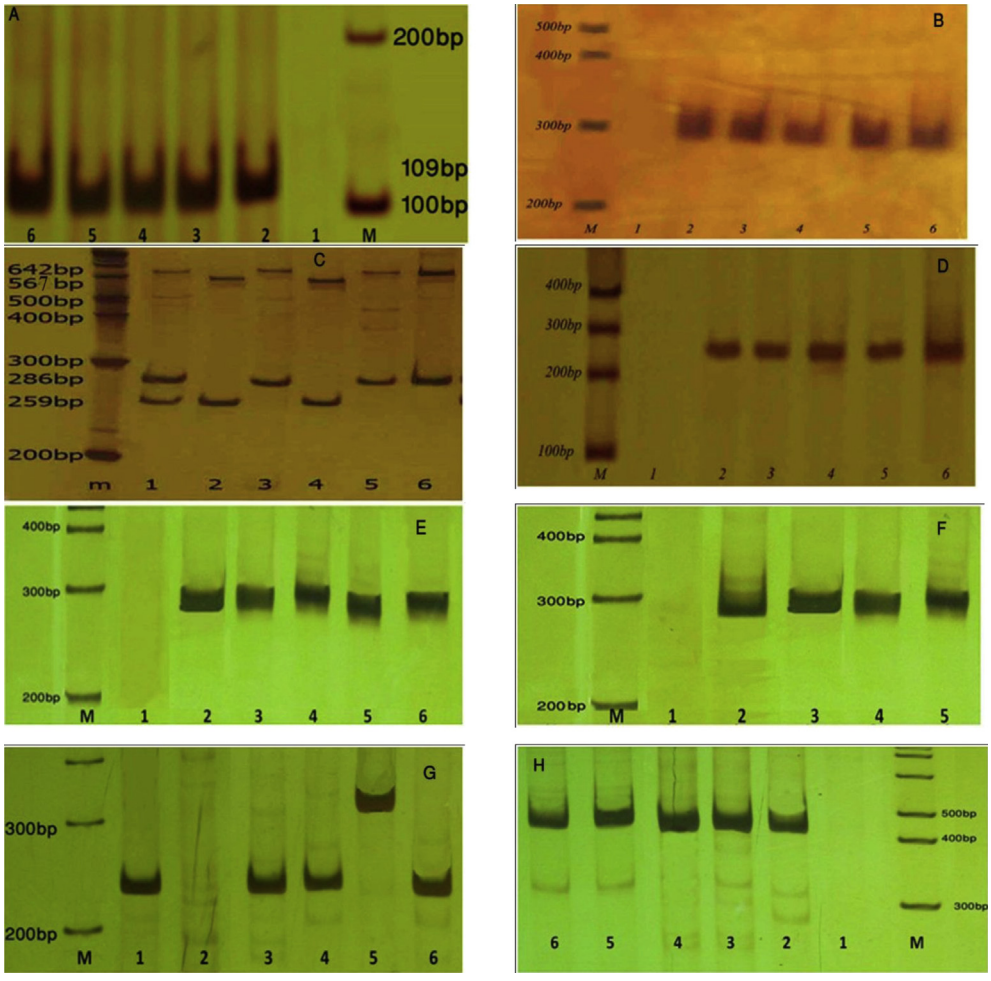

Fig. 2. *H. pylori* diagnostic by PCR and vacA, cagA, iceA1/2, oipA and babA2 genotypes. (A) and (B) *H. pylori* diagnostic: (A) M, 100-bp ladder marker. (2–6) 109 bp *H. pylori* 16srRNA and (B) M, 100-bp ladder marker. (2–6) 294 bp *H. pylori* glmM. (C) *H. pylori* vacA (middle sequence) genotype: M, 100-bp ladder marker. 567 bp (m1 allele); 642 bp (m2 allele) and vacA (signal sequence) genotype: 259 bp (s1 allele); 286 bp (s2 allele). (D) *H. pylori* cagA genotype: M, 100-bp ladder marker. (2–6) 232 bp, cagA-positive strains. (E) *H. pylori* babA2 genotype: M, 100-bp ladder marker. (2–6) 271 bp, babA2-positive strains. (F) *H. pylori* iceA1 genotype: M, 100-bp ladder marker. (2–5) 247 bp, iceA1-positive strains. (G) *H. pylori* iceA2 genotype: M, 100-bp ladder marker. (1 and 3–6) 229 or 334 bp, iceA2-positive strains. (H) *H. pylori* oipA genotype: M, 100-bp ladder marker. (2–6) 430 bp, oipA-positive strains.

Table 2
Prevalence of *H. pylori* genotypes detected in patients.

