Characterization and genetic diversity of Helicobacter pylori type IV secretion system components, CagI and CagN and its association with clinical outcomes among Iranian patients

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**Research**

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Abstract

Background

A number of cagPAI genes in H. pylori genome was proposed to be the most probably evolved under a diversifying selection and evolutionary pressure. Among them, CagI and CagN are described as a part of the two different-operon of cagPAI that are involved in the T4SS, but the definite association of these factors with clinical manifestations is unclear.

Methods

A total of 70 H. pylori isolates were obtained from different gastroduodenal patients. All isolates were examined for the presence of primary H. pylori virulence genes by PCR analysis. Direct DNA sequence analysis was performed for the cagI and cagN genes. The results were compared with reference strain.

Results

The cagI, cagN, cagA, cagL, vacA s1m1, vacA s1m2, vacA s2m2, babA2, sabA and dupA genotypes were detected in 80%, 91.4%, 84%, 91.4%, 32.8%, 42.8%, 24.4%, 97.1%, 84.3%, and 84.3% of the total isolates, respectively. The most variable codon usage in cagI was observed at residues 20 to 25, 55 to 60, 94, 181 to 199, 213 to 221, 241 to 268, and 319 to 320, while the most variable codon usage in CagN hypervariable motif (CagNHM) was observed at residues 53 to 63. This CagNHM region is postulated to contain GDEEITEEEKK sequence in the P12 reference strain. Sequencing data analysis of cagN revealed a conserved hypothetical hexapeptide repeat (EAKDEN/K) in residues of 278–283 among six H. pylori isolates, which needs further studies to evaluate its putative function.

Conclusion

The present study demonstrated a high prevalence of cagI and cagN genes among Iranian H. pylori isolates with gastroduodenal diseases. Furthermore, no significant correlation between cagI and cagN variants and clinical outcomes was observed. However, all patients had high prevalence of cagPAI genes including cagI, cagN, cagA and cagL that indicates more potential role of these genes in disease outcome.

Introduction

Helicobacter pylori (H. pylori) is a Gram-negative, microaerophilic bacterium that can be chronically colonized in the human stomach. This organism infects more than 50% of the world's population, and is the main cause of chronic active gastritis, gastric and duodenal ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma [1, 2]. Although H. pylori is recognized as the
major risk factor for development of gastric malignancy, most \textit{H. pylori}-infected individuals are asymptomatic and may tolerate the presence of this organism in their stomach lifelong [3, 4]. The severity of \textit{H. pylori}-induced gastric diseases seems to be associated with several parameters, including host genetic polymorphism, inflammatory responses, environmental factors, and bacterial virulence genotype [5, 6].

\textit{H. pylori} is associated with high genetic variability including virulence genes due to genetic plasticity, rearrangement of DNA and, high transformation and recombination frequency. Thereby, \textit{H. pylori} infected patients greatly varies in the disease progression and clinical outcomes geographically. To date, several virulence factors have been identified in the genome of \textit{H. pylori} such as CagA, VacA, BabA, SabA, and DupA [6, 7]. CagA, an oncoprotein, is the best studied virulence-associated factor of \textit{H. pylori} that is translocated into the host gastric epithelial cells via the type 4 secretion system (T4SS). The \textit{H. pylori} T4SS machinery is constituted by a cluster of gene products located on an approximately 40 kb chromosomal region named \textit{cag} Pathogenicity Island (\textit{cagPAI}) [8, 9]. \textit{cagPAI} encodes about 27–31 genes, by which a subset of these genes encodes the main components of the T4SS apparatus spanning bacterial membranes. Moreover, possibly 15 to 16 different proteins of the T4SS are required for translocation of CagA and peptidoglycan fragments into the host cells, and also secretion of IL-8 from gastric epithelial cells [10]. Once CagA is translocated then it modulates the host cell signaling which results the loss of membrane polarity, cell elongation, induction of inflammatory cytokines and development of gastric adenocarcinoma [11]. \textit{cagPAI} encodes several unique Cag components that have no sequence similarities to any other bacterial proteins involved in T4SS. However, a number of \textit{cagPAI} genes such as \textit{cagI} and \textit{cagN} were proposed to be most probably evolved under a diversifying selection and evolutionary pressure [12]. CagI, a small protein (41.5 kDa) encoded by \textit{cagI} (\textit{cag19/hp0540}) gene, does not share any sequence and topological homology to any other known proteins [13, 14], whereas CagN, a 32–35 kDa protein also termed as Cag17/HP0538 encoded by \textit{cagN} gene (\textit{hp0538}), is a poorly characterized component of the T4SS that appears to be localized to the bacterial inner membrane rather than the periplasm [10, 13, 15, 16].

There are some conflicting reports about the role of CagI and CagN in CagA translocation, IL-8 induction from gastric epithelial cells, and \textit{H. pylori} T4SS machinery [15, 17–21]. Reviewing subsequent and more recent literatures have revealed that CagI is capable of binding to $\beta$1 integrins of the host cell and is essential for CagA translocation, and is also involved in pilus biogenesis of T4SS [22, 23]. On the other hand, deletion of \textit{cagN} can reduce the phosphorylation degree of CagA into host cell and it is not considered as a substrate for the T4SS [15]. To understand the precise role of CagI and CagN in CagA translocation and pathogenesis need to be further investigated. The oncogenic potential of \textit{H. pylori} strains is associated with their virulence capacity, genetic diversity and specific sequence polymorphisms within the key genes involving in translocation and phosphorylation of T4SS effectors [24–27]. Therefore, the present study aimed to determine the prevalence of \textit{cagI} and \textit{cagN} genes and their amino acid sequence polymorphisms in Iranian \textit{H. pylori}-infected patients with various gastroduodenal diseases. The probable association between the genetic variants of \textit{cagI} and \textit{cagN} and other virulence genotypes of \textit{H. pylori} with clinical consequences were also investigated.
Methods

H. pylori clinical isolates and biopsy specimens

Seventy gastric biopsy specimens were obtained from patients with different gastroduodenal diseases who referred for upper gastroduodenal endoscopy at Research Institute for Gastroenterology and Liver Diseases, Tehran, Iran, between January 2017 and May 2019. Three antral biopsies were taken from each patient and examined for culture and histopathology. The biopsy specimens were immediately placed in transport medium containing Thioglycolate supplemented with 3% yeast extract (Oxoid Ltd., Basingstoke, UK) and 1.3 g/L agar (Merck, Germany). All patients gave their written informed consent under a local protocol approved by the Institutional Ethical Review Committee of Research Institute for Gastroenterology and Liver Diseases at Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.RIGLD.REC.1398.023).

H. pylori culture and identification

Biopsy specimens were carefully homogenized and inoculated onto the surface of Brucella agar plates (Merck, Germany) supplemented with 7% (v/v) horse blood, 10% fetal calf serum (FCS), Campylobacter-selective supplement (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg), and amphotericin B (2.5 mg/l). The incubation was performed at 37°C for 3-7 days under a microaerophilic atmosphere (5% O₂, 10% CO₂ and 85% N₂) in a CO₂ incubator (Innova® CO-170; New Brunswick Scientific, USA). The suspected colonies were identified as H. pylori based on colony morphology, Gram staining, positive reaction for oxidase, catalase and urease tests, and also by H. pylori gene-specific PCR following the previously described protocols [28, 29]. Pure cultures from confirmed isolates were kept in 0.5 ml of Brain heart infusion (BHI) medium (Merck, Germany) containing 15% glycerol plus 20% FCS, and stored at -80°C until further analysis.

