Association of *cagA* Positive *Helicobacter pylori* Infection and Laryngeal Squamous Cell Carcinoma: A PCR Approach

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Abstract *Helicobacter pylori* is a gram-negative bacillus causing benign and malignant gastric diseases such as gastroesophageal reflux (GER). In larynx, *H. pylori* causes chronic inflammation and mucosal destruction that may lead to malignant changes. Although, *H. pylori* poses several virulence factors, *cagA* is probably the main factor in this regard. To evaluate the role of *cagA* gene in laryngeal squamous cell carcinoma (LSCC), a case–control study was conducted on patients with laryngeal complaints during 2010–2012. Seventy-two patients with LSCC (case group) and 72 patients without malignancy (control group) were included in the study. The *H. pylori* and *cagA* factor were assessed in laryngeal specimen of patients with PCR technique. 33 % of patients in case group (24 patients) and 45.8 % (33 patients) of control group were positive for *H. pylori*. *CagA* gene was present in 13.8 % (10 patients) of case group specimens and 31.9 % (23 patients) of control group. This difference was statistically significant with Mantel–Haenszel statistical test analyses. The results showed that patients with LSCC have significantly lower incidence of laryngeal *H. pylori* infection and *cagA* virulence factor than those without LSCC. Findings from this study support the protective effect of *H. pylori* infection against laryngeal cancer.

Keywords *Helicobacter pylori* · *cagA* ·
Laryngeal cancer · SCC

Introduction

Helicobacter pylori (*H. pylori*) is a gram negative bacillus colonized in humans' stomach [1] and yet most of cases may remain asymptomatic. *H. pylori* can cause benign and malignant gastric diseases [2]. Studies have also detected the presence of *H. pylori* in dental plaque, saliva and adenotonsillar tissue. Different virulence factors such as urease, flagella, adhesion factors, vacuole cytotoxins and *cagA* have been identified for *H. pylori*. However, it seems that *cagA* gene is the main virulence factor of *H. pylori* [3]. *CagA* gene encodes a 120–145 kDa protein and has been located on the 40 kb *cag* pathogenicity island [4]. *In vitro* experiments on animal models of *H. pylori* infection have shown that infection with *cagA* positive *H. pylori* is more likely to lead to peptic ulcer and malignancy [5]. *CagA* is not seen in all types of *H. pylori* but most of East Asian *H. pylori* isolates are positive for *cagA* [6]. However, it is used as a marker for pathogenicity [7]. Although, *cagA* positive *H. pylori* is mostly reported in gastric tissue, *cagA* is also detected in adenotonsillar tissue, nasal polyps and laryngeal diseases. Cirak et al. have reported the presence of *cagA* in 71 % of adenotonsillar tissue samples [8]. It can be hypothesized that *H. pylori* along with other risk factors such as smoking, alcohol and gastroesophageal reflux (GER) play a role in the pathogenesis of laryngeal squamous cell carcinoma (LSCC). Chronic inflammation of infected tissue with *H. pylori* followed by epithelial cell proliferation in the laryngeal mucosa might cause laryngeal cancer as in the pathogenesis of *H. pylori* in gastric cancer

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[8, 9]. *H. pylori* exists in oral cavity and stomach independently. *H. pylori* can be colonized in larynx too [10]. Studies have shown the presence of *H. pylori* in specimens of benign and malignant laryngeal diseases with serologic, histochemistry and PCR techniques. Most of these studies have utilized the serologic tests of *H. pylori* with ELISA technique and have evaluated the presence of IgG antibody against *H. pylori*. Serologic tests measure antibodies (IgA, IgM and IgG) against *H. pylori* antigens. Although tests for measuring different types of immunoglobulins are available, FDA has only approved the serum IgG-based tests for detection of *H. pylori* infection. Serologic tests accurately reflect the presence or absence of active *H. pylori* infection [11]. There are few studies using PCR technique for detection of *H. pylori* infection, and one study that has used PCR technique suffers from limited number of cases [12]. Laryngeal cancer is one of the most common malignancies of head and neck region.

There are few data available on *cagA* gene condition of *H. pylori* variants in Iran. This study aimed to assess the association between *cagA* positive *H. pylori* infection and LSCC using a specific PCR technique.

