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Research paper

Carrying a 112 bp-segment in *Helicobacter pylori dupA* may associate with increased risk of duodenal ulcer

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ABSTRACT

The discovery of *Helicobacter pylori* in 1983 challenged researchers around the world to identify this pathogen's major virulence factors. The main rationale for this kind of research was to identify a biomarker associated with specific diseases following *H. pylori* colonization. Among different investigated virulence factors, *duodenal ulcer promoting gene A* (*dupA*) has been found to be associated with duodenal ulcer (DU), but its effect was different in various geographical regions. To determine the prevalence of *dupA*, we applied both classic primer pairs and our newly developed primers producing a highly conserved segment in PCR method. In our survey, 143 (47%) *H. pylori* isolates were obtained from 304*H. pylori*-colonized individuals [age range of 19–92; 113 (37%) males with the mean age of 50 and 191 (63%) females with the mean age of 49]. The presence of the *dupA* gene was investigated by using the different specific primers. The prevalence of the 112 bp segment isolated from *H. pylori* strains recovered from DU, GU and atrophy groups were significantly higher (81%, p value = .002, 64%, p = .065, 68% and p = .047 38%, respectively) than our control group, where the prevalence of the 112 bp segment was only 38%. Interestingly, a significant relationship was observed between the occurrence of DU and the presence of the 112 bp segment [p = .002; OR: 6.98; (95% CI: 1.94–25.00)]. Taken as a whole, we believe the 112 bp region of *H. pylori dupA* may serve as the first detected biomarker for the early detection of DU in patients admitted to hospitals.

1. Introduction

Helicobacter pylori (*H. pylori*) is a pathogenic spiral microorganism that colonizes the human stomach (Warren and Marshall, 1983). The gastric colonization of this bacterium was found to be highly associated with a wide range of ailments including acute superficial gastritis, peptic ulcer diseases (PUD), gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (Sgouras et al., 2015; Suerbaum and Michetti, 2002). The prevalence of the bacterium in developed countries ranges 20–30%; however, this rate is about 50–95% in many developing countries with lower socioeconomic levels (Okuda et al., 2015; Leja et al., 2016). Regardless of huge progress made so far, such as the development of prophylactic vaccines and optimal treatments, some issues such as the virulence factors of this bacterium are left poorly understood (Sutton and Boag, 2018). It has been frequently stated that many bacteria with specific virulence factors induce specific pathologic outcomes in human carriers (Kao et al., 2016; Yamaoka and Graham, 2014; Graham and Yamaoka, 2000; Höcker and Hohenberger, 2003). In fact, defining an accurate relationship between *H. pylori* infection and specific virulence determinants and related gastroduodenal disease is a complicated query (Höcker and Hohenberger, 2003).

1.1. New dupA-based biomarker for H. pylori

The aim of discovering a biomarker associated with ulcers or gastric cancer has attracted the attention of researchers over the last decade. However, the current body of knowledge is showing that we lack a definite conclusion. With this regard, Lu et al. investigated the novel sequences of *jhp0917-jhp0918* as a possible virulence determinant for this pathogen (Lu et al., 2005). They termed this genetic area located in an accessory region of the *H. pylori* genome as *duodenal promoting gene* A (*dupA*). This putative *H. pylori* virulence factor has been found to be linked with duodenal ulcer (DU) occurrence in certain human populations (Lu et al., 2005), but this association was not confirmed in later studies from other regions of the world (Jung et al., 2012; Nguyen et al., 2010; Hussein, 2010; Schmidt et al., 2009; Zhang et al., 2008; Gomes

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Table 1
PCR primer sets used for identification of the different *dupA* gene-specific fragments in our study

Primer	Sequence 5 ′to 3′	Product length	Annealing Tm	Annealing Tm	References
glmM (FWR)	AAG CTT TTA GGG GTG TTA GG	294 bp	58	45 s	(Douraghi et al., 2008)
glmM (REV)	AAG CTT ACT TTC TAA CAC TA				
jhp0918 (FWR)	CCTA TAT CGC TAA CGC GCG CTC	276 bp	62	45 s	(Schmidt et al., 2009)
jhp0918 (REV)	AAG CTG AAG CGT TTG TAA CG				
jhp0917 (FWR)	TGGTTTCTACTGACAGAGCGC	307 bp	64	45 s	(Schmidt et al., 2009)
jhp0917 (REV)	AACACGCTGACAGGACAATCTCCC				
112 bp (FWR)	CAT GGC GTT TCA AAA AAT ATC TCA A	112 bp	60	45 s	Current study
112 bp (REV)	TTC ATC AGT ATC TTT TGT GGG GTA				

F: forward, R: reverse, bp: base pair, s: second.

et al., 2008; Douraghi et al., 2008). Based on available data in indexing databases, *H. pylori dupA* positivity was reported to be 7.1%–89.5% around the world (Hussein, 2010).