Genomic DNA extraction

Genomic DNA was extracted from freshly harvested colonies on agar plates, using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The quality of DNA was checked by using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The extracted DNA samples were stored at -20°C until PCR assay.

Genotyping of H. pylori virulence-associated genes

PCR analysis was performed to detect virulence target genes including cagL, cagA, vacA alleles (s1/s2 and m1/m2), babA2, sabA and dupA genes using specific primers (Table 1). Briefly, PCR mixtures in a volume of 25 µl consisted of 2 µl of template DNA (approximately 200 ng), 0.1 mM of each primer, 2.5 µl of a 10-fold concentrate PCR buffer, 100 mM of deoxynucleotide triphosphates, 1 mM MgCl₂, and 1.5 U of Super-Taq™ DNA polymerase (HT Biotechnology Ltd., Cambridge, UK). PCR amplifications were performed in a thermocycler (Eppendorf, Hamburg, Germany) 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 at the indicated temperature for each reaction in Table 1 for 45 s, extension at 72°C for 1 min. A final extension step was performed at 72°C for 10 min to ensure full extension of the PCR products. PCR amplicons were electrophoresed on a 1.2% TBE agarose gel, stained with ethidium bromide, and examined under a UV transilluminator. *H. pylori* J99 (CCUG 47164) and a no-template mixture served as positive and negative controls in each PCR experiment, respectively.

**Primer designation for cagI and cagN genotyping**

The NCBI GenBank database ([http://www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)) and the DNA Data Bank of Japan ([http://www.ddbj.nig.ac.jp/](http://www.ddbj.nig.ac.jp/)) were searched for all available complete and partial cagI and cagN sequences of *H. pylori* strains. Based on pairwise and multiple nucleotide sequence alignments of cagI and cagN genes from different *H. pylori* strains and using the complete relevant sequence of *H. pylori* P12 (CP001217.1) as the reference strain, two pairs of specific primers were designed from the conserved regions for detection of complete related sequences using CLC Sequence Viewer 8 software ([https://www.qiagenbioinformatics.com/](https://www.qiagenbioinformatics.com/)). The selected primer target sites were compared to all available complete and partial cagI and cagN sequences of *H. pylori* strains with the Basic Local Alignment Search Tool ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)).

**Analysis of cagI and cagN diversity by PCR sequencing**

For DNA sequencing of cagI and cagN, PCR amplification was carried out in a final volume of 25 µl using designed specific primers including 5‘-CATTTGACTTACCTTGATTAC-3’ (cagIF) and 5‘-TTTGAGCACTTGTGGTTGG-3’ (cagIR), 5‘-GAGCGACAAAACAACTATGC-3’ (cagNF) and 5‘-GATCCCTAGAACAAGTAAGC-3’ (cagNR) yielding DNA fragments of about 1377 and 1192 bp in length, respectively. The PCR products were purified using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific, Fermentas, USA) followed by sequencing on both strands using an automated sequencer (Macrogen, Seoul, Korea). DNA sequences were edited by Chromas Lite version 2.5.1 (Technelysium Pty Ltd, Australia) and BioEdit version 7.2.5 [30]. All of complete and partial cagI and cagN nucleotide and amino acid sequences were aligned to the relevant sequences of *H. pylori* strain P12 (GenBank: CP001217.1) as a reference sequence after in-frame translation. The single nucleotide variations and codon usage of the sequences were examined using BioEdit version 7.2.5.

**Phylogenetic analysis**

Phylogenetic trees were generated for CagI and CagN nucleotide and amino acid sequences using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) [31]. Evolutionary history was inferred by the Maximum Likelihood trees using Tamura 3-parameter model and Poisson correction method for nucleotide and amino acid sequences, respectively.

**Nucleotide sequence accession numbers**
The complete and partial nucleotide sequences of \textit{cagI} and \textit{cagN} genes from \textit{H. pylori} strains determined in this study were deposited in the NCBI GenBank database under the accession numbers MG573078-MG573107 (\textit{cagI}) and MG559675-MG559720 (\textit{cagN}).

**Statistical analysis**

The statistical associations between \textit{H. pylori} virulence genotypes and different clinical status were determined by the Chi-square and Fisher's exact tests. A two-sided \(P\) value of less than 0.05 was regarded statistically significant. The IBM SPSS Statistics for Windows version 21.0 (Armonk, NY: IBM Corp.) was used for all statistical analyses.

**Results**

**Demographic and clinical characteristics of patients**

The median age of the patients was 45.6 years (ranging from 14 to 75 years). Of the study cohort, 32.8\% \((n = 23)\) was male and 67.1\% \((n = 47)\) was female. According to the endoscopic and histopathology findings, 39 (55.7\%) patients were diagnosed with were non-ulcer dyspepsia (NUD), 23 (32.8\%) patients had peptic ulcer disease (PUD), 7 (10\%) patients had intestinal metaplasia (IM), and one (1.4\%) had gastric cancer. Three patients (4.3\%) suffered from gastritis and duodenitis simultaneously. Table 2 indicates the demographic characteristics and clinical status of the included subjects. In each of the 70 cases, \textit{H. pylori} was isolated by culture and the isolates were approved by detection of the \textit{glmM} and \textit{16s rRNA} genes.

**Virulence genotypes and variants**

The molecular analysis revealed that the \textit{cagA}, \textit{cagI}, \textit{cagN}, \textit{cagL}, \textit{vacA s1m1}, \textit{vacA s1m2}, and \textit{vacA s2m2} positive strains had a prevalence of, respectively, 84\% \((n = 59)\), 80\% \((n = 56)\), 91.4\% \((n = 64)\), 91.4\% \((n = 64)\), 32.8\% \((n = 23)\), 42.8\% \((n = 30)\), and 24.4\% \((n = 17)\) in our study, whereas \textit{babA2}, \textit{dupA}, and \textit{sabA} were detected in, respectively, 97.1\% \((n = 68)\), 84.3\% \((n = 59)\), and 84.3\% \((n = 59)\) of the isolates included in this investigation (Table 3). There was no statistically significant relationship between the \textit{H. pylori} virulence genotypes and clinical status of the patients \((P > 0.05)\). On the other hand, the results obtained are compatible with the clinical status of the patients. In present study, 100\% \((23/70)\) of the PUD and 94.9\% \((37/70)\) of the NUD strains were positive for \textit{babA2} gene by PCR. Furthermore, the prevalence of \textit{cagN} and \textit{cagL} genes for PUD strains is attributed to 95.6\% \((22/70)\) and 91.3\% \((21/70)\), respectively. In the meantime, patients suffering from NUD showed the frequency of 89.7\% \((35/70)\) and 94.9\% \((37/70)\) for the same genes as PUD. When it comes to \textit{vacA} allelic combinations, \textit{vacA s1m2} was found to be the most common allele among the strains recovered from the PUD patients (52.2\%), whereas 42.8\% and 33.3\% of allelic combinations were assigned to \textit{vacA s1m1} and \textit{vacA s2m2}, within the IM and NUD strains, respectively.

