Human papillomavirus–related oropharyngeal cancer: HPV and p16 status in the recurrent versus parent tumor

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ABSTRACT: *Background.* Although typically associated with a favorable prognosis, a minority of human papillomavirus (HPV)-related (+) oropharyngeal cancers recur after chemoradiation. We postulated that a minor HPV-negative tumor subfraction may be responsible for recurrences of HPV+ oropharyngeal cancer.

Methods. Paired untreated primary and recurrent tumor specimens were identified for 37 patients with oropharyngeal cancer who received definitive chemoradiotherapy at our institution. Concordance in HPV/p16 expression between primary and recurrent tumors was assessed.

Results. Among 31 patients with HPV+/p16+ primary tumors, 30 (97%) retained evidence of both HPV and p16 expression at recurrence (27 HPV+/p16+; 3 HPV+/p16-partial). One (3%) initially HPV+/p16+ patient developed an HPV-negative/p16-negative lung squamous cell carcinoma (SCC), representing either a discordant oropharyngeal cancer metastasis or second primary tumor.

Conclusion. HPV-related oropharyngeal cancers retain HPV+/p16+ expression at recurrence. Our results fail to provide evidence that a minor HPV-negative tumor subfraction is responsible for biologically aggressive behavior of HPV+ oropharyngeal cancer that recurs after chemoradiation. © 2014 Wiley Periodicals, Inc. *Head Neck* **37:** 8–11, 2015

KEY WORDS: human papillomavirus, p16, oropharyngeal cancer, expression profiling, chemoradiation

INTRODUCTION

Human papillomavirus (HPV) is the primary causative factor in the majority of oropharyngeal cancers in developed countries and is present in over 70% of newly diagnosed oropharyngeal cancer.[1] HPV-associated oropharyngeal cancer represents a distinct disease entity from non-HPV-associated oropharyngeal cancer, more commonly affecting younger patients and former or nonsmokers who often present with smaller primary tumors and cystic-appearing nodal metastases.[2–4] Although HPV-related oropharyngeal cancer is associated with a more favorable prognosis than its HPV-negative counterpart, those patients with a history of heavy tobacco use, matted lymph nodes, and more advanced tumor (T4) and nodal (N3) stages remain at significant risk for both locoregional and distant failure.[2,5–7] The molecular basis of the uncharacteristically aggressive tumor behavior in these poorer prognosis HPV+ oropharyngeal cancer subgroups has yet to be determined.

Overexpression of p16 is a surrogate marker for HPV infection that can be readily determined by immunohistochemistry (IHC) and is frequently used to determine HPV-positivity in oropharyngeal cancer.[8,9] Although IHC for p16 is typically unequivocally positive or negative, partial or even absent p16 immunostaining may occasionally be encountered in HPV+ oropharyngeal cancer.[10,11] Similarly, heterogeneous HPV-expression within tumors has been described.[12,13] Such reports have stimulated speculation that heterogeneous or discordant HPV and p16 expression may identify tumors in which HPV is present merely as a "bystander," which may be associated with a worse prognosis than "HPV-driven" oropharyngeal cancer that diffusely expresses both HPV and p16.[12] It may further be hypothesized that the minority of HPV+ oropharyngeal cancers, which demonstrate biologically aggressive behavior, may be driven by a minor HPV-negative subpopulation of tumor cells within an otherwise HPV+ tumor, and would therefore manifest an HPV-negative/p16-negative phenotype at the time of recurrence. The poorer prognosis of patients with HPV+ oropharyngeal cancer who have a history of heavy smoking

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supports this hypothesis, given the established causal relationship between smoking and HPV-negative head and neck squamous cell carcinoma (SCC).[2,5,6,14,15] Data to assess this possibility, however, remain lacking. We therefore analyzed HPV and p16 expression in recurrent oropharyngeal cancer to determine whether HPV-associated oropharyngeal cancer expresses HPV and p16 at the time of recurrence.

