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Relationship between histopathological status of the *Helicobacter pylori* infected patients and proteases of *H. pylori* in isolates carrying diverse virulence genotypes

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ABSTRACT

Helicobacter pylori is the main cause of several gastroduodenal diseases in Humans. Among various virulence factors of *H. pylori*, proteases may also be involved in its pathogenicity. In this study, relationship between proteolytic activity of *H. pylori* strains and histopathological changes of the stomach was investigated in the patients infected with strains carrying diverse virulence factors. *H. pylori* strains were isolated from the biopsies of 116 patients who referred to hospital for their gastroduodenal disorders, in Tehran, Iran. Biopsies were sent to microbiology and pathology laboratories for further analysis. All the suspected grown colonies were characterized by both biochemical tests and polymerase chain reaction (PCR). Presence of seven protease genes, *htrA*, *clpP*, *hp0169*, *hp1012*, *hp*0382, *hp1350* and *hp1435*, and distinct allelic variants of *H. pylori* virulence factors, *cagA*, *vacA*, *iceA*, *babA2* and *sabA*, were analyzed in each strain. Protease activity of the strains was assessed using spectrophotometric assay. Furthermore, association between diversity in protease genes and virulence genes, protease activity, as well as pathological changes was estimated statistically. Proteases genes, *htrA*, *clpP*, *hp0169*, *hp1012*, *hp*0382, *hp1350*, *hp1435*, were detected among 100%, 100%, 98%, 98%, 98%, 98%, and 8% of fifty *H. pylori* strains isolated from the patients, respectively. Status of *cagA*, *vacA s1*, *vacA s2*, *vacA m1*, *vacA m2*, *iceA1*, *iceA2*, *babA2* and *sabA* genes in isolates were 64%, 68%, 30%, 26%, 74%, 48%, 52%, 100%, and 96%, respectively. Predominant (84%) combined status for protease genes was: *htrA*/*clpP*/*hp0169*/*hp1012*/*hp0382*/*hP1350*/*hp1435*, while the prevalent combined status (16%) for virulence genes was: *cagA*+/*vacA s1m2*/*iceA1*$^{+}$/*sabA*$^{+}$/*babA2*$^{+}$. Although most of the strains (91.4%) presented moderate protease activity in vitro, lowest activity was measured in strains isolated from the patients with chronic gastritis (4.25%). Present study provide the new data on diversity of protease genes in *H. pylori*, as well as the proteolytic activity of these genes in *H. pylori* strains from the sick patients. Presence of significant association between lower protease activity of the strains and mildness of the pathological changes propose involvement of these proteases in the pathogenesis of *H. pylori* in vivo.

1. Introduction

More than half of the world's population are chronically infected with *Helicobacter pylori* [1,2]. In developing regions infection occurs mainly in childhood and in the absence of appropriate treatment, the infected individuals may carry on *H. pylori* in their stomach for lifelong [3,4]. Establishment of a long-term infection, favor

Abbreviations: CG, chronic gastritis; IM, intestinal metaplasia; SAG, severe active gastritis; PUD, peptic ulcer disease; NUD, non-ulcer disease.

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development of various clinical disorders of upper gastrointestinal tract, such as chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [3]. Clinical outcomes of *H. pylori* infection depends on complex host-pathogen interactions, where host genetic and genetic diversity of the strains play the important roles. Disease development is associated with functional activity of a multitude of bacterial virulence factors [2,3]. Among them Urease, bacterial adhesins such as BabA (Blood group antigen binding adhesin), OipA (Outer inflammatory protein A), and SabA (Sialic acid-binding adhesin), the Cag pathogenicity island (Cag-PAI), VacA toxin, and IceA (Induced by contact with epithelium gene A) might be more important [2,5]. The role for some other virulence factors have also proposed of which bacterial proteases may especially be noted [6]. Pathogenic bacteria exploit their diverse proteases for immune evasion, providing nutritional demands, or invasion into host tissues [7,8]. More than 20 putative proteases were described in *H. pylori* strain 26695. Among them, high temperature requirement A (HtrA,HP1019), ATP-dependent caseinolytic proteases (ClpP, HP0794), Collagenase (HP0169), PqqE (HP1012), Zinc metalloprotease (HP0382), PspA (HP1435), and protease 1350 (HP1350) may be the most common members in *H. pylori* strains [9]. There are some evidences about the roles of proteases in pathogenesis of *H. pylori*, especially those of HtrA and ClpP, which can play a role in tissue invasion of gastric epithelium [10,11]. However not much data has been published concerning these activities and their relationship with the gastric pathological changes of the infected individuals as well as their clinical manifestations.

In this study, we explored relationship between diverse proteases of *H. pylori,* some important virulence genes, including *cagA/vacA* status/genotype, *babA*, *sabA*, *iceA* and histopathological changes of the gastric tissue using clinical *H. pylori* strains isolated in this area.

