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Risk assessment of gastric cancer in the presence of *Helicobacter pylori cag*A and *hop*QII genes

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ABSTRACT

Helicobacter pylori bacterium is one of the most common bacterial infections globally and is the leading cause of indigestion, gastric and duodenal ulcers, and gastric cancer. This bacterium can escape the antibacterial effects of stomach acid by adapting to the inner layers of the stomach. It combines with the natural sugars in the gastric mucosa. The compound is so effective that it makes bacterium resistant. For genes related to the pathogenesis of *H. pylori*, using the existence of genes such as *cag*A, *hop*QI, and *hop*QII, PCR is performed on some of these genes to amplify fragments of different lengths. One of the less-studied cases is that two or more pathogenic genes are simultaneously associated with *H. pylori*. This study examined the frequency of diseases and healthy individuals infected with *H. pylori* and *cag*A and *hop*QII genotypes. To diagnose *H. pylori* infection in healthy and stomach cancer patients, the PCR products are electrophoresed on the agarose gel after glmM gene amplification by PCR. To this aim, stomach tissue biopsies were used for patients, and saliva was used for healthy individuals. For this purpose, 150 gastric biopsy samples from stomach cancer patients and 150 saliva samples from healthy people were collected. Data showed a significant relationship between the coexistence of two genes, *cag*A and *hop*QII, and stomach cancer. 34.2% of patients and 10.1% of healthy individuals showed two genotypes, while other healthy people (89.9%) infected with *H. pylori* did not have this genotype. Therefore, the simultaneous presence of these two bacterial genes in human societies can be an essential biomarker for the diagnosis and prognosis of gastric cancer.

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Introduction

Helicobacter pylori bacterium is the most common microorganism that infects humans in the world. More than half of the world's population is infected with this bacterium. This bacterium is the main cause of diseases such as stomach ulcers and stomach and intestinal diseases. This type of bacteria in European countries and North America is ten times higher than in other countries (approximately 74% of the US population) (1, 2).

H. pylori is basically a type of spiral bacteria, but it can also be spherical, habitable and pathogenic, but cannot be cultured (in vitro) and attached to the gastric mucosa (generally there are two forms. Biological and Pathogenic Sick) (3, 4).

Another critical point about *H. pylori* is the presence of a pump on the surface of these bacteria. This protein pump (K^+/H^+ ATPase) is similar to the pump naturally occurring in parietal cells. The existence of this pump in bacteria is unusual. The role of this pump is that it can keep the gradient of protons (positive ions) on both sides of the wall at a ratio of one million and transmit any positive ions that enter the bacteria. In the stomach environment, there are too many proton ions due to the presence of stomach acid. If they enter the bacteria, they will destroy it. To solve the problem, this pump is created on the surface of the bacteria, and the positive ions that enter the bacteria kill the bacteria quickly (5-7).

H. pylori bacterium is a slow-growing microaerobic gram-negative bacterium that exists in the stomach and duodenum and is associated with many gastrointestinal diseases. This bacterium is characterized by high production of urease, which is a virulence factor and can be used for diagnosis (8-10).

This bacterium uses a needle-like organ to inject *cag*A positive and *vac*A (vacuum cytotoxin) positive at the junction of two cells in the stomach wall. The

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c*ag*A is a gene that can produce cytotoxins related to the A gene. Only some of *H. pylori* strains carry the c*ag*A and *vac*A genes. These genes change the structure of stomach cells, making it easier for bacteria to attach to them. Long-term exposure to *cag*A can cause chronic tissue inflammation. There are considerable variations in the four regions of the *vac*A gene, which may play an important role in leading to different clinical outcomes in patients infected with HP infection (11-13).

The less studied *hop*Q gene encodes the extracellular protein *Hop*Q, which can regulate the binding of some *Helicobacter pylori* strains to gastric epithelial cells, so it may play an important role in early bacterial colonization and long-term resistance to the gastrointestinal tract. 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 alleles in Iran has not yet been studied. Recently, it has been shown that there is a certain degree of covariance between *hop*Q and *cag*PAI. It has been reported that *hop*QI alleles are usually present in Helicobacter strains containing *cag*PAI, although some strains have both alleles (9, 10, 14-16)

So far, there are many studies on the relationship between *H. pylori* genotype and gastric cancer. This study aimed to investigate the relationship between *cag*A and *hop*QII genes and gastric cancer.

