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HPV16 E6 seropositivity among cancer-free men with oral, anal or genital HPV16 infection

Daniel C. Beachler [a,*], Tim Waterboer [b], Christine M. Pierce Campbell [c], Donna J. Ingles [d], Krystle A. Lang Kuhs [a], Alan G. Nyitray [e], Allan Hildesheim [a], Michael Pawlita [b], Aimée R. Kreimer [a,1], Anna R. Giuliano [c,1]

[a] Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD, United States
[b] German Cancer Research Center (DKFZ), Heidelberg, Germany
[c] Center for Infection Research in Cancer, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, United States
[d] Vanderbilt Institute for Global Health, Nashville, TN, United States
[e] University of Texas School of Public Health, Houston, TX, United States

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ABSTRACT

Antibodies against the Human papillomavirus 16 (HPV16) E6 oncoprotein appear years prior to clinical diagnosis of anal and oropharyngeal cancer, but whether they develop around the time of HPV infection is unclear. Serum samples from 173 cancer-free men from the Human Papillomavirus Infection in Men (HIM) Study were tested for HPV antibodies and DNA. HPV16 E6 seropositivity was low among men with oral HPV16-infection (1/28; 3.6%, 95%CI=0.0–18.4%), anal HPV16-infection (1/61; 1.6%, 95%CI=0.0–8.8%), and 24-month persistent genital HPV16-infection (1/84; 1.2%, 0.0–6.5%). This suggests E6 seroconversion may not occur around the time of oral, anal, or genital HPV16 acquisition.

1. Introduction

Overexpression of the E6 and E7 oncoproteins are believed to drive the transition from benign human papillomavirus (HPV) infection to carcinogenesis, as E6/E7 overexpression has been observed in HPV-driven cancers, including cervical, penile, anal, and oropharyngeal cancer [1]. Recent reports found that seropositivity against the HPV16 E6 oncoprotein has a high sensitivity and specificity for HPV16-driven oropharyngeal and anal cancer[2–4] and is often detected in pre-diagnostic serum among individuals with these cancers [5,6]. These analyses suggest that while HPV16 E6 seropositivity may not be commonly present prior to cervical or penile cancer [6], it is often induced within five years prior to anal cancer [6] and ten or more years prior to oropharyngeal cancer [5].

Despite the detection of these antibodies years prior to anal and oropharyngeal cancer, it is unclear at what point during the natural history of HPV16, E6 seropositivity is induced by infection at these different anatomic sites. So far, this biomarker has not been examined in the earliest stages of the disease process (i.e. when HPV infections are acquired). E6 seropositivity at the time of oral HPV16 infection is of particular interest, as the oropharynx is comprised of lymphoid tissue that may be more likely to induce early antibody responses to infection. We therefore examined the presence of HPV16 E6 antibodies among cancer-free individuals with genital, anal, or oral HPV16 infection in the Human papillomavirus Infection in Men (HIM) Study.

2. Material and methods

2.1. Study population and design

This study is nested within the ongoing HIM Study, a semi-annual prospective cohort of individuals recruited from Brazil, Mexico, and the United States starting in 2005 [7]. The HIM Study includes 4123 men aged 18–70 years who reported no prior diagnosis of penile or anal cancer, had never been diagnosed with genital warts, and reported no treatment or symptoms of a sexually transmitted infection. All consenting participants had genital, anal, and serum samples collected at semi-annual HIM Study visits, and have been followed for a median of four years. Oral rinse and gargle sample collection was initiated in 2007 for all

* Correspondence to: Infections & Immunoepidemiology Branch, Division of Cancer Epidemiology and Genetics, NCI, 9609 Medical Center Drive, RM 6-E220, Bethesda, MD 20892, United States.
E-mail address: daniel.beachler@nih.gov (D.C. Beachler).
[1] Co-senior authors.

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consenting participants. To date, oral samples were tested for HPV DNA from 1626 HIM men who reported no history of head and neck cancer and provided oral samples on two or more semi-annual study visits [8,9]. All HIM participants gave written informed consent, and the Human Subject Committees of the University of South Florida (Tampa, FL), Ludwig Institute for Cancer Research (São Paulo, Brazil), Centro de Refêrencia e Treinamento em Doencas Sexualmente Transmissiveis e AIDS (São Paulo, Brazil), and Instituto Nacional de Salud Pública de Mexico (Cuernavaca, Mexico) approved all study procedures.

