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Biologic Predictors of Serologic Responses to HPV in Oropharyngeal Cancer: The HOTSPOT Study

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Abstract

Objectives—We hypothesized that viral and host factors impact the serologic responses to HPV early antigens in HPV-positive oropharyngeal cancer (HPVOPC).

Materials and methods—We conducted a multicenter study to measure HPV16-specific IgG among patients with HPVOPC, their long-term sexual partners, and healthy volunteers. Risk factor surveys and rinse and gargle specimens were collected. Peripheral blood samples at diagnosis were evaluated for IgG Abs to HPV16 antigens using a programmable ELISA assay. Predictors for HPV16 serologic responses were evaluated using univariate and multivariable linear regression.

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CONFLICT OF INTEREST STATEMENT

Dr. Anderson is a consultant and has stock options with Provista Diagnostics. Dr. Anderson and Dr. Posner are on institutional patent submissions for HPV serologic testing. Other authors report no conflicts.

Results—116 patients with HPVOPC, 43 partners, and 81 healthy volunteers were enrolled and had baseline sera for analysis. Cases were primarily male (90%), with a median age of 56 years. Abs to E1, E2, E6 or E7 antigens were detected more often in HPVOPC compared with volunteers or partner sera ($p<0.0001$). HPV16 Abs to at least one early protein (E1, E2, E4, E5, E6, or E7) were detected in the sera of 90.6% of cases, 0% of partners and 7.4% of healthy volunteers. Gender, race, sexual behavior, and viral integration were not associated with antibody response. Younger age and higher oral HPV16 copy number were associated with higher HPV16 E6 and NE2 antibody levels.

Conclusions—HPV16 seroreactivity is commonly detected among patients with HPVOPC at diagnosis, but not among partners or healthy volunteers. Seroreactivity among cases are correlated with viral load and stage and not with other demographic or behavioral factors. Positive HPV16 serology was strongly associated with HPV 16 oropharyngeal cancer.

Keywords

Antibodies; serology; biomarker; HPV; oropharyngeal cancer

Introduction

The incidence of human papillomavirus-positive oropharyngeal squamous cell cancer (HPVOPC) has been markedly increasing in the US and Europe [1-4], with a higher rate in men than in women in North America [5]. Oral infection with HPV16 has been detected in the majority of cases of oropharyngeal squamous cell cancer (OPC) [6-9], but little is known about transmission, immunogenicity, and oncogenicity of HPV within the oropharynx. There is a strong association with having performed oral sex and numbers of lifetime partners [10-13], suggesting that initial infection of HPV in the oropharynx is related to sexual activity. Unlike HPV-related cancer in the cervix, precursor lesions have not been identified in the oropharynx, hence the natural history and pathogenesis of oropharyngeal HPV infection is not known [14]. Examination of rinse and gargle samples for HPV nucleic acid suggests a prevalence of any oncogenic HPV infection of 4.7% in the general population ages 45-65 years old although the implications of this finding for developing clinically apparent OPC is not known [15]. Since HPVOPC remains an uncommon cancer (prevalence of 2-3/100,000) compared to the prevalence of HPV in the saliva of the general population, this suggests that there are host factors that limit HPV mediated oncogenesis in the oropharynx.

Patients with HPVOPC develop serum IgG antibodies (Abs) to the oncogenic E6 and E7 proteins derived from HPV16 [6, 16, 17] (reviewed in [18]). Of note, Abs to E6 protein have been detected years prior to clinical diagnosis in a minority of patients [19]. Since these Abs are rarely present in healthy blood donors [20], they may represent biomarkers of risk for oncogenesis. In a pilot study using sera from HPVOPC patients, we detected a heterogeneous serologic response to multiple HPV16-derived early antigens, including E1, E2, E4, as well as E6 and E7 Abs [16]. These data suggest that there may be differences in viral exposure history, viral load, or immunogenicity of the viral antigens based on the host immune system in HPVOPC patients, resulting in differential serologic responses.

The purpose of this study was to investigate the serologic immune responses to multiple HPV16 antigens in HPVOPC patients and their sexual partners in a prospective cohort from four academic medical centers in the United States [15]. We examined clinical, pathologic and viral criteria associated with the development of HPV16-specific Abs in HPVOPC patients and explored the utility of the unique heterogeneous serologic responses as diagnostic biomarkers for HPVOPC.

Methods

Study design and subjects

The HOTSPOT (Human Oral Papillomavirus Transmission in Partners over Time) study design and enrollment have been previously reported [15]. This was a subset analysis of a multicenter prospective study of HPVOPC cases and their spouses/long-term partners (defined by sexual activity for two or more years). Subjects with incident, previously untreated OPC with tumor testing indicating HPV-positivity and with blood collected at baseline for testing were included in this analysis: 116 of 164 cases (70.7%), 43 of 93 partners (46.2%), and 81 of 85 healthy volunteers (95.3%) met these criteria and were included. Subjects were enrolled between October 2009 and May 2013 in head and neck cancer clinics at four study sites: Johns Hopkins Hospital (JHH), Mount Sinai Medical System (MSMS), Dana Farber Cancer Institute (DFCI), and Oregon Health and Science University (OHSU). At two study sites (OHSU and MSMS) a convenience sample of “healthy volunteers” (N=85) were enrolled from individuals attending free oral cancer screening events in May 2011 and April 2012, both of which occurred during the study period. Two volunteers were eliminated from analysis due to a history of OPC and another two were eliminated due to significant sexual history (>100 contacts) as they were not representative of enrolled cases. The two eliminated volunteers did not have HPV16 Abs. As this was a convenience sample, healthy volunteers may not represent the general population. All participants provided an oral rinse and gargle sample and a blood sample at baseline and answered the same baseline survey. The computer assisted self-interview (CASI) risk factor survey included questions on demographics, tobacco and alcohol use, and detailed sexual behaviors. Written informed consent was obtained from all subjects under institutional review board approval. Clinical Trials number: NCT01342978.