Materials and methods

In this research, to study the *hop*QII type genotypes of *Helicobacter pylori* and its relationship with *cag*A virulence factor, 150 gastric biopsy specimens were collected from patients with gastric cancer and 150 saliva samples from healthy individuals. Agarose and polymerase chain reaction (PCR) materials were prepared from Fermentas (USA).

Participants

All samples were collected through an endoscope by the internist with the informed consent of the subjects. For all participants in the questionnaire, include information such as age, gender, past medical history or family members, eating habits and lifestyle. The rest of the research was done in the molecular research laboratory.

DNA purification and PCR reaction

After DNA purification from biopsy specimens (taken 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) (Bio-Rad, USA) and the use of a pair of specific primers for a conserved area in the *glm*M gene of this bacterium. Genotyping of *cag*A and *hop*QII genes was carried out by PCR using a specific primer pair. Pairs of specific primers are shown in Table 1.

After purifying DNA from the saliva of biopsy specimens (from patients with gastric cancer) and healthy individuals (without any history or symptoms of stomach upset), the DNA was purified by polymerase chain reaction (PCR) and a pair of conserved regions in the *glm*M gene of the bacteria-specific primers. Genotyping of *cag*A and *hop*QII genes was performed by PCR using specific primer pairs. See Table 1 for specific primer pairs.

Table 1. Specific primer pairs for the detection of glmM, *cag*A and *hop*QII genes in gastric cancer and healthy individuals; gene (A), accession No (B), primer sequence (C), amplified fragment length (D)

A	B	C	D
glmM	900169	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' 5'-AAGCTTACTTTCTAACACTAACGC -3'	294 bp
*cag*A	889201	5'-TTGACCAACAACCACAAACCGAAG -3' 5'-CTTCCCTTAATTGCGAGATTCC-3'	422 bp
*hop*QII	8208107	5'-AC*AG*CCACTCCAATC*CAG*AA-3' 5'-TTGACCAACAACCACAAACCGAAG-3'	160 bp

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

To discover healthy people infected with *H. pylori* and extract DNA from them, gastric juice, stool, or saliva can be used to extract DNA (17). The method of DNA extraction from saliva was used due to the low risk to volunteers, hygiene, and ease of sampling (18). For this purpose, after identifying the target persons, cleaning the mouth and keeping 5 ml of 3% sucrose solution in the mouth of the volunteers for one

minute, the DNA was extracted from the saliva according to the following instructions.

First, TNE, lysis and AE buffers were prepared. Then the following operation was carried out. The sample tubes were centrifuged at 3000 rpm for 10 minutes. To eliminate the supernatant, 1ml of TNE buffer and 20µl of lysozyme was added and kept at 37°C for one hour. The samples were centrifuged at 2000 rpm for 5 minutes. The supernatant was removed, and the sample was shaken for 5 seconds. 1.3 ml of lysis buffer and 10µl of proteinase k were added, and the samples were shaken for 5 seconds, and they were incubated at 55°C overnight. 1.4 ml of the mixture was transfer to the 2ml tube, 500µl Buffer AE was added and the samples were shaken for 5 seconds, and centrifuged at 17,000 g for 10 minutes. 900 µl of supernatant was transferred to a 2 ml tube and 540µl of cold isopropanol was added and the samples were inverted about 20 times. Again, the samples were centrifuged at 14,000 rpm for 5 minutes and removed the supernatant. 70% ethanol was added to samples, and they were centrifuged at 8000 rpm for 5 minutes. Finally, the supernatant was removed. The resulting precipitate was dried, and 50µl of double distilled water (DDW) was added.

