HHS Public Access

Author manuscript

Oral Oncol. Author manuscript; available in PMC 2022 April 01.

Published in final edited form as:

Oral Oncol. 2021 April ; 115: 104958. doi:10.1016/j.oraloncology.2020.104958.

cfDNA detection for HPV+ squamous cell carcinomas

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Abstract

High-risk human papillomavirus (HPV) is an etiologic factor in a spectrum of squamous cell carcinomas including anal, cervical, and oropharyngeal. HPV cell free DNA (cfDNA) is shed from the primary tumor into systemic circulation and can be detected using several platforms including quantitative PCR, digital droplet PCR, or next generation sequencing. Levels of HPV cfDNA at time of initial presentation is associated with known poor prognostic clinicopathologic variables, such as advanced stage and, locoregional and distant metastases. Moreover, longitudinal sampling revealed that persistent or increasing HPV cfDNA levels are indicative of treatment relapse and, in some studies, HPV cfDNA detection predicted treatment failures prior to routine post- treatment clinical imaging. A liquid biopsy platform using HPV cfDNA offers unique advantages over traditional approaches and may have clinical utility for detection of minimum residual disease, treatment response, and disease progression in patients with HPV+ cancers.

Keywords

HPV; liquid biopsy; ddPCR; early detection; biomarker; squamous; cell carcinoma

Introduction

Human papillomaviruses (HPVs) are 8-kb, non-enveloped, circular, double-stranded DNA viruses which preferentially infect basal cells of the squamous epithelium. There are more than 200 different HPV genotypes divided into low-risk, such as HPV6 and HPV11 which cause benign hyperproliferative diseases, and high-risk, such as HPV16 and HPV18, which drive tumorigenesis in distinct anatomical sites. HPV-associated squamous cell carcinomas

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Conflicts of Interest: None declared.

(HPV+ SCC) account for 4.5% of cancers worldwide, and predominantly occur in the cervix (SCCC), oropharynx (SCCOP) and anus (SCCA)[1]. SCCC is almost universally driven by HPV, whereas ~80% of SCCOP and SCCA cases are HPV+[1–3]. These HPV+ SCCs are primarily driven by HPV16, however, other high-risk HPV genotypes have been detected in these malignancies[2–5]. Technologies for early detection and, monitoring of treatment response and disease progression are needed to optimally manage HPV+ SCC patients.

The clinical benefit of screening tests for early detection of SCCC is unquestioned. Widespread utilization of Papanicolaou (Pap) tests, cytologic examination of cells from the uterine cervix, in the United States resulted in a dramatic reduction in the incidence of early stage SCCC between 1976 and 2009[6]. Alternatively, HPV testing can be used for early detection of cervical intraepithelial neoplasia (CIN) lesions; an advantage of this method is fewer false negatives than Pap smears but the trade-off is higher rates of false positive[7]. Current guidelines from the American College of Obstetrics and Gynecology and the US Preventive Services Task Force are: women aged 21 to 29 should have a Pap test every 3 years and women aged 30 and above should have a Pap test combined with HPV test every 5 years as long as the test results are normal. Similar to early detection of SCCC, Pap tests have been used to identify anal dysplastic lesions which are associated with HPV infection and increased risk of SCCA[8]. However, this cytology approach is not utilized in the oropharynx since HPV infection in this anatomical site is not associated with dysplasia[9]. Since SCCOP and SCCA are primarily driven by HPV, HPV testing may have utility to identify dysplastic disease and individuals at high-risk for these carcinomas. At this time, routine standard of care screening strategies for SCCOP and SCCA have not been introduced.

Treatment relapse and disease progression are recalcitrant challenges in the management of HPV+ SCC patients. In SCCOP, 10 to 25% of patients will develop disease recurrence or distance metastases within 5-years after chemoradiation therapy[10,11]. The National Comprehensive Cancer Network (NCCN) guidelines recommend a follow up every 1 to 3 months for the first year, every 2 to 6 months for the second year, every 4 to 8 months for years 3 to 5, and annually thereafter. However, computed tomography scans performed as post-definitive treatment follow-up can miss residual or recurrent disease due to resolution limitations and furthermore, it may be challenging to discern post-treatment inflammation and scar tissue from tumor. Regarding SCCC, 15% of patients relapse within the 2-years after treatment completion. The 2013 American College of Obstetricians and Gynecologists (ACOG) recommends a follow up visits at 12 and 24 months during which cytology should be performed, however, this is a highly invasive and uncomfortable procedure. In case of SCCA, 20–25% of HPV+ cases develop local recurrence within 3 years after surgery and/or chemoradiation[12]. The American Society of Colon and Rectal Surgeons (ASCRS) suggest that surveillance should be undertaken every 3–6 months for the first 24 months. Each patient’s visit includes a digital rectal examination (DRE), anoscopy, inguinal lymph node palpation, and thoracoabdominal CT scan. These procedures are resource-intensive, invasive, and may require multiple visits.