For this study, we selected 173 individuals with the putative highest likelihood of HPV16 E6 seropositivity. This included all individuals who had a detectable oral or anal HPV16 infection during the HIM Study and individuals who had a genital HPV16 infection that persisted for at least 24 months. We restricted to these individuals given the very low prevalence of E6 seropositivity in the general population [2]. While oral and anal samples have been collected on most HIM participants, HPV DNA testing has only been performed on a subset and thus there were fewer men with known anal and oral infections. As such, the study included 28 individuals with an oral HPV16 infection [8], 61 with an anal HPV16 infection, and 84 individuals with 24-month persistent genital HPV16 infection.

For the 28 oral HPV16 infected individuals, we selected the serum sample corresponding to the visit when oral HPV16 DNA was last detected, as well as two other serum samples collected one and two years after that visit. For participants that were oral HPV16 DNA-positive during their last HIM Study visit, a serum sample was utilized from the last visit as well as a serum sample collected one year prior to the last visit. Sampling was similar for those who were anal HPV16 infected or had a 24-month persistent genital HPV16 infection, except that only two serum samples were tested for all of these men.

2.2. Laboratory and statistical analyses

Oral, anal, and genital sampling methods for the HIM Study have been described previously [7,9,10]. HPV DNA was extracted using the Qiagen QIAamp Media MDx kit according to the manufacturer's instructions. Samples were tested for DNA of 37 alpha HPV types, including HPV16, using the Roche Linear Array with PGMY09/11 PCR primers. A prevalent HPV16 infection was defined as detection at the first tested visit, while an incident HPV16 infection was defined as being first detected after having at least one negative test at a prior visit.

Serum samples from the HIM Study were shipped on dry ice to the German Cancer Research Center (Heidelberg, Germany) and stored at $-20\,^{\circ}C$ until analysis. Serologic testing was performed using multiplex assays [11] by laboratory staff blinded to the oral/anal/genital HPV16 status of the participants. Antigens were affinity-purified, bacterially expressed fusion proteins with N-terminal glutathione S-transferase. Samples were analyzed for antibodies to the early oncoproteins E6 and E7, as well as the major capsid protein (L1), and other early proteins (E1, E2, E4) of HPV16. We used the same median fluorescence intensity (MFI) cutoffs for L1, E1, E2, E4, and E7 seropositivity as in our previous analyses among cancer patients [2,5]. For E6 seropositivity, we utilized both the initial cutoff of $\geq$484 MFI and the recently employed more stringent cutoff of $\geq$1000 MFI [2,5].

A subset of samples from this study was randomly chosen and included as blinded duplicates. Quality control of this assay has been previously described [12], and the intra-individual correlation coefficient for E6 seropositive duplicates in this study was 1.00. The three groups (oral HPV16-infected, anal HPV16-infected, and 24-month persistent genital HPV16-infected men) were compared through chi-square, Fisher's exact, or Wilcoxon-Mann-Whitney tests when appropriate. HPV16 E6 seropositivity prevalence and binomial 95% confidence intervals (95%CIs) were calculated for each risk group.

3. Results

The mean age of the 173 HPV16-infected men was 35 years (IQR=27–42), approximately 51% were white, 16% had ever had sex with a man, and the median lifetime number of sexual partners was 15 (IQR=8–31). Oral, anal, and genital HPV16-infected men were similar to each other with regard to most characteristics examined (Table 1), except anal HPV16-infected men were more likely to be younger and to have ever had sex with a man (p-values <0.05).

Among the three groups, 1 of the 28 (3.6%, 95%CI=0.0–18.4%) oral HPV16-infected men, 1 of the 61 (1.6%, 95%CI=0.0–8.8%) anal HPV16-infected men, and 1 of the 84 (1.2%, 95%CI=0.0–6.5%) men with 24-month persistent HPV16 genital infection was HPV16 E6 seropositive (Table 2). HPV16 E6 seroprevalence was similarly low across these three groups (p=0.70), and all 34 men with incident oral or anal HPV16 infection were E6 seronegative (Table 2). All three E6 seropositive men had MFIs above the stricter definition of positivity (MFI >1000). No other men had MFIs between 484–1000. In comparison, seroprevalences of HPV16 L1 and E4 were significantly higher than HPV16 E6 seroprevalence (12.7% & 13.3% vs. 1.2%, respectively; p-values <0.05), while the seroprevalences of HPV16 E2 and E7 were non-significantly higher than HPV16 E6 seroprevalence (4.0% & 6.4% vs. 1.2%, p-values >0.05). The seroprevalence of HPV16 L1 was 10.7% (95%CI=2.3–28.2%) among oral HPV16-infected men, 16.4% (95%CI=8.2–28.1%) among anal HPV16-infected men, and 10.7% (95%CI=5.0–19.4%) among men with 24-month persistent HPV16 genital infection.