Materials and Methods

Study Population and Specimen Collection

A cross-sectional study was designed and local ethics committee approved the study protocol. Data were collected from patients admitted to otolaryngology-head and neck clinic in Shafa Hospital of Kerman with complaints of laryngeal disease between March 2010 and March 2012. A complete history was obtained and risk factors were assessed for patients with laryngeal complaints. The patients were asked to read, fill out and sign the informed consent form. The exclusion criteria of the study were the followings: rejection to participate in the study, serious gastrointestinal diseases that were confirmed by endoscopy like severe gastritis or gastrointestinal cancers, recent use of drugs against *H. pylori* activity such as omeprazole, antibiotics (metronidazole, amoxicillin and bismuth), pathologic diagnosis of dysplasia and patients under 30 years of age.

Sample Preparation

In patients with suspicious lesion in indirect laryngoscopy, patients with risk factors for laryngeal malignancy (opium addiction/cigarette smoking) or a history of hoarseness more than 2 weeks, direct laryngoscopy in operation room was done and samples were taken for pathology. From the fresh specimen 3–4 mm² of tissue was separated, coded,

kept at –80 °C and the reminder of the specimen was sent for pathology study with the same coding. If the pathologic diagnosis was in favor of malignancy, the specimen was then placed in case group and if it was in favor of benign lesions (nodule, polyp, cyst or granulation), it was then assigned to control group. If the pathologic diagnosis was in line with dysplasia, the patient was excluded from the study. To prevent any bias in PCR testing of the specimens, the coded samples were sent for PCR technician to blind the lab staff from pathologic diagnosis.

PCR Detection of *H. pylori* and *cagA*

The standard strain of *H. pylori* was provided by Iran Pasteur Institute, Liver and Gastrointestinal department, *H. pylori* center. The samples were placed in sterile normal saline; DNAs were extracted using High Pure A, DNA Tissue Extract Kit (High Media company). The samples were investigated for existence of *H. pylori* by performing a real time PCR using Detection Pylori Kit (DNA Technology company, Russia). Positive samples for *H. pylori* infection were investigated for *cagA* gene using CagA Kits (Kiagen company, Germany). The Multiple PCR test was carried out on the samples and the results were registered for further investigation.

Statistical Analyses

The sample size with consideration of a relative accuracy of 10 %, type I error ($\alpha = 5\%$) was computed as 72 persons for each group. SPSS V. 20 and STATA V. 10 were used to calculate odds ratio (OR), relative ratio (RR) and performing Mantel–Haenszel test. Mantel–Haenszel test was performed to consider the confounding effect of opium addiction and cigarette smoking in LSCC and adjust OR for *H. pylori* and *cagA*.

Results

In this study 144 men and women with laryngeal complaints aging 30–88 years old were selected. The average age was 52.6. The most common age group in this study was 50–59 followed by 40–49 with frequencies of 22.9 and 28.5 % respectively. Twenty-six patients were female (18.1 %) and 118 were male (81.9 %). Eighty-three percent of patients were opium addicted (inhalation, oral use or both). Of these 83 addicted patients, 75 (90.4 %) were also cigarette smokers. The duration of opium consumption and cigarette smoking ranged from 1 to 50 (MEAN $\pm$ SD) years and 2 to 50 years (MEAN $\pm$ SD), respectively.

Of 144 patients in study, 72 had benign laryngeal lesion (control group) and 72 had LSCC. *H. pylori* was detected

in 57 patients (39.6 %) of which 24 (42.1 %) patient had LSCC and 33 (57.9 %) patient had benign laryngeal lesion (Table 1). In patients with positive samples for *H. pylori* (57 patients), 33 had positive PCR results for *cagA* gene (57.9 % in *H. pylori* positive group and 22.9 % in total patients). *CagA* was positive in 10 patients with LSCC and 23 patients with benign laryngeal lesions. Comparison of *H. pylori* frequency between the two groups showed that in control group the presence of *H. pylori* was more frequent than case group. However, this difference was not statistically significant ($P > 0.05$). Comparing the age distribution of patients in this study showed no significant difference between the case and control group with the exception of patients aging 60–69 years of old. In this later group, *H. pylori* was positive in 88.9 % of patients in control group and 42.9 % of patients in case group. This difference was statistically significant ($P = 0.027$).

