Published in final edited form as:
Oral Oncol. 2014 April ; 50(4): 306–310. doi:10.1016/j.oraloncology.2014.01.006.

Human papillomavirus (HPV) status of non-tobacco related squamous cell carcinomas of the lateral tongue

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Abstract

Objectives—The human papillomavirus (HPV) is an important cause of some head and neck squamous cell carcinomas (HNSCCs), but its role in cancer of the lateral tongue is debatable. Suspicion of HPV causation is heightened when these lateral tongue carcinomas arise in patients that are young and/or have never smoked. The purpose of this study was to determine the incidence of transcriptionally active high risk HPV in these tumors, with a particular emphasis on non-smoking patients who are often presumed to have HPV-positive tumors.

Methods—We evaluated 78 HNSCCs of the lateral tongue for the presence of HPV using p16 immunohistochemistry and an RNA in situ hybridization assay targeting HPV E6/E7 mRNA. The study population was enriched for patients without traditional risk factors such as smoking and drinking.

Results—P16 overexpression was detected in 9 (11.5%) of 78 cases, but HPV E6/E7 mRNA transcripts were detected in only 1 (1.3%) case (positive predictive value of p16 staining for the presence of transcriptionally active HPV = 0.12). HPV mRNA transcripts were not detected in any patient under 40 (n = 11), or in patients who had never smoked (n=44), had quit smoking (n=15), and/or were only light consumers of alcohol (n = 57).

Conclusions—HPV is not detected in the vast majority of lateral tongue carcinomas. In light of the observation that HPV plays little if any role in the development of these cancers, routine HPV testing is unwarranted , even for patients without traditional risk factors. P16 staining is not a reliable marker for the presence of transcriptionally active HPV at this particular anatomic site.

Keywords

head and neck cancer; oral cancer; RNA in situ hybridization; p16; smoking; tobacco

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Introduction

Most squamous cell carcinomas of the head and neck (HNSCC) are caused by smoking, but an important subset is caused by high risk human papillomavirus (HPV) (1, 2). These HPV-related HNSCCs are distinct from smoking related cancers. Not only do they arise independent of smoking activity, but they involve younger patients and are associated with more favorable clinical outcomes (3–5). Due to the prognostic and therapeutic implications of tumor causation, testing HNSCCs for the presence of HPV has become an increasingly common clinical practice.

A growing experience with HPV testing has confirmed that most HPV-related HNSCCs arise in the oropharynx (1, 6–9). HPV is detected in up to 80% of HNSCCs arising at this particular site. The role of HPV in HNSCC arising in the oral cavity proper is more ambiguous. Indeed, previously published HPV detection rates in oral cavity cancers have shown widely disparate results, ranging from 0% to 100% (10). This astounding variation reflects the combination of various factors including non-uniform methods of HPV detection. Most notably, those assays that merely detect the presence of HPV, including passenger virus and viral contaminants, may yield incident rates that highly inflate the presence of clinically relevant virus. Variation in detection rates may also be influenced by differences in stringency for defining anatomic tumor site. For example, the common practice of combining carcinomas of the oral tongue and base of tongue would certainly inflate the incidence of HPV-related oral carcinoma. Finally, detection rates could also be impacted by differences in patient population. A disproportionate number of HNSCCs of the oral tongue arise in non-smokers and in patients under 40 years of age (11–14) – a clinical profile that, on the face of it, points to HPV causation. To the degree that these tumors are in fact HPV-positive, overrepresentation of this patient population could also potentially drive up HPV incidence rates at this site.

The purpose of this study was to determine the incidence of transcriptionally active high risk HPV in oral squamous cell carcinoma using RNA in situ hybridization technology (RNAscope®) – a methodology that permits direct visualization of RNA in formalin-fixed, paraffin-embedded tissue - with a particular emphasis on non-smoking patients who are often presumed to have HPV-positive tumors. The RNAscope® technology confirms the presence of integrated and transcriptionally-active virus in FFPE samples with a performance that is comparable to Reverse transcriptase-PCR in matched fresh frozen samples (15, 16). Clarification of the HPV incidence in HNSCCs of the lateral tongue could help inform the HPV evaluation process in a way that moves wide scale, indiscriminant and non-standardized testing towards a more directed, clinically relevant and standardized approach.