Methods for HPV DNA detection in rinse and gargle samples

Oral exfoliated cells were collected using a 30-second oral rinse and gargle method with 10 ml of Scope (P&G, Cincinnati, OH). Baseline rinse and gargle samples were evaluated for HPV16 viral load using TaqMan quantitative real-time-PCR (qPCR) using the 7300 real-time PCR system (Applied Biosystems, Foster City, CA), as previously described [21, 22].

Methods for tumor HPV detection and classification

The 116 HPVOPC cases included in this analysis were classified as HPV-related based upon centralized testing for HPV type 16 (HPV16) by in situ hybridization (ISH, N=34, 29.3%), local HPV16 ISH positivity and local p16 immunohistochemistry (N=21, 18.1%), local high-risk HPV ISH probe positivity and local p16 immunohistochemistry (n=28, 24.1%) or local p16 immunohistochemistry alone (n=33, 28.4%; 19 of these cases also had local PCR

testing demonstrating HPV16 DNA was present), of the tumor biopsy. Thus, of the 116 HPVOPC cases, 55 were known to be specifically HPV16-positive because they either had centralized HPV16 (N=34) or local (N=21) HPV16 ISH results.

Measurement of HPV integration

Real-time quantitative PCR of the E2 and E6 genes was performed using sequence specific primers and probes at a final concentration of 0.3 and 0.1 μ M, respectively, in a total volume of 10 μ l. 50 ng of target DNA from saliva specimens was added to the reaction mixture. Two standard curves were generated by amplification of the E6 gene from the CaSki cell line which was serially diluted from 30 to 0.03 ngs of genomic DNA (gDNA). A linear relationship between the threshold cycle values plotted against the log of the copy number over the entire range of dilutions was observed. At least three no-template control reaction mixtures were included in each run. All experiments were performed twice in triplicate with similar ratios. The polymerase chain reaction (PCR) reactions were run on a Taqman 7900HT machine and SDS v 2.4 software was used for quantitative analysis. A ratio of E2:E6 less than 1 indicated the presence of both integrated and episomal forms of HPV DNA. A ratio of E2:E6 greater than 1 indicated a predominance of the episomal form of HPV DNA.

Serologic Assays

HPV16 programmable ELISA assays were performed as previously described [15]. Individual HPV16 genes were expressed as full-length C-terminal GST fusion proteins except for the E2 protein, which was subcloned as N (NE2) and C (CE2)-terminal fragments for optimal protein expression. The HPV and GST control proteins were expressed in vitro using the human HeLa cell lysate transcription/translation (IVTT) system (Thermo Scientific, Waltham, MA) [23] and captured onto anti-GST plates. Sera were diluted 1:100 and bound IgG was detected using a 1:10000 dilution of HRP anti-human IgG secondary Abs (Life Technologies, Carlsbad, CA), and Supersignal ELISA Femto Chemiluminescent substrate (Thermo Scientific). Luminescence was measured in Relative Light Units (RLU). To control for non-specific and GST-specific Abs, the ratio of RLU for individual HPV-specific Abs to the RLU for the control GST-antigen was calculated.

Statistical Methods

Characteristics of cases, partners and volunteers were compared using χ^2 test for categorical variables and Fisher's Exact Test when the assumptions of the χ^2 test were violated (race only). Cut-off values for positive serology were previously established, and defined as the mean + 3 standard deviations of the RLU ratio observed among an independent set of healthy volunteers from MD Anderson Cancer Center (N=247). Healthy control sera were collected from genetically unrelated visitors or companions of the patients who presented to the head and neck clinic [24].

Comparisons of RLU ratios between groups were performed using Mann-Whitney nonparametric analysis (GraphPad Prism version 5.0c, San Diego, CA). MFI ratios were compared between cases and healthy volunteers and cases and partners. Predictors of HPV serology titer were evaluated using linear regression to model the increase in log titer of each

antibody type separately. Multivariable analysis considered all variables statistically significant in univariate analysis; those that were statistically significant in any model at $p < 0.05$ level were retained. Final multivariable models were only adjusted for age (continuous). Using an independent dataset from a previous study of 111 HPV16+ OPC cases and 247 controls[24], we used binary logistic regression classifier modeling to compute the area under the receiver operating characteristic curve (ROC) as the basis for comparing the performance for the diagnosis of OPC cancer of all the combinations of sets of antibodies. The optimal classifier used Abs to all early gene proteins and was pre-defined for this study. We then used the classifier to construct an ROC curve for the HOTSPOT cases and healthy controls.