2. Materials and methods

2.1. Patients and biopsy specimens

This study was approved by ethical committee of Shahid Beheshti University of Medical Sciences (RIGLD 760). A total of 116 adult dyspeptic patients who referred to the endoscopy unit of Taleghani hospital of Tehran, Iran were subjected to routine diagnostic endoscopy. Biopsy specimens from antral and/or stomach body were obtained for culture and histological examinations. Informed consent forms were obtained from all the patients and their data, including gender, age, body mass index (BMI) and types of the gastric diseases, were recorded. BMI was calculated as body weight divided by the square of body height in meters (kg/m2). Hematoxylin and eosin staining were used for histological examinations of the biopsy sections in the pathology unit of Research Institute for Gastroenterology and Liver Diseases. Pathological status of the patients was interpreted based on the updated Sydney System [12].

2.2. H. pylori culture and identification

Biopsy specimens of antral or body from each patient were transported in thioglycolate agar medium (Merck, Germany) supplemented with 1.3 g/L agar and 3% yeast extract (Merck, Germany). The biopsies were cut into small pieces, homogenized and were cultured on Brucella agar plates supplemented with 10% horse blood, 10% fetal bovine serum, *Campylobacter* selective supplement (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg) and amphotericin B (2.5 mg/L). The plates were incubated at 37 °C under microaerobic atmosphere containing approximately 5% O_2, 10% CO_2 and 85% N_2 (InnovaCO-170; New Brunswick Scientific, USA) for 3–7 days. Characterization of the isolates was performed based on conventional biochemical (Catalase, oxidase, urease) tests [4,13,14] and *H. pylori*-specific polymerase chain reaction (PCR), as described below.

2.3. Genomic DNA extraction and genotyping of the isolates for virulence determinants

Fresh colonies of *H. pylori* strains on Brucella Blood agar plates were used for DNA extraction using QIAamp DNA Mini Kit, according to the manufacturer's instructions (Qiagen, Germany). Specific *glmM* primers [15] was employed for confirmation of the biochemically characterized isolates. The specific PCR primers for *cagA*, *vacA*(s/m), *babA* (A2) and *sab*A genes were selected for genotyping of the strains [14]. PCRs were performed in a volume of 25 μl containing 1 × PCR buffer, 1 μmol/L of each primer, 1 μL of genomic DNA (approximately 150 ng), 200 μmol/L of dNTPs mix, 2 mmol/L of $MgCl_2$, and 0.05 U/μL Taq DNA polymerase.

2.4. Detection and characterization of protease genes

H. pylori strains were screened for presence of seven protease genes: *htrA*, *clpP*, *hp0169*, *hp1012*, *hp*0382, *hp1350* and *hp1435*. Primers were designed on the basis of more conserved nucleotide sequences of the genes deposited in Gen Bank database by using the Gene Runner software (version 3.05; Hastings Software Inc.). Details of the primers sequences, annealing temperatures and their predicted products are shown in Table 1. The PCR amplifications were carried out in 25 μl reaction mixtures under the following conditions: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing for 45 s at the indicated temperature for each primer, extension at 72 °C for a time selected on the basis of predicted fragment size (1 min/kb), and final extension at 72 °C for 10 min. PCR products were electrophoresed on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator. To confirm the protease gene sequences, the PCR products of 7 strains were randomly selected and their DNA sequences (Gene Fanavaran, Iran) were compared with the National Center for Biotechnology Information (NCBI) database.

2.5. Protease activity assay (protease secretion assays)

Tissue culture flasks (Orange, Belgium) containing10 mL of Brucella broth (pH, 7.5) supplemented with 10% horse serum, antibiotic supplements (Merck, Germany) and ferrous chloride hydrate ($FeCl_2 \cdot 6H_2O$) were inoculated with bacterial suspension adjusted to number 2 of McFarland standard, corresponding to 2.5×10^8 CFU/ml. The culture plates were incubated at 37 °C for 4 days under microaerobic conditions. The contents of the culture flaks were centrifuged for 15 min at 13,000 g at 22 °C and the supernatant was used for detection of proteolytic activity according to the method described by Mohapatra *et al* [16]. For this purpose, 150 μl of freshly prepared supernatant was added to 750 μl of substrate solution containing 1.2% [w/v] soluble bovine milk casein (sigma, USA) in 10 mM Tris-HCl buffer (pH 7.5), 100 μl of 10 mM Tris-HCl buffer (pH 7.5). Changes in optical density (OD) of mixture was recorded after incubation at 37 °C for 30 min in 405 nm using ELISA Reader (Bioteck, USA). Supernatant of inoculated Brucella broth medium with *Bacillus subtilis* natto strain (World Intellectual Resource Co., Taiwan) and the uninoculated medium were used as positive and negative controls, respectively. Cut-off values for classification of the protease activities were calculated based on the mean of three independent tests for all strains as follow: Mean