Oral Oncology 95 (2019) 120–126

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Salivary HPV DNA informs locoregional disease status in advanced HPV-associated oropharyngeal cancer

Glenn J. Hanna[a,*], Christie J. Lau[b], Umair Mahmood[a], Julianna G. Supplee[b], Abhishek R. Mogili[b], Robert I. Haddad[a], Pasi A. Jänne[a,b], Cloud P. Paweletz[b]

[a] Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, USA
[b] Robert and Renée Belfer Center for Applied Cancer Science, Dana-Farber Cancer Institute, Boston, USA

ARTICLE INFO

Keywords:
HPV
Oropharyngeal cancer
Saliva
DNA
Head and neck cancer
Biomarker

ABSTRACT

Objectives: Quantifying tumor DNA in tissue and circulating in blood permits high-quality molecular monitoring to detect and track cancer progression. Evaluating tumor DNA in both blood and saliva in human papillomavirus (HPV)-associated oropharyngeal cancer (OPC) could provide a non-invasive and clinically actionable method for real-time disease detection.

Methods: We previously validated an ultrasensitive droplet-digital (dd)PCR assay targeting the dominant high-risk HPV subtypes causally linked to OPC. Here we enrolled an observational cohort to evaluate the predictive and prognostic potential of paired plasma-salivary tumor DNA among 21 patients with advanced HPV + OPC.

Results: In patients with recurrent, persistent locoregional (LR) disease, median baseline normalized salivary HPV DNA was 10.9 copies/ng total DNA, nearly 20x higher compared with those with distant disease only ($p = 0.01$). A cutoff of 5 copies/ng yielded 87% sensitivity and 67% specificity for accurately predicting LR disease. Total tumor burden among those with LR disease strongly correlated with salivary HPV DNA levels ($R = 0.83$, $p = 0.02$). The rise and fall of salivary HPV DNA predicted treatment failure and response, respectively, in all patients with LR disease, and predated imaging findings. Among paired salivary-plasma (cell-free) cfDNA samples, only higher plasma HPV cfDNA levels were associated with poor outcomes ($p < 0.01$), suggesting that each bodily fluid provides unique information about HPV disease status.

Conclusions: Salivary HPV DNA provides valuable information about tumor burden and predicts treatment response in advanced HPV + OPC. Paired blood-saliva samples could be used to monitor HPV DNA with broad applications to inform diagnosis, prognosis, and surveillance in HPV-associated diseases.

Introduction

While decreasing tobacco use in the United States since the 1980 s has coincided with a decline in certain subtypes of head and neck cancer, human papillomavirus (HPV)-associated oropharyngeal cancers (OPCs) are increasing in incidence [1]. Representing a distinct biologic disease, HPV + OPC often portends an excellent prognosis in those patients without high-risk features (tobacco use or retropharyngeal adenopathy) with 5-year overall survival rates exceeding 80% [2,3]; and even in the setting of recurrence or advanced disease HPV + OPC portends better outcomes [4]. Physical or endoscopic examination, and radiographic techniques are currently the only available tools to monitor for disease response and recurrence, and there is no approved method of screening.

Blood-based or "liquid" biopsies are increasing in popularity as they provide a less invasive and cost-effective method with which to monitor the molecular features of cancer, as cell-free or circulating tumor DNA (cfDNA) is often present in blood and allows genotyping or detection of certain oncogenic mutations [5,6]. HPV + OPC is similar in that high-risk subtypes of HPV DNA integrate into host epithelial cells and produce viral specific oncoproteins that lead to carcinogenesis [7,8]. Therefore, platforms aimed at capturing and quantifying tumor shed HPV DNA in HPV-associated cancers are of interest given their widespread potential for clinical application.

Early approaches, including our own, focused on antibody detection of HPV early (E) oncogenic proteins and real-time or quantitative polymerase chain reaction (qPCR) to detect amplified HPV-specific sequences [9–11]. These methods are limited by their detection

* Corresponding author at: Department of Medical Oncology, Center for Head & Neck Oncology, Dana-Farber Cancer Institute, 450 Brookline Ave, Dana building, Room 2-140, Boston, MA 02115, USA.
E-mail address: glenn_hanna@dfci.harvard.edu (G.J. Hanna).

https://doi.org/10.1016/j.oraloncology.2019.06.019
Received 6 April 2019; Accepted 13 June 2019
Available online 20 June 2019
1368-8375/

capabilities and lack of specificity. We recently developed a droplet-digital (dd)PCR assay that permits ultrasensitive, quantitative, single-copy detection of high-risk HPV subtype DNA targets. We published data showing that plasma HPV cell-free DNA levels correlate with burden of disease, provide an early indication of response or resistance to cancer-directed therapies, and offer prognostic information in a pilot HPV + OPC population with advanced disease [12].

Beyond detection of plasma or HPV cfDNA in circulation, we hypothesized that these patients may have detectable and quantifiable HPV DNA in salivary secretions, representing tumor shed from the primary or local tumor source in the oropharynx. We sought to understand if salivary HPV DNA levels were a surrogate for disease burden or recapitulated disease response, in considering locoregional disease among HPV + cancer patients. To test these hypotheses, we enrolled an observational pilot cohort of advanced HPV + OPC patients and prospectively collected paired plasma and salivary HPV DNA samples over time to evaluate their individual and combined predictive or prognostic potential, building on our prior work. A saliva and blood-based assay to evaluate disease burden and response in HPV + oropharyngeal cancer could be transformative in screening, monitoring, or predicting disease relapse.