Methods

Patients and specimens

Study approval was obtained from the Johns Hopkins Medical Institutions Review Board. The Head and Neck Cancer data base at the Johns Hopkins Hospital was searched for cases of squamous cell carcinoma (SCC) of the lateral tongue in patients who were non-smokers or formers smokers. The lateral location of the cancer and its distinction from a ventral origin was a matter of clinical judgment. For comparison, SCCs of the lateral tongue from 14 current smokers were also included. With the exception of 3 patients (2 current smokers and 1 non-smoker), this was a distinct study group that was not included in a prior report of HPV status in head and neck cancers. For each patient, the medical records were carefully reviewed to confirm smoking status as well as age at diagnosis, gender, tumor site and stage, and use of alcohol. A patient was considered a smoker if exposure to tobacco was estimated

to be at least 10 pack years and he or she was either currently smoking or had ceased smoking less than 10 years before diagnosis. Light smokers (individuals with less than 10 pack year history) were not included in this study. Individuals who had quit smoking more than 10 years earlier were considered to be former smokers. Nonsmokers had never used tobacco on a regular basis. Alcohol use was estimated in terms of units of alcohol (12 ounces of beer, 3.6 ounces of wine, or 1 ounce of hard liquor). Alcohol use was categorized as “light”, indicating <10 units per week, “moderate”, indicating 10 to 20 units per week, and “heavy”, indicating more than 20 units per week. Patients were included only if histologic slides and corresponding formalin-fixed and paraffin-embedded tumor block was available for HPV analysis.

P16 immunohistochemistry

Five-micron sections from the tissue blocks were evaluated by immunohistochemistry for expression of a biomarker of HPV E7 oncoprotein activity, the CDK-inhibitor p16. Sections of formalin-fixed and paraffin-embedded tissues were deparaffinized and subjected to antigen retrieval using 10 mM citrate buffer (92° C for 30 minutes). P16 expression was evaluated by use of a mouse monoclonal antibody against p16 (MTM Laboratories, Heidelberg, Germany) visualized using the Ultra view polymer detection kit (Ventana Medical Systems, Inc. Tucson, AZ) on a Ventana BenchmarkXT autostainer (Ventana). P16 expression was scored using a 4-tiered system: 0 = completely negative staining; 1 = focal staining (less than 20% of tumor cells); 2 = patchy staining (20 – 70% of tumor cells); 3 = diffuse staining (nuclear and cytoplasmic staining in greater than 70% of tumor cells). As a surrogate marker of HPV infection, only staining that was diffuse was regarded as positive for p16 overexpression.

HPV RNA In situ hybridization (ISH)

RNA ISH for high risk HPV E6/E7 mRNA was performed fully automated on the Ventana Discovery XT slide autostaining system (Ventana Medical System, Tuccson, AZ) using the RNAscopeVS™ HPV kit (Advanced Cell Diagnostics, Inc., Hayward, CA). Briefly, 4 μm formalin fixed, paraffin embedded tissue sections were pretreated with heat and protease prior to hybridization with a cocktail of target probes to the high risk HPV genotypes 16, 18, 31, 33, 35, 52, and 58. The preamplifier, amplifier and HRP-labeled probes were then hybridized sequentially, followed by color development with DAB. Probes to the bacterial gene dapB and the endogenous UBC mRNA were used as negative and positive controls, respectively, for each case. Specific staining signals were identified as brown, punctate dots present in the cytoplasm and/or nucleus. All three stained slides of each case, HPV, UBC and dapB, were examined together to determine HPV status. HPV status was scored qualitatively as either positive or negative, using the dapB-stained slides as reference. HPV positive cases had definitive punctate brown staining present in at least a subset of tumor cells.

