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The presence of *Helicobacter pylori* in the larynx pathologies

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Abstract

Objective: The well-known relation of *Helicobacter pylori* to the MALT-lymphoma and gastric carcinoma suggested a possible presence in the laryngeal tissues of patients with squamous cell carcinoma of the larynx by using Polymerase Chain Reaction (PCR) investigations, and PCR results of benign laryngeal pathologies were compared.
Methods: Polymerase Chain Reaction investigations were carried out in biopsy samples of 21 patients with squamous cell carcinoma of the larynx and of 19 patients with benign laryngeal pathologies like vocal polyp and nodules.
Results: The PCR results of 17 out of the 21 samples (80.9%) of the patients with laryngeal squamous cell carcinoma were positive for *H. pylori*. Moreover, any genomic material of *H. pylori* was not found in the PCR results of the 19 patients with benign laryngeal pathology. It was also determined that the presence of the *H. pylori* in the patients with laryngeal squamous cell carcinoma was statistically significant ($p = 0.0001$).

In the patients with laryngeal squamous cell carcinoma, the comparison of the *H. pylori* presence within the normal and tumoral tissues revealed that 16 normal tissue samples (76.19%) were positive, while 9 of the tumoral tissue samples (42.85%) were positive. The ratio of bacterial presence in both the normal and tumor tissue samples was 38.09% (8 patients). The rates of presence revealed a statistically significant difference between the normal and tumoral tissue samples ($p = 0.039$).
Conclusions: The presence of the genomic material of *H. pylori* within the laryngeal tissue of patients with squamous cell carcinoma of the larynx is a proof of the colonization of the bacterium in that tissue. While this may suggest a possible relation of the bacterium to the development of laryngeal squamous cell carcinoma, no conclusion is possible to be drawn about the mechanism of the process.

Keywords: *Helicobacter pylori*; Larynx; PCR

1. Introduction

The squamous cell carcinoma of the larynx is the most frequent malignancy encountered in the upper aero-digestive system. Larynx carcinoma comprises 25% of the cancers of the head-and-neck region, and 2–3% of all body cancers, and approximately 1% of all newly diagnosed cancers [1]. Though important developments have evolved in the treatment of the disease nowadays, early diagnosis increases the chances of therapy and thus, affects positively the duration and quality of life. Smoking and alcohol are the two major risk factors in the development of laryngeal carcinoma. Besides, some viruses, bacteria, radiation exposure, gastroesophageal reflux, genetic predisposition, diet and occupational factors are considered to comprise to the multifactorial mechanism in laryngeal carcinogenesis. *Helicobacter pylori* (*H. Pylori*) is one of those factors and is known for its close relation to the carcinogenesis in gastric cancer and mucosa associated lymphoid tissue lymphoma (MALT-lymphoma). *H. pylori* was approved as a Type-1 carcinogen by WHO in 1994 [2]. Considerations about *H. pylori*'s effects for predisposition to carcinoma of the upper aero-digestive tracts by destroying mucosal and immune barriers and thus, facilitating exposure to carcinogens, are under investigation [3].

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Though there are multiple studies in the medical literature showing the presence of *H. pylori* within laryngeal cancer tissue, the tests specificities used in those are considered to be insufficient and false-positive results are to be considered [4].

In this study, the presence of *H. pylori* within laryngeal carcinoma tissue and the comparison of them with the benign laryngeal pathologies were investigated using the Polymerase Chain Reaction (PCR) technique, which can produce more specific and definitive results, and the relation of this microorganism to the carcinogenesis of laryngeal carcinoma was discussed.

