Investigating the Prevalence of Human Papilloma Virus in Squamous Cell Carcinoma of the Larynx and Its Correlation with Disease Prognosis

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Abstract

Introduction:
The human papilloma virus (HPV) can play a role in the development of head and neck squamous cell carcinoma (SCC). Our aim was to assess the prevalence of HPV DNA in SCC of the larynx. The impact of HPV infection on patient survival was also evaluated.

Materials and Methods:
This case-control study was performed in 44 patients with SCC of the larynx (case group), while the control group comprised samples obtained from cadavers with no previous history of malignancy. A preliminary pathologic evaluation was performed on all samples in the control group (36 samples) to ensure the absence of dysplasia or malignancy. Polymerase chain reaction (PCR) was used to detect HPV DNA. After completing the treatment protocol, patients were followed to assess the impact of HPV infection on overall survival (OS).

Results:
PCR evaluation in the case group showed that HPV DNA was successfully isolated from 11 (25%) samples, while only two (5.6%) HPV DNA-positive were obtained from cadavers. According to these results, a significant difference was obtained in the prevalence of HPV DNA and laryngeal SCC between cases and controls (P=0.031). No statistically significant difference was observed in the OS of patients with or without HPV infection in the case group (P=0.235).

Conclusion:
Based on these results, we suggest that the prevalence of HPV infection is higher in laryngeal SCC subjects compared with healthy individuals. Although a longer OS was seen in HPV-positive patients, survival analysis did not show a significant difference in the comparison of HPV-positive and negative findings in SCC patients.

Keywords:
Human Papilloma Virus (HPV), Larynx, Survival, Squamous Cell Carcinoma, Risk.

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Introduction

Squamous cell carcinoma (SCC) is the most common malignancy of the head and neck and is estimated to account for 90% of all tumors arising from this anatomic structure (1,2). Alcohol ingestion and tobacco smoking have been identified as the most common risk factors for head and neck SCC (HNSCC) (3). However, up to approximately 20% of patients with laryngeal cancer lack these risk factors, suggesting a role of other risk factors in the development of laryngeal SCC (4).

Although human papilloma virus (HPV) is considered a conventional risk factor for cervical cancer, several studies more than two decades ago have revealed the presence of HPV DNA in samples of HNSCC and it has been postulated that the virus may also increase the risk of HNSCC (5). In the past decade, there has been a sharp increase in the incidence of HPV-positive oropharyngeal cancer in both European countries and North America (6). It has been suggested that the prevalence of HPV in tonsil and oropharyngeal cancer is higher than that in laryngeal or oral cavity tumors (7,8). The scientific proposition put forward is that, even in the absence of conventional risk factors, HPV might induce tumorigenesis, and that oropharyngeal cancer associated with HPV may have distinct clinicopathological characteristics (9-11). Chronic HPV infection may be associated with head and neck cancer (HNC) by interfering with tumor suppressor gene functions (12,13). Furthermore, viruses may act as cofactors enhancing activation, amplification, and overexpression of oncogenes in neoplastic tissues. Molecular and epidemiological evidence has demonstrated the role of HPV in HNCs (14-16).

In this regard, most previous studies reported the isolation of HPV DNA from the oropharynx and tonsils and assessed the impact of HPV on patient survival (17-20). Our aim was to assess the prevalence of HPV DNA in laryngeal cancer samples and compare it with samples obtained from cadavers with no history of malignancy. Additionally, after completing the treatment protocol, live patients were followed to evaluate the impact of HPV on survival.

