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Research Article

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Serum Antibodies to HPV16 Early Proteins Warrant Investigation as Potential Biomarkers for Risk Stratification and Recurrence of HPV-Associated Oropharyngeal Cancer

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Abstract

Human papillomavirus (HPV) is responsible for increasing incidence of oropharyngeal cancer. At present, there are no biomarkers in the surveillance algorithm for HPV-positive oropharyngeal cancer (HPV-OPC). HPV16 E6 antibody precedes oropharyngeal cancer diagnosis. If HPV16 E6 indeed precedes primary diagnosis, it is similarly expected to precede disease recurrence and may have a potential role as a biomarker for surveillance of HPV-OPC. To determine whether HPV antibody titers have a potential role as early markers of disease recurrence or prognosis, a retrospective pilot study was designed to determine whether HPV16 early antibody titers E6, E7, E1, and E2 decrease after treatment of HPV16-positive OPC. Trends in pretreatment, early ($\leq$6 months after treatment), and late posttreatment (>6 months after treatment) HPV16 antibody titers were examined. There were 43, 34, and 52 subjects with serum samples available for pretreatment, early, and late posttreatment intervals. Mean pretreatment antibody levels were higher than posttreatment antibody levels. Average antibody levels decreased significantly over time for E6 ($P_{trend} = 0.001$) and E7 ($P_{trend} < 0.001$). Six disease recurrences were observed during the follow-up period (median, 4.4 years). In univariate analysis, a log-unit increase in pretreatment E6 titer was significantly associated with increased risk of disease recurrence (HR, 5.42; 95% CI, 1.1–25.7; $P = 0.03$). Therefore, levels of antibodies to HPV16 early oncoproteins decline after therapy. Higher E6 titers at diagnosis are associated with significant increases in the risk of recurrence. These data support the prospective evaluation of HPV16 antibodies as markers of surveillance and for risk stratification at diagnosis. *Cancer Prev Res; 9(2); 135–41. ©2015 AACR.*

Introduction

The incidence of oropharyngeal squamous cell carcinomas (OPC) is rapidly increasing in the United States, as well as other countries around the world (1, 2). Human papillomavirus (HPV), a sexually transmitted infection, is the recognized etiologic agent for this growing majority of OPCs (3, 4). In the United States, HPV is the demonstrated oncogenic agent responsible for these incidence trends (4) and currently accounts for approximately 80% of OPCs diagnosed (5, 6). The presence of HPV in oropharyngeal tumors confers improved overall and progression-free survival (PFS), relative to HPV-negative tumors (5, 6). Despite improved prognosis, up to 30% of HPV-positive patients still experience recurrence of disease, the majority of which occurs in the first two years after treatment (7–9).

Historically, even 1-year survival of patients with recurrent OPC was dismal (5%–30%; ref. 10). However, recent data suggest that at the time of disease recurrence, HPV-positive tumor status and surgical salvage are independently associated with improved overall survival (OS; ref. 8). Two-year OS is 25% greater for recurrent HPV-positive patients who undergo surgical salvage as compared with those who do not (7, 8). Therefore, if recurrent HPV-positive oropharyngeal cancer (HPV-OPC) is detected at an early stage when surgical salvage is possible, patients may have a significant improvement in OS, although whether improved lead time afforded by any potential biomarker would change the outcome is unknown.

At present, National Comprehensive Cancer Network guidelines for surveillance recommend history and physical examination at routine intervals with anatomic and metabolic imaging as clinically indicated (11). In contrast to malignancies of other anatomic sites for which biomarkers are integral to recurrent disease surveillance (e.g., prostate surface antigen titer), there are no analogous or validated biomarkers for HPV-OPC. Therefore, we were interested in identifying a candidate biomarker for disease status in HPV-OPC.

The presence of antibodies to HPV16 early oncoprotein E6 is strongly associated with diagnosis of OPC [OR, 58.4; 95% confidence interval (CI), 24.2–138.3; refs. 12, 13] and precedes

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diagnosis of OPC by 10 years (14). HPV16-specific E1, E2, and E7 antibodies are similarly associated with incident HPV-OPC years before diagnosis of malignancy (14).