Genotype	Prevalence
cagA	
cagA positive	139 (71.2%)
cagA negative	56 (28.8%)
vacA	
s1 positive	151 (77.4%)
s2 positive	30 (15.3%)
s1s2 positive	9 (4.6%)
s1s2 negative	5 (2.5%)
m1 positive	48 (24.6%)
m2 positive	125 (64.1%)
m1m2 positive	18 (9.2%)
m1m2 negative	4 (2.0%)
s1m1	55 (28.2%)
s1m2	79 (40.5%)
s2m1	29 (14.8%)
s2m2	0 (00.0%)
Other genotype	32 (16.4%)
iceA	
iceA1 positive	100 (51.2%)
iceA2 positive	61 (31.2%)
iceA1/iceA2 positive	16 (8.2%)
iceA1/iceA2 negative	18 (9.2%)
oipA	
oipA positive	187 (95.9%)
oipA negative	8 (4.1%)
babA2	
babA2 positive	168 (86.1%)
babA2 negative	27 (13.9%)

56 (28.8%) were negative (Table 2). In the m-region, 18 patients (9.2%) contained both m1 and m2 alleles. In the patients containing one single vacA m allele, the m1 allele was found in 48 patients (24.6%) and m2 in 125 one (64.1%). In the s-region, 9 patients (4.6%) contained both m1 and m2 alleles. In the patients containing one single vacA s allele, the s1 allele was found in 151 patients (77.4%) and s2 in 30 one (15.3%). The vacA genotype s1/m1, s1m2 and s2m1 were detected in 55 (28.2%), 79 (40.5%) and 29 (14.8%) patients. The s2m2 genotype was not found in our study. The oipA and babA2 genes were detected in 187 (95.9%) and 168 (86.1%) patients. Overall, iceA1 was detected in 100 patients (51.2%) of all 281 patients and iceA2 was found in 61 patients (31.2%). 16 patients (8.2%) were positive for both iceA1 and iceA2 and 18 patients (9.2%) were negative for both iceA1 and iceA2 (Table 2).

3.2. Correlation of vacA alleles and presence virulence factors

The cagA gene was detected in 139 (71.2%) patients with *H. pylori* infection. The association between vacA alelles and presence virulence factors is described in Table 3. Of the 139 patients infected with *H. pylori* that were positive for cagA, 115 patients were associated with the toxin-producing vacA s1 and only 33 patients with cagA-positive were vacA s2. Of the 139 patients with *H. pylori* infection that were positive for cagA, 43 patients were associated with vacA s1m1, 61 patients were associated with vacA s1m2 and only 13 patients with cagA-positive were vacA s2m2. Also vacA genotypes in patients infected with *H. pylori* were associated with iceA1 and iceA2 (Table 3).

3.3. H. pylori density

The density of *H. pylori* was scored in gastric biopsy specimens. The density scores of *H. pylori* in patients with cagA and oipA positive were higher than in the cagA and oipA negative (Table 4). There was no significant relationship between other virulence factors and *H. pylori* density.

3.4. Neutrophil activity

CagA, oipA and vacA s1 positive genotypes were strongly associated with higher neutrophil activity in gastric biopsy specimens (Table 4). No relationships were found between neutrophil activity and other virulence factors in gastric biopsy specimens.

3.5. Chronic inflammation

There was an association between cagA positivity and high inflammation scores in gastric biopsy specimens ($p = 0.013$). There was no relationship between other virulence factors and chronic inflammation in gastric biopsy specimens (Table 4).

4. Discussion

H. pylori infection results in chronic gastritis and, eventually, diseases, such as peptic ulcer, gastric cancer, and MALT lymphoma [1,3]. Genotypic alterations of *H. pylori* are thought to be responsible for the various clinical manifestations and for infection without symptoms or with symptoms of gastric carcinoma and MALT lymphoma. In our study, the presence of virulence factors, which is thought to be associated with severe diseases, was investigated in patients with functional dyspepsia and compared with histological findings. Although an association between *H. pylori* infection and chronic gastritis is clear, development of severe gastric diseases is rare. These variations in the clinical consequences are because of factors such as duration of the infection, inflammatory response of the patient, virulence of *H. pylori* strains. Infection with less virulent strains is associated with mild symptoms whereas infection with more virulent strains is thought to be associated with more severe gastric inflammation and, eventually, peptic ulcer, gastric adenoma, and MALT lymphoma. The

Table 3
Correlation of vacA alleles with the cagA, babA2, and iceA genotype of the samples studied.

vacA genotype	cagA		babA2		iceA1		iceA2	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
s1m1	43	9	48	5	37	16	16	37
s1m2	61	19	63	17	48	32	34	46
s2m2	13	16	26	3	9	20	17	12
P value	0.001		0.128		0.003		0.042	
s1	115	33	125	23	96	52	52	96
s2	13	17	27	3	10	20	17	13
P value	0.000		0.576		0.001		0.027	
m1	43	10	48	5	38	15	15	38
m2	78	40	98	20	61	57	57	61
P value	0.046		0.198		0.014		0.014	

Table 4
Relationship between histological parameters determined in gastric biopsy specimens and *H. pylori* cagA status.

