Helicobacter pylori hrgA, A Novel Discriminatory Biomarker for Duodenal Ulcer Patients

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ABSTRACT

Background: *Helicobacter pylori* is a major human gastric for various gastro duodenal diseases. A number of putative virulence factors such as *dupA*, *homB*, *tnpA* have been described. To date, none were found to be significantly associated with specific *H. pylori*-related diseases (e.g. gastric cancer and duodenal ulcer).
***Methods*:** the primary aim of this study was to test the *H. pylori hrgA* genotype isolated from 253 Iranian symptomatic patients to investigate possible association with clinical outcomes. The positive culture results were confirmed by *glmM* (genetic control for *H. pylori*) PCR assay.
Results: The results showed *hrgA* gene was detected in 44/253 strains (17.3%). Prevalence of the *hrgA* gene was relatively high in strains isolated from duodenal ulcer patients (P=0.0063; Odd ratio: 3.54; CI 95%: 1.42-8.77).
***Conclusions*:** In contrast our findings showed that the prevalence of *hrgA* in our control group (gastritis patients) was 22.7% (*P*>0.05). Conclusively, *hrgA* gene is a good candidate as a discriminatory biomarker for patients with duodenal ulcer

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Introduction

Helicobacter pylori is the most prevalent human pathogen with over half of the world's population is colonized with this microaerophilic bacterium. It is now firmly established that *H. pylori* infection is associated with gastritis (G), duodenal ulcer (DU), gastric ulcer (GU), gastric cancer (GC), and gastric mucosa-associated lymphoid tissue lymphoma (MALT) (1). Interestingly, *H. pylorus has* been recognized as a traditional resident of the human stomach that persists for the lifetime, if untreated. Colonization in the stomach activates a multifaceted mucosal inflammatory response and causes severe gastroduodenal outcomes (2). Etiologically, disease outcome is a major result of the complex interplay between the H. pylori putative virulence and its host (2). During the relatively short period of time that we have known about H. pylori, there have been many different virulence factors (i.g., cagA, babA2, iceA, oipA, homBand dupA) thought to be associated with certain gastroduodenal disorders (3-7). However, currently, none have been consistently found to be specific *H. pylori*-related digestive disease. More recently, the restriction endonuclease-replacing gene A (*hrgA*), which is mainly responsible for inducing severe gastroduodenal diseases (with an unknown biologic mechanism), has been suggested as a novel virulence factor (8, 9). Additionally, isolation of many *H. pylori* strains possessing *hrgA* from gastric cancer patients supported this hypothesis, suggesting its involvement in bacterial pathogenesis. Therefore, *hrgA* may be considered as a new *H. pylori* virulence factor (10-12). Additionally, there is a report indicating the importance of *H. pylori hrgA* among gastric cancer patients from Japan. However, no reports describing the association between the *hrgA* gene and *H. pylori*-related diseases in different countries are available yet. Strikingly, it has been declared that the *H. pylori hrgA* is the first disease-specific virulence factor for an *H. pylori*-related digestive disease (i.e., duodenal ulcer and gastric cancer) (9). In fact, *hrgA* may be a clinical biomarker to detect or predict *H. pylori*-linked digestive diseases. Broadly defined, there is no biomarker to detect specific *H. pylori* linked with certain gastroduodenal disease. Consequently, a novel biomarker seems urgently necessary for screening the *H. pylori* related disease, at least in high risk populations. The Mazandaran is the most densely-populated province in the north of Iran, with one of the highest rates of *H. pylori*-induced disease in the world (3, 13). Frequently reported digestive disorders from this state, in addition to the high prevalence of the infection (5), prompted us to testify the association between the *hrgA* genotype and *H. pylori*-induced disease severity in a large collection of isolates from Northern Iran (14). Undoubtedly, significant association of *hrgA* with each digestive disease can be a promising for finding a reliable and simple discriminatory biomarker.

Material and method

Participants

Two hundred-fifty three patients [72 with gastritis (G), 67 with duodenal ulcer (DU), 49 with gastric cancer (GC) and 65 with gastric ulcer (GU)] were enrolled in this study. Antral biopsy specimens were taken for histopathological analysis (15), culture, and genotyping from each patient. Exclusion criteria were: (1) age less than 15 years old and (2) consumption of antibiotics or anti-secretory drugs during the last 4 months. Patients in this investigation were from the same ethnic origins, socioeconomic level and had similar cultural habits. In this study, diagnosis of disease was based on endoscopic examination and pathology laboratory findings. Prior to endoscopy, all subjects or their parents signed an informed consent form approved by the ethics committee at Tarbiat Modares University, Tehran, Iran.