Data from cervical cancer literature, the paradigm of HPV-related malignancy, demonstrate a significant reduction in titer of antibodies after treatment of disease, and antibody status is a significant predictor of prognosis (15, 16). Although similar reductions in E6 and E7 titers have been observed in head and neck cancer, the clinical relevance is limited by heterogeneity of HPV tumor status, histology types, and anatomic sites (17, 18).

To explore whether HPV16 antibodies to E6, E1, E2, and E7 have potential as biomarkers of disease status for patients with HPV-OPC, we hypothesized that titers will decrease after treatment with curative intent.

Materials and Methods

This was a retrospective study designed to determine whether HPV16 antibody titers change after treatment. Participants with HPV-OPC and two or greater serology specimens available were eligible. Serology samples had been collected from patients enrolled in the Molecular Surveillance Protocol, an Institutional Review Board–approved study at Johns Hopkins (Baltimore, MD). Clinical characteristics of interest including age, gender, race, alcohol and smoking history, primary site of diagnosis, staging, primary treatment modality, date of last clinical visit, presence, and date of first recurrence were abstracted from the electronic medical record. HPV16 tumor status was obtained from previously reported data which included qPCR for HPV16 genomic DNA ($\geq$0.1 copy per genome) and high risk HPV *in situ* hybridization (19). A subset analysis was restricted to participants with clinically available HPV16-positive *in situ* hybridization tumor status.

Serologic methods

Antibodies to HPV16 E1, E2, E6, and E7 were measured by ELISA using the GST capture method (20) with some modifications. The following reagents were generously provided by Michael Pawlita (German Cancer Research Center, Heidelberg, Germany): cleared lystae from *E. coli* over-expressing GST-HPV16 E1, GST-HPV16 E2, GST-HPV16 E6, GST-HPV16 E7, and GST alone. Briefly, 96-well polystyrene flat bottom MaxiSorp plates (Nunc) were coated overnight at 4°C with 200 ng/well of glutathione-casein in PBS, pH 7.2 (PBS) and blocked for 1 hour at 37°C with 0.5% (w/v) polyvinyl alcohol, MW 30,000–70,000 (Sigma-Aldrich) in Blocker Casein in PBS (casein PVA buffer; Thermo Scientific). The blocked plates were incubated for 1 hour at room temperature with GST-HPV antigen lysate diluted in casein PVA buffer to 0.25 μg/μL total lysate protein. Control wells were coated with GST alone at the same protein concentration. Before addition of serum samples and following each incubation step, the plates were washed 4 times with PBS containing 0.05% (v/v) Tween 20 (Sigma-Aldrich) in an automatic plate washer (Skanwasher 300, Skatron). Serum samples diluted 1:100 in casein PVA buffer containing GST alone lysate (0.50 mg/mL) were left to react for 1 hour at 37°C. Samples were tested in duplicate on the same day but in different microtiter plates. Antigen-bound immunoglobulin was detected with peroxidase-conjugated goat antibodies against human IgG, γ-chain–specific, (SouthernBiotech) diluted 1:4,000 in casein PVA buffer containing 0.8% (w/v) polyvinylpyrrolidone, MW 3,60,000 (Sigma-Aldrich), and 0.05% (v/v) Tween 20. After 30 minutes at 37°C, color development was initiated by the addition of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) hydrogen peroxide solution (Kirkegaard and Perry). The reaction was stopped after 20 minutes by the addition of 1% dodecyl sulfate and optical density (OD) is measured at 405 nm, with a reference wavelength of 490 nm, in an automated microtiter plate reader (Molecular Devices). The mean OD in wells with GST alone was subtracted from the mean OD in wells with the GST-HPV protein to give an antigen-specific OD value.

Antibody titers to BK polyomavirus (BKPyV) were used as a control. Antibody to BKPyV capsids were measured by virus-like particle (VLP) ELISA as previously described (21). The rationale for performing this assay was to understand whether the observed changes in antibody levels were specific to HPV or applicable to antibody levels in general, and the choice of BKPyV ELISA was based on the knowledge that a majority of individuals are expected to be seropositive, and thus changes in antibody level could be measured.

