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Original Research

The relationship between the simultaneity present of *cag*A and *hop*QI genes in *Helicobacter pylori* and the risk of gastric cancer

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Abstract: *Helicobacter pylori* is a bacterium that causes infections in the gastrointestinal tract. This type of bacterium is very common and contagious at the same time. *H. pylori* enters the mouth and continues its course along the gastrointestinal tract. *H. pylori* infection induces an inflammatory response that leads to the activity of neutrophils, lymphocytes, plasma cells, and macrophages. In addition to the bacterial role in gastric mucosa, the host's inflammatory response may also play a role in disease outcome. In inflammation, the risk of carcinogenesis increases due to DNA damage increased proliferation and the creation of an environment rich in cytokines and growth factors. Genetic methods and diagnosis of *H. pylori* genes are used to identify healthy and healthy gastric cancer patients infected with *H. pylori*. In relation to the genes associated with *H. pylori* pathogenesis, the presence of genes such as *cag*A, *hop*QI, *hop*QII and so on is used, and PCR of a part of these genes amplified fragments of different lengths. One of the less-studied cases is the association of two or more pathogenic genes simultaneously with *H. pylori*. In this research, the frequency of disease and healthy individuals who are infected with *H. pylori* and have two genotypes *cag*A and *hop*QI at the same time, was examined. In order to diagnose *H. pylori*-infected individuals in healthy and gastric cancer patients, after PCR of glmM gene, PCR product electrophoresis on agarose gel was used. For this purpose, gastric tissue biopsy was used in patients and saliva was used in healthy individuals. For this purpose, 100 gastric biopsy samples were collected from patients with gastric cancer and 100 saliva samples from healthy individuals. According to the data, there is a significant relationship between the simultaneous presence of two genes *cag*A and *hop*QI and gastric cancer. In patients, 45.3% showed both genotypes, while in healthy individuals only 10.5% have this genotype and other healthy but infected with *H. pylori* (90.8%) do not have this genotype. To be. No report was observed on the simultaneous study of *cag*A and *hop*QI genes. No report was observed regarding the simultaneous study of *cag*A and *hop*QI genes.

Key words: *cag*A; Genotyping; Gastric cancer; Helicobacter pylori; *hop*QI.

Introduction

Gastric cancer is a condition in which cancer cells have formed on the lining of the stomach. This type of cancer can spread from the stomach to other parts of the abdomen, especially the liver, lungs, bones, abdominal wall, and lymph nodes. The disease has no clinical signs in the early stages (1-5).

Symptoms of gastric cancer include (6-11): Pain in the upper abdomen and a feeling of heaviness in the upper abdomen, difficulty swallowing (dysphagia), especially in tumors of the primary gastric region, feeling full stomach with pain before eating, nausea, anorexia, weight loss and jaundice, the presence of blood in the stool and black stools, bloody vomiting, especially when the tumor is in the lower part of the stomach, the discovery of a palpable abdominal mass, which in most cases indicates long-term growth and local spread of the tumor (the most common site for the spread of gastric tumors is the liver) and anemia, iron deficiency in men, and occult blood in the stool can be possible symptoms.

To prevent the disease, the following can be mentioned (12-18): 1) Lifestyle modification and avoidance of the mentioned risk factors in the diet are effective in the incidence of gastric cancer. 2) Consistent consumption of fresh fruits and vegetables in multiple meals throughout the day. 3) The effect of taking one 80 mg aspirin tablet daily with a doctor's prescription, for men over 40 and women over 50. Important global research has shown that taking this pill is very effective not only in preventing stomach cancer but also in preventing other cancers, and in the United States, as a country where the incidence of gastric cancer is greatly reduced, continuous use of this pill is recommended. People who tolerate a daily dose of 80 mg aspirin tablets, use it from the age of 50, permanently and for at least 10 years. 4) Periodic endoscopy and eradication of *Helicobacter pylori* in people whose first-degree relatives have died of gastric cancer.