The HPV16 E6 seropositive man who was oral HPV16-infected had a prevalent oral infection at baseline that persisted throughout the study. His E6 seropositivity was detectable at the first visit and persisted throughout the following two yearly visits (Online-only Table 1). This participant was also HPV16 L1 and E2 seropositive, but negative for the other HPV16 proteins tested and for genital and anal HPV16 at each visit. The HPV16 E6 seropositive man with an anal HPV16 infection also had a prevalent anal infection at baseline, although his anal HPV16 infection cleared by the following one year visit, and his E6 MFI declined from 1724 to 816. He was HPV16 E7 seropositive, but negative for the other HPV16 proteins, and for oral and genital HPV16 infection. The HPV16 E6 seropositive man who had a 24-month persistent genital HPV16 infection had an incident genital infection that persisted >3 years, with the antibody detected 3.5 years following HPV acquisition. His MFI increased from 962 to 1150 during his last two annual visits (Online-only Table 1). He was also L1 seropositive, but negative for the other HPV16 early proteins and for oral and anal HPV16.

4. Discussion

In this study of early HPV infection events, HPV16 E6 seropositivity was rare among cancer-free individuals with genital, anal, or oral HPV16 infection. None of the participants with incident oral or anal HPV16 were E6 seropositive at the visit their HPV infection was first detected. Only two of 55 men with prevalent oral or anal HPV16 infection were E6 seropositive, and only one man with a long-term genital HPV 16 infection was E6 seropositive. This suggests it may require long-term persistence of an oral/anal HPV16 infection or further progression to induce E6 seropositivity, or that seroconversion only occurs in a small subset

Table 1
Characteristics of the 173 HIM participants with oral, anal, or 24-month persistent genital HPV16 infection.

Baseline characteristics of selected HIM's participants	Oral HPV16 positive N=28	Anal HPV16 positive N=61	Genital HPV16 (24+ mo.) positive N=84
Type of HPV16 Infection			
Prevalent	11 (39%)	44 (72%)	52 (62%)
Incident	17 (61%)[#]	17 (28%)	32 (38%)
Age, median (IQR)	38 (28–44)	29 (25–40)[^]	34 (29–43)
Younger than 30	7 (25%)	31 (51%)[^]	23 (27%)
30–39	9 (32%)	13 (21%)	34 (40%)
40 or older	12 (43%)	17 (28%)	27 (32%)
Country			
United States	12 (43%)	26 (43%)	29 (35%)
Brazil	8 (29%)	17 (28%)	31 (37%)
Mexico	8 (29%)	18 (29%)	24 (29%)
Race/Ethnicity			
White	15 (54%)	32 (52%)	42 (50%)
Black	4 (14%)	8 (13%)	17 (20%)
Mixed	7 (25%)	19 (31%)	19 (23%)
Other	2 (7%)	2 (3%)	5 (6%)
Refused	0 (0%)	0 (0%)	1 (1%)
Cigarette Smoker			
Current	5 (18%)	20 (33%)	23 (27%)
Former	6 (21%)	10 (16%)	10 (12%)
Never	17 (61%)	31 (51%)	51 (61%)
Sexual Orientation[a]			
Men who have sex with women (MSW)	26 (93%)	42 (68%)	78 (93%)
Men who have sex with men and women (MSMW)	1 (4%)	11 (18%)[^]	3 (4%)
Men who have sex with men (MSM)	1 (4%)	8 (13%)[^]	3 (4%)
Oral sex in last six months			
No	5 (18%)	8 (13%)	18 (21%)
Yes	22 (79%)	7 (11%)	63 (75%)
Not reported	1 (4%)	46 (75%)	3 (4%)
Lifetime number of sex partners (male+female), median(IQR)	18 (10–40)	13 (6–29)	19 (10–35)
0–5	2 (7%)	11 (18%)	3 (4%)
6–20	11 (39%)	26 (43%)	34 (40%)
21 or more	9 (32%)	18 (30%)	31 (37%)
Not reported	6 (21%)	6 (10%)	16 (19%)
HIV-infection			
No	28 (100%)	59 (97%)	83 (99%)
Yes	0 (0%)	0 (0%)	1 (1%)
Not reported	0 (0%)	2 (3%)	0 (0%)

[#] Oral HPV16 infections were more likely to be incident than the anal HPV16 (p=0.003) or persistent genital HPV16 infections (p=0.02).

