HUMAN PAPILLOMAVIRUS INFECTIONS IN LARYNGEAL CANCER

Mariela C. Torrente, MD,[1] Juan P. Rodrigo, MD, PhD,[2,3] Missak Haigentz, Jr, MD,[4] Frederik G. Dikkers, MD, PhD,[5] Alessandra Rinaldo, MD, FRCSEd *ad hominem*, FRCS (Eng, Ir) *ad eundem*, FRCS Glasg,[6] Robert P. Takes, MD, PhD,[7] Jan Olofsson, MD,[8] Alfio Ferlito, MD, DLO, DPath, FRCSEd *ad hominem*, FRCS (Eng, Glasg, Ir) *ad eundem*, FDSRCS *ad eundem*, FHKCORL, FRCPath, FASCP, IFCAP[6]

[1] Departamento de Otorrinolaringologia, Universidad de Chile, Santiago de Chile, Chile
[2] Instituto Universitario de Oncología del Principado de Asturias, Oviedo, Spain
[3] Department of Otolaryngology, Hospital Universitario Central de Asturias, Oviedo, Spain
[4] Division of Oncology, Department of Medicine, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, New York
[5] Department of Otorhinolaryngology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[6] Department of Surgical Sciences, ENT Clinic, University of Udine, Udine, Italy. E-mail: a.ferlito@uniud.it
[7] Department of Otolaryngology – Head and Neck Surgery, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands
[8] Department of Otolaryngology – Head and Neck Surgery, Haukeland University Hospital, Bergen, Norway

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Abstract: Although the association and clinical significance of human papillomavirus (HPV) infections with a subset of head and neck cancers, particularly for oropharyngeal carcinoma, has recently been well documented, the involvement of HPV in laryngeal cancer has been inadequately evaluated. Herein we review the currently known associations of HPV infections in diseases of the larynx and their potential for oncogenicity. Using several methods of detection, HPV DNA has been detected in benign (papillomatosis), indolent (verrucous carcinoma), and malignant (squamous cell carcinoma) lesions of the larynx. Consistent with the known oncogenic risk of HPV infections, common HPV types associated with laryngeal papillomatosis include low-risk HPV types 6 and 11, with high-risk HPV types 16 and 18 more commonly present in neoplastic lesions (verrucous carcinoma and squamous cell carcinoma). Although a broad range of prevalence has been noted in individual studies, approximately 25% of laryngeal squamous cell carcinomas harbor HPV infections on meta-analysis, with common involvement of high-risk HPV types 16 (highest frequency) and 18. Preliminary results suggest that these high-risk HPV infections seem to be biologically relevant in laryngeal carcinogenesis, manifested as having viral DNA integration in the cancer cell genome and increased expression of the p16 protein. Despite this knowledge, the clinical significance of these infections and the implications on disease prevention and treatment are unclear and require further investigation. © 2010 Wiley Periodicals, Inc. *Head Neck* **33:** 581–586, 2011

Correspondence to: A. Ferlito
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The relationship between human papillomavirus (HPV) and pathology of the upper airway has been known for almost a century,[1] but only in the last 3 decades has its potential oncogenic activity been acknowledged in the literature.[2–4] Among head and neck cancers, HPV infection has a known role in oropharyngeal carcinogenesis, particularly for tonsillar cancer, with prognostic significance. Both therapeutic response and survival are better in HPV-positive cases.[5,6] In other anatomic sites of the upper aerodigestive tract, HPV infection has been noted in several studies, but controversy exists regarding its role in carcinogenesis.[4] Herein we discuss the possible association of HPV infections in laryngeal cancer.