Methods

Study subjects

We enrolled 21 individuals with advanced or incurable, metastatic HPV + oropharyngeal cancer between July 2018 and March 2019. All patients consented to an existing institutional review board-approved protocol permitting collection of samples and review of demographic information (separate from any therapeutic protocol consent). Immunostaining for p16 (diffuse staining pattern > 70% considered positive) and in situ hybridization (ISH) or polymerase chain reaction (PCR) testing was required from all tumor samples prior to enrollment, either from the primary tumor or a metastatic site. Patient enrollment was open to those individuals undergoing active surveillance or any form of cancer-directed therapy.

Monitoring salivary and plasma HPV cfDNA

Subjects were prospectively enrolled on a rolling basis and their first blood and saliva sample collection was deemed time 0 (Supplemental Fig. 1). All plasma and saliva collections took place on the same day at each time point. Following time 0, subjects were followed over time and up to 6 additional paired collections (average 5.7 per patient) were arranged at a median 14-day interval during the observational period, over a maximum of 210 elapsed days (accounting for 116 total paired plasma and saliva samples, or 232 specimens). Patients therefore entered the study already on active surveillance or on an existing therapy, and based on their disease course, then changed treatments when deemed clinically necessary. When possible, plasma and salivary samples were coordinated with treatment or protocol visits and timed around restaging scans and/or changes in therapy. Plasma HPV cfDNA values (copies/mL plasma) and normalized salivary HPV DNA values (copies of HPV DNA/ng of total DNA, copies/ng) were then recorded and analyzed.

Multiplexed ddPCR for high-risk HPV subtypes

Whole blood samples were collected in EDTA vacutainer tubes and centrifuged for 10 min at 1500 g to isolate plasma, which was stored at −80 °C in crysostat tubes. cfDNA was extracted from 2 mL plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to manufacturer protocol. One to 3 mL of saliva was collected in 50 mL conical tubes, diluted in 4 mL PBS and centrifuged for 5 min at 1800 g. The resulting pellet was stored at −80 °C until subsequent DNA extraction, upon which the pellet was resuspended and purified using the QIAamp Blood and Tissue Kit (Qiagen) according to manufacturer protocol. Extracted plasma and salivary DNA was stored at −80 °C until further use.

We previously developed and validated a ddPCR assay for common high-risk HPV subtypes: 16, 18, 31, 33, and 45 [12]. Briefly, E7 genes from each high-risk HPV subtype were cloned into pUC57 plasmids (GenScript) as positive controls. Custom Taqman Copy Number Variation assays (Applied Biosystems) were designed to recognize each of the five unique HPV E7 sequences. Standard emulsification and ddPCR cycling conditions were maintained (10 min at 95 °C, followed by 40 cycles of a two-step thermal profile of 15 s at 94 °C denaturation and 60 s at 59 °C annealing, followed by a 10 °C hold). Finally, a 96-well plate was loaded and read by a QX100/QX200 Droplet Reader (Bio-Rad), and data was analyzed using QuantaSoft software (Bio-Rad). Of importance, since our multiplexed ddPCR design permits identification of multiple HPV subtypes in a single assay, reflex tumor testing to confirm the causal HPV variant was employed.

Specimens were assayed in technical triplicates and viral load was calculated as the mean of estimated target DNA concentration (copies/mL of plasma or copies/ng of total salivary DNA) across merged reactions. In order to control for volumetric variance across clinical saliva specimens, salivary HPV DNA viral load was normalized to total salivary DNA concentration as measured by fluorometric quantification using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) and scaled to units of copies/ng. Assay technicians were blinded to all clinical and outcome data.

Statistical analysis

Tumor burden was assessed using the largest lesion(s) (≥2 mm in any axis, up to three per organ, and up to five total per subject) for each participant, as previously described [13]. Lymph nodes with a short axis ≥ 8 mm were also considered evaluable. We then calculated the total tumor burden (TTB) per subject as the sum of the largest diameter of all detectable lesions on imaging (threshold of ≥ 2 mm, up to 5 sites) using restaging scans closest to the date of enrollment. Correlation coefficients were determined using Spearman's rho (ρ) to test strength of association. A Mann-Whitney *U* test was used to evaluate differences in DNA viral load by subgroups (such as those with locoregionally persistent disease with or without distant disease). A Kruskal-Wallis test or one-way ANOVA on ranks was used to compare HPV DNA viral levels by subsite of disease and cancer prognostic scores based on DNA values. A receiver operating characteristics (ROC) curve was formulated to assess the accuracy of normalized salivary HPV DNA in detecting locally recurrent or persistent oropharyngeal disease using the three normalized salivary DNA values timed closest to clinical and radiologic evaluations. A p-value < 0.05 (*) was considered statistically significant; two-sided. Data were analyzed using Stata 14.2 (StataCorp LP, College Station, TX, USA).

Results

Subject clinical characteristics

Twenty-one patients with incurable or metastatic HPV + OPC treated at our institution were enrolled. The cohort was comprised of almost all males (90.4%) with the majority being (≥10 pack-year) former smokers (11, 52.4%) (Table 1). Nearly all had evidence of primary oropharyngeal cancer and all had strong and diffuse p16 immunoreactivity with tumor HPV confirmatory testing. Fifteen (71.4%) had evidence of locoregionally recurrent or persistent disease in the head and neck, while 17 (80.9%) had distant disease (not necessarily mutually exclusive; six or 28.6% had only distant disease). All patients had previously received some combination of surgery, radiation, and/or systemic therapy prior to enrollment.

