



## HPV16 antibodies as risk factors for oropharyngeal cancer and their association with tumor HPV and smoking status



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### SUMMARY

**Background:** Antibodies (Abs) to the HPV16 proteome increase risk for HPV-associated OPC (HPVOPC). The goal of this study was to investigate the association of a panel of HPV16 Abs with risk for OPC as well as the association of these Abs with tumor HPV and smoking status among patients with OPC.

**Methods:** IgG Abs to the HPV16 antigens E1, E2, E4, E5, E6, E7, L1, L2 were quantified using a programmable ELISA assay. Sera were obtained from 258 OPC patients at diagnosis and 250 healthy controls. HPV16 tumor status was measured by PCR for 137 cases. Multivariable logistic regression was used to calculate odds ratios for the association of HPV16 Abs with risk for OPC.

**Results:** HPV16 E1, E2, E4, E5, E6, E7 and L1-specific IgG levels were elevated in OPC patients compared to healthy controls ( $p < 0.05$ ). After multivariable adjustment, Ab positivity for NE2, CE2, E6, and/or E7 was associated with OPC risk (OR [95% CI], 249.1 [99.3–624.9]). Among patients with OPC, Ab positivity for these antigens was associated with tumor HPV status, especially among never or light smokers (OR [95% CI], 6.5 [2.1–20.1] and OR [95% CI], 17.5 [4.0–77.2], respectively).

**Conclusions:** Antibodies to HPV16 proteins are associated with increased risk for HPVOPC. Among patients with OPC, HPV16 Abs are associated with tumor HPV status, in particular among HPV positive patients with no or little smoking history.

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### Introduction

Oropharyngeal cancer (OPC) is a subset of head and neck cancer, which is ranked as the sixth most common cancer worldwide with 405,000 new cases and 200,000 deaths annually [1]. Tobacco-associated OPC, which is associated with somatic mutations in p53, is decreasing in incidence, but human papillomavirus-associated OPC (HPVOPC) has increased in the U.S. by 225% between 1984 and 2004 [2]. HPV type 16 accounts for 85–90% of HPV-associated cases of OPC [3,4]. Epidemiological evidence

supports a causal role for HPV in OPC, including the association with lifetime numbers of vaginal and oral sex partners [3,5] and presence of HPV DNA in oral exfoliated cells [3,6–10]. HPV is detectable and persistent in tumors [11–14], contains viral oncogenes [15–17], can transform target cells [18,19], and induce tumors in transgenic mice [20,21].

Unlike cervical cancer, there exists no sensitive and selective screening method for the early detection of HPVOPC. The HPV16 genome consists of six early genes (E1, E2, E4, E5, E6, and E7) and two late genes (L1 and L2) that constitute the viral capsid. Serum antibodies (Abs) to HPV16 E6 and E7 have been detected in a subset of patients with OPC [22,23], with Abs to E6 and/or E7 present in 67% of HPV-positive OPC cases [24]. Seropositivity for HPV16 E6 and E7 are strongly associated with increasing odds of HPV-positive OPC (OR 58–67) [25,26] and with improved prognosis [27,24,28]. HPV16 Abs to E6 have been detected in 34.8% of OPC patients up to 10 years prior to clinical diagnosis [11],

*Abbreviations:* Abs, antibodies; CV, coefficient of variation; HR HPV, High Risk Human Papilloma Virus; ISH, in situ hybridization; IVTT, in vitro transcription/translation; OPC, oropharyngeal cancer; OR, odds ratio; SD, standard deviation.

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suggesting that HPV serology may yield biomarkers for early detection of OPC. In a pilot study, we detected Abs to both E6 and E7 proteins in OPC patient sera, but also detected Abs to the HPV16 E1 and E2 proteins [22]. This suggests that serology of additional HPV antigens may improve detection of HPVOPC.

In this study, we investigated the association of a panel of HPV16 Abs with risk of OPC. We used an extensive collection of sera from newly-diagnosed OPC patients and cancer-free controls to evaluate the association between HPV16 proteome-wide serology and disease status as well as tumor HPV and smoking status among cases.

