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Human papillomavirus infection in laryngeal lesions in Northeast Bosnia and Herzegovina

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ABSTRACT

Introduction: Various studies conducted in the past few years have shown a causal relationship between human papillomavirus (HPV) and benign and malignant lesions on the laryngeal mucosa. There are no other studies on the association between HPV and laryngeal lesions in Bosnia and Herzegovina. The aim of the study was to investigate the influence of HPV infection on the development of benign and malignant lesions in the larynx.

Methods: Samples for the recommended HPV analysis were taken using cotton swabs from Digene's Specimen Collection Kit (Sample Collection Kit) from the surface of the affected area of the laryngeal mucosa with a gentle rotational movement through the directoscope. Detection of the presence of HPV in the endolaryngeal swab was performed using Digene's HPV test-Hybrid Capture II, and results were obtained on a DML 2000 luminometer.

Results: In this study, a total of 40 patients were enrolled. Among them, 17 cases (42.5%) presented with benign lesions of the larynx, whereas 23 cases (57.5%) had malignant lesions. Within the group of 17 cases with benign lesions, 5 tested positive for HPV: 2 for high-risk HPV (HR HPV), 2 for low-risk HPV (LR HPV), and 1 for both high-risk and LR HPV. Among the 23 cases with malignant lesions, 11 (47.83%) were HPV positive: 5 for HR HPV, 4 for LR HPV, and 2 for both high-risk and LR HPV.

Conclusion: Our findings underscore the noteworthy influence of HPV infection on both benign and malignant changes in the laryngeal mucosa. However, future studies necessitate larger sample sizes and the identification of specific virus types.

Keywords: Human papillomavirus; larynx; benign; laryngeal cancer

INTRODUCTION

The association of infection with the human papillomavirus with head-and-neck cancers has been unequivocally proven. Various studies conducted in the past few years have shown a causal relationship between human papillomavirus (HPV) and benign and malignant lesions on different mucosa. HPV DNA was isolated from malignant tissue of the oropharynx, oral cavity and, to a lesser extent, from laryngeal carcinoma (1). The virus shows the strongest inclination or preference for the mucous membrane of the reproductive tract, specifically the cervix, followed by the skin, and then the mucous membrane of the oropharynx and larynx. High-risk HPV (HR HPV) types were isolated in 99.7% of women with cervical cancer (2). At the end of the past century, the association between HPV and squamous cell carcinoma of the head and neck squamous cell carcinoma (HNSCC) was proven with an infection rate of 8-83% and an average prevalence of 25% (1, 3). Previous studies have confirmed that low-risk HPV (LR HPV), primarily types 6 and 11, are associated with recurrent respiratory papillomatosis (4), while HR HPV is associated with laryngeal cancer (5). HPV DNA has also been proven in benign changes such as polyps, nodules, dysplasia, and oral papillomatosis, but to a much smaller extent (4).

However, the association between laryngeal squamous cell carcinoma (LSCC) and HPV infections still remains controversial due to inconsistent results (6, 7). Even within the same country, the prevalence of HPV in head-and-neck cancer varies greatly. Potential reasons for variations in outcomes include demographic and racial factors, sample quality, the location of cancer, and the method used for virus detection (8).

The most common source of data is retrospective studies, which are based on formalin-fixed and paraffin-embedded

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tissue. Though the research has mostly involved small groups, often fewer than 100 patients, the data are very valuable for understanding why individual changes occur and for improving treatment, monitoring, and outcomes for each patient. There are no other studies on the association between HPV and laryngeal lesions in Bosnia and Herzegovina.

METHODS

The research included 40 patients who had confirmed alterations in the larynx requiring surgery and were experiencing specific symptoms related to voice problems (dysphonia). Some patients had non-cancerous changes in the larynx and were tested for different types of HPV, while others had malignant changes (squamous cell carcinoma) and were also screened for HPV. All participants were informed about the purpose and details of the study and willingly agreed to participate. Tissue samples were collected for diagnosis and swabs were taken from inside the larynx to detect HPV DNA.

Throughout the study various information was collected, including gender, age, risk factors (smoking, alcohol consumption, and family history), HPV status, and the clinical and pathological stage of the disease based on the TNM classification system. It's worth noting that all patients tested negative for COVID-19 on a polymerase chain reaction (PCR) test.

The study was approved by the Ethical Committee of University Clinic Center Tuzla (21 May 2020. No: 02-09/2-30/20).