Since it remains unclear why certain populations develop DU whereas others do not, we investigated the *H. pylori dupA* gene isolated from the biopsies of DU patients. To our knowledge, the 112 bp segment within *dupA* is not universally held by all *H. pylori* lines infecting humans. Our main goal was to compare the prevalence of three segments located in *dupA*, including two classic sequences called 307 bp and 276 bp and our new proposed region (called 112 bp segment) among *H. pylori*-carrying subjects with different gastric ailments.

2. Materials and methods

2.1. Sampling procedures

Gastric biopsy specimens were obtained from 304 Iranian patients (191 females and 113 males) aged 19–91 years (mean 49 years) undergoing routine upper gastrointestinal endoscopy for the evaluation of symptoms related to digestive tract at Imam Khomeini, Mehrad, Labaphi-Nejad and Seda-Sima's Medical Centers, in Tehran, Iran. Patients with a history of gastric surgery, active gastrointestinal bleeding, consumption of immunosuppressive drugs, antibiotics, proton pump inhibitors, and bismuth drugs in the previous two weeks as well as pregnant women were excluded (our main exclusion criteria). Two antral biopsies (for bacterial culture and pathology) were obtained from patients admitted to the hospitals because of their gastroduodenal pain or problems. The first biopsy sample was collected in a small tube containing Thioglycollate-broth medium and was shipped to our microbiology laboratory within three hours for culture. The other biopsy sample was fixed in 10% formalin for histological examination and then was stained with Giemsa stain to ascertain the presence of *H. pylori* and the degree of inflammation and gastritis (Dixon et al., 1996; Laine et al., 1997). Based on endoscopic and histological examinations, patients were diagnosed with chronic gastritis, atrophy, peptic ulcer and DU. This study was approved by the ethic committee of Tarbiat Modares University, Tehran, Iran (IR.TMU.REC.1395.442) and all patients gave their written informed consent to participate in the survey.

2.2. Bacterial culture and phenotypic characterization

For bacterial culture in our laboratory, biopsy specimens were homogenized and then inoculated in Brucella Agar supplemented with 5% defibrinated sheep's blood, 7% fetal calf serum and selective antibiotics (3 μg vancomycin per ml, 50 μg nalidixic acid per ml and 5 μg trimethoprim per ml). Nearly all known *H. pylori* strains are naturally resistant to these antibiotics. Culture plates were incubated for 4 to 10 days at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) (Mégraud and Lehours, 2007). Typical colonies were selected for the identification of *H. pylori* with phenotypic characteristics (translucent colonies varying in size from barely detectable with the naked eyes to approximately 3 mm); colonies consisted of gram-negative, curved rods and confirmed positive for urease, catalase and oxidase tests (Bermejo et al., 2002). Rapid urease test results were observed within 3 h after endoscopic surgery (Mégraud and Lehours, 2007; Raissy et al., 2016). Color change from yellow to pink, orange or red (based on alkalization) was considered as positive (Mégraud and Lehours, 2007). Bacterial colonies were harvested and stored at −80 °C in tubes containing brain heart-infusion broth medium (BHI), 18% glycerol, 10% fetal calf serum and 5% DMSO.

2.3. DNA extraction

Bacterial colonies from sub-cultured plates were harvested and genomic DNA was extracted using YTA DNA extraction commercial kit, according to the manufacturer's instructions (Yekta-TajhizAzma, Tehran, Iran). In brief, bacterial colonies were harvested from cultured plates and washed with PBS (pH 8.0) by centrifugation at 14000 rpm for 2 min (G-force: 15338.96). The pelleted cells were suspended in 200 μl TG1 buffer (pH 8) and 20 μl proteinase K (10 mg ml $^{-1}$). The mixture was incubated at 60 °C for 2–3 h, followed by the addition of 200 μl TG2, then incubation at 70 °C for 10 min, and finally the addition of 200 μl ethanol (96–100%) and vortexed; then the mixture was centrifuged at 8000 rpm for 1 min. Then several washing steps with W_1 and buffer were performed and finally DNA was collected with 50–200 μl eluted buffer. In the final step, the quality and quantity of DNA were checked by loading 5–10 μl extracted DNA to 1% agarose gel electrophoresis. DNA was stored at −20 °C for further analysis.