Due to these challenges, there is a need to develop technologies to augment standard of care diagnostic and imaging approaches to optimally manage HPV+ SCC patients. In this review,

we discussed the application of HPV cfDNA as a liquid biopsy platform for disease detection in HPV+ SCC.

Liquid biopsy

Liquid biopsy is the sampling and analysis of specific bodily fluids to assist clinicians in patient management. In contrast to tissue biopsies, liquid biopsies are safer, less invasive, and quicker to obtain[13–15]. Blood is one of the most commonly used source for liquid biopsies[16]. Tumors shed circulating tumor cells (CTCs), which are found in circulation as single cells or in clusters[17]. A small subset of CTCs have the ability to survive in plasma, migrate and invade to distant sites thus, CTCs may be the tumor cells responsible for disease progression and distant metastasis[17,18]. Extracellular vesicles (EVs) are abundant in blood and carry molecules such as proteins, lipids, microRNAs, mRNAs, long non-coding RNA, from a donor cell to a recipient cell[19,20]. Studies show elevated levels of EVs in the serum of cancer patients compared to healthy subjects[21,22]. EVs are secreted by tumor cells into the microenvironment, especially under hypoxic conditions, and carrying pro-migratory and inflammatory signals to drive tumorigenesis, tumor invasion, and metastasis,[23–29] in part through modulation of immune cells, such as CD8+ T cells[30]. Cell-free DNA (cfDNA) was originally discovered by Mandel and Métais in the blood of healthy individuals in 1948[31]. When released from tumor or CTCs[16,32–34], cfDNA are often referred to as circulating tumor DNA (ctDNA). cfDNA are more fragmented, typically 160–180 basepairs, in cancer patients than in healthy individuals[35,36]. The half-life of cfDNA in plasma is estimated at 114 minutes; this characteristic of cfDNA allows for longitudinal sampling to monitor disease burden over time[37–39]. Compared to the other liquid biopsy constituents, cfDNA offers several advantages including ease of isolation and storage, and robust detection sensitivity[40,41].

Collection, detection, and quantification of cfDNA

cfDNA is not stable in whole blood and, therefore, isolate of cfDNA should be performed within two hours of blood collection. The National Cancer Institute (NCI) published a Biospecimen Evidence-Based Practices (BEBP) on March 2020 in order to standardize cfDNA collection and processing, between organizations and institutions[42]. For blood collection, Streck BCT, PAXgene cfDNA, Roche cfDNA, CellSave, or Blood Exo DNA ProTeck collection tubes were highly recommended to ensure cfDNA stability.

Three primary cfDNA detection platforms have been developed: quantitative PCR (qPCR), droplet digital PCR (ddPCR), and next generation sequencing (NGS). The limit of detection of cfDNA using qPCR is at the picomolar range and may not have the sensitivity for utility in low cfDNA settings. ddPCR is a more recent technology that offers direct and independent quantification of DNA, including cfDNA, without the need for a housekeeping gene control. ddPCR is more sensitive and reproducible than qPCR, and allows absolute quantification of cfDNA present in a sample; the detection threshold can be as low as a single copy per milliliter of DNA. The strength of ddPCR resides in the fractionation of a sample into thousands of droplets containing DNA, which are then individually amplified resulting in high detection sensitivity and specificity. A third approach, NGS, is the most

expensive and has been used to sequence cfDNA globally or in part using targeted gene capture. NGS is more time and resource intensive than ddPCR; actionable results using ddPCR can be obtained within a day, whereas, NGS data may take weeks to materialize.