## Material and methods

### Patient sera

Patients with newly diagnosed, histopathologically confirmed, and previously untreated OPC who were participating in a large ongoing molecular epidemiology study of head and neck cancer at the University of Texas MD Anderson Cancer Center in Houston, TX were eligible for the study. All participants were recruited between January 2006 and September 2008. Participants provided demographic and exposure history, including smoking and alcohol use, using a standardized questionnaire and provided a blood sample for biological testing. Sera used in this analysis were collected from OPC patients prior to initiation of treatment ( $n = 258$ ). Healthy control sera were collected from genetically unrelated visitors or companions of patients to the head and neck clinic during the same time period. Controls were frequency matched to cases on age ( $\pm 5$  years), gender, and race ( $n = 250$ ). Approximately 93% of cases and 85% of controls who were eligible agreed to participate in the study. All samples were collected using a standardized sample collection protocol and stored at  $-80^{\circ}\text{C}$  until use. Written informed consent was obtained from all subjects under institutional review board approval.

### HPV DNA cloning and expression

Plasmids containing HPV16 genes [29] were expressed as a C-terminal GST-fusion protein using human HeLa cell lysate [30] (Thermo Scientific, Waltham, MA) per manufacturer's instructions. The HPV16 E2 gene was expressed as N- and C-terminal fragments for optimal protein expression [22]. GST was expressed as a negative control protein. All recombinant DNA research was performed in accord with NIH guidelines under institutional biological safety review and approval.

### Programmable protein (RAPID) ELISA

ELISAs were performed essentially as described [31], with modifications [32]. Protein was expressed from template cDNA and captured onto 96-well plates coated with anti-GST Ab (GE Healthcare, Piscataway, NJ). Sera were diluted 1:100 and blocked with *E. coli* lysate. Cases and controls were analyzed simultaneously in duplicate. Horseradish peroxidase (HRP) anti-human IgG Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) were added at 1:10,000, and detected using Supersignal ELISA Femto Chemiluminescent substrate (Thermo Scientific). Luminescence was detected as relative light units (RLU) on a Glomax 96 Microplate Luminometer (Promega, Madison, WI) at 425 nm. To control for non-specific and GST-specific antibodies, the ratio of RLU for individual HPV-specific Abs to the RLU for the control GST-antigen was measured. To establish cut-off values, an RLU ratio  $>$  (the mean + 3 standard deviations) of 125 randomly chosen control samples was designated positive. These levels were E1: 2.66; NE2: 2.29; CE2: 4.35; E4: 2.58; E5: 1.69; E6: 2.01; E7: 1.99;

L1: 1.83; L2: 3.57. The controls chosen to establish cut-off values were not statistically significantly different with respect to age, sex, race, smoking, or alcohol use from the controls not used.

### Reproducibility of RAPID ELISA

To determine the reproducibility of the RAPID ELISA assay, replicates for E6 serology were performed with 8 replicates on 4 consecutive days. RLU ratios (E6-GST: GST control) among RAPID ELISAs processed on the same day showed intra-assay CVs of 0.2–7%, and RLU ratios between plate-based arrays processed on 4 consecutive days demonstrated inter-assay CVs of 12–17%.

### Tumor HPV DNA detection by PCR

Diagnostic in-house paraffin-embedded tissue was obtained following histopathologic confirmation of the diagnosis for determination of tumor HPV status [33]. DNA was extracted using a tissue DNA extraction kit (Qiagen Inc., Valencia, CA). Tumor tissue from the study subjects was tested for the presence of HPV16 E6 or E7 regions using PCR-based type-specific assays and each subject was classified as HPV-positive or HPV-negative based on these results. Samples were run in triplicate with positive (Siha cell line) and negative (TPC-1 cell line) controls and  $\beta$ -actin as DNA quality control.

### Statistical analysis

Categorical variables were created to describe study subjects' demographic, clinical, and exposure (smoking and alcohol) history. A subject was considered an ever-smoker if they had smoked at least 100 cigarettes during their lifetime and an ever-drinker if they had drunk alcoholic beverages at least once a week for a year or more during their lifetime. Subjects who previously smoked or drank alcohol but had not done so in the year prior to their diagnosis were considered former-smokers and former-drinkers, respectively.

