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Presence of the *Helicobacter pylori* in Esophageal Squamous Cell Carcinoma Samples

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Abstract

Background: The main causes of esophageal squamous cell carcinoma (ESCC) in developing countries differ from developed countries. In developing countries, approximately one-fourth of cancer cases are caused by infectious agents. In terms of infectious etiology of esophageal cancer, *Helicobacter pylori* has been among the most widely investigated, but its role in etiology of ESCC remains unclear.

Objectives: The present study aimed to investigate the presence of *H. pylori* in the pathogenesis of ESCC.

Materials and Methods: In total, 277 formalin-fixed paraffin-embedded esophageal samples (177 with ESCC, and 107 without esophageal malignancy) were examined for *H. pylori* infection. After removing of paraffin from tissue samples, DNA was extracted and polymerase chain reaction (PCR) was performed to investigate the presence of *H.pylori*.

Results: *H. pylori* was not detected in any of the cancerous and non-cancerous esophageal sample.

Conclusion: In the present study, there was no association between *H. pylori* and ESCC.

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Background

Esophageal cancers, including esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (ADC), are among the most lethal cancers. These cancers have different risk factors and their prevalence varies in different parts of the world.[1] Esophageal cancer has a specific geographic distribution, as some countries such as Iran, China, South Africa, Singapore, the former Soviet Union, Chile, Puerto Rico, Brazil, France, and Switzerland are at greater risk. The highest prevalence was reported from northern China, coasts of the Caspian Sea, and the Transkei region of South Africa.[2]

The main causes of ESCC in developed countries are different from the ones of the developing countries. Several lines of evidence revealed that unlike the developed countries, cigarette smoking and alcohol drinking are not the main ethological factors of ESCC in the developing countries, and other factors may be contributed).[3,4] In the developed countries, about 7% of the risk factors for cancer are related to infections, while infections are responsible for 26% of cancers in the developing countries. Among the infectious factors effective in cancer in Iran, papillomavirus, hepatitis B virus, hepatitis C virus, and *Helicobacter pylori* are of a high degree of contamination.[5-7]

As one of the most common bacteria in the world, *H. pylori* was considered carcinogenic in 1994.[8] Different factors are involved in the pathogenesis of gram-negative bacteria, which include urease, vacuolating cytotoxin (VacA), and cytotoxin-associated gene A (CagA) protein, as well as the host immune system response.[9] Based on the reports, *H. pylori* infection in stomach, which causes atrophic gastroenteritis and reduction of gastric acid and serum pepsinogen, reduces the risk of esophageal ADC.[10]

Recent studies showed an inverse relationship between *H. pylori* and ADC of the esophagus.[11] Preliminary studies showed that *H. pylori* infection was higher in ESCC tissue than esophageal normal tissue.[12] Several studies have been conducted in different regions mainly based on serological findings; however, they show contradictory results on the relationship between *H. pylori* and ESCC. Therefore, the relationship between *H. pylori* infection

and ESCC is still unknown and requires further studies.[13]

Iran is located in a geographic area with high prevalence and incidence of ESCC, especially in northern and northwestern areas. Therefore, it is important to identify the environmental factors of esophageal cancer.[14]

Objectives

The high prevalence of esophageal cancer in Iran, especially in northern areas, carcinogenicity of *H. pylori*, and reports of some studies on the presence of the bacteria in the tissue of esophageal cancer led us to examine the relationship between *H. pylori* and esophageal cancer in Mazandaran province.

Materials and Methods

Patients and Samples

One hundred seventy cancerous samples and 107 non-cancerous esophagus samples were collected from the archive of pathology centers (pathology laboratory of Shahid Beheshti hospital in Babol and central laboratory of pathobiology in Amol). The data were collected by questionnaire and analyzed from the patient's medical records. All the cancerous samples were diagnosed by the pathologist and they were reviewed before including in the study to approve their diagnosis. Five-micron cuts were prepared and collected from each paraffin-embedded tissue block.