H. pylori isolation

Three samples from each patient were taken from the gastric antrum, one for genotyping and detection of *H. pylori* by polymerase chain reaction (PCR), one for pathology (if necessary), and the last one for histopathological analyses. In this survey, antral biopsy specimens were placed in sterile thioglycolate broth (Merck, Germany), and then immediately shipped in a container at 4°C. For bacterial culture, 100 µl of suspension from homogenized biopsy was added onto the surface of Colombia agar plates (MAST, UK), containing 10% defibrinated sheep blood, 8% FCS (Fetal Calf Serum) (Gibco, USA) and *H. pylori* selective supplement (Oxoid-SR147E). Agar plates were incubated for 7-11 days in 10% CO2 provided by anaerobic candle jar (4). H. pylori biochemical identification was performed by routine tests including urease, catalase and oxidase. The bacterial culture for H. pylori was considered to be positive by observing small, typical and translucent colonies, in conjunction with Gram-negative staining (16).

DNA extraction and PCR

Using single colonies for genotyping from plates was a strategy to avoid H. pylori mix infection. Genomic DNA was extracted from a single colony per patient using an Accu Prep genomic DNA extraction kit (Bioneer, South Korea) according to the manufacturer's directions. PCR was performed according to a previously reported method with minor modifications (17). An aliquot (0.25 µl) of Taq DNA polymerase and deoxynucleoside triphosphates (Bioneer, South Korea) was mixed with 2 µl of a genomic DNA sample of each strain and primer. The primers used were hrgA and glmM (18) as genetically confirmation of H. pylori and hrgA among these strains (Table 1). PCR was performed using a thermal cycler (Roche, Germany) under the following conditions with minor modification: an initial denaturation for 10 min at 93°C; 40 cycles of 1 min at 93°C, 1 min at 52°C, and 1 min at 72°C; and a final extension at 72°C for 10 min.

Table 1. Oligonucleotide primers selected to detect the *glmM* and *hrgA* genes of *H. pylori*

Primer	equence	size (bp)	PCR condition	Ref
hrgA	TCTCGTGAAAGAGAATTTCC TAAGTGTGGGTATATCAATC	594	94_°C for 30 sec, 52_°C for 1 min, 72_°C for 1 min (40 cycles)	18
glmM	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294	93° C, 1 min; 55 °C, 1 min; 72 °C, 1 min (35 cycles)	9

The amplified products were subsequently visualized by electrophoresis in 2 % agarose gel stained with ethidium bromide.

Statistical analysis

We used SPSS version 18.0 software for statistical analysis. P values < 0.05 were regarded as statistically significant. In current study, the logistic regression values are exhibited for associations considered to be statistically significant according to univariate analysis.

Result

A total of 253 strains isolated from *H. pylori*-positive Iranian patients (139 males, 114 females; mean age, 39 years; age range, 16 to 69 years), which were obtained from antral biopsy specimens; confirmed by culture. The culture results were confirmed by *glmM* (genetic control for *H. pylori*) PCR assay. Indeed, our PCR analyses showed that all 253 strains contained *glmM* (294 bp). In addition, *H. pylori* were evaluated for the relation of age, gender, and ethnic group with the severity of disease as shown in table 2. No significant associations were observed between the presence of the *hrgA* gene and histological findings, age and gender (P>0.05). In total, the *hrgA* gene was detected in 44/253 strains (17.3%). The frequency of the *hrgA* gene was higher in strains isolated from DU patients (P=0.0063) (Table 3). No statistical significance was observed between *H. pylori* infection and any of the examined disease groups (Table 2). A univariate analysis showed that the prevalence of *hrgA* in DU patients is 36.3%, which is significantly higher than that in our control group (gastritis patients) (22.7%; *P*>0.05) (Table 3). In contrast, the presence of the *hrgA* gene was relatively low in both strains isolated from GU and gastritis G patients (15.9% and 22.7%; respectively).

Discussion

After the ground breaking discovery of *H. pylori* in 1983, the bacterium became an interesting focus of scientific attention worldwide. Clinically, identification of virulent *H. pylori* strains is not highly necessary yet, but knowing the specific virulent strains which are linked with more severe digestive diseases can help clinicians to improve their strategies to better manage *H. pylori* infection. Undeniably, there is continuing interest in determining *H. pylori* specific virulence factors that might predict the risk for symptomatic digestive outcomes (18, 19). Yet, numerous studies found certain determinants, which are relatively associated with gastroduodenal diseases. In 2002, *hrgA* was primary reported as potential factor involved in *H. pylori* restriction endonuclease system (20). We investigated whether an association between *H. pylori hrgA* exists among symptomatic patients with different digestive disorders including GC, DU and GU. Analysing the Iranian population, we detected the *H. pylori hrgA* gene among the 44/253 (17.3%) patients. In the past, studies have determined that the presence of *hrgA is* highly associated with occurrence of gastric cancer (9, 21). Lu *et al*. detected the *hrgA* gene was present in 29% of gastric cancers and 29% of ulcers with no significant association (P>0.05)(22). In contrast, we observed a positive correlation between *hrgA* and occurrence of duodenal ulcer (P=0.0063; Odd ratio= 3.54; 95% CI: 1.42-8.77). Inversely, in comparison with Ando *et al.*, our finding is informative that the presence of *hrgA* can be a surrogate biomarker for duodenal ulcer patients (Table 3). *H. pylori* possesses a genome of approximately 1,600 genes, hence the possibility of involvement of other novel virulence factors is not unlikely. To the best of our knowledge, this is the first study to report such significant association between *H. pylori hrgA* gene and duodenal ulcer patients.