Demographic and clinical variables of interest were analyzed using standard descriptive statistical methods. Differences between groups were compared using chi-square or Fisher's exact (when cell frequencies  $< 5$ ) tests for categorical variables and Student's *t*-test, with adjustment for unequal variances where appropriate, for continuous variables. Mean Ab values were plotted for cases and controls and compared using Mann–Whitney non-parametric analysis (GraphPad Prism version 5.0c, San Diego, CA). Odds ratios (OR) with 95% confidence intervals (CI) were calculated using logistic regression models with adjustment for possible confounding factors to determine the association between pre-treatment Ab status and OPC. The association between pre-treatment Ab status and tumor HPV16 and smoking status among cases was also evaluated (Stata 12.0, StataCorp, College Station, TX). A *p*-value of  $< .05$  was considered significant and all tests were 2-sided.

## Results

### Subject characteristics

Ab levels specific for HPV16 proteins and GST control protein were compared in sera from 258 cases of OPC and 250 age-, gender- and race-matched controls. Two cases were omitted from further study because they presented with distant metastases at diagnosis, leaving a sample size of 256 cases. The demographics of cases and controls are presented in Table 1. There were no significant differences between cases and controls with respect to age, gender, and race (Table 1). However, cases were more likely

to be current smokers while controls were less likely to be current alcohol drinkers ( $p = 0.007$  and  $p < 0.001$ , respectively; Table 1). The majority of cases was derived from the tonsils or base of tongue (95%) and was stage III/IV (92.6%) at presentation. Among the 256 cases, HPV16 status (as determined by tumor HPV16 PCR for E6 and E7) were available for 137 cases, with 111 cases positive for HPV16 (81%).

#### Detection of HPV16 Abs by programmable ELISA

Results of the RAPID ELISA for serum IgG Abs to HPV16 antigens for OPC cases and healthy controls are shown in Fig. 1. The medians and ranges of these values for each individual antigen are presented in Supplementary Table 1. In the total OPC samples (unselected by HPV status), all HPV16 Abs except L2 were significantly higher among patients with OPC than healthy controls ( $p < 0.05$ , Supplementary Table 1 and Fig. 1). A higher proportion of patients with OPC than healthy controls were seropositive for each of these Abs (Table 2). Using cut-off values derived from the healthy controls ( $n = 125$ ,  $>3$  SDs over the mean), at least one HPV16 early gene Ab was detected in the sera of 210/256 (82.0%) of OPC cases, compared with 10/250 (4.0%) of healthy controls (Table 2). A high proportion of patients were positive for IgG Abs specific for HPV16 E1 (144/256, 56.3%), NE2 (104/256, 40.6%), CE2 (137/256, 53.5%), E4 (61/256, 23.8%), E6 (148/256, 57.8%), and/or E7 (148/256, 57.8%). In comparison, only 9 cases (3.5%) were positive for E5 Abs, 15 cases (5.9%) were positive for L1 Abs, and none were positive for L2 Abs.

Risk for OPC was estimated by calculating OR (95% CI), adjusted for age, smoking, and alcohol drinking status (Table 2). The OR for E1, E2, E6, and E7 Abs ranged from 86 to 171. While the 95% CI are wide, an extremely high risk of OPC was associated with seropositivity for the E1 protein (OR = 171), the C-terminal portion of E2 (OR = 158), the E6 protein (OR = 131), and for the E7 protein (OR = 147). Being seropositive to any antigen was associated with a 194-fold increased risk of OPC, while being seropositive to an early protein was associated with a 244-fold increased risk of OPC, and being seropositive to a late protein was associated with a 3-fold increased risk of OPC.

