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Association between infection of virulence *cagA* gene *Helicobacter pylori* and laryngeal squamous cell carcinoma

1,2 **Paweł Krzysztof Burduk**

1 Chair of Otolaryngology and Laryngological Oncology Collegium Medium in Bydgoszcz, Nicolaus Copernicus University of Toruń, Bydgoszcz, Poland
2 Biomedical Engineering, Mechanical Engineering Department University of Technology and Life Sciences in Bydgoszcz, Bydgoszcz, Poland

Corresponding Author: Paweł K. Burduk, e-mail: pburduk@wp.pl
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Background: The aim of the study was to evaluate the presence of *cagA* gene *Helicobacter pylori* in etiopathogenesis of initiation and development of larynx squamous cell carcinoma (LSCC) and its predictable role as a prognostic factor.

Material/Methods: The prospective, controlled study involved a series of 75 patients (65 male, 10 female, mean age 59.1 years, range 43 to 79 years) with larynx cancer. Samples of larynx cancerous tissue, each of 10–15 mg, were obtained from fresh tissues and were used for nucleic acid purification. DNA was extracted from 225 samples (larynx tumor – I (75), margin of tumor and normal tissue – II (75) and normal larynx tissue from opposite side to the tumor – III). All samples were subjected to *H. pylori* ureA detection by the PCR *H. pylori* diagnostic test. Samples that were positive for ureA *H. pylori* gene were evaluated for *cagA H. pylori* gene.

Results: Presence of *H. pylori cagA* gene was identified in 46,7% to 49,3% of 75 *H. pylori* ureA gene-positive larynx cancer depending of tissue location. There was a correlation of high incidence of positive *cagA* gene in larynx cancer tissue in supraglottic versus subglottic and glottic location. We observed a predominance of *cagA* gene in LSCC in patients with positive cervical lymph nodes and clinical stage T3 and T4.

Conclusions: *H. pylori* is present in larynx tissue and may be a possible carcinogen or co-carcinogen in LSCC development, but that must be addressed by future investigations. The presence of *cagA* gene in larynx cancer tissues significantly decreases survival rate and increases the disease recurrence possibilities.

Key words: ***Helicobacter pylori*** **•** ***cagA*** **gene • laryngeal cancer • PCR • survival • recurrence**

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Background

Laryngeal squamous cell carcinoma is one of the most common malignant neoplasms of the head and neck [1]. The potentially high incidence of morbidity and incommensurably low cure rate, as expected, require searching for new diagnostic procedures and carcinogenic factors [2–4]. The most important carcinogenic agents for epithelial larynx cancer are cigarette smoking and alcohol consumption [1]. Other exogenic and endogenic factors are mostly promoters or carcinogens in multiplied carcinogenesis processes. The other factors, such as the human papillomavirus and *Helicobacter pylori* (*H. pylori*) may be etiologically involved in carcinogenesis [2,5–9]. *H. pylori* is known to be one of the most virulent bacteria worldwide [10]. The models of transmission for extra-gastric involvement are still under investigation. The possible routes of colonization are person-to-person, oral-oral, gastric-oral, and fecal-oral [5,11,12]. Several bacterial virulence factors are responsible for the pathogenicity of *H. pylori*. The most important is cag pathogenicity island (cagPAI), which encodes a CagA protein [13–16]. The presence of high oncogenic bacterial tribe of *H. pylori* in larynx tissue is considered to be a potential carcinogenic agent. *H. pylori* with expression of CagA protein coded by *cagA* gene have the ability to developed gastric or duodenal ulcer and greater expression of atrophic inflammation or gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma (MALT) [17–21]. However, the presence of *H. pylori* in extra-gastric MALT lymphoma has not been demonstrated [22]. CagA protein is a well known bacterial oncoprotein in human and animal models [3,11,13].

The aim of the study was to evaluate the presence of cag A gene *H. pylori* in etiopathogenesis of LSCC and its relationship with clinical, pathological, and prognostic factors.

