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

Yasaman Azizimoghaddam

Shaheed Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases

Sadaf Kermanpour

Shaheed Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases

Nasrin Mirzaei

Shaheed Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases

Hamidreza Hour

Shaheed Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases

Abbas Yadegar (✉ a.yadegar@sbmu.ac.ir)

Shaheed Beheshti University of Medical Sciences <https://orcid.org/0000-0002-2135-7581>

Navin Kumar

Gautam Buddha University

Hamid Asadzadeh Aghdaei

Shaheed Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases

Mohammad Reza Zali

Shaheed Beheshti University of Medical Sciences Research Institute for Gastroenterology and Liver Diseases

Research

Keywords: *Helicobacter pylori*, CagI, CagN, cagPAI, genetic diversity, virulence genotype

Posted Date: September 1st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-64096/v1>

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 GDEEITEEEEKK 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 *H. pylori*-infected individuals are asymptomatic and may tolerate the presence of this organism in their stomach lifelong [3, 4]. The severity of *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].

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, *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 *H. pylori* such as CagA, VacA, BabA, SabA, and DupA [6, 7]. CagA, an oncoprotein, is the best studied virulence-associated factor of *H. pylori* that is translocated into the host gastric epithelial cells via the type 4 secretion system (T4SS). The *H. pylori* T4SS machinery is constituted by a cluster of gene products located on an approximately 40 kb chromosomal region named *cag* Pathogenicity Island (*cagPAI*) [8, 9]. *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]. *cagPAI* encodes several unique Cag components that have no sequence similarities to any other bacterial proteins involved in T4SS. However, a number of *cagPAI* genes such as *cagl* and *cagN* were proposed to be most probably evolved under a diversifying selection and evolutionary pressure [12]. Cagl, a small protein (41.5 kDa) encoded by *cagl* (*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 *cagN* gene (*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 Cagl and CagN in CagA translocation, IL-8 induction from gastric epithelial cells, and *H. pylori* T4SS machinery [15, 17–21]. Reviewing subsequent and more recent literatures have revealed that Cagl 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 *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 Cagl and CagN in CagA translocation and pathogenesis need to be further investigated. The oncogenic potential of *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 *cagl* and *cagN* genes and their amino acid sequence polymorphisms in Iranian *H. pylori*-infected patients with various gastroduodenal diseases. The probable association between the genetic variants of *cagl* and *cagN* and other virulence genotypes of *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/>) and the DNA Data Bank of Japan (<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/>). 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>).

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' (*cagI*-F) and 5'-TTTGAGCACTTGTTGGTTGG-3' (*cagI*-R), 5'-GAGCGACAAAACAACTATGC-3' (*cagN*-F) and 5'-GATCCCTAGAACAAAGTAAGC-3' (*cagN*-R) 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 *cagI* and *cagN* genes from *H. pylori* strains determined in this study were deposited in the NCBI GenBank database under the accession numbers MG573078-MG573107 (*cagI*) and MG559675-MG559720 (*cagN*).

Statistical analysis

The statistical associations between *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, *H. pylori* was isolated by culture and the isolates were approved by detection of the *glmM* and 16s rRNA genes.

Virulence genotypes and variants

The molecular analysis revealed that the *cagA*, *cagI*, *cagN*, *cagL*, *vacA* s1m1, *vacA* s1m2, and *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 *babA2*, *dupA*, and *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 *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 *babA2* gene by PCR. Furthermore, the prevalence of *cagN* and *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 *vacA* allelic combinations, *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 *vacA* s1m1 and *vacA* s2m2, within the IM and NUD strains, respectively.

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 GDEEITEEEKK 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 two times 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 *cagPAI* (*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 *cagPAI* (*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 *cagPAI* 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 *cagPAI*-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 *cagl* gene, whilst 91.4% of strains had *cagN* gene. To the best of our knowledge, the *cagl* 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 *cagPAI* 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 GDEEITEEEEKK in the P12 reference strain. Our findings revealed that there was no significant correlation between clinical outcomes and *cagl* 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 *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 *H. pylori* clinical strains contained *cagL* 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 *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 *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 *H. pylori* T4SS activity. Despite the alterations in various *cag* sequences, it is noticeable that 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.