#### Association of serology with tumor HPV status and smoking

Among the 137 cases with tumor tissue tested for HPV16 E6 or E7 DNA by PCR, 111 (81.0%) were determined to be tumor HPV positive, and 26 (19.0%) were tumor HPV negative. The association between serology and tumor HPV status among the cases are presented in Table 3. Of the tumor HPV positive cases, 94/111 (84.7%) were serologically positive for at least one HPV early antigen. However, 16/26 (61.5%) of tumor HPV negative cases were also serologically positive for at least one HPV early antigen ( $p = 0.008$ ). The presence of Abs to NE2, CE2, E6, and/or E7 was associated with tumor HPV status (OR [95% CI], 6.5 [2.1–20.1]; Table 3), and this was particularly true among those who smoked less than or equal to 10 pack years (OR [95% CI], 17.5 [4.0–77.2]; Table 3). Being seropositive to the early proteins CE2 or E7 was associated with HPV tumor status among the cases with  $\leq 10$  pack-years of smoking but this association was not observed for late proteins (Table 3). Among smokers ( $>10$  pack-years), no consistent association between seropositivity and HPV tumor status was identified (Table 3).

#### Discussion

Due to the rising incidence of HPV-associated oropharyngeal cancers, there is an urgent clinical need for biomarkers for early

**Table 1**  
Demographic, exposure, and clinical characteristics of cases and controls.

	Cases $n = 256$	Controls $n = 250$	$p$
Age, mean (SD)	56.5 (9.6)	56.5 (9.6)	.963
Age, median	55	55.5	.930
	N (%)	N (%)	
Sex			.913
Male	220 (85.9)	214 (85.6)	
Female	36 (14.1)	36 (14.4)	
Race			.691
White	233 (91.0)	230 (92.0)	
Other	23 (9.0)	20 (8.0)	
Alcohol			<.001
Never	71 (27.7)	111 (44.4)	
Former	66 (25.8)	47 (18.8)	
Current	119 (46.5)	92 (36.8)	
Smoking			.007
Never	116 (45.3)	131 (52.4)	
Former	93 (36.3)	97 (38.8)	
Current	47 (18.4)	22 (8.8)	
Smoking <sup>a</sup>			
$\leq 10$ pack years	149 (59.6)		
$>10$ pack years	101 (40.4)		
HPV status <sup>b</sup>			
Negative	26 (19.0)		
Positive	111 (81.0)		
Subsite			
Tonsil	122 (47.7)		
Base of tongue	121 (47.3)		
Other oropharynx	9 (3.5)		
Other site (not oropharynx)	4 (1.6)		
Stage			
I–II	19 (7.4)		
III–IV	237 (92.6)		
T category			
0–1	76 (29.7)		
2	110 (43.0)		
3	41 (16.0)		
4	29 (11.3)		
N category			
0	29 (11.3)		
1–2a	42 (16.4)		
2b	126 (49.2)		
2c–3	59 (23.1)		
Grade <sup>c</sup>			
Well to moderate	76 (34.5)		
Moderately poor to poor	144 (65.5)		