DNA purification from samples of patients with gastric cancer infected with *H. pylori*

First, the lysis buffer was prepared. Then the following operations were accomplished. The gastric biopsy tissue sample was put into a 2ml tube, and 1ml lysis buffer, 100µl of 10% SDS, and 20µl proteinase K (40 mg/ml) were added. After vortexing, samples were incubated at 58°C for 1 hour. After cooling the sample on ice, 350µl chloroform and 350µl NaCl (5M) were added and mixed. The sample was centrifuged at 6000 rpm for 10 minutes to form 3 phases. the supernatant was separated and transferred to a new 2 ml microtube. 1ml of cold absolute ethanol was added to the sample and centrifuged at 2000rpm, 4°C for 15min. The supernatant was removed, 1ml of 70% ethanol was added to the precipitate, and samples were centrifuged at 12000rpm, 4°C for 15 minutes. The supernatant was removed and they were kept at room temperature until the pellet became dry. 50 ml of distilled water was added twice to each sample. 0.8% agarose gel (horizontal electrophoresis) was used to assess DNA quality.

Polymerase chain reaction (PCR)

In polymerase chain reactions, the reagents were mixed at the concentrations listed in Table 2, and the final volume of the mixture reached to 25µl. Then it was placed in a thermal cycler for PCR reaction.

Table 2. 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 µl
Taq DNA Polymerase	1 unit
Sterilized water	16.25µl
Total volume	25µ l

According to Table 3, the PCR reaction program was defined for the thermal cycler of each locus. In the DNA sample obtained from biopsy, 35 cycles were defined, and in the case of DNA obtained from saliva, 40 repeated cycles from steps 2 to 4 were defined.

Table 3. Thermal cycling of PCR reaction and primer names for research 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)
*hop*QII	94ºC (5 min)	94ºC (30 sec)	54ºC (30 sec)	72ºC (30 sec)	72ºC (5 min)

Data analysis

To compare the different genotypes of the genes in different groups, χ^2 test was applied and to match the underlying variables, χ^2 and t-test were used. SPSS V26 software was also applied for statistical analysis.

Results and discussion

Finding of genes used to recognize *H. pylori*

To identify healthy gastric cancer patients infected with *H. pylori*, genetic methods and detection of genes related to *H. pylori* were applied. In this research, after purifying genomic DNA, the bacterial *glm*M gene was used to find infected individuals (Fig. 1). The PCR reaction produced a 294bp fragment resulting from

primers designed for this gene in the current experiment (Fig. 2).