Results

The clinical characteristics of the study populations are shown in **Table 1**. Cases (N=116) were primarily male (89.7%), had performed oral sex (96.6%), were never-smokers (56.8%) with a median age of 56 years (IQR 50-62). Most cases had a tonsil (45.0%) or base of tongue (46.8%) sub-site and were primarily stage 4 (80.6%) at the time of diagnosis. Partners (N=43) were primarily female (93.0%), had performed oral sex (100%), and were never-smokers (52.5%) with a median age of 51 years (IQR 46-60). Healthy volunteers (N=81) were primarily female (60.5%), had performed oral sex (92.5%), and were never-smokers (56.3%) with a median age of 51 years (IQR 42-60).

HPV16 Antibody Prevalence

IgG Abs to seven HPV16 antigens (E1, E4, E5, E6, E7, L1, and L2 antigens), as well as the N- and C-terminal fragments of E2 (NE2 and CE2) were measured in baseline serum from 116 HPVOPC cases and 43 partners, and compared to 81 healthy volunteers (**Figure 1**). The median of all HPV16 Abs except L2 were increased in cases compared to volunteers ($p = 0.01$). There was no significant difference observed between healthy volunteers and partners of HPVOPC cases for any HPV16 antibody (**Table 2**). The median Ab levels are shown in **Supplemental Table 1**.

The prevalence of serum IgG Abs among HPVOPC cases, partners, and volunteers specific for HPV16 antigens is summarized in **Table 2**. Abs to most E proteins were common in cases, including E1-Abs (63%), NE2-Abs (46%), and CE2-Abs (63%), E6-Abs (46%) and E7-Abs (68%). Abs to E4 (24%) and E5 (4%) were less prevalent among cases. Most HPVOPC cases had Abs to at least one early antigen (E1, E2, E4, E5, E6, and/or E7) (105/116, 91%). The sensitivity [proportion of HPVOPC cases with at least one HPV16 early antigen-specific Ab detected] was similar (87.3%) when restricted to 55 cases known to have HPV16 (as opposed to any other oncogenic HPV) detected in the tumor. In contrast, no partners (0/43, 0%) and six controls (6/81, 7.4%) had any HPV16 early antigen-specific Abs detected.

We evaluated the combination of early antigen-specific Abs, as a panel, based on their individual association with tumor status, to predict tumor status in this cohort. A 7-Ab panel (E1, NE2, CE2, E4, E5, E6, and E7) that was determined to be the optimal classifier in a previous study ([24]) was pre-defined for use in this study. Using the pre-determined

classifier, we constructed a receiver operating characteristic curve for the HOTSPOT HPVOPC cases (N=116) and healthy volunteers (N=81) in this study. At 95% specificity, the sensitivity of the combination panel was 87% (AUC=0.927, **Figure 2**). Use of the classifier improved the specificity of the panel, compared with using cut-off values alone.

Predictors of HPV16 Antibody Level

We evaluated the demographic, clinical and pathologic predictors of each HPV16 Ab level (**Table 3**). Older age was associated with reduced log titer of E6 Abs ($\beta=-0.28$ per 10 years of age, $p=0.01$) and E4 ($\beta=-0.24$, $p=0.05$), with similar but non-significant inverse association with age observed for E1 and NE2 (**Table 3**). Gender, race and ethnicity, family history of head and neck cancer, and sexual behavior were not associated with antibody response. Inconsistent associations were observed in univariate analysis between study site with E5 and L1 levels ($p=0.002$ and $p=0.03$, respectively), alcohol status with E4 and L2 levels ($p=0.003$ and $p=0.04$), and cigarette smoking with E5 levels ($p=0.02$; **Table 3**); however, these variables were no longer predictors of antibody response after adjustment for other factors. (**Table 4**). There was no association with marijuana status, number of lifetime oral sex partners or having ever had sex with someone of the same sex (data not shown).

Association of HPV16 Antibodies with Viral and Pathologic Parameters

Oral HPV DNA prevalence in rinse and gargle specimens from the original study cohort has been previously reported [15]. Of the HPVOPC cases reported in that study, 61% (100/164) had oncogenic HPV DNA detected at diagnosis. Most (88/100) of the oncogenic oral HPV DNA detected among HPVOPC cases was HPV16. The median HPV16 copy number detected in 88 rinse and gargle samples was 74 copies, IQR=8-470. Serology was not available for the current study from all subjects who had rinse and gargle samples. Given the high incidence of oncogenic HPV previously detected in rinse and gargle samples, we evaluated the association between the log titers of individual HPV16 Abs in sera with cancer stage, oral HPV16 copy number, and integration status of HPV (**Table 3**). E1, E6, and L1 Abs were associated with higher cancer stage ($p=0.01$ for E1, $p=0.02$ for E6, and $p=0.04$ for L1), and a similar but weaker association for CE2 and E4 Abs ($p=0.07$ for both).

