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Relationship between *ure*B Sequence Diversity, Urease Activity and Genotypic Variations of Different *Helicobacter pylori* Strains in Patients with Gastric Disorders

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Abstract

Association of the severity of *Helicobacter pylori* induced diseases with virulence entity of the colonized strains was proven in some studies. Urease has been demonstrated as a potent virulence factor for *H. pylori*. The main aim of this study was investigation of the relationships of *ure*B sequence diversity, urease activity and virulence genotypes of different *H. pylori* strains with histopathological changes of gastric tissue in infected patients suffering from different gastric disorders. Analysis of the virulence genotypes in the isolated strains indicated significant associations between the presence of severe active gastritis and *cag*A$^+$ (P = 0.039) or *cag*A/*ice*A1 genotypes (P = 0.026), and intestinal metaplasia and *vac*A m1 (P = 0.008) or *vac*A s1/m2 (P = 0.001) genotypes. Our results showed a 2.4-fold increased risk of peptic ulcer (95% CI: 0.483–11.93), compared with gastritis, in the infected patients who had *dup*A positive strains; however this association was not statistically significant. The results of urease activity showed a significant mean difference between the isolated strains from patients with PUD and NUD (P = 0.034). This activity was relatively higher among patients with intestinal metaplasia. Also a significant association was found between the lack of *cag*A and increased urease activity among the isolated strains (P = 0.036). While the greatest sequence variation of *ure*B was detected in a strain from a patient with intestinal metaplasia, the sole determined amino acid change in UreB sequence (Ala201Thr, 30%), showed no influence on urease activity. In conclusion, the supposed role of *H. pylori* urease to form peptic ulcer and advancing of intestinal metaplasia was postulated in this study. Higher urease activity in the colonizing *H. pylori* strains that present specific virulence factors was indicated as a risk factor for promotion of histopathological changes of gastric tissue that advance gastric malignancy.

Key words: *Helicobacter pylori*, virulence, factor urease activity, histopathological changes

Introduction

Helicobacter pylori is a Gram-negative spiral bacterium that infects at least half of the world's population and is a known carcinogen (WHO, 1994). This bacterium is responsible for different gastrointestinal diseases, including duodenal and gastric ulcer diseases (Ribeiro *et al.*, 2003), gastritis, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer (Kusters *et al.*, 2006). Infiltration of immune cells, particularly polymorphonuclear leukocytes, commonly occurs after colonization of *H. pylori* strains in the gastric tissue (D'Elios *et al.*, 2007). Association of the severity of *H. pylori* induced diseases with virulence entity of the colonized strains was reported in several studies. CagA, VacA, IceA, DupA and urease are among the most important virulence factors whose involvement in the progression of these diseases has been established (Rathbone and Rathbone, 2011). Early colonization of *H. pylori* strains in childhood, expression

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of more virulent allelic variants of the virulence factors, and chronic induction of the inflammatory responses caused histopathological changes that are associated with poor clinical outcomes (Kusters *et al.*, 2006; Yahav *et al.*, 2000). Urease of *H. pylori* accounts for about 10% of the total cell protein that is expressed in most of the strains (Suzuki *et al.*, 2007). This enzyme, a nickel-requiring metalloenzyme, consists of two subunits, UreA and UreB (Hu *et al.*, 1992), which hydrolyze urea to ammonia and carbon dioxide within the gastric mucus layer to facilitate its initial interaction in this acidic environment (McGee and Mobley, 1999).

There are some other proposed roles for *H. pylori* urease, including its involvement in colonization of the gastric tissue, chemotactic activity for human monocytes, inhibition of the phagocytosis, intracellular survival of the bacterium, induction of inflammatory cytokines and inducible NO synthase (iNOS) (Shimoyama *et al.*, 2003). Urease can bind to class II MHC on gastric epithelial cells and induces apoptosis, a phenomenon that may explain its involvement in ulcer formation (Fan *et al.*, 2000). Beyond the proposed roles for *H. pylori* urease, the association of its activity with clinical results and the pathological changes of the gastric tissue is not so clear. The activity of this enzyme has been shown to be dependent on nickel availability and the promoter of the *ure*A gene. However, little information is known about the effects of genetic variation of the enzyme subunits or its expression level on its catalytic activity. It is recognized that the active site of the enzyme is located in the B subunit that is involved in restoration of urease activity, induction Th17 cell response, induction of NF-κB and interleukin-8 production (Lee *et al.*, 2001; Eaton *et al.*, 2002; Beswick *et al.*, 2006; Zhang *et al.*, 2011). The aim of this study was to determine the relationships among *ure*B sequence diversity, urease activity of the collected strains and virulence property of *H. pylori* strains in patients with different histopathological changes.