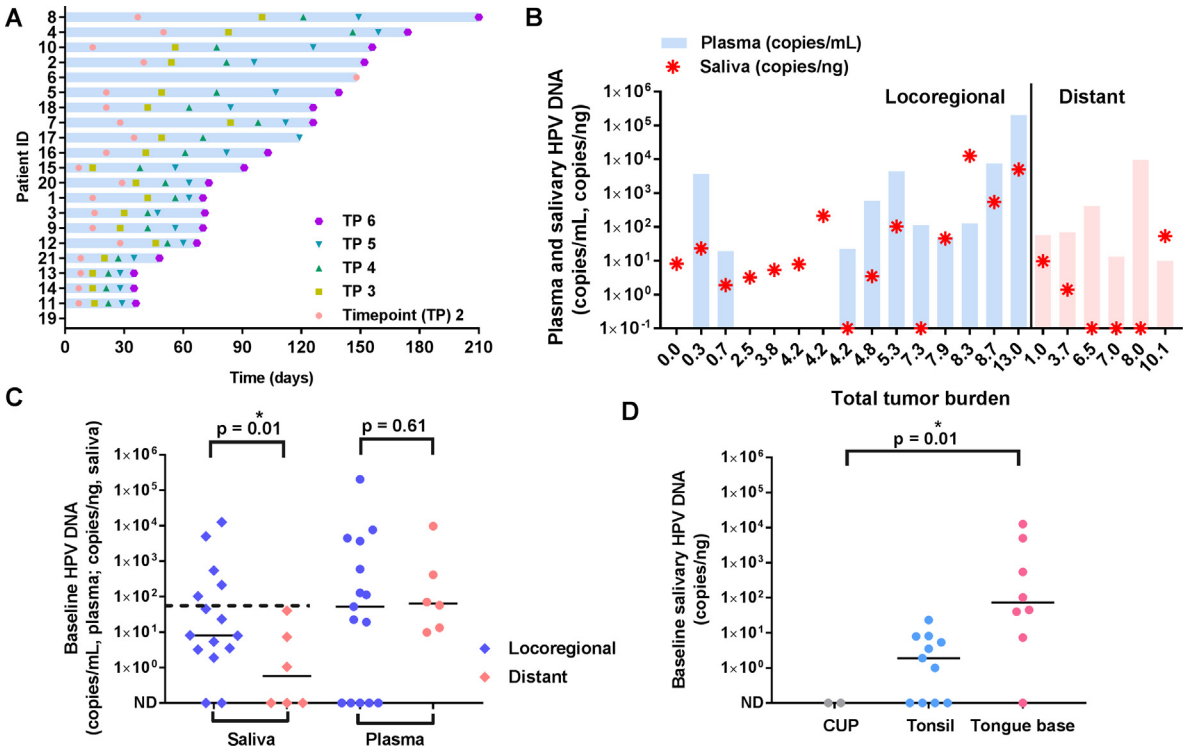

Fig. 1. (A) Graphically depicts the paired plasma and salivary collection timepoints (TP) for each participant over a maximum of 210 elapsed days, arranged in order of longer to shorter follow-up. (B) Logarithmic normalized, baseline salivary HPV and plasma HPV cell-free (cf)DNA values are shown corresponding to increasing tumor burden among each patient (n = 21), separated by those patients with locoregional disease (n = 15) and those with distant disease only (n = 6). (C) Baseline normalized salivary HPV and plasma HPV cfDNA values are compared in those subjects with locoregional and distant disease (not necessarily mutually exclusive) [Mann-Whitney test, two-sided; median depicted by the solid lines]. No patients with only distant disease had a salivary DNA level above 40 copies/ng (dotted line) at baseline. (D) Baseline normalized salivary HPV DNA values correlate to oropharyngeal subsites of disease [one-way ANOVA on ranks, two-sided; median depicted by the solid lines]. (*) $p < 0.05$.

Table 1
Patient demographics and clinical characteristics.

Characteristic	(%)[A], N = 21
Age (median, y)	57 (46–74)
Gender	
Male	19 (90.4)
Female	2 (9.5)
Smoking status	
Never or < 10 pack-year	10 (47.6)
Former (≥10 pack-year)	11 (52.4)
Current	0
ECOG[B] performance status	
0–1	20 (95.2)
2 or greater	1 (4.8)
Primary site of disease	
Base of tongue	11 (52.4)
Tonsil	8 (38.1)
Unknown	2 (9.5)
Initial staging at diagnosis	
Stage I, II	2 (9.5)
Stage III, IV	19 (90.4)
Initial treatment regimen	
Surgery + radiation	2 (9.5)
Surgery + CRT	2 (9.5)
Definitive CRT	16 (76.2)
Chemotherapy or immunotherapy	1 (4.8)

[A] Except for age.
[B] Eastern Cooperative Oncology Group; CRT = concurrent chemoradiation.

