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RESEARCH ARTICLE

Helicobacter pylori infection of the larynx may be an emerging risk factor for laryngeal squamous cell carcinoma

Hongli Gong · Yi Shi · Liang Zhou · Lei Tao · Yong Shi · Wenjun Cao · Lei Cheng

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Abstract
Introduction Several studies have implicated *Helicobacter pylori* as a risk factor in laryngeal cancer, but other studies disagree. It is fundamental that the relationship between *Helicobacter pylori* and laryngeal cancer be verified in order to provide evidence of ways to prevent the initiation and development of this carcinoma.
Materials and methods In total, 81 patients with laryngeal squamous cell carcinoma and 75 control subjects were enrolled in a case–control study. Semi-nested polymerase chain reaction techniques were applied to detect *Helicobacter pylori* in the laryngeal mucosa and enzyme-linked immunosorbent assays were used to detect serum antibodies against *Helicobacter pylori*. Risk factors associated with laryngeal carcinoma were analyzed using logistic regression models.
Results The presence of *Helicobacter pylori* in the larynx was higher in patients with laryngeal cancer than in control subjects (71.6 vs. 25.3 %, $p < 0.001$). Among patients with laryngeal carcinoma, rates of *Helicobacter pylori* infection were higher in normal laryngeal tissues than in tumor tissues. After adjusting for confounding factors, regression analysis indicated that the microbe was an independent risk factor for laryngeal cancer (OR = 7.15, 95 % CI [3.29, 15.53], $p < 0.001$).
Conclusions This study suggests that *Helicobacter pylori* is present in the mucosa of the larynx. The microorganism may be an independent risk factor for laryngeal squamous cell carcinoma. The laryngeal mucosa thus provides a reservoir for the bacteria possibly, and is a likely staging place for its transmission to other areas.

Keywords Laryngeal squamous cell carcinoma · Helicobacter pylori · Semi-nested polymerase chain reaction · Enzyme-linked immunosorbent assays

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the most common tumor histology of the larynx, the second most common malignancy of the head and neck, and the 11th most common form of cancer worldwide [1]. Because there is no pain until the late stages, laryngeal carcinomas tend to be diagnosed late and the overall 5-year relative survival rate is 64.2 % [2]. The primary treatments for this disease include surgery, chemotherapy, and radiotherapy, either individually or in combination [2]. However, disease prevention and early diagnosis are the most efficient ways of enhancing survival rates, preserving functionality, and improving quality of life.

It has been found that transition from normal epithelium to squamous intraepithelial lesions and then LSCC was a sequential, comprehensive, and multiplied process, as well as related to genetic mutation and aberration. Risk factors

H. Gong and Y. Shi are the joint first authors because of the equal contribution to this study.

H. Gong · L. Zhou (✉) · L. Tao · Y. Shi · L. Cheng
Department of Otolaryngology, Eye, Ear, Nose, and Throat Hospital, Fudan University, Shanghai, China
e-mail: zhlwc@online.sh.cn

H. Gong
e-mail: gonghongli2008@yahoo.cn

Y. Shi · W. Cao
Department of Clinical Laboratory, Eye, Ear, Nose, and Throat Hospital, Fudan University, Shanghai, China

Y. Shi
Department of Clinical Laboratory, Nanhui Central Hospital, Shanghai, China

contributing to the process include tobacco smoking and alcohol consumption [1, 2]. Nevertheless, a substantial proportion of head and neck cancers cannot be attributed to tobacco and alcohol use, suggesting that other factors, such as infection with human papillomavirus, may be etiologically involved [3]. More recently, several studies reported the presence of *Helicobacter pylori* (*H. pylori*) in the middle ear [4, 5], the nasal sinus [6], the oral cavity [7, 8], and the laryngeal mucosa [9] and implicated this microbe as a risk factor in laryngeal cancer, but other studies disagree. First described in 1982, *H. pylori* is thought to be a causative agent in gastric carcinoma and has been classified as a group I carcinogen by the International Agency for Research on Cancer. The infection causes persistent chronic gastritis, which for some individuals may develop into gastric atrophy, followed by metaplasia, dysplasia, and eventually gastric tumors [10–12].

Among the Chinese population, the rate of *H. pylori* infection has been reported to range from 34.5 to 80.6 %, with a mean infective rate of 58.1 % [13]. Several studies have suggested a relationship between *H. pylori* infection and laryngeal cancer [14–16], but others hold negative views [17, 18]. It is important to examine the risk factors for laryngeal cancer in order to provide new treatment options and establish preventive measures against the initiation and development of laryngeal carcinoma.