Materials and Methods

Study design and participants

This case-control study was designed to assess the prevalence of HPV in patients with SCC of the larynx in Shahid Sadoughi Hospital in Yazd, Iran between 2007 and 2012. Patients who underwent laryngectomy and whose final pathological evaluation confirmed the SCC nature of the tumor were included. Tissue samples were obtained from fresh cadavers with no history of malignancy (control group). If a suspicious lesion was encountered in the larynx of cadavers during biopsy, the corresponding control was not considered eligible for study enrollment. Finally, samples from cadavers were reviewed by a pathologist and cases of dysplasia or malignancy were excluded from the study. Written informed consent was obtained from patients in the case group and relatives of the deceased prior to study enrollment. Condolences were given to the family members of the deceased patients. The study protocol was performed in accordance with the latest Declaration of Helsinki for investigation on human subjects and was approved by the local ethics committee.

Laboratory assessment

Paraffin blocks were cut into 8 to 10 slices with a thickness of 5 µm. In order to remove the paraffin, samples were dissolved in xylene for 5 min, and then were centrifuged at a speed of 14000g for 10 min. Next, genomic DNA was isolated using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). The presence of HPV DNA was assessed using a
MY09/My11/HMB01- polymerase chain reaction (PCR) system with Gold AmpliTaq DNA polymerase that amplifies a conserved 450 base-pair segment in the L1 sequence of HPV, plus nested-PCR that uses primers specific for the HPV16E6 gene and the upstream regulatory region. Samples testing positive with consensus PCR were further genotyped using biotinylated oligonucleotide probes for four HPV subtypes, including 16,18,6 and 11.

Follow-up and statistical analysis

Patients were followed up after the procedure in our hospital clinic. Overall survival (OS) was defined as the date of pathological diagnosis to death or last date known alive. Patients who were not willing or able to attend the clinic were excluded from study. The health status of patients was obtained via telephone or by examination and interview in the clinic. All statistical analyses were performed using statistical package for the social sciences (SPSS) software version 20.0 (IBM Inc., New York, NY, USA). Continuous variables are presented as mean and standard deviation, while categorical variables such as gender and frequency of HPV-positive patients in each group are shown as a percentage. A Chi-square test, Fisher exact test and independent samples t-test were used to compare the variables of interest between the two groups, as applicable. A Kaplan-Meier test and log-rank test was used to compare the survival of patients with or without HPV infection.

Results

A total of 53 patients with laryngeal cancer were recruited, of whom four had a non-SCC tumor in the final pathology assessment and five were lost to follow-up. Therefore, 44 patients were included in the final assessment as the case group. Biopsy samples were obtained from 37 cadavers, but the first pathologic review revealed intermediate dysplasia in one sample, and that sample was consequently excluded from the study. Hence, the control group comprised 36 samples. The mean age of participants in the case and control groups was 57.84±5.83 and 56.44±13.83 years, respectively (P=0.545). The case group comprised 40 (90.9%) male and four (9.1%) female patients, while the control group included 27 (75%) male and nine (25%) female participants. No statistically significant difference was observed regarding the gender distribution of the two groups (P=0.071) (Table 1).

|                      | Case group (N=44) | Control group (N=36) | P-value |
|----------------------|-------------------|----------------------|---------|
| Mean age±SD          | 57.84±5.83        | 56.44±13.83          | 0.545   |
| Gender               |                   |                      | 0.071   |
| Male                 | 40 (90.9%)        | 27 (75%)             |         |
| Female               | 4 (9.1%)          | 9 (25%)              |         |

PCR assessment of samples isolated HPV DNA from 11 (25.0%) and two (5.6%) specimens in the case and control groups, respectively. Comparison of the prevalence of HPV DNA between the two study groups showed a significant difference (P=0.031).

The most common subtypes of HPV in the case group were HPV type 16, which was detected in seven (63.63%) samples, followed by HPV type 18 detected in three (27.27%) samples and HPV type 6 isolated from only one sample. As mentioned previously, two samples from the control group had a positive HPV DNA test and HPV types 6 and 11 were identified in two separate samples.

Patients in the case group were followed up to determine OS and assess the impact of HPV diagnosis on survival in patients with laryngeal cancer. The median follow-
up time was 28.50 months (ranging, 2–69 months).

