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

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Submitted 26 August 2015, revised 19 October 2015, accepted 3 November 2015

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 ureB 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 cagA+ (P = 0.039) or cagA/iceA1 genotypes (P = 0.026), and intestinal metaplasia and vacA m1 (P = 0.008) or vacA s1/m2 (P = 0.001) genotypes. Our results showed a 2.4-fold increased risk of peptic ulcer (95% CI: 0.48–11.93), compared with gastritis, in the infected patients who had dupA 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 cagA and increased urease activity among the isolated strains (P = 0.036). While the greatest sequence variation of ureB was detected in a strain from a patient with intestinal metaplasia, the sole determined amino acid change in UreB sequence was 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
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 activity of H. pylori accounts for about 10% of the total cell protein that is expressed in most of the strains (Suzuki et al., 2006). The 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 ureA 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-kB 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 ureB 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 at 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, severe 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 (Yektahajiz, Tehran, Iran) according to the manufacturer’s instructions. 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 16sRNA and glmM 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 MgCl₂, 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 cagA, vacA (s and m alleles), iceA (A1/A2 alleles) and dupA 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.

**ureB sequencing and sequence alignment.** To study sequence diversity of ureB 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
Urease activity, sequence diversity, virulence factors and gastric disorders

Table I
Specific primers used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence (5'3')</th>
<th>Amplicon size (bp)</th>
<th>Annealing temp.</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>glmM</td>
<td>F: GGATAAGCTTTTACGGGGTGTTAGGGG&lt;br&gt;R: GCTTACTTTTTCAACTAAGCGGC</td>
<td>269</td>
<td>58°C</td>
<td>(Kaiser et al., 2005)</td>
</tr>
<tr>
<td>iceA1</td>
<td>F: TATTTCGGAACGTGCGACACCTGAT&lt;br&gt;R: GGCTCAACACCCGGTGATAT</td>
<td>–900</td>
<td>57°C</td>
<td>(Mukhopadhyay et al., 2000)</td>
</tr>
<tr>
<td>iceA2</td>
<td>F: CGGCTGAGGACATTAAGGCTA&lt;br&gt;R: TCAATCCCTATGGAACAAATGATCGTT</td>
<td>–800</td>
<td>57°C</td>
<td>(Mukhopadhyay et al., 2000)</td>
</tr>
<tr>
<td>vacA (s1/s2)</td>
<td>F: CTGCCATTGAAATGCCCAACAC&lt;br&gt;R: ATGGGAATAACAAACACAC</td>
<td>259/286</td>
<td>57°C</td>
<td>(Pariz et al., 2014)</td>
</tr>
<tr>
<td>vacA (m1/m2)</td>
<td>F: CAATTGTCAAACTCAAGGAG&lt;br&gt;R: GGGTCAAAATATTTCAAAGG</td>
<td>567–42</td>
<td>57°C</td>
<td>(Qiao et al., 2003)</td>
</tr>
<tr>
<td>cagA</td>
<td>F: AACAGGACAGAAGCGTACGCC&lt;br&gt;R: TATAATGGCCTGGTGCTG</td>
<td>500</td>
<td>57°C</td>
<td>(Russo et al., 1999)</td>
</tr>
<tr>
<td>ureB seq</td>
<td>F: AGCGGATATTGGTATTAAAG&lt;br&gt;R: TGACGGGAACATTTCTCTG</td>
<td>1056</td>
<td>58°C</td>
<td>this study</td>
</tr>
<tr>
<td>dupA</td>
<td>F: ATGGAAATACAACAAACAC&lt;br&gt;R: AGGCTGAAACGGTTTGAACGA</td>
<td>1598</td>
<td>57°C</td>
<td>this study</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>F: GGCTATGACGGGTATCCGGC&lt;br&gt;R: GGATAAGCTTTTAGGGGTGTTAGGGG</td>
<td>764</td>
<td>58°C</td>
<td>(Bohr et al., 2002)</td>
</tr>
</tbody>
</table>

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 electro-phoresed in 1.8% agarose gel and finally bidirectional sequence analysis of the amplicons was performed using the strains after purification. Diversity of the obtained sequences was determined after their alignment using the MEGA6.06 software in comparison 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 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, ureB 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 100%, 60%, 83.3%, and 63% of the patients, respectively. 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, vacA, cagA, iceA and dupA were detected in 100%, 60%, 83.3%, and 63% of the strains, respectively. The vacA s1/m1 accounted for 36% of the strains, while the s2/m1, s1/m2, and s1/m – allelic
variants accounted for 33%, 23% and 6% of the strains. Among the iceA positive strains, different allelic types were detected in these patients, including iceA1/iceA2+ (36%), iceA1/iceA2- (33%), iceA1/iceA2- (13%). Statistically significant associations were found between the presence of cagA and SAG, and also vacA s1/m1 and vacA s1/m2 genotypes and IM. Also significant association was found between the iceA1/cagA+ genotype and IM (Table II). However, no correlation was determined between ulcer formation (GU and DU) and virulence genotypes in these patients.

UreB sequence diversity. The ureB was detected in all the strains and the diversity in their sequences was analyzed. The ureB sequence variants were assigned by GenBank (accession numbers: KP401951-KP401975). Based on the ureB reference sequence (Strain J99), different point mutations were found in the ureB 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.

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 cagA 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.
PUD was reported in some studies, our results didn’t confirm such a relationship. In the case of *iceA*, 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, *cagA/iceA*1 genotype was in correlation with the occurrence SAG, in a study by Nishiya et al. (2000) it was concluded that *iceA*1-positive strains can induce more increased active gastritis in *cagA*-positive and *vacA* s1/m1 strains. The association between inflammatory cell infiltration and *H. pylori* virulence factors was also detected for *dupA* (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 *dupA* positive strains; however, this association was not statistically significant. There are other controversial results for the noted association between gastrointestinal disorders and *dupA* 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* *ureA*-urel 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 *ureB* 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 *cagA* 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

**Discussion**

In this study, the relationships of *ureB* 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 *vacA* m1 or *s1m2* allelic variants. The higher expression VacA in *H. pylori* strains conferring *s1m1* genotype compared with those presenting *s1m2* 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 *vacA* *s1* and *m1* allelic variants are significantly associated with atrophy and IM. While the association of *vacA* *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 *vacA* *s* and *m* regions and PUD was similarly established by Aydin et al. (2004). The interplay between *vacA* and *cagA* signaling pathways may explain this incongruity (Argent et al., 2008). The association between *cagA* status and pathological changes was identified in our study. In this subject, SAG was dominantly found in patients infected with *cagA* positive strains that was agreed to in earlier reports (Plummer et al., 2007). Although the association of *cagA* with
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, cagA, cagViceA1, or vacA 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 dupA 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 ureB 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 cagA 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.

Acknowledgments
This article was part of a PhD thesis that supported financially by Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The authors like to thank all the staffs who help us for doing this study.

Conflict of interest
The authors declare no conflict of interest.

Literature


1264–1269.


1264–1269.