In the preset study, we also investigated the presence of various *H. pylori* virulence genotypes. In accordance with our previous study in Iranian populations, we detected a high prevalence of *vacA* s1 (77.1%) and *vacA* m2 (65.7%) allelic genotypes [39]. The *vacA* s1 allele has been reported to be associated with more severe atrophic gastritis in peptic ulcer patients [40, 41]. In our study, the *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 *H. pylori* [42, 43]. Accordingly, type s1m1 *H. pylori* strains express large amounts of VacA toxin and are strongly associated with a higher level of inflammation and mucosal ulceration, while *vacA* s1m2-harboring strains produce moderate amount of toxin and *vacA* s2m2 strains are virtually non-toxic and rarely associated to clinical outcome [44]. A majority of *H. pylori* strains in the current study contained *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 *H. pylori* in other parts of the world, and the hypervirulent *vacA* s1m1 genotype was commonly associated with PUD patients [45]. Hence, it can be inferred that correlation between *H. pylori* genotyping and clinical outcome of the patients vary in different geographical regions.

Conclusion

In summary, a large body of evidence indicates that certain *cagPAI* components are correlated with the risk of gastric carcinogenesis. Here, we investigated the diversity of CagI and CagN sequences in clinical *H. pylori* isolates from Iranian patients with different clinical status. We detected several putative variants of CagI and CagN sequences in *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 *cagPAI* 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

Acknowledgments

The authors wish to thank the laboratory staff of Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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.

Funding

This study was supported financially by a grant (no. RIGLD 969) from Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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.

References

1. Amieva MR, El-Omar EM. Host-bacterial interactions in *Helicobacter pylori* infection. *Gastroenterol*. 2008;134(1):306–23.
2. Naumann M, Sokolova O, Tegtmeyer N, Backert S. *Helicobacter pylori*: A paradigm pathogen for subverting host cell signal transmission. *Trends Microbiol*. 2017.
3. Cover TL, Blaser MJ. *Helicobacter pylori* in health and disease. *Gastroenterol*. 2009;136(6):1863–73.
4. Eusebi LH, Zagari RM, Bazzoli F. Epidemiology of *Helicobacter pylori* infection. *Helicobacter*. 2014;19(Suppl 1):1–5.
5. Malfertheiner P, Link A, Selgrad M. *Helicobacter pylori*: perspectives and time trends. *Nat Rev Gastroenterol Hepatol*. 2014;11(10):628–38.
6. Polk DB, Peek RM Jr. *Helicobacter pylori*: gastric cancer and beyond. *Nature reviews Cancer*. 2010;10(6):403.
7. Backert S, Neddermann M, Maubach G, Naumann M. Pathogenesis of *Helicobacter pylori* infection. *Helicobacter*. 2016;21(Suppl 1):19–25.
8. Hatakeyama M. *Helicobacter pylori* CagA and gastric cancer: a paradigm for hit-and-run carcinogenesis. *Cell Host Microbe*. 2014;15(3):306–16.
9. Kwok T, Zabler D, Urman S, Rohde M, Hartig R, Wessler S, Misselwitz R, Berger J, Sewald N, König W, et al. *Helicobacter* exploits integrin for type IV secretion and kinase activation. *Nature*. 2007;449(7164):862–6.
10. Backert S, Tegtmeyer N, Fischer W. Composition, structure and function of the *Helicobacter pylori* cag pathogenicity island encoded type IV secretion system. *Future Microbiol*. 2015;10(6):955–65.
11. Tegtmeyer N, Wessler S, Backert S. Role of the cag-pathogenicity island encoded type IV secretion system in *Helicobacter pylori* pathogenesis. *FEBS J*. 2011;278(8):1190–202.
12. Olbermann P, Josenhans C, Moodley Y, Uhr M, Stamer C, Vauterin M, Suerbaum S, Achtman M, Linz B. A Global Overview of the Genetic and Functional Diversity in the *Helicobacter pylori* cag Pathogenicity Island. *PLOS Genetic*. 2010;6(8):e1001069.
13. Cendron L, Zanotti G. Structural and functional aspects of unique type IV secretory components in the *Helicobacter pylori* cag-pathogenicity island. *FEBS J*. 2011;278(8):1223–31.
14. Merino E, Flores-Encarnacion M, Aguilar-Gutierrez GR. Functional interaction and structural characteristics of unique components of *Helicobacter pylori* T4SS. *FEBS J*. 2017;284(21):3540–9.