Experimental

Materials and Methods

Patients and sampling. Isolation of *H. pylori* strains was performed from 75 patients suffering from gastric disorders that referred to an endoscopy unit in Taleghani Hospital in Tehran, Iran. The study received ethical approval from the ethics committee of Shahid Beheshti University of Medical Sciences. Informed consent forms were signed by all the patients. Gastric biopsy specimens from antrum and corpus were collected from each patient. The biopsies were used for both histopathological and microbiological studies. The homogenized biopsies were cultured on supplemented Brucella agar medium supplemented with 7% sheep blood, 10% FBS and selective antimicrobials. The cultures were incubated up to 5 days in 37°C under microaerobic conditions. The entity of the grown colonies was characterized by both biochemical (urease activity, catalase, oxidase) and molecular tests (see below). The characterized strains were stored at –70°C for further examination.

Histopathological examination. The histological sections were evaluated and graded according to the features suggested by the updated Sydney Classification system (Dixon *et al.*, 1996). The formalin fixed paraffin embedded biopsy samples were cut in 5-μm-thick sections on a microtome with a disposable blade. Patients were classified based on the determined pathological changes and clinical data into three following groups: chronic gastritis, sever active gastritis (SAG), and intestinal metaplasia (Nishiya *et al.*, 2000).

DNA extraction. Genomic DNA extraction of the freshly grown *H. pylori* colonies was performed using YTA Genomic DNA Extraction Mini Kit (Yektahtajhiz, Tehran, Iran) according to the manufacturer's instructors. The DNA samples were stored at –20°C until used for gene amplification.

Characterization of *H. pylori* isolates and genotyping. Genus and species specific primer pairs for 16srRNA and *glm*M were used to characterize the initially detected isolates (Table I). Therefore, a final reaction volume of 25 μl, including 2 μl of the template DNA, 0.01 μM of each primer, 1X PCR buffer, 200 mM deoxynucleoside triphosphates, 4 mM $MgCl_2$ and 0.5 unit U Taq DNA polymerase. The amplification were performed at following conditions: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at the indicated temperatures for each reaction in Table I for 45 s, extension at 72°C for 1 min, and then final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in agarose gel after staining with ethidium bromide.

Multiplex-PCR genotyping. Four different genes *cag*A, *vac*A (*s* and *m* alleles), *ice*A (A1/A2 alleles) and *dup*A were investigated for virulence genotyping of the collected strains. The used primers, length of PCR products, and annealing temperatures are shown in Table I. The PCR was performed in a multiplex assay as described by Farzi *et al.* (2014) under the following conditions: 35 cycles of 1 min at 94°C, 40 seconds at 57°C, and 1 min at 72°C.

***ure*B sequencing and sequence alignment.** To study sequence diversity of *ure*B among *H. pylori* strains from patients presented different pathological changes, a pair of primers covering 1056 bp of the gene that encodes functionally important regions of