Higher oral HPV16 copy number was significantly associated with E1, NE2, and E6 Ab levels ($\beta=0.19-0.32$ per 1000 copies, $p<0.05$ for all; **Table 3**) and was similarly associated, although statistically significant, with CE2, and L1 Abs ($p=0.1$ for both) in univariate analysis. We did not observe any association between serology and integration status as measured by qPCR ($n=54$; **Table 3**). In multivariable analysis, age ($\beta=-0.24$, $p=0.02$) and oral HPV16 copy number ($\beta=0.24$, $p=0.01$) remained associated with E6 levels.

Discussion

HPV16 has been identified as the primary cause of OPC in Europe and the United States [25]. While the marked increase in incidence of HPVOPC is strongly associated both with number of lifetime sexual partners and oral sex practices other factors may contribute to susceptibility [10, 11]. The mechanisms of viral transmission, carcinogenesis and biologically at-risk populations are not known. We recently reported that oral HPV DNA

detection and serologic responses to HPV16 E6 and E7 proteins were common among HPVOPC patients; however, this was not the case among sexual partners of HPVOPC patients despite presumed chronic exposure [15]. Here, we demonstrate that IgG antibodies specific for five HPV16 early proteins (E1, E2, E4, E6, and E7) are common and heterogeneously present among HPVOPC cases. These Abs are rarely detected in partners or in healthy volunteers. This study demonstrates that the majority (90.6%) of HPVOPC patients have generated an immunologic response against the virus and have detectable levels of Abs at diagnosis to at least one HPV16 early protein. In contrast, the prevalence of detectable HPV16 Abs in partners of HPVOPC patients is similar to that in a control population (0-7.4%). Applying a pre-determined multivariable algorithm of all early antigen IgG levels, the sensitivity of detection of HPVOPC by multiplexed antibody detection and the utility tool at the time of diagnosis was 87% at a specificity of 95%; however, the healthy volunteer population was not matched to the cases by age, gender, race, ethnicity, or study site.

Given the high frequencies and heterogeneity of serologic responses to HPV16 early proteins among HPVOPC patients, we assessed the association between individual HPV16-specific serologic responses (measured as a continuous variable) with clinical, epidemiologic, and pathologic parameters, as well as oral viral load and viral integration rates. We observed an inverse correlation between Ab status and age, suggesting that older patients have lower levels of Ab responses, particularly to E6, which is consistent with published data that weak lymphocyte proliferation is significantly associated with HPV appearance among women 45 years of age and older [26]. A weak, inverse association between age and lower E1, NE2, and E4 serologic responses was also observed. Age-related declines in Ab responses (quantity, not specificity) have been observed for the influenza virus vaccine, with significant reductions over age 65 [27]. Within cases of HPVOPC, we did not observe a correlation of serology with the number of lifetime sexual partners or oral sexual partners. In other words, when cancer is established, early antigen serology appears independent of sexual history.

As expected, we observed that HPV16 serologic responses to multiple antigens correlated with tumor stage, similar to Abs to tumor antigens such as p53 [28]. HPV16 serologic responses to multiple antigens also correlated with oral HPV16 copy number, however this was confirmed in multivariable analysis specifically only for E6 although a weak association was observed for NE2. Further studies will be necessary to evaluate the relationship and utility of oral HPV copy number [15], tissue HPV copy number [29, 30] combined with serologic status for detection and prognosis of HPVOPC. The biologic implications of this limited correlation of oral HPV copy number with E6 and NE2 Abs are not known at this time.

A critical step in the pathophysiology of HPV related malignancies is the integration of the virus into the host genome. This is accompanied by loss of E2 expression and de-repression of E6 and E7. However, the relative contributions of episomal vs. integrated virus to the progression of carcinoma in HPVOPC has not been explored and episomal virus is commonly found in oropharyngeal tumors [31, 32]. Furthermore, E2 expression in OPC is not always lost upon integration [33], which may explain the detection of high-level E2 IgG

Abs in the majority of HPVOPC patients. In contrast, we rarely observed E1 and E2 Abs in cervical carcinoma or precancer, suggesting significant differences in pathophysiology and carcinogenesis of HPV between these tumor sites (data not shown). Viral integration status has been conventionally determined by the ratio of E2 to E6 DNA, crudely representing the amount of the episomal form in relation to the integrated form [34]. Here, we did not observe a correlation between E2/E6 ratio and serologic status, however further studies are warranted given the limited sample size evaluated.

Because the prevalence of HPVOPC within the general population is low, serum biomarkers for detection would need very high specificity in order to be useful for screening the general population. Identification of people at higher risk of development of HPVOPC will be critical for any targeted screening plan and preventative therapy. Identification of at-risk populations and development of rapid, cost-effective biomarkers will be essential for early detection of HPVOPC. The utilization of a serologic test in patients presenting with head and neck cancer or unknown primary tumors may be used in addition to p16 or ISH testing to identify HPVOPC as opposed to environmental OPC in immunologically normal populations. HPV in oropharyngeal tumors is associated with reduced risk of cancer progression [35, 36] and improved overall survival [37]. In addition, serologic responses precede frank malignancy by a considerable period. Therefore, further exploration of serologic responses as screening tools in larger populations is warranted [38]. Specific patterns of antibodies based on the heterogeneity of responses may even have biological relevance for prognosis.