\textit{cagI} variants in patients with different clinical status
Out of 56 cagI-positive H. pylori strains, the cagI gene of 30 strains were randomly selected and sequenced. The full-length cagI gene was successfully sequenced in 27 H. pylori strains. Moreover, the cagI gene was partially sequenced in three strains due to poor quality of sequence data or sequencing errors. According to our sequencing data, there was no insertion or deletion in the full-length cagI fragment from 27 H. pylori studied, and sequence alignments were therefore straight forward. In addition, we performed in-frame translation for cagI gene into amino acid sequences, and investigated rates and locations of CagI variants. The distribution of synonymous and nonsynonymous polymorphisms in cagI of H. pylori strains are represented in Figure 1 and Table 4. The most variable codon usage was observed at residues 20 to 25, 55 to 60, 94, 181 to 199, 213 to 221, and 241 to 268. The CagI E22, E221, and V268 amino acid variants occurred at higher rate in H. pylori isolates from NUD patients compared to that isolated from PUD individuals, however, this difference was not statistically significant. Moreover, CagI variants A23, S57, and S94 were found to occur at non-significantly higher rates in H. pylori isolates from individuals with PUD compared to NUD patients. As we expected, the SKVIVK hexapeptide motif (376-381) located at the C-terminal of CagI was completely conserved among the cagI sequenced H. pylori strains.

**cagN variants in patients with different clinical status**

Regarding cagN sequence analysis, 46 strains were randomly sent for direct DNA sequencing from 64 cagN-positive H. pylori strains. The complete cagN gene was successfully sequenced in 43 H. pylori strains. Furthermore, the cagN gene fragments of three strains were partially sequenced as the same reasons for the cagI gene. The cagN sequencing findings showed a high level of variability in CagN nucleotide and protein sequences. The most variable codon usage was observed at residues 53 to 63, so-called as CagN hypervariable motif (CagNHM), which includes many missense mutations and it is postulated to contain GDEEITSEEK in the P12 reference strain. Moreover, a conserved hypothetical hexapeptide repeat (EAKDEN/K) was inserted in residues 278-283 among six H. pylori strains. Interestingly, this motif was repeated twice in a row in one of these clinical strains (EAKDENEAKDEN). The other insertion sequences were detected between residues 224-225 and 234-235 for VK and KN amino acids in one of the strains. The sequencing data analysis revealed that these insertion sequences in cagN gene caused no frameshift mutations as compared to the P12 reference strain. Figure 2 and Table 5 showed the distribution of synonymous and nonsynonymous polymorphisms of CagN among 43 H. pylori strains in this study.

**Phylogenetic analysis of H. pylori CagI and CagN**

The phylogenetic trees of cagI nucleotide and amino acid sequences from H. pylori isolates are illustrated in Figures 3 and 4, respectively. Generally, no characteristic clusters were observed between DNA and amino acid sequences of CagI and different clinical status. Furthermore, on the basis of the CagN nucleotide and amino acid sequences, a phylogenetic tree was reconstructed by using the Maximum Likelihood method, which are illustrated in Figures 5 and 6, respectively. Similar to CagI sequences, the CagN phylogenetic analysis indicated no characteristic clusters with regard to the clinical status.
Discussion

Virulent *H. pylori* strains harbor the *cag*PAI (*cag*+) encoding a type IV secretion apparatus, which has been shown to inject CagA and possibly also other virulence effectors into infected gastric epithelial cells [32]. It has been well documented that *cag*+ *H. pylori* strains augment the risk for severe gastritis, peptic ulceration, atrophic gastritis, dysplasia, and gastric adenocarcinoma compared to strains that lack the *cag*PAI (*cag*−) [33–35]. Previously, it has been described that CagI forms a functional protein complex at the bacterial cell surface by interacting with CagL, which is another important Cag secretion apparatus component. Accordingly, some evidence suggested that CagI can interact with CagL protein and let to bind to integrin receptors on the target cell surface [9, 18]. CagI and CagL proteins contain N-terminal signal peptide, and therefore they can be supposed to be transported to the periplasm, however, the two proteins are not distributed equally on the bacterial cell surface [36]. Regarding different views on CagI, Kumar et al. [37] found that CagI does not participate in CagA translocation from cytoplasm to bacterial cell surface. On the other hand, it has been discovered that mutation in *cagN* did not interrupt CagA delivery or IL-8 secretion and the CagN-deficient *H. pylori* strains could cause an infection similar to wild-type *H. pylori* strains. Some experiments also have indicated that CagN is not conclusively required for *H. pylori* T4SS function [17]. In another study conducted by Kutter et al., CagN was established to interact with two other *cag*PAI proteins, including CagV and CagY [36]. Thus, the biological function of CagN is yet to be investigated. In the current study, the attempts were made to detect possible variants of CagI and CagN, as uncharacterized *cag*PAI-encoded factors, on both nucleotide and amino acid sequence levels among *H. pylori* isolates in Iran. We also investigated the distribution and variations in *H. pylori* virulence factors. Our findings revealed that 80% of *H. pylori* isolates harbored *cagI* gene, whilst 91.4% of strains had *cagN* gene. To the best of our knowledge, the *cagI* and *cagN* variants in *H. pylori* isolates in the subset of patients with different gastroduodenal diseases are not available in the literature. Based on our molecular findings, CagI E22, E221, and V268 amino acid polymorphisms occurred at higher rate in *H. pylori* isolates from NUD individuals compared to that isolated from PUD patients, however, this difference was not statistically significant. On the other hand, CagI amino acid changes A23, S57, and S94 were detected at non-significantly higher rates in *H. pylori* isolates from PUD patients compared to NUD subjects.

Despite the fact that Olbermann et al. found that *cagN* and *cagM* were demonstrated to be conserved in the *cag*PAI throughout all *cag*+ *H. pylori* strains that have been sequenced so far [12], a high level of variability in CagN nucleotide and protein sequences was observed in present study. In the meantime, the most variable region in CagN amino acid sequence was found at residues 53 to 63, which includes many missense mutations and it is postulated to contain GDEEITEEEKK in the P12 reference strain. Our findings revealed that there was no significant correlation between clinical outcomes and *cagI* and *cagN* variants at both nucleotide and amino acid levels (*P* > 0.05), which is in consistent with previous study reported by Ogawa et al. [26]. Pham et al., stated that C-terminal motif (SKVIVK) in CagI is essential for T4SS function, and thus is completely conserved among *H. pylori* strains. Remarkably, the C-terminal motif of CagI is reported to be similar to the C-terminal motifs of CagL SK(I/V)IVK and CagH TKIIVK,
representing the possibility that the amino acid sequences essentially act as binding motifs for a common interaction partner of all three proteins [18]. In agreement with above mentioned study, our findings also confirmed that the CagI C-terminal motif was completely conserved among all \textit{H. pylori} isolates. Ogawa et al. discovered complete RGD motifs in CagL sequences were observed from all isolates, which possibly imply the importance of the RGD motif for CagL function [26]. A recent investigation on this topic was performed by Yadegar et al. in Iran, in which almost 97\% of \textit{H. pylori} clinical strains contained \textit{cagI} gene [29]. Furthermore, their findings highlighted the importance of a common CagL hypervariable motif (CagLHM) such as NEIGQ along with multiple C-type EPIYA repeats, which was linked to PUD, GE, and GC with more severity compared to NUD. In fact, it is believed that the over mentioned CagLHM motif played a key role in the pathogenesis of \textit{H. pylori} strains. Besides, sequencing analysis of the present study also showed that a conserved hypothetical hexapeptide repeat (EAKDEN/K) was detected in residues 278–283 in CagN among 13.9\% of \textit{H. pylori} isolates. Although Bats et al. [38] implied that the mutations and truncations in CagN sequence was irrelevant to folding properties or the overall shape of CagN, further studies are required to assess the impact of this hexapeptide motif on CagN protein structure and its role in \textit{H. pylori} T4SS activity. Despite the alterations in various \textit{cag} sequences, it is noticeable that all patients had high prevalence of \textit{cagPAI} genes including \textit{cagI}, \textit{cagN}, \textit{cagA} and \textit{cagL} that indicates more potential role of these genes in disease outcome.