The causes of stomach cancer are: severe hypertrophy (overgrowth of cells) in the gastric folds, gastric ulcers and some polyps (polyps that tend to be malignant), atrophic gastritis (a disease in which the stomach wall is degenerated), past gastric surgery (removal of the end of the stomach), decreased gastric acidity, prolonged consumption of high concentrations of nitrates in smoked, dry and salty foods, use salty and salty diets, smoking, hookah, opium, high consumption of red meat, especially grilled meat, high consumption of high-fat cream, consumption of hot drinks, including hot tea, which is very common in the northern regions of the country, family history and a microbial infection called

H. pylori. The role of this microbe in stomach cancer is very important, but getting this germ is not enough to cause stomach cancer alone, and there must be other risk factors; however, it can be said that 90% of people with gastric cancer are infected with *H. pylori* (19-22).

H. pylori is a gram-negative spiral curved bacterium that was identified by Marshall and Warren in 1983 by microscopic examination of the gastric epithelium of patients with chronic active gastritis (23). *H. pylori* is the most common cause of chronic bacterial infection and the main cause of the peptic ulcer, chronic active gastritis - is known in the world that it is accumulated in the stomach of more than 50% of the world's population (24). The World Health Organization (WHO) classifies *H. pylori* as a carcinogen from group 1. The method of transmission of this infection is person to person through fecal-oral or oral-oral contact (25).

Epidemiologically, *H. pylori* is one of the most common infectious diseases in the world. This bacterium causes 95% of chronic gastritis, 70-80% of gastroduodenal and also the development of gastric cancer. Chronic H.pylori infection may be associated with chronic gastritis, peptic ulcer disease, adenocarcinoma gastritis. Approximately 20% of people infected with *H. pylori* develop gastroduodenal disorders during their lifetime. Infection with this bacterium can play a significant role in gastric cancer. The annual incidence of *H. pylori* infection is 4 to 15% in developing countries but 0.5% in industrialized countries (26).

The doctors prescribe special antibiotics to kill *H. pylori*. Most common wound healing medications are also prescribed. Most of the time, killing this bacterium will prevent the wound from coming back. Sometimes not all bacteria are killed or come back. If this happens, another wound will form (27).

In general, *H. pylori* is associated with about 70% of all gastric cancers. Seroepidemiological studies in different parts of Iran have shown nearly 90% infection with this bacterium in people over 35 years of age. *H. pylori* in the stomach leads to general gastritis and decreased production of gastric acid. Acute inflammation can directly damage the peripheral cells that are responsible for secreting acid. In addition, inflammatory responses lead to the secretion of IL-1β (a pro-inflammatory cytokine with the property of inhibiting acid secretion). Acute inflammation is progressively replaced by chronic inflammation and acid secretion resumes. Gastritis is usually associated with acid secretion. Excess acid secretion reduces inflammation of the corpus and induces inflammation of the antrum, anthrax stimulates acid secretion, so antral gastritis is associated with high acid secretion and corpus gastritis is associated with decreased acid secretion. Only a small percentage of people infected with *H. pylori* develop gastric carcinoma. Scientists believe that the mismatch between the number of infected people and the number of people with cancer is due to environmental factors, genetic predisposition to inflammation in the host, and bacterial species diversity (13,20,28).

H. pylori regulate apoptosis of gastric epithelial cells through several mechanisms. Following the attachment of bacteria to the surface of epithelial cells, signaling through the *cag* secretory system activates an unknown factor called nuclear factor B (NF-B).) are activated. NF-B is transported into the nucleus to activate the transcription of pre-apoptotic genes. *H. pylori* can also induce apoptosis by stimulating the expression of Fas. *H. pylori* urease protein can induce apoptosis by binding to MHC class II tissue compatibility complex molecules (29-32).

The risk of developing gastric carcinoma is associated with a heterogeneity of *H. pylori* virulence factors, especially *cag*PAI (Pathogenesis Island). *cag*PAI is a cluster of 45 kb (or 45,000 bp) and 31 genes, containing the *cag*A terminal gene, which typically acts as a marker for the whole island. Following binding to *H. pylori* to host epithelial cells, the *Cag*A protein enters the cytoplasm of host cells through the secretory *cag*PAI coding secretory system (T4SS) (28,33,34).

In host cells, *Cag*A interacts with several proteins and through signaling leads to increased expression of proinflammatory cytokines, skeletal actin markets, changes in cell polarity, and increased invasive power. The presence of T4SS, a *Cag*A protein, is highly associated with gastrointestinal ulcer inflammation and an increased risk of gastric carcinoma (14,28,30).