Statistical analysis

The primary outcome of interest was change of OD values over time from pretreatment to posttreatment. The overall average trend in the OD values over time was visualized using locally weighted regression (lowess) curves. The time of serum sample collection was considered as a categorical variable with respect to diagnosis and treatment. Serology samples collected from date of diagnosis and up to initiation of therapy were considered "pretreatment". If repeated measurements were available on the same day for an individual, an average value was used for analysis. Samples obtained up to 6 months after therapy and after 6 months were categorized as "early posttreatment" and "late posttreatment", respectively. If a subject had greater than one sample available during a posttreatment period ("early" and "late"), an average value of the available measurements was used. Descriptive statistics of the serology data based upon the average values by treatment periods were calculated. A linear mixed-effects model was used to estimate the means of log-transformed OD values across treatment periods, as well as mean differences between treatment periods (e.g., pre and posttreatment periods), where correlated measurements within the same subject were accounted for by assuming an exchangeable correlation structure.

The timing of clinical recurrence was used to determine prognosis group. The analysis was restricted to subjects who were followed for at least 2 years, and none of the early dropouts were due to death. Recurrence within two years of therapy was considered poor prognosis, whereas no evidence of recurrence during this time period or recurrence long after 2 years post-treatment was categorized as good prognosis.

Recurrence-free survival (RFS) was defined as the date of end treatment until the date of first-documented disease recurrence. Patients who did not recur but died were censored at the time of death. Patients who remained alive without recurrence were censored at the time of last contact. For analyses of RFS, mean OD values were log-transformed. Association of baseline serology with RFS was evaluated using Cox proportional hazards model by univariate and multivariate analyses adjusting for alcohol consumption, smoking history, and tumor–node–metastasis (TNM) stage. All tests were considered statistically significant at $P<0.05$. All analyses were performed with the use of SAS 9.2 (SAS Institute) and R version 3.1.0 (available at http://www.r-project.org/).

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Results

The overall study population consisted of 60 HPV16-positive OPC patients. The characteristics of the study population are summarized in Table 1. The majority of individuals were male ($n = 53$, 88.3%) and white ($n = 59$, 98.3%) with advanced overall stage ($n = 45$, 75%). Pretreatment serology was available for 43 participants (71.7%).

Antibody levels over time were evaluated for the study population overall. There were 43, 34, and 52 subjects with serum samples available for pretreatment, early, and late posttreatment intervals. Average antibody levels were higher pretreatment than posttreatment for E6, E7, E1, and E2 (Fig. 1; Supplementary Table S1). For E6, the median pretreatment antibody level was 0.31 (range, 0.03–1.2) and declined after treatment. In the early posttreatment interval (up to 6 months after treatment), median E6 level was 0.26 (range, 0.07–1.3), and in the late posttreatment interval (6 months or greater after treatment) was 0.21 (range, 0–0.78). Similar patterns of declining antibody levels per treatment interval were observed for E7, E1, and E2 over time, although the mean levels for E6 were consistently lower.

The trajectory of mean antibody levels over time was further modeled. Within-participant correlation of antibody levels over time was accounted for (Table 2). This analysis was restricted to 43 individuals with available pretreatment serum samples. Compared with pretreatment levels, the average antibody level declined in the early posttreatment interval for E6, E2, and E1, although not statistically significantly ($P > 0.05$). For E7, average early posttreatment antibody levels were significantly lower than pretreatment levels ($P = 0.03$). However, when average late posttreatment antibody levels were compared with pretreatment levels, there were significant decreases for E6, E7, and E1 ($P < 0.02$ for all). For E2, the decrease remained nonsignificant ($P = 0.09$). Overall, the average antibody levels decreased significantly over time for E6 ($P_{trend} = 0.001$) and E7 ($P_{trend} < 0.001$). In a subset analysis restricted to subjects with ISH16-positive tumors, E6 and E7 declined significantly in the early posttreatment period (Table 3; E6, $P = 0.001$; E7, $P < 0.001$; $P_{trends} < 0.001$).