DNA Extraction

After transferring the samples to the laboratory, paraffin was removed and DNA was extracted using High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) from esophageal cancer tissue. The quality and integrity of extraction was evaluated by real-time polymerase chain reaction (PCR) analysis for ribonuclease P gene, as described previously.[15]

Polymerase Chain Reaction

After setting up the experiment, PCR was used for detecting *H. pylori* DNA. A reaction mixture of 25 µL, including 14.85 µL of deionized water, 2.5 µL of buffer, 1 µL of $MgCl_2$, 0.5 µL of each primer, 0.5 µL of dNTP, and 0.15 µL of Taq enzyme, was prepared in a micro tube. Next, 5 µL of DNA was added to the sample. Thermo cycler's program was as follows: (*a*) Heat start for 2 minutes at 95°C, (*b*) 30 cycles at 60, 90, and 72°C each for 30 seconds, and (*c*) Final extension at 72°C for 5 minutes. PCR products were then analyzed by a 1.5% agarose gel with electrophoresis. The PCR was carried out using the specific primers for *glmM* gene of *H. pylori* strain. Table 1 shows the sequence of these primers. The PCR product of this gene with glmM primer is a piece with a length of 294 bp.[16]

Results

The samples were examined in terms of age, gender, anatomical location of the esophagus, and histopathologic diagnosis and residence status. Of 170 cancerous and 107

Table 1. Sequences of Primers Used for the *glmM* Gene

Primers	Sequences 5′→3′	Product of PCR
ureC F	5′-AGGCTTTTAGGGGTGTTAGGGGTTT-3′	294bp
ureC R	5′-AAGCTTACTTTCTAACACTAACGC-3′	294bp

Abbreviation: PCR, polymerase chain reaction.

Table 2. Demographic Features in Non-cancerous Esophageal Lesions

Characteristics	Anatomical Sites			
	Upper Third	Middle Third	Lower Third	Total
	No. (%)	No. (%)	No. (%)	No. (%)
Gender				
Male	17 (26.2)	21 (32.3)	27 (41.5)	65 (62.5)
Female	7 (17.9)	11 (28.2)	21 (53.8)	39 (37.5)
Age groups				
<45	3 (12.5)	5 (15.6)	6 (12.5)	14 (13.5)
45 to 60	5 (20.8)	13 (40.6)	21 (43.8)	39 (37.5)
61 to 75	11 (45.8)	8 (25.0)	11 (22.9)	30 (28.8)
<75	5 (20.8)	6 (18.8)	10 (20.8)	21 (20.2)
Resident				
Urban	20 (24.7)	26 (32.1)	35 (43.2)	81 (77.9)
Rural	4 (17.4)	6 (26.1)	13 (56.5)	23 (22.1)
Histopathological diagnosis				
Ulcer	5 (20.8)	5 (15.6)	10 (20.8)	20 (19.2)
Esophagitis	10 (41.7)	12 (37.5)	22 (45.8)	44 (42.3)
Barretts	0 (00.0)	3 (9.4)	1 (2.2)	4 (3.8)
Dysplasia	6 (25.0)	3 (9.4)	4 (8.3)	13 (12.5)
Normal tissue	3 (12.5)	9 (9)	11 (22.9)	23 (22.1)

control samples, 84 (49.4%) and 67 (62.6%) were male, respectively. The mean age of patients and control group was 66.50 ± 11.05 and 62.50 ± 14.50, respectively. Similarly, among patients, 77.1% and 22.9% lived in the cities and rural area, respectively (Table 3).

The analysis of lesions for different anatomical sites is shown in tables 2 and 3. In cancer patients, 35 (21.3%), 65 (39%) and 64 (39.6%) cases belong to upper third, middle third and lower third of the anatomical sites.

Of the 104 control samples, 24 (23.1%), 32 (30.8%) and 48 (46.2%) samples were in the upper, middle and lower third of the esophagus, respectively. 124 (72.9%) of esophageal cancer samples had been collected by biopsy and 46 patients (27.1%) by surgery. Tables 2 and 3 shows the frequency distribution of the samples in both groups in different parts of the esophagus in terms of gender, type of lesion, age, and place of residence.

Ribonuclease P gene was examined using the real-time PCR technique through examining and controlling the presence of DNA in the tissue extracted from samples while all the samples had sufficient DNA. In this study, no band indicating the presence of *H. pylori* DNA was seen in the agarose gel of the samples (Figure 1).

Discussion

Many studies examined ADC and most of them concluded the protective role of *H. pylori* and reported a reverse relationship between ADC and *H. pylori*. The cause of

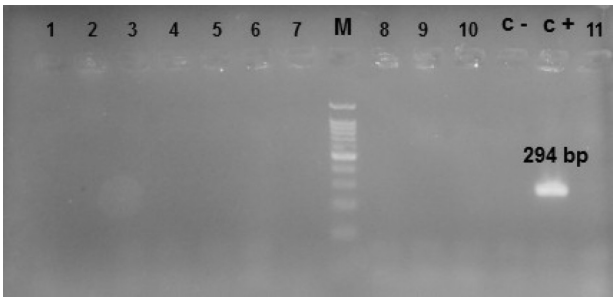

Figure 1. Agarose Gel Electrophoresis of Polymerase Chain Reaction Products.
M: 100 bp DNA lader, C+: positive control C-: negative control, No. 1-11 samples.