Human Papillomavirus Biology and Detection

Viral Biology. More than 200 different HPV genotypes have been described, which, according to their association with cervical carcinogenesis, are classified as high-, intermediate-, or low-oncogenic risk.[7] On the basis of mounting epidemiologic and molecular evidence, in 1995, the International Agency for Research on Cancer recognized that high-risk HPV types 16 and 18 were carcinogenic in humans.[8] Together, these 2 HPV types are responsible for approximately 70% of cervical cancer cases, and other intermediate-risk HPV types (31, 33, and 35) account for virtually all of the remaining cases of this disease. HPV 16 is the

predominant (90% to 95%) genotype detected in head and neck tumors, with different prevalence between anatomic sites.[9] HPV is a double-stranded DNA epitheliotrophic virus of almost 8000 base pairs, organized in early (E) and late (L) transcriptional genes. It is an obligatory intranuclear virus that must infect mitotically active cells to establish itself in epithelia. The infection can access basal and parabasal cells of multilayered epithelium in 3 different sites: at the site of mucosal injury, metaplastic epithelium, or the squamocolumnar junction. When the virus infects a mitotically active epithelial cell, it may result in a latent infection with viral replication connected with the cell cycle.[10] The viral DNA is maintained in the nuclei of infected basal epithelial cells as a low-copy-number plasmid. During the viral replication phase, viral episomes proliferate in the suprabasal post-mitotic cells, and the early proteins E1, E2, E6, and E7 are produced at low levels. The late viral genes, encoding L1 and L2 proteins that constitute the virus particle, are expressed only in highly differentiated cells. Thus, these infected cells accumulate virions that will be released to the media along with the hosting cells.[11] The viral replication is accompanied with a number of morphologic cellular and tissue alterations, including koilocytosis, multinucleation, cellular atypia, acanthosis, and epithelial thickening. The proliferative process initiated by the episomal form of HPV begins at the basal and parabasal layers of squamous cell epithelium of the oral cavity and oropharynx, or alternatively at the reserve cell layer in the respiratory epithelium of the larynx.

The laryngeal epithelium is, therefore, susceptible to HPV infection. In the larynx, the HPV-induced reserve cell proliferation usually undergoes a metaplastic alteration with a formation of multilayered squamous cell epithelium, and finally papilloma can develop. Clinically, papillomas tend to arise from the junction of squamous and respiratory epithelium and areas of iatrogenic-induced squamous metaplasia.[12] However, they can also arise in adjacent areas predominantly within a few centimeters of such transitional zones.

Integration of HPV DNA into the host cell genome is a crucial step in HPV-induced carcinogenesis. Integration usually disrupts or deletes either the E1 or the E2 open reading frame, which results in loss of expression of the corresponding gene product and also leads to upregulation of viral oncoproteins E6 and E7.[13] This loss of transcriptional regulation can occur when viral particles replicate in basal layer cells. Oncogenic activity mediated by viral oncoproteins E6 and E7 can lead to DNA instability.[14] The E6 protein of high-risk HPV binds and induces the degradation of the p53 tumor suppressor protein via an ubiquitin-mediated process. The E7 protein binds to pRb, which dissociates pRb/E2F complexes and thereby facilitates the expression of DNA replication proteins. The E6 and E7 proteins produced by low-risk HPV types are not as effective in binding with p53 and pRb as those from high-risk HPV types. In the presence of DNA errors, an epithelial cell infected by HPV, particularly high-risk HPV, will not enter apoptosis, leading to the accumulation of DNA aberrations. In addition, the E6 oncoprotein interferes with DNA repair enzymes, and the E7 oncoprotein can induce structural and numerical chromosome abnormalities by the disruption of centrosome synthesis. This genetic instability can cause the emergence of tumorigenic cells.[15] While these events are taking place at the molecular level, parallel morphologic alterations also occur. The malignant transformation is accompanied by a significant increase in the mitotic rate, aneuploidy with alterations in chromosome number and/or structure, and an increased rate of mutation in the host cell genome.