The hypothesis of this research was that *H. pylori* infection is present in the laryngeal mucosa and is a risk factor for LSCC. To examine the hypothesis we designed a case–control study in which we enrolled 81 patients with laryngeal squamous cell carcinoma and 75 control subjects. We then used the semi-nested polymerase chain reaction (SN-PCR) techniques to detect *H. pylori* infection in the laryngeal mucosa and enzyme-linked immunosorbent assays (ELISA) to detect serum antibodies to *H. pylori*. Statistical analyses were applied to identify whether *H. pylori* is an independent risk factor for LSCC.

Materials and methods

Subjects and study design

In the present study, 81 patients with laryngeal squamous cell carcinoma and 75 controls (50 patients with vocal cord polyps and 25 patients with epiglottic cysts) from the Eye, Ear, Nose, and Throat Hospital, Fudan University, were enrolled between July 2009 and January 2011. Each patient's diagnosis was confirmed using histopathology and assessment of tumor location and TNM staging was performed according to the criteria of the Union for International Cancer Control [19, 20]. All of the study subjects completed a questionnaire detailing their sex, age, marital status, occupation, annual salary income, education level, tobacco usage, alcohol intake, and digestive health history. To exclude the confounding factors confuse to verify the hypothesis in current study, exclusion criteria for both patients with LSCC and control subjects included (1) history of gastroesophageal reflux disease, chronic gastritis, peptic ulcer disease or gastric resection; (2) prior use of anti-*H. pylori* antibiotic therapy or antireflux therapy, such as proton pump inhibitors or H_2 receptor antagonists; (3) presence of reflux symptoms; (4) use of hormones or antibiotics during the previous 3 months; (5) seropositivity for human immunodeficiency virus; and (6) reception of a blood transfusion in the previous 6 months. All procedures in this study were approved by the ethical review board of Fudan University.

Subjects were divided into three groups and scored depending on age: (0) 49 years of age or younger; (1) 50–64 years; (2) 65 years or older. Depending on marital status the subjects were divided into four groups, never married; married; widowed; and divorced. For annual salary income there were three groups, low ($\leq$7,500 Chinese yuan per person/year); medium (7,500–45,000 Chinese yuan per person/year); and high ($\geq$45,000 Chinese yuan per person/year) (based on data from the Development Research Center of the State Council of People's Republic of China). For education level the groups were low (no experience of school or elementary school only); medium (finished high school); and high (went to university). For occupational status the groups were blue collar; white collar; and others (included farmers, retired persons, housewives and undefined) [21].

According to their smoking habits, members of the study population were classified as never smokers (no history of smoking); former smokers (having not smoked for at least 12 months); and current smokers (who were smoking at the time of admission). Former and current smokers were also combined into a single category, as "have smoked", for comparison with those who had never smoked. Data concerning the number of cigarettes per day were also recorded and subjects were divided into a high consumption group (>180 packs per year) and a low consumption group (180 packs per year).

To classify drinking habits mean weight of the alcohol content consumed per day was recorded (1 standard drink contains 12 g of absolute alcohol). Based on alcohol consumption, patients were then divided into "non-drinkers," which included subjects who do not drink or who only occasionally drank, and "drinkers". Drinkers were further divided into two groups low (fewer than 3 drinks per day for males or fewer than 2 drinks per day for females) and high (3 or more drinks per day for males and 2 or more drinks per day for females) [21].

Sample collection

Tissue samples were collected in a laminar flow operation room to avoid contamination. For patients with LSCC

samples of tumor and normal tissue were taken simultaneously with separate instruments. Normal tissue was obtained from areas at least 1 cm away from the tumor site. Specimens from the control group, which included polyps from vocal cords or epiglottic cysts, were obtained using a direct laryngoscope. Serum samples were separated from blood drawn aseptically by venipuncture. All tissue specimens and serum for each patient were conserved at −80 °C.

Semi-nested polymerase chain reaction (SN-PCR) and sequence analyses

DNA was extracted from tissue samples using a QIAGEN DNA extraction kit and SN-PCR performed as has been previously described [22]. Primers were as follows: primer-1 (5′ CTG GAG AGA CTA AGC CCT CC 3′), primer-2 (5′ ATT ACT GAC GCT GAT TGT GC 3′), and primer-3 (5′ AGG ATG AAG GTT TAA GGA TT 3′) (Sangon, Shanghai, China). The first step in the SN-PCR process used primer-1 and primer-3 to produce 423 bp fragments and the second step engaged primer-1 and primer-2 to produce 110 bp fragments from the product of first step. SN-PCR products were analyzed by electrophoresis on a 2 % agarose gel at 90 V and stained with GelRed (Biotium, Canada). To verify the specificity of this SN-PCR method, the positive PCR products were purified using a gel extraction kit (Takara, Dalian, China) and then sent for sequence analyses (Invitrogen, Shanghai, China).