Material and Methods

The prospective, controlled study involved a series of 75 patients with LSCC, undergoing total laryngectomy at the Department of Otolaryngology and Laryngological Oncology, Collegium Medicum, Nicolaus Copernicus University. The study was approved by the ethics review board of the Nicolaus Copernicus University. Informed consent was obtained from all subjects.

For LSCC, a total 75 patients (65 male, 10 female, mean age 59.1 years, range 43 to 79 years) were included in the study. The staging system for tumor, regional lymph nodes, and distant metastasis (TNM) classification was consistent with the International Union Against Cancer (UICC) criteria [23]. Histological grading was performed according to the World Health Organization (WHO) criteria: well differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3). Patients with LSCC were treated with adjuvant postoperative radiotherapy (50–60 Gy). The follow-up period was 3 and 5 years. The clinicopathological and epidemiological features of LSCC patients and *cagA* gene *H. pylori* are shown in Table 1. All the subjects had similar educational level and material status. The patients who had previous endolaryngeal surgery and were on antibiotics, bismuth-containing drugs, H_2 receptor blockers, proton pump inhibitors, or antacids 4 weeks before the surgery were excluded from the study. Samples were obtained during total laryngectomy under general anaesthesia. All biopsy specimens were also evaluated by a pathologist to confirm the diagnosis.

Sample preparation and nucleic acid extraction

Samples of 10–15 mg obtained from fresh tissues were used for nucleic acid purification. DNA was extracted from 225 samples (larynx tumor – I (75), margin of tumor and normal tissue – II (75) and normal larynx tissue from opposite side of the tumor - III (75) using commercial Genomic DNA-Prep Plus (A&A Biotechnology, Gdansk, Poland) extracting kit according to the manufacturer's recommendations [24]. During sample collection and preparation, great care was taken to avoid contamination. Extracted DNA was stored at –20°C for up to 2 weeks prior to PCR analysis.

Polymerase chain reaction for detection of *H. pylori* ureA and *cagA* genes

All samples were subjected to *H. pylori* ureA detection by the PCR *Helicobacter pylori* diagnostic test (DNA – Gdańsk II, Poland). The lower limit of detection (LOD) of this assay is 1×10^3 cfu/ml. The amplification was carried out according to the manufacturer's recommendations. The 315-bp PCR products were analyzed on 2% agarose gel containing ethidium bromide and visualized with UV light. As a negative control, a reaction mixture without DNA was included in the experiment. Because of the lack of positive control in the PCR *Helicobacter pylori* diagnostic test, sequencing of 1 obtained 315-bp product was performed (Figure 1). The comparison between the query sequence and those deposited in the GenBank database was carried out using the BLAST program available from the National Centre for Biotechnology Information (*http://www.ncbi.nlm.nih.gov*). The interpretation of the sequence data confirmed that the amplified fragment of DNA was homologous to *H. pylori* ureA gene. DNA that contained the target sequence was included as a positive control to prove the functionality of the reaction mix for amplification of the ureA gene.

Samples that were positive for ureA *H. pylori* gene were evaluated for *cagA H. pylori* gene. This was accomplished by the PCR *Helicobacter pylori* CagA$^+$ diagnostic test (DNA – Gdańsk II,

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Table 1. Epidemiological and clinicopathological features of 75 patients with laryngeal cancer and *cagA* gene *Helicobacter pylori* using PCR.