Table I
Specific primers used in this study

Target	Primer sequence (5'3')	Amplicon size (bp)	Annealing temp.	References
***glm*M**	F: GGATAAGCTTTTAGGGGTGTTAGGGG R: GCTTACTTTCTAACACTAACGCGC	269	58°C	(Kauser *et al*., 2005)
***ice*A1**	F: TATTTCTGGAACTTGCGCAACCTGAT R: GGCCTACAACCGCATGGATAT	~900	57°C	(Mukhopadhyay *et al*., 2000)
***ice*A2**	F: CGGCTGTAGGCACTAAAGCTA R: TCAATCCTATGTGAAACAATGATCGTT	~800	57°C	(Mukhopadhyay *et al*., 2000)
***vac*A (*s1*/*s2*)**	F: CTGCTTGAATGCGCCAAAC R: ATGGAAATACAACAAACACAC	259/286	57°C	(Farzi *et al*., 2014)
***vac*A (*m1*/*m2*)**	F: CAATCTGTCCAATCAAGCGAG R: GCGTCAAAATAATTCCAAGG	567–42	57°C	(Qiao *et al*., 2003)
***cag*A**	F: AACAGGACAAGTAGCTAGCC R: TATTAATGCGTGTGTGGCTG	500	57°C	(Russo *et al*., 1999)
ure*B* seq	F: AGCGGATATTGGTATTAAAG R: TGAGCGAACATTTCTCTG	1056	48°C	this study
***dup*A**	F: ACGATTGAGCGATGGGAATA R: AAGCTGAAGCGTTTGTAACGA	1598	57°C	this study
16S rRNA	F: GGCTATGACGGGTATCCGGC R: GCCGTGCAGCACCTGTTTTC	764	58°C	(Bohr *et al*., 2002)

UreB subunit, including its active site, were designed (Table I). PCR was performed under the following conditions: 30 cycles of 1 min at 94°C, 45 seconds at 48°C, and 1 min at 72°C. The PCR products were electrophoresed in 1.8% agarose gel and finally bidirectional sequence analysis of the amplicons was performed using the same primers after purification. Diversity of the obtained sequences was determined after their alignment using the MEGA6.06 software in compare to reference sequence strain j99 (ATCC 700824).

Urease activity assay. In this study, the urease activity was determined among different strains of *H. pylori* by colorimetric assay according to the method of Onal Okyay and Frigi Rodrigues (2013) with some modifications. All the strains were cultured on Brucella agar medium supplemented with 5% defibrinated sheep blood and 7% fetal bovine serum and incubated for 5 days at microaerophilic conditions. The grown bacterial colonies were suspended in phosphate buffer solution (PBS, pH 7.4) and then adjusted to an optical density (Ribeiro *et al*., 2003) of 0.08 at 620 nm with an Elisa plate reader. Rapid urease broth medium was used for the proposed assay. Suspensions of 100 µl of each strain at determined OD were inoculated into 96-well microplate containing 300 µl of rapid urease broth medium in duplicate. Changes of color and absorbance during 20 min were recorded for all the strains. To estimate any significant difference in the obtained urease activity rates and for the grading the activities, a cut-off value was determined as follows: Mean OD + 2 SD (standard deviation).

Statistical analysis. The correlations between *H. pylori* genotypes, *ure*B sequence diversity, urease activity (color change rate) and the clinical and pathological findings were estimated using either the chi-square or fisher exact tests. Student's t-test and Man-Whitney test were used to analyze significant difference of the estimated mean activity of urease in *H. pylori* strains in comparison with severity of pathological changes and disease status (p value < 0.05).

Results

Out of 75 examined patients, *H. pylori* isolates were obtained from 30 patients with different gastric disorders (age range, 28 to 79 years). Among these patients, 12 patients presented peptic ulcer disease (Duodenal ulcer (DU)), 4/12, and gastric ulcer, (8/12), while non-ulcer diseases (NUD) was detected in 18 patients. The entity of all the isolates was confirmed by both biochemical and PCR methods. Analysis of the pathological findings showed the presence of CG, SAG, and IM in 11 (36.6%), 14 (46.6%), and 5 (16.6%) patients, respectively. No significant correlation between age, nutrition, smoking and the pathological changes was determined in the infected patients. Among the 30 isolated strains, different virulence genotypes were detected. In total, *vac*A, *cag*A, *ice*A and *dup*A were detected in 100%, 60%, 83.3%, and 63% of the strains, respectively. The *vac*A *s1*/*m1* accounted for 36% of the strains, while the *s2*/*m1*, *s1*/*m2*, and *s1*/*m* – allelic