There are several limitations to interpreting the findings in our study. This prospective study was designed to evaluate potential biologic causes of the heterogeneity of serologic responses within HPVOPC patients. It was not designed as a phase II biomarker study. HPVOPC is more common among males, and both the partners and healthy volunteers in this study were predominantly female. They were not matched to the demographics of the cases. Since few female cases and male partners were enrolled, the generalizability of our findings to these sub-groups is limited. As a strength, the observed results were similar across the four geographically distinct academic medical centers where subjects were enrolled [15]. Centralized testing of rinse and gargle samples, viral integration, and antibody HPV data as well as some centralized tumor testing are also strengths of our report. Furthermore, this study used a standardized risk factor survey at each study site, collected risk factor information directly from subjects using CASI, and was restricted to incident cases with confirmed HPV-related disease. The biologic consequence of Abs and of antibody heterogeneity to HPV early antigens is unknown. Serologic biomarkers may identify a population of early HPVOPC patients for trials of targeted immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Abs	antibodies
ELISA	enzyme-linked immunosorbent assay
IVTT	in vitro transcription/translation
HPVOPC	HPV-positive oropharyngeal cancer
RAPID	rapid antigenic protein in situ display

References

1. Chaturvedi AK, et al. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. *J Clin Oncol*. 2008; 26(4):612–9. [PubMed: 18235120]
2. Hammarstedt L, et al. The incidence of tonsillar cancer in Sweden is increasing. *Acta Otolaryngol*. 2007; 127(9):988–92. [PubMed: 17712680]
3. Hammarstedt L, et al. Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer. *Int J Cancer*. 2006; 119(11):2620–3. [PubMed: 16991119]
4. Chaturvedi A, et al. Human papillomavirus (HPV) and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. 2011; 29(32):4294–4301. [PubMed: 21969503]
5. Combes JD, Chen AA, Franceschi S. Prevalence of human papillomavirus in cancer of the oropharynx by gender. *Cancer Epidemiol Biomarkers Prev*. 2014; 23(12):2954–8. [PubMed: 25205515]
6. D'Souza G, et al. Case-control study of human papillomavirus and oropharyngeal cancer. *The New England journal of medicine*. 2007; 356(19):1944–56. [PubMed: 17494927]
7. Nasman A, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer*. 2009; 125(2):362–6. [PubMed: 19330833]
8. Smith EM, et al. Human papillomavirus in oral exfoliated cells and risk of head and neck cancer. *J Natl Cancer Inst*. 2004; 96(6):449–55. [PubMed: 15026470]
9. Zhu C, et al. The relationship between oral squamous cell carcinoma and human papillomavirus: a meta-analysis of a Chinese population (1994-2011). *PloS one*. 2012; 7(5):e36294. [PubMed: 22570701]
10. Gillison ML, et al. Prevalence of oral HPV infection in the United States, 2009-2010. *JAMA : the journal of the American Medical Association*. 2012; 307(7):693–703. [PubMed: 22282321]
11. D'Souza G, et al. Oral sexual behaviors associated with prevalent oral human papillomavirus (HPV) infection. *Journal of Infectious Disease*. 2009; 199:1–7.
12. Kreimer AR, et al. Oral human papillomavirus infection in adults is associated with sexual behavior and HIV serostatus. *The Journal of infectious diseases*. 2004; 189(4):686–98. [PubMed: 14767823]
13. Beachler DC, et al. Risk factors for oral HPV infection among a high prevalence population of HIV-positive and at-risk HIV-negative adults. *Cancer Epidemiology, Biomarkers & Prevention*. 2012; 21(1):122–33.
14. Gillison ML, et al. Eurogin Roadmap: comparative epidemiology of HPV infection and associated cancers of the head and neck and cervix. *Int J Cancer*. 2014; 134(3):497–507. [PubMed: 23568556]