When considering average antibody levels by prognosis group, the majority were in the good prognosis group at pretreatment (33/37) and early posttreatment (23/28). At pretreatment, E6 antibody level was lower for the good prognosis as compared with the poor prognosis group (0.32 vs. 0.61, $P = 0.048$) and early posttreatment (0.25 vs. 0.64, $P = 0.045$) intervals. Similar trends were observed for E7, E1, and E2, although they were not statistically significant.

Given the declines in antibody levels over time with respect to therapy, the prognostic significance of pretreatment serology levels was explored (Table 3). There were only 6 disease recurrences observed in this study population with a median follow-up time of 4.4 years (range, 0.08–11.9). In univariate analysis, higher pretreatment E6 level (per log unit) was significantly associated with increased risk of disease recurrence (HR, 5.4; 95% CI, 1.1–25.7; $P = 0.03$). After adjustment for clinically relevant factors, the robust association between increasing level of E6 antibody at pretreatment and risk of disease recurrence remained significant (HR, 7.1; 95% CI, 1.2–43.2; $P = 0.04$). Pretreatment levels of E7, E1, and E2 were not of prognostic significance ($P > 0.05$ for all). In the subset analysis restricted to 31 subjects with ISH16-positive tumors and 5 events of recurrence, a similar magnitude of the association with increased risk of recurrence for E6 was observed, albeit not statistically significant (HR, 5.5; 95%CI, 0.66–46.7).

In addition, the prognostic implication of E6 antibody in the first 3 months after therapy was explored. In univariate analysis, each log-unit increase in level of E6 antibody level was associated with a 7-fold increased risk of recurrence, although nonsignificant (HR, 6.9, 95% CI, 0.50–95.9). To determine whether the observed declines in antibody titers were HPV-specific or systemic immune-related, BK virus titers were evaluated over time. In contrast to declines in HPV16 early oncoproteins, BK virus titers remained stable over time ($P = 0.30$).

Table 1. Clinical characteristics of study population ($n = 60$)

Characteristics	Number of patients (%)
Age at diagnosis (years)	
Median (range)	56 (29–84)
Gender	
Male	53 (88.3)
Female	7 (11.7)
Race	
White	59 (98.3)
Black	1 (1.7)
Alcohol consumption	
<14 drinks/week	45 (75.0)
≥14 drinks/week	12 (20.0)
Unknown	3 (5.0)
Smoking history	
Never	25 (41.7)
Ever	32 (53.3)
Unknown	3 (5.0)
Primary site at diagnosis	
Oropharynx	58 (96.7)
Unknown primary	2 (3.3)
Tumor stage	
I	30 (50.0)
II	21 (35.0)
III	4 (6.7)
IV	2 (3.3)
Unknown	3 (5.0)
Nodal stage	
N0-N1	9 (15.0)
N2a	13 (21.7)
N2b	30 (50.0)
N3+	6 (10.0)
Unknown	2 (3.3)
Overall stage	
Stage 0-III	12 (20.0)
Stage IV	45 (75.0)
Unknown	3 (5.0)
Primary treatment	
RT ± chemotherapy	23 (38.3)
Surgery ± RT ± chemotherapy	34 (56.7)
Unknown	3 (5.0)

Abbreviation: RT, radiotherapy.

Discussion

Levels of antibodies against HPV16-specific oncoproteins declined after therapy in a study population of HPV-OPC patients. Notably, higher levels of E6 antibody at diagnosis were associated with significantly increased risk of recurrence. These observations provide critical initial data to support further investigation of serum antibodies of HPV as biomarkers of prognosis and disease status.