2.4. Polymerase chain reaction (PCR)

A 294 bp segment of the *glmM* gene was amplified to confirm the identity of the 152 isolated strains as *H. pylori*. This was followed by amplification of three segments of the *dupA* gene (See Table 1). Amplification of the target genes was carried out in a total volume of 16 μl, containing 5 μL distilled water (DW), 0.5 μL of each primer (10 pmol μL^{-1}) (synthesized by Takapozist company, Tehran, Iran), 8 μL master mix 2x (Ampliqon, Copenhagen, Denmark; containing 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.5 U Taq polymerase) and 2 μL DNA extract. PCR was performed in a T100 Thermal Cycler (BioRad, Berkeley, California) as shown in Table 1. In this study, 2% agarose gel (Sina-clon, Tehran, Iran) stained with ethidium bromide and transilluminated with UV illuminator (Biometra, Germany) was used to visualize the PCR products. The sizes of amplicons were checked to confirm their identity. All PCR reactions to evaluate the parts of *dupA* included a negative control (26695, ATCC 700392) and a positive control (J99, ATCC 700824). PCRs targeted *dupA* regions including two sequences of *dupA* studied by primers introduced in 2005 (Lu et al.) and one sequence targeting by a new designed primer based on the alignment of all *dupA* gene sequences which was present in NCBI DNA database until the end of 2017. Finally, we sequenced all PCR products for the 112 bp, 276 bp, and 307 bp segments when they were amplified successfully, confirming the validity of our reactions. (See Fig. 1.)

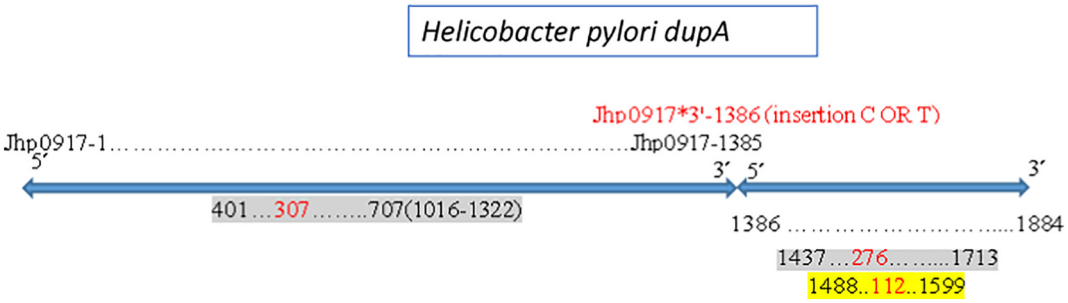

Fig. 1. Schematic locations of various amplified regions in our study.

2.5. Statistical analysis

A chi-square test was used to calculate the association of our three segments with the various gastric symptoms we studied. The effect of the presence of each *dupA* segment and the report of particular gastroduodenal diseases was presented as an odds ratio (OR) with a 95% confidence interval (CI). A p value of < 0.05 was considered statistically significant in our analysis.

3. Results

Out of our 304 patients, 113 were males and 191 were females, with an age range of 19 to 91 years (mean, 49 years). Altogether, 143 (47%) patients were determined *H. pylori* positive using the rapid urease test and further histology examination. Then patients were assessed for *H. pylori* infection through attempts to culture the bacteria and amplifying the *glmM* gene (Table 2). With this regard, we had 99*H. pylori* samples which were confirmed by urease, culture and *glmM* PCR methods. These 99 isolates were entered in this study for analyzing *dupA* segments. The histological findings had no statistical association with the sex, disease types and the *H. pylori dupA* segments (data not shown). The prevalence of the 112 bp segment isolated from *H. pylori* strains recovered from DU, GU and atrophy groups were significantly higher (81%, p value = .002, 64%, p = .065, 68% and p = .047, respectively) than our control group, where the prevalence of the 112 bp segment was only 38%. The prevalence of the isolates possessing *dupA* (according to the presence of 307 bp and 276 bp representative segments) was very high in DU and considerable in GU and atrophy samples compared to gastritis patients (52%, 41%, 37% and 30%, respectively) (Table 3), but none of them were statistically significant (Table 2). Current findings showed a significant association between the presence of *dupA* 112 bp segment and the development of DU disease (Tables 3, 4). However, we did not find significant differences in the presence of 276 bp and 307 bp sequences (for *jhp0918* and *jhp0918*, respectively) among our clinical isolates in different disease groups (p value > .05) (Table 4). The statistical analysis on various *dupA* segments was also performed to find significant associations between disease groups (Table 4). An odds ratio (OR) analysis showed that patients with the 112 bp segment were 6.98 times more likely to have duodenal ulcers than those patients lacking the segment (95% CI, 1.94–25.00). Also, our findings are showing that subjects carrying the 112 bp segment in the *H. pylori dupA* are at higher risk to have GU and atrophy (2.87 and 3.55 times), respectively. However, ORs were not statistically significant among the different disease groups and 276 bp and 307 bp segments in this survey (See Table 4).