HPV Detection. The diagnosis of HPV infection is based entirely on the detection of the viral DNA in clinical samples. Polymerase chain reaction (PCR)-based methods have high sensitivity, identifying low viral loads (even 1 copy of HPV per cell). Type-specific or broad spectrum primer sets can be used depending on the final purpose. Broad spectrum primers amplify the widely conserved viral gene L1 open reading frame. The MY09/11 amplifies a 450 base pair fragment, and GP5+/GP6+ amplifies a 150 base pair fragment; both methods are highly sensitive and specific.[16] However, type-specific primers that amplify E6 or E7 regions might be more effective, because a region of L1 can be lost during integration, so generic primers can underestimate true HPV prevalence.[17] In general, however, secondary, type-specific probe hybridization is required for HPV typing, involving dot-blot hybridization, reverse line probe hybridization, or bead-based arrays.

A positive result for HPV DNA PCR amplification only demonstrates the presence of HPV and does not necessarily imply its role in carcinogenesis. A key factor that correlates with viral carcinogenesis is integration into the host genome. In situ hybridization techniques permit identification of infections in which viral DNA is integrated into the host genome, revealing a nuclear pattern of staining.[18] Expression of p16 can also aid in identifying integrated forms because functional inactivation of pRb by E7 results in an upregulation of the nuclear protein p16, resulting in a diffuse immunohistochemical staining pattern. This technique has reportedly 100% sensitivity, and is generally considered a good initial choice in discriminating HPV-positive from HPV-negative samples.[19] By contrast, loss of p16 protein expression is a common and early event in tobacco carcinogen-related head and neck cancers.[20–22] Thus, tobacco and alcohol-associated head and neck squamous cell carcinomas are associated with downregulation of p16 protein and *TP53* gene mutations, whereas HPV-associated cancers are associated with wild-type *TP53* and *RB1* genes and upregulation of p16 protein levels. Immunohistochemical determinations of p16 expression

could serve as a reasonable surrogate marker for biologically relevant high-risk HPV infection; however, standardization of technique and interpretation of stained slides is required for widespread clinical application.

A wide range of values of HPV prevalence in normal oral cavity mucosa has been reported, from 0%[23–25] up to 70%.[26] The variability in results might be the effect of different methods of sample collection, the anatomic sites of sample collection (base of tongue or elsewhere), and the sensitivity of the detection technique used.[27] From studies of cervical carcinoma, HPV infection of the genital tract in women is very common: high-risk genotypes can be identified in 15.7% to 27.4% of sexually active females, and most with identified infection will have cleared up within 6 to 12 months.[28] Only a minority of infections progress to preneoplastic lesions.

Human Papillomavirus and Laryngeal Cancer

Although recently overshadowed by its involvement in oropharyngeal carcinogenesis, the classical location of HPV infection in the upper aerodigestive tract has been the larynx, with laryngeal papillomatosis caused by low-risk HPV types 6 and 11. Despite its historically low-risk oncogenicity, recurrent respiratory papillomatosis did develop into squamous cell carcinoma in 3 of 241 cases in which the original papilloma revealed HPV type 6[29]; coinfection with HPV type 11 was noted in 1 case and coinfection with HPV type 16 was noted in another case. In their discussion, Jeong et al[29] reviewed 15 studies describing 44 patients in whom recurrent respiratory papillomatosis transformed to carcinoma. Although not all cases were HPV typed as benign papillomas, 25 papillomas contained HPV 11 DNA, 4 harbored HPV 6, and another 6 cases revealed low-risk type HPV, with the exact type not designated.