In the present study, we also investigated the presence of various \textit{H. pylori} virulence genotypes. In accordance with our previous study in Iranian populations, we detected a high prevalence of \textit{vacA} s1 (77.1\%) and \textit{vacA} m2 (65.7\%) allelic genotypes [39]. The \textit{vacA} s1 allele has been reported to be associated with more severe atrophic gastritis in peptic ulcer patients [40, 41]. In our study, the \textit{vacA} s1 genotype was found to be more prevalent among PUD patients, however, there was no significant association between the presence of other virulence genes and clinical disease outcomes. The mosaic combination of s- and m-region allelic genotypes also has been established to be associated with the pathogenicity of \textit{H. pylori} [42, 43]. Accordingly, type s1m1 \textit{H. pylori} strains express large amounts of VacA toxin and are strongly associated with a higher level of inflammation and mucosal ulceration, while \textit{vacA} s1m2-harboring strains produce moderate amount of toxin and \textit{vacA} s2m2 strains are virtually non-toxic and rarely associated to clinical outcome [44]. A majority of \textit{H. pylori} strains in the current study contained \textit{vacA} s1m2 genotype and this was mainly observed in NUD patients. On the contrary, allelic combination s1m1 or s2m2 genotypes were detected among the majority of clinical isolates of \textit{H. pylori} in other parts of the world, and the hypervirulent \textit{vacA} s1m1 genotype was commonly associated with PUD patients [45]. Hence, it can be inferred that correlation between \textit{H. pylori} genotyping and clinical outcome of the patients vary in different geographical regions.

\textbf{Conclusion}

In summary, a large body of evidence indicates that certain \textit{cagPAI} components are correlated with the risk of gastric carcinogenesis. Here, we investigated the diversity of CagI and CagN sequences in clinical \textit{H. pylori} isolates from Iranian patients with different clinical status. We detected several putative variants of CagI and CagN sequences in \textit{H. pylori} isolates, however, there was no significant relevance between
these variants and clinical phenotypes. Our findings also demonstrated that the C-terminal SKVIVK motif within the CagI protein is conserved among all tested *H. pylori* strains. Meanwhile, the repeating motif EAKDEN was a typical attribute identified in C-terminal sequence of CagN protein among some of the *H. pylori* strains, however its potential impact on T4SS activity and translocation of effectors requires further investigations. Despite the present study has successfully demonstrated the genetic diversity of *cagI* and *cagN* genes, it has certain limitations in terms of insufficient sample size. Accordingly, the possible effects of CagI and CagN variants on the T4SS activity as well as their possible interactions with other *cag*PAI components in a large number of *H. pylori* isolates needs to be explored. Also, the probable relevance of overmentioned variants with different clinical outcomes should not be ignored.

**Declarations**

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**Authors contribution**

YA and SK cultured the isolates and performed the PCR test. AY worked on concepts and designed the study. AY, NM and HH participated in data analysis and wrote the manuscript. NK, HAA and MRZ critically revised the manuscript. All authors approved the final version of the manuscript.

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**Availability of data and materials**

The available data used and/or analyzed during the current study are all included in the manuscript.

**Ethical approval**

This work deals with clinical bacterial strains isolated from human gastric biopsies. No tissue material or other biological material was stored from the patients, only subcultured bacterial isolates. Informed consent was obtained from all individual participants included in the study. All procedures performed were in accordance with the ethical standards retrieved from the Institutional Ethical Review Committee of Research Institute for Gastroenterology and Liver Diseases (RIGLD) at Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.RIGLD.REC.1398.023).

**Consent for publication**
Not applicable.

**Competing interests**

The authors declare that they have no conflicts of interest.

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### Tables

**Table 1.** Oligonucleotide sequences used for amplification of the *H. pylori* virulence genes of interest.

| Target gene | Primer designation | Oligonucleotide sequence (5′-3′) | Annealing temperature (°C) | PCR product (bp) | Reference |
|-------------|---------------------|---------------------------------|---------------------------|-----------------|-----------|
| 16S rRNA    | C97-20              | GGCTATGACGGGTATCCGGC            | 58                        | 764             | [39]      |
|             | H3A-20              | GCCGTGCAACACCTTGTTC             |                           |                 |           |
| glmM        | GlmM2-F             | GGATAAGCTTTAGGGGTAGGGG          | 56                        | 296             | [39]      |
|             | GlmM1-R             | GCTTACTTTCTAACAATAACGGGCC       |                           |                 |           |
| cagL        | cagL-B4             | GCAGAATTCATAACAAGCGGCTAAAG     | 60                        | 695             | [39]      |
|             | cagL-B5             | ATTAGAATTCATAGGCCTTAGTCAG      |                           |                 |           |
| cagA        | 93089               | AATACACCAACGCCTCAAG            | 57                        | 400             | [39]      |
|             | 93261               | TTGTGCGCTTTTGTCTTC             |                           |                 |           |
| vacA s1/s2  | VA1-F               | ATGGAATACAAACACACAC             | 57                        | 259/286         | [39]      |
|             | VA1-R               | CTGGTTGAATGCSCCAAC             |                           |                 |           |
| vacA m1/m2  | VAG-F               | CAATCTGTCCAATCAAGGAGG          | 57                        | 570/645         | [39]      |
|             | VAG-R               | GCGGCAAAATAATCCAGG             |                           |                 |           |
| babA2       | bab7-F              | CCAAAAGGAAACAAAAAGGT           | 52                        | 271             | [46]      |
|             | bab7-R              | GCTTGTGAAATGCGGTCGT            |                           |                 |           |
| sabA        | F1-HP726-jhp663     | TTTTGTCAGCTACGCGGTC            | 56                        | 487             | [46]      |
|             | R1-HP725-jhp662     | ACCGAAGTGAATACGCGGTT           |                           |                 |           |
| dupA        | DupA-F              | ATTCACGCTAAGACCTCA             | 55                        | 581             | [46]      |
|             | DupA-R              | CTGGAAGGCCTTATATCTGTGG         |                           |                 |           |