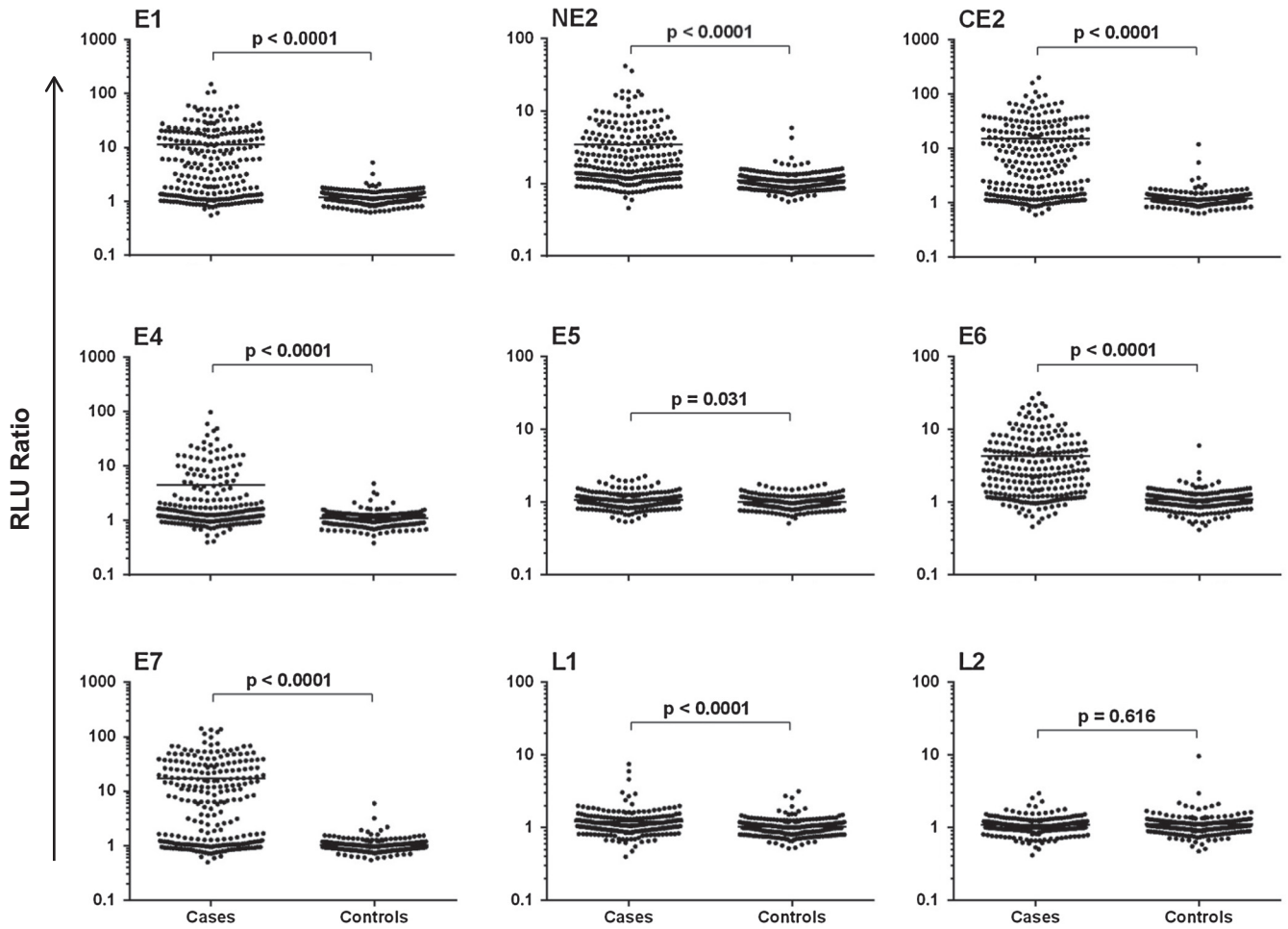
<sup>a</sup> Total  $n = 250$  cases due to missing data ( $n = 6$ ).

<sup>b</sup> Total  $n = 137$  cases due to missing data ( $n = 119$ ).

<sup>c</sup> Total  $n = 220$  cases due to missing data ( $n = 36$ ).

detection, diagnosis, prognosis, and monitoring of these patients. Prior studies have demonstrated that a subset of patients (~64–74%) with HPVOPC have detectable Abs to HPV16 E6 and/or E7 Abs in their sera [26,34]. In the present study we evaluated Abs specific for a panel of eight HPV16 antigens as potential biomarkers for the diagnosis of OPC. We demonstrate that Abs to multiple HPV16 early antigens (E1, E2, E4, E5, E6, and E7) are specifically detected in the sera of patients compared with age- gender- and race-matched controls. Using these stringent cutoffs, Abs to the capsid protein L1 are rarely observed (and L2 Abs are not observed) in HPVOPC. Our data support the hypothesis that HPV16 antibody signatures may be specific and clinically useful biomarkers of HPVOPC, and potentially for other HPV-associated malignancies.