Establishing salivary HPV DNA detection

Fig. 1 depicts sample collection time point intervals during the observational period. First, we sought to understand the range of HPV DNA which could be detected in salivary secretions: median baseline normalized salivary HPV DNA was 10.6 copies/ng in saliva secretions, ranging from 0 to 12,656 copies/ng among the entire cohort. Fig. 1 depicts baseline, matched plasma-salivary HPV DNA values arranged by increasing tumor burden and degree of disease spread. In our subgroup with evidence of recurrent or persistent locoregional disease in the oropharynx and/or necks, median baseline normalized salivary HPV DNA was 10.9 copies/ng, or nearly twenty times higher compared with 0.6 copies/ng among those with distant disease only ($p = 0.01$). We compared baseline plasma and salivary HPV DNA values as this permitted correlation with recent clinical and radiographic findings at study entry. We did observe 2/15 (13.3%) cases where locoregional disease was present without detectable salivary HPV DNA at baseline; however, their primary or neck disease burden was low comparatively. Further, 1/6 (16.7%) patients had a baseline normalized salivary HPV DNA level of 40 copies/ng without clinical or radiographic evidence of locoregional disease.

Tumor burden and local disease subsite correlate with salivary HPV DNA

Next, we sought to understand whether quantitative tumor burden and disease site impacted normalized salivary HPV DNA or plasma HPV cfDNA levels. The median total tumor burden (TTB) for the entire cohort was 4.8 (range: 0–13). Median TTB was similar among those subjects with only distant vs. locoregional disease (6.8 vs. 4.2, $p = 0.56$). TTB among those patients with locoregional disease correlated strongly with salivary HPV DNA viral load ($R = 0.83$, $p = 0.02$) at

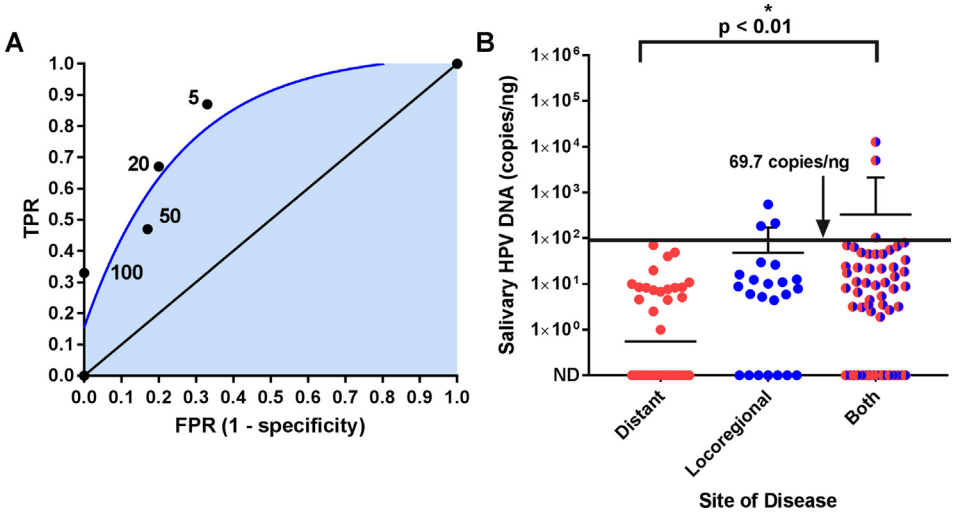

Fig. 2. (A) Receiver operator characteristics (ROC) curve to assess the predictive accuracy of normalized salivary HPV DNA values (expressed as copies/ng DNA) at different cutoffs (at 100, 50, 20, and 5 copies/ng) in diagnosing clinical or radiographic locoregional disease in advanced HPV + oropharyngeal cancer patients. TPR = true positive rate, FPR = false positive rate. (B) All logarithmic, normalized salivary HPV DNA values compared by site of disease [one-way ANOVA on ranks, two-sided; medians are shown by the short solid lines]. (*) $p < 0.05$. The horizontal solid line indicates the maximum salivary DNA value observed among patients with only distant disease (< 70 copies/ng DNA).

this cohort size, but not among those with distant disease only ($p = 0.21$). Further, plasma HPV cfDNA strongly correlated with TTB ($R = 0.79$, $p < 0.01$) among the entire cohort and with distant sites of disease (pulmonary vs. extra-pulmonary) ($p = 0.01$) (not shown). Moreover, when we considered specific subsites of oropharyngeal disease (e.g. tonsil or base of tongue), we observed a significant increase in salivary HPV DNA levels among base of tongue tumors (median: 73.9 copies/ng, $p = 0.01$). Baseline plasma HPV cfDNA values were similar among subjects regardless of site(s) of disease ($p = 0.61$).

Accuracy of salivary HPV DNA in predicting locoregional disease

We used these data to generate a ROC curve shown in Fig.2 which depicts the accuracy of predicting locoregional disease at four cutoffs; for example, using a cutoff of 5 copies/ng yielded a sensitivity of 87% and specificity 67%. Fig. 2 plots all data points for salivary HPV DNA among the cohort by disease specific site, showing that locoregional disease alone or in combination with distant disease corresponds to higher HPV DNA values over time ($p < 0.01$). We observed that in patients with only clinical or radiographically detectable distant disease, salivary HPV DNA values failed to exceed 70 copies/ng DNA at any time point during the study. Of note, plasma HPV cfDNA values among those with only locoregional disease never exceed 18.5 copies/mL of plasma at any time point during the observational period.