As a very uncommon, indolent lesion of the larynx, verruca vulgaris has also been associated with HPV types 6 and 11.[30] However, negative immunoperoxidase staining and in situ DNA hybridization for HPV antigen for this entity has been reported.[31] Several different HPV types have been identified in laryngeal verrucous carcinoma using various methods (including PCR coupled with blot hybridization, Southern blot hybridization, or Southern blot hybridization of genomic DNA) and an etiologic relationship has been suggested.[32–36] Using histologic findings, immunohistochemistry, and DNA hybridization, Abramson et al[33] studied tissue specimens from 5 patients with verrucous carcinoma of the larynx and clearly demonstrated HPV 16-related DNA sequences in the tumor and in adjacent healthy tissues in all cases. In 1993, Kasperbauer et al[34] studied the incidence of HPV DNA in archival formalin-fixed, paraffin-embedded tissue sections from verrucous carcinoma of the larynx using PCR with consensus primers and by in situ hybridization designed to detect HPV types 6/11, 16/18, and 31/33/35. HPV DNA was detected in 17 of 20 (85%) tissue samples by PCR; none of the 20 samples were positive for the 7 genotype types tested by in situ hybridization. It is not clear whether this discrepancy in results represents true presence of different HPV types or merely a lack of sensitivity of the in situ hybridization method used. Fliss et al[35] examined HPV infections in formalin-fixed, paraffin-embedded tissue samples from 29 patients with laryngeal verrucous carcinoma by PCR using DNA primers specific for HPV types 6b/11, 16, and 18. Overall, HPV DNA was detected in 13 (45%) of the cases. Of these, HPV 16 DNA, HPV 18 DNA, and both HPV 16 DNA and HPV 18 DNA were detected in 4 (14% overall; 31% of positive cases), 4, and 5 (17% overall; 38% of positive cases) samples, respectively. HPV 6b/11 DNA was not detected in any laryngeal verrucous carcinomas. Furthermore, in 16 cases, no HPV DNA was detected. Although there was a trend toward HPV DNA detection in higher stage tumors, HPV DNA detection was unrelated to patient age, tumor site, or radiotherapeutic responsiveness. The detection of HPV DNA in 45% of laryngeal verrucous carcinomas suggests an association between the presence of HPV 16 DNA and HPV 18 DNA, and some laryngeal verrucous carcinomas. In 1996, López-Amado et al[36] studied 10 tissue samples of verrucous carcinoma of the larynx and found HPV expression in 4 cases.

The relationship of HPV with conventional squamous carcinoma in the larynx is not well established. In laryngeal squamous carcinomas, the frequency of HPV varies in different series. A few studies report on more than 80 patients with PCR-based techniques for detection. Table 1[37–44] summarizes the largest studies indicating the prevalence of HPV infections in laryngeal squamous carcinoma. These larger studies tended to show overall HPV prevalence lower than the average. In 2005, a total of 5046 head and neck cancers were pooled in a meta-analysis[9]; of these, 1435 were laryngeal cancers. HPV DNA was identified in 24% of tested laryngeal tumors (95% confidence interval [CI], 21.8–26.3). On the other hand, the general prevalence of HPV in normal laryngeal mucosa has not been determined because sampling methods for HPV detection in healthy cells have not been standardized, and prevalence estimates remain inconsistent. The reported incidence of HPV infection in normal laryngeal mucosa has been as high as 19%.[45–48] These results suggest that the number of HPV-positive cancers observed might reflect the prevalence of latent HPV infections in the vocal cord epithelium.

Hobbs et al[49] performed a systematic review and meta-analysis of observational studies that tested the presence of HPV 16 exposure in cases with head and neck squamous cell carcinomas and in a control group. They found that the association between HPV and cancer was strongest for tonsil primaries (odds ratio [OR], 15.1; 95% CI, 6.8–33.7), intermediate for oropharynx primaries (OR, 4.3; 95% CI, 2.1–8.9) and

Table 1. Human papillomavirus detection rates in large series of laryngeal squamous carcinomas.

