Human papillomavirus and tobacco use in tongue base cancers

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Abstract

Human papillomavirus 16 (HPV-16) infection and tobacco use are associated with human oropharyngeal cancers. We conducted a study of the role of HPV and tobacco use in base of the tongue (BOT) cancers. DNA from 34 such cancers was subjected to HPV-16 and HPV-18-specific polymerase chain reaction analysis. Demographic and clinicopathologic data were obtained from each patient's medical record. HPV-16 was detected in 68% of tumors. Tobacco use was the only factor found to be significantly associated with HPV status. Tumors from 100% of patients who had never *used tobacco tested positive for HPV, compared with only 56% of those who had* ever *used tobacco (Fisher exact test,* p = *0.024). All tumors were associated with either tobacco use or HPV infection. These findings are consistent with the hypothesis that either tobacco use or HPV infection is necessary to the etiology of BOT tumors, and they suggest that tongue base carcinoma may be prevented by combining HPV vaccination with tobacco avoidance.*

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Introduction

The causal role of tobacco use in most head and neck squamous cell carcinomas (HNSCCs) has been well demonstrated.[1-3] Public health efforts to regulate the sale and use of tobacco products in the United States have led to a significant reduction in per capita cigarette consumption over the past 40 years to levels not seen since the 1940s.[4] This decline in smoking is reflected in an overall reduction in the incidence of HNSCC in some sites of the upper aerodigestive tract (e.g., the oral cavity, larynx, and hypopharynx), but not in the oropharynx. This exception is particularly notable in individuals younger than 45 years.[5,6] From 1974 through 1999, the proportion of oropharyngeal cancers relative to all HNSCCs rose by nearly 30%.[5]

The human papillomavirus (HPV) is known to cause nearly all uterine cervical cancers worldwide.[7] More recently, HPV's role in HNSCC has become increasingly evident. Only 15 to 20% of HNSCC cases occur in nonsmokers and nondrinkers. Evidence is mounting to support the role of HPV, particularly HPV-16, as a significant etiologic agent in these cancers.[8-10] In addition, several studies have reported that the prognosis for HPV-positive HNSCC patients is more favorable than it is for HPV-negative patients. HPV-positive patients may have a 60 to 80% reduction in the risk of death caused by their cancer compared with HPV-negative patients.[8,9,11-13]

We conducted a study to evaluate the incidence of HPV infection in oropharyngeal tumors, focusing on one subsite in the oropharynx: the base of the tongue (BOT). In addition, we also examined the relation-

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ship between the occurrence of HPV infection and our patients' clinicopathologic variables. We directed particular attention to the relationship between HPV-related cancers and tobacco use.

Patients and methods

Patients and tumor samples. We analyzed the tissue samples of 34 patients who had been treated for SCC of the tongue base from January 1996 through December 2007 in the Department of Head and Neck Surgery at the Roswell Park Cancer Institute in Buffalo, N.Y. The study population included 30 men and 4 women, aged 46 to 82 years (mean: 60.3; median: 58). Of this group, 31 were white and 3 were black.

In addition to demographic data, we obtained information on tobacco and alcohol use, use of illicit drugs, disease severity, and survival.

Tobacco use. Patients were categorized as *current, former,* or *never* tobacco users. The distinction between *current* and *former* was based on the definition of *former smoker* provided in the National Cancer Institute Thesaurus: "A [former smoker is a] person who was not smoking at the time of the interview but has smoked at least 100 cigarettes in their life."[14] At the time of their diagnosis, 15 patients were current tobacco users, 10 were former users, and 9 were never users.

Alcohol use. Patients' alcohol use was classified as either *significant* or *not significant* based on the definition provided by the Centers for Disease Control and Prevention (CDC).[15] According to the CDC, significant alcohol use entails more than two drinks per day on average for men and more than one drink per day on average for women. Alcohol use was not significant in 22 patients, significant in 6, and unknown in 6.

Illicit drugs. The use of illicit drugs was ascertained as part of each patient's medical history. None of these patients reported using any illicit drugs, including marijuana.