15. Bourzac KM, Satkamp LA, Guillemin K. The *Helicobacter pylori* cag pathogenicity island protein CagN is a bacterial membrane-associated protein that is processed at its C terminus. *Infect Immun*. 2006;74(5):2537–43.
16. Ta LH, Hansen LM, Sause WE, Shiva O, Millstein A, Ottemann KM, Castillo AR, Solnick JV. Conserved transcriptional unit organization of the cag pathogenicity island among *Helicobacter pylori* strains. *Front Cell Infect Microbiol*. 2012;2:46.
17. Fischer W, Püls J, Buhrdorf R, Gebert B, Odenbreit S, Haas R. Systematic mutagenesis of the *Helicobacter pylori* cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. *Mol Microbiol*. 2001;42(5):1337–48.
18. Pham KT, Weiss E, Soto LFJ, Breithaupt U, Haas R, Fischer W. CagI is an essential component of the *Helicobacter pylori* Cag type IV secretion system and forms a complex with CagL. *PloS one*. 2012;7(4):e35341.
19. Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiß S, Sittka A, Chabas S, Reiche K, Hackermüller J, Reinhardt R. The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature*. 2010;464(7286):250–5.
20. Li SD, Kersulyte D, Lindley IJ, Neelam B, Berg DE, Crabtree JE. Multiple Genes in the Left Half of the cagPathogenicity Island of *Helicobacter pylori* Are Required for Tyrosine Kinase-Dependent Transcription of Interleukin-8 in Gastric Epithelial Cells. *Infect Immun*. 1999;67(8):3893–9.
21. Selbach M, Moese S, Meyer TF, Backert S. Functional analysis of the *Helicobacter pylori* cag pathogenicity island reveals both VirD4-CagA-dependent and VirD4-CagA-independent mechanisms. *Infect Immun*. 2002;70(2):665–71.
22. Jimenez-Soto LF, Kutter S, Sewald X, Ertl C, Weiss E, Kapp U, Rohde M, Pirch T, Jung K, Retta SF, et al. *Helicobacter pylori* type IV secretion apparatus exploits beta1 integrin in a novel RGD-independent manner. *PLoS Pathog*. 2009;5(12):e1000684.
23. Shaffer CL, Gaddy JA, Loh JT, Johnson EM, Hill S, Hennig EE, McClain MS, McDonald WH, Cover TL. *Helicobacter pylori* exploits a unique repertoire of type IV secretion system components for pilus assembly at the bacteria-host cell interface. *PLoS Pathog*. 2011;7(9):e1002237.
24. Gorrell RJ, Zwickel N, Reynolds J, Bulach D, Kwok T. *Helicobacter pylori* CagL hypervariable motif: a global analysis of geographical diversity and association with gastric cancer. *J Infect Dis*. 2016;213(12):1927–31.
25. Linz B, Windsor HM, Gajewski JP, Hake CM, Drautz DI, Schuster SC, Marshall BJ. *Helicobacter pylori* genomic microevolution during naturally occurring transmission between adults. *PloS one*. 2013;8(12):e82187.
26. Ogawa H, Iwamoto A, Tanahashi T, Okada R, Yamamoto K, Nishiumi S, Yoshida M, Azuma T. Genetic variants of *Helicobacter pylori* type IV secretion system components CagL and CagI and their association with clinical outcomes. *Gut Pathog*. 2017;9(1):21.
27. Rizzato C, Torres J, Plummer M, Muñoz N, Franceschi S, Camorlinga-Ponce M, Fuentes-Pananá EM, Canzian F, Kato I. Variations in *Helicobacter pylori* cytotoxin-associated genes and their influence in