2. Material and methods

Between the December 2004 and January 2007 time period, biopsy materials obtained from, both benign laryngeal pathologic tissues and laryngeal carcinoma tissues of 40 patients were preserved in 1.5 ml Eppendorf test tubes containing bovine brain-heart infusion broth and transported to the Microbiology Department. 21 patients with laryngeal carcinoma were included to the study group. Of the 11 specimens were obtained from laryngectomy patients, and 10 were obtained during diagnostic direct laryngoscopic interventions. In the patients with laryngeal carcinoma biopsy materials were taken both from the tumoral tissue and from the healthy laryngeal mucosa far away from the tumoral area. Normal tissue specimens were obtained using different surgical instruments to avoid contamination with tumoral tissues. Tumoral and normal tissues were preserved in different test tubes for each patient. For laryngectomised patients, specimens were obtained from the most obvious tumoral tissue regions, and normal tissue specimens were obtained from the most distant part from the tumor regions upon inspection of the tissue, using through-cut technique. Direct laryngoscopic specimens were obtained from the tumoral tissue, and normal tissue specimens were obtained from the most distant part of the larynx from the tumor upon inspection and without distortion of the normal laryngeal anatomy and function, using punch-biopsy technique.

In the control group, 12 patient with vocal cord polyp and 7 patients with vocal cord nodule, totally of the 19 patients with benign laryngeal pathology were selected for the investigation of the pathologic tissue excisions that were performed by direct laryngoscopy.

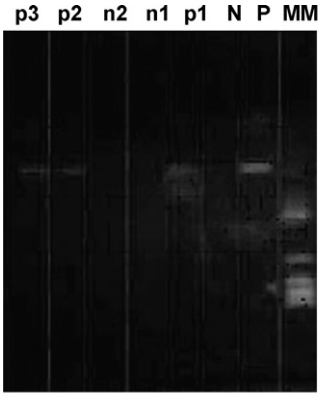
This study is approved by the Institutional Review Board.

The specimens were inoculated on Brain Heart Infusion agar (Oxoid), containing 7% horse blood and *H. pylori* selective supplement (Oxoid-SR 147E) and then incubated under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37 °C for until 7 days. Gram's stain, catalase, oxidase and urease tests were performed for suspected colonies.

2.1. PCR amplification

The specimens preserved in Tris EDTA at −20 °C were used for PCR amplification. Chromosomal DNA was extracted from all samples by the cetyltrimethyl-ammonium bromide (CTAB) method according to the DNA miniprep protocol of Wilson [5,6]. This method is known to remove complex polysaccharides which may inhibit PCR amplification. The HP1 (5′ CTG GAG AGA CTA AGC CCT CC 3′) and HP3 (5′ AGG ATG AAG GTT TAA GGA TT 3′) primers (Metis Biotecnology Ltd., Ankara) that amplify 446 bp fragment of 16S rRNA gene of *H. pylori* were used. After the initial reaction HP1 and HP2 (5′ ATT ACT GAC GCT GAT TGT GC 3′) primers (Metis Biotecnology Ltd.) were used for the amplification of the nested 109 bp fragment [6]. Twenty-five microliters Master mix was prepared using H_2O: 16.5 μl, 10× buffer: 2.6 μl, Mg: 1.5 μl, dNTP: 0.2 μl, Primer 1: 0.25 μl, Primer 2: 0.25 μl, Taq DNA Polymerase: 0.13 μl, DNA: 2 μl. (10). Thermal Cycler Program for PCR amplification was; 2 min at 95 °C, 30 s at 94 °C, 30 s at 55 °C, 6 min at 72 °C. All of the so produced PCR products (10 μl of each aliquots) were electrophoresed in 2% agarose gels with 1× TBE running buffer at 100 V for 4 h. A Φ × 174 Hae molecular marker was used as a size marker to calculate the absolute lengths of the bands in individual sample lanes in all gels. After the gels were stained with ethidium, the bands were examined by ultra violet products (UVPs) white/UV transluminator and Grab-IT 2.0 annotating grabber programme.

H. pylori National Collection of Type Cultures (NCTCs) 11,637 was used as a positive control in all PCR reactions and cultures. The microbiologists were blinded to the pathology and clinical status of the patients. The gel electrophoresis of the 109 bp PCR product of *H pylori* is shown in laryngeal carcinoma (Picture 1).



