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The Significant Association of the *dupA* and *cagA* genes of *Helicobacter pylori* with Peptic Ulcer

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ABSTRACT

Background: *Helicobacter pylori* play a significant etiological role in various digestive diseases. Peptic ulcer is caused by *H. pylori*, which destroys the duodenum mucus and is often observed in the individuals consuming tobacco, spicy and heavy meals, alcohol, coffee, and tranquilizers. Several studies have indicated that duodenal ulcer promoting genes *dupA* and *cagA* are involved in *H. pylori* etiology. The present study aimed to evaluate the correlation between these genes and peptic ulcer.

Methods: In this study, 500 stomach biopsy samples were assessed based on the rapid urease test and polymerase chain reaction (PCR) for *H. pylori* infection, followed by histological and microscopic examinations.

Results: The *dupA* and *cagA* genes were subjected to PCR. Although *dupA* showed no significant correlation with peptic ulcer, the *cagA* genotype had a significant association with peptic ulcer ($P < 0.05$). Similar to the *dupA* gene, blood group was not observed to be correlated with *H. pylori* infection.

Conclusion: According to the results, there are significant correlations between tobacco use ($P < 0.05$), tranquilizer use ($P < 0.05$), and meteorism ($P < 0.05$) with peptic ulcer. In addition, the expression of the *cagA* and *dupA* genes was investigated in patients with non-ulcer dyspepsia and peptic ulcer.

1. Introduction

Helicobacter pylori are gram-negative, spiral bacillus with the approximate length of 3.5 microns and width of 0.5 microns [1, 2]. *H. pylori* settle in the human gastrointestinal tract and are of great etiological importance in the incidence of digestive diseases, such as gastritis, stomach ulcer, gastric cancer, and peptic ulcer (PU)[1, 3, 4]. PU is a mucosal damage with an outward hole in the stomach, duodenum, and gullet, which is more prevalent in men based on the average age of onset compared to women [5].

The rate of PU breakout has been estimated at 5-10% during lifetime [6]. It often causes a single ulcer and rare multiple ulcers in the stomach, as well as the Zollinger-Ellison syndrome in some cases [7]. Use of tobacco, tranquilizers, and alcohol, meteorism, age of more than 50 years, long-term work, family history, and use of nonsteroidal anti-inflammatory drug (NSAIDs) aspirin are among the most important risk factors for PU [7].

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The *Jhp0917* and *Jhp0918* genes of *H. pylori* are significant features in its virulence. There are three classes of virulence genes in *H. pylori*, including lineage-specific genes, phase-variable genes, and genes with variable structures/genotypes. The cag pathogenic island of *H. pylori* contains genes encoding the gears of type IV secretion, and this lineage is mostly associated with the incidence of cancer [3, 7]. The *Jhp0947* and *dupA* genes are classified in this category [8]. Another cluster contains six genes with a transitive phase, including *oipA*, *sabA*, *sabB*, *babB*, *babC*, and *hopz*, which encrypt a protein that is located at the outer bacterial sheath [8-10]. The additional groups of pathogenic genes are the genes with variable structures/genotypes (e.g., *vacA*). Various genotypes of these genes consist of the mosaic compounds of the signal and middle regions, which are associated with various digestive diseases [1, 3].

In 2005, Lu et al. (2005) [11], investigated the *dupA*, *JhPO917*, and *Jhpo918* genes in various types of *H. pylori*, introducing them as the appropriate markers of PU in Japan, Korea, and Colombia. Evidently, *dupA* is more prevalent in patients with PU, while it is less common in patients with gastric cancer [11, 12]. Several studies have confirmed the association of this gene with the incidence of digestive diseases [12- 15]. The host blood type is considered to be another plausible risk factor for PU. Furthermore, several reports have indicated that individuals with the O type blood are at a higher risk of PU compared to others [5, 16]. The possible risk factors for PU include drinking coffee [17], alcohol consumption [18], smoking [19], stress [20], spice consumption [21], and poor nutrition [22]. The present study aimed to examine the correlation between the *Jhp0917*, *Jhp0918*, and *cagA* genes with the ABO blood type, as well as some environmental factors, with the incidence of PU.