Genotype	Neutrophil activity[a]	Chronic inflammation[a]	*H. pylori* density
cagA (+)	0.99 (0–3)	1.88 (0–3)	1.80 (0–3)
cagA (+)	0.59 (0–3)	1.61 (0–3)	1.56 (0–3)
P[b]	**0.004**	**0.013**	**0.034**
vacA m1	1.00 (0–3)	1.75 (0–3)	1.51 (0–3)
vacA m2	0.79 (0–3)	1.50 (0–3)	1.49 (0–3)
	0.135	0.057	0.869
vacA s1	0.94 (0–3)	1.61 (0–3)	1.47 (0–3)
vacA s2	0.59 (0–3)	1.34 (0–3)	1.47 (0–3)
P[b]	**0.042**	0.1	0.998
s1m1	1.00 (0–3)	1.72 (0–3)	1.55 (0–3)
s1m2	0.88 (0–3)	1.56 (0–3)	1.54 (0–3)
s2m2	0.59 (0–3)	1.28 (0–3)	1.53 (0–3)
P[b]	0.06	0.576	0.996
iceA1 (+)	0.86 (0–3)	1.64 (0–3)	1.54 (0–3)
iceA1 (−)	0.90 (0–3)	1.44 (0–3)	1.42 (0–3)
P[b]	0.787	0.079	0.236
iceA2 (+)	0.85 (0–3)	1.53 (0–3)	1.55 (0–3)
iceA2 (−)	0.90 (0–3)	1.57 (0–3)	1.45 (0–3)
P[b]	0.658	0.731	0.309
babA2 (+)	0.89 (0–3)	1.57 (0–3)	1.51 (0–3)
babA2 (−)	0.81 (0–3)	1.44 (0–3)	1.41 (0–3)
P[b]	0.673	0.431	0.475
oipA (+)	0.91 (0–3)	1.55 (0–3)	1.51 (0–3)
oipA (−)	0.25 (0–3)	1.62 (0–3)	1.12 (0–3)
P[b]	**0.032**	0.797	**0.018**

[a] The histopathological parameters were scored as: 0, none; 1, mild; 2, moderate; 3, severe.
[b] Mean scores were compared with the *t* test.

relationship between *H. pylori* genotypes and infection with gastric inflammatory response varies within nationalities. In Western countries it has been demonstrated that more severe gastric inflammation develops after infection with cagA-positive strains; in Asian countries there is no such difference. Studies from Turkey on cagA positivity and the histopathological findings of gastritis led to conflicting results. Demirturk et al. suggested that cagA positivity is associated with more severe glandular atrophy, inflammation, and activity, whereas Saruc et al. demonstrated a relationship between cagA positivity with inflammation, *H. pylori* density, and intestinal metaplasia but not with glandular atrophy [21,22]. In our study, we found 71.2% infected patients with cagA-positive which are similar to some reports from Iran [23] and different from reports of South and East Asian where the presence of cagA strain and its association with clinical outcomes is more than 90% [24,25]. It has been shown that the prevalence of cagA-positive strains in USA and Europe is 60–70% [26]. Our results also demonstrate that cagA-positive patients showed more severe neutrophil infiltration, chronic inflammation and *H. pylori* density in patients infected with *H. pylori*. The variability of the vacA s and m regions is thought to have effects over the secretion of vacuolating toxin. s1/m1 strains produce huge amounts of toxins and are highly detrimental. s2 strains do not produce such toxins. In many studies, the variability of the s genotypes is associated with disease formation but not proven. In our study, we find that there is statistically significant relationship between vacA s1 genotype and neutrophil infiltration. Warburton et al. reported no relationship between vac s1 and s2 regions and histological findings [27]. This finding is controversial, because it is known that strains with s1 genotype produce much greater amounts of toxins. Subtypes of vacA s1 region may be closely related to histological parameters. Nevertheless, Atherton et al. suggested that s1a genotype is associated with peptic ulcer and more severe gastritis [28]. Our results indicate that oipA-positive patients were strongly associated with higher neutrophil activity and *H. pylori* density in gastric biopsy specimens. oipA expression was reported to be linked to severe inflammation and the induction of IL-8 secretion and the presence of a functional gene is significantly associated with the presence of duodenal ulcers, gastric cancer, and increased neutrophil infiltration [29,30]. Recent work using Mongolian gerbils infected with wild-type *H. pylori* and an isogenic oipA mutant strain demonstrated a role for oipA in induction of the mucosal cytokines IL-1, IL-17, and tumor necrosis factor alpha (TNF-α) and in gastric mucosal inflammation [31]. In the present study, iceA1 allele was predominant and was not associated with the severity of the gastritis. Besides the virulence factors of *H. pylori*, variations of acid production, the genetics of the infected subject, tobacco and alcohol intake may affect both clinical outcomes and the histopathological findings. More studies are needed to evaluate other factors besides the *H. pylori* genotypes, because *H. pylori* is thought to be a cause of serious diseases.

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