All patients who tested positive for HPV survived to the end of the follow-up period. Among 33 patients who tested negative for HPV, five died. The median OS of HPV-negative patients was 27 months (interquartile range, 15.5–44.5 months), while the corresponding figure in HPV-positive patients was 31.0 months (interquartile range, 22–51). Although the median OS was higher in the HPV-positive group, Kaplan-Meier analysis showed no significant difference between HPV-positive and negative patients in terms of survival (P=0.235) (Fig.1).

In recent years, several studies have suggested an association between the presence of HPV DNA and an increased risk of HNSCC. A systematic review of articles published up to 2004 demonstrated that the prevalence of HPV in SCC of the larynx can be as high as 46.9%. A prevalence of HPV in the control group (comprising patients with benign lesions of the larynx) was between 0 and 5.7% (21). The corresponding figures in the current study were 25% in the case group and 5.6% in the control group. It should be noted that a wide variety of HPV DNA prevalence rates has been reported in previous studies. For instance, in a study conducted by Laskaris et al., the total prevalence of HPV in SCC of the larynx was 18.5% and HPV 16 was the most common subtype, detected in 7.5% of samples (22). In a study conducted by Roshana et al. investigating the prevalence of HPV in 60 formalin-fixed, paraffin-embedded samples of laryngeal cancer and 22 normal larynx tissue samples, PCR results detected no HPV-16/18 DNA in either group (23). In another recent study conducted by Rodrigo et al., only two paraffin-embedded tumor specimens (one larynx and one hypopharynx) of 124 SCC patients surgically treated for laryngeal (62 cases) and hypopharyngeal (62 cases) were HPV positive (24). These differences might be attributed to variations in techniques used to detect HPV DNA. While quantitative PCR can detect clinically insignificant viral DNA, enzyme-linked immunosorbent assay (ELISA)-based techniques lack the sensitivity required to detect carcinogenic HPV infections (7).

Several reports indicated that HPV 16 is the most common subtype of HPV that can be isolated from tumor samples (3). Our results were in agreement with the mentioned findings; among the 11 HPV-positive samples, HPV 16 was found in

**Discussion**

In the current study, a higher prevalence of HPV DNA isolated from laryngeal SCC samples was observed compared with HPV DNA extracted from control group samples. The most common subtype was HPV 16, detected in 15.9% of patients in the case group. Only two samples from the control group (i.e., cadavers with no history of previous malignancy) were positive for HPV DNA, and the subtypes were HPV 6 and 11.

**Fig 1:** Comparison of survival of patients with laryngeal SCC with respect to the presence of HPV DNA in tumor samples

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seven cases, accounting for 63.6% of all isolated HPV DNA.

There are major inconsistencies between the results of published articles regarding the effect of HPV on survival of patients with laryngeal cancer. In a study comprising 73 patients with HNSCC who underwent a median follow-up of 28 months (0.3–94 months), median survival among HPV-positive and HPV-negative patients was 31.9 and 25.2 months, respectively. Authors demonstrated that HPV cannot be considered an independent prognostic factor for patient survival (25). In another study enrolling 253 patients with newly diagnosed HNSCC, a 40% reduction in risk of death was observed in HPV-positive patients after adjustment for nodal status, age, and alcohol consumption (P=0.07) (4). In the current study, HPV-positive patients had longer OS; however, no statistically significant difference existed in patient survival between the two groups.

One important limitation of this study is the lack of data on conventional risk factors for laryngeal SCC, such as alcohol consumption and tobacco smoking. In addition, the relatively small number of patients that were recruited could contribute to the failure of finding significant differences in patient survival.

**Conclusion**

Based on our findings, it could be proposed that the prevalence of HPV infection is higher in laryngeal SCC subjects compared with healthy individuals. HPV 16 was the most common subtype of HPV isolated from our tumor samples. Our results failed to show a difference between the OS of patients with laryngeal cancer who were HPV-positive and those who were HPV-negative, although the HPV-positive participants had a longer median OS.

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