

# Chapter 18

## Targeting Molecular Residual Disease Using Novel Technologies and Clinical Trials Design in Head and Neck Squamous Cell Cancer



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### Introduction

Despite therapeutic advances in locoregionally advanced head and neck squamous cell carcinoma (LA-HNSCC), there are patient subsets who remain at risk for disease relapse and death from their malignancies. For instance, the five-year overall survival (OS) of patients with stage III human papillomavirus (HPV)-positive oropharyngeal cancer, classified by the 8th edition of the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) criteria, is about 55–60% [1]; whereas the three-year OS for HPV-negative oropharyngeal cancer is about 46% [2]. Patients with LA-HNSCC who develop clinical or radiological disease progression or relapse have limited curative options if salvage treatments are not possible. Although the immuno-oncology era has led to long-term survival in a small proportion of patients with recurrent or metastatic HNSCC [3], this only constitutes an incremental advancement in the field. In recent years, emerging technology has enabled the detection of microscopic quantities of nucleic acids (i.e. DNA, RNA), proteins, metabolites and other molecules secreted or shed by tumor cells into the blood stream. Such techniques, collectively referred to as “liquid biopsy”, have garnered increasing attention due to their potential to detect the presence of cancer and associated molecular changes at a microscopic level [4].

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## Liquid Biopsy and Molecular Residual Disease

There are multiple clinical applications of liquid biopsy to measure circulating tumor DNA (ctDNA) in body fluids, most frequently in plasma. These applications include the early detection of cancer before it becomes macroscopically visible; the assessment of molecular residual disease (MRD) after definitive treatment; the monitoring of response to treatment; and the evaluation of emerging resistance mechanisms. In all of these applications, the overarching hypothesis is that molecular detection of ctDNA precedes the clinical event (i.e. the development of cancer, clinical relapse, antitumor response, disease progression and resistance development, respectively). In this chapter, the focus will be on MRD, which describes the state post-definitive treatment such as surgery or (chemo)radiotherapy for LA-HNSCC in which conventional investigations such as physical examination and radiological imaging are unable to diagnose the presence of cancer, but residual disease is detectable by the presence of cancer-derived molecular biomarkers, using highly sensitive and specific assays [4].

### Assays for MRD Detection

The detection of MRD can be performed using different types of ctDNA assays. Mutation-based assays rely on the detection of cell-free DNA (cfDNA) molecules that bear genomic alterations, suggesting that their source is likely from cancer cells rather than normal cells. Bespoke (i.e. personalized), mutation-based, tumor-informed assays rely on whole genome or exome sequencing of tumor tissue from which a limited number of somatic variants is selected based on proprietary algorithms to create patient-specific panels. These personalized panels are then used to track such variants in plasma samples of patients to assess quantitative changes in variant allele frequencies (VAF) or in number of mutant molecules per milliliter. There are also fixed gene panels that are not derived from next generation sequencing (NGS) of patients' tumor tissue, which propose to have faster turnaround time but harbor the risk of missing relevant aberrations which may thus lower their sensitivity and specificity [5]. In addition to mutation-based ctDNA assