Sexually Transmitted Diseases, Publish Ahead of Print
DOI: 10.1097/OLQ.0000000000001802

Associations of HPV-16 gene methylation with oral HPV-16 persistence among a multinational sample of men

Jessica Y. Islam, PhD, MPH[1], Deepti Bettampadi, PhD, MBBS[1], Richard R. Reich, PhD[2], Belinda Nedjai, PhD[3], Anna R. Giuliano, PhD[1]

[1]Center for Immunization and Infection Research in Cancer, Cancer Epidemiology Program, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL

[2]Biostatistics and Bioinformatics Shared Resource, H. Lee Moffitt Cancer Center and Research Institute, Tampa FL

[3]Center for Cancer Prevention, Queen Mary University of London, London, UK

Corresponding Author:

Anna R. Giuliano, PhD

Center for Infection and Immunization Research Center

Cancer Epidemiology Program

H. Lee Moffitt Cancer Center and Research Institute

12902 Magnolia Drive MRC-CANCONT, Tampa, FL 33612

Email: anna.giuliano@moffitt.org

Downloaded from http://journals.lww.com/stdjournal by BhDMf5ePHKav1zEoum1tQfN4a+kJLhEZgbsIHo4XMi0hCywCX1AWnYQp/IlQrHD3i3D0OdRyi7TvSFl4Cf3VC1y0abggQZXdtwnfKZBYtws= on 03/21/2023

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

Abstract

Using data from the HPV Infection in Men cohort study, we demonstrate HPV 16 methylation associations with persistent oral HPV infection, the obligate precursor to oropharyngeal cancer (OPC). HPV16 persistence was significantly associated with methylation of HPV16 L2 CpG-4268 (Wilcoxon p=0.04), and methylation of HPV16 E2 CpG-Pos-4 (Wilcoxon p=0.04).

List of Abbreviations:

AIN: Anal intraepithelial neoplasia

CI: Confidence interval

CIN: Cervical intraepithelial neoplasia

DNA: Deoxyribonucleic acid

CpG: 5'—C—phosphate—G—3'

FL: Florida

HIM: HPV Infection in Men Study

HPV: Human papillomavirus

MSM: Men who have sex with men

MSW: Men having sex with only women

OPC: Oropharyngeal cancer

RHA: Reverse hybridization assay

SD: Standard deviation

TMD: Trimmed mean difference

USA: United States of America

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

Background

Human papillomavirus type 16 (HPV 16) infection is one of the most important human carcinogens known globally, and can lead to the development of several cancers including oropharyngeal cancer (OPC) [1]. Globally, approximately 38,000 or one-third of head and neck cancer cases, including OPC, are attributable to oncogenic HPV infection, with the majority of the global burden occurring in high-resourced settings[2]. Unlike other HPV-associated cancers, currently no screening tests are available for OPC. As such, most cases of OPC are diagnosed at an advanced stage leading to poor outcomes and poor quality of life associated with treatment side effects[3].

Although our understanding of the natural history of oral HPV remains unclear, persistent oncogenic HPV infection at other anatomical sites is known to lead to invasive cancer. HPV viral methylation is a novel biomarker that may distinguish HPV infections that are short lived, or transient, from those that may persist and potentially progress to OPC. For example, persistent high-risk HPV infections in the cervix, particularly HPV-16, results in progression to high grade lesions, and in the absence of screening and treatments, to invasive cancer[4]. At the cervix methylation of HPV-16 L1, L2, and E2 CpG sites have been validated as biomarkers for detection of high-grade cervical intraepithelial lesions. Methylation of HPV L1 has also been previously detected in oral squamous cell carcinoma[6], suggesting that early detection of HPV-16 methylation may be useful to detect oral HPV infections at risk of persisting. Our objective was to evaluate the association between methylation of HPV-16 L1, L2, and E2 and persistent HPV-16 infections among men residing in Brazil, Mexico, and the United States participating in the HPV Infection in Men (HIM) Study.

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

Methods

The study population was nested within an oral sub-cohort of the HIM study, a prospective cohort of men residing in Sao Paulo, Brazil, Cuernavaca, Mexico, and Tampa, FL, USA. Pertinent study eligibility criteria for the HIM were as follows: (1) age 18-73 years; (2) Resident of Brazil, Mexico, or United States; (3) no prior diagnosis of anal or penile cancer, and genital or anal warts; (4) no current sexually transmitted infection; (5) never participated in a HPV vaccine study; and (6) no history of human immunodeficiency virus or acquired immune deficiency syndrome. Further details about recruitment of participants for the HIM study and study procedures have been previously published[7]. All study participants provided informed consent.

The HIM Study protocol included a pre-enrollment visit, a baseline or enrollment visit, and up to 13 study visits 6 months apart after enrollment. Oral gargle samples were collected every 6 months, for a median of 38.7 months, from 2009 to 2015. The oral gargle sample was collected in 15 ml of Scope or equivalent brand mouthwash and processed within twenty-four hours of collection. Participants were asked to perform energetic washing of the oral cavity including the throat by swishing the mouthwash in their mouth vigorously for approximately 15 seconds and were instructed to cover all surfaces of their mouth. The participant was then instructed to tip their head back and gargle in the throat for another fifteen seconds and spit the mouthwash back into the collection tube. Oral gargle samples were placed at 20 °C until processing. Within twenty-four hours of collection the gargle specimen was centrifuged at 2000×*g* for 15 min at 4 °C. The supernatant was decanted, and the resulting cell pellet resuspended in 20 ml cold PBS (4 °C). Centrifugation and pellet washing was repeated twice

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

with the final cell pellet resuspended in 1.2 ml of PBS and maintained at −80 °C until DNA extraction when 300 µl was used for extraction.

Oral gargle specimens from the oral sub-cohort of HIM study (n=3200) were HPV genotyped using the SPF_{10} PCR-DEIA-$LiPA_{25}$ system, an *in vitro* reverse hybridization assay (RHA). To evaluate biomarkers of HPV-16 persistence, methylation of HPV-16 L1, L2, and E2 was evaluated among men who were oral HPV-16 positive at their baseline oral gargle specimen (n=50). Further details regarding DNA extraction and purification procedures have been provided elsewhere[8]. Following DNA isolation and bisulfite conversions, oral cell samples were tested for methylation of five CpG sites of E2, two CpG sites of L1, and five CpG sites of L2 genes of HPV 16 using a previously validated pyrosequencing method.

Our primary outcome of HPV persistence was defined as follows: men who were HPV 16 positive at 6- and 12-month visits; or HPV-16 negative at the 12th month follow-up, but HPV-16 positive at 6th, 18th and 24 month visits (i.e., + - + +). Trimmed mean differences (TMD with 95% CI) of methylation levels of each CpG site of three HPV 16 among participants with and without persistence were used with trim of 0.05. Wilcoxon rank sum tests compared methylation between these two groups. Based on the exploratory nature of this analysis, we did not include an adjustment for multiple comparisons[10]. All analyses were performed using R software version 4.0 (R Foundation, Vienna, Austria).

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

Results

At the first oral gargle collection visit, mean age of the participants was 33.9 (SD:9.1) years. Residents in Brazil, Mexico, and the US were 40%, 36%, and 24% of the sample, respectively. Fifty-two percent of participants were married or cohabiting, 76% were MSW (men having sex with only women), 34% reported more than 19 lifetime number of sexual partners, and 36% reported that their last oral sex was performed more than 30 days ago. Forty-four percent of the participants were never-smokers and 36% reported having consumed more than 30 alcoholic drinks in the past month. Twenty percent of the participants reported having swollen gums or consistent bleeding of gums after brushing teeth.

Forty-nine participants were available for follow-up after 12 months (i.e., data were available for baseline, six- and 12-month visits). Overall, 14 (28%) participants had persistent HPV-16 infection. Higher percent methylation among certain CpG sites in the E2 and L2 genes of HPV-16 were observed among those who persisted compared to those who did not persist. Methylation of HPV 16 E2 CpG site Pos 4 was associated with HPV persistence with a greater TMD (-3.46%, 95% CI: -4.21, -1.05) in the group of participants with persistent HPV16 compared to those who cleared HPV16 infection (Table 1). In addition to methylation of HPV 16 E2 Pos 4 CpG site methylation of CpG 4268 of HPV16 L2 was also significantly associated with HPV-16 persistence (TMD: -2.25, 95% CI: -6.35, -0.16).