Authors	Publication year	No. of samples	Detection technique	HPV +	HPV genotypes
Syrjänen et al[37]	1987	116	ISH for HPV types 6, 11, 16, and 30	12.9%	HPV 11: 7.8% HPV 16: 5.2%
Salam et al[38]	1995	87	HPV: CP Genotyping: RFLP	22.2%	HPV 6: 37.5% HPV 11: 12.5% HPV 16: 25%
Ma et al[39]	1998	102	HPV: CP Genotyping: Southern blot hybridization	58.8%	HPV 6: 42% HPV 11: 3% HPV 16: 50% HPV 18: 37% HPV 33: 1.6%
Gorgoulis et al[40]	1999	91	HPV: nested CP Genotyping: type-specific primers for HPV 6, 11, 16, 18, 31, 33, and 35	21%	HPV 6: 11% HPV 16: 68% HPV 18: 16% HPV 33: 16%
Gillison et al[41]	2000	86	HPV: CP, E7 type-specific for HPV 16 and 18 Genotyping: ISH	19%	
de Oliveira et al[42]	2006	110	HPV: CP Genotyping: multiplex PCR for HPV 6, 11, 16, and 18	37.3%	HPV 16: 37% HPV 18: 44% Not identified: 20%
Gungor et al[43]	2007	95	HPV: CP Genotyping: multiplex PCR for HPV 6, 11, 16, 18, 31, 33, 52, 58	7.4%	HPV 11: 4% HPV 6 & 11: 2% HPV 11 & 16: 1%
Morshed et al[44]	2008	93	HPV: CP Genotyping: INNO-LiPA	35.5%	HPV 16: 81.8% HPV 18: 18.2% HPV 33: 15.1%

Abbreviations: HPV, human papillomavirus; ISH, in situ hybridization; CP, consensus primers (MY09/11, GP5+/GP6+, SPF_{10}); RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; INNO-LiPA, reverse hybridization of L1 region with specific oligonucleotides.

weakest for oral (OR, 2.0; 95% CI, 1.2–3.4) and larynx primaries (OR, 2.0; 95% CI, 1.0–4.2). The method of detection of HPV 16 may be an important source of heterogeneity in the results of individual studies: those that detected HPV 16 using serum antibody testing by enzyme-linked immunosorbent assay (ELISA) are consistent with the idea that HPV is a strong risk factor for tonsillar carcinoma but less important elsewhere; studies that use tumor-based DNA amplification as the method of detection suggest that for oral, laryngeal, and oropharyngeal cancer, the association with HPV was greater. The inconsistency between studies using serum-based ELISA and tumor-based PCR analyses may reflect differences in sensitivity and specificity between the 2 techniques. Indeed, they are completely different methodologies: serum-based assays testing the presence of antibody against HPV are an indirect, systemic measure of prior HPV infection, whereas tumor-based assays are direct and have the ability to implicate a viral role in carcinogenesis. However, the study underscores the importance of performing case-control studies to estimate the risk attributable to HPV infection, as evidence of prior HPV infection has been detected in up to 38% of control tissues.[49]

The reported prevalence of HPV in laryngeal cancer varies significantly. Why are these figures so different? Reports that include small sample sizes are subject to potential selection bias. The methods used for case identification are often unclear; it is difficult to differentiate studies that enrolled consecutive patients from studies that used alternative inclusion criteria. As mentioned, HPV prevalence seems to be inversely proportional to the study sample size. These findings may be indicative of a selection bias in which certain cases were preferentially included in the study, or certain studies were published based on especially high HPV prevalence. Poor quality of some of the cancer specimens may also have affected the prevalence estimates. Another consideration is methodological differences. Gillison et al[41] identified HPV by consensus primers MY09/11, and to avoid false-negative results, all samples were also tested with type-specific primers for the E7 regions of HPV 16 and HPV 18. Of 253 head and neck squamous cell carcinomas analyzed, 55 tumors harbored HPV infection according to MY09/11 primer-based PCR, and 7 additional cases were positive only for type-specific primers. Because other HPV types were not included for type-specific E7 analysis, the true prevalence of viral DNA could also be underestimated.

The presence of viral DNA is not important by itself; rather, oncologic activity must be evident to suggest causality. As previously stated, there is a solution to the question of what constitutes a biologically relevant infection. A causal role for HPV in head and neck cancer can be defined by the presence of E6/E7 mRNA, viral integration with an intact E6 gene and p16 overexpression. A specific genetic profile of the tumor has been found when HPV is transcriptionally active, which can be interpreted as proof for active viral involvement in carcinogenesis. As a consequence, the