- progression to gastric cancer: implications for prevention. PLoS One. 2012;7(1):e29605.
28. Yadegar A, Alebouyeh M, Lawson AJ, Mirzaei T, Mojarad EN, Zali MR. Differentiation of non-pylori *Helicobacter* species based on PCR–restriction fragment length polymorphism of the 23S rRNA gene. World J Microbiol Biotechnol. 2014;30(6):1909–17.
 29. Yadegar A, Mohabati Mobarez A, Zali MR. Genetic diversity and amino acid sequence polymorphism in *Helicobacter pylori* CagL hypervariable motif and its association with virulence markers and gastroduodenal diseases. Cancer Med. 2019;8(4):1619–32.
 30. Hall TA. BioEdit: a user-friendly biological sequence alignment editor. and analysis program for Windows 95/98/NT. In: Nucleic Acids Symp Ser. 1999: [London]: Information Retrieval Ltd., c1979-c2000.; 1999: 95–98.
 31. Kumar S, Stecher G, Tamura K. MEGA7. molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–4.
 32. Noto JM, Peek RM. The *Helicobacter pylori* cag pathogenicity island. In: Helicobacter Species. edn.: Springer; 2012: 41–50.
 33. Crabtree J, Taylor J, Heatley R, Shallcross T, Rathbone B, Wyatt J, Tompkins D. Mucosal IgA recognition of *Helicobacter pylori* 120 kDa protein, peptic ulceration, and gastric pathology. Lancet. 1991;338(8763):332–5.
 34. Crabtree J, Wyatt J, Sobala G, Miller G, Tompkins D, Primrose J, Morgan A. Systemic and mucosal humoral responses to *Helicobacter pylori* in gastric cancer. Gut. 1993;34(10):1339–43.
 35. Torres J, Pérez-Pérez GI, Leal-Herrera Y, MUNoz O. Infection with CagA + *Helicobacter pylori* strains as a possible predictor of risk in the development of gastric adenocarcinoma in Mexico. Int J cancer. 1998;78(3):298–300.
 36. Kutter S, Buhrdorf R, Haas J, Schneider-Brachert W, Haas R, Fischer W. Protein Subassemblies of the *Helicobacter pylori* Cag Type IV Secretion System Revealed by Localization and Interaction Studies. J Biotechnol. 2008;190(6):2161–71.
 37. Kumar N, Shariq M, Kumari R, Tyagi RK, Mukhopadhyay G. Cag type IV secretion system: CagI independent bacterial surface localization of CagA. PLoS One. 2013;8(9):e74620.
 38. Bats SH, Bergé C, Coombs N, Terradot L, Josenhans C. Biochemical characterization of the *Helicobacter pylori* Cag Type 4 Secretion System protein CagN and its interaction partner CagM. Int J Med Microbiol. 2018;308(4):425–37.
 39. Yadegar A, Mobarez AM, Alebouyeh M, Mirzaei T, Kwok T, Zali MR. Clinical relevance of cagL gene and virulence genotypes with disease outcomes in a *Helicobacter pylori* infected population from Iran. World J Microbiol Biotechnol. 2014;30(9):2481–90.
 40. Chiurillo MA, Moran Y, Cañas M, Valderrama E, Granda N, Sayegh M, Ramírez JL. Genotyping of *Helicobacter pylori* virulence-associated genes shows high diversity of strains infecting patients in western Venezuela. Int J Infect Dis. 2013;17(9):e750–6.
 41. van Doorn L-J, Schneeberger P, Nouhan N, Plaisier A, Quint W, De Boer W. Importance of *Helicobacter pylori* cagA and vacA status for the efficacy of antibiotic treatment. Gut. 2000;46(3):321–6.

42. Chauhan N, Tay ACY, Marshall BJ, Jain U. *Helicobacter pylori* VacA, a distinct toxin exerts diverse functionalities in numerous cells: An overview. *Helicobacter*. 2019;24(1):e12544.

43. Palframan SL, Kwok T, Gabriel K. Vacuolating cytotoxin A (VacA), a key toxin for *Helicobacter pylori* pathogenesis. *Front cell Infect Microbiol*. 2012;2:92.

44. Atherton JC, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori* association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem*. 1995;270(30):17771–7.

45. Miernyk K, Morris J, Bruden D, McMahon B, Hurlburt D, Sacco F, Parkinson A, Hennessy T, Bruce M. Characterization of *Helicobacter pylori* cagA and vacA genotypes among Alaskans and their correlation with clinical disease. *J Clin Microbiol*. 2011;49(9):3114–21.

46. Farzi N, Yadegar A, Sadeghi A, Asadzadeh Aghdai H, Marian Smith S, Raymond J, Suzuki H, Zali MR. High prevalence of antibiotic resistance in Iranian *Helicobacter pylori* Isolates: importance of functional and mutational analysis of resistance genes and virulence genotyping. *J Clin Med*. 2019;8(11):2004.