CagA was less frequent in both control and case group. A comparison between the two groups of the study revealed that the different frequency of *cagA* positive samples between the control group (23 samples) and case group (10 samples) was statistically significant ($P = 0.008$). Overall 39.6 % of patients in this study had positive laryngeal PCR results for *H. pylori* infection. About 45.8 % of patients in control group (33/72) had positive samples for *H. pylori* in comparison to 33 % (24/72) of samples in case group. Statistical analyses revealed a non-significant ($P > 0.09$) OR of 0.56. In order to adjust the confounding effect of cigarette smoking and opium addiction, Mantel–Haenszel test was applied (Table 2).

This table shows that considering cigarette smoking and opium addiction as confounding factors and using a proper statistical technique for elimination of this confounding effect, the relationship between the two groups and *H. pylori* remains statistically significant. Table 3 shows the cofounding effect of cigarette smoking and opium consumption on relationship between type of disease and existence of *cagA* gene. OR less than 1 even after adjustment with Mantel–Haenszel test shows that the presence of *cagA* gene like *H. pylori* infection is higher in control group.

Discussion

GER is associated with laryngeal symptoms of which the most commons are posterior glottis erythema and edema. However, hyperkeratosis, thickening (pachyderma laryngitis) and granulation are also reported. Hoarseness, globus sensation and clearing the throat are common. Reflux can lead to contact ulcers in vocal folds, granuloma, leukoplakia and laryngeal malignancy [13]. The relationship between *H. pylori* and larynx cancer is not fully understood. Some studies have shown reductions in esophagitis and Barrett's esophagitis in the presence of *H. pylori* infection. As *H. pylori* infection reduces gastric acidity and reflux, it might also pose a protective effect against laryngeal cancer [14]. In this study, 144 patients with laryngeal complaints, were assessed by PCR technique for laryngeal infection with *H. pylori*. The results showed that

Table 1 Comparing the frequency of *H. pylori* and *CagA* in two groups

	Cag A gene existence					
	No		Yes		Total	
	Count	Row N %	Count	Row N %	Count	Row N %
Group						
Control						
<i>Helicobacter pylori</i> existence						
No	38	100.0	0	0.0	38	100.0
Yes	10	30.3	23	69.7	33	100.0
Total	48	67.6	23	32.4	71	100.0
Case						
<i>Helicobacter pylori</i> existence						
No	49	100.0	0	0.0	49	100.0
Yes	14	58.3	10	41.7	24	100.0
Total	63	86.3	10	13.7	73	100.0
Total						
<i>Helicobacter pylori</i> existence						
No	87	100.0	0	0.0	87	100.0
Yes	24	42.1	33	57.9	57	100.0
Total	111	77.1	33	22.9	144	100.0

Table 2 Confounding effect of cigarette and opium on correlation between type of disease and *H. pylori*

Group/HBP existence	Crude OR	CI 95 %	P value	Adjusted OR*	CI 95 %	P value
No confounding factor	0.564	0.287–1.108	0.095	–	–	–
Use of cigarette as confounding factor	–	–	–	0.383	0.166–0.880	0.024**
Use of opium as confounding factor	–	–	–	0.257	0.104–0.633	0.003**

OR odd ratio, CI 95 % confidence interval 95 %

* Mantel–Haenszel common odd ratio estimate

** Statistically significant

Table 3 Confounding effect of cigarette and opium on correlation between type of disease and cagA

Group/CagA Existence	Crude OR	CI95 %	P value	Adjusted OR*	CI 95 %	P value
No confounding factor	0.331	0.144–0.761	0.008**	–	–	–
Use of cigarette as confounding factor	–	–	–	0.186	0.066–0.522	0.001**
Use of opium as confounding factor	–	–	–	0.194	0.071–0.530	0.001**

OR odd ratio, CI 95 % confidence interval 95 %

* Mantel–Haenszel common odd ratio estimate

** Statistically significant

only 33 % of patients with malignant changes had *H. pylori* in comparison to 45.8 % of patients with benign lesion. Mantel–Haenszel test was considered to assess the confounding effect of cigarette smoking and opium addiction along with the role of *H. pylori* infection in laryngeal pathologies. The results suggest that *H. pylori* may play a role as a protective factor against laryngeal malignancy. However, this finding might be due to other factors like consumption of drugs, nutritional diets (low fat) or individual habits and requires further investigations.