Cancer staging. The extent of disease was determined for each patient in accordance with current American Joint Committee on Cancer guidelines for head and neck cancer of the oropharynx.[16]

DNA preparation. The patients' tissue samples included 32 formalin-fixed, paraffin-embedded sections and 2 fresh-frozen tissue specimens. They were obtained through the Department of Pathology's Paraffin Archive or Tissue Procurement Facility. All specimens were primary mucosal SCCs of the BOT.

DNA was extracted from three 10-micron paraffin sections. Paraffin was removed by xylene treatment, and the specimen was rehydrated in a decreasing ethanol gradient. DNA from the remaining tissue and the fresh-frozen biopsies was extracted as described by Basik et al.[17] Briefly, an overnight digestion at 60°C in a cocktail containing 50 millimolars (mM) of KCl, 1.5 mM of $MgCl_2$, 10 mM of Tris-HCl, 0.5% TWEEN 20, and 200 µg/ml of proteinase K was followed by RNase A (100 µg/ml) digestion, phenol/chloroform extraction, and ethanol precipitation. Plasmids containing the HPV-16 or HPV-18 E6 genes (p3524 pGEM HPV-16 E6 and p3035 GST-HPV-18 E6) were purchased from the Addgene Repository[18] and used as positive controls in polymerase chain reaction (PCR) testing.

HPV detection. Of the more than 100 genotypes of human papillomavirus, HPV-16 and HPV-18, which are both high risk for promoting cancer, are associated with more than 85% of all virus-positive cancers of the oropharynx.[19] Therefore, our assessment of HPV status was limited to these two HPV types. PCR primers and conditions were as described by Stephen et al.[20] Reactions 1 and 2 contain 0.25 mM forward and reverse type-specific primers for the E6 regions of HPV-16 or HPV-18, 1.5 mM of $MgCl_2$, 2.0 mM of dNTPs, and 1 unit of Taq DNA polymerase (Invitrogen; Life Technologies; Carlsbad, Calif.) in a 1× amplification buffer supplied by the manufacturer. The forward and reverse primers for these reactions were:

- HPV-16F: 5′-ATTAGTGAGTATAGACATTA-3′
- HPV-16R: 5′-GGCTTTTGACAGTTAATACA-3′
- HPV-18F: 5′-ATTAGAGAATTAAGACATTA-3′
- HPV-18R: 5′-GGTTTCTGGCACCGCAGGCA-3′

Reaction 3, a control that amplifies a DNA fragment from the housekeeping gene β-globin, differs from reactions 1 and 2 only in the primers added and in that it contains 2.5 of mM $MgCl_2$. The forward and reverse primers for this reaction were:

- β-globinF: 5′-CAACTTCATCCACGTTACCC-3′
- β-globinR: 5′-GAAGAGCCAAGGACAGGTAC-3′

Triplicate amplifications were performed in a thermal cycler (PTC-100 Thermal Cycler; MJ Research; St. Bruno, Que.) as follows: initial denaturation at 95°C for 7 minutes was followed by 40 cycles at 95°C for 45 seconds (denaturation), at 52°C for 1 minute (annealing), at 72°C for 1 minute (extension), and for a 5-minute final extension at 72°C.

Five µl of each PCR reaction were resolved by elec-

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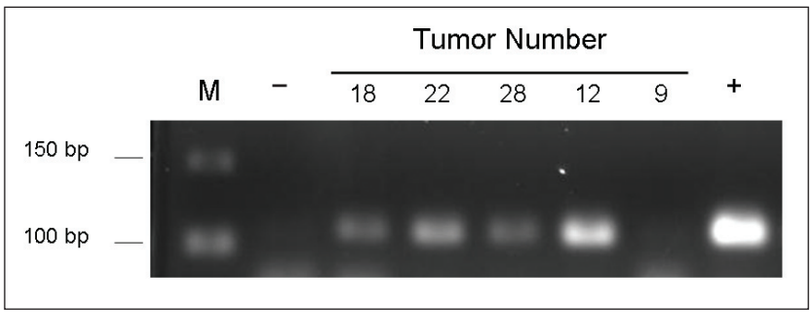