The less studied *hop*Q gene encodes the extracellular protein *Hop*Q, which can regulate the binding of some strains of *H. pylori* to gastric epithelial cells, so it may play an important role in early colonization and long-term resistance of bacteria to the gastric stroma. The *hop*Q gene exists in two forms, type I and type II. It should be noted that the prevalence of I&II *Hop*Q type alleles in Iran has not been studied so far. Recently, a degree of covariance between *hop*Q and *cag*PAI has been shown, and it has been reported that the *hop*QI allele is commonly present in *cag*PAI-containing strains of *Helicobacter*, although some strains have both alleles (35,36).

The *hop*Q gene is one of the newly identified genes involved in *Cag*PAI protein translocation. Then, this experiment aimed to determine the relationship between *cag*A+ strains of *hop*QI genotypes and its relationship with gastric cancer.

Materials and Methods

Statistical population

In this study, in order to investigate the *hop*Q type genotypes of Helicobacter pylori and its relationship with *cag*A virulence factor, 100 gastric biopsy specimens were collected from patients with gastric cancer and 100 saliva samples from healthy individuals, from Affiliated Yueqing Hospital, Wenzhou Medical University, Wenzhou, China.

All samples were collected endoscopically by a physician with the informed consent of the subjects. For all participants in the questionnaire, information including age, sex, history of previous illness or family members, eating habits and lifestyle were considered. The rest of the work was done in a molecular research laboratory.

DNA extraction, PCR reaction and primers used

After DNA extraction from biopsy specimens (from patients with gastric cancer) and saliva from healthy individuals (without any history or symptoms of gastric upset), the presence of *H. pylori* was investigated by polymerase chain reaction (PCR) and the use of a pair

Table 1. Pairs of specific primers for detection of glmM, *cag*A and *hop*QI genes in gastric cancer and healthy individuals.

Gene	Accession No.	Primer sequence	Amplified fragment length
glmM	900169	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' 5'-AAGCTTACTTTCTAACACTAACGC -3'	294 bp
***cag*A**	889201	5'-TTGACCAACAACCACAAACCGAAG -3' 5'-CTTCCCTTAATTGCGAGATTCC -3'	183 bp
***hopQ*1**	7010294	5'- ACGAACGCGCAAAAACTTTA-3' 5'-TTGCCATTCTCATCGGTGTA-3'	187 bp

of specific primers for one conserved area in the *glm*M gene of this bacterium. Genotyping of *cag*A and *hop*Q type I genes was performed by PCR using a specific primer pair. Pairs of specific primers are shown in Table 1.

DNA extraction from healthy individuals infected with *H. pylori*

In order to find healthy people infected with *H. pylori* and extract DNA from them, gastric juice, feces or saliva can be used to extract DNA. Due to the low risk for volunteers, hygiene and simplicity of sampling, the DNA extraction method was used from saliva. For this purpose, after identifying the target individuals, cleaning the mouth and holding 5 ml of 3% sucrose solution for one minute in the mouths of volunteers, DNA was extracted from saliva according to the following instructions.

At first TNE, lysis and AE Buffers were prepared. Then the following operation was performed. Centrifuge sample tubes at 3000 rpm for 10 minutes. Removal of supernatant. Adding 1 ml of TNE buffer and 20 µl of lysozyme and keep at 37 °C for one hour. Centrifuging the samples at 2000 rpm for 5 minutes. Removing the supernatant and the sample vortex for 5 seconds. Adding 1.3 ml of lysis buffer and 10 µl proteinase k and sample vortex for 5 seconds. Incubating the samples at 55 °C overnight. Transferring 1.4 ml of the mixture to the 2 ml tube, adding 500 µl of AE buffer and vortexing of sample for 5 seconds. Centrifuging the samples in 17000 g for 10 minutes. Transfer 900 µl of supernatant to 2 ml tube, and adding 540 µl of cold isopropanol and turn the samples upside down about 20 times. Centrifuging the samples at 14000 rpm for 5 minutes and remove the supernatant. Adding 70% ethanol and centrifuge the samples at 8000 rpm for 5 minutes and remove the supernatant. Drying the resulting precipitate and adding 50 µl of double distilled water (DDW).

DNA extraction from biopsy specimens of patients with gastric cancer infected with *H. pylori*

At first lysis Buffer was prepared. Then the following operation was performed.