Table II
The relationship between variables and pathological outcomes

variables	CG	SAG	IM	*p* value	OR	95%CI
***cag*A$^+$**		*		0.034	8.8	0.9–38.35
***vac*A**						
s1/m1						
s1/m2			*	0.001	9.3	2.4–57.4
m1			*	0.08	13	1.36–24.2
***ice*A1$^+$**						
***ice*A2$^+$**						
***ice*A1$^+$/A2$^+$**						
***dup*A$^+$**						
***ice*A1$^+$/*cag*A$^+$**		*		0.026	6.4	1.15–35.43
variation Ala < Thr						
Cutoff-point						

variants accounted for 33%, 23% and 6% of the strains. Among the *ice*A positive strains, different allelic types were detected in these patients, including *ice*A1$^+$/*ice*A2$^+$ (36%), *ice*A1$^+$/*iec*A2$^-$ (33%), *ice*A1$^-$/*ice*A2$^+$ (13%). Statistically significant associations were found between the presence of *cag*A and SAG, and also *vac*A *m1* and *vac*A *s1/m2* genotypes and IM. Also significant association was found between the *ice*A1$^+$/*cag*A$^+$ genotype and IM (Table II). However, no correlation was determined between ulcer formation (GU and DU) and virulence genotypes in these patients.

***ure*B sequence diversity.** The *ure*B was detected in all the strains and the diversity in their sequences was analyzed. The *ure*B sequence variants were assigned by GenBank (accession numbers: KP401951-KP401975). Based on the *ure*B reference sequence (Strain J99), different point mutations were found in the *ure*B sequence, with one of them being responsible for Ala→Thr amino acid change at position 201. This mutation was detected in 30% (9/30) of the strains. No correlation was detected between this mutation and the clinical or pathological data. The neighbor-joining method was used for investigation of relationships between the obtained sequences. Comparison of the nucleotide sequences with reference sequence J99 showed the highest diversity (3% difference) in a strain (HC452), which was isolated from a patient with IM.

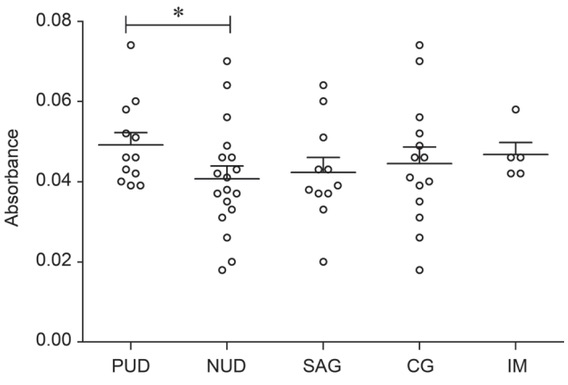

Fig. 1. Diagram of frequency distribution of urease activity with mean ± S.E.M bars. Among clinical findngs isolated strains of *H. pylori* from peptic ulcer patients had higher urease activity than from gastritis patients (0.049 ± 0.01 *vs.* 0.04 ± 0.013). Comparing urease activity based on pathological outcomes showed isolated strains from patients with IM had highest urease activity than SG and CG patients respectively (0.046 ± 0.006 vs. 0.042 ± 0.015 and 0.042 ± 0.012).

Abbrev: IM – intestinal metaplasia, SG – severe active gastritis, CG – chronic moderate active gastritis. Graphs depicted by GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA, USA). * = Statistically significant, ─ = mean bar ,┬ = S.E.M bar.

Urease activity. Urease activity was evaluated in all the strains. Analysis of mean values of urease activity (Absorbance change/Δt) showed diversity of this activity among different strains of *H. pylori* in different patients groups. The highest activity was detected among the strains isolated from patients with PUD compared with those presenting NUD (P = 0.043) (Fig. 1). The strains isolated from patients with IM had higher urease activity than those from patients with other pathological changes. However, the obtained mean difference was not statistically significant. The results of our study showed significant association between the lack of *cag*A and increased urease activity (P = 0.031) (Fig. 2). No significant mean difference between urease activity of *H. pylori* strains carrying Ala > Thr amino acid mutation and wild type strains (P = 0.525) as detected (Fig. 2). A cut-off value of 0.056 was estimated for qualitative analysis of urease activity according to the obtained absorbance values. Considering the cut-off value, our data didn't show any significant relationship between urease activity and clinical or pathological data.