Figure 1. Image shows an electrophoretic anaylsis of HPV-16 E6 gene PCR products in 5 representative cases. The presence of a 109-base-pair (bp) fragment is indicative of the presence of HPV-16 DNA in the tumor (M = DNA size markers; – = no DNA control; + = HPV-16–positive control plasmid DNA containing the E6 gene).

trophoresis on a 3% LE agarose gel containing 1 mg/ml of ethidium bromide. The presence of a 109-base-pair fragment in either of the first two reactions confirms the presence of HPV in the sample, while a 268-base-pair β-globin fragment in reaction 3 establishes the DNA's ability to be amplified in the absence of either HPV band. Amplified DNA is visualized by ultraviolet transillumination. For HPV-negative tumors, an additional 40 cycles of PCR were performed on 5-µl aliquots of the previous PCR product, for a total of 80 cycles, in order to minimize false negatives.

Statistical analysis. The Fisher exact test was used for analyses of differences between the categorical (proportional) data, such as sex, tobacco use, alcohol consumption, race, stage, and tumor differentiation with respect to HPV status. The nonparametric Mann-Whitney *U* test was used to test for significant differences between HPV-positive and -negative tumors with respect to age at diagnosis and years of tobacco use (continuous data). Survival analyses were conducted with the Kaplan-Meier method. Statistical significance was accepted at a p value of ≤0.05.

Ethical considerations. Patients were enrolled in this study prior to surgery, and they provided written consent for the research use of their tissues and their clinical histories. The tissue samples were procured under the supervision of the Roswell Park Cancer Institute's Institutional Review Board (IRB), and a waiver of consent was granted by the IRB for use of the archived samples.

Table 1. Association between tobacco status and HPV-16 status

Tobacco status	Positive, n	Negative, n	*p* Value*
Current	7	8	
Former	7	3	
Never	9	0	0.024

** Fisher exact test for the distribution of current, former, and never tobacco users.*

Results

HPV-specific PCR coupled with gel electrophoresis was used to identify those tumors that harbored either HPV-16 or HPV-18 (figure 1). Of the 34 BOT tumors tested for HPV-16 and -18, 23 specimens (68%) tested positive for HPV-16 as evidenced by the amplification of the 109-base-pair fragment of the E6 gene; the remaining 11 tumors (32%) remained negative for HPV even after reamplification of the initial PCR reaction products (a total of 80 PCR cycles). None of the tumors was positive for HPV-18.

Of the clinicopathologic factors examined, only the use of tobacco products was found to be significantly associated with HPV status. Tumors from 9 of 9 patients (100%) who had never used tobacco tested positive for the presence of HPV, compared with 14 of 25 (56%) of those who had ever used cigarettes or tobacco products (table 1). Of these 14 patients, 7 were currently using tobacco and 7 were former users. Statistical analysis of the relationship between patients' history of tobacco use and the presence of HPV within their tumors using the Fisher exact test revealed this distribution to be statistically significant (p = 0.024, table 1).

A most interesting finding was that all 34 tumors occurred in patients who either had a tobacco history or were HPV-16 positive. No patient was both tobacco-negative and HPV-negative.

Analyses of the distribution of pack-years according to the Mann-Whitney *U* test indicated that patients with HPV-positive tumors had significantly fewer pack-years when current (p = 0.01), current and former (p = 0.02), and current, former, and never (p = 0.0006) smokers were compared with their HPV-negative counterparts (table 2).

No patient reported using marijuana, which has previously been shown to be associated with HPV-16–positive tumors.[21]

Among the 28 patients for whom information was available on alcohol use, we found no significant association between alcohol consumption and HPV-16 status with the Fisher exact test (p = 0.2). We also found no

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significant associations between tumor HPV status and sex, race, age at diagnosis, stage, or degree of tumor differentiation.

Finally, while an improved prognosis has been reported for patients with HPV-16–positive HNSCC in general and in BOT tumors in particular,[9,11,12] our Kaplan-Meier analyses found no differences in survival between patients with HPV-16-positive tumors and HPV-16-negative tumors (figure 2).