Placing the gastric biopsy tissue sample in a 2 ml tube and add 1 ml of lysis buffer, 100 µl of 10% SDS and 20 µl of proteinase K (40 mg / ml). After vortexing, incubation was performed for one hour at 58 °C. After cooling the samples on ice, 350 µl chloroform and 350 µl NaCl (5 M) were added to them and mixed. The samples were centrifuged at 6000 rpm for 10 min, in which 3 phases were formed. Separating the supernatant and transfer to a new 2 ml microtube. Adding 1 ml of cold absolute ethanol to the samples and centrifuge for 15 min at 12000 rpm and 4 °C. The supernatant was discarded and 1 ml of 70% ethanol was added to the precipitate and centrifuged for 15 min at 12000 rpm and 4 °C. Discarding the supernatant and keep the tubes at room temperature until the pellets are dry. 50 ml of distilled water was added to each sample twice.

Evaluation of DNA quality was performed using 0.8% agarose gel (horizontal electrophoresis).

Polymerase chain reaction (PCR)

In all polymerase chain reactions, the reaction materials were mixed with the concentrations listed in Table 2 and the final volume of the mixture reached 25 µl. It was then placed in a thermocycler to perform a PCR reaction.

The PCR reaction program was defined for the thermocycler for each locus according to Table 3. In DNA samples obtained from biopsy 30 cycles and in the case of DNA obtained from saliva 40 cycles of repetition from steps 2 to 4 were defined.

Data analysis

To compare the different genotypes of the genes in

Table 2. Consumables materials in all polymerase chain reactions (PCR).

Materials	Amount
$MgCl_2$	1.5 mM
dNTP	200 mM
PCR Buffer	50 mM
F-Primer	50 pmol
R-Primer	50 pmol
Template DNA	2 l
Taq DNA Polymerase	1 unit
Sterilized water	16.25µl
Total volume	25µ l

Table 3. Thermal cycle of PCR reaction and names of primers for the studied genes.

Primer name	Thermal cycle of PCR reaction				
	1	2	3	4	5
glmM	94ºC (5 min)	94ºC (30 sec)	58ºC (30 sec)	72ºC (30 sec)	72ºC (5 min)
*cag*A	94ºC (5 min)	94ºC (30 sec)	55ºC (30 sec)	72ºC (30 sec)	72ºC (5 min)
*hopQ*1	94ºC (5 min)	94ºC (30 sec)	54ºC (30 sec)	72ºC (30 sec)	72ºC (5 min)

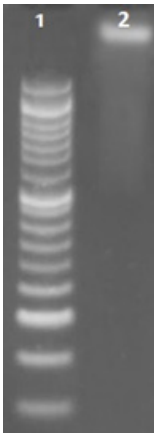

Figure 1. Agarose gel electrophoresis for genomic DNA. Wells: 1: Size marker and 2: Genomic DNA.

different groups, χ^2 test was used and to match the background variables, χ^2 and t-test were used. SPSS V26 software was also used for statistical analysis.

Results and discussion

Detection of genes used to identify and pathogenicity of *H. pylori*

In order to identify healthy gastric cancer patients infected with *H. pylori*, genetic methods and detection of genes related to *H. pylori* were used. In this study, after extracting genomic DNA, bacterial *glm*M gene was used to identify infected individuals (Figure 1). The PCR reaction resulting from primers designed for this gene in this study produced a 294 bp fragment (Figure 2).

In relation to the genes associated with *H. pylori* pathogenesis, the presence of *cag*A and *hop*QI genes was used, and PCR of these genes was amplified into fragments of 183 and 187 bp, respectively (Figure 2).

Identify people infected with *H. pylori*

In order to diagnose *H. pylori*-infected individuals in healthy and gastric cancer patients, after PCR of glmM gene, PCR product electrophoresis on an agarose gel and observation of 294 bp fragment was used (Figure 4-2). For this purpose, gastric tissue biopsy was used in patients and saliva was used in healthy individuals (Figure 3).

Figure 3 shows the agarose gel electrophoresis for the glmM gene to detect *H. pylori*-infected individuals in gastric cancer patients and healthy individuals. Infected samples were used for further analysis and identification of genes associated with pathogenicity.

Relationship between co-presence of *cag*A and *hop*QI genes in *H. pylori* and risk of gastric cancer

In this section, the simultaneous presence of *cag*A and *hop*QI genes in *H. pylori*, the frequencies of these two genes are shown in Table 4.