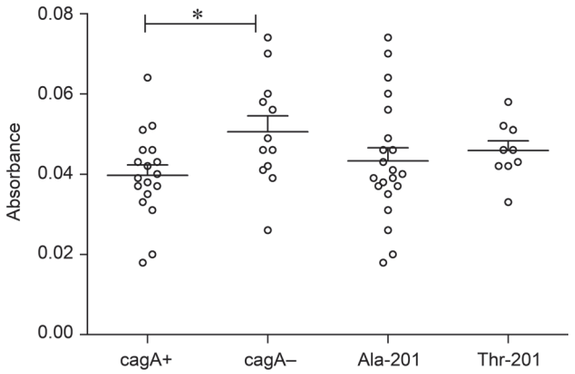

Fig. 2. Diagram of frequency distribution of urease activity with mean ± S.E.M bars. The obtained results this study showed strains of positive *cag*A had higher urease activity than of negative cagA (0.039 ± 0.01 *vs.* 0.05 ± 0.013). The mean of urease activity of strains with aminoacid variations Ala↔Thr-201 had no statistical differences (0.043 ± 0.014 *vs.* 0.045 ± 0.007). Graphs depicted by GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA, USA).

* = Statistically significant, — = mean bar, ┬ = S.E.M bar

Discussion

In this study, the relationships of *ure*B sequence diversity, urease activity and genotypes of different *H. pylori* strains were investigated in patients with different gastric disorders. Pathological changes of the infected stomach tissue could be in association with virulence entity of the colonizing strains in this organ. The results of our study showed an association between occurring IM and *vac*A *m1* or *s1m2* allelic variants. The higher expression VacA in *H. pylori* strains conferring *s1*/*m1* genotype compared with those presenting *s1*/*m2* variant, could explain the observed pathological changes in patients with IM (Evans *et al.*, 1998). In a study by Nogueira *et al.* (2001) it was shown that higher degrees of lymphocytic and neutrophilic infiltrates were seen in gastric biopsy specimens of patients infected with strains encoding distinct genotypes. They showed that *vac*A *s1* and *m1* allelic variants are significantly associated with atrophy and IM. While the association of *vac*A *s1* allele with PUD in the infected patients was established in some studies (Atherton, 1997), this association was not supported by our results. The lack of an association between variability of the *vac*A s and m regions and PUD was similarly established by Aydin *et al.* (2004). The interplay between *vac*A and *cag*A signaling pathways may explain this incongruity (Argent *et al.*, 2008). The association between *cag*A status and pathological changes was identified in our study. In this subject, SAG was dominantly found in patients infected with *cag*A positive strains that was agreed to in earlier reports (Plummer *et al.*, 2007). Although the association of *cag*A with PUD was reported in some studies, our results didn't confirm such a relationship. In the case of *ice*A, the results was similar to other studies (Nogueira *et al.*, 2001), its allelic variants were not significantly associated with neutrophil infiltration in the studied biopsy samples. However, *cag*A/*ice*A1 genotype was in correlation with the occurrence SAG. In a study by Nishiya *et al.* (2000) it was concluded that *ice*A1-positive strains can induce more increased active gastritis in *cag*A-positive and *vac*A *s1*/*m1* strains. The association between inflammatory cell infiltration and *H. pylori* virulence factors was also detected for *dup*A (Wang *et al.*, 2015). This virulence factor is in correlation with the occurrence of duodenal ulcer (Arachchi *et al.*, 2007; Lu *et al.*, 2005). Our results showed a 2.4-fold increased risk of peptic ulcer (95% CI: 0.483–11.93), compared with gastritis in the infected patients who had *dup*A positive strains; however, this association was not statistically significant. There are other controversial results for the noted association between gastrointestinal disorders and *dup*A status (Lu *et al.*, 2005; Arachchi *et al.*, 2007; Argent *et al.*, 2007; Nguyen *et al.*, 2010). Although an increased risk of DU was detected in our study, the lack of significance difference in these patients could be explained by the probable lack in the function of DupA or its secretion in the responsible strains (Jung *et al.*, 2012). The *H. pylori ure*A-*ure*I genes play important role in urease production. Urease has two major subunits A and B and five accessory subunits E, F, G, I and D. There are six copies of UreA and UreB subunits in the holoenzyme, whose active site is located within the UreB subunit (at position 322) (Mobley *et al.*, 1995). Urease can express on the bacterial surface or release into the gastric mucosa. UreB subunit is composed of 569 amino acids and is nearly conserved among different strains. In a study by Muller *et al.* (2002), they compared obtained *ure*B sequences and found more than 98% identity among the sequences, which is similar to our results. Theoretically, it seems that diverse urease activity affect survival of the bacterium and its pathogenesis in the gastric tissue. In our study, increased urease activity in the patients with peptic ulcer than those with gastritis was indicated. This diversity was not explained by the sole determined amino acid change (Ala > Thr) in the *H. pylori* strains isolated from the studied patients groups. This mutation is not located near the active site, which refuses its influence on urease activity. The lack of urease accessory proteins may explain the noted variations that were detected in these strains (Benoit *et al.*, 2007; Fong *et al.*, 2013). The inverse association that was found between *cag*A status and urease activity was a new finding in our experiments. It is well known that *H. pylori* possesses proton-dependent intrabacterial transportation systems that transport CagA and urease toward the type-IV secretion