2. Materials and Methods

2.1. Sample Preparation

In this study, 500 stomach biopsy samples were collected from patients with digestive diseases in spring 2014. Questionnaire containing data on the age, gender, occupation status, and history of drug consumption were completed by physicians, and the clinical parameters were determined by experts. Since the inhibitory effects of antibiotics, NSAIDs, H^+ pump inhibitors, and bismuth compounds may lead to false negative results on RUT, the patients using one of these agents within the past 14 days before the biopsy were excluded from the study.

2.2. Rapid Urease Test (RUT)

Biopsy samples were prepared from the antrum of the stomach. *H. pylori* release urease, which was used to hydrolyze urea in the urea cristiyance solution (Dako Corporation SL029) by incubation at the temperature of 37 º C for one hour. In this process, the color of ammonia and its compounds in the solution changed from yellow to red.

2.3. Histological Tests

The morphological features and aggregation of *H. pylori* in the tissue of each sample were meticulously examined after the treatment of each sample by formaldehyde solution for fixation and Hematoxylin-Eosin staining (Dako Corporation SL029) in accordance with the instructions of the manufacturer. By performing histological tests, the chronic inflammation in *H. pylori* infection could be specified by counting the inflammatory cells via conventional microscopy.

2.4. DNA Extraction and PCR

Genomic DNA was isolated from the tissue samples using a DNA extraction kit (Fermentas) in accordance with the instructions of the manufacturer. The *Jhp0917* and *Jhp0918* genes were amplified via PCR amplification with sequence-specific primers (PCR-SSP) (Table 1). The primers of *Jhp0917* and *Jhp0918* were designed using the Primer3 online software.

Initially, the PCR mixture was pre-heated at the temperature of 94 ºC for six minutes before 35 cycles of amplification, which consisted of incubation at the temperatures of 94 ºC for 60 seconds, 60 ºC for 60 seconds, 72 ºC for 60 seconds, and 72 ºC for seven minutes. In addition, electrophoresis was performed using 2% agarose gel and visualized under ultraviolet light after ethidium bromide staining.

To avoid false negative results in the PCR due to the variations in the primer annealing sites, a dot blot was performed on all the samples. Per each single dot, 100 nanograms of the sample DNA was mixed with 100 microliters of denaturing buffer (0.8 M NaOH, 1.5 M NaCl) and spotted on to a Hybond N^+ membrane (Amersham Biosciences, Little Chalfont, UK) using a 96-well Bio-Dot apparatus (Bio-Rad, Ivry-sur-Seine, France). The DNA extracted from the reference strain 26695, J99, SS1 (GenBank accession number AE001439) and peripheral blood cells were used as positive and negative controls, respectively.

Table 1: Primer sequences for PCR amplification

Gene	Primer sequences	TM
16S rDNA	Forward primer 5´- TAGCCACTGGAAACGGTGAT -3´ Reverse primer 5´- GTGTACTAGGCCCGGGAACGTATTC -3´	62 ºC
cagA	Forward primer 5´- GATCTCGGTGGGTCTTTC -3´ Reverse primer 5´- TCTTTTACGGCATTGTTCA -3´	57 ºC
Jhp0917	Forward primer 5´- TGGTTTCTACTGACAGAGCGC -3´ Reverse primer 5´- AACACGCTGACAGGACAATCTCCC -3´	57 ºC
Jhp0918	Forward primer 5´- CCTATATCGCTAACGCGCGCTC -3´ Reverse primer 5´- AAGCTGAAGCGTTTGTAACG -3´	63 ºC

2.5. RNA Extraction and cDNA Synthesis

RNA was extracted from the tissue samples using Sinaclon RNA extraction kit (CAT: RN7713C) in accordance with the instructions of the manufacturer and stored at the temperature of -80 ˚C. The RNA obtained from the individual samples was reverse-transcribed into cDNA using a reverse transcription system. In this process, nine microliters of RNA was mixed with one microliter of random hexamer (100 µM), incubated at the temperature of 65 ˚C for five minutes, and chilled on ice. Afterwards, 10 microliters of RevertAid M-Mul-V reverse transcriptase (200 U/µl) was added to provide the total volume of 20 microliters. The reaction mixture was incubated at the temperature of 25 ˚C for five minutes, followed by incubation at the temperature of 60 ˚C for 50 minutes and 70 ˚C for 10 minutes, and placed on ice. Afterwards, cDNA was either used directly for real-time PCR or stored at the temperature of -20 ˚C.