MATERIALS AND METHODS

Patients

This study was approved by the University of Michigan Institutional Review Board. The records of 231 consecutive patients with histologically confirmed, previously untreated American Joint Committee on Cancer stage III or IV oropharyngeal SCC who received definitive radiotherapy and concomitant cytotoxic chemotherapy at the University of Michigan between May 2003 and October 2010 were retrospectively reviewed. Thirty-eight patients who experienced biopsy-proven locoregional or distant recurrence were identified. After excluding 1 patient without available tissue from the time of recurrence, 37 patients with paired tumor tissue from both the time of primary diagnosis and recurrence were included in the present study.

Treatment

After routine staging, consisting of clinical examination, direct laryngoscopy, contrast-enhanced CT, or fluorodeoxyglucose-positron emission tomography-CT, and chest imaging, all patients underwent CT simulation in a 5-point thermoplastic mask for immobilization. All patients received intensity-modulated radiotherapy (IMRT) with concurrent cytotoxic chemotherapy, consisting of either weekly carboplatin and paclitaxel ($n = 36$) or daily cisplatin and 5-fluorouracil during weeks 1 and 5 ($n = 1$), with hydration and antiemetics administered per standard of care. IMRT in all patients consisted of a single differentially dosed plan with 70 Gy prescribed to the gross tumor volume and 56 to 64 Gy prescribed to clinical target volumes at risk for subclinical disease. Planning target volumes were created by a 3- to 5-mm uniform expansion of the gross tumor volumes and clinical target volumes. IMRT was delivered in either 2 Gy daily fractions ($n = 35$) or 1.25 Gy twice-daily fractions ($n = 2$). All patients were routinely seen in follow-up in the University of Michigan Departments of Radiation Oncology, Otolaryngology – Head and Neck Surgery, and Hematology/Oncology, with clinical examination performed every 6 to 12 weeks and head and neck imaging (either contrast-enhanced CT or positron emission tomography/CT) obtained at 3 months after completion of chemoradiation and every 3 to 6 months thereafter.

Human papillomavirus and p16 testing

HPV and p16 testing was prospectively performed on untreated primary tumor tissue either as part of either an institutional review board-approved tissue microarray study ($n = 35$) or as part of routine clinical practice ($n = 2$). For specimens collected in the tissue microarray, HPV expression was determined by an ultrasensitive method using HPV-MultiPlex Polymerase Chain Reaction-MassArray (PCR-MA) real time competitive polymerase chain reaction (PCR) after isolation of DNA from cored tissue samples using the QIAamp DNA Formalin-Fixed Paraffin-Embedded Tissue kit (Qiagen, Valencia, CA), with DNA concentration and purity confirmed via NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). PCR amplification of the E6 region of 15 discrete high-risk HPV types and matrix-assisted laser desorption/ionization-time of flight mass spectroscopy with separation of products on a matrix-loaded silicon chip array were performed as previously described.[16] For clinically determined cases, HPV expression tested by in situ hybridization (ISH) on 4-μm sections from paraffin-embedded tissue blocks containing a representative sample of primary tumor using the INFORM HPV ISH assay (Ventana Medical Systems, Tucson, AZ) by a cocktail directed against a subset of high-risk HPV genotypes (HPV-16, 18, 33, 35, 39, 45, 51, 52, 56, and 66), with positive reactions detected using the ISH I View Blue Plus Detection Kit (Ventana Medical Systems) in accordance with the manufacturer's instructions. For all cases, IHC for p16 was performed per protocol supplied by the kit (CINtec ^{p16INK4a} Histology Kit; mtm Laboratories, Westborough MA). p16 expression was classified based upon percentage of tumor staining in both the nucleus and cytoplasm, with >75% staining classified as positive, 20% to 75% scored as partial, and <20% scored as negative.

For recurrent tumors, HPV and p16 status was retrospectively determined using the HPV ISH and p16 IHC procedures described above. Concordance between the molecular characteristics of the primary and recurrent tumor was determined by the expression of HPV and p16 in each specimen. For cases with discordance between HPV PCR-MA in the untreated primary tumor and HPV ISH in the recurrence, confirmatory testing for HPV was performed on both the primary and recurrent tumor using ISH for primary tumors initially tested by PCR-MA and PCR-MA for recurrent tumors initially tested by ISH.