Clinical applications

HPV cfDNA detection have been studied in several clinical settings: at time of diagnosis for prognosis and, during and after treatment for disease monitoring. Numerous studies in the past five years have demonstrated that HPV cfDNA is detectable in the plasma or serum of patients with HPV+ SCCs, with multiple studies achieving greater than 90% sensitivity (Table 1)[43–49]. Baseline HPV cfDNA levels correlate with clinical features and patient outcomes in SCCA, SCCC, and SCCOP[48–55]. Work in these three malignancies revealed that HPV cfDNA levels tend to be lower in SCC patients with lower N stage or tumor burden. SCCC patients with ≥20 HPV cfDNA positive droplets at baseline using ddPCR showed an increased relative risk of disease progression (RR = 1.69, p=0.030) and mortality (RR = 1.70, p=0.007)[55]. In another study, baseline level of ≥3 HPV cfDNA positive droplets was not predictive of progression free survival in SCCA patients; however, there was a significant association (HR = 2.1, p=0.04) between low HPV cfDNA levels at baseline and longer progression free survival when data were analyzed using a receiver operating characteristic curve[44]. In contrast, work by Cabel et al. [2018] did not show a relationship between baseline HPV cfDNA levels using ddPCR and patient outcomes in SCCA. In agreement, using a qPCR approach, HPV+ SCCOP patients with detectable HPV cfDNA had similar progression-free survival to patients with undetectable HPV cfDNA[50]. Another group [2016] used NGS to demonstrate the feasibility of detecting host-viral DNA integration patterns in cfDNA from SCCC patients. These integration patterns were classified based on the number, position, and orientation of the junctions between viral and human genomic DNA[46]. There was a correlation between viral load and the integration pattern, with episomal HPV having the highest viral load and those with only two junctions having the lowest viral load. However, integration patterns did not show a significant impact on patient outcomes[46]. Based on these studies, there is no consensus on whether baseline cfDNA levels can be utilized as a prognostic biomarker in HPV+ SCC. However, key experimental differences, such as HPV probe design and treatment modalities, preclude direct comparison between these published studies. Additional work using a standardized HPV cfDNA detection platform in an uniformly-treated patient population is needed to conclusively determine the value of baseline cfDNA as a prognostic biomarker in HPV+ SCC.

At this time, the most promising clinical application for HPV cfDNA detection is monitoring treatment response and disease progression. In HPV+ SCCOP, post-treatment levels of HPV cfDNA, assessed using plasma and saliva, were strongly predictive of recurrence free survival (sensitivity: 69.5%; specificity: 90.7%; HR, 10.5; 95% CI, 2.84–57.10 [$P < .001$] [56]. In this study, the sensitivity of HPV16 detection within 3-years post-treatment was 18.8% with saliva, 55.1% with plasma, and 69.5% with combined plasma and saliva. Recent work by Nguyen et al. [2020] using ddPCR showed that HPV cfDNA was more sensitive than HPV cfRNA and EV. HPV cfDNA levels decreased after treatment in all cases and was undetectable after treatment in 81% patients without metastatic disease, most of whom

received chemoradiation[40]. Other studies using ddPCR have confirmed these results and showed that detection of HPV cfDNA at the end of chemoradiation is associated with worse outcomes, including shorter progression free and overall survival in SCCOP, as well as in SCCC and SCCA[44,48,49,54]. In addition to its use with patients receiving chemoradiation, the complete and persistent clearance of HPV cfDNA post-treatment in SCCC patients treated with tumor-infiltrating lymphocytes immunotherapy was able to predict the response to therapy[45]. In patients with recurrent HPV+ SCCOP, HPV cfDNA levels correlated with recurrence location; locoregional recurrences had the lowest levels whereas pulmonary and extra-pulmonary metastasis had medium and high HPV cfDNA levels, respectively[57]. In addition, the median level of HPV cfDNA at all of the measured time points was predictive of overall survival[57].

Integration of HPV cfDNA sampling in the clinical management of SCCOP is an area of active investigation. In a cohort of SCCOP patients, HPV cfDNA detection and standard imaging were performed 3-months post-treatment, and 28% (19/66) of these patients showed discrepant results between these two modalities. Presence of HPV cfDNA at the 12 week timepoint predicted residual disease at 9 months post-treatment with a positive predictive value (PPV) of 83% and a negative predictive value (NPV) of 100%[58]. This is in contrast to the imaging performed at twelve weeks which had a PPV of 17.4% and an NPV of 100%. Performance of HPV cfDNA detection is at least competitive and perhaps better than standard imaging, with PPV ranging between 8–90% from multiple HPV+ SCCOP[59–61]. Another HPV+ SCCOP study used the presence of two consecutive HPV cfDNA positive samples as an strategy to limited false positives during transitive HPV cfDNA spikes, and revealed a PPV of 94% and NPV of 100%[62]. Moreover, HPV cfDNA detection offered a time advantage and predicted progression 0.37–12.9 (median 3.9) months prior to biopsy confirmation.

