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Human Papillomavirus-related Oropharyngeal Cancer: Are Recurrences Similar to the Parent Tumor?

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Abstract

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

Methods—Paired untreated primary and recurrent tumor specimens were identified for 37 OPC patients 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 a HPV-/p16- lung squamous cell carcinoma, representing either a discordant OPC metastasis or second primary tumor.

Conclusion—HPV-related OPCs retain HPV+/p16+ expression at recurrence. Our results fail to provide evidence that a minor HPV-negative tumor sub-fraction is responsible for biologically aggressive behavior of HPV+ OPC that recurs after chemoradiation.

Keywords

Human papillomavirus; p16; Oropharyngeal Cancer; Expression profiling; Chemoradiation

Background

Human papillomavirus is the primary causative factor in the majority of oropharyngeal cancers (OPC) in developed countries, and is present in over 70% of newly diagnosed OPC [(1)]. HPV-associated OPC represents a distinct disease entity from non-HPV-associated OPC, more commonly affecting younger patients and former or non-smokers who often present with smaller primary tumors and cystic-appearing nodal metastases [(2-4)]. Although HPV-related OPC 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+ OPC 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 OPC [(8, 9)]. Although IHC for p16 is typically unequivocally positive or negative, partial or even absent p16 immunostaining may occasionally be encountered in HPV+ OPC [(10, 11)]. Similarly, heterogeneous HPV-expression within tumors has been described [(12, 13)]. Such reports have stimulated speculation heterogeneous or discordant HPV and p16 expression may identify tumors in which HPV is present merely as a “bystander”, which may be associated with worse prognosis than “HPV-driven” OPC that diffusely expresses both HPV and p16 [(12)]. It may further be hypothesized that the minority of HPV+ OPCs 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 a HPV-negative/p16-negative phenotype at the time of recurrence.

The poorer prognosis of patients with HPV+ OPC who have a history of heavy smoking supports this hypothesis, given the established causal relationship between smoking and HPV-negative head and neck squamous cell carcinoma [2, 5, 6, 14, 15]. Data to assess this possibility, however, remain lacking. We therefore analyzed HPV and p16 expression in recurrent OPC to determine whether HPV-associated OPC expresses HPV and p16 at the time of recurrence.

Methods and Materials

Patients

This study was approved by University of Michigan Institutional Review Board (IRB). The records of two-hundred thirty one consecutive patients with histologically confirmed, previously untreated AJCC stage III or IV oropharyngeal squamous cell carcinoma (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 one 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 computed tomography (CT) or FDG-positron emission tomography-CT (PET/CT), and chest imaging, all patients underwent CT simulation in a 5-point thermoplastic mask for immobilization. All patients received intensity modulated radiotherapy 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 anti-emetics 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 (GTV) and 56-64 Gy prescribed to a clinical target volumes (CTVs) at risk for subclinical disease. Planning target volumes were created by a 3-5 mm uniform expansion of the GTVs and CTVs. 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 6-12 weeks and head and neck imaging (either contrast-enhanced CT or PET/CT) obtained at 3 months following completion of chemoradiation and every 3-6 months thereafter.

HPV and p16 Testing

HPV and p16 testing was prospectively performed on untreated primary tumor tissue either as part of either an IRB-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 ultra-sensitive method using HPV-MultiPlex PCR-MassArray (PCR-MA) real time competitive polymerase chain reaction (PCR) after isolation of DNA from cored tissue samples using the QIAamp DNA FFPE 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 4μm sections from paraffin-embedded tissue blocks containing a representative sample of primary tumor using the INFORM HPV ISH assay (Ventana Medical Systems Inc., 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 Inc., Tucson, AZ) according to the manufacturer's instructions. For all cases, immunohistochemistry for p16 was performed per protocol supplied by the kit (CINtec ^{p16INK4a} Histology Kit; mtm Laboratories, Westborough MA). p16 expression was scored based upon percentage of tumor staining in both the nucleus and cytoplasm, with >75% staining classified as positive, 20-75% scored as partial, and <20% scored as negative.

For recurrent tumors, HPV and p16 status was retrospectively determined using the HPV ISH and p16 immunohistochemistry 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 a HPV-positive/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, this 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 concordant HPV expression but demonstrated only partially positive (20-75%) staining for p16, compared with the diffusely positive staining (>75%) pattern observed in the initial untreated primary tumor. In one of these initially HPV+/p16+ cases, ISH for HPV on the recurrent tumor was negative, though subsequent PCR on the recurrent tumor revealed the presence of HPV16

DNA. Therefore, 30 of 31 (97%) cases of initially HPV+/p16+ OPC retained evidence of HPV and p16 expression at time of recurrence.

Among the two remaining cases with potential discordance between the untreated primary and recurrent tumor, one 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 tongue, who experienced a suspected local recurrence within the base of tongue immediately adjacent to his original primary tumor 5 years after completion of chemoradiation. The untreated original 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 five years later was negative for HPV by both ISH and PCR and negative for p16 expression by immunohistochemistry. 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 ½ years after completing chemoradiation. The untreated primary tumor was a poorly differentiated squamous cell carcinoma that contained HPV by PCR, though ISH for HPV was negative and p16 testing was also negative. The pulmonary lesion that subsequently developed was also a poorly differentiated squamous cell carcinoma, which tested negative for HPV by both PCR and ISH and negative for p16 by immunohistochemistry. If this second case is re-classified as a second primary lung cancer rather than an OPC 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 (i.e. >10 pack-years) was not predictive of discordant HPV or p16 expression in the recurrent tumor (Fisher's exact test p=0.63).

Discussion

In our study comparing concordance of HPV and p16 expression in primary and recurrent OPC, 97% of recurrences from initially HPV+/p16+ OPC demonstrated evidence of HPV and p16 impression, including 87% with identical HPV and p16 expression profiles. If the lone patient with discordant HPV-/p16-squamous cell carcinoma at recurrence is re-classified 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+ OPC are driven by a HPV-negative tumor sub-fraction. 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 a HPV-negative subpopulation, given the dual relationships between smoking and the development of HPV-negative OPC as well as smoking and risk of tumor progression and death in HPV+ OPC [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 one case of initially HPV+/p16+ OPC recurred with an HPV-negative/p16-negative expression profile. The recurrent tumor in this case demonstrated a moderate degree of histological differentiation, as compared with 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 OPC. 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-negative 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 ½ 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 three cases of initially HPV+/p16+ OPC which 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-positive primary tumors at 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-positive 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 OPC. 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-Mass Array 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 due to 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 OPC and the initial primary tumor. These findings fail to support the hypothesis that chemoradioresistance in HPV-related OPC is driven by a HPV-negative tumor subfraction.

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Table 1

Baseline Characteristics

CHARACTERISTIC	N (%)
Gender	
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 pack-years	15 (41)
≥ 10 pack-years	22 (49)
HPV & 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)	
HPV16	26 (81.2%)
HPV18	2 (6.2%)
HPV33	2 (6.2%)

CHARACTERISTIC	N (%)
n/a	2 (6.2%)

n/a = not available