RESULTS

Characteristics for the 37 patient cohort are displayed in Table 1. The initial primary tumor was HPV+ in 32 patients and HPV-negative in 5 patients. Concordance between HPV and p16 expression in the untreated primary tumor was 97% (36 of 37 cases); the only discordant case was in an HPV+/p16-negative T4 N2c tonsillar SCC in a former 25 pack-year smoker.

Recurrent tumor tissue was obtained from locoregional recurrence in 16 patients (43%) and distant metastasis in 21 patients (57%), including lung metastases in 9 patients, mediastinal or hilar lymph nodes in 7 patients, bony metastases in 4 patients, and a paravertebral soft tissue metastasis in 1 patient.

Expression of both HPV and p16 in the recurrent tumor identically matched that of the initial primary tumor in 32 of 37 patients (86%). Among initially HPV+/p16+ primary tumors the rate was 87% (27 of 31). Three of the 5 cases without complete concordance in HPV and p16 expression between the initial and recurrent tumors retained

TABLE 1. Baseline characteristics.

Characteristic	No. of patients (%)
Sex	
Male	35 (95)
Female	2 (5)
Tumor site	
Base of tongue	17 (46)
Tonsil	20 (54)
T classification	
1	3 (8)
2	8 (22)
3	6 (16)
4	20 (54)
N classification	
0	2 (5)
1	1 (3)
2a	1 (3)
2b	14 (38)
2c	12 (32)
3	7 (19)
AJCC stage	
III	1 (3)
IV	36 (97)
Smoking status	
Never	11 (30)
Former	11 (30)
Current	15 (40)
Smoking pack-years	
<10	15 (41)
>10	22 (49)
HPV and p16 status at diagnosis	
HPV+/p16+	31 (84)
HPV-negative/p16-negative	5 (13)
HPV+/p16-negative	1 (3)
HPV-negative/p16+	0 (0)
HPV genotype among HPV+ patients, $n = 32$	
HPV-16	26 (81.2)
HPV-18	2 (6.2)
HPV-33	2 (6.2)
N/A	2 (6.2)

Abbreviations: AJCC, American Joint Committee on Cancer; HPV, human papillomavirus; N/A, not available.

concordant HPV expression but demonstrated only partially positive (20% to 75%) staining for p16, compared with the diffusely positive staining (>75%) pattern observed in the initial untreated primary tumor. In 1 of these initially HPV+/p16+ cases, ISH for HPV on the recurrent tumor was negative, although subsequent PCR on the recurrent tumor revealed the presence of HPV-16 DNA. Therefore, 30 of 31 cases (97%) of initially HPV+/p16+ oropharyngeal cancer retained evidence of HPV and p16 expression at the time of recurrence.

Among the 2 remaining cases with potential discordance between the untreated primary and recurrent tumor, 1 demonstrated discordance in both HPV and p16 expression, whereas the second demonstrated discordance in only HPV expression. The first case was a 67-year-old male 80 pack-year former smoker with a poorly differentiated T4N2cM0 SCC of the base of the tongue, who experienced a suspected local recurrence within the base of the tongue immediately adjacent to his original primary tumor 5 years after completion of chemoradiation. The original poorly differentiated primary tumor demonstrated the presence of HPV DNA by both ISH and PCR and diffuse positive immunostaining for p16, whereas the moderately differentiated tumor that developed 5 years later was negative for HPV by both ISH and PCR and negative for p16 expression by IHC. The second case was a 51-year-old male 25 pack-year former smoker with a T4N2c tonsillar SCC, who developed an isolated pulmonary metastasis 3.6 years after completing chemoradiation. The original primary tumor was a poorly differentiated SCC that contained HPV by PCR, although ISH for HPV was negative and p16 testing was also negative. The pulmonary lesion that subsequently developed was also a poorly differentiated SCC, which tested negative for HPV by both PCR and ISH and negative for p16 by IHC. If this second case is reclassified as a second primary lung cancer rather than an oropharyngeal cancer recurrence, the resulting rate of identical HPV and p16 expression between primary and recurrent tumors is 90% (27 of 30) for those patients with initially HPV+/p16+ primary tumors, with HPV and at least partial expression of p16 detected in 100% of recurrences. In patients with initially HPV+/p16+ primary tumors ($n = 31$), a history of moderate or heavy smoking (ie, >10 pack-years) was not predictive of discordant HPV or p16 expression in the recurrent tumor (Fisher's exact test, $p = .63$).