[^] Anal HPV16-infected men were more likely to be younger and to have ever had sex with a man than men with oral HPV16 infections or 24-month genital HPV16 infections (p-values < 0.05).

[a] Sexual orientation defined by self-report lifetime sexual behavior data, MSMW report having at least one lifetime female and at least one male sex partner and MSM only reported having at least one lifetime male sex partner.

of infected individuals shortly after infection.

Among women, seroconversion to the HPV16 L1 capsid protein occurs soon after cervical HPV16 infection acquisition [13], however, E6/E7 seroconversion does not occur until much later in the disease process [6]. Less is known about oral and anal HPV natural history, but initial nested case-control studies suggest that HPV16 E6 seroconversion may occur earlier in the natural history of oral or anal HPV16 infection than for genital HPV16 infection [5,6]. HPV16 E6 seropositivity of oral HPV16-infected individuals is of particular interest, given that E6 antibodies are often present at least ten years prior to oropharyngeal cancer diagnosis [5], suggesting that HPV infection of the lymphatic tissue of the oropharynx may be suited to induce an earlier humoral response to infection compared to other anatomic sites. While it is unclear exactly when in the infection/disease process HPV16 E6 antibodies are induced, the results of this current study may suggest that HPV16 E6 seroconversion does not occur immediately following acquisition of oral (or anal and genital) HPV16 infection. Alternatively, E6 seroconversion may occur soon after HPV16 infection, but preferentially among individuals pre-destined to develop oropharyngeal or anal cancer. Further follow-up is necessary to distinguish between these two theories.

Table 2
E6 antibody status among individuals who were oral, anal, or genital HPV16 DNA positive during the HIM study.

HPV16 status	E6 positive[a]	Total	% E6 positive	95% CI
Oral HPV16 positive	1	28	3.6%	0.0–18.4%
Incident oral HPV16 infection	0	17	0.0%	0.0–19.5%
Anal HPV16 positive	1	61	1.6%	0.0–8.8%
Incident anal HPV16 infection	0	17	0.0%	0.0–19.5%
Genital HPV16 positive (2+ year persistent)	1	84	1.2%	0.0–6.5%
Incident 2+ year pers. genital HPV16 inf.	1	32	3.1%	0.0–16.2%

[a] E6 seropositivity evident at all three visits for the oral HPV16 infected man (MFI: 3498, 5414, 3466), but only at one visit for the anal HPV16 infected man and the genital HPV16 infected man.

Oral HPV16 infection is relatively uncommon in the US, with an overall prevalence of 1.6% in US men [14]. Initial studies have suggested that the clearance of HPV infections may differ by anatomic site [8,15], but many of these infections at each anatomic site appear to clear (or become undetectable) within two years [9,15,16]. Given this clearance and the modest incidence of oropharyngeal cancer (~4 per 100,000 person-years) [17], rapid E6 seroconversion after oral HPV16 acquisition would have suggested that this biomarker has a poor specificity for subsequent oropharyngeal cancer/pre-cancer. Instead, this study is consistent with recent findings that HPV16 E6 seropositivity is rare among individuals without a diagnosed cancer (~0.3% in >4000 individuals with unknown HPV status, representing a specificity of over 99%) [2]. This is consistent with the notion that HPV16 E6 seropositivity in healthy individuals may have a high specificity for subsequent oropharyngeal cancer/pre-cancer [2]. However, further study is needed to determine this biomarker's sensitivity, specificity, and positive predictive value for oropharyngeal and other HPV-driven cancers.

While this is one of the first studies to examine whether HPV16 E6 seropositivity is evident among individuals with HPV at several anatomic sites using a well validated assay [11,12], there were limitations. There were limited number of oral HPV16 infections, and DNA at these different sites may sometimes represent deposition rather than true infection that is required to develop a serologic response. Larger studies are necessary to better assess the interaction of HPV infections and E6 seropositivity. We also lacked anal and oral HPV DNA testing on many of the participants including some men who had 24 month persistent genital HPV16 infection. In addition, cancer history was self-reported, so it is possible that some participants could have had undiagnosed HPV-driven cancer.

5. Conclusions

Results from this study suggest E6 seroconversion does not occur around the time of oral, anal, or genital HPV16 acquisition in most HPV16 infected individuals. Further research is needed to

understand how long HPV16 needs to persist or progress at each anatomic site to increase the likelihood of HPV16 E6 antibody production.

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Conflicts of Interest

All 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.pvr.2016.07.003.

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