Epidemiological and clinicopathological features	*cagA* gene-positive				*cagA* gene-negative				Statistical significance
	I No	II No	III No	I+II+III No	I No	II No	III No	I+II+III No	
Sex									
Men	30	31	31	32	35	34	34	33	p<0.7684
Women	5	6	4	6	5	4	6	4	
Age									
≤60 years	19	20	19	20	21	20	21	20	p<0.3328
>60 years	16	17	16	18	19	18	19	17	
Smoking									
Yes	35	37	35	38	39	37	39	36	p<0.9892
No	0	0	0	0	1	1	1	1	
≤20 cigarettes/day	28	30	27	30	27	25	28	25	p<0.3160
>20 cigarettes/day	7	7	8	8	12	12	11	11	
Alcohol abuse									
No	11	12	10	12	7	6	8	6	p<0.0724 heavy *vs.* sporadic+no abuse
Sporadic	11	11	10	11	18	18	19	18	
Heavy drinker	13	14	15	15	15	15	14	13	
Localization									
Glottic	7	8	8	8	16	15	15	15	p<0.0096 supraglottic *vs.* glottic
Supraglottic	23	24	23	25	24	23	24	22	
Subglottic	5	5	4	5	0	0	0	0	
T stage									
T2	3	3	3	3	4	4	4	4	p<0.9010 T3 *vs.* T4
T3	23	25	24	26	28	26	27	25	
T4	9	9	8	9	8	8	9	8	
N stage									
N0	8	9	8	10	15	14	15	13	p<0.4071
N+	27	28	27	27	25	24	25	25	
N1	5	5	5	6	5	5	5	4	p<0.2160
N2	2	3	2	3	5	4	5	4	
N3	1	1	1	1	5	5	5	5	
Clinical stage									
II	3	3	3	3	4	4	4	4	p<0.8015
III	22	23	22	23	22	21	22	21	
IV	10	11	10	8	14	13	14	10	
Histology grade									
G1	2	2	2	2	7	7	7	7	p<0.1694
G2	29	31	29	32	29	27	29	26	
G3	4	4	4	4	4	4	4	4	

I – larynx tumour; II – margin of tumour and normal tissue; III – normal larynx tissue from opposite side to the tumour.

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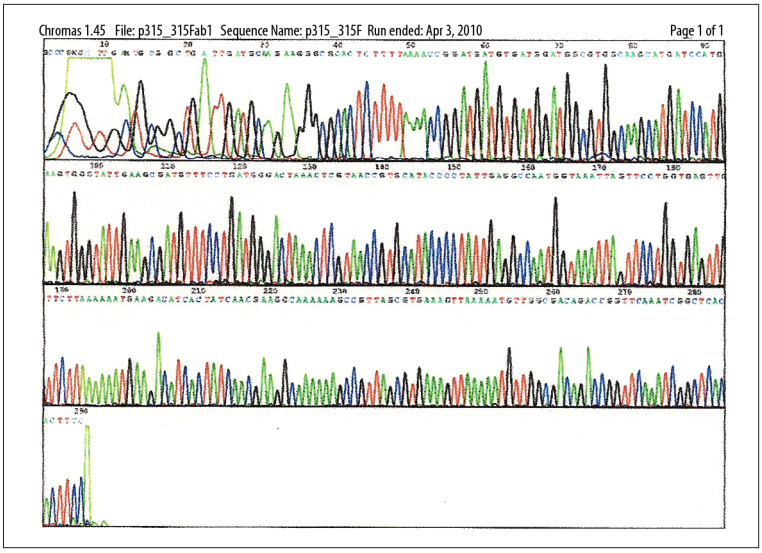

Figure 1. The results of sequencing of gene ureA *H. pylori* (315-bp) for confirmation of obtained amplification product.

Poland). The LOD of this assay is 5×10^3 cfu/ml. DNA amplification was carried out according to the manufacturer's recommendations. All of the PCR assays contained positive and negative controls. The 445-bp PCR product was analyzed by electrophoresis on 2% agarose gel.

The amplification was performed in the GeneAmp PCR System 2700 Thermal cycler (Applied Biosystem).

Statistical analyses employed the following tests according to specific requirements: the Mann-Whitney test, Fisher's test, and paired-samples t-test. Survival analysis was undertaken using the Kaplan-Meier method. For all, a significance level was set at $p < 0.05$. Data analyses were performed by Statistica version 8.0 (StatSoft, Poland).

Results

H. pylori DNA (ureA gene) was detected by PCR method in all locations of LSCC patients (100% of samples). The electrophoregram for positive ureA gene 315-bp is presented in Figure 2.