Table 2. Demographic data for patients

Disease type	Sample size	Male (%)	Pathology findings	Age range detailed data for each disease groups				
				<30	31-40	41-50	51-60	>60
G	72	38 (52.7)	Mild (n=8)	3	3	2	0	0
			Moderate (n=59)	19	31	16	4	2
			Atrophic (n=5)	1	3	1	0	0
GU	65	37 (56.9)	Mild (n=15)	3	10	1	1	0
			Moderate (n=39)	11	21	4	2	0
			Atrophic (n=11)	4	3	3	1	0
DU	67	35 (52.2)	Mild (n=13)	3	8	1	1	0
			Moderate (n=37)	5	12	12	7	1
			Atrophic (n=17)	3	8	2	3	1
GC	49	29 (59.1)	Mild (n=9)	2	2	3	1	1
			Moderate (n=28)	7	13	5	2	1
			Atrophic (n=12)	3	5	3	1	0

Yet, the *hrgA* gene was associated with an increased risk of gastric cancer in Japan, but current results from Iran are controversial, and there may be variability depending partially on the geographic regions studied. When using the gastritis group as controls for gene distribution (Table 3), we found an increased prevalence of the *hrgA* gene in the DU group. From this point of view, the presence of the *hrgA* gene is thought to be less prevalent in strains from gastric cancer patients (P=0.0136; Odds Ratio: 2.06; CI 95%: 0.79-5.36). Our work has a few limitations; sample size might be a potential limiting factor in present study, as contradictory results were previously described (22-25). In this particular case, it may be due to the low number of patients included in their study. Observed discrepancy between frequency of *hrgA* in DU and GC can be considered as a new predictive tool among those mentioned digestive diseases.

In conclusion, our current data did not support the current hypothesis that the virulence factors of *H. pylori*, *hrgA* is strongly associated with gastric cancer, at least among the Iranian population.

Conclusion

Overall, the development of severe *H. pylori* disease is clearly determined by the virulence of the colonizing strain. In fact, studies on the genetic diversity of *H. pylori* genes are important for predicting the clinical outcomes of the infection (26). As such, finding a novel biomarkers seem necessary to detect *H. pylori* related to certain severe diseases such as gastric cancer and duodenal ulcer. It is clear that *H. pylori has* extreme genetic variability, and this heterogeneity is contributing in the ability of bacterium to survive longer in human gastric mucosa.

Table 3. Univariate analysis demonstrating the association of *hrgA* genotype and DU, GU and GC

Disease group		Univariate analysis		
	Positive (%)	*P* value	Odds ratio	95% CI
G*	10(22.7)	(Control group)		
GC**	11(25)	0.136	2.06	0.79-5.36
DU***	16(36.6)	0.0063	3.54	1.42-8.77
GU****	7(15.9)	0.76	1.17	0.41-3.34

* G: Gastritis (accepted as control group) **GC: Gastric cancer
***DU: Duodenal ulcer
****GU: Gastric ulcer
Statistical difference was considered as $P<0.05$.

Similarly, new candidate virulence factors, such as *homB, dupA and tnpA* have also been suggested to be associated with certain digestive diseases, but contradictory data still exists. Moreover, it has been speculated that genotyping of *hrgA* might offer a predictive tool for identification of patients with gastric cancer (20, 22). As a general rule, an acceptable specific disease prediction tool should be useful in different regions. Traditionally, *H. pylori* infection is thought to contribute to both duodenal ulcer and gastric cancer which are at conflicting ends of the disease spectrum. Collectively, we were unable to confirm the reports of association of *hrgA* presence and gastric cancer. We speculate that the study with larger sample size investigating *H. pylori hrgA* can elucidate to answer left questions about feasibility of this gene as predictive tool for detecting patients with gastric cancer harbouring *H. pylori*. Altogether, larger disease groups from different regions especially western countries will be required to investigate the current hypotheses. Designing valid biomarkers, which can be used in different genetic pools, is a promising area in *H. pylori* research. Duodenal ulcer patients are one of main groups who are suffering from *H. pylori* infection. Certainly, having an accurate biomarker to detect *H. pylori* in those patients would be useful in clinical settings. Due to our findings, *hrgA* can be suggested as a candidate biomarker in DU patients. Overall, the recent advances in sequencing technology have enabled massive sequence comparisons (27). Hopefully, in the near future, whole genome analysis will enable researchers to identify additional virulence factors for *H. pylori*. Taking all findings into account, much work remains to identify which biomarkers, or panels of biomarkers, will provide the best prognostic and predictive information about those digestive disease.

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Conflict of interest

None declared conflicts of interest.

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