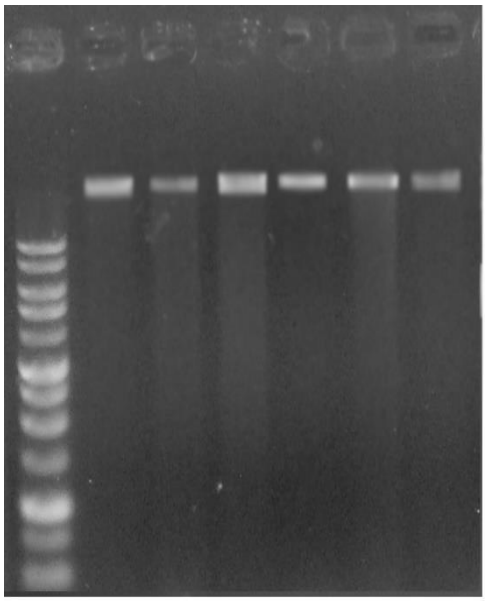

Figure 1. Extracted genomic DNA from *H. pylori*-infected samples

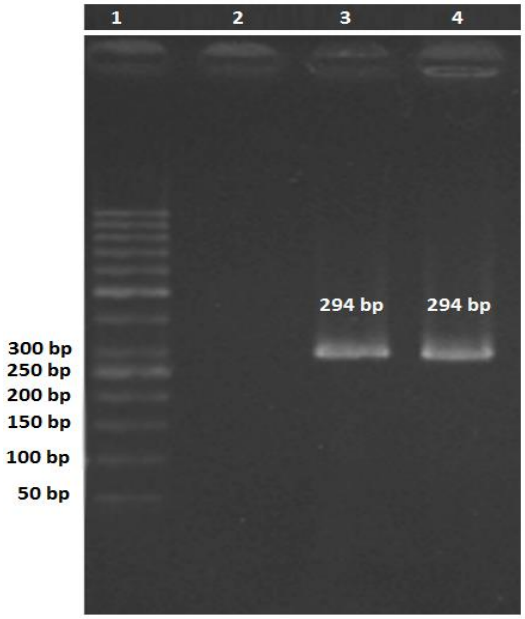

Figure 2. Detection of *H. pylori* from biopsy specimens and normal samples. Agarose gel electrophoresis of PCR products and for *glm*M gene from *H. pylori*-infected samples. Lane 1: DNA size marker, Lane 2: Negative control, Lane 3: *glm*M gene amplification in gastric cancer patients, Lane 4: PCR product for *glm*M gene in cancer-free individuals

Gene amplification for *hopQ*II and *cag*A genes

In relation to the genes associated with *H. pylori* pathogenesis, the occurrence of *cag*A and *hop*QII genes was used, and PCR of these genes was amplified into 422 bp and 160 bp, respectively.

The PCR agarose gel electrophoresis has been shown in Figure 3for *hopQ*II and *cag*A genes recognition in the *H. pylori* infectious individuals.

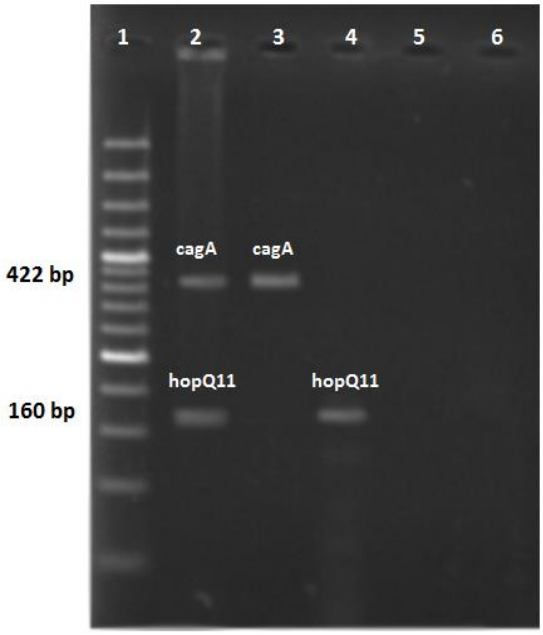

Figure 3. The PCR product agarose gel electrophoresis for *hopQ*II and *cag*A genes in the *H. pylori* infections. Lane 1: DNA size marker, Lane 2: *hopQ*II$^+$ and *cagA*$^+$, Lane 3: *cag*A$^+$, Lane 4: *hopQ*II$^+$. Lane 5: *cag*A$^-$, and Lane 6: *hopQ*II$^-$

The frequency of *hopQ*II gene in the *H. pylori* infections

The *hopQ*I and *cag*A gene frequency in *H. pylori* infections has been shown in Table 4 and Figure 4. The χ^2 analysis showed that there was a significant difference between healthy individuals and gastric cancer presence of alleles in their strains (P<0.05).

Table 4. The *hopQ*II and *cag*A gene frequency in the *H. pylori* infections

	*hopQ*II$^+$, *cag*A$^+$ (%)	*hopQ*II$^-$, *cag*A$^-$ (%)
Case	34.2	65.8
Normal	10.1	89.9

P value = 0.006

Among patients, 34.2% showed two genotypes, while only 10.1% of healthy people had this genotype, while other healthy people infected with *H. pylori* (89.9%) did not have this genotype.

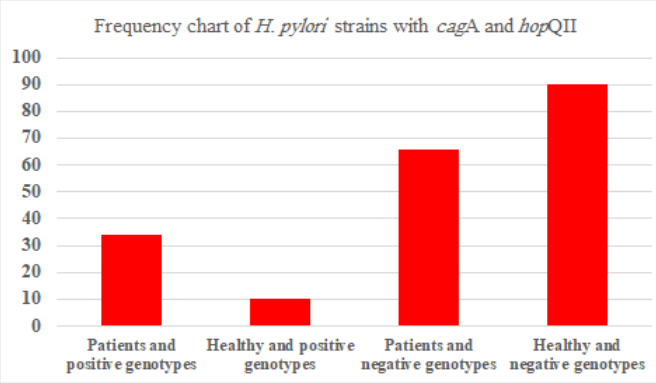

Figure 4. Frequency of *H. pylori* strains with *cag*A and *hop*QII genes in patients with gastric cancer and healthy individuals infected with *H. pylori*