The growing interest in therapeutic deintensification of HPV-OPC has highlighted the need to identify the subsets of HPV-positive patients who are at decreased risk of recurrence and

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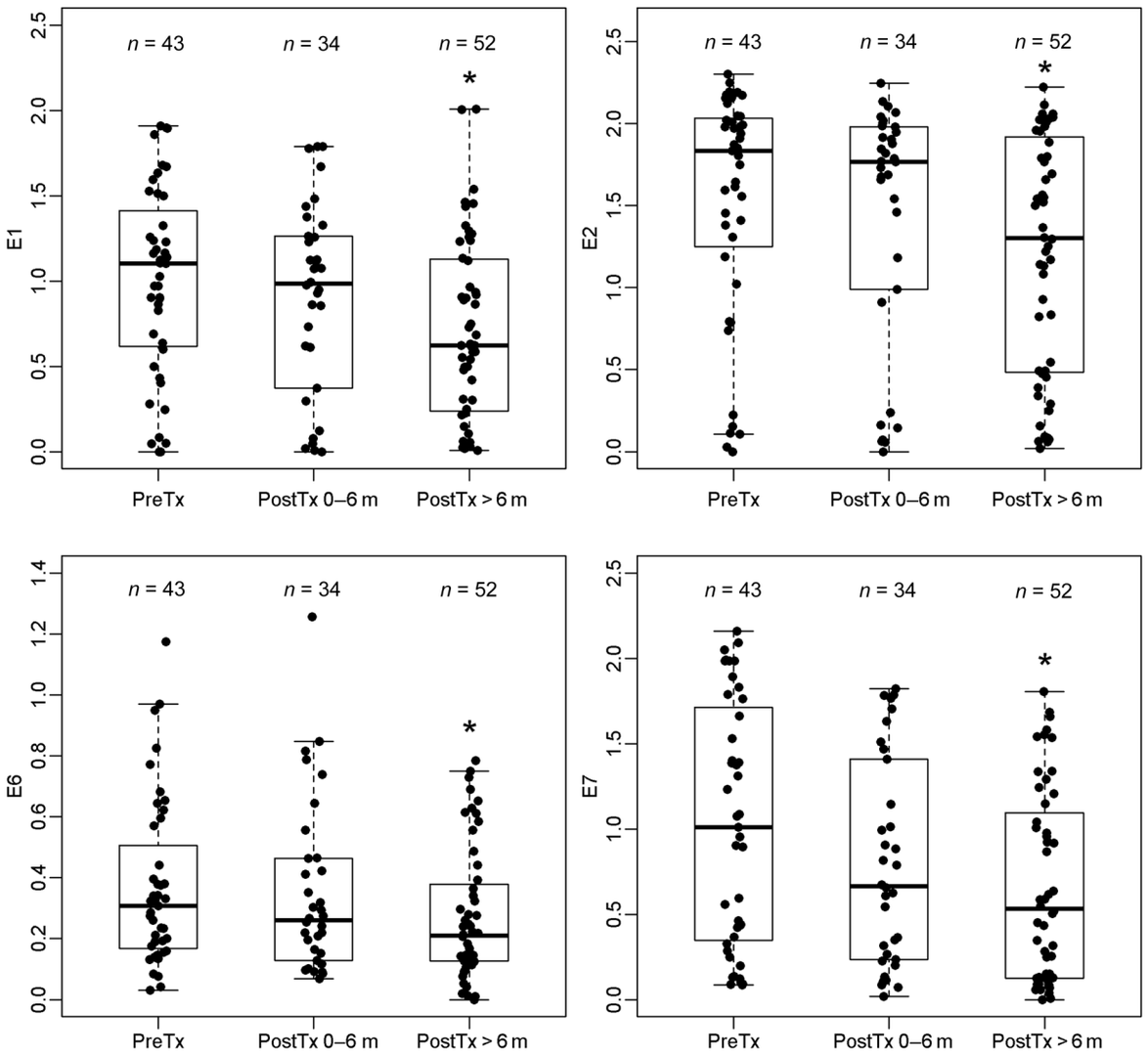

Figure 1.
Antibody levels by treatment period. The length of the box is the interquartile range and represents the middle 50% of antibody levels. The horizontal line inside the box depicts the median. The bottom and top hinges of the box represent the 25th and 75th percentiles, respectively. The vertical dashed lines extend from the box to the top and bottom 1.5 interquartile values from the top and bottom hinges. The filled circles represent the actual antibody levels. Significant change from pretreatment (PreTx; $P < 0.05$) is indicated by asterisk (*). Posttreatment, PostTx.

therefore appropriate candidates for de-intensification (22). Present prognostic risk stratification for HPV-positive patients is limited to lifetime tobacco exposure and nodal disease (6) in the RTOG 0129 model and age, TNM stage, and lifetime tobacco exposure in the Princess Margaret–proposed prognostic groups (23). Other than p16 tumor status, there are currently no other prognostic biomarkers available to refine deintensification eligibility. In this study, the magnitude of the baseline antibody level is strongly associated with recurrence. If this finding is validated in a rigorous prospective study, then HPV serology may offer a novel prognostic biomarker to further stratify the risk categorization currently used for clinical trials.