Tables

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

Target gene	Primer designation	Oligonucleotide sequence (5′-3′)	Annealingtemperature (°C)	PCR product (bp)	Reference
16S rRNA	C97-20	GGCTATGACGGGTATCCGGC	58	764	[39]
	H3A-20	GCCGTGCAGCACCTGTTTTC			
<i>glmM</i>	GlmM2-F	GGATAAGCTTTTAGGGGTGTTAGGGG	56	296	[39]
	GlmM1-R	GCTTACTTTCTAACACTAACGCGC			
<i>cagL</i>	cagL-B4	GCAGAATTCATAACAAGCGGCTTAAAG	60	695	[39]
	cagL-B5	ATTAGAATTCATAGCCTATCGTCTCAG			
<i>cagA</i>	93089	AATACACCAACGCCTCCAAG	57	400	[39]
	93261	TTGTTGCCGCTTTTGCTCTC			
<i>vacA</i> s1/s2	VA1-F	ATGGAAATACAACAAACACAC	57	259/286	[39]
	VA1-R	CTGCTTGAATGCGCCAAAC			
<i>vacA</i> m1/m2	VAG-F	CAATCTGTCCAATCAAGCGAG	57	570/645	[39]
	VAG-R	GCGTCAAAATAATTCCAAGG			
<i>babA2</i>	bab7-F	CCAAACGAAACAAAAGCGT	52	271	[46]
	bab7-R	GCTTGTGTAAAAGCCGTCGT			
<i>sabA</i>	F1-HP726-jhp663	TTTTTGTCAGCTACGCGTTC	56	487	[46]
	R1-HP725-jhp662	ACCGAAGTGATAACGGCTTG			
<i>dupA</i>	DupA-F	ATTCACGCCTAAGACCTCA	55	581	[46]
	DupA-R	CTGAGAAGCCTTATTATCTTGTGG			

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)	<i>cagI</i> GenBank no. ^a	<i>cagN</i> GenBank no.
1	HC3	PUD	Male	46	Negative ^b	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 ^c	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

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

IC, 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 (n=70)
	NUD (n=39)	PUD (n=23)	IM (n=7)	GC (n=1)	
<i>cagI</i> -positive	32 (82%)	17 (73.9%)	6 (85.7%)	1 (100%)	56 (80%)
<i>cagI</i> -negative	7 (18%)	6 (26%)	1 (14.3%)	0 (0%)	14 (20%)
<i>cagN</i> -positive	35 (89.7%)	22 (95.6%)	6 (85.7%)	1 (100%)	64 (91.4%)
<i>cagN</i> -negative	4 (10.3%)	1 (4.3%)	1 (14.3%)	0 (0%)	6 (8.6%)
<i>cagA</i> -positive	33 (84.6%)	19 (82.6%)	6 (85.7%)	1 (100%)	59 (84.3%)
<i>cagA</i> -negative	6 (15.4%)	4 (17.4%)	1 (14.3%)	0 (0%)	11 (15.7%)