Picture 1. The 109 bp 16 S rRNA of *Helicobacter pylori* is viewed in the p1, p2, p3 bars of electrophoresis in the patients with laryngeal carcinoma.

Table 1
The patient profiles

	Cigarette smokers (*n*) (%)	Average smoking duration (year)	Age (year) (mean)
Laryngeal carcinoma	21/21 (100)	27	44–72 (56.7)
Benign laryngeal pathology	15/19 (80)	24	33–56 (47.6)

Table 2
The presence of *H. pylori* in the patients with laryngeal carcinoma

	PCR positive cases	
	n	(%)
Tumoral tissue	1/21	(4.76)
Normal tissue	8/21	(38.09)
Tumoral + normal tissue	8/21	(38.09)
Total	17/21	(80.9)

In the study group, the McNemar test was used for the statistical evaluation of the relationship between the normal and tumoral tissues produced with PCR. The relation between the results of all the study group specimens and the results of the control group were compared with the significance of the difference between two independent proportions. Also the relation between the results of only the tumoral subgroup of the study group and the results of the control group were compared with the significance of the difference between two independent proportions.

3. Results

All patients were males, ages were between 33 and 72 years, mean age of 52.4 years. All the patients with laryngeal carcinoma and 80% of the patients with benign laryngeal pathology were cigarette smokers, and average smoking duration was 26 years, equivalent to 15 cigarettes/day (Table 1). None of our patients in the study or the control group had the habit of alcohol consumption. None of them complained of gastric ulcer pain, vomiting, pyrosis, and none of them was on antireflux therapy. Of the patients with laryngeal carcinoma supraglottic tumor was diagnosed in 8, glottic tumor in 3, and 10 patients had a tumor disseminated transglottically. Subglottic tumor extension was diagnosed in 5 of the patients. Staging of tumors was as follows: T1–3 patients (14.2%), T2–10 patients (47.6%), T3–4 patients (19.1%), and T4–4 patients (19.1%).

PCR results for *H. pylori* genetic material were positive in 17 (80.9%) of the 21 patients with laryngeal carcinoma. Of those, 8 (38.09%) were positive in normal tissue, 8 (38.09%) were positive both in tumor and normal tissue, and 1 (4.06%) was positive in tumor tissue only (Table 2). None of the tissue cultures proved positive for *H. pylori*.

Staging of the 8 patients in which *H. pylori* was positive in normal tissue only revealed the following: T1a–1, T2–5, T3–1, T4–1. The single patient with *H. pylori* positivity in tumor tissue only, was stage T2. Staging of the 8 patients in which *H. pylori* was positive in both normal and tumoral tissues revealed the following: T1b–1, T2–3, T3–2, T4–2. Evaluation of *H. pylori* positive all 17 patients revealed that 11 patients (64.8%) were stage T1 and T2. From the 16 *H. pylori* positive normal tissue samples 2 were stage T1, 8 were stage T2, 3 were stage T3 and 3 were stage T4. Out of the 16 normal tissue *H. pylori* positive patients 10 (62.5%) were of the T1 and T2 stages, considered to be early stages (Table 3).

In the PCR investigation results of the control group patients with chord vocal polyp or nodule any *H. pylori* genomic material was not found.

The statistical evaluation of the relation between bacterial presence within normal and tumor tissues of patients with laryngeal squamous cell carcinoma reveals that out of 5 patients whose normal tissue samples were negative for bacterial presence, 4 (80%) had negative tumor tissue samples, as well. Out of the 16 patients with positive normal tissue samples, 8 (50%) had positive tumor tissue samples, too; and 8 (50%) had negative tumor tissue samples. As a result, a statistically significant difference ($p = 0.039$) was seen concerning *H. pylori* genomic material presence in tumor and normal tissues of patients.

There was no statistically significant relation between bacterial presence either normal or tumoral tissues and the stage of the tumor ($p = 0.429$).

When the presence of the *H. pylori* in only the tumoral subgroup of the study group and the results of the control group were compared a statistically meaningful difference was found ($p = 0.0001$). Also, when the results of all the specimens in the study group and the results of the control group were compared it was determined that the difference was statistically meaningful ($p = 0.0001$).