Presence of *H. pylori cagA* gene (Figure 1) was identified in 35 (46.7%) of 75 *H. pylori* ureA gene-positive larynx tumor (I) and normal larynx tissue from the opposite side to the tumor (III) and in 37 (49.3%) of 75 positive *H. pylori* ureA gene in margin samples of the tumor and normal tissue (II). A statistically significant dependence of *cagA* gene positive and negative was revealed among LSCC ($p<0.0275$).

Twenty-three (65.7%) LSCC samples of I localization, 24 (64.9%) of II localization, and 23 (65.7%) of 35 of III localization with positive *cagA* gene were localized in the supraglottic region of the larynx. Seven (20%) LSCC samples of I localization, 8

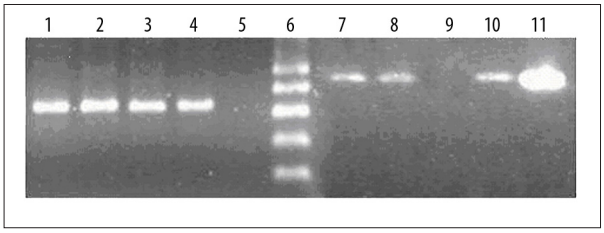

Figure 2. Amplification products obtained using the PCR *Helicobacter pylori* diagnostic test (lanes 1–4) and the PCR *Helicobacter pylori* CagA+ diagnostic test (lanes 7–11). Lanes: 1 – ureA positive control, 2–4, 7–10 – tested DNA samples, 5 – negative control, 6 – M100–500 DNA size standard (DNA – Gdańsk II, Poland), 11 – *cagA* positive control.

(21.6%) of II localization, and 8 (22.9%) of III localization positive for the *cagA* gene were localized in the glottic region of the larynx. Five (14.3%) LSCC samples of I localization, 5 (13.5%) of II localization, and 4 (11.4%) of III localization positive for the *cagA* gene were localized in the subglottic region of the larynx. We found a correlation of high incidence of positive *cagA* gene in larynx cancer tissue in supraglottic versus subglottic and glottic localization ($p<0.0096$). We observed a positive detection of *cagA* gene from 2.1% to 8.6% for T2, from 65.7% to 68.6% for T3 and from 22.8% to 25.7% for T4 stages. We recorded an increase of *cagA* gene presence in LSCC in patients with positive cervical lymph nodes (N+): 27 (77.1%) of group I *vs.* 28 (75.6%) of group II *vs.* 27 (77.1%) of group III compared to negative lymph nodes (N0) (8 (22.9%) *vs.* 9 (24.3%) *vs.* 8 (22.9%)). Most of the patients with LSCC and positive *cagA* gene were at clinical stage III (62.2–62.8%) and IV (28.6–29.7%). Fewer were at clinical stage II (8.1–8.6%) and we did not observe clinical stage I in our patients.

Twenty-nine (82.9%) of group I and III and 31 (83.8%) of group II samples with positive *cagA* gene were classified as G2. Four

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Table 2. Recurrence and presence of *cagA* gene *Helicobacter pylori* of 75 patients with laryngeal cancer.

		Recurrence		Statistical significance
		Present – N (%)	Absent – N (%)	
cagA I	+	17 (22.6%)	18 (24.0%)	p<0.5982
	–	17 (22.6%)	23 (30.8%)	
cagA II	+	17 (22.6%)	20 (26.8%)	p<0.9162
	–	17 (22.6%)	21 (28.0%)	
cagA III	+	16 (21.3%)	19 (25.3%)	p<0.9505
	–	18 (24.0%)	22 (29.4%)	
cagA I+II+III	+	17 (22.6%)	17 (22.6%)	p<0.9162
	–	21 (28.0%)	20 (26.8%)	

(+) – *cagA* gene positive; (–) – *cagA* gene negative; I – larynx tumour; II – margin of tumour and normal tissue; III – normal larynx tissue from opposite side to the tumour.

Table 3. Type of recurrence and presence of *cagA* gene *Helicobacter pylori* of 75 patients with laryngeal cancer.