**Table 2.** Demographic data and clinical characteristics of patients colonized with *H. pylori* strains (n=70) in this study.
| No. | Strains | Clinical status | Gender | Age (years) | cagI GenBank no. | cagN GenBank no. |
|-----|---------|----------------|--------|-------------|-----------------|-----------------|
| 1   | HC3     | PUD            | Male   | 46          | Negative        | NA              |
| 2   | OC4     | PUD            | Female | 58          | MG573078        | MG559675        |
| 3   | OC30    | NUD            | Male   | 56          | MG573079        | MG559676        |
| 4   | HC114   | PUD            | Female | 49          | Negative        | NA              |
| 5   | OC149   | NUD            | Female | 31          | MG573080        | NA              |
| 6   | HC168   | PUD            | Male   | 49          | NA              | NA              |
| 7   | HC175   | NUD            | Female | 27          | Negative        | NA              |
| 8   | OC175   | NUD            | Female | 30          | MG573081        | MG559677        |
| 9   | OC179   | GC             | Female | 63          | NA              | MG559678        |
| 10  | OC180   | IM             | Female | 39          | Negative        | NA              |
| 11  | OC217   | NUD            | Female | 42          | Negative        | Negative        |
| 12  | OC235   | PUD            | Female | 75          | NA              | NA              |
| 13  | OC245   | IM             | Male   | 48          | NA              | Negative        |
| 14  | OC250   | PUD            | Male   | 57          | NA              | NA              |
| 15  | OC485   | NUD            | Male   | 54          | NA              | MG559679        |
| 16  | OC494   | NUD            | Female | 42          | MG573082        | MG559680        |
| 17  | OC505   | NUD            | Female | 39          | NA              | NA              |
| 18  | OC557   | PUD            | Female | 50          | Negative        | Negative        |
| 19  | OC562   | NUD            | Female | 43          | MG573083        | NA              |
| 20  | OC571   | NUD            | Female | 49          | Negative        | MG559681        |
| 21  | OC573   | NUD            | Female | 36          | MG573084        | MG559682        |
| 22  | OC576   | NUD            | Female | 42          | MG573085        | MG559683        |
| 23  | OC606   | PUD            | Male   | 60          | NA              | MG559684        |
| 24  | OC639   | PUD            | Male   | 25          | Negative        | MG559685        |
| 25  | OC656   | PUD            | Male   | 41          | NA              | MG559686        |
| 26  | OC658   | NUD            | Female | 33          | NA              | MG559687        |
| 27  | OC661   | NUD            | Male   | 33          | MG573086        | MG559688        |
| 28  | OC688   | IM             | Female | 42          | MG573087        | MG559689        |
| 29  | OC722   | NUD            | Female | 43          | MG573088        | MG559718        |
| 30  | OC723   | NUD            | Male   | 47          | NA              | MG559690        |
| 31  | OC728   | NUD            | Female | 23          | NA              | MG559691        |
| 32  | OC731   | NUD            | Female | 24          | MG573089        | NA              |
| 33  | OC734   | NUD            | Male   | 50          | MG573090        | MG559692        |
| 34  | OC743   | NUD            | Male   | 60          | Negative        | MG559693        |
| 35  | OC749   | NUD            | Male   | 70          | NA              | MG559694        |
| 36  | OC751   | NUD            | Female | 44          | MG573091        | MG559695        |
| 37  | OC770   | NUD            | Female | 73          | MG573092        | MG559696        |
| 38  | OC775   | PUD            | Female | 39          | NA              | MG559697        |
| 39  | OC776   | NUD            | Male   | 34          | MG573093        | NA              |
| 40  | OC785   | NUD            | Male   | 60          | Negative        | MG559719        |
| 41  | OC790   | NUD            | Male   | 26          | NA              | MG559720        |
| 42  | OC793   | NUD            | Female | 41          | MG573094        | MG559698        |
| No. | Accession | Status | Gender | Age | Sex | Accession |
|-----|-----------|--------|--------|-----|-----|-----------|
| 43  | OC796     | PUD    | Female | 51  |     | MG573095  |
|     |           |        |        |     |     | MG559699  |
| 44  | OC797     | IM     | Female | 28  |     | NA        |
|     |           |        |        |     |     | NA        |
| 45  | OC803     | NUD    | Male   | 52  |     | NA        |
|     |           |        |        |     |     | MG559700  |
| 46  | OC805     | NUD    | Female | 48  |     | MG573096  |
|     |           |        |        |     |     | MG559701  |
| 47  | OC808     | NUD    | Female | 65  |     | NA        |
|     |           |        |        |     |     | MG559702  |
| 48  | OC810     | NUD    | Female | 53  |     | NA        |
|     |           |        |        |     |     | NA        |
| 49  | OC814     | PUD    | Female | 25  |     | NA        |
|     |           |        |        |     |     | MG559703  |
| 50  | OC815     | NUD    | Female | 34  |     | MG573105  |
|     |           |        |        |     |     | Negative  |
| 51  | OC816     | NUD    | Male   | 14  |     | NA        |
|     |           |        |        |     |     | MG559704  |
| 52  | OC819     | PUD    | Female | 32  |     | NA        |
|     |           |        |        |     |     | MG559705  |
| 53  | OC824     | PUD    | Female | 43  |     | MG573097  |
|     |           |        |        |     |     | NA        |
| 54  | OC840     | IM     | Male   | 54  |     | NA        |
|     |           |        |        |     |     | MG559706  |
| 55  | OC846     | NUD    | Female | 52  |     | MG573098  |
|     |           |        |        |     |     | MG559707  |
| 56  | OC852     | IM     | Male   | 45  |     | NA        |
|     |           |        |        |     |     | MG559708  |
| 57  | OC854     | NUD    | Female | 71  |     | MG573099  |
|     |           |        |        |     |     | Negative  |
| 58  | OC884     | NUD    | Female | 60  |     | Negative  |
|     |           |        |        |     |     | Negative  |
| 59  | OC897     | PUD    | Female | 60  |     | Negative  |
|     |           |        |        |     |     | MG559709  |
| 60  | OC912     | PUD    | Female | 64  |     | MG573100  |
|     |           |        |        |     |     | MG559710  |
| 61  | OC913     | PUD    | Male   | 42  |     | MG573106  |
|     |           |        |        |     |     | NA        |
| 62  | OC937     | NUD    | Female | 48  |     | MG573101  |
|     |           |        |        |     |     | MG559711  |
| 63  | OC939     | PUD    | Male   | 54  |     | MG573102  |
|     |           |        |        |     |     | MG559712  |
| 64  | OC975     | IM     | Female | 31  |     | MG573107  |
|     |           |        |        |     |     | MG559713  |
| 65  | OC978     | PUD    | Female | 45  |     | MG573103  |
|     |           |        |        |     |     | MG559714  |
| 66  | OC983     | PUD    | Female | 55  |     | Negative  |
|     |           |        |        |     |     | NA        |
| 67  | OC996     | PUD    | Female | 52  |     | MG573104  |
|     |           |        |        |     |     | MG559715  |
| 68  | OC1021    | PUD    | Female | 50  |     | NA        |
|     |           |        |        |     |     | MG559716  |
| 69  | OC1028    | NUD    | Female | 27  |     | Negative  |
|     |           |        |        |     |     | MG559717  |
| 70  | OC1031    | NUD    | Female | 52  |     | NA        |
|     |           |        |        |     |     | NA        |

GC, gastric cancer; IM, intestinal metaplasia; NUD, nonulcer dyspepsia; PUD, peptic ulcer disease; NA, not assigned.

The accession numbers are deposited in GenBank database for cagI and cagN gene sequences of the H. pylori strains in this study.

The cagI-negative or cagN-negative H. pylori strains.