Table 3. Demographic Features in Cancerous Esophageal Lesions

Characteristics	Anatomical Sites			
	Upper Third	Middle Third	Lower Third	Total
	No (%)	No (%)	No (%)	No (%)
Gender				
Male	19 (23.2)	34 (41.5)	29 (35.4)	82 (50)
Female	16 (19.5)	30 (36.6)	36 (43.9)	82 (50)
Age groups				
<45	0 (00.0)	3 (50)	3 (50)	6 (3.7)
45 to 60	11 (23.9)	20 (43.5)	15 (32.6)	46 (28)
61 to 75	18 (22.8)	28 (35.4)	33 (41.8)	79 (48.2)
<75	6 (18.2)	13 (39.4)	14 (42.4)	33 (20.1)
Resident				
Urban	30 (24)	48 (38.4)	47 (37.6)	125 (76.2)
Rural	5 (12.8)	16 (41)	18 (46.2)	39 (23.8)
Histopathological diagnosis SCC				
Insitu carcinoma	23 (65.7)	40 (62.5)	50 (76.9)	113 (68.9)
Poorly differentiated SCC	2 (5.7)	1 (1.6)	3 (4.6)	6 (3.7)
Moderately differentiated SCC	1 (2.9)	1 (1.6)	2 (3.1)	4 (2.4)
Well differentiated SCC	5 (14.3)	12 (18.8)	6 (9.2)	23 (14)
Well moderately	4 (11.3)	9 (14.1)	3 (4.6)	16 (9.8)
differentiated SCC	0 (0)	1 (1.6)	1 (1.5)	2 (1.2)
Type of samples				
Biopsy	26 (22)	39 (33.1)	53 (44.9)	118 (72)
Surgery	9 (19.6)	25 (54.3)	12 (26.1)	46 (28)

Abbreviation: SCC, squamous cell carcinoma.

this relationship has not been realized yet; however, one reason may be the presence of *H. pylori*, which neutralize the acidity of gastro-esophageal reflux, which is one of the major risk factors for ADC.[11] Some studies reported an inverse relationship between *H. pylori* and ESCC, including a study in Taiwan in 2005 which showed a protective role for *H. pylori* in ESCC development.[17] Similarly another study in Taiwan in 2009 used the same method and reported a protective role for *H. pylori* in ESCC occurrence.[18] Findings of the separate studies in Urmia and Tabriz using serological methods showed an inverse relationship between *H. pylori* and ESCC development.[19,20] A positive relationship was reported between *H. pylori* and ESCC in some countries, such as Sweden[12] and China[13] studies.

A study, which was conducted using Western blot analysis in Sweden in 2007, reported no relationship between infection by this bacterium and ESCC. A study using a serological method in China in the same year also showed no relationship.[21,22]

Another study in Australia in 2010 examined the prevalence of *H. pylori* in esophageal cancer using serological methods and polymorphism. The study showed an inverse relationship between *H. pylori* infection in ADC and EGJAC (esophageal-gastric junction), but showed no relationship between *H. pylori* and ESCC.[10] Some studies were conducted in this concern in Finland and Germany in 2011, which reported no relationship between *H. pylori* and ESCC.[23,24] The present study did not show the presence of *H. pylori* in any esophageal cancerous and non-cancerous sample of the patients of Mazandaran province and no relationship was found between *H. pylori* and esophageal squamous cell carcinoma (ESCC) ($P > .05$).

With respect to the results and importance of the issue, further extensive epidemiological studies are recommended in different parts of Iran, preferably on fresh samples, to clarify whether *H. pylori* is really linked to esophageal cancer.

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Authors Contributions

Yousef Yahyapour, Ramazan Rajabnia and Aynaz Khademian; developed the study concept, performed experimental protocols and prepared the manuscript. Soraya Khafri performed statistical analysis. Javad Shokri Shirvani, Elaheh Ferdosi-Shahandashti, and Farzin Sadeghi; carried out administrative, technical, and sample support.

Conflict of Interest Disclosures

None.

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