		Type of recurrence			Statistical significance
		Local N (%)	Nodal N (%)	Local and nodal N (%)	
cagA I	+	4 (11.7%)	7 (20.6%)	6 (17.7%)	p<0.3571
	–	3 (8.8%)	11 (32.4%)	3 (8.8%)	
cagA II	+	4 (11.7%)	7 (20.6%)	6 (17.7%)	p<0.3571
	–	3 (8.8%)	11 (32.4%)	3 (8.8%)	
cagA III	+	4 (11.7%)	6 (17.7%)	6 (17.7%)	p<0.2139
	–	3 (8.8%)	12 (35.3%)	3 (8.8%)	
cagA I+II+III	+	4 (11.8%)	7 (20.6%)	6 (17.6%)	p<0.3571
	–	3 (8.8%)	11 (32.4%)	3 (8.8%)	

(+) – *cagA* gene positive; (–) – *cagA* gene negative; I – larynx tumour; II – margin of tumour and normal tissue; III – normal larynx tissue from opposite side to the tumour.

(11.4%) of group I and III and 4 (10.8%) of group II were classified as G3 and only 2 patients were at G1. All the patients were cigarette smokers. We recorded a presence of cag A gene from 77.1% to 81% among patients who had smoked 20 cigarettes per day and approximately 19% to 22.9% in heavy smokers (over 20 cigarettes/day). Eleven (31.4%) of 35 group I patients with positive *cagA* gene were non-drinkers, 11 (31.4%) consumed alcohol occasionally, and 13 (37.2%) frequently. We observed a higher incidence of positive *cagA* gene in group I among non-drinking patients or frequent drinkers (31.4% to 37.2%) compared to those who declared occasionally drinking (31.4%), (p<0.0817). Twelve (32.4%) of group II patients with positive *cagA* gene were non-drinkers, 11 (29.7%) occasionally drank, and 14 (37.8%) drank frequently. Among this group, we observed relatively higher presence of *cagA* gene among patients with no alcohol consumption *vs.* occasionally and frequent consumption (p<0.0915). In the third group with positive *cagA* gene, 10 (28.6%) patients declared no alcohol consumption, 10 (28.6%) were occasional drinkers, and 15 (42.8%) declared heavy drinking. Patients who were heavy alcohol drinkers had higher incidence of positive *cagA* gene compared to occasional drinkers and non-drinkers (p<0.0724).

No significant correlation was found between the incidence of *cagA* gene presence and the histological and clinical stage of tumors. Univariate analysis of the association between the presence of *cagA* gene in all localizations of LSCC and recurrence revealed a correlation with high incidence of recurrences at 3-year follow-up; however, the correlation was not statistically significant (Table 2). We observed more frequent regional nodal recurrence *vs.* regional or nodal and regional together (Table 3). The difference between 3-year and 5-year survival rates to the deaths for all reasons between the *cagA*-positive and *cagA*-negative groups in all tissues localisation was not significant. Nerveless, we observed a lower survival rate in all patients with positive *cagA* gene. The differences in

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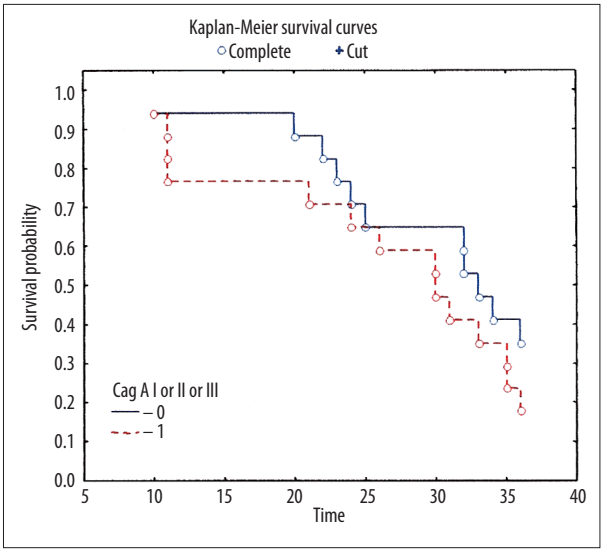

Figure 3. Analysis of survival rate of patients with positive *cagA* gene and recurrence in 3-year time.