The cagI-positive or cagN-positive H. pylori strains that either were not sequenced or sequenced unsuccessfully.

Table 3. Distribution of virulence genotypes in relation to clinical status among 70 H. pylori strains.
| Virulence genotypes | Clinical status | Total |
|---------------------|----------------|-------|
|                     | NUD (n=39)     | PUD (n=23) | IM (n=7) | GC (n=1) |
| cagI-positive       | 32 (82%)       | 17 (73.9%) | 6 (85.7%) | 1 (100%) | 56 (80%) |
| cagI-negative       | 7 (18%)        | 6 (26%)    | 1 (14.3%) | 0 (0%)   | 14 (20%) |
| cagN-positive       | 35 (89.7%)     | 22 (95.6%) | 6 (85.7%) | 1 (100%) | 64 (91.4%) |
| cagN-negative       | 4 (10.3%)      | 1 (4.3%)   | 1 (14.3%) | 0 (0%)   | 6 (8.6%) |
| cagA-positive       | 33 (84.6%)     | 19 (82.6%) | 6 (85.7%) | 1 (100%) | 59 (84.3%) |
| cagA-negative       | 6 (15.4%)      | 4 (17.4%)  | 1 (14.3%) | 0 (0%)   | 11 (15.7%) |
| cagL-positive       | 37 (94.9%)     | 21 (91.3%) | 5 (71.4%) | 1 (100%) | 64 (91.4%) |
| cagL-negative       | 2 (5.1%)       | 2 (8.7%)   | 2 (28.6%) | 0 (0%)   | 6 (8.6%) |
| vacA s1m1           | 12 (30.8%)     | 8 (34.8%)  | 3 (42.8%) | 0 (0%)   | 23 (32.8%) |
| vacA s1m2           | 14 (35.9%)     | 12 (52.2%) | 3 (42.8%) | 1 (100%) | 30 (42.8%) |
| vacA s2m2           | 13 (33.3%)     | 3 (13%)    | 1 (14.3%) | 0 (0%)   | 17 (24.3%) |
| babA2-positive      | 37 (94.9%)     | 23 (100%)  | 7 (100%)  | 1 (100%) | 68 (97.1%) |
| babA2-negative      | 2 (5.1%)       | 0 (0%)     | 0 (0%)    | 0 (0%)   | 2 (2.9%) |
| sabA-positive       | 32 (82%)       | 20 (87%)   | 6 (85.7%) | 1 (100%) | 59 (84.3%) |
| sabA-negative       | 7 (18%)        | 3 (13%)    | 1 (14.3%) | 0 (0%)   | 11 (15.7%) |
| dupA-positive       | 32 (82%)       | 20 (87%)   | 6 (85.7%) | 1 (100%) | 59 (84.3%) |
| dupA-negative       | 7 (18%)        | 3 (13%)    | 1 (14.3%) | 0 (0%)   | 11 (15.7%) |

GC, gastric cancer; IM, intestinal metaplasia; NUD, nonulcer dyspepsia; PUD, peptic ulcer disease.

Table 4. The frequency of amino acid and nucleotide substitutions of CagI among clinical strains of *H. pylori* (n = 27) from patients with different clinical status.
| CagI polymorphic residues\(^a\) | Amino acid diversity | NUD (\(n=19\)) | PUD (\(n=7\)) | IM (\(n=1\)) |
|-------------------------------|---------------------|----------------|----------------|--------------|
| 2                             | K/N                 | 18:1           | 7:0            | 1:0          |
|                               | AA(A/G):AAT         | AAA            | AAA            |              |
| 3                             | C/S/F               | 17:1:1         | 7:0:0          | 1:0:0        |
|                               | TGT:TCG:TTT         | TGT            | TGT            |              |
| 5                             | S/D                 | 18:1           | 7:0            | 1:0          |
|                               | AGC:GAC             | AGC            | AGC            |              |
| 9                             | S/F                 | 18:1           | 7:0            | 1:0          |
|                               | TCT:TTT             | TCT            | TCT            |              |
| 12                            | T/I                 | 18:1           | 7:0            | 1:0          |
|                               | ACT:ATT             | ACT            | ACT            |              |
| 22                            | E/G                 | 18:1           | 4:3            | 1:0          |
|                               | GA(A/G):GGG         | GAA:GGG        | GAA            |              |
| 23                            | V/A/I               | 17:1:1         | 3:3:1          | 1:0:0        |
|                               | GTA:GCA:ATA         | GTA:GCA:ATA    | GTA            |              |
| 25                            | I/M                 | 17:2           | 6:1            | 1:0          |
|                               | ATA:ATG             | ATA:ATG        | ATA            |              |
| 34                            | I/N                 | 18:1           | 7:0            | 1:0          |
|                               | ATT:AAT             | ATT            | ATT            |              |
| 40                            | A/V                 | 18:1           | 7:0            | 1:0          |
|                               | GCC:GTC             | GCC            | GCC            |              |
| 44                            | T/A                 | 18:1           | 7:0            | 1:0          |
|                               | ACC:GCC             | ACC            | ACC            |              |
| 51\(^b\)                      | A/V                 | 17:2           | 7:0            | 1:0          |
|                               | GCC:GTC             | GCC            | GCC            |              |
| 57                            | N/S                 | 10:9           | 2.5            | 0:1          |
|                               | AAT:AGT             | AAT:AGT        | AGT            |              |
| 94                            | G/S                 | 9:10           | 1:6            | 0:1          |
|                               | GCC:AGC             | GCC:AGC        | AGC            |              |
| 116                           | A/G                 | 18:1           | 7:0            | 1:0          |
|                               | GCC:GGG             | GCC            | GCC            |              |
| 162                           | A/T                 | 18:1           | 7:0            | 1:0          |
|                               | GCT:ACT             | GCT            | GCT            |              |
| 166                           | A/V                 | 19:0           | 6:1            | 1:0          |
|                               | GCG:GCG:GTG         | GCG            | GCG            |              |
| 182                           | E/K                 | 18:1           | 7:0            | 1:0          |
|                               | GAA:AAA             | GAA            | GAA            |              |
| 187                           | A/T                 | 18:1           | 7:0            | 1:0          |
|                               | GCT:ACC             | GCT            | GCT            |              |
| 190                           | S/N                 | 19:0           | 6:1            | 1:0          |
|                               | AGT                 | AGT:AAT        | AGT            |              |
| 192                           | S/F                 | 18:1           | 7:0            | 1:0          |
|                               | TCT:TTT             | TCT            | TCT            |              |
| 195                           | A/T                 | 17:2           | 6:1            | 1:0          |
|                               | GCT:ACT             | GCT:ACT        | GCT            |              |
| 199                           | A/T                 | 18:1           | 7:0            | 1:0          |
|                               | GCT:ACT             | GCT            | GCT            |              |
| 204                           | G/S                 | 18:1           | 7:0            | 1:0          |
|                               | GGT:AGT             | GGT            | GGT            |              |
| 213                           | K/E                 | 16:3           | 7:0            | 1:0          |
|                               | AAA:GAA             | AAA            | AAA            |              |
| 221                           | T/E                 | 15:4           | 7:0            | 1:0          |
|                               | ACA:GAG             | ACA            | ACA            |              |
| Position | Amino Acid Residue | Amino Acid Frequency | Nucleotide Frequency | Nucleotide Substitution |
|----------|-------------------|----------------------|---------------------|------------------------|
| 243      | A/T               | 15:4                 | 5:2                 | GCC:ACC GCC:ACC GCC    |
| 246      | A/V               | 19:0                 | 6:1                 | GCG:GCG:GCG:GCG       |
| 254      | S/N               | 17:2                 | 7:0                 | AGC:AAGC AGC AGC      |
| 257      | A/T               | 18:1                 | 7:0                 | GCA:ACA GCA GCA       |
| 262      | I/F               | 18:1                 | 7:0                 | ATT:TTT ATT ATT       |
| 263      | E/Q               | 18:1                 | 7:0                 | GAA:CAA GAA GAA       |
| 268      | A/V/E             | 13:5:1               | 6:1:0               | GCA:GTC:GAG GCA:GTC GCA:GTG |
| 305      | D/G/N             | 18:1:0               | 5:1:1               | GAT:GTT GAT:GTT:AAT GAT |
| 319      | G/E               | 18:1                 | 5:2                 | GGA:GAA GGA:GAA GGA   |
| 320      | E/Q               | 18:1                 | 5:2                 | GAA:CAA GAA:CAA GAA   |
| 351      | L/F               | 18:1                 | 7:0                 | CTT:TTT CTT CTT       |
| 353      | K/T               | 18:1                 | 7:0                 | AAA:ACA AAA AAA       |
| 368      | T/M/K             | 17:1:1               | 7:0                 | ACG:ATG:AAG ACG ACG   |
| 375      | S/G               | 19:0                 | 5:2                 | AGC AGC:GGC GGC       |