Enzyme-linked immunosorbent assays (ELISA)

The detection and quantitative determination of human IgG antibodies against *H. pylori* in serum was performed using an ELISA kit (Demeditec Company, Germany), according to the manufacturer's instructions.

Statistical analyses

The variables of sex, age, marital status, occupation, annual salary income, education, tobacco smoking, alcohol consumption, *H. pylori* infection, LSCC TNM staging, and tumor location were analyzed using Pearson's Chi-Square test, the Mann–Whitney *U* test and Fisher's Exact test. Binary logistic regression models were applied to account for the variables that were potential confounding factors in the relationship between *H. pylori* infection and LSCC. Results derived from the logistic regression models are expressed as Odds Ratios (ORs) with their accompanying 95 % confidence intervals (95 % CIs) and *p* values. Statistical significance was defined by a *p* value below 0.05. SPSS software (version 12.0) was used to conduct all analyses.

Results

The present study contained 156 subjects who were divided into two groups, one containing 81 patients with laryngeal cancer and the other containing 75 control subjects. There was no significant difference in the marital status between patients with LSCC and control subjects ($p = 0.24$). In contrast, annual salary income, occupation, and education level were statistically different between patients with LSCC and control subjects ($p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively). Prevalence of tobacco use and level of alcohol intake were also significantly higher in the LSCC population than in the control population ($p < 0.001$) (Table 1).

For *H. pylori* infection of laryngeal tissue, as detected by SN-PCR, the rate of infection was significantly higher in patients with LSCC than in control subjects (71.6 vs. 25.3 %, $p < 0.001$). In contrast, using ELISA to detect *H. pylori* antibodies in serum samples indicated that there was no significant difference between the two groups ($p = 0.79$) (Table 2).

In the 58 patients with LSCC who were identified by SN-PCR as being infected with *H. pylori*, 27 subjects (46.6 %) were positive for infection in normal tissue samples only, 8 subjects (13.8 %) were positive for infection in tumor tissues samples only, and 23 subjects (39.7 %) were positive for infection in both tumor and normal tissues. This analysis indicates that the occurrence of *H. pylori* infection was significantly higher in normal tissue samples compared with tumor tissue samples from patients with LSCC ($p < 0.001$) (Table 3).

From the sequence analyses data, we observed that the use of basic nucleotide BLAST alignment (Blastn) indicated that the 110 bp PCR sequences detected in the tissue samples completely matched the 16S rRNA gene of the *H. pylori* strain LPB423-04 (EU033951.1) found in GenBank.

Statistical analyses were performed to identify risk factors associated with LSCC. The application of binary logistic regression analysis was used to evaluate the individual factor of *H. pylori* infection and LSCC which suggested the association was statistically significant (crude OR = 7.43, 95 % CI [3.46, 16.13], $p < 0.001$) (Table 4). Since the variables of sex, smoking, drinking, marriage status, salary income, and education experience were not match among current data (Table 1), we applied two binary logistic regression models to adjust these risk factors that may confuse *H. pylori* infection and LSCC. The first binary logistic model included sex, age, smoking, drinking, and *H. pylori* infection. After the adjustment of these factors, *H. pylori* infection identified by SN-PCR was an independent risk factor for LSCC (adjusted OR = 7.15, 95 % CI [3.29, 15.53], $p < 0.001$). A second logistic analysis model included the subgroups within the variables of age, sex,

Table 1 Profiles of cases of laryngeal squamous cell carcinoma patients and controls in present study

	Cases		Controls		*p*
	N	%	*N*	%	
Sex					<0.001*
Male	79	97.5	56	74.7	
Female	2	2.5	19	25.3	
Age groups (years)					0.06†
≤49	9	12.2	15	18.9	
50–64	46	56.1	44	59.5	
≥65	26	31.7	16	21.6	
Marriage status					0.24‡
Never married	1	1.2	4	1.3	
Married	77	95.1	70	93.3	
Widowed	3	3.7	1	5.3	
Divorced	0	0.0	0	0.0	
Occupation status					0.004*
Blue color	27	33.3	20	26.7	
White color	15	18.5	32	42.7	
Others	39	48.1	23	30.7	
Salary income[a]					<0.001†
Low	23	28.4	5	6.7	
Medium	29	35.9	17	22.7	
High	29	35.9	53	70.7	
Education experience					<0.001†
Low	28	34.6	8	10.7	
Medium	46	56.8	46	61.3	
High	7	8.6	21	28.0	
Smoking					<0.001†
Never smokers	11	13.6	36	48.0	
Former smokers	10	12.3	11	14.7	
Current smokers					
≤182 packs/year	5	6.2	4	5.3	
≥182 packs/year	55	67.9	24	32.0	
Drinking					<0.001†
Non-drinkers	38	46.9	56	74.7	
Drinkers					
Low	1	1.2	7	9.3	
High	42	51.9	12	16.0	

* *p* value was tested from Pearson Chi-Square test,† *p* value was tested from Mann–Whitney test, ‡ *p* value was tested from Exact test

[a] Renminbi

marital status, occupation, annual salary income, education, smoking, and drinking, using the forward LR method which also indicated that *H. pylori* infection was an independent risk factor for LSCC (adjusted OR = 8.12, 95 % CI [3.07, 21.45], $p < 0.001$) (Table 4).

The potential association of *H. pylori* infection with T classification, tumor locations (supraglottic, glottic, and subglottic), and TNM staging (I, II, III, IVa, IVb) were also

Table 2 *H. pylori* infection was tested by SN-PCR and ELISA methods within case and control groups

	H. pylori positive		*H. pylori* negative		Total	*p**
	N	%	*N*	%		
SN-PCR						<0.001
Cases	58	71.6	23	28.4	81	
Controls	19	25.3	56	74.7	75	
ELISA						0.79
Cases	63	77.8	18	22.2	81	
Controls	57	76.0	18	24.0	75	

* *p* value were tested from Pearson Chi-Square test

Table 3 Colonization of *H. pylori* in normal and tumor tissues of positive *H. pylori* infection LSCC cases

	H. pylori positive		*H. pylori* negative		Total	*p**
	N	%	*N*	%		
Normal tissues	27	46.6	0	0.0	27	<0.001
Tumor tissues	8	13.8	0	0.0	8	
N and *T*[a]	23	39.7	23	100	46	

* *p* value was tested from Exact test

[a] Normal and tumor tissues

examined. All 81 cases of LSCC were analyzed and there were no statistical differences in the tumor characteristics of patients who were *H. pylori* positive compared with those who were *H. pylori* negative ($p = 0.13$, $p = 0.44$, and $p = 0.52$, respectively, for T classification, locations, and TNM staging) (Table 5).

Discussion

Half of the world's population is found to be infected with *H. pylori*, the first bacterium observed to behave as a carcinogenic microorganism. The effects of *H. pylori* on the function of gastric epithelial cells have been shown to be both indirect, through inflammation, and direct, through the induction of protein modulation and genetic mutation [12]. What has been shown to be true of the action of *H. pylori* in the gastric mucosa may also be true for other tissues of the body (e.g., the larynx).

The present study demonstrated that *H. pylori* infection in patients positively correlated with LSCC comparing with control subjects. The use of two logistic regression models to exclude the influence of potential confounding factors indicates that *H. pylori* infection is associated with a seven to eightfold increase in the risk of LSCC independent of other risk factors (Table 4). The control samples used in the study were vocal cord polyps or epiglottic cysts

Table 4 Logistic regression analyses estimating risk factors for LSCC

	Cases		Controls		p* crude OR 95 % CI	$p^{\dagger}$ adjusted OR 95 % CI	$p^{\ddagger}$ adjusted OR 95 % CI
	N	%	*N*	%			
H. pylori infection					<0.001	<0.001	<0.001
No infection	23	28.4	56	74.7	7.43	7.15	8.12
Infection	58	71.6	19	25.3	3.46–16.13	3.29–15.53	3.07–21.45

Binary logistical regression method: forward LR

* Variable included the individual risk factor with *H. pylori* infection

† Variables included age, sex, tobacco smoking, alcohol drinking, and *H. pylori* infection

‡ Variables included sex, age, marriage status, education experience, salary income, occupation status, smoking, drinking, and *H. pylori* infection

Table 5 Diverse *H. pylori* infection within T classification, TNM staging and tumor location of LSCC cases

	H. pylori positive	*H. pylori* negative	Total	*p*
T classification				0.13*
T1	4	3	7	
T2	24	12	36	
T3	21	7	28	
T4a	9	1	10	
TNM staging				0.44*
I	4	3	7	
II	17	12	29	
III	22	5	27	
IVa	14	3	17	
IVb	1	0	1	
Tumor location				0.052†
Supraglottic	22	3	25	
Glottic	35	19	54	
Subglottic	1	1	2	

* p value was tested from Mann–Whitney Rank test; † p value was tested from Exact test (2-sides)

as this is the only way to collect non-tumor tissues from the larynx in the clinic and is a recognized method that has been used previously [9, 15]. The vocal cord polyps or epiglottic cysts were confirmed to be cancer free with no evidence of epithelial dysplasia. In order to fully confirm the specificity of the SN-PCR technique, the 110 bp SN-PCR product was sequenced and analyzed. Target fragments completely matched the 16S rRNA gene sequence of *H. pylori* strain LPB423-04 (EU033951.1).

In previous research, Rezaii et al. [14] used ELISA to determine the presence or absence of *H. pylori* IgG antibodies in 105 healthy control subjects and 98 cases of laryngohypopharyngeal carcinoma and concluded that *H. pylori* is an independent risk factor for laryngohypopharyngeal carcinoma. Similarly, Titiz et al. [15] used PCR to detect genetic material from *H. pylori* in the larynx and their results suggested a possible relation of the bacterium to the development of LSCC [positive results in 17/21 (80.9 %) cancer patients vs. 0/19 (0 %) control subjects, ($p < 0.001$)]. In contrast, Nurgalieva et al. [18] used ELISA to detect antibodies to *H. pylori* in 119 patients with laryngopharyngeal cancer and 111 control subjects and concluded that *H. pylori* infection do not either protects against or promotes laryngopharyngeal carcinoma. In this study, we used SN-PCR in combination with logistic regression models to identify the strength of the relationship between *H. pylori* infection and LSCC. As a comparison, we simultaneously used ELISA on serum samples from the same patients, subjected those results to the same analysis and observed no significant relationships.

Using ELISA the ratio of positive to negative results was high in patients with LSCC and also in control subjects, an outcome that was not observed with the use of SN-PCR. Seropositivity for *H. pylori* is usually considered as an indication of current or previous infection, but the relevance of such a finding to *H. pylori* colonization of the larynx is unknown [23]. The ELISA assay was considered to have a low specificity for detection of *H. pylori* infection in larynx, as positive serological outcomes may also be related to infection of the middle ear, nasal sinus, oral cavity, or stomach. In addition, even following successful eradication of the bacteria, antibodies persist for a long duration after *H. pylori* infection, and the ratio of seropositive results increases with age. Hence, compared to ELISA, the use of PCR is a more accurate method with strong sensitivity and specificity for the detection of *H. pylori* in laryngeal mucosa.

Through its secreted proteins *H. pylori* affects the function of gastric mucosa and destroys the gastric epithelial defense [11], a process that is also likely to occur in the laryngeal mucosa [23]. The transition from normal epithelium to laryngeal carcinoma has been observed to be a lengthy, comprehensive and multistage process, possibly occurring in the face of a progressive accumulation of

genetic mutations. The selection of a population of transformed epithelium can be related to tobacco smoking, alcohol intake, the human papillomavirus, and other as yet unknown factors [3]. Consequently, a very likely scenario is that *H. pylori*, acting as a cooperative causative agent, destroys the mucosal lining of the larynx and adds to the complex process underlying the development of laryngeal carcinoma.

It has been observed that *H. pylori* is also present in stool, water, and the oral cavity, the potential routes of transmission have been proposed to include oral–oral, gastric–oral, or fecal–oral [24–26]. As there is a relationship between gastric *H. pylori* infection and the presence of *H. pylori* in the mouth it was surmised that the oral presence of *H. pylori* constitutes a permanent or transient reservoir for (re-)infection of the stomach [27]. The data in this study suggest that this microbe also exists in larynx, which ties in with the potential transmission model. Several studies have implicated that gastroesophageal reflux disease (GERD) may be a significant risk factor for laryngeal cancer, and *H. pylori* could be transmitted from the stomach to the larynx by gastric reflux [28]; however, this study excluded subjects with GERD and still detected the microorganism in laryngeal mucosa. Thereby, these data indicate that the laryngeal mucosa is also a reservoir for *H. pylori* and may be a staging place for its routes of transmission.

Taken together, the current data suggest that *H. pylori* is able to infect the mucosa of the larynx, resulting in a reservoir of the bacteria and a staging place for its transmission to other areas of the body possibly. In addition, *H. pylori* infection may be an independent risk factor for laryngeal squamous cell carcinoma. Further studies are required with larger numbers of subjects and the definition of the exact mechanisms which lead to disease.

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Conflict of interest The authors disclose no conflicts.

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