Table 2. Smoking and quitting history according to HPV-16 status

	Smoking history, pack-years, mean (range)	Time from quitting to diagnosis, years, mean (range)
Current smokers*		
HPV-positive (n = 7)	35 (17 to 60)	
HPV-negative (n = 8)	70 (29 to 129)	
Former smokers		
HPV-positive (n = 7)	24 (8 to 50)	21 (6 to 34)
HPV-negative (n = 3)	24 (15 to 40)	20 (2 to 40)
Current and former smokers†		
HPV-positive (n = 14)	30 (8 to 60)	
HPV-negative (n = 11)	59 (15 to 129)	
Current, former, and never smokers‡		
HPV-positive (n = 23)	18 (0 to 60)	
HPV-negative (n = 11)	59 (15 to 129)	

** Mann-Whitney U test,* p = *0.01.*
† Mann-Whitney U test, p = *0.02.*
*‡ Mann-Whitney U test,*p = *0.0006.*

Discussion

The incidence of HPV in tumors of the head and neck has been generally reported to range from 25 to 35% overall and 40 to 65% in oropharyngeal cancers.[19,22,23] Our highly sensitive PCR analysis showed a 68% prevalence of HPV-16 DNA in these BOT tumors, a finding that is consistent with similar studies of HPV that focused on the oropharynx. For example, in studies of tonsillar SCC by Charfi et al[22] and by Hammarstedt et al,[23] comparable rates of HPV positivity in tumors were observed—62% positivity in the former study and between 57 and 65% positivity in the latter. We did not find HPV-18 to be present in any of our tumors.

Gillison et al reported a strong association between HPV-16–positive HNSCC and the use of marijuana.[21] None of our patients reported any history of marijuana use prior to diagnosis, and we conclude that marijuana did not play a role in this cohort.

Tobacco exposure has long been regarded as the major causative factor in oropharyngeal SCC.[1-3] Our data suggest that a significant subset (26%) of BOT SCCs are linked to HPV infection and are independent of tobacco use (i.e., the 9 never smokers in our study).

Two additional subsets of tumors were identified, one in tobacco users who were HPV-negative (32%) and a second in tobacco users who were HPV-positive (41%). In addition, we found that among ever smokers, the number of pack-years prior to diagnosis was significantly less in those whose tumors were HPV-positive than in those whose tumors were

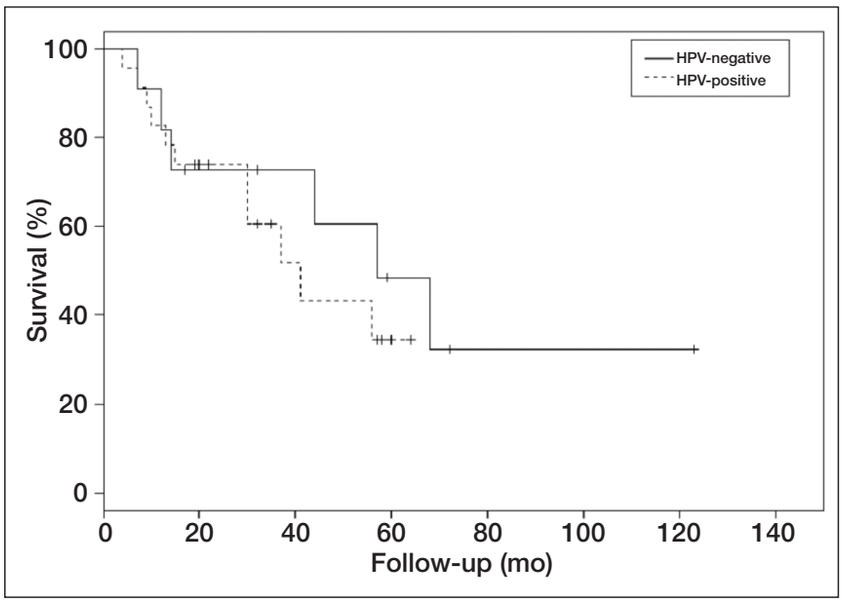

*Figure 2. Kaplan-Meier plots compare survival between patients with HPV-16–positive BOT SCCs and HPV-16–negative tumors (N = 34). A log-rank test of the data determined that there was no significant difference between the two groups (*p = *0.499). Vertical lines represent censored patients.*