NGS has also been used to assess HPV cfDNA levels in response to definitive treatment[47]. Lee et al. [2017] observed perfect agreement in HPV E7 cfDNA detection with NGS and qPCR in a cohort of advanced SCCOP patients treated with chemoradiation. The study had 37 patients with confirmed HPV+ disease as well as a complete set of serial plasma samples which were divided into test (27) and validation (10) cohorts. There was 84% agreement between the response as assessed by ^{18}F-FDG PET-CT and HPV cfDNA at 12 weeks post-treatment. Thirty of 37 patients had complete radiological response and were below threshold levels of HPV cfDNA. A further six of 37 patients had increased ^{18}F-FDG uptake at the primary site or cervical lymph nodes, but were negative for HPV cfDNA at 12 weeks post-treatment. Subsequent biopsies, however, showed no evidence of residual disease which was in agreement with the assessment based on HPV cfDNA levels. The remaining patient had an HPV+ liver metastasis that was observed through both ^{18}F-FDG PET-CT and HPV cfDNA levels. This study, while limited in sample size, provides initial evidence that HPV cfDNA has a better positive predictive value of treatment response and disease progression when compared to standard imaging. A recognized advantage of NGS is signal sensitivity, however, in this study with stage III/IV patients, qPCR was as robust as NGS at detecting HPV cfDNA. This observation argues that PCR-based platforms offer sufficient sensitivity in advance stage disease, but comparative NGS performance in early stage disease remains to be examined. Since PCR approaches are less resource intensive than NGS, PCR-based

approaches for HPV cfDNA detection, in particular ddPCR, should be prioritized in future studies for treatment response and prognosis.

Conclusion

Treatment failure and disease progression remain recalcitrant challenges in HPV+ SCC despite advances in multi-disciplinary treatment modalities. Development of non-invasive assays to monitor treatment response in a longitudinal manner will detect disease progression in real-time and be a value-added tool for clinicians to optimally manage HPV+ SCC patients. Accumulating evidence supports HPV cfDNA as a blood biopsy platform to monitor treatment response in the setting of HPV+ malignancies. Compared to standard of care post-treatment imaging, HPV cfDNA detection offers several advantages, including improved sensitivity and the opportunity for serial sampling during treatment and follow-up periods.

As the field of HPV cfDNA detection continues to evolve toward ddPCR, there is a need to develop standard operating procedures (SOPs) for blood collection, cfDNA isolation, HPV probe design, and threshold determination. The National Cancer Institute published a SOP for blood collection and cfDNA processing to set a uniform protocol for cfDNA detection. At this time, there has been limited efforts to set field standards for probe design and signal threshold. ddPCR probes used to detect HPV cfDNA are varied in sequence and, most often, unique amongst various research groups. Preference has been to target the E7 viral oncogene alone or in tandem with the E6 viral oncogene. Based on our experience, signal amplitude and sensitivity for HPVE6 cfDNA detection is appreciably higher than HPVE7 cfDNA (unpublished data). However, it is unclear if this is a consequence of increased HPVE6 cfDNA levels, probe design characteristics, or a combination of these two possibilities. Another variable that remains to be standardized is threshold determination. Most research groups set a minimum number of positive droplets or absolute concentration as the threshold. However, this variable is likely driven by specific probes and instruments. Further work to address these questions will result in a set of probes with threshold cut-offs for HPV cfDNA detection with optimal sensitivity and specificity.