DISCUSSION

In our study comparing concordance of HPV and p16 expression in primary and recurrent oropharyngeal cancer, 97% of recurrences from initially HPV+/p16+ oropharyngeal cancer demonstrated evidence of HPV and p16 impression, including 87% with identical HPV and p16 expression profiles. If the lone patient with discordant HPV-negative/p16-negative SCC at recurrence is reclassified as a second primary lung cancer, the rate of concordance of HPV and p16 expression increases to 100%. Our results, therefore, do not support the hypothesis that recurrent or metastatic HPV+ oropharyngeal cancers are driven by an HPV-negative tumor subfraction. This is true even among those HPV+ patients with a history of moderate or heavy smoking, whose oropharyngeal tumors may be postulated to be most likely to harbor an HPV-negative subpopulation, given the dual relationships between smoking and the development of HPV-negative oropharyngeal cancer, as well as smoking and risk of tumor progression and death in HPV+ oropharyngeal cancer.[2,5,6] Nonetheless, even rare instances of discordance in HPV/p16 expression at recurrence among initially HPV+/p16+ patients warrant further discussion.

In this cohort, only 1 case of initially HPV+/p16+ oropharyngeal cancer recurred with an HPV-negative/p16-negative expression profile. The recurrent tumor in this case demonstrated a moderate degree of histological differentiation, as compared to poor differentiation in the initial primary, suggesting the possibility that this recurrent tumor may, in fact, be a second primary tumor rather than a recurrence of the original oropharyngeal cancer. This circumstance is further supported by the clinical picture in which the second tumor arose after a 5-year disease-free interval in a former heavy smoker. A similar explanation

also seems likely in the second instance of discordant HPV expression at recurrence, which occurred in the patient with p16- primary tonsillar SCC that was HPV+ by PCR, but HPV-negative by ISH. The absence of both HPV expression (by either PCR or ISH testing) and p16 staining in a suspected solitary lung metastasis 3.6 years after completion of chemoradiation suggests the possibility of second primary lung tumor, rather than a distant metastasis, in this former heavy smoker. The alternative hypothesis, namely that the initial positive PCR for HPV represents a false-positive and that this patient was rather HPV-negative at presentation and developed a solitary distant metastasis with matching HPV-negative characteristics, remains equally plausible.

The 3 cases of initially HPV+/p16+ oropharyngeal cancer that demonstrated a partial p16 expression pattern at recurrence are additionally noteworthy. In all of these recurrences, partial p16 expression below the current standard 75% threshold for p16-positivity was observed, which stands in contrast to the diffusely positive p16 expression pattern observed in the initial primary tumor. Such heterogeneous expression of p16 has been previously described in HPV+ primary tumors at the time of diagnosis, although not at the time of recurrence, to the best of our knowledge.[10] In contrast to these previous reports, we did not observe any cases of partial or heterogeneous p16 expression at initial presentation in HPV+ tumors in this cohort.