We obtained samples for HPV analysis using cotton swabs from Digene's Specimen Collection Kit (Sample Collection Kit LOT M2163). The swabs were gently rotated on the surface of the altered laryngeal mucosa using a directoscope. The collected swab was immediately placed in a plastic tube containing a special transport solution designed to prevent bacterial growth and protect DNA integrity. Samples were stored at 2–8°C for analysis within 7 days or at –20°C for longer periods.

The presence of HPV in the endolaryngeal swab was detected using Digene's HPV test - Hybrid Capture II, and the results were obtained using a DML 2000 luminometer.

The observed morphological changes were examined using an Olympus BX 41 microscope with light microscopy. These changes were categorized according to the World Health Organization classification of laryngeal lesions. Biopsy samples formalin-fixed and paraffin-embedded, were cut into standard 5 μm-thick sections and stained using a standard hematoxylin-eosin staining procedure, and then mounted using Canada balsam. All specimens were initially fixed in 10% formalin solution and embedded in paraffin. Immunohistochemical analysis of three-step immunoperoxidase with streptavidin was performed on histological sections of tissue samples with a thickness of 4 μm. Histological sections were transferred to organosilane-pre-treated glass slides and incubated at 37°C overnight.

Statistical analyses were performed using Easy Fit software. The significance of differences between cases and controls in demographic variables, tobacco smoking, alcohol consumption, and HPV-16 status was determined using the Chi-square test, t-test, Mann–Whitney test, and Krusklal–Wallis test. $p \leq 0.05$ were considered statistically significant.

RESULTS

A total of 40 cases were prospectively gathered for analysis. Among these, 57.5% (23) presented with malignant laryngeal lesions, while 42.5% (17) exhibited benign laryngeal lesions such as vocal cord polyps and nodules, chronic laryngitis, and dysplasia.

Table 1 provides a demographic and clinical description of the patients. The median age of the patients at the time of diagnosis was 61 years (range 39-80), with a male-to-female ratio of 3:1. There was no statistically significant difference in the ages of male and female respondents (p = 0.3476). Out of the 17 patients with benign laryngeal lesions, 9 were male and 8 were female. 3 Patients were non-smokers, and 14 were smokers. Among the 23 malignant lesions, 21 occurred in males, and 2 in females. One of the females was a non-smoker, while the remaining 22 patients with malignant lesions were smokers (Table 1).

Archived tissue blocks were obtained from 19 patients with benign laryngeal lesions (Figure 1). Two samples were excluded due to the existence of another malignancy and exposure to radiation. Consequently, 17 cases were analyzed using Digene's HPV test – Hybrid Capture II. Among these patients, 29.41% tested positive for HPV infection, while 70.59% were HPV-negative. Out of the 5 patients with HPV-positive tumors, 2 were positive for LR HPV, 2 were positive for HR HPV, and 1 was positive for both LR HPV and HR HPV.

Archived tissue blocks were obtained from 24 patients who underwent curative surgery for laryngeal carcinoma (Figure 2). One sample was excluded due to previous radiation. Thus, 23 cases were examined using Digene's HPV test - Hybrid Capture II. Among these patients, 21.73% tested positive for infection with one or several types of HR HPV, 17.39% were positive for LR HPV, 8.69% were positive for both LR HPV and HR HPV, and 52.17% were HPV-negative.

9 samples, out of 40 cases, are positive for LR HPVs or 22.5%, with male-female ratio 77.78%: 22.22%. LR

TABLE 1. Demographic and clinical description of the patients

Variables	Benign	Malignant
Number	17 (42.5%)	23 (57.5%)
Age		
<40	1	0
41-55	5	4
56-70	10	15
>70	1	4
Sex		
Male	9	21
Female	8	2
Tobacco smoking		
Yes	14	22
No	3	1
Alcohol drinking		
Yes	5	10
No	12	13

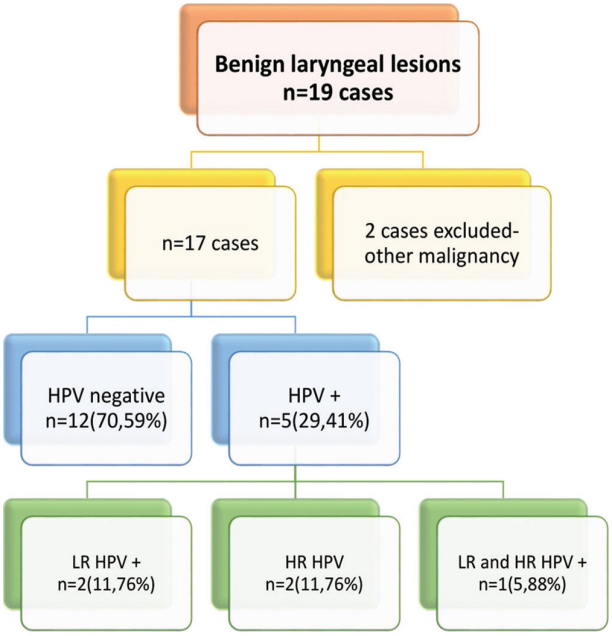

FIGURE 1. Flow diagram of the human papillomavirus results from benign laryngeal lesion specimens included in this study.