<i>cagL</i> -positive	37 (94.9%)	21 (91.3%)	5 (71.4%)	1 (100%)	64 (91.4%)
<i>cagL</i> -negative	2 (5.1%)	2 (8.7%)	2 (28.6%)	0 (0%)	6 (8.6%)
<i>vacA</i> s1m1	12 (30.8%)	8 (34.8%)	3 (42.8%)	0 (0%)	23 (32.8%)
<i>vacA</i> s1m2	14 (35.9%)	12 (52.2%)	3 (42.8%)	1 (100%)	30 (42.8%)
<i>vacA</i> s2m2	13 (33.3%)	3 (13%)	1 (14.3%)	0 (0%)	17 (24.3%)
<i>babA2</i> -positive	37 (94.9%)	23 (100%)	7 (100%)	1 (100%)	68 (97.1%)
<i>babA2</i> -negative	2 (5.1%)	0 (0%)	0 (0%)	0 (0%)	2 (2.9%)
<i>sabA</i> -positive	32 (82%)	20 (87%)	6 (85.7%)	1 (100%)	59 (84.3%)
<i>sabA</i> -negative	7 (18%)	3 (13%)	1 (14.3%)	0 (0%)	11 (15.7%)
<i>dupA</i> -positive	32 (82%)	20 (87%)	6 (85.7%)	1 (100%)	59 (84.3%)
<i>dupA</i> -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 (<i>n</i> =19)	PUD (<i>n</i> =7)	IM (<i>n</i> =1)
2	K/N	18:1 AA(A/G):AAT	7:0 AAA	1:0 AAA
3	C/S/F	17:1:1 TGT:TCG:TTT	7:0:0 TGT	1:0:0 TGT
6	S/D	18:1 AGC:GAC	7:0 AGC	1:0 AGC
9	S/F	18:1 TCT:TTT	7:0 TCT	1:0 TCT
12	T/I	18:1 ACT:ATT	7:0 ACT	1:0 ACT
22	E/G	18:1 GA(A/G):GGG	4:3 GAA:GGG	1:0 GAA
23	V/A/I	17:1:1 GTA:GCA:ATA	3:3:1 GTA:GCA:ATA	1:0:0 GTA
25	I/M	17:2 ATA:ATG	6:1 ATA:ATG	1:0 ATA
34	I/N	18:1 ATT:AAT	7:0 ATT	1:0 ATT
40	A/V	18:1 GCC:GTC	7:0 GCC	1:0 GCC
44	T/A	18:1 ACC:GCC	7:0 ACC	1:0 ACC
51 ^b	A/V	17:2 GCC:GTC	7:0 GCC	1:0 GCC
57	N/S	10:9 AAT:AGT	2:5 AAT:AGT	0:1 AGT
94	G/S	9:10 GGC:AGC	1:6 GGC:AGC	0:1 AGC
116	A/G	18:1 GCG:GGG	7:0 GCG	1:0 GCG
162	A/T	18:1 GCT:ACT	7:0 GCT	1:0 GCT
166	A/V	19:0 GCG	6:1 GCG:GTG	1:0 GCG
182	E/K	18:1 GAA:AAA	7:0 GAA	1:0 GAA
187	A/T	18:1 GCT:ACC	7:0 GCT	1:0 GCT
190	S/N	19:0 AGT	6:1 AGT:AAT	1:0 AGT
192	S/F	18:1 TCT:TTT	7:0 TCT	1:0 TCT
195	A/T	17:2 GCT:ACT	6:1 GCT:ACT	1:0 GCT
199	A/T	18:1 GCT:ACT	7:0 GCT	1:0 GCT
204	G/S	18:1 GGT:AGT	7:0 GGT	1:0 GGT
213	K/E	16:3 AAA:GAA	7:0 AAA	1:0 AAA
221	T/E	15:4 ACA:GAG	7:0 ACA	1:0 ACA