Salivary HPV DNA is a predictor of locoregional treatment response and prognosis

We followed participants for a median of 103 days (range: 36–210). Fig. 3 graphically depicts the trend in normalized salivary HPV DNA values over time, in order to understand the predictive potential of salivary HPV DNA in response to different therapies. While salivary HPV DNA levels appeared to fluctuate among individual participants at different time points, again we did not observe levels > 70 copies/ng among subjects with clinical or radiographic evidence of solely distant disease. We show that salivary HPV DNA levels decline in all patients with locoregional disease response while on treatment ($n = 6$). Similarly, those individuals who developed clinical evidence of locoregional recurrence while on study ($n = 4$) all demonstrated increasing salivary HPV DNA levels (and all eventually achieved salivary HPV DNA levels > 40 copies/ng). In most cases, the salivary HPV DNA levels increased or fell prior to confirmatory imaging or biopsy among the cohort (occurring a median of 5.6 days prior to clinical detection). We also observed rapid assay kinetics whereby those subjects who responded to locoregional cytotoxic therapy with or without radiation had a median decline in salivary HPV DNA level by an estimated 3.8% per day (range 1.9–20.3%).

We then used a previously validated prognostic scoring system (which has been used in advanced head and neck cancer patients) to understand the relationship between HPV DNA levels and survival [14]. As expected, a higher RMH (Royal Marsden Hospital) prognostic score (based on albumin, lactate dehydrogenase, and number of metastatic sites of disease) correlated with higher median plasma HPV cfDNA levels ($p < 0.01$), but this was not significant for normalized salivary HPV DNA levels.

Discussion

Detecting cell-free tumor DNA has proven to be a clinically meaningful surrogate for monitoring *EGFR*-mutated lung cancers [15,16], and there remains a strong interest in developing this application in other tumor types. HPV-associated cancers are unique in that tumor viral shed permits quantification and serial monitoring of HPV DNA in the bloodstream using ultrasensitive techniques, with the added potential of studying salivary secretions in oropharyngeal tumors causally linked to high-risk HPV subtypes. These methods hold significant clinical potential for early disease detection, genotyping, guiding treatment or monitoring response, and for minimal residual disease detection. We have previously shown that plasma HPV cfDNA can provide useful information about disease burden and treatment response in HPV + OPC [12] but further aimed to explore the utility of HPV DNA in oral secretions.

We demonstrate that salivary HPV DNA is detectable and quantifiable among an advanced HPV + OPC population. We normalized HPV DNA copy number in saliva given the volumetric differences among saliva samples using ng of total salivary DNA as our unit of comparison. Further, we established that levels of salivary DNA are over 20 times higher among patients with clinicoradiographic evidence of locoregional disease, compared with individuals who have only distant disease outside the head and neck. We sought to determine diagnostic test characteristics and showed that using a cutoff of 5 copies/ng in saliva achieved a true positive rate approaching 90% (false positive rate, 33% at this cutoff), with a specificity of 80% when the cutoff was increased to 20 copies/ng in saliva or greater. These data could therefore serve as a benchmark for future studies in HPV + OPC patients, whereby a cutoff of 5 copies/ng could be applied to screening exercises given the sensitivity of nearly 90%, but appreciating the risk of false positive results. Other recent studies have utilized saliva-based DNA collection methods and PCR technology to detect high-risk HPV DNA [17–21] (Supplemental Table 1), with comparable operating characteristics when obtained at diagnosis. Despite our ultrasensitive detection, our results could have been impacted by the acidic pH of saliva or a lack of rinsing