Results

The clinical features of the 78 patients with HNSCC of the lateral tongue are summarized in Table 1. Forty-two patients (54%) were female and 36 (46%) were male. The relationship between gender and tobacco history in head and neck squamous cell carcinomas is well recognized. Large epidemiological studies have noted that the proportion of female subjects among nonsmokers is significantly higher than among smokers (17, 18). The median age was 55 years (range, 22 to 84 years). Eleven (14%) patients were under the age of 40 at the time of diagnosis. Forty-four (56%) were non-smokers, 15 (19%) were former smokers, and 14 (18%) were active smokers at the time of diagnosis. For 5 (6%) patients, smoking status could not be confirmed on review of the medical records. None of the patients were chewers

of tobacco. As for alcohol consumption, 57 (73%) were classified as light alcohol consumers, 5 (6%) as moderate consumers, and 6 (8%) as heavy consumers. Of the 44 non-smokers, 41 (93%) were light alcohol consumers. For 10 (13%) patients, an accurate assessment of alcohol use could not be determined on review of the medical records.

Using the RNAscope method, mRNA E6/E7 transcripts were detected in just 1 (1.3%) of the 78 HNSCCs of the lateral tongue. This singular case was from a 62 year old white male who was a current smoker and a moderate consumer of alcohol at the time of diagnosis. Notably, this singular HPV-positive SCC displayed histologic features reminiscent of "warty carcinoma" – an HPV-related carcinoma of the female vulva (19). These features included marked epithelial proliferation with parakeratosis and hyperkeratosis, broad rete pegs, and squamous cells with koilocytic atypia, cytoplasmic cavitation, and marked nuclear pleomorphism characterized by enlargement, wrinkling of the nuclear membrane and even multinucleation (Figure 1). The tumor demonstrated high expression of p16 by immunohistochemistry, and abundant mRNA E6/E7 transcripts by RNA in situ hybridization (Figure 1). mRNA E6/E7 transcripts were not detected in any of the other 77 lateral tongue cancers including the 44 cancers from non-smokers, 15 cancers from former smokers and 11 cancers from young patients (under the age of 40 years).

Nine (12%) of the 78 lateral tongue carcinomas were p16 positive by immunohistochemistry. P16 staining did not correlate with patient age, smoking history or alcohol consumption. P16 expression was not strongly associated with the presence of HPV E6/E7 mRNA (Figure 2). Of the 9 lateral tongue carcinomas that were p16 positive by immunohistochemistry, only 1 (11%) was HPV positive using the RNAscope method. Using HPV E6/E7 mRNA expression to measure the prevalence of HPV-related HNSCC for HNSCCs of the lateral tongue, the positive predictive value of p16 positivity as a surrogate marker of HPV was just 0.12 (0.06 – 0.21, 95% confidence interval).

Discussion

HPV is an important cause of oropharyngeal carcinoma, but highly disparate rates of HPV detection have caused considerable uncertainty regarding its role in the development of squamous cell carcinomas of the oral cavity. Recent studies using sensitive PCR methods have identified HPV DNA in a high proportion (35–55%) of oral squamous cell carcinomas (20, 21), but detection rates tend to plummet in those studies that require evidence of oncologic activity of HPV. Using expression of HPV E6 and E7 mRNA transcripts as a true measure for the presence of oncologically active virus, HPV is detected in only a small fraction (1–5%) of oral squamous cell carcinomas (8, 22). Taking advantage of recently developed RNA in-situ hybridization probes complementary to E6/E7 mRNA that now permit direct visualization of viral transcripts in routinely processed tissues, we were able to confirm that transcriptionally active HPV is seldom encountered in squamous cell carcinomas of the lateral tongue. E6/E7 mRNA viral transcripts were detected in only 1 (1.3%) of the carcinomas using RNA in situ hybridization. This low detection rate cannot be dismissed on the basis of low sensitivity of the RNAscope method. In formalin-fixed and paraffin-embedded samples of oropharyngeal carcinomas, the sensitivity of this method has been shown to match the sensitivity of p16 immunohistochemical staining and exceed that of HPV DNA in situ hybridization (8, 16, 23, 24).