Table 3
Stage of the tumor of the PCR positive cases with laryngeal carcinoma

	T1	T2	T3	T4	Total
PCR (+) cases in only normal tissues	1	5	1	1	8
PCR (+) cases in the normal and tumoral tissues	1	3	2	2	8
PCR (+) cases in only tumoral tissues	1	–	–	–	1
Total	3 (17.6%)	8 (47.2%)	3 (17.6%)	3 (17.6%)	17 (100%)

4. Discussion

It is stated that about 70% of the normal population is seropositive for *H. pylori* and can remain asymptomatic throughout life [7,8]. *H. pylori* is also known to colonize in the gastric mucosa causing chronic gastritis and following this, diffuse and intestinal type of gastric adenocancer and MALT-lymphoma [4]. *H. pylori* was shown to induce apoptosis and cellular proliferation of the gastric mucosa in the early phases of the chronic inflammation and during the process of malignant transformation apoptosis is inhibited and cellular proliferation increases progressively. Increased gastric epithelial cell proliferation secondary to *H. pylori* infection is one of the necessary factors for the process of carcinogenesis [4,9]. Similarly, in the process of laryngeal carcinogenesis, the epithelial cell proliferation is shown to be increased by the Human Papilloma Virus (HPV) which is suggested to be an etiologic agent in the epidemiology of the laryngeal carcinoma. Beside other factors contributing to laryngeal carcinogenesis like cigarette smoking, alcohol, and gastroesophageal reflux, *H. pylori* is suggested to colonize in the laryngeal mucosa and like in the case of the gastric mucosa, may cause increased epithelial cell proliferation and lead, thus, to carcinoma of the larynx [4,1].

There are literature studies suggesting a possible contribution of *H. pylori* in the laryngeal carcinoma considering the mentioned mechanisms, as well as studies denying such a relation.

There are many serological studies relating *H. pylori* and laryngeal cancer, but their results are both supporting, as well as opposing each other. In their clinical studies Grandis et al. investigated 21 head-and-neck region cancer cases (9 of those being laryngeal cancers) and 21 healthy controls for *H. pylori* seropositivity and found similar results in both groups (57 and 62%), respectively [10]. Rubin et al. conducted a similar study and found that 63% of the 61 cases with head-and-neck region carcinomas or severe laryngeal dysplasia were positive for *H. pylori* antibodies and suggested that the bacterium could have reached and colonized the larynx via gastroesophageal reflux [11].

Aygenc et al. conducted a controlled study with 26 squamous cell laryngeal carcinoma patients identifying *H. pylori* presence serologically: the IgG antibodies in 73% of the 26 laryngeal cancer patients, and 40% of the 32 control group patients were serologically positive. This difference was statistically significant and the authors suggested that *H. pylori* infection could eventually play a role in laryngeal carcinogenesis by causing mucosal damage ($p < 0.05$) [7]. However, this study has not considered the bacterial colonization of the gastric mucosa, which might have caused serological positivity in both groups.

Nurgalieva et al. investigated the serological *H. pylori* positivity in laryngopharyngeal carcinoma patients and suggested a different hypothesis: ''*H. pylori* causes corpus gastritis and this leads to a decrease in gastric acid production, which may have a preventive role against gastroesophageal reflux complications, including distal esophageal adenocarcinoma. The same may be true in preventing development of laryngopharyngeal carcinoma.'' The *H. pylori* IgG positivity of the laryngeal carcinoma group was 32.8%, while being 30% in the control group and there was no statistically significant difference between the two groups. Thus, they suggested that: ''There is no relation between *H. pylori* infection and laryngopharyngeal squamous cell carcinomas, but serological *H. pylori* positivity in carcinoma cases is 1.5 times more when compared with the serologically negative group. Thus, laryngeal carcinoma predisposes to *H. pylori* infection, so *H. pylori* is an opportunistic infection rather than being a carcinogenic factor'' [3].