Discussion

In this study we demonstrated the potential utility of HPV-16 DNA methylation as a biomarker for the detection of persistent oral HPV16 infection. With the recent rise in OPC

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

burden, particularly in high-resourced settings, there has been growing interest in developing OPC screening interventions to reduce the morbidity and mortality associated with OPC. Although questions remain regarding the natural history of oral HPV, oncogenic oral HPV infection has been established as the precursor to HPV-associated OPC, with persistent oral HPV infection the obligate precursor to HPV related OPC. Most recently, a prospective study of oncogenic oral HPV infection demonstrated the first temporal description of persistent oral HPV-16 infection followed until clinical presentation of HPV-related OPC over a 7-year follow-up period [11]. Novel biomarkers, such as DNA methylation, as a screening tool for early detection of persistent HPV16 infection are needed.

In our study, we focused on HPV-16 positive participants as measured at baseline to identify DNA methylation markers of persistent HPV infection. To our knowledge, this is the first study to leverage oral gargle specimens to evaluate DNA-methylation based biomarkers for the detection of persistent oral HPV16 infection. At the cervix, DNA methylation of human genes has been evaluated as a screening and triage test for the detection of cervical intraepithelial lesions (CIN), an important precursor to invasive cervical cancer, as well as the progression of HPV-16 to CIN[12]. Among HPV-positive women, prior research suggests DNA methylation of HPV-16 L1/L2 may increase with increasing CIN grade, with significantly higher methylation in CIN3 compared to CIN2, and universally high methylation in invasive cervical cancer cases[12]. Unlike cervical cancer, the progression of high-risk HPV infection to invasive OPC does not include a readily identifiable precursor lesion, which limits our ability to leverage cytological samples as an endpoint. Therefore, persistent HPV-infection may be an important endpoint to

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

evaluate for early detection of OPC. OPC early detection will be dependent on the development of informative biomarkers along the natural history of HPV-associated OPC.

The strength of this study includes the use of diverse participants residing in three different countries and belonging to a wide range of age groups. The HIM study is a longitudinal cohort, allowing us to evaluate the natural history of OPC and develop novel biomarkers for detection of persistent infections that may lead to invasive disease. However, several limitations should be considered when contextualizing the results of this analysis. First, this study included a relatively small sample size and therefore, we were only able to make simple comparisons across HPV-16 persistent infection groups. Further, although our results demonstrate a potential difference in methylation levels, the difference in methylation between cleared and persistent infections is rather small underscoring the importance of future work to confirm and replicate our findings in a larger population. Future work should be conducted to evaluate the analytic performance, including sensitivity and specificity, of HPV-16 DNA methylation as a biomarker for the detection of persistent HPV-16 infection.

In conclusion, our study demonstrates the potential use of HPV DNA methylation as a novel biomarker associated with persistent HPV-16 infections. As this is the first study to identify DNA methylation markers associated with persistent infection using oral gargle specimens, future research should be conducted to replicate these findings in a larger cohort and including a wider array of methylation markers to optimize validity of a OPC screening modality.

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

Footnotes

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement: Approval of study procedures before the commencement of the HIM study was obtained from the Centro de Referência e Treinamento de Doenças Sexualmente Transmissíveis e AIDS, Brazil, National Institute of Public Health of Mexico and the Human Subject Committees of the University of South Florida. All participants gave written consent.

Conflict of Interest: A. R. G. reports grants from Merck & Co, Inc, and personal fees (advisory board member and consultant) from Merck & Co, Inc, during the conduct of the study. All other authors report no potential conflicts.