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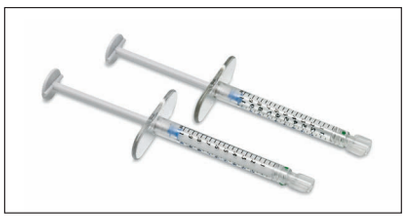
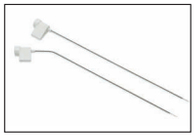


RADIESSE Voice and RADIESSE Voice Gel implants are sold with either a malleable transoral needle or a non-coring percutaneous needle.



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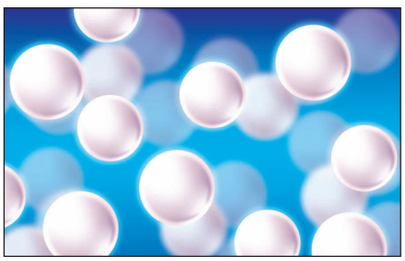
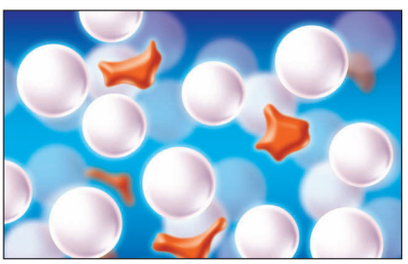
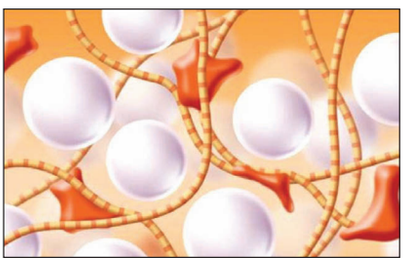
In clinical testing and routine clinical use for over 6 years, no implant migration or evidence of granuloma formation has been observed.



RADIESSE Voice implant (CaHA + gel carrier) initially performs as a filler.



Macrophages start to degrade gel carrier (2-3 months).



Macrophages dissolve gel carrier as new collagen forms.

Clinically proven results

- Proven effective – in clinical testing with 12 months follow up, the majority of patients treated with the RADIESSE Voice implant reported that their voice was greatly or significantly improved.[1]
- Proven safe – no granuloma formation or major complications have been reported.[1]
- Proven long-lasting – results typically last more than 12 months.[1,4]

Convenient and easy-to-use

- No fat harvesting or processing
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Injection techniques

RADIESSE products can be injected through a 25-gauge needle in the operating room or in the office using a trans-thyroid cartilage, trans-cricothyroid membrane or thyrohyoid approach.[2,3]

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In Europe, availability of the RADIESSE Voice implant varies by country, and the RADIESSE Voice Gel implant is currently not available.

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HPV-negative. Although this suggests that HPV-16 infection is more likely to be the primary cause of BOT cancer in these patients, it is not possible to ascertain if one factor was more responsible for the onset of cancer than any other factor in our study. In the future, other analyses such as genomic profiling may shed light on this question.

It is significant that we found that all tongue base cancers were associated with either tobacco use or HPV-16 infection, and often both. Similarly, the study by Charfi et al demonstrated that 100% (10/10) of tonsillar SCCs in never smokers were found to be HPV-16-positive, with a high degree of statistical significance.[22] Based on our data and that of other researchers, we conclude that HPV-16 infection and tobacco use are two separate but not mutually exclusive etiologic agents that underlie nearly all cases of oropharyngeal SCC.

The quadrivalent HPV vaccine for HPV-16, -18, -6, and -11 has been shown to be effective in preventing cervical cancer associated with HPV-16 and HPV-18.[24] Since HPV-16 appears to play an important role in some tongue base and other oropharyngeal cancers, it is anticipated that this vaccine may have a significant effect on reducing the incidence of these cancers. If this is correct, then we further anticipate that HPV vaccination in combination with successful tobacco avoidance may profoundly impact the incidence of oropharyngeal cancer.

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