Our study has several strengths, most notably that it is the first published report to compare HPV and p16 expression profiles between primary and recurrent oropharyngeal cancer. Additionally, HPV and p16 expression was prospectively ascertained in all untreated primary tumors using largely uniform methodology, with the exception of 2 cases for which ISH rather than PCR was used. Our study also has several potential limitations, including its retrospective nature, relatively small size, and the use of different methods used to determine HPV status in recurrent tumors (ISH) than was used for the majority of the initially untreated primary tumors (PCR). We have recently performed an analysis of nearly 300 head and neck cancer cases evaluated by PCR-MA and ISH for HPV expression, in which discordant cases were resolved by a second viral L1 consensus PCR followed by Sanger sequencing of the PCR product to identify the HPV type. In that study, we show that ISH is less sensitive than PCR-MA and L1 consensus PCR, which is largely because of the low viral copy number in such tumors and occasional examples of HPV types not represented in the assay (unpublished data).

In summary, our study demonstrates a high rate of concordant HPV and p16 expression between recurrent oropharyngeal cancer and the initial primary tumor. These findings fail to support the hypothesis that chemoradioresistance in HPV-related oropharyngeal cancer is driven by an HPV-negative tumor subfraction.

REFERENCES

1. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol* 2011;29:4294–4301.
2. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med* 2010;363:24–35.
3. Corey AS, Hudgins PA. Radiographic imaging of human papillomavirus related carcinomas of the oropharynx. *Head Neck Pathol* 2012;6 Suppl 1: S25–S40.
4. Maxwell JH, Kumar B, Feng FY, et al. Tobacco use in human papillomavirus-positive advanced oropharynx cancer patients related to increased risk of distant metastases and tumor recurrence. *Clin Cancer Res* 2010;16:1226–1235.
5. O'Sullivan B, Huang SH, Siu LL, et al. Deintensification candidate subgroups in human papillomavirus-related oropharyngeal cancer according to minimal risk of distant metastasis. *J Clin Oncol* 2013;31:543–550.
6. Gillison ML, Zhang Q, Jordan R, et al. Tobacco smoking and increased risk of death and progression for patients with p16-positive and p16-negative oropharyngeal cancer. *J Clin Oncol* 2012;30:2102–2111.
7. Spector ME, Gallagher KK, Light E, et al. Matted nodes: poor prognostic marker in oropharyngeal squamous cell carcinoma independent of HPV and EGFR status. *Head Neck* 2012;34:1727–1733.
8. Kumar B, Cordell KG, Lee JS, et al. EGFR, p16, HPV Titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer. *J Clin Oncol* 2008;26:3128–3137.
9. Mellin Dahlstrand H, Lindquist D, Björnestål L, et al. P16(INK4a) correlates to human papillomavirus presence, response to radiotherapy and clinical outcome in tonsillar carcinoma. *Anticancer Res* 2005;25: 4375–4383.
10. Lewis JS Jr, Chernock RD, Ma XJ, et al. Partial p16 staining in oropharyngeal squamous cell carcinoma: extent and pattern correlate with human papillomavirus RNA status. *Mod Pathol* 2012;25:1212–1220.
11. Robinson M, Schache A, Sloan P, Thavaraj S. HPV specific testing: a requirement for oropharyngeal squamous cell carcinoma patients. *Head Neck Pathol* 2012;6 Suppl 1:S83–S90.
12. Evans MF, Matthews A, Kandil D, Adamson CS, Trotman WE, Cooper K. Discrimination of 'driver' and 'passenger' HPV in tonsillar carcinomas by the polymerase chain reaction, chromogenic in situ hybridization, and p16(INK4a) immunohistochemistry. *Head Neck Pathol* 2011;5: 344–348.
13. Lewis JS Jr, Thorstad WL, Chernock RD, et al. p16 positive oropharyngeal squamous cell carcinoma: an entity with a favorable prognosis regardless of tumor HPV status. *Am J Surg Pathol* 2010;34:1088–1096.
14. [No authors listed]. Cumulative Index to IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. *IARC Monogr Eval Carcinog Risk Chem Hum* 1986;39:379–403.
15. Blot WJ, McLaughlin JK, Winn DM, et al. Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res* 1988;48:3282–3287.
16. Tang AL, Hauff SJ, Owen JH, et al. UM-SCC-104: a new human papillomavirus-16-positive cancer stem cell-containing head and neck squamous cell carcinoma cell line. *Head Neck* 2012;34:1480–1491.