machinery and UreI (Wu *et al.*, 2014). Although UreI dependent translocation of CagA within the cytoplasm of *H. pylori* was established by Wu *et al.* (2005), it remained unclear how CagA interacts with UreI to modulate its activity. The development of ulcers in the antral mucosa caused by the urease of *H. pylori* due to apoptosis was suggested by several studies (Kohda *et al.*, 1999; Fan *et al.*, 2000). In our study, the highest activity was found among the strains isolated from patients with PUD compared with those presented NUD. This activity was also relatively higher among patients with IM. The association between urease activity and peptic ulcer was described by several studies. It seems that ammonia produced by the urease can induce apoptosis, whose action promotes tissue damage and ulcer formation (Igarashi *et al.*, 2001). Although there is no report about higher level of urease activity in patients with peptic ulcer, the increased activity was previously established in strains from cancer patients (Ito *et al.*, 1995). In a study by Xu *et al.* (1990) it was shown that urease inhibitor can cause a 75% drop in vacuolating gastric cells that had been induced by defined concentration of urease. While our results showed higher activity of this enzyme in the strains collected from ulcerative tissue, it remains to clarify its effect on gastric acid secretion and ulcer formation in these patients. Urease dependent NO production in the gastric tissue and its involvement in mucosal damage may explain its immunological role in the pathogenesis of *H. pylori* mediated gastritis and carcinogenesis (Gobert *et al.*, 2002).

Based on the analyzed strains, the characterized relationships between *H. pylori* virulence genotypes, *cag*A, *cag*A/*ice*A1, or *vac*A *s1m2* allelic forms, and IM or SAG proposed role of these virulence genes in forming histopathological changes that advance gastric malignancy. While an increased risk of peptic ulcer, compared with gastritis, was seen in the infected patients with the *dup*A positive strains, no statistically significant relationship was found for the studied virulence factors in these patients. Analysis of the association between urease activity of the *H. pylori* strains and *ure*B nucleotide polymorphisms showed that this subunit is conserved among most of the strains. The sole amino acid change (Ala > Thr) in these strains didn't show any possible influence on enzymatic activity in these strains. The putative role of *H. pylori* urease in the progression of ulcer formation was postulated in this study, since greater urease activity was seen among the strains that were isolated from patients with PUD compared with those from NUD patients. The noted activity seems to be affected by the CagA cytoplasmic translocation, so the highest activity was determined in the *cag*A negative strains. Because urease comprises 10% of total *H. pylori* cell proteins, it is important to realize the association between the higher inflammatory response and pathological changes of gastric tissue and risk of *H. pylori*-associated gastric cancer because of its activity in the stomach.

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Conflict of interest

The authors declare no conflict of interest.

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