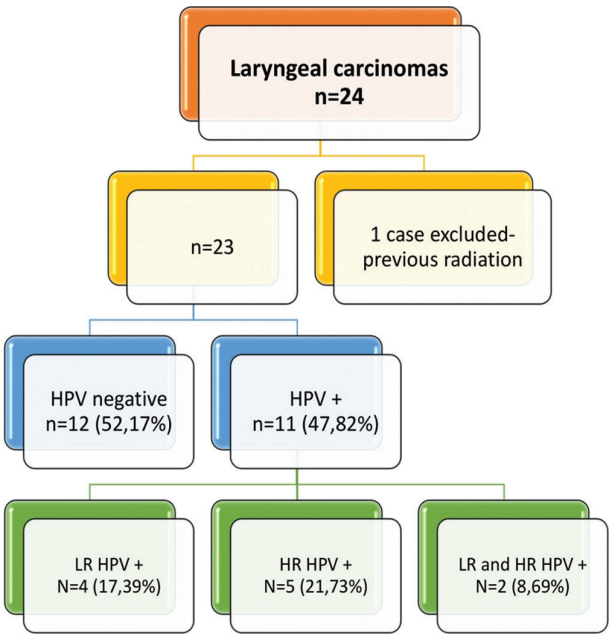

FIGURE 2. Flow diagram of the human papillomavirus results laryngeal carcinoma specimens included in this study.

HPV is statistically more common in men than in women. 10 patients are positive for HR HPVs, or 25%, and all are males.

DISCUSSION

Over 40 years ago, Gissmann et al. (9) first reported the correlation between certain types of HPV infection and the emergence of head-and-neck tumors. Subsequent research has consistently reinforced this association, with the latest studies affirming the significant impact of HPV infection on the development of laryngeal cancer.

In the available literature, there is no data on the frequency of HPV infection in laryngeal lesions in our country. Therefore this study, despite its small sample size, represents part of one of the first investigations of this type in Bosnia and Herzegovina.

The prevalence of HPV DNA in laryngeal cancer in the literature ranges between 0% and 85% (10, 11). The large difference in the prevalence of HPV detection in LSCC is explained by the different sensitivity of HPV DNA detection methods, ethical and geographical differences in the population, small study samples, sample conditions, and differences in sample storage methods and lesion localization (12, 13). Detection by DNA *in situ* hybridization, which is more commonly used, is less sensitive than PCR techniques (14). In our study, we proved the presence of HPV in 47.83% of malignant alterations in the larynx using Digene's HPV test-Hybrid Capture II.

A large difference in the frequency of HPV detection is seen even with the results obtained using the PCR reaction and ranges from 3-85%. The differences depend on the primers used in the reactions, the genomic localization of the length of the PCR-amplified product, the reaction conditions, and the false positive results obtained due to contamination of the sample with a viral particle (15).

The etiology of laryngeal carcinoma is extremely complex and is associated with smoking tobacco products and consumption of alcohol, which together increase the risk of developing cancer of the larynx many times over. One of the most important factors for the development of the disease is smoking, with a relative risk of 1-9 times, and regular alcohol consumption, which increases the risk of the disease 2-5 times. If they are consumed together the risk increases even more. It is interesting that about 5% of all LSCC occurs in non-smokers and non-drinking patients, in 15-20% of these patients co-infection with HPV is found (16, 17).

In earlier studies, it was proven that smoking is a cofactor in HPV infection in pre-cancerous and cancerous lesions of the cervix, similarly to the laryngeal mucosa. Smoking impairs cellular immunity and the production of antibodies against HPV, thereby favoring the development of pre-cancerous and cancerous lesions on the mucosa (18).

In our study, we identified a strong correlation between malignant laryngeal lesions, tobacco smoking, and HPV. The percentage of smokers was notably high at 95.65%, with 47.82% of them testing positive for HPV. This finding aligns with previous research in this field. Although it has been observed in some studies, that HPV is more common in non-smokers and non-alcoholics and in the subglottic region; we did not prove this in our study (11).