M, intestinal metaplasia; NUD, nonulcer dyspepsia; PUD, peptic ulcer disease.

Positions of amino acid residues correspond to the *H. pylori* P12 reference strain.

The underlined nucleotides denote that all of our *H. pylori* strains had C in the third-base position of this codon compared to *H. pylori* P12 that had T.

**Table 5.** The frequency of amino acid and nucleotide substitutions of CagN among clinical strains of *H. pylori* (*n*=43) from patients with different clinical status.
| CagN polymorphic residues | Amino acid diversity | NUD (n=24) | PUD (n=14) | IM (n=4) | GC (n=1) |
|---------------------------|----------------------|------------|------------|----------|----------|
| 8                         | L/I                  | 24:0       | 13:1       | 4:0      | 1:0      |
|                           |                      | (C/T)TA    | CTA:ATA    | CTA      | CTA      |
| 15                        | S/F                  | 22:2       | 14:0       | 4:0      | 1:0      |
|                           |                      | TCT:TCT    | TCT        | TCT      | TCT      |
| 17                        | V/A/I                | 20:3:1     | 12:1:1     | 4:0:0    | 1:0:0    |
|                           |                      | GTT:GCT:ATT| GTT        | GTT      | GTT      |
| 18                        | I/V                  | 13:11      | 7:7        | 2:2      | 1:0      |
|                           |                      | ATT:GTT    | ATT:GTT    | ATT:GTT  | ATT:GTT  |
| 32                        | S/N                  | 24:0       | 13:1       | 4:0      | 1:0      |
|                           |                      | AGT        | AGT:AAT    | AGT      | AGT      |
| 33                        | E/K                  | 23:1       | 14:0       | 4:0      | 1:0      |
|                           |                      | GAA:AAA    | GAA        | GAA      | GAA      |
| 36                        | E/K                  | 15:9       | 13:1       | 2:2      | 1:0      |
|                           |                      | GAA:AAA    | GAA:AAA    | GAA:AAA  | GAA:AAA  |
| 38b                       | A/V                  | 0:24       | 0:14       | 0:4      | 0:1      |
|                           |                      | GTG        | GCG:GT(G/A)| GTG      | GTG      |
| 39                        | A/V                  | 24:0       | 13:1       | 4:0      | 1:0      |
|                           |                      | GCT        | GCT:GTT    | GCT      | GCT      |
| 46                        | K/T                  | 24:0       | 13:1       | 3:1      | 1:0      |
|                           |                      | AA(A/G)    | AAA:ACA    | AAA:ACA  | AAA:ACA  |
| 48                        | L/F                  | 16:8       | 8:6        | 3:1      | 0:1      |
|                           |                      | CTC:TTC    | CTC:TTC    | CTC:TTC  | TCC      |
| 49                        | H/Y                  | 17:7       | 10:4       | 3:1      | 0:1      |
|                           |                      | CAT:TAT    | CAT:TAT    | CAT:TAT  | TAT      |
| 52                        | H/R                  | 24:0       | 13:1       | 4:0      | 1:0      |
|                           |                      | CAT        | CAT:CGT    | CAT      | CAT      |
| 53                        | G/D                  | 0:24       | 0:14       | 0:4      | 0:1      |
|                           |                      | GAC        | GAC        | GAC      | GAC      |
| 54                        | D/N                  | 23:1       | 14:0       | 4:0      | 1:0      |
|                           |                      | GAC:AA(C/T)| GAC        | GAC      | GAC      |
| 55                        | E/K                  | 17:7       | 11:3       | 4:0      | 1:0      |
|                           |                      | GAA:AAA    | GAA:AAA    | GAA      | GAA      |
| 57                        | I/V                  | 8:16       | 4:10       | 2:2      | 1:0      |
|                           |                      | ATT:GTT    | ATT:GTT    | ATT:GTT  | ATT:GTT  |
| 59                        | E/K                  | 7:17       | 1:13       | 1:3      | 1:0      |
|                           |                      | GAA:AAA    | GAA:AAA    | GAA:AAA  | GAA:AAA  |
| 61                        | E/K                  | 21:3       | 14:0       | 4:0      | 1:0      |
|                           |                      | GAA:AAA    | GAA        | GAA      | GAA      |
| 63                        | K/E                  | 8:16       | 2:12       | 1:3      | 1:0      |
|                           |                      | AAA:GAA    | AAA:GAA    | AAA:GAA  | AAA:GAA  |
| 80                        | A/V                  | 24:0       | 12:2       | 3:1      | 1:0      |
|                           |                      | GCA        | GCA:GT(G/A)| GCA:GT(G/A)| GCA      |
| 98                        | V/I                  | 6:18       | 5:9        | 3:1      | 0:1      |
|                           |                      | GTT:ATT    | GTT:ATT    | GTT:ATT  | ATT      |
| 102                       | A/V                  | 22:2       | 14:0       | 4:0      | 1:0      |
|                           |                      | GCG:GTG    | GCG        | GC(G/A)  | GCG      |
| 103                       | A/T/S                | 16:8:0     | 8:5:1      | 3:1:0    | 1:0      |
|                           |                      | GCT:ACT    | GCT:ACT:TCT| GCT:ACT  | GCT      |
| 106                       | K/R                  | 21:3       | 13:1       | 4:0      | 1:0      |
|                           |                      | AAA:AGA    | AAA:AGA    | AAA      | AAA      |
| 114                       | I/T                  | 21:3       | 8:6        | 4:0      | 1:0      |
|                           |                      | ATT:ACT    | ATT:ACT    | ATT      | ATT      |
| NUCLEOTIDE | T/N/H | P/S | D/N | S/G | A/T | N/H | D/G | D/N | E/G | A/S | A/T/V | A/T | N/D | E/K | I/V | C/G | D/N | G/D | D/E | A/T/V | E/K | I/V | C/G | D/N | G/D | D/E | A/T/V | E/K | I/V | C/G | D/N | G/D | D/E | A/T/V | E/K | I/V | C/G |
|------------|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|
| 117        | T/N/H | 3:7:14 | 2:3:9 | 0:3:1 | 0:1:0 |
| 118        | P/S   | 24:0 | 13:1 | 4:0 | 1:0 |
| 121        | D/N   | 22:2 | 14:0 | 4:0 | 1:0 |
| 125        | S/G   | 21:3 | 12:2 | 4:0 | 1:0 |
| 129        | A/T   | 8:16 | 3:11 | 0:4 | 0:1 |
| 134        | N/H   | 22:2 | 14:0 | 4:0 | 1:0 |
| 137        | D/G   | 23:1 | 14:0 | 4:0 | 1:0 |
| 140        | D/N   | 22:2 | 12:2 | 4:0 | 1:0 |
| 148        | E/G   | 11:13 | 8:6 | 2:2 | 1:0 |
| 149        | A/S   | 17:7 | 10:4 | 2:2 | 1:0 |
| 154        | A/T/V | 21:2:1 | 10:4:0 | 3:1:0 | 0:1:0 |
| 155        | A/T   | 24:0 | 13:1 | 4:0 | 1:0 |
| 160        | N/D   | 6:18 | 3:11 | 0:4 | 1:0 |
| 161        | E/K   | 24:0 | 13:1 | 4:0 | 1:0 |
| 170        | I/V   | 21:3 | 14:0 | 4:0 | 1:0 |
| 174        | C/G   | 23:1 | 14:0 | 4:0 | 1:0 |
| 182        | D/N   | 24:0 | 13:1 | 3:1 | 1:0 |
| 191        | G/D   | 24:0 | 12:2 | 3:1 | 1:0 |
| 194        | D/E   | 23:1 | 14:0 | 4:0 | 1:0 |
| 199        | A/T/V | 19:5:0 | 8:5:1 | 2:2:0 | 1:0:0 |
| 203        | E/K   | 23:1 | 14:0 | 3:1 | 1:0 |
| 208        | I/V   | 23:1 | 14:0 | 4:0 | 1:0 |
| 221        | S/N   | 22:2 | 14:0 | 4:0 | 1:0 |
| 224        | K/R   | 23:1 | 14:0 | 4:0 | 1:0 |
| 225        | L/F   | 23:1 | 14:0 | 4:0 | 1:0 |
| 226        | A/V   | 23:1 | 14:0 | 4:0 | 1:0 |
| 227        | L/F   | 24:0 | 12:2 | 3:1 | 0:1 |
|     |                  | CT(C/T) | CT(C/T):TTT | CT(C/T):TTT | TTT |
|-----|------------------|---------|-------------|-------------|-----|
| 228 | N/H              | 24:0    | 13:1        | 4:0         | 1:0 |
| 232 | N/S              | 24:0    | 13:1        | 4:0         | 1:0 |
| 233 | R/K              | 24:0    | 13:1        | 4:0         | 1:0 |
| 241 | T/A              | 2:22    | 1:13        | 0:4         | 0:1 |
| 248 | K/R              | 24:0    | 13:1        | 4:0         | 1:0 |
| 259 | T/I              | 23:1    | 14:0        | 4:0         | 1:0 |
| 262 | A/T              | 22:2    | 13:1        | 4:0         | 1:0 |
| 263 | S/G              | 24:0    | 14:0        | 3:1         | 1:0 |
| 264 | K/E              | 1:23    | 0:14        | 0:4         | 0:1 |
| 267 | T/A              | 9:15    | 3:11        | 2:2         | 0:1 |
| 268 | T/A              | 23:1    | 12:0        | 4:0         | 1:0 |
| 273 | N/S              | 23:1    | 14:0        | 4:0         | 1:0 |
| 279 | T/A/V            | 16:7:1  | 9:5:0       | 2:2:0       | 0:1 |
| 280 | F/S              | 23:1    | 13:1        | 3:1         | 1:0 |
| 284 | R/H              | 20:4    | 12:2        | 4:0         | 1:0 |
| 285 | S/F/P            | 22:2:0  | 14:0:0      | 3:0:1       | 1:0 |
| 287 | S/F              | 23:1    | 131:0       | 4:0         | 1:0 |
| 288 | E/D              | 23:1    | 14:0        | 4:0         | 1:0 |
| 302 | A/V              | 23:1    | 14:0        | 4:0         | 1:0 |
| 304 | E/G              | 0:24    | 0:14        | 0:4         | 0:1 |