Our data confirms and extends published studies from the Pawlita laboratory demonstrating detection of E6 and E7 Abs in patients with newly diagnosed HPVOPC [26,34]. E6 Abs have been detected over 10 years prior to clinical diagnosis [35,36], but in only 35% of patients with undefined tissue HPV status, limiting



**Fig. 1.** Specific detection of multiple HPV16 antibodies in patients with HPV-associated OPC HPV16 proteins were expressed as GST-fusion proteins and captured onto anti-GST coated plates. The RLU ratio (RLU of HPV antigen/RLU of GST-control) of IgG detected in HPV-associated OPC sera ( $n = 256$ ) and controls ( $n = 250$ ) is shown.

**Table 2**  
Association of pre-treatment antibody status with case-control status.

	Cases $n = 256$ No. + (%)	Controls $n = 250$ No. + (%)	$p^b$	Crude OR (95% CI)	Adjusted OR <sup>c</sup> (95% CI)
E1	144 (56.3)	2 (0.8)	<0.001	159.4 (38.8–655.1)	170.6 (41.3–705.0)
NE2	104 (40.6)	2 (0.8)	<0.001	84.8 (20.6–348.8)	85.7 (20.7–354.2)
CE2	137 (53.5)	2 (0.8)	<0.001	142.8 (34.7–586.5)	157.5 (38.0–653.2)
NE2 and/or CE2 <sup>a</sup>	144 (56.3)	2 (0.8)	<0.001	159.4 (38.8–655.1)	177.5 (42.8–736.9)
E4	61 (23.8)	3 (1.2)	<0.001	25.8 (8.0–83.3)	26.9 (8.3–87.4)
E5	9 (3.5)	2 (0.8)	0.063	4.5 (1.0–21.1)	4.7 (1.0–22.8)
E6	148 (57.8)	3 (1.2)	<0.001	112.8 (35.2–361.8)	130.6 (40.1–425.5)
E7	148 (57.8)	3 (1.2)	<0.001	112.8 (35.2–361.8)	147.4 (45.0–483.1)
E6 and/or E7 <sup>a</sup>	194 (75.8)	6 (2.4)	<0.001	127.2 (53.9–300.4)	241.4 (92.6–629.4)
NE2, CE2, E6, and/or E7 <sup>a</sup>	203 (79.3)	7 (2.8)	<0.001	133.0 (59.2–298.9)	249.1 (99.3–624.9)
Any E <sup>a</sup>	210 (82.0)	10 (4.0)	<0.001	109.6 (53.9–222.5)	243.7 (101.4–586.0)
L1	15 (5.9)	4 (1.6)	0.017	3.8 (1.3–11.7)	4.0 (1.3–12.3)
L2	0	1 (0.4)	0.494	NC	NC
Any L <sup>a</sup>	15 (5.9)	5 (6.3)	0.038	3.0 (1.1–8.5)	3.1 (1.1–8.8)
Any E and/or L <sup>a</sup>	210 (82.0)	12 (4.8)	<0.001	90.5 (46.7–175.5)	193.8 (85.0–441.5)

NC, not calculable due to zero cells.

<sup>a</sup> Any positive vs. all negative.

<sup>b</sup> Fisher's exact test.

<sup>c</sup> Adjusted for age, smoking, and alcohol status.

clinical applicability. Here, we detect E6 and/or E7 Abs in 79% of HPVOPC patients ( $n = 111$ ). Our results are similar to our findings of E6 and/or E7 Abs in 76% of patients ( $n = 119$ ) with HPVOPC from the independent, multicenter HOTSPOT study [32], suggesting that

there is limited regional or technical variation in serologic detection of HPVOPC. This also suggests that differences in the definition of HPV status (PCR used in this study vs p16/ISH in HOTSPOT) do not significantly impact these results. Using the full panel of early