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

References

1. de Sanjosé S, Serrano B, Tous S, et al. Burden of Human Papillomavirus (HPV)-Related Cancers Attributable to HPVs 6/11/16/18/31/33/45/52 and 58. JNCI Cancer Spectr **2018**; 2:pky045.
2. de Martel C, Plummer M, Vignat J, Franceschi S. Worldwide burden of cancer attributable to HPV by site, country and HPV type. Int. J. Cancer **2017**; 141:664–670.
3. Høxbroe Michaelsen S, Grønhøj C, Høxbroe Michaelsen J, Friborg J, von Buchwald C. Quality of life in survivors of oropharyngeal cancer: A systematic review and meta-analysis of 1366 patients. Eur. J. Cancer **2017**; 78:91–102.
4. Muñoz N, Bosch FX, de Sanjosé S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N. Engl. J. Med. **2003**; 348:518–527.
5. Zhang L, Tan W, Yang H, Zhang S, Dai Y. Detection of host cell gene/HPV DNA methylation markers: a promising triage approach for cervical cancer. Frontiers in Oncology **2022**;
6. Balderas-Loaeza A, Anaya-Saavedra G, Ramirez-Amador VA, et al. Human papillomavirus-16 DNA methylation patterns support a causal association of the virus with oral squamous cell carcinomas. Int. J. Cancer **2007**; 120:2165–2169.
7. Giuliano AR, Lazcano-Ponce E, Villa LL, et al. The human papillomavirus infection in men study: human papillomavirus prevalence and type distribution among men residing in Brazil, Mexico, and the United States. Cancer Epidemiol. Biomarkers Prev. **2008**; 17:2036–2043.

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

8. Kreimer AR, Villa A, Nyitray AG, et al. The epidemiology of oral HPV infection among a multinational sample of healthy men. Cancer Epidemiol. Biomarkers Prev. **2011**; 20:172–182.

9. Vasiljević N, Wu K, Brentnall AR, et al. Absolute quantitation of DNA methylation of 28 candidate genes in prostate cancer using pyrosequencing. Dis. Markers **2011**; 30:151–161.

10. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology **1990**; 1:43–46.

11. D'Souza G, Clemens G, Strickler HD, et al. Long-term Persistence of Oral HPV Over 7 Years of Follow-up. JNCI Cancer Spectr **2020**; 4:pkaa047.

12. Kelly H, Benavente Y, Pavon MA, De Sanjose S, Mayaud P, Lorincz AT. Performance of DNA methylation assays for detection of high-grade cervical intraepithelial neoplasia (CIN2+): a systematic review and meta-analysis. Br. J. Cancer **2019**; 121:954–965.

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

Table 1: Trimmed mean differences and their 95% confidence intervals among study participants whose oral HPV 16 cleared at <12 months compared to those who persisted

Gene	CpG site	Cleared <12 months (N = 35)		HPV Persistence* (N = 14)		Trimmed Mean Difference with 95% Confidence Intervals (CI)	Wilcoxon test ***P***
		N-methylation zero n (%)	**Mean methylation**	**N methylation zero n (%)**	**Mean methylation**		
HPV16 E2	Pos 1	14 (40.0)	1.49	4 (28.6)	3.04	-2.02 (-3.17, 0.07)	0.59

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

	)					
Pos 2	22 (62.9)	0.92	8 (57.1)	2.37	-1.28 (-3.86, 0.96)	0.68
Pos 3	25 (71.4)	0.81	7 (50.0)	1.38	-1.17 (-1.58, 0.43)	0.30
Pos 4	17 (48.6)	1.82	1 (7.1)	4.46	**-3.46 (-4.21, -1.05)**	**0.02**
Pos 5	29 (82.9	0.69	8 (57.1)	1.36	-1.37 (-1.67, 0.33)	0.10

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

		)					
HPV16 L1	6389	11 (31.4)	20.99	2 (14.3)	23.11	-0.33 (-15.05, 10.81)	0.50
	6367	10 (28.6)	22.15	3 (21.4)	26.40	-0.62 (-18.20, 9.71)	0.47
HPV16 L2	4275	25 (71.4)	3.61	8 (57.1)	6.90	-1.13 (-9.26, 2.69)	0.33
	4268	31 (88.6	0.49	9 (64.3)	3.74	**-2.25 (-6.35, -0.16)**	**0.04**

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

	)					
4259	26 (74.3)	2.84	9 (64.3)	5.77	-1.08 (-8.56, 2.71)	0.45
4247	30 (85.7)	1.73	1 (92.9)	0.52	1.10 (-1.02, 3.44)	0.50
4238	30 (85.7)	1.50	12 (85.7)	3.35	-1.14 (-5.23, 1.53)	0.99

*We defined HPV persistence as positive at 6- and 12-month visits; or negative at 12th month but positive at 6th, 18th and 24 month visits (i.e., + - + +))

ACCEPTED

Copyright © 2023 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.