The low rate of HPV detection was upheld in a population enriched for patients without traditional risk factors of oral carcinoma. HPV was not detected in any patients under 40 years of age (n = 10), or in patients who never smoked (n = 44), had quit smoking (n = 15) and/or were light consumers of alcohol (n = 55), even though this profile depicts the prototypic patient with HPV-related HNSCC. Liang et al.(25) also found the incidence of

HPV to be very low in oral tongue cancer across a wide range of patient ages including patients under 45 years. The rising trend of oral tongue cancer in patients without traditional risk factors, it would seem, cannot be attributed to HPV. This observation expands an evolving view of the makeup of HNSCC. HNSCC has long been regarded as a single disease entity, but a growing awareness of the causal role of HPV has advanced a perspective that HNSCC is a more heterogeneous group that can be further divided along the lines of HPV status into smoking related and HPV related categories. The absence of transcriptionally active HPV in the abstaining population supports the presence of yet a third important category of HNSCC. Not surprisingly, this third category is known to carry a distinct molecular profile (26). Focus on this group may provide fresh insights into new risk factors and molecular targets that may aid in the cancer prevention and management in this abstaining population.

Confirmation of human papillomavirus (HPV) as a causative agent for a subset of HNSCC has resulted in a growing expectation for HPV testing of head and neck cancers. At the same time, clinical algorithms for HPV testing must be informed in a way that moves wide scale, indiscriminant and nonstandardized testing towards a more directed, clinically relevant and standardized approach. Our observation supports a growing body of evidence that high risk HPV has a strong site predilection for the oropharynx, and the likelihood of detecting its presence in carcinomas of non-oropharyngeal sites, including the adjacent oral cavity, is very low (1, 6–9). This low incidence, together with the lack of sufficient evidence to establish HPV as a strong prognostic factor for non-oropharyngeal HNSCCs, would not support routine testing of all HNSCCs. Indeed, the common clinical practice of using young age and tobacco abstinence as trigger points for HPV testing of lateral tongue carcinomas is not valid.

Immunostaining for p16 protein has recently been regarded as a practical alternative for HPV testing based on a high correlation between HPV detection and p16 overexpression in many studies (27–29). Our observations, however, caution against the indiscriminate use of p16 immunohistochemistry in a way that does not take into account tumor site. In sites like the oral cavity that are not preferentially targeted by HPV, the likelihood that p16 over-expression truly reflects the presence of transcriptionally active HPV (i.e. positive predictive value) is very low (8, 22). Given the low positive predictive value of p16 staining for HNSCCs of the lateral tongue, the possibility that elevated p16 expression may be induced by non-viral related mechanisms must be a strong consideration.

In summary, HPV is not a common cause of lateral tongue carcinomas in patients without significant risk factors. Accordingly, this group must remain a focus of continued efforts to discern the etiology and mechanisms of cancer development in ways that would facilitate a rational approach to tumor prevention and management. In light of the observation the HPV plays little if any role in the development of these cancers, routine HPV testing is unwarranted.

Acknowledgments

Conflicts of Interest and Sources of Funding: The RNA in situ hybridization assays in the study were funded by and performed by the coauthors from Advanced Cell Diagnostics, Inc. (S.B., X-J.M., and Y.L.), who have stocks in this company and stands to profit by any use of this testing through the company.