Table 2
The characteristics of the study population.

Characteristic	Gastritis	Peptic ulcer	Atrophy	Duodenal ulcer	Normal
Age(years) in means	45.29	47.5	59	50.23	44.94
Sex M/F	30/42	14/27	17/23	11/15	23/16
H. pylori positive	37(51%)	22(54%)	19(47%)	21(80%)	13(33%)
Total number	72	41	40	26	39

Table 3
The prevalence of the *glmM*, 276 bp, 307 bp and 112 bp sequences of *dupA*.

Fragment	Gastritis	Duodenal ulcer	Gastric ulcer	Atrophy	Total
294 bp(glmM gene)	37/72(51%)	21/26(81%)	22/41(54%)	19/40(47%)	55%
276 bp	11/37(30%)	11/21(52%)	9/22(41%)	7/19(37%)	38%
307 bp	12/37(32%)	12/21(57%)	9/22(41%)	7/19(37%)	40%
112 bp	14/37(38%)	17/21(81%)	14/22(64%)	13/19(68%)	59%

Table 4
Association of different *dupA* fragments with disease types in our patients included in the study.
Sutton and Boag (2018).

dupA fragment	Disease groups	P value
		OR (95% CI)
112 bp	Duodenal ulcer	0.002 6.98 (1.94–25.00)
	Gastric ulcer	0.065 2.87 (0.96–8.58)
	Atrophy	0.047 3.55 (1.10–11.50)
276 bp	Duodenal ulcer	0.10 2.6 (0.85–7.88)
	Gastric ulcer	0.40 1.63 (0.54–4.93)
	Atrophy	0.76 1.37 (0.42–4.44)
307 bp	Duodenal ulcer	0.09 2.77 (0.92–8.38)
	Gastric ulcer	0.57 1.44 (0.48–4.30)
	Atrophy	0.77 1.21 (0.38–3.87)

4. Discussion

In recent years, various studies have been conducted to identify genetic biomarkers as indicators of pathogenicity used by *H. pylori* during colonization in the human gastric microniche (Graham and Yamaoka, 2000; Höcker and Hohenberger, 2003; Lu et al., 2005; Shiota and Yamaoka, 2014; Yu et al., 2019; Begg and Tavassoli, 2017). At first glance, it was assumed that these determinants identified thus far played a key role in determining the virulence capacity of *H. pylori* strains (Sugimoto et al., 2012). However, markers that have seemed promising based on one human population have not held up as indicative of disease in other populations. The main biologic rationale

behind many failed attempts is various recognized genetic instabilities observed in the *H. pylori* genome (Machado et al., 2010). The identification of an *H. pylori* virulence biomarker that could predict duodenal ulcer risk would be important for screening subjects for further medication and other treatments in clinical practice. Thus, we may pay attention to the use of clinical biomarkers for screening the *H. pylori* colonized subjects to track the high risk populations. The high rates of gastroduodenal disorders, including duodenal ulcer in the Iranian population, creates a demand for a novel biomarker (Douraghi et al., 2008). Many studies in different populations have investigated some candidates for possible significance (Yamaoka and Graham, 2014; Graham and Yamaoka, 2000; Douraghi et al., 2008). Among the various disease types associated with *H. pylori*, some studies from various geographical regions have demonstrated that the prevalence of *dupA* in DU patients was higher than that in GU patients; Zhang et al. (2008) in a Chinese population, Arachchi et al. (2007) in an Indian population, Hussein et al. (2008) in an Iraqi group and Douraghi et al., 2008 in Iranian patients. However, contradictory results have been reported by Schmidt et al. (2009) for an Australian group and a group in Malaysian and Singaporean (Chinese, Indians and Malaysian) populations which showed no association between *dupA* and GC and DU, as well as consistent results from South America, Brazil, (Gomes et al., 2008), East Asia (Douraghi et al., 2008), Japan and United States (Sung woo Jung, 2011) (Jung et al., 2012; Nguyen et al., 2010; Schmidt et al., 2009; Zhang et al., 2008; Gomes et al., 2008; Douraghi et al., 2008; Arachchi et al., 2007; Hussein et al., 2008). Interestingly, in other studies by Argent et al. in Belgium, South Africa, China and United States (the prevalence of *dupA* was 85% in South Africa and 32–44% in other three countries), there was not only such an association between *dupA* and duodenal ulcer disease, but also as a precursor to gastric cancer (Argent et al., 2007).