Infection by *H. pylori* is more common in developing countries, whereas GER is relatively less common in Asia and Africa than North America and Western Europe countries. Reduction of acid secretion after infection with *H. pylori* can be explained via three mechanisms: severe gastritis of corpus region, increased secretion of gastric bicarbonate and ammonium production by the bacteria itself. After eradication of *H. pylori*, rising acid secretion and probably reflux can lead to esophagitis, a condition well known to predispose to premalignant conditions like Barrett's esophagitis [14]. In an epidemiologic study by EL-Serag, lower prevalence of *H. pylori* infection was seen in patients with esophagitis [15]. Wu et al. reported that infection with *H. pylori* was associated with a decrease in Barrett's esophagitis [16]. In a study by Majidi et al. in Iran on 45 patients with laryngeal cancer and 42 controls, anti *H. pylori* antibody titer was significantly higher in controls than cases. It was concluded that infection with *H. pylori* has an anti secretory effect in stomach which leads to decreased level of gastric acidity. Thus, *H. pylori* infection can pose a protective effect against diseases associated with GER [17].

In a study by Nurgalieva et al. in USA on 120 patients with larynx cancer and 120 persons in control group, the amount of anti *H. pylori* IgG antibody was similar between the two groups (32.8 % in patients and 27 % in controls) [18]. In the above study, *H. pylori* had no association with larynx malignancy and was neither a risk factor nor a protective factor for it. Lower infection rate of *H. pylori* in both groups of the mentioned study is acceptable due to the geographical distribution of *H. pylori* infection. Moreover, the presence of strong risk factors of laryngeal cancer such as cigarette smoking and alcohol usage further complicates the finding about the role of *H. pylori* in laryngeal cancer in this geographical region. In Aygene study in 2001 in Turkey on 26 patients with laryngeal cancer and 32 healthy persons, serum IgG antibodies against *H. Pylori* antigens were assessed. The results showed that IgG antibody was positive in 73.07 % of cases and 40.62 % of controls. This study supported the role of *H. pylori* in laryngeal cancer [19]. In 1996, Jenifer et al. conducted a study on 21 cases with laryngeal cancer and 21 controls, serum IgG antibodies against *H. pylori* antigens were assessed and no significant difference was found [20]. Mokhtari (2003) studied on 30 patients with laryngeal cancer and 30 healthy controls serum IgG antibodies against *H. pylori* antigens were evaluated. Sixty-three percent of cases and 26 % of controls had positive serologic results [21]. The findings of the study by Mokhtari is in line with Aygene study but not similar to the findings of the present study [19]. It seems that the main cause of controversial findings in these studies may result from ELIZA technique in detection of serum IgG antibodies against *H. pylori* that is not specific to *H. pylori* infection of larynx. An investigation was done

in 2004, seeking for *H. pylori* in specimens of larynx cancer using histopathological and immunohistochemical approaches; however, no correlation was found [7]. In 2009, a study used PCR detection of *H. pylori* in 29 patients with nasal polyps and laryngeal disease (benign or malignant) and 29 healthy persons. Nevertheless, this study failed to show significant correlation between the disease and *H. pylori* [12]. However, this study suffered from few numbers of cases. Ozyurt et al. in 2009 identified *H. pylori cagA* in 78.9 % of the *H. pylori* positive nasal polyp samples, 89.5 % of normal nasal mucosa samples, and 82.4 % of normal larynx samples [12]. The findings of the study by Ozyurt et al. are not congruent with higher *cagA* factor in benign lesions in this study.

In this study, the frequency of *H. pylori* infection and *cagA* gene was significantly higher in case group than control group. This finding supports the protective effect of *H. pylori* against laryngeal malignancy. However, this finding merit further investigation with prospective study design, parallel assessment of gastric disease and evaluation of more risk factors of *H. pylori* infection and laryngeal malignant disease.

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