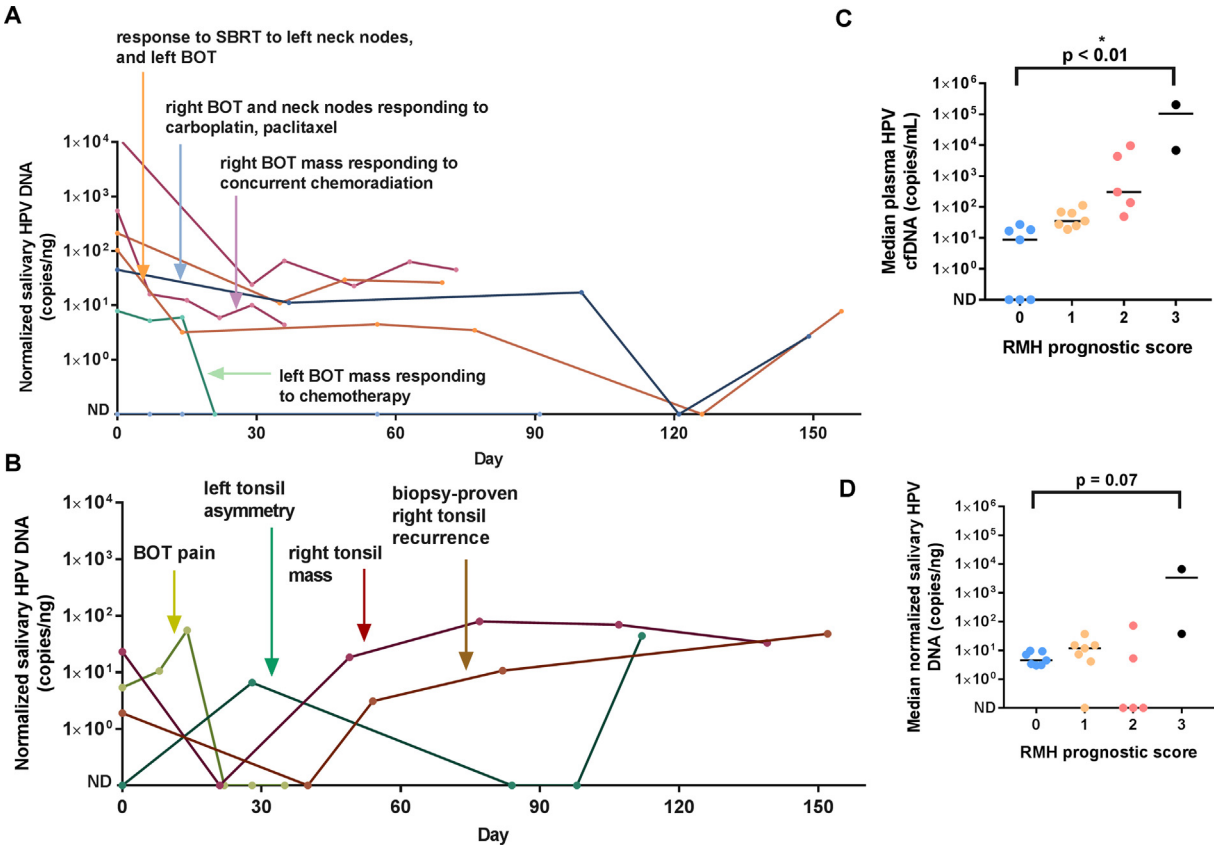

Fig. 3. (A) Normalized salivary HPV DNA levels decreasing over time in response to therapy (n = 6) and (B) increasing over time in patients with both biopsy-proven and clinical recurrence (n = 4) of disease. NED = no evidence of disease. (C) Median plasma HPV cell-free and (D) normalized salivary DNA values plotted by RMH (Royal Marsden Hospital) prognostic score [Kruskal-Wallis, two-sided; median shown by short solid lines].

solution to promote collection, behaviors just prior to sample collection (although our subjects were not allowed to brush their teeth, eat or drink anything 30 min prior), and our unique patient subgroup (many of whom experience prolonged xerostomia and hyposalivation from prior radiotherapy).

We show that greater tumor burden among our HPV + patients with locoregional oropharyngeal or neck disease resulted in higher detectable salivary HPV DNA levels at this cohort size. While prior studies have shown that quantitative circulating tumor DNA levels correlate with stage and tumor size among HPV + carcinomas [9,12], we are the first to report similar findings with salivary HPV DNA values in the context of locoregional disease. Additionally, those with base of tongue tumors had the highest levels of detectable salivary HPV DNA among our cohort. Of note, locoregional disease burden among tonsil and tongue base cases was similar (3.4 vs 4.6, p = 0.71). While speculative, the HPV virus can be encased in biofilms within the tonsil crypts [22] which may explain why viral shed is lower compared to base of tongue or lingual tonsil regions.

While three prior studies have monitored salivary HPV DNA before and after treatment in the curative HPV + OPC setting [17–19], we followed subjects with advanced HPV + disease for nearly a year to understand salivary HPV DNA trends. We included patients with either incurable locoregional disease, distant disease, or both. We show that salivary HPV DNA levels rise and fall among those with clinicoradiographic locoregional disease progression or response, respectively; and that changes in HPV DNA predated clinical disease detection in most cases. Salivary HPV DNA might provide complimentary information between restaging scans to inform treatment response and guide more timely therapeutic decision-making. We also highlight that none of the subjects with only distant disease had salivary HPV DNA levels above 70 copies/ng at any time point, but 91.3% (73/80 data points) of salivary HPV DNA values were below 70 copies/ng among those with known locoregional disease. Those individuals in our cohort with only distant disease may have detectable salivary HPV DNA resulting from subclinical HPV DNA shedding from the oropharynx. We attribute the modest fluctuations in HPV DNA values among our cohort to differences in disease kinetics, and variations in therapeutic potential or efficacy among this advanced cohort.

To our knowledge, few studies have reported on matched plasma-salivary HPV DNA values in HPV + OPC [18,23]. Ahn and colleagues evaluated those undergoing curative treatment and assessed only HPV16 disease with the aim of improving the sensitivity of HPV detection before and after treatment (the sensitivity of plasma HPV16 surveillance testing for recurrence outperformed salivary-based testing in their study, 55.1 vs. 18.8%). Wang, et al. investigated tumor-derived DNA among a diverse head and neck squamous cell carcinoma population, showing a high fraction of mutated tumor DNA in saliva (mean: 3.5%) among oral cavity cancers, specifically those that were localized. Among OPC, mutated tumor DNA detection rates were only 47%. Our viral detection rates were higher likely reflecting our probe sensitivity for high-risk HPV, which detected 86.7% of those with HPV + locoregional disease at baseline. They reported increased sensitivity when both plasma and saliva DNA detection was combined, and we too report 100% detection of HPV DNA when considering both bodily fluids in tandem.

As reported in a separate advanced HPV + OPC cohort [12], we previously showed that plasma HPV cfDNA values are positively correlated with overall tumor burden, but this correlation is also observed for locoregional disease burden when measuring salivary HPV DNA. Since our cohort continues to mature and all subjects are alive, we could not generate survival statistics, rather we utilized a validated cancer prognostic scoring system to show that only high plasma HPV

cfDNA values were linked to worse outcomes, as opposed to salivary levels. These findings were expected as pre-treatment serum HPV and Epstein-Barr virus (EBV) DNA values are strong predictors of outcomes in head and neck cancer [24,25].