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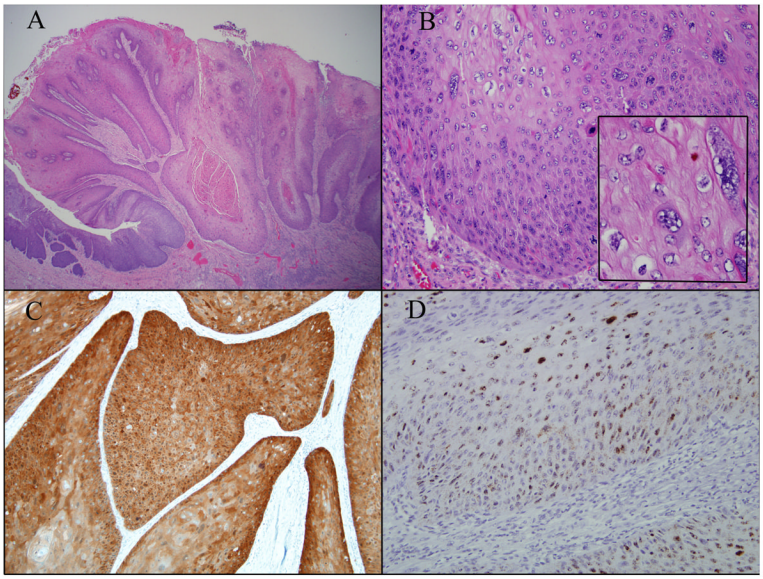

Figure 1.

An HPV-related squamous cell carcinoma of the lateral tongue. The tumor demonstrates features of “warty carcinoma” including a thickened surface epithelium with downward extension as broad pushing rete pegs (A, hematoxylin and eosin). The tumor cells exhibit varying degrees of koilocytic atypia including perinuclear halos and wrinkled nuclear contours (B, hematoxylin and eosin). Some of the cells have enlarged and multilobulated nuclei (B, inset). The tumor is strongly p16 positive by immunohistochemistry (C) and high risk HPV positive by RNA in situ hybridization (D).

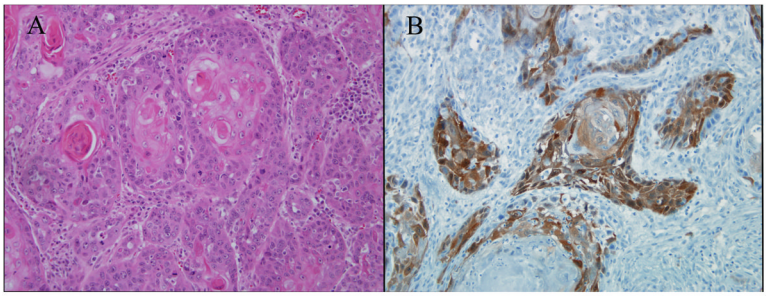

Figure 2.
This keratinizing squamous cell carcinoma (A, hematoxylin and eosin) was HPV negative by in situ hybridization even though it overexpressed p16 (B, p16 immunohistochemistry).

Table 1

Summary of clinical findings and HPV analysis in 78 squamous cell carcinomas of the lateral tongue

	P16 IHC		HPV ISH		Total
	+	−	+	−	
Gender					
male	6	30	1	35	36 (46)
female	3	39	0	42	42 (54)
Age					
< 40	1	10	0	11	11 (14)
≥ 40	8	59	1	66	67 (86)
Smoking status					
Smoker	2	12	1	13	14 (18)
Former-smoker	2	13	0	15	15 (19)
Non-smoker	5	39	0	44	44 (56)
Undocumented	0	5	0	5	5 (6)
Alcohol consumption					
Light	5	52	0	57	57 (73)
Moderate	2	3	1	4	5 (6)
Heavy	2	4	0	6	6 (8)
Undocumented	0	10	0	10	10 (13)
Tumor differentiation					
Well	2	14	1	15	16 (21)
Moderately	7	44	0	51	51 (65)
Poorly	0	11	0	11	11 (14)
TNM stage					
T Stage					
1	5	47	1	51	52 (67)
2	4	18	0	22	22 (28)
3	0	4	0	4	4 (5)

	P16 IHC		HPV ISH		Total
	+	−	+	−	
4	0	0	0	0	0 (0)
N stage					
0	7	47	1	53	54 (69)
1	0	10	0	10	10 (13)
2	2	12	0	14	14 (18)
M stage					
0	9	68	1	76	77 (99)
1	0	1	0	1	1 (1)
Total	9 (12)	69 (88)	1 (1)	77 (99)	78 (100)

IHC, immunohistochemistry; HPV, high risk human papillomavirus; ISH, in situ hybridization