2.6. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

RT-PCR amplification was performed using specific primers (20 pM) (Tabel 1) in the total volume of 25 microliters. The RT-PCR reactions included incubation at the temperature of 95 °C for five minutes, followed by 40 cycles at the temperatures of 95 °C for 50 seconds, 62 °C for 20 seconds, 72 °C for 30 seconds, and a final extension step at the temperature of 72 °C for 10 minutes.

2.7. Statistical Analysis

Data analysis was performed in ANOVA, Chi-Square SPSS version 16.0 considering a two-sided *P*-value of less than 0.05 as the significance level.

3. Results and Discussion

In the present study, 500 stomach biopsy samples were collected from patients with digestive diseases, and 237 patients were positive for PU. Among these patients, 204 cases were positive based on the RUT and 16S rDNA PCR test, and the remaining 300 patients were negative for PU and manifested other diseases (e.g., non-ulcer dyspepsia [NUD]). Patients with NUD were classified into two categories of gastritis (80%) and duodenitis (20%) (Figure 1), and their data are presented in Table 2.

Table 2: The patients' information with PUD and NUD

Gene	Symptom	Number of patients	Personal habits
PUD	Stomach pain	256	Alcohol***
	Meteorism	244	Coffee**
	Puke	230	Tea**
	Distaste	110	Tranquilizers*
NUD	Stomach pain	123	Alcohol
	Meteorism*	75	Coffee
	Puke	15	Tea
	Distaste	48	Tranquilizers*

*Use the drugs for 2 years, 3 times a day (mostly Aspirin)
**More than one cup a day
*** Consumption of more than 75 g of ethanol a week

Among 204 patients who were positive for PU based on the RUT and 16 srDNA, 109 cases were male, and 95 cases were female with the average age of 43 and 41 years, respectively. According to the findings, 76% of the patients who were positive for H. pylori infection had PU, and the others had NUD (24%). The patients with PU were classified into three categories of stomach ulcer (61%), duodenal ulcer (38%), and gastric cancer (1%).

All the H. pylori positive samples (n = 204) were positive for 16S rDNA. In addition, the presence of the *dupA* and *cagA* genes was investigated in all the isolates using the PCR, and 67 cases were *dupA*-positive, while 161 cases were *cagA*-positive. Also, 31 samples were positive for both *dupA* and *cagA*, 38 of which had PU, and 13 cases had NUD. RT-PCR was used to evaluate gene expression. The PCR amplification of *cagA*, *Jhp0917*, and *Jhp0918* genes was estimated at 506, 307, and 276 bp fragments, respectively (Figures 1& 2).

In the current research, the expression of the *cagA* and dupA genes was investigated in the patients with PU and NUD. Evaluation of 16SrRNA expression was also used as control. In the patients with PU, *cagA* had a high expression, while in the patients with NUD, *dupA* had expression levels. In addition, the expression of *cagA* and *dupA* was observed to be higher in the patients with PU compared to those with NUD.

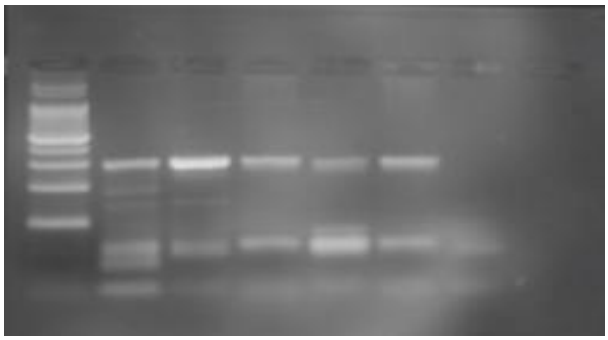
According to the results of the present study, *H. pylori* infection is a significant predisposing factor for duodenal ulcer disease. *H. pylori* infection was detected in 76% of the patients with PU and 6% of gastritis, non-PUD patients (odds ratio [OR]: 49.6, 95% confidence interval: 27.8-88.2; $P < 0.05$).

Several studies have reported significant correlations between PU and ABO blood group [4, 22, 24], which is inconsistent with the results of the similar studies conducted in the Netherlands [6], Brasilia[18], and Taiwan[25]. In the present study, no significant correlation was observed between PU and ABO blood group. Furthermore, our findings indicated that tobacco consumption is a potent risk factor for duodenal ulcer disease, and the rate of smoking habits was higher in the patients with PUD (85%) compared to those with non-PUD gastritis (14%; OR: 21.3, 95% CI: 12.7-35.7; $P < 0.05$). This finding is inconsistent with the studies performed in the United States and Japan [26], in which no such correlation was reported with smoking habits and functional dyspepsia. This is in congruence with the study by Rosenstock et al. (2003) [19].