detection of HPV and an immunohistochemical assessment of the p16 expression may allow the tumor to be classified as biologically HPV-positive or biologically HPV-negative.[19,50] In 1999, the series by Gorgoulis et al[40] noted viral DNA integration by in situ hybridization for HPV types 16, 18, and 31. Two recent studies address this issue by also measuring p16 expression by immunohistochemistry.[51,52] The first study included 24 laryngeal carcinomas, and p16 was detected in 58%, with 100% correlation with HPV identification by chromogen in situ hybridization. In 2009, Baumann et al[52] analyzed 38 early laryngeal carcinomas (T1 or carcinoma in situ) with a positive result for PCR consensus primers in 16% (6 tumors). Of these 6 cases, 5 had enough material for immunohistochemistry, and all of them demonstrated overexpression of p16. These results suggest an active role for HPV infection in laryngeal carcinogenesis.

Potential Clinical Implications

In HPV-associated laryngeal cancer, HPV genotype distribution is expected to resemble that of high-risk HPV present in cervical lesions, mainly HPV 16 and HPV 18, which together account for more than 70% of worldwide cervical carcinomas, because anogenital contamination is the proposed route of transmission.[53] In Table 1, the prevalence of the most common HPV genotypes are described, but special consideration should be given to the method of HPV detection. The importance of genotyping can perhaps be justified given the current prophylactic vaccines for cervical carcinoma (Gardasil [Merck] and Cervarix [Glaxo Smith Kline]), which, apart from low-risk genotypes HPV 6 and HPV 11 (in Gardasil only), only include high-risk genotypes HPV 16 and HPV 18.[54] However, the role of these vaccines in preventing HPV-associated head and neck cancers is currently unknown.

The association of biologically relevant HPV infections in some laryngeal cancers also suggests an opportunity for future intervention with novel therapies targeting HPV-infected cells. Additionally, the implications of HPV infections in laryngeal cancer on clinical outcomes are certainly of interest in light of the known improved therapeutic responsiveness and prognosis of patients with HPV 16-associated oropharyngeal cancers.[5,6] In the largest series addressing this concern in HPV-associated laryngeal cancer, Morshed et al[44] observed no significant difference between HPV-positive and HPV-negative tumors in patient overall and disease-specific survival at 3 and 5 years. In the series of 78 laryngeal and hypopharyngeal cancers reported by Clayman et al,[53] detection of HPV DNA (in 46% of cases) was actually related to decreased survival, independent of disease stage. However, these analyses did not select for individual specific HPV types, and the stage distribution of patients and treatments administered were not homogeneous. As has been proposed for HPV-associated oropharyngeal cancers, identification of a subset of patients with laryngeal cancers with improved clinical outcomes can lead to the development of novel curative treatment approaches with the intent of improving quality of life and preserving excellent therapeutic results. Demonstration of improved chemosensitivity in a subset of laryngeal cancers would be of particular interest given the recently demonstrated potential of definitive chemotherapy for highly selected patients.[55,56] The role of HPV infection in therapeutic outcomes for patients with laryngeal cancer requires further investigation.

CONCLUSIONS

Although preliminary results suggest biological oncogenic activity, the role of HPV infection in squamous carcinoma of the larynx has not been clearly established. There is insufficient data on eventual carcinogenicity of low-risk HPV 6 and 11 infections, and biopsy specimens from patients with recurrent respiratory papillomatosis and transformed carcinomas should, therefore, be HPV typed. Although high-risk HPV DNA has been detected in a large proportion of laryngeal cancers, perhaps more commonly in verrucous carcinomas, whether HPV infection is etiologic for this cancer remains unclear. As was studied in oropharyngeal cancers, larger epidemiologic series and case-control studies are needed to definitively answer questions in laryngology that are still controversial, that is, the true prevalence of HPV infection in laryngeal mucosa and laryngeal cancers, its associated epidemiology and clinicopathologic characteristics, evidence of its carcinogenic intracellular activity, and its interactions with established risk factors like tobacco and alcohol consumption. Additionally, the prognostic significance for HPV infection in laryngeal cancer remains to be established, as well as the future, long-term impact of currently available HPV-based prophylactic vaccinations on the development of this disease.

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