To consider HPV antibody levels as a candidate biomarker for surveillance of HPV-OPC after treatment, an important first question is whether antibody levels change after treatment. In this data, significant declines are observed for HPV antibodies E6, E7, and E1 after treatment. This study builds upon a previous report of significant declines in mean antibody levels after treatment in a patient population including oral cavity, an anatomic site not relevant to HPV (17). In contrast, to evaluate the question of whether HPV antibody levels decline after therapy, this study was restricted to OPCs with HPV16-positive tumor status. Indeed, other head and neck cancers which are not HPV-related are not expected to be HPV-seropositive (24), and therefore any associations observed may be driven by the majority seronegative and low levels of antibodies. A similar approach of restricting to HPV-OPCs (determined by PCR) was recently used to demonstrate that the presence of HPV E6 serum antibodies at diagnosis was significantly associated with improved overall and PFS (25). In contrast to the findings of the current study, the median antibody levels of individuals who recurred or did not recur were statistically similar and appeared to be (nonsignificantly) lower for

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Table 2. Changes in antibody levels in patients with HPV-OPC over time[a]

	Overall study population				Subset: ISH HPV16-positive subjects			
Serology	***N***	**Mean ± SE[b]**	***P*[c]**	***P*$_{trend}$[c]**	***N***	**Mean ± SE[b]**	***P*[c]**	***P*$_{trend}$[c]**
E1				0.066				0.168
Pretreatment	43	−0.508 ± 0.223	—		31	−0.323 ± 0.228	—	
Early posttreatment	29	−0.559 ± 0.235	0.730		21	−0.326 ± 0.242	0.985	
Late posttreatment	37	−0.816 ± 0.227	0.024		27	−0.584 ± 0.232	0.082	
E2				0.166				0.019
Pretreatment	43	0.032 ± 0.191	—		31	0.296 ± 0.166	—	
Early posttreatment	29	0.047 ± 0.200	0.896		21	0.199 ± 0.170	0.212	
Late posttreatment	37	−0.148 ± 0.194	0.092		27	0.092 ± 0.167	0.005	
E6				0.001				<0.0001
Pretreatment	43	−1.272 ± 0.148	—		31	−1.175 ± 0.143	—	
Early posttreatment	29	−1.484 ± 0.163	0.114		21	−1.471 ± 0.149	0.001	
Late posttreatment	37	−1.736 ± 0.153	0.0003		27	−1.594 ± 0.145	<0.0001	
E7				<0.0001				<0.0001
Pretreatment	43	−0.381 ± 0.187	—		31	−0.265 ± 0.188	—	
Early posttreatment	29	−0.692 ± 0.201	0.031		21	−0.530 ± 0.194	0.006	
Late posttreatment	37	−1.049 ± 0.192	<0.0001		27	−0.822 ± 0.190	<0.0001	

[a]This analysis was restricted to subjects with available pretreatment serology measurements.
[b]Estimated log-transformed mean.
[c]Comparison relative to pretreatment measurements based on linear mixed-effects model taking into account the within-subject correlation.

patients who recurred. Of note, consistent with our findings, a decline in median antibody levels is apparent graphically when comparing pretreatment and posttreatment levels (25).