GC, gastric cancer; IM, intestinal metaplasia; NUD, nonulcer dyspepsia; PUD, peptic ulcer disease.

*a*Positions of amino acid residues correspond to the *H. pylori* P12 reference strain.

*b*The underlined nucleotides denote that all of our *H. pylori* strains had T in the second-base position of this codon compared to *H. pylori* P12 that had C.

*c*The underlined nucleotides denote that all of our *H. pylori* strains had G in the second-base position of this codon compared to *H. pylori* P12 that had A.

The inserted sequences are not indicated in the table.
Figure 1

Complete alignment of CagI sequences among H. pylori strains (n=27) from patients with different clinical status. The amino acid sequences were compared with the CagI sequences of H. pylori strain P12 (is shown on the top line), as a reference strain. The variable and infrequent amino acid residues are
surrounded by black borders. Notably, the C-terminal hexapeptide motif consisting of the SKVIVK sequence were highly conserved among the strains.

Figure 2

Complete alignment of CagN sequences among 43 H. pylori strains from patients with different clinical status. The amino acid sequences were compared with the CagN sequences of H. pylori strain P12 (is shown on the top line), as a reference strain. The variable and infrequent amino acid residues are
surrounded by black borders. Notably, the CagN hypervariable motif (CagNHM) and the conserved hypothetical hexapeptide repeat (EAKDEN/K) are indicated at residues 53-63 and 278-283, respectively.

Figure 3

Phylogenetic tree of H. pylori clinical strains (n=27) based on cagI nucleotide sequences. Maximum likelihood tree of concatenated sequences was constructed using MEGA7 software with bootstrap
method at 1000 replications. The evolutionary distances were computed using the Tamura 3-parameter model.

Figure 4
Phylogenetic tree of H. pylori clinical strains (n=27) based on translated CagI amino acid sequences. Maximum likelihood tree of concatenated sequences was constructed using MEGA7 software with
bootstrap method at 1000 replications. The evolutionary distances were computed using the Poisson correction method.

Figure 5

Phylogenetic tree of H. pylori clinical strains (n=43) based on cagN nucleotide sequences. Maximum likelihood tree of concatenated sequences was constructed using MEGA7 software with bootstrap method at 1000 replications. The evolutionary distances were computed using the Poisson correction method.
method at 1000 replications. The evolutionary distances were computed using the Tamura 3-parameter model.

Figure 6

Phylogenetic tree of H. pylori clinical strains (n=43) based on translated CagN amino acid sequences. Maximum likelihood tree of concatenated sequences was constructed using MEGA7 software with
bootstrap method at 1000 replications. The evolutionary distances were computed using the Poisson correction method.