15. D'Souza G, et al. Oral Human Papillomavirus (HPV) Infection in HPV-Positive Patients With Oropharyngeal Cancer and Their Partners. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2014
16. Anderson KS, et al. Serum antibodies to the HPV16 proteome as biomarkers for head and neck cancer. *British journal of cancer*. 2011; 104(12):1896–905. [PubMed: 21654689]
17. Smith EM, et al. Human papillomavirus seropositivity and risks of head and neck cancer. *Int J Cancer*. 2007; 120(4):825–32. [PubMed: 17131312]
18. Gillison ML, et al. Human papillomavirus and diseases of the upper airway: head and neck cancer and respiratory papillomatosis. *Vaccine*. 2012; 30(Suppl 5):F34–54. [PubMed: 23199965]
19. Kreimer AR, et al. Evaluation of Human Papillomavirus Antibodies and Risk of Subsequent Head and Neck Cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2013
20. Lang Kuhs KA, et al. Human Papillomavirus 16 E6 Antibodies in Individuals Without Diagnosed Cancer: A Pooled Analysis. *Cancer Epidemiol Biomarkers Prev*. 2015
21. Koshiol J, et al. Assessment of human papillomavirus in lung tumor tissue. *Journal of the National Cancer Institute*. 2011; 103(6):501–7. [PubMed: 21293027]
22. Agoston ES, et al. Polymerase chain reaction detection of HPV in squamous carcinoma of the oropharynx. *American journal of clinical pathology*. 2010; 134(1):36–41. [PubMed: 20551264]
23. Festa F, et al. Robust microarray production of freshly expressed proteins in a human milieu. *Proteomics. Clinical applications*. 2013; 7(5-6):372–7. [PubMed: 23027544]
24. Anderson KS, et al. HPV16 antibodies as risk factors for oropharyngeal cancer and their association with tumor HPV and smoking status. *Oral oncology*. 2015
25. Chaturvedi AK, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2011; 29(32):4294–301. [PubMed: 21969503]
26. Gonzalez P, et al. Behavioral/lifestyle and immunologic factors associated with HPV infection among women older than 45 years. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2010; 19(12):3044–54.
27. Sasaki S, et al. Limited efficacy of inactivated influenza vaccine in elderly individuals is associated with decreased production of vaccine-specific antibodies. *The Journal of clinical investigation*. 2011; 121(8):3109–19. [PubMed: 21785218]
28. Anderson KS, et al. Application of protein microarrays for multiplexed detection of antibodies to tumor antigens in breast cancer. *Journal of proteome research*. 2008; 7(4):1490–9. [PubMed: 18311903]
29. Kumar B, 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(19):3128–37. [PubMed: 18474878]
30. Worden FP, et al. Chemoselection as a strategy for organ preservation in advanced oropharynx cancer: response and survival positively associated with HPV16 copy number. *J Clin Oncol*. 2008; 26(19):3138–46. [PubMed: 18474879]
31. Se-Heon Kim B-SK, Kang Suki, Park Kyeongmee, Kim Haeryoung, Ryul Lee Kyung, Joo Lee Moo, Man Kim Jong, Chang Choi Eun, Hoon Cho Nam. HPV integration begins in the tonsillar crypt and leads to the alteration of p16, EGFR and c-myc during tumor formation. *Int. J. Cancer*. 2007; 120:1418–1425. [PubMed: 17205528]
32. Olthof, Nadine C.; E.-J.M.S.; Kolligs, Jutta; Haesevoets, Annick; Henfling, Mieke; Ramaekers, Frans C. S.; Preuss, Simon F.; Drebber, Uta; Wieland, Ulrike; Silling, Steffi; Lam, Wan L.; Vucic, Emily A.; Kremer, Bernd; Klussmann, Jens-P.; Huebbers, Christian U. Comprehensive Analysis of HPV16 Integration in OSCC Reveals No Significant Impact of Physical Status on Viral Oncogene and Virally Disrupted Human Gene Expression. *PLOS ONE*. 2014; 9(2)
33. Keiko Akagi JL, Broutian Tatevik R. Padilla-Nash Hesus, Xiao Weihong, Jiang Bo, Rocco James W. Teknos Theodoros N. Kumar Bhavna, Wangsa Danny, He Dandan, Ried Thomas, Symer David E. Gillison Maura L. Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. *Genome Research*. 2013; 24:185–199. [PubMed: 24201445]

34. Peitsaro P, Johansson B, Syrjanen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *Journal of clinical microbiology*. 2002; 40(3):886–91. [PubMed: 11880410]
35. Ang KK, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. 2010; 363(1):24–35. [PubMed: 20530316]
36. Fakhry C, Gillison ML. Clinical implications of human papillomavirus in head and neck cancers. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2006; 24(17):2606–11. [PubMed: 16763272]
37. Fakhry C, et al. Human papillomavirus and overall survival after progression of oropharyngeal squamous cell carcinoma. *J Clin Oncol*. 2014; 32(30):3365–73. [PubMed: 24958820]
38. Kreimer AR, et al. Evaluation of human papillomavirus antibodies and risk of subsequent head and neck cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2013; 31(21):2708–15. [PubMed: 23775966]

Highlights

- Abs to the HPV16 proteome were measured in 256 cases of OPC and 250 controls.
- Serum Abs to HPV16 early Ags were detected in 85% of HPVOPC cases and 4% of controls.
- Seropositivity was strongly associated with OPC risk (OR 249).
- Seropositivity to E6 and E2 was associated with oral HPV 16 copy number.

Figure 1.

Detection of HPV16 antibodies in serum from 116 HPVOPC cases, 43 partners, and 81 controls. The RLU ratio of IgG to specific HPV16 protein/control GST protein detected in sera is shown. The black line in each group represents the median value in that group. HPV16-specific Abs to E1, NE2, CE2, E4, E5, E6, E7 and L1 are detected in sera from patients with HPVOPC compared to partners and controls.

Figure 2.

Receiver operating characteristic curve illustrating the performance of the 7-Ab biomarker panel (E1, NE2, CE2, E4, E5, E6, E7) for distinguishing HPVOPC cases compared with controls. The optimal operating point for this panel was 87% at a specificity of 95% (AUC=, 927).

Table 1

Demographic characteristics of HPVOPC cases, partners, and controls.