Analogous trends have been observed in cervical cancer; however, a substantial proportion of women with cervical cancer have no immunologic response to HPV-specific antibodies and the broad distribution of HPV types responsible for cervical cancer precludes from examination of antibody response to one antibody type (26). In contrast, the majority of HPV-OPC patients are seropositive to HPV; in a case–control study in which HPV16 DNA was present in 72 tumors, 64 (88.9%) of these cases were seropositive to HPV16 E6 or E7 (12). As a biomarker, the increased prevalence of this marker at diagnosis in OPC as compared with cervical cancer is appealing. In addition, the vast majority of HPV-OPCs are HPV16-related (27). Recent data suggest that although approximately 97% of HPV-OPC are seropositive, presence of antibodies to specific antigens (E1, E2, E4, E5, E6, and E7) is highly variable (2%–66%) and is affected by age (28). Realistically, HPV serology is only useful in patients with an antibody response, and therefore it can be expected that for some, E7 will be a better marker of disease status than E6 and vice versa.

Clinical surveillance for disease recurrence is currently similar for HPV-positive and HPV-negative OPC patients. However, whether surveillance strategies should differ for HPV-OPC changes is an area of controversy (9, 29). While most HPV-OPC patients are "cured" of their disease, up to 30% of patients still experience recurrence. To date, there is no method to identify the patients who will unfortunately experience disease recurrence after therapy. Indeed, HPV-positive patients at the time of recurrence retain the phenotype of HPV-positive patients; those who recur are not the HPV-positive patients with characteristics more similar to HPV-negative patients (7–9). Therefore, serology at the time of diagnosis may be a biomarker to identify patients who need more or less intense follow-up. This could influence imaging recommendations and clinical examinations in the current surveillance paradigm.

The possibility that the decreases in HPV16 antibodies were nonspecific and applicable to any antibody was explored. Others have shown that L1 serology, a measure of cumulative lifetime exposure to HPV, does not change with therapy (17). In this study, BK virus levels were used as controls and were stable over time ($P = 0.30$). Therefore, the level of HPV16 antibody titers appears to be specific to the disease status of HPV-OPC and may be an index of the tumor or antigen "load" (13). This extends prior provocative data in a European case–control, which showed the presence of E6 antibodies a decade or more prior to OPC diagnosis at the time of early carcinogenesis and antigen presentation (14).

There are several limitations of this study that warrant discussion. Most importantly, this is a retrospective pilot study performed to determine whether this hypothesis should be considered prospectively. Available samples were collected under a prospective collection protocol, however the timing and number of available samples is variable and dictated by clinical follow-up, availability of patients and prior use of serum. HPV16 ISH, which is the clinical gold standard for HPV16 detection, was only clinically available for a limited subset of subjects. However, HPV high-risk ISH in combination with qPCR for HPV16 oncoproteins was available for all the cases. HPV16 is responsible for >95% of HPV-OPC, and estimates were similar in the subset analysis; therefore, it is unlikely that cases included were attributed to other high-risk HPV infections. It is important to note that not all HPV-positive OPC patients are E6 or E7 seropositive. Therefore,

Table 3. Association of pretreatment antibody levels with RFS in patients with HPV-OPC

	Univariate analysis[a] (*n* = 43)		Multivariable analysis[b] (*n* = 41)	
Serology parameter	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Log E1	1.01 (0.60–1.69)	0.982	0.97 (0.59–1.59)	0.912
Log E2	1.00 (0.56–1.79)	0.999	0.92 (0.48–1.73)	0.785
Log E6	5.42 (1.14–25.7)	0.033	7.06 (1.15–43.2)	0.035
Log E7	0.71 (0.32–1.58)	0.403	0.46 (0.16–1.32)	0.149

[a]Cox proportional hazards model.
[b]In multivariable analysis, alcohol consumption, smoking history, and TNM stage at diagnosis were adjusted for.

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future prospective analyses should determine baseline antibody status to HPV16 and other HPV high-risk types. It was not feasible in this study to examine trends among individuals seropositive at pretreatment. In addition, for the few HPV-OPC tumors, due to other high-risk HPV types, there may be cross-reactivity. With regard to the association of pretreatment HPV16 E6 titer and recurrence, there were limited recurrences, which may explain the wide CIs of the risk estimates. In addition, the calculation of risk at the completion of therapy was not possible given the lack of samples consistently collected at the end of treatment.

These data support further study of HPV16 serology as a candidate biomarker of prognosis at the time of diagnosis and surveillance after treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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