Helicobacter pylori can cause diseases such as duodenal ulcers, gastric ulcers, and gastric cancer. Among the diseases caused by this bacterium, cancer is critical (19). Although this bacterium is known as a pathogen, by applying the strategy and techniques of genetic engineering and biotechnology, this pathogen can be used to treat cancer (20). For example, the factors in this bacterium that induce apoptosis can be used directly to kill cancer cells. Primary attachment to host cell surface receptors is required to cause disease (21). The interaction between the bacterium and the cell not only causes the establishment of bacteria, but also some of these targeted connections initiate a series of intracellular signaling cascade pathways that cause changes in the cell and damage the host cell and tissue (22).

Helicobacter pylori are initially attached to type IV collagen and binds to the lamina propria tissue (23). Another essential protein that bacteria can bind to is laminin. Laminin is the major protein in the basement membrane (24). After damaging the cell, Helicobacter pylori is exposed to the basement membrane and attaches to the laminin using its surface receptors such as LPS and 25 and 67 kDa proteins. This connection results in better placement of the bacterium in the damaged areas and wounds. After the bacterium attaches and settles on the cell surface, other pathogens of the bacterium begin. The bacterial secretory system plays a significant role in the production of pathogenesis (25). The secretory system of four Helicobacter pylori species injects the essential CagA protein directly into the cell during its infection. This molecule is phosphorylated after entering the cell, increasing cell proliferation and destroying strong connections between adjacent cells (23, 26).

About 4% of the Helicobacter pylori genome has significantly more coding genes of Outer inflammatory protein-A (oipA) than any other bacterium (27). In this bacterium, there are 32 outer membrane proteins. These proteins are involved in bacterial pathogenicity and are highly correlated with OMP and Helicobacter pylori density, gastric mucosal injury, high IL-8 levels, and neutrophil leakage at the site of inflammation. The gene encodes one of the outer membrane proteins and the inflammation-related gene located at 100kb from the PAI cag on the Helicobacter pylori chromosome (28). The cytotoxic function of the protein is strongly related to the VacA and CagA phenotypes. As suggested, the mode of action of oipA is associated with the rate of cell death. Because oipA probably acts as a sticky agent, the sides that are oipA-enabled have a stronger attachment to the stomach (29).

OipA is a component of Hop outer membrane proteins belonging to the Helicobacter pylori family of outer membrane proteins. The presence of this protein has been linked to duodenal ulcers, gastric cancer, and neutrophil accumulation (30, 31). These outer membrane proteins play a role in host adaptation. Their expression is under the control of the repair strand-slipped mechanism. In the N-terminal part of these proteins, there is a signal sequence that, with the addition of CT bases to this region, affects the expression of the gene and usually causes Helicobacter pylori to pass through the ovary after passing through it several times. The expression of this protein has been reported to be related to the CagA factor, and most of the strains that are considered on have CagA, and most of the off strains are CagA negative (32, 33).

Previous studies have examined the effect of *cag*A (10, 12), *hop*QI (11), *cag*A and *hop*QI Simultaneous (16) and *hop*QII (14) genes. In the current study, the simultaneous effect of two genes (*cag*A and *hop*QII) was investigated. For additional study, it is needed to organize more experiments on more genes as well as to study a wide range of gene networks (34-36). The occurrence of *H. pylori* infection in developing countries is 4 to 15% and this is a thoughtful cautionary for more attention and prevention (37, 38).

In the current study, the frequency of diseased and healthy individuals infected with *H. pylori* and both genotypes *cag*A and *hop*QII were investigated. According to the data, there is a significant relationship between the two genes *cag*A and *hop*QII and gastric cancer. Consequently, the simultaneous presence of these two bacterial genes (*cag*A and *hop*QII) in human societies can be an important biomarker for the diagnosis and prognosis of gastric cancer.

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