243	A/T	15:4	5:2	1:0
		GCC: <u>ACC</u>	GCC: <u>ACC</u>	GCC
246	A/V	19:0	6:1	1:0
		GCG	GCG:GTG	GCG
254	S/N	17:2	7:0	1:0
		AGC: <u>AAC</u>	AGC	AGC
257	A/T	18:1	7:0	1:0
		GCA: <u>ACA</u>	GCA	GCA
262	I/F	18:1	7:0	1:0
		ATT: <u>TTT</u>	ATT	ATT
263	E/Q	18:1	7:0	1:0
		GAA: <u>CAA</u>	GAA	GAA
268	A/V/E	13:5:1	6:1:0	0:1:0
		GCA:GTG: <u>GAG</u>	GCA:GTG	GCA:GTG
305	D/G/N	18:1:0	5:1:1	1:0
		GAT:GGT	GAT:GGT: <u>AAT</u>	GAT
319	G/E	18:1	5:2	1:0
		GGA: <u>GAA</u>	GGA: <u>GAA</u>	GGA
320	E/Q	18:1	5:2	1:0
		GAA: <u>CAA</u>	GAA: <u>CAA</u>	GAA
351	L/F	18:1	7:0	1:0
		CTT: <u>TTT</u>	CTT	CTT
353	K/T	18:1	7:0	1:0
		AAA: <u>ACA</u>	AAA	AAA
368	T/M/K	17:1:1	7:0	10:0
		ACG:ATG: <u>AAG</u>	ACG	ACG
375	S/G	19:0	5:2	0:1
		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 ^a	Amino acid diversity	NUD (<i>n</i> =24)	PUD (<i>n</i> =14)	IM (<i>n</i> =4)	GC (<i>n</i> =1)
8	L/I	24:0 (C/T)TA	13:1 CTA:ATA	4:0 CTA	1:0 CTA
15	S/F	22:2 TCT:TTT	14:0 TCT	4:0 TCT	1:0 TCT
17	V/A/I	20:3:1 GTT:GCT:ATT	12:1:1 GTT:GCT:ATT	4:0:0 GTT	1:0:0 GTT
18	I/V	13:11 ATT:GTT	7:7 ATT:GTT	2:2 ATT:GTT	1:0 ATT
32	S/N	24:0 AGT	13:1 AGT:AAT	4:0 AGT	1:0 AGT
33	E/K	23:1 GAA:AAA	14:0 GAA	4:0 GAA	1:0 GAA
36	E/K	15:9 GAA:AAA	13:1 GAA:AAA	2:2 GAA:AAA	1:0 GAA
38 ^b	A/V	0:24 GTG	0:14 GCG:GT(G/A)	0:4 GTG	0:1 GTG
39	A/V	24:0 GCT	13:1 GCT:GTT	4:0 GCT	1:0 GCT
46	K/T	24:0 AA(A/G)	13:1 AAA:ACA	3:1 AAA:ACA	1:0 AAA
48	L/F	16:8 CTC:TTC	8:6 CTC:TTC	3:1 CTC:TTC	0:1 TTC
49	H/Y	17:7 CAT:TAT	10:4 CAT:TAT	3:1 CAT:TAT	0:1 TAT
52	H/R	24:0 CAT	13:1 CAT:CGT	4:0 CAT	1:0 CAT
53	G/D	0:24 GAC	0:14 GAC	0:4 GAC	0:1 GAC
54	D/N	23:1 GAC:AA(C/T)	14:0 GAC	4:0 GAC	1:0 GAC
55	E/K	17:7 GAA:AAA	11:3 GAA:AAA	4:0 GAA	1:0 GAA
57	I/V	8:16 ATT:GTT	4:10 ATT:GTT	2:2 ATT:GTT	1:0 ATT
59	E/K	7:17 GAA:AAA	1:13 GAA:AAA	1:3 GAA:AAA	1:0 GAA
61	E/K	21:3 GAA:AAA	14:0 GAA	4:0 GAA	1:0 GAA
63	K/E	8:16 AAA:GAA	2:12 AAA:GAA	1:3 AAA:GAA	1:0 AAA
80	A/V	24:0 GCA	12:2 GCA:GT(G/A)	3:1 GCA:GT(G/A)	1:0 GCA
98	V/I	6:18 GTT:ATT	5:9 GTT:ATT	3:1 GTT:ATT	0:1 ATT
102	A/V	22:2 GCG:GTG	14:0 GCG	4:0 GC(G/A)	1:0 GCG
103	A/T/S	16:8:0 GCT:ACT	8:5:1 GCT:ACT:TCT	3:1:0 GCT:ACT	1:0 GCT
106	K/R	21:3 AAA:AGA	13:1 AAA:AGA	4:0 AAA	1:0 AAA
114	I/T	21:3 ATT:ACT	8:6 ATT:ACT	4:0 ATT	1:0 ATT