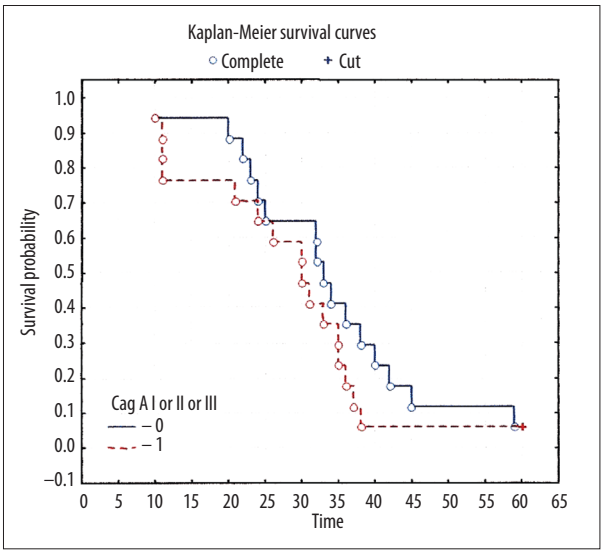

Figure 4. Analysis of survival rate of patients with positive *cagA* gene and recurrence in 5-year time.

3-year and 5-year survival rates and deaths due to neoplasms between the groups of *cagA*-positive and *cagA*-negative in all samples was not significant at 3-year follow-up and demonstrated a significant correlation in 5-year follow-up ($p<0.0058$).

Out of 75 patients with LSCC, 46 (61.3%) survived for 3 years and 39 (51.9%) survived for 5 years. Univariate analysis of the association between 3- and 5-year survival rate of patients with positive *cagA* gene and recurrence had worse survival prognosis compared to those with negative *cagA* gene (Figures 3 and 4)

Multivariate (Cox's proportional hazards) analysis of 3- and 5-years survival rate and deaths due to neoplasms showed a

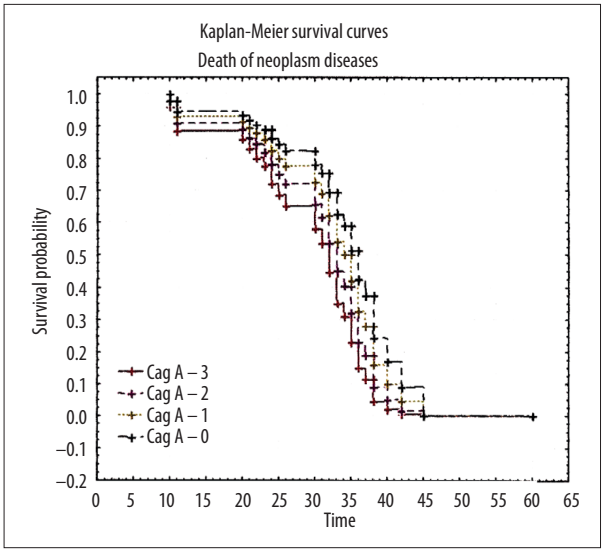

Figure 5. Analysis of survival rate and deaths of neoplasm disease in 5-year time among patients with *cagA* gene in all tissues localizations.

significant decrease in 5-year survival among patients with *cagA* gene presence in all tissues localizations ($p<0.0069$) (Figure 5)

Discussion

The role of *H. pylori* in the pathogenesis of chronic diseases and carcinoma of the digestive tracts is well known [10,11]. The potential for destroying mucosal and epithelial barriers and inflammation could cause chronic injury and epithelial cell proliferation leading to larynx pathology [4,25]. The *cagA* protein, a major *H. pylori* virulence factor, demonstrates carcinogenic activity and could be involved in LSCC development [8,25,26]. Multiple studies have evaluated the presence and correlation of *H. pylori* with laryngeal disorders. Sensitivity of the tests used in studies can be insufficient and false-positive as well as false-negative results can be obtained [4,25]. There are studies suggesting a possible role of *H. pylori* in the LSCC, as well as denying them. There are many serological studies relating *H. pylori* and laryngeal disorders, but results and conclusions vary [27,28,30]. The fast urease test, immunohistochemical methods, light microscopy, and tissue cultures can also be false-positive, as well as false-negative [7,31,32]. The best method for detection of *H. pylori* in larynx tissue is PCR with high sensitivity and specificity [29].