Our study was limited by enrolling an observational cohort with a smaller sample size, but we attribute this to the relative infrequency of advanced HPV + OPC. We are currently investigating salivary and plasma HPV DNA monitoring approaches in other HPV + OPC populations, but we felt an advanced HPV + OPC population would provide valuable information about detection among those with locoregional or distant disease, DNA kinetics, and therapeutic response. Despite implementation of a standardized collection protocol, we observed variation in volume and content across salivary specimens. Similar investigations into oral sampling of cancer-associated HPV have circumvented this issue by focusing on binary detection status rather than viral load, using a standardized volume of an oral rinse [18,26], performing parallel next-generation sequencing (NGS) to quantify mutant allele fraction [23], or standardizing an acceptable volumetric range of salivary input for DNA extraction. In order to maximize translatability of oral liquid biopsies, we chose to normalize viral load (copies/volume salivary DNA) to the total concentration of salivary DNA (ng/uL) as measured by fluorometric analysis – a rapid and cost-effective strategy in comparison to NGS. While the concentration of plasma cfDNA is known to demonstrate a positive correlation with tumor burden [27,28], it remains unclear whether viral DNA demonstrates similar kinetics in both plasma and saliva.

In conclusion, we add to an existing small body of literature suggesting that novel ddPCR technologies have the potential to revolutionize clinical management in HPV-associated cancers, including OPC. We show that salivary HPV DNA may provide complimentary, yet specific information about the burden of disease and response to treatment among those with locoregional tumor confined to the oropharynx. These data warrant large-scale, prospective validation in the setting of clinical trials – research that could prove impactful in a wide range of HPV-associated cancers, which impacts upwards of 43,000 people each year in the United States [29].

Funding

This work was supported by the Expect Miracles Foundation to C.P.P.; and the American Cancer Society [grant number CRP-17-111-01-CDD to P.A.J.]; and the Robert A. and Renée E. Belfer Foundation.

Financial support

research support to institution from Bristol-Myers Squibb (BMS), EMD Serono; consulting honoraria from BMS, Maverick, Regeneron, Sanofi [GJH]; research support and consulting for BMS, Merck, Pfizer, Genentech; in addition to consulting for Nanobiotix, AstraZeneca, Bayer, LOXO, and GlaxoSmithKline; member of the NCCN head and neck cancer panel [RH]; receives commercial research grants from Astellas Pharmaceuticals, AstraZeneca, Daiichi Sankyo, Eli Lilly and Company, and PUMA, holds ownership interest (including patents) in Lab Corp, and is a consultant/advisory board member for ACEA Biosciences, Araxes Pharmaceuticals, Ariad Pharmaceuticals, AstraZeneca, Boehringer Ingelheim, Chugai Pharmaceuticals, Eli Lilly and Company, Ignyta, LOXO Oncology, Merrimack Pharmaceuticals, Pfizer, and Roche/Genentech [PAJ]; honorarium from AstraZeneca, BioRad. Scientific advisory board for DropWorks, Inc. [CP]. PAJ and CP are also co-inventors on a pending patent held by the Dana-Farber Cancer Institute (DFCI) for the use of EGFR cfDNA genotyping, and receive a share of post-market licensing revenue distributed by the DFCI. All remaining authors have declared no conflicts of interest.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.06.019.