	Total N=240	Cases N=116	Partners N=43	Healthy Volunteers N=81	
Characteristic	N (%)	N (%)	N (%)	N (%)	P-value ^I
Sex					<0.001
Male	139 (57.9%)	104 (89.7%)	3 (7.0%)	32 (39.5%)	
Female	101 (42.1%)	12 (10.3%)	40 (93.0%)	49 (60.5%)	
Age in years					
Median (IQR)	54 (47-60)	56 (50-62)	51 (46-60)	51 (42-60)	
Race					0.850
White	219 (91.3%)	107 (92.2%)	40 (93.0%)	72 (88.9%)	
Black	11 (4.6%)	5 (4.3%)	2 (4.7%)	4 (4.9%)	
Other	10 (4.2%)	4 (3.4%)	1 (2.3%)	5 (6.2%)	
Study Site					<0.001
DFCI	58 (24.2%)	43 (37.1%)	15 (34.9%)	0 (0.0%)	
JHH	46 (19.2%)	36 (31.0%)	10 (23.3%)	0 (0.0%)	
OHSU	85 (35.4%)	7 (6.0%)	3 (7.0%)	75 (92.6%)	
MSMS	51 (21.3%)	30 (25.9%)	15 (34.9%)	6 (7.4%)	
Cancer Stage²					
II		4 (3.5%)			
III		18 (15.9%)			
IVa (N2MO)		81 (71.7%)			
IVb (N3MO)		8 (7.1%)			
IVc (any N; metastasized)		2 (1.8%)			
Cancer Sub-site³					
Tonsil		49 (45.0%)			
BOT		51 (46.8%)			
Tonsil & BOT		3 (2.8%)			
Other OP/unknown primary OP		6 (5.5%)			
Family History of HNC					0.187
No	176 (73.3%)	80 (70.0%)	30 (69.8%)	66 (81.5%)	
Yes	17 (7.1%)	6 (5.2%)	1 (2.3%)	10 (12.3%)	
Unknown	47 (19.6%)	30 (25.9%)	12 (27.9%)	5 (6.2%)	
Alcohol status⁴					<0.001
Current	133 (56.8%)	63 (55.8%)	19 (47.5%)	51 (63.0%)	
Former	42 (17.9%)	31 (27.4%)	4 (10%)	7 (8.6%)	
Never	59 (25.2%)	19 (16.8%)	17 (42.5%)	23 (28.4%)	
Smoking status⁵					0.955
Current	31 (13.4%)	14 (12.6%)	5 (12.5%)	12 (15.0%)	
Former	71 (30.7%)	34 (30.6%)	14 (35.0%)	23 (28.8%)	

	Total N=240	Cases N=116	Partners N=43	Healthy Volunteers N=81	
Characteristic	N (%)	N (%)	N (%)	N (%)	P-value ^I
Never	129 (55.8%)	63 (56.8%)	21 (52.5%)	45 (56.3%)	
Cigarette Pack-years⁶					0.091
<20 pack-years	57 (57.6%)	24 (50.0%)	13 (81.3%)	20 (57.1%)	
20 pack-years	42 (42.4%)	24 (50.0%)	3 (18.8%)	15 (42.9%)	
Lifetime oral sex partners⁷					0.002
0	10 (4.4%)	4 (3.7%)	0 (0.0%)	6 (7.5%)	
1	25 (11.1%)	6 (5.6%)	6 (15.4%)	13 (16.3%)	
2-5	81 (35.8%)	30 (28.0%)	18 (46.2%)	33 (41.3%)	
6-15	60 (26.5%)	36 (33.6%)	12 (30.8%)	12 (15.0%)	
16	50 (22.1%)	31 (29.0%)	3 (7.7%)	16 (20.0%)	
Lifetime vaginal sex partners⁸					0.004
0	3 (1.4%)	0 (0.0%)	0 (0.0%)	3 (3.8%)	
1	20 (9.1%)	6 (5.8%)	5 (13.2%)	9 (11.5%)	
2-5	46 (20.9%)	17 (16.3%)	14 (36.8%)	15 (19.2%)	
6-15	79 (35.9%)	35 (33.7%)	12 (31.6%)	32 (41%)	
16	72 (32.7%)	46 (44.2%)	7 (18.4%)	19 (24.4%)	

Abbreviations: DFCI, Dana-Farber Cancer Institute; JHH, Johns Hopkins Hospital; OHSU, Oregon Health and Sciences University; MSMS, Mount Sinai Medical School; BOT, Base of Tongue; OP, Oropharyngeal; HNC, Head and Neck Cancer

^IP-values were estimated using χ^2 test for categorical data and excluded those with missing data for each variable; for all variables except for race as the assumptions of χ^2 test were not satisfied. The P-value for race was therefore estimated using Fisher's Exact Test

²Cancer stage missing for 3 cases

³Cancer sub-site missing for 7 cases

⁴Alcohol status missing for 3 cases and 3 partners

⁵Smoking status missing for 5 cases, 3 partners, and 1 healthy volunteer

⁶Cigarette pack-years missing for 3 partners among current and former smokers

⁷Lifetime number of oral sex partners missing for 9 cases, 4 partners, and 1 healthy volunteer

⁸Lifetime number of vaginal sex partners missing for 12 cases, 5 partners, and 3 healthy volunteers