HPV16 is the predominant high-risk genotype that drives HPV+ malignancies. Unsurprisingly, ddPCR probes to detect HPV cfDNA have largely been designed against HPV16 in a majority of studies to date. Non-HPV16 genotypes were reported to have an aggressive clinical course in SCCC and SCCOP and thus, may be responsible for an inordinate number of treatment failures[4,63–65]. Therefore, development of probes to target non-HPV16 genotypes is a clinical need that has yet to be fully explored. Since the distribution of non-HPV16 genotypes in SCCC and SCCOP is dependent on geographical location, the spectrum of non-HPV16 genotypes necessary for a comprehensive HPV cfDNA detection assay remains an open question. Hybrid Capture 2, which detects HPV16 and twelve other high-risk HPV genotypes, is the only FDA approved HPV test for women with abnormal Pap smears. Development of a multi-HPV genotype ddPCR platform to mirror the Hybrid Capture 2 assay would be a good first step and move the field forward.

Cancer prevention is, perhaps, the most impactful but also, the most challenging strategy to develop and implement to the general population. Screening assays to identify women at high risk for SCCC have been successfully implemented, however, these diagnostic tools are not available for SCCA or SCCOP. HPV cfDNA have been detected in early stage disease and post-treatment disease progression suggesting that this platform may have sufficient sensitivity to be utilized as a screening assay. Indeed, HPV cfDNA was detected in women with low grade or precancerous cervical dysplasia[66]. An important question that still needs to be addressed is whether patients with active HPV infections will shed fragmented HPV cfDNA in circulation resulting in a false positive signal. Individuals with active infection may have positive HPV cfDNA but this will likely be a transient event and as the infection is cleared, the expectation is that the initial positive test will transition to a negative test. Therefore, one approach to mitigate this issue is to perform follow-up testing in individuals with an initial positive test.

Liquid biopsy for HPV cfDNA detection has clear value for monitoring treatment response and disease progression in HPV+ SCC patients. ddPCR is the platform of choice due to cost and resource efficiency, ease of use, and expedient turnaround time for test results. Additional work to optimize and standardize HPV cfDNA isolation and detection is needed to continue to push this field forward.

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- Development of non-invasive assays to monitor treatment response and disease progression is a critical need to optimally manage HPV+ carcinoma patients.
- HPV cfDNA detection using ddPCR has emerged as the liquid biopsy platform of choice in the field.

Table 1:

HPV cfDNA detection in SCCOP.

Author and year	Source	Detection	Genes	HPV subtype	Baseline Sensitivity
Chera et al. 2020	Plasma	ddPCR	E6,E7	Panel	--
Nguyen et al. 2020	Plasma & EV	ddPCR	E7	16	91% & 42%
Rutkowski et al. 2020	Plasma	qPCR	E6, E7	16	--
Damerla et al. 2019	Plasma	ddPCR	E6, E7	16, 33	95.6%
Chera et al. 2019	Plasma	ddPCR	E7	Panel	89%
Hanna et al. 2018	Plasma	ddPCR	E7	Panel	--
Lee et al. 2017	Plasma	NGS	E7	16	95.7%
Mazurek et al. 2016	Plasma	qPCR	E6, E7	16, 18	--
Jeannot et al. 2016	Plasma and serum	ddPCR	E7	16, 18	100%
Dahlstrom et al. 2015	Serum	qPCR	E6, E7	16	60.5%
Wang et al. 2015	Saliva and plasma	PCR	E7	16, 18	86%
Ahn et al. 2014	Saliva and plasma	qPCR	E6, E7	16	76.1%

Table 2:

HPV cfDNA detection in SCCA.

Author and year	Source	Detection	Genes	HPV subtype	Baseline Sensitivity
Damerla et al. 2019	Plasma	ddPCR	E6, E7	16, 33	87.5%
Bernard-Tessier et al. 2018	Serum	ddPCR	E7	16	91.1%
Cabel et al. 2018	Plasma and serum	ddPCR	E7	16, 18	88%
Jeannot et al. 2016	Plasma and serum	ddPCR	E7	16, 18	93%

Table 3:

HPV cfDNA detection in SCCC and cervical dysplasia.

Author and year	Source	Detection	Genes	HPV subtype	Baseline Sensitivity
Cheung et al. 2019	Plasma	ddPCR	E7, L1	16, 18	61.6%
Han et al. 2018	Plasma	ddPCR	E6, E7	Panel	100%
Cocuzza et al. 2017	Plasma	qPCR	E1, E2	Panel	34.2%
Kang et al. 2017	Serum	ddPCR	E7	16, 18	90.5%
Holmes et al. 2016	Plasma and serum	NGS	Whole	Panel	100%
Jeannot et al. 2016	Plasma and serum	ddPCR	E7	16, 18	83%