References

[1] Vokes EE, Agrawal N, Seiwert TY. HPV-Associated Head and Neck Cancer. J Natl Cancer Inst 2015;107(12):djv344.
[2] O'Sullivan B, Huang SH, Su J, Garden AS, Sturgis EM, Dahlstrom K, et al. Development and validation of a staging system for HPV-related oropharyngeal cancer by the International Collaboration on Oropharyngeal cancer Network for Staging (ICON-S): a multicentre cohort study. Lancet Oncol 2016;17(4):440.
[3] Samuels SE, Vainshtein J, Spector ME, Ibrahim M, McHugh JB, Tao Y, et al. Impact of retropharyngeal adenopathy on distant control and survival in HPV-related oropharyngeal cancer treated with chemoradiotherapy. Radiother Oncol 2015;116(1):75–81.
[4] Fakhry C, Zhang Q, Nguyen-Tan PF, Rosenthal D, El-Naggar A, Garden AS, et al. Human papillomavirus and overall survival after progression of oropharyngeal squamous cell carcinoma. J Clin Oncol 2014;32(30):3365.
[5] Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. Cancer Discov 2014;4(6):650.
[6] Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res 2014;20(6):1698–705.
[7] Lechner M, Fenton TR. The genomics, epigenomics, and transcriptomics of HPV-associated oropharyngeal cancer-understanding the basis of a rapidly evolving disease. Adv Genet 2016;93:1–56.
[8] Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsagué X, Laporte L, et al. HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. Lancet Oncol 2014;15(12):1319–31.
[9] Jeannot E, Becette V, Campitelli M, Calméjane MA, Lappartient E, Ruff E, et al. Circulating human papillomavirus DNA detected using droplet digital PCR in the serum of patients diagnosed with early stage human papillomavirus-associated invasive carcinoma. J Pathol Clin Res 2016;2(4):201–9.
[10] Hanna GJ, Sridharan V, Margalit DN, La Follette SK, Chau NG, Rabinowits G, et al. Salivary and serum HPV antibody levels before and after definitive treatment in patients with oropharyngeal squamous cell carcinoma. Cancer Biomark 2017;19(2):129–36.
[11] Liang C, Marsit CJ, McClean MD, Nelson HH, Christensen BC, Haddad RI, et al. Biomarkers of HPV in head and neck squamous cell carcinoma. Cancer Res 2012;72(19):5004–13.
[12] Hanna GJ, Supplee JG, Kuang Y, Mahmood U, Lau CJ, Haddad RI, et al. Plasma HPV cell-free DNA monitoring in advanced HPV-associated oropharyngeal cancer. Ann Oncol 2018;29(9):1980–6.
[13] Aerts HJ, Velazquez ER, Leijenaar RT, Parmar C, Grossmann P, Carvalho S, et al. Decoding tumour phenotype by noninvasive imaging using a quantitative radiomics approach. Nat Commun 2014;3(5):4006.
[14] Garrido-Laguna I, Janku F, Vaklavas C, Falchook GS, Fu S, Hong DS, et al. Validation of the Royal Marsden Hospital prognostic score in patients treated in the Phase I clinical trials program at the MD Anderson cancer center. Cancer 2012;118(5):1422–8.
[15] Remon J, Caramella C, Jovelet C, Lacroix L, Lawson A, Smalley S, et al. Osimertinib benefit in EGFR-mutant NSCLC patients with T790M-mutation detected by circulating tumour DNA. Ann Oncol 2017;28(4):784–90.
[16] Sacher AG, Paweletz C, Dahlberg SE, Alden RS, O'Connell A, Feeney N, et al. Prospective validation of rapid plasma genotyping for the detection of EGFR and KRAS mutations in advanced lung cancer. JAMA Oncol 2016;2(8):1014–22.
[17] Chuang AY, Chuang TC, Chang S, Zhou S, Begum S, Westra WH, et al. Presence of HPV DNA in convalescent salivary rinses is an adverse prognostic marker in head and neck squamous cell carcinoma. Oral Oncol 2008;44(10):915–9.
[18] Ahn SM, Chan JY, Zhang Z, Wang H, Khan Z, Bishop JA, et al. Saliva and plasma quantitative polymerase chain reaction-based detection and surveillance of human papillomavirus-related head and neck cancer. JAMA Otolaryngol Head Neck Surg 2014;140(9):846–54.
[19] Rettig EM, Wentz A, Posner MR, Gross ND, Haddad RI, Gillison ML, et al. Prognostic implication of persistent human papillomavirus Type 16 DNA detection in oral rinses for human papillomavirus-related oropharyngeal carcinoma. JAMA Oncol 2015;1(7):907–15.
[20] Chai RC, Lim Y, Frazer IH, Wan Y, Perry C, Jones L, et al. A pilot study to compare the detection of HPV-16 biomarkers in salivary oral rinses with tumour p16INK4a expression in head and neck squamous cell carcinoma patients. BMC Cancer 2016;16(1). https://doi.org/10.1186/s12885-016-2217-1.
[21] Wasserman JK, Rourke R, Purgina B, Caulley L, Dimitroulakis J, et al. HPV DNA in saliva from patients with SCC of the head and neck is specific for p16-positive oropharyngeal tumours. J Otolaryngol Head Neck Surg 2017;46(1):3.
[22] Rieth KKS, Gill SR, Lott-Limbach AA, Merkley MA, Botero N, Allen PD, et al.

Prevalence of high-risk human papillomavirus in tonsil tissue in healthy adults and colocalization in biofilm of tonsillar crypts. JAMA Otolaryngol Head Neck Surg 2018;144(3):231–7.
[23] Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Sci Transl Med 2015;7(293):293ra104.
[24] Dahlstrom KR, Li G, Hussey CS, Vo JT, Wei Q, Zhao C, et al. Circulating human papillomavirus DNA as a marker for disease extent and recurrence among patients with oropharyngeal cancer. Cancer 2015;121(19):3455–64.
[25] Lo YM, Chan AT, Chan LY, Leung SF, Lam CW, Huang DP, et al. Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. Cancer Res 2000;60(24):6878–81.
[26] Parker KH, Pan Y, Yang Z, Giuliano AR, Pinto LA. Evaluation of HPV-16 and HPV-18 specific antibody measurements in saliva collected in oral rinses and merocel sponges. Vaccine 2018;36(19):2705–11.
[27] Lee Y, Park S, Kim WS, Lee JC, Jang SJ, Choi J, et al. Correlation between progression-free survival, tumor burden, and circulating tumor DNA in the initial diagnosis of advanced-stage EGFR-mutated non-small cell lung cancer. Thoracic Cancer 2018;9(9):1104–10.
[28] McEvoy AC, Warburton L, Al-Ogaili Z, Celliers L, Calapre L, Pereira MR, et al. Correlation between circulating tumour DNA and metabolic tumour burden in metastatic melanoma patients. BMC Cancer 2018;18(1):726.
[29] Centers for Disease Control and Prevention, Cancers Associated with Human Papillomavirus, United States—2011–2015. 2018(USCS data brief, no 4. Atlanta, GA: Centers for Disease Control and Prevention).