In the literature, there are few studies showing direct bacterial presence in laryngeal tissue. One of those studies was conducted by Borkowski et al., investigates *H. pylori* infection in chronic laryngitis using only the fast urease test, but without applying histological methods, tissue cultures, immunohistochemical methods or PCR. In 6 out of the 35 chronic laryngitis patients included in the study test results were positive. Besides *H. pylori*, there are several other urease activity possessing agents colonizing in laryngeal tissue (some *Campylobacter* sp., *Corynobacterium* sp., *Haemophylus* sp., *Streptococcus salivarius*, *Candida* sp., *Klebsiella pneumonia* and *Proteus*). Thus, urease positivity is not specific enough to prove the presence of *H. pylori* in laryngeal tissue [4,12].

Debates are present on the issue of showing *H. pylori* presence in laryngeal tissue using immunohistochemical methods. Akbayir et al. investigated *H. pylori* presence in laryngeal tissue of 50 squamous cell carcinoma patients and 50 patients with benign laryngeal lesions using histopathological and immunohistochemical methods. No *H. pylori* presence was shown in the tissues of patients with benign laryngeal lesions. However, in 28 laryngeal carcinoma patients *H. pylori* was identified under light microscopy. Unfortunately, immunohistochemical evaluation of the samples failed to provide *H. pylori*-specific staining. It is well known that *H. pylori* is spontaneously eradicated from gastric cancer tissue and the surrounding atrophic mucosa. The same may be true for the absence of *H. pylori* in laryngeal carcinoma tissues. The investigators stated that the absence of *H. pylori* in laryngeal carcinoma tissue suggests lack of its role in the carcinogenesis; and absence in benign laryngeal tissue suggests lack of bacterial colonization [4].

The results of all these studies suggest that serological tests, light microscopy, fast urease test or immunohistochemical tests may provide false-positive results for *H. pylori* presence in upper aero-digestive tissues including the larynx. Thus, this is the most proper way to show the *H. pylori* presence in tissues is culturing and PCR.

As far as we know, there are no studies in the literature in which PCR technique was used to show the presence of *H. pylori* within the laryngeal tissue. In our study, however, in the PCR results genomic material of *H. pylori* was not

present in the patients with benign laryngeal pathology and *H. pylori* was positive in the laryngeal tissues of the 80.9% of the patients with larynx carcinoma. When normal and tumoral tissues of patient with laryngeal carcinoma were compared, 16 (76.19%) of normal and 9 (42.85%) of tumoral tissues were found to be positive for the bacterium. Bacteria were present in both normal and tumoral tissues in 38.09% of the cases. There was a statistically significant difference between normal and tumoral tissues in regard of presence of *H. pylori* ($p = 0.039$). However, the ratio of positivity in normal tissue was greater than the positivity in tumoral tissue. This can be explained with disappearance of the bacterium from the tumoral tissue in time, as in the case with gastric carcinoma [4]. The relation between the positivity for *H. pylori* and stage of the tumor was not statistically significant ($p = 0.429$).

Since PCR detects the genetic material of both living and dead microorganisms, we used bacterial cultures to show active bacterial infection. None of the cultures were positive for *H. pylori*. One possible explanation for this may be the death of the microorganism during the transfer to the laboratory because of its microaerophilic nature [5], or the problems during the incubation period. Apart from the technical problem of culturing microaerophilic *H. Pylori*, it may be commented that the microorganism may be an innocent bystander in the patients with laryngeal carcinoma. However, since we have not detected *H. pylori* in any of the control subjects, it is more likely that the microorganism plays a role in the carcinogenesis of the laryngeal cancer.

The high ratio of identification of *H. pylori*'s genomic material within the laryngeal tissues of the patients with squamous cell carcinoma of the larynx may constitute a proof for the relation of this bacteria with the laryngeal cancer. However, our study does not illuminate the relation between *H. pylori* and laryngeal carcinogenesis. We need further studies on this subject.

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