**Table 3**  
Association of pre-treatment antibody status with tumor HPV and smoking status among cases.

	HPV– (reference) n = 26 No. + (%)	HPV+ n = 111 No. + (%)	p	Adjusted OR <sup>c</sup> (95% CI)	HPV+ >10 pack years <sup>d</sup> n = 47 No. + (%)	p <sup>e</sup>	Adjusted OR <sup>ef</sup> (95% CI)	HPV+ ≤10 pack years <sup>d</sup> n = 63 No. + (%)	P <sup>e</sup>	Adjusted OR <sup>ef</sup> (95% CI)
E1	13 (50.0)	66 (59.5)	0.380	1.1 (0.4–2.9)	21 (44.7)	0.663	0.7 (0.2–1.9)	44 (69.8)	0.076	1.5 (0.5–4.2)
NE2	8 (30.8)	50 (45.1)	0.185	1.5 (0.6–4.0)	19 (40.4)	0.413	1.6 (0.5–4.8)	31 (49.2)	0.111	1.5 (0.5–4.3)
CE2	11 (42.3)	68 (61.3)	0.078	1.9 (0.7–5.0)	20 (42.6)	0.984	1.0 (0.3–2.8)	47 (74.6)	0.004	2.9 (1.0–8.1)
NE2 and/or CE2 <sup>a</sup>	11 (42.3)	72 (64.9)	0.034	2.3 (0.9–6.0)	22 (46.8)	0.711	1.2 (0.4–3.5)	49 (77.8)	0.001	3.4 (1.2–9.6)
E4	6 (23.1)	27 (24.3)	0.893	0.9 (0.3–2.6)	10 (21.3)	0.859	0.8 (0.2–2.6)	16 (25.4)	0.818	0.7 (0.2–2.4)
E5	2 (7.7)	4 (3.6)	0.319 <sup>b</sup>	0.3 (0–1.9)	3 (6.4)	1.0 <sup>b</sup>	0.6 (0.1–4.3)	1 (1.6)	0.203 <sup>b</sup>	0.2 (0–2.4)
E6	10 (38.5)	66 (59.5)	0.052	2.1 (0.8–5.3)	24 (51.1)	0.301	1.6 (0.6–4.6)	42 (66.7)	0.014	2.5 (0.9–7.0)
E7	11 (42.3)	68 (61.3)	0.078	1.8 (0.7–4.7)	20 (42.6)	0.984	0.9 (0.3–2.6)	47 (74.6)	0.004	2.9 (1.0–8.1)
E6 and/or E7 <sup>a</sup>	12 (46.2)	88 (79.3)	0.001	4.4 (1.6–12.4)	30 (63.8)	0.143	2.0 (0.7–5.7)	57 (90.5)	<0.001	8.4 (2.5–27.8)
NE2, CE2, E6, and/or E7 <sup>a</sup>	13 (50.0)	94 (84.7)	<0.001	6.5 (2.1–20.1)	33 (70.2)	0.087	2.5 (0.8–7.5)	60 (95.2)	<0.001	17.5 (4.0– 77.2)
Any E <sup>a</sup>	16 (61.5)	94 (84.7)	0.008	3.4 (1.0– 11.4))	33 (70.2)	0.450	1.4 (0.4–4.4)	60 (95.2)	<0.001	8.0 (1.8–35.7)
L1	3 (11.5)	6 (5.4)	0.371 <sup>b</sup>	0.4 (0.1–1.8)	3 (6.4)	0.659 <sup>b</sup>	0.5 (0.1–2.7)	3 (4.8)	0.352 <sup>b</sup>	0.3 (0.1–2.0)
L2	0	0	NC	NC	0	NC	NC	0	NC	NC
Any L <sup>a</sup>	3 (11.5)	6 (5.4)	0.371 <sup>b</sup>	0.4 (0.1–1.8)	3 (6.4)	0.659 <sup>b</sup>	0.5 (0.1–2.7)	3 (4.8)	0.352 <sup>b</sup>	0.3 (0.1–2.0)
Any E and/or L <sup>a</sup>	16 (61.5)	94 (84.7)	0.008	3.4 (1.0–11.5)	33 (70.2)	0.450	1.4 (0.4–4.4)	60 (95.2)	<0.001	8.0 (1.8–35.7)

NC, not calculable due to zero cells.

<sup>a</sup> Any positive vs. all negative.

<sup>b</sup> Fisher's exact test.