Laryngeal cancer primarily impacts men, as indicated by the study's ratio of 21:2 between male and female cases. It is more prevalent during middle age, with 65.21% of cases occurring in individuals aged between 56 and 70 years. Remarkably, only four patients in our study are younger than 55 years, while another four are older than 70 years. The incidence of laryngeal carcinoma in middle-aged patients is associated with smoking and the number of cigarettes per day (19). In a study from India, it was proven that HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) is not associated with smoking and alcohol, but with high-risk sexual habits. Unlike OPSCC, LSCC is not

associated with sexual habits because HPV does not come into direct contact with the laryngeal mucosa (20).

The importance of determining the HPV status in patients with HNSCC is also reflected in the prognosis of cancer. Some studies have found that HPV-positive HNSCC, especially HPV-16 positive, are more radiosensitive and have a complete response to therapy and long-term survival. These studies are usually related to OPSCC, but more research is needed for LSCC (11, 19). A meta-analysis of 1422 patients showed better overall survival in the HPV-positive group than in the HPV-negative group in terms of short-term survival. There was no statistically significant difference in 5-year and 10-year disease-free survival between HPV-positive and HPV-negative LSCCs (21).

This is one of the initial studies of its type in Bosnia and Herzegovina. In this research, our primary focus was on the association between HPV infection and laryngeal lesions. The correlation and incidence of specific HPV types will be the goal of further research.

Research from 2017 found a significant association of HPV16 positivity with LSCC in non-smokers, non-alcoholics, and men over the age of 55. Other researches have proven similar results (22).

Apparently, the impact of HPV infection on the development of LSCC is unquestionable. Even more, its influence on the development of benign lesions on the laryngeal mucosa is not negligible. While laryngeal papillomatosis is the most common clinical manifestation of HPV infection, with an incidence ranging from 83-100%, HPV DNA can also be present in other benign changes on the laryngeal mucosa and even in normal mucosa (23). However, our results revealed that 5 (29.41%) cases of benign laryngeal lesions were positive for HPV. This finding is consistent with the results of other studies where HPV was detected in 23.1% of the polyp group and in another study where HPV was found in 19% of benign laryngeal lesions other than papilloma (4). Some studies have proven that there is no relationship between HPV prevalence and vocal cord polyps in sections with normal mucosa (22), while in another study HPV was detected post-mortem in as many as 18% of samples of normal laryngeal and hypopharyngeal mucosa (24). In their study, Iravan et al. proved a higher prevalence of HPV infection in smokers with laryngeal polyps (4).

Detection of HPV on the normal lining of the larynx is not a standardized process, but it would be of great importance to determine the influence of "latent infection" on the development of laryngeal cancer (13), which might also be the focus of further studies.

In our research, we found that HPV is in about 29.41% of benign lesions on the larynx, which matches a study by Iravani et al. in 2021 (4). However, other studies like George et al. in 2020 didn't find any HPV infection in similar non-cancerous changes on the larynx (19). In many previous studies, the association of HPV-16 with changes in the laryngeal mucosa was found, but the technical limitation in sequestering HPV to types was limited in our study.

While LR HPV is typically linked to benign changes on the larynx, such as polyps, nodules, dysplasia, and recurrent respiratory papillomatosis (RRP), our study identified 4 samples of laryngeal cancer that tested positive for LR HPV. This suggests the malignant potential of these genotypes, particularly in cases of long-term infections where transformations leading to malignancy can occur. Cases of malignant transformation of head-neck papillomas caused by LR HPV have been described earlier, and research with long-term follow-up is needed to investigate the malignant potential.

Until now, similar research has not been conducted in our region, except the study from 2015, which was conducted in 29 countries, including head-and-neck cancer (HNC) samples from our Clinical Center. At the time 1042 laryngeal cancer samples were analyzed, 59 HPV-positive samples were found, 56 single, 1 multiple, and 2 undetermined genotypes (25). Therefore, new research with a larger number of samples is needed.

CONCLUSION

We identified a substantial correlation, accounting for 47.83%, between HPV infection and malignant alterations in the larynx. Numerous studies have demonstrated the association of HR HPV with malignant changes and LR HPV with benign changes. Interestingly, our study also revealed a significant link between LR HPV and malignant lesions in the larynx.

Our findings underscore the noteworthy influence of HPV infection on both benign and malignant changes in the laryngeal mucosa. However, future studies necessitate larger sample sizes and the identification of specific virus types. Further research, along with prolonged patient monitoring, is imperative to delineate HPV's impact on treatment response and survival rates.

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DECLARATION OF INTERESTS

Authors declare no conflict of interest.

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