As shown in Table 4 and Figure 4, there is a significant relationship between the simultaneous presence of *cag*A and *hop*QI genes and gastric cancer. In patients' 45.3% show both genotypes, while in healthy individuals only 10.5% have this genotype and the rest of the healthy but infected with *H. pylori* (89.5%) do not have this genotype.

Previous studies have examined the effect of *cag*A

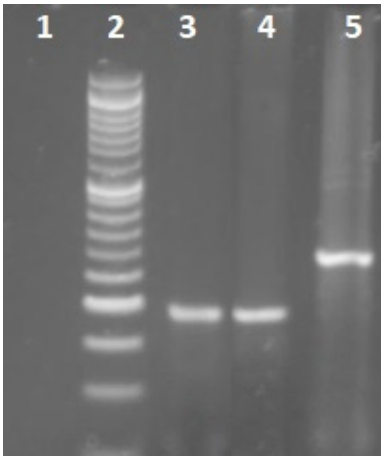

Figure 2. Agarose gel electrophoresis for genes involved in the identification and pathogenicity of *H. pylori*. Wells: 1: Negative control, 2: Size marker, 3: *hop*QI gene PCR product, 4: *cag*A gene PCR product and 5: PCR product of *glm*M gene.

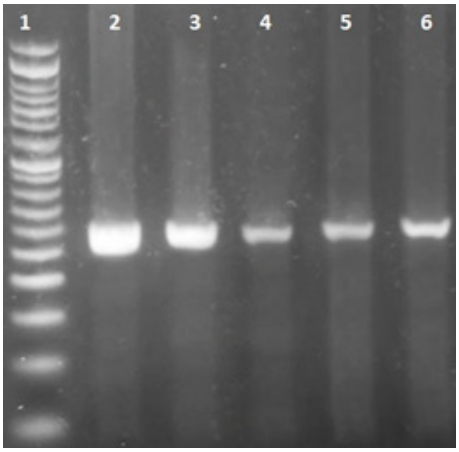

Figure 3. Agarose gel electrophoresis for the glmM gene to detect *H. pylori*-infected individuals in healthy and gastric cancer patients. Wells: 1: Size marker, 2 and 3: Samples of patients infected with *H. pylori*, 4-6: Samples of healthy individuals infected with *H. pylori*.

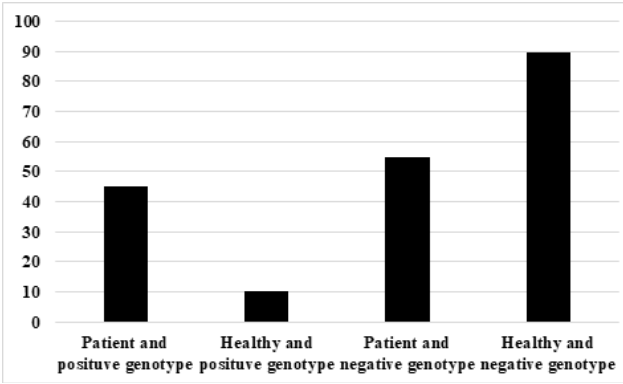

Figure 4. Frequency chart of *H. pylori* strains with *cag*A and *hop*QI genes in patients with gastric cancer and healthy individuals infected with *H. pylori.*

Table 4. Association between the presence of *H. pylori* and the presence of *cag*A and *hop*QI genes in gastric cancer patients and healthy individuals.

Case	*cag* A^+ , *hopQI*$^+$(%)	Other modes
Patient	45.3	54.7
Healthy	10.5	89.5

p-value =0.000

(28,30), *hop*QI (36) and *hop*QII (35) genes. However, in this study, the simultaneous effect of two genes (*cag*A and *hop*QI) was studied. For further study, it is necessary to do more studies on more genes as well as to study a wide range of gene networks (37-42). The prevalence of H. pylori infection in developing countries is 4 to 15% and this is a serious warning for more attention and prevention (43-50).

A less researched case is that two or more pathogenic genes are associated with *H. pylori* at the same time. In this section, the frequency of diseased and healthy individuals infected with *H. pylori* and both genotypes *cag*A and *hop*QI are studied. According to the data, there is a significant relationship between two genes *cag*A and *hop*QI and gastric cancer.

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