<sup>c</sup> Adjusted for age, smoking (≤10 pack years vs. >10 pack years), N category (N0–N2a vs. N2b–N3), and subsite (tonsil/base of tongue vs. other).

<sup>d</sup> One HPV+ case is missing pack years.

<sup>e</sup> Referent group is HPV– cases.

<sup>f</sup> Adjusted for age, N category (N0–N2a vs. N2b–N3), and subsite (tonsil/base of tongue vs. other).

antigens, the proportion of patients seropositive was increased to 85%, strongly supporting the use of a multiparametric signature for HPVOPC detection.

The OPC cases presented here are representative of the head and neck clinic at MD Anderson Cancer Center, as over 90% of eligible patients consented to the study. In subset analysis, there were no correlations between age, gender, and race with seropositivity. Patients who were heavy smokers (>10 pack-years) had both lower frequencies of seropositivity and no association of serology with tumor HPV16 status. Because smoking has a higher association of tumors with p53 mutation, this may represent a sub-population where serologic biomarkers are less reliable.

One limitation of this study is the rapid evolution of methods and standards for the detection of HPV in tumor tissue in the 10 years during which these patients were enrolled [37]. There is no current standard of care for tissue biomarkers of HPV, although p16 expression detected by immunohistochemistry (IHC) is commonly used as a surrogate marker, with or without ISH for HR HPV nucleic acid [38]. Assignment of HPV status in this study was determined by PCR for HPV16 E6 and E7 [33]. Only a small subset of these cases had tissue available for p16 IHC ( $n = 22$ ). Therefore, we did not have the ability to confirm the tissue HPV status with p16 testing.

Within the subset of cases with tissue HPV16 status identified by PCR ( $n = 137$ ), Abs to HPV16 early antigens (E1, E2, E4, E5, E6, E7) were detected in 85% of cases who also had tumors that tested HPV16 positive by PCR, but 62% of cases that were negative by tissue HPV PCR were still positive by serology. These serologic responses were similar in strength and in antigenic specificity as the responses in tissue HPV PCR positive cases (data not shown).

Since 10–15% of HPVOPC are associated with HPV subtypes other than HPV16, we cannot exclude the possibility that the serologic assay is cross-reacting with other subtypes of HPV in the seropositive, PCR negative population. A second possibility is that the HPV16 PCR assay used in this study has limited sensitivity (false negatives), which cannot be confirmed by p16 testing in this study. We cannot exclude that we are detecting false-positive serologic responses in the HPV-negative OPC cases, but we observe no significant differences in sero-reactivity between these healthy controls from MD Anderson ( $n = 250$ ) and Oregon Health Sciences University ( $n = 78$ , data not shown).

A notable finding from our data is the heterogeneity of the serologic response to HPV16 in these patients, which is of unknown biologic or clinical significance. The majority (79%) of patient sera have Abs to E6 and/or E7, but Abs to multiple other early genes, including E1, E2, and E4 are also specifically detected. This is unique to HPVOPC, as these Abs are rarely detected in sera from patients with invasive cervical cancer (not shown). E1 Abs are strongly correlated with E2 Abs, and E4 Abs were only detected in a subset (25.7%) of patients with E7 Abs. Five percent of patients had isolated Abs to E1/E2 antigens, without E6/E7 Abs.

A key observation from our study is the high frequency of E1 and E2 Abs in HPVOPC sera, which has not been observed in cervical disease [39]. Because humoral immunity is induced by antigen expression, we predict that the variation in the serologic response to individual HPV antigens is a result of differences in antigen expression in the tumor. Since expression of E2 decreases due to viral integration and de-repression of E6/E7 [40], we predict that Abs to E2 may inversely correlate with viral integration. As a result, E1/E2 Abs may be detected earlier in HPVOPC development than

E6/E7 Abs. In summary, panels of antibodies to HPV16 early proteins may be highly specific biomarkers for the diagnosis of HPVOPC.

### Conflict of interest statement

Dr. Anderson is a consultant, hold stock options, and serves on the scientific advisory board of Provista Diagnostics. All other authors report no conflicts of interest.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.oraloncology.2015.04.011>.

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