Many genes had become potential candidates for this purpose but the data about *H. pylori dupA* is lacking (Hussein, 2010; Douraghi et al., 2008). Thus, analysis of the non-universal 112 bp segment of *dupA* appears promising to serve as a novel virulence marker at least in our population. These reported mutations and other type of genetic instabilities are suspected to be responsible for the induction of different bacterial virulence types associated with specific gastroduodenal diseases in human subjects carrying those *H. pylori* strains.

In our study, the prevalence of two common sequences of *dupA* (representatives of *jhp0917* and *jhp0918* ORF as *dupA)*, which were primarily assumed to be associated with DU, was compared to the 112 bp segment among 99 patients with different disease groups.

Current evidence indicated that there was a relationship between DU disease and the existence of 112 bp segment [*p* value = .002, OR: 6.98 (95% CI = 1.94–25.00)], but not with two classic sequences (276 bp and 307 bp segments) previously reported (Lu et al., 2005). To the best of our knowledge, our study is the first to report such an association between this conserved region of the *dupA* gene and increased statistical risk of developing DU. Previous studies have shown that there were wide differences in the reported prevalence of *H. pylori dupA* recovered from different groups and geographical regions which could be due to many mutations occurring in these two classic regions, especially at the beginning of their primer bind.

Interestingly, it will be important to study how the 112 bp segment correlates with duodenal ulcer also in different ethnic groups and geographical areas. Sequence analysis confirmed our claim about the presence conserved regions in the whole *dupA* gene. It seems that the reported variations in classic primers were neglected by Lu et al. and it was the main reason of different significant and non-significant associations disclosed in investigations conducted in the last decade by various groups. In this study we sequenced the PCR products for those amplified regions and we found all expected sequences. In a meta-analysis, the prevalence of *dupA* (according to the presence of 276 bp and 307 bp sequences) in *H. pylori* strains was reported to be 47.9% (in DU patients 67%) (Hussein, 2010). Although there are many regions believed to be related to the occurrence of DU patients colonized by *H. pylori*, none of these regions have been well studied in different geographical areas. In our survey, *dupA* (according to the presence of 276 bp and 307 bp sequences) was found to be 52% *H. pylori dupA* positive among DU patients. Meantime, we had just 38% isolates positive for *dupA* gene using 112 bp segment introduced in this study. Thus, our analysis of a 112 bp segment within *dupA* appears promising to serve as a novel biomarker, at least in our population. We report that the presence of 112 bp sequence was significantly associated with DU [*p* value = .002, OR: 6.98 (95% CI = 1.94–25.00)], thus we suggest this segment as predictive of duodenal ulcer. To date, there is no report on the prevalence of *dupA* using this region of *H. pylori* genome. Our findings show that the rates for the mentioned segments were higher in DU patients than other groups. We suggest further sophisticated molecular studies on sequences next to 112 bp segment to determine the relationship between the new defined *dupA*-positive *H. pylori* and clinical manifestation. However, current study had several limitations and strengths. This survey was designed as cross-sectional, thus we were unable to measure causal relationship between DU patients and 112 bp *dupA*-positive *H. pylori*.

The most important advantages of our study are as follows:

i) We surveyed a large, cross-sectional, clinical sample of Iranian dyspeptic patients; therefore, our conclusion can be a starting point for other groups from different regions.
ii) There was no possible biological bias in recruiting clinical samples and performing PCR experiments. We have also sequenced our positive samples for 112 bp segment with confirmatory results. Nonetheless, current novel results can be used for further analysis in designing and developing biomarkers for patients with gastroduodenal diseases, particularly DU.

Conducting a study with a larger sample size in different ethnic groups and inclusion of high risk population offers more reliable results and realistic hopes for better management of duodenal ulcer. It is crucial to be clear on what exactly we want from a biomarker for duodenal ulcer patients. A desirable biomarker would identify the most vulnerable individuals to develop duodenal ulcer. Of course, the application of such a biomarker in the diagnosis and management of severe gastroduodenal disorders like DU is well-known and ideal for clinicians and microbiologists. In conclusion, our findings can serve as the first step in developing a universal biomarker for the early detection of DU in patients admitted to the hospitals and private clinics.

Conflicts of interest

The authors declare no conflict of interest.

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