117	T/N/H	3:7:14 ACC:AAC:CAC	2:3:9 ACC:AAC:CAC	0:3:1 AAC:CAC	0:1:0 AAC
118	P/S	24:0 CCT	13:1 CCT:TCT	4:0 CCT	1:0 CCT
121	D/N	22:2 GAC:AAC	14:0 GAC	4:0 GAC	1:0 GAC
125	S/G	21:3 AGC:GGC	12:2 AGC:GGC	4:0 AGC	1:0 AGC
129	A/T	8:16 GCT:ACT	3:11 GCT:ACT	0:4 ACT	0:1 ACT
134	N/H	22:2 AAT:CAT	14:0 AAT	4:0 AAT	1:0 AAT
137	D/G	23:1 GAT:GGA	14:0 GAT	4:0 GAT	1:0 GAT
140	D/N	22:2 GAT:AAT	12:2 GAT:AAT	4:0 GAT	1:0 GAT
148	E/G	11:13 GAA:GGA	8:6 GAA:GGA	2:2 GAA:GGA	1:0 GAA
149	A/S	17:7 GCT:TCT	10:4 GCT:TCT	2:2 GCT:TCT	1:0 GCT
154	A/T/V	21:2:1 GCT:ACT:GTT	10:4:0 GCT:ACT	3:1:0 GCT:ACT	0:1:0 ACT
155	A/T	24:0 GCT	13:1 GCT:ACT	4:0 GCT	1:0 GCT
160	N/D	6:18 AAT:GAT	3:11 AAT:GAT	0:4 GAT	1:0 AAT
161	E/K	24:0 GAG	13:1 GAG:AAA	4:0 GAG	1:0 GAG
170	I/V	21:3 AT(T/C):GTT	14:0 AT(T/C)	4:0 ATT	1:0 ATT
174	C/G	23:1 TGT:GGT	14:0 TGT	4:0 TGT	1:0 TGT
182	D/N	24:0 GAT	13:1 GAT:AAT	3:1 GAT:AAT	1:0 GAT
191	G/D	24:0 GG(C/T)	12:2 GGC:GAC	3:1 GGC:GAC	1:0 GGC
194	D/E	23:1 GAT:GAA	14:0 GAT	4:0 GAT	1:0 GAT
199	A/T/V	19:5:0 GCC:ACC	8:5:1 GCC:ACC:GTC	2:2:0 GCC:ACC	1:0:0 GCC
203	E/K	23:1 GAA:AAA	14:0 GA(A/G)	3:1 GAA:AAA	1:0 GAA
208	I/V	23:1 ATA:GTA	14:0 ATA	4:0 ATA	1:0 ATA
221	S/N	22:2 AGT:AAT	14:0 AGT	4:0 AGT	1:0 AGT
224	K/R	23:1 AAA:AGA	14:0 AAA	4:0 AAA	1:0 AAA
225	L/F	23:1 CT(C/T):TTC	14:0 CTC	4:0 CTC	1:0 CTC
226	A/V	23:1 GCT:GTT	14:0 GCT	4:0 GCT	1:0 GCT
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
		AAT	AAT:CAT	AAT	AAT
232	N/S	24:0	13:1	4:0	1:0
		AAT	AAT:AGT	AAT	AAT
233	R/K	24:0	13:1	4:0	1:0
		AGA	AGA:AAA	AGA	AGA
241	T/A	2:22	1:13	0:4	0:1
		ACT:GC(G/T)	ACT:GC(G/T)	ACT:GCT	GCT
248	K/R	24:0	13:1	4:0	1:0
		AAA	AAA:AGA	AAA	AAA
259	T/I	23:1	14:0	4:0	1:0
		ACT:ATT	ACT	ACT	ACT
262	A/T	22:2	13:1	4:0	1:0
		GCA:ACA	GCA:ACA	GCA	GCA
263	S/G	24:0	14:0	3:1	1:0
		AG(C/T)	AG(C/T)	AG(C/T):GGT	AGT
264	K/E	1:23	0:14	0:4	0:1
		AAG:GA(G/A)	GA(G/A)	GA(G/A)	GAG
267	T/A	9:15	3:11	2:2	0:1
		ACT:GCT	ACT:GCT	ACT:GCT	GCT
268	T/A	23:1	2:12	4:0	1:0
		ACT:GCT	ACT:GCT	ACT	ACT
273	N/S	23:1	14:0	4:0	1:0
		AA(T/C):AGC	AA(T/C)	AA(T/C)	AAT
279	T/A/V	16:7:1	9:5:0	2:2:0	0:1:0
		ACA:GC(A/G):GTG	ACA:GC(A/G)	ACA:GC(A/G)	GCA
280	F/S	23:1	13:1	3:1	1:0
		TT(C/T):TCC	TTC:TCC	TTC:TCC	TTC
284	R/H	20:4	12:2	4:0	1:0
		CGC:CAC	CGC:CAC	CGC	CGC
285	S/F/P	22:2:0	14:0:0	3:0:1	1:0:0
		TCT:TTT	TCT	TCT:CCT	TCT
287	S/F	23:1	13:1	4:0	1:0
		TC(T/C):TTT	TCT:TT	TC(T/C)	TCT
288	E/D	23:1	14:0	4:0	1:0
		GA(A/G):GAC	GA(A/G)	GAA	GAA
302	A/V	23:1	14:0	4:0	1:0
		GCT:GTT	GCT	GCT	GCT
304 ^c	E/G	0:24	0:14	0:4	0:1
		GAA:G <u>G</u> A	GAA:G <u>G</u> A	GAA:G <u>G</u> A	GAA:G <u>G</u> A

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

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

^bThe 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.

^cThe 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.

Figures

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 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.