Table 2Prevalence of positive antibody response¹ to each HPV16 protein

HPV16 Antibodies	No. + (%)			
	Cases		Partners	Healthy Volunteers
	All HPVOPC N=116	Tumor known to be HPV16+ N=55	N=43	Total N=81
E1	74 (63.3%)	34 (61.9%)	0 (0%)	2 (2.5%)
NE2	54 (46.2%)	23 (41.8%)	0 (0%)	2 (2.5%)
CE2	74 (63.3%)	33 (60.0%)	0 (0%)	2 (2.5%)
E4	28 (23.9%)	10 (18.2%)	0 (0%)	3 (3.7%)
E5	5 (4.3%)	1 (1.8%)	0 (0%)	2 (2.5%)
E6	54 (46.2%)	24 (43.6%)	0 (0%)	1 (1.2%)
E7	80 (68.4%)	36 (65.5%)	0 (0%)	1 (1.2%)
Any E ²	105 (90.6%)	48 (87.3%)	0 (0%)	6 (7.4%)
L1	10 (8.6%)	3 (5.5%)	0 (0%)	2 (2.5%)
L2	3 (2.6%)	1 (1.8%)	0 (0%)	1 (1.2%)
Any E and/or L ²	105 (90.6%)	48 (97.3%)	0 (0%)	7 (8.6%)

¹ Cut-off values defined as average RLU ratio +3 standard deviations for each antigen in serum of healthy controls (n=247)² Any positive vs. all negative

Table 3

Univariate Analysis of Predictors for HPV Antibody Positivity Among HPV+ OPC¹

Predictors	Target HPV16 Antigen											
	E1		NE2		CE2		E4		E5		E6	
	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²
Sex												
Male	1	0.54	1	0.22	1	0.32	1	0.70	1	0.42	1	0.96
Female	0.29		0.35		0.44		0.13		-0.12		0.02	
Age (per 10 years) ³	-0.28	0.08	-0.17	0.09	-0.14	0.39	-0.24	0.05	-0.09	0.09	-0.28	0.01
Race and Ethnicity												
White Non-Hispanic	1		1	0.77	1	0.39	1	0.87	1	0.66	1	0.05
Other	-0.16	0.75	-0.09		-0.40		0.06		-0.07		-0.62	
Cancer Stage ⁴												
II-III	1		1	0.15	1	0.07	1	0.07	1	0.22	1	0.02
IVa-IVc	0.94	0.01	0.32		0.63		0.47		0.14		0.56	
Alcohol Status ⁶												
Never	1	0.73	1	0.09	1	0.05	1	0.003	1	0.43	1	0.11
Former	-0.20		-0.16		-0.61		0.10		0.16		0.10	
Current	0.07		0.28		0.18		0.79		0.17		0.45	
Cigarette Smoking Status ⁷												
Never	1	0.98	1	0.87	1	0.62	1	0.79	1	0.02	1	0.44
<20 pack-years	-0.06		-0.11		0.01		-0.10		-0.01		-0.13	
20 pack-years	-0.06		0.02		-0.32		-0.18		0.16		-0.29	
Oral HPV16 Copy Number (per 1000 copies) ¹¹						0.10				0.22		
E2/E6	0.32	0.03	0.19	0.04	0.24	0.10	0.07	0.56	0.06	0.22	0.25	0.01
qPCR ¹²												
											0.10	0.07
											-0.04	0.33

Target HPV16 Antigen																		
Predictors	E1		NE2		CE2		E4		E5		E6		E7		L1		L2	
	N=116		N=116		N=116		N=116		N=116		N=116		N=116		N=116		N=116	
	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²	β	P-value ²
No E2/E6	1	0.14	1	0.38	1	0.30	1	0.20	1	0.21	1	0.72	1	0.26	1	0.66	1	0.34
Integrated dominant	-0.16		0.15		-0.42		-0.58		0.30		-0.23		0.70		0.05		0.11	
Episomal dominant	1.10		0.55		0.58		0.20		-0.05		-0.02		-0.35		0.24		0.09	

Abbreviations: HPVOPC, Human Papillomavirus Oropharyngeal Cancer; HNC, Head and Neck Cancer

⁵N=86

⁸N=109

⁹N=107

¹⁰N=108

¹³N=107

¹The natural logarithm of each antibody result was used as the outcome variable for each model

²P-values were estimated using Likelihood Ratio Tests

³ β represents the increase per every 10 years of age

⁴N=113

⁶N=113

⁷N=111

¹¹N=112; β represents the increase per every 1000 oral HPV16 copy numbers

¹²N=54

Table 4
Multivariable Analysis of Predictors for HPV Antibody Positivity Among HPVOPC¹

Predictors	Target HPV16 Antigen											
	NE2			CE2			E6			E7		
	β	SE	N=112	β	SE	N=112	β	SE	N=112	β	SE	N=112
Age (per 10 years) ²	-0.16	0.10	0.11	-0.10	0.16	0.51	-0.24	0.10	0.02	-0.18	0.18	0.32
Oral HPV16 Copy Number (per 1000 copies) ³	0.18	0.09	0.05	0.23	0.14	0.10	0.24	0.09	0.01	0.12	0.16	0.47

Abbreviations: HPVOPC, Human Papillomavirus-positive Oropharyngeal Cancer; SE, Standard Error

⁴ β represents the increase per every 1000 HPV16 copies

¹The natural logarithm of each antibody result was used as the outcome variable for each model

²P-values were estimated using Likelihood Ratio Tests

³ β represents the increase per every 10 years of age