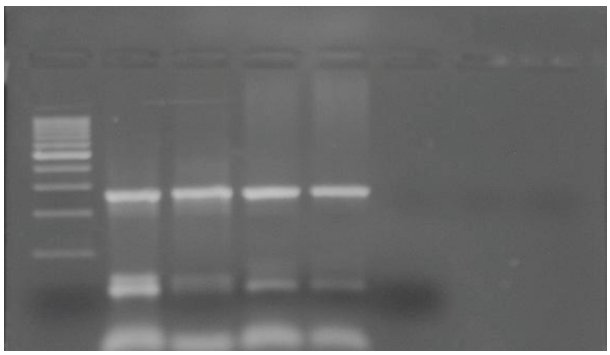
In line with the previous studies in this regard [19, 20, 28, 29], our findings showed no significant association between alcohol consumption and duodenal ulcer. On the same note, caffeine in coffee instigates the secretion of acid, thereby reducing the compression of the bottom sphincter [19]. However, the results obtained by Rosenstock et al. (2003) [19] and Shiota et al. (2010) have shown no association between coffee consumption and duodenal ulcer [30].

Excessive consumption of tranquilizers (e.g., aspirin) causes damage to the coating of the stomach and leads to bleeding [28- 30]. Consistent with the study by Ji et al. (2006) [27], our findings indicated that NSAIDs cause PU and the associated complications in a vast majority, which is inconsistent with the study by Fletcher et al. (2010) (OR: 61.6, 95% CI: 34.9-109; $P < 0.05$) [29].

According to the research conducted in Japan, Korea, and Colombia, *dupA* is associated with the increased risk of duodenal ulcer and reduced risk of gastric atrophy and cancer [30].



a) M C+ 01 02 03 04 C-



b) M C+ 01 02 03 C-

Figure1: Amplification of for *dupA* genes: a) Amplification of jhp0917 gene Of *H. pylori* (307 bp). M: 100 bp ladder size marker, C-positive: 26695, J99, SS, 01 to 04: positive samples and C-negative control. b) Amplification of *Jhp0918* gene of *H. pylori* (276 bp). M: 100 bp Ladder, size marker, C-positive: 26695, J99, SS, 01 to 04: positive samples and C-negative control.

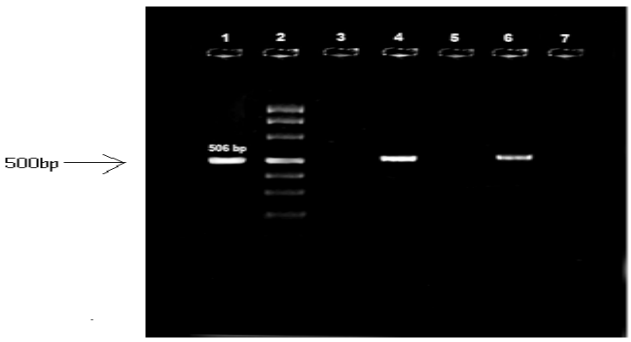


Figure 2: Electrophoresis of *cag A* gene PCR products on 2% agaros gel: Lane 2; Marker, line3; negative control, 1, 4 and 6; *cag A* positive (506 bp). M: 100 bp Ladder, size marker

4. Conclusion

However, this association has not been reported in Western countries [18], in the present study, no correlation was observed between *dupA* and duodenal ulcer. The frequency of *dupA* was 27% in the *H. pylori*-infected patients with PU and 44% of the *H. pylori*-infected patients without PU (OR: 0.47, 95%CI: 0.17-1.29). In addition, the expression of *dupA* and *cagA* was observed to be higher in the patients with PU compared to those with NUD. Finally, a significant correlation was denoted between meteorism and PU in the patients ($P < 0.05$).

Authors' Contributions

Study was designed by H.R.Z.G., Sh.T. and E.M., sampling were done by E.M., P.B. and V.B. and finally data were statically analyzed by Sh.T.

Conflict of Interest

The authors report no conflicts of interest.

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