We have detected *H. pylori* ureA gene (315-bp) by PCR method in all our cases. The high percentage of positive ureA gene *H. pylori* is because nearly 100% of the Polish population is infected with *H. pylori*. To determine the presence of *cagA H. pylori* gene, the major virulence factor, we used PCR method as well. We detected *cagA* gene (445-bp) in 46,5% to 49,3% of LSCC.

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The differences in detection rates were statistically significant (p<0.0275). In an attempt to associate *H. pylori* with LSCC, Titiz et al employed PCR (targeted a 446-bp of the 16rRNA gene) to revealed that 80.9% of the patients had laryngeal carcinoma, but did not observe *H. pylori* DNA with benign laryngeal pathologies [23]. Grbesa et al employed PCR (410 and 361-bp target for ureA gene) and found *H. pylori* in 26% of LSCC patients [8]. An investigation similar to ours was done by Ozurt et al, who identified *cagA* gene of *H. pylori* (128-bp target for *cagA* gene) in 78.9% of nasal polyps, 89,5% of normal nasal mucosa, and 82.4% of larynx samples (9 cases of LSCC) [25].

To our knowledge, ours is the first study to identify the presence of *cagA* gene *H. pylori* in large samples of LSCC tissue. We found a relatively high presence of *cagA* gene in larynx cancer tissues, which suggests its possible role as a carcinogenic agent. This is also the first comparison between and analysis of presence of *cagA* gene *H. pylori* in LSCC and relationship with clinical, pathological, and prognostic factors. We observed more expressed presence of *cagA* gene *H. pylori* in supraglottic tumors (64.7–65.7%) compared to glottic (20–22.9%) and subglottic (11.4%–14.3%) tumors. The high incidence of *H. pylori* presence in supraglottic tumor localisation was also confirmed by another study using serologic methods [27]. The predilection for supraglottic localization could be promoted by coexistence of gastroesophageal reflux [4,9] because the acid injury of the tissue and lower pH could create a better microenvironment for bacteria [4,12]. Our analysis also showed that *cagA* gene *H. pylori* was more frequently observed in higher clinical stages of LSCC (T3 and T4 *vs.* T2) and with nodal metastasis. This could be the reason why *H. pylori* can increase proliferation and inhibit cell apoptosis and, hence, may be correlated with carcinogenesis and progression of the tumor [4,16,17,29], but this mechanism remains to be investigated in future studies. We did not find any significant correlation between presence of *cagA* gene in LSCC and smoking, alcohol consumption, and histopathology grading of the tumor. However, there was a trend for higher *cagA* gene presence among heavy alcohol drinkers compared to occasional drinkers and non-drinkers. Our study is the first describing the analysis of *cagA* gene presence in LSCC and the recurrence and survival rate. We observed nodal recurrence more frequently in patients with *cagA* gene at 3-year follow-up. The analysis of 3- and 5-year survival rate to deaths due to all reasons between the groups with and without *cagA* gene was not significant. However, we observed a lower survival rate among patients with the *cagA* gene. A significant correlation (p<0.0581) was found in 5-year survival rate and deaths due to neoplasms. The group o LSCC and *cagA* gene had lower survival rate. Patients with recurrence of the disease and positive *cagA* gene had worse survival prognosis.

Conclusions

Our results confirmed the presence of *Helicobacter pylori* in squamous cell carcinoma of the larynx. *H. pylori* infection may be a possible carcinogen or co-carcinogen in LSCC development. The presence of the *cagA* gene in larynx cancer tissues significantly decreases patient survival rate and increases possibility of disease recurrence. Analysis of the *H. pylori cagA* gene in larynx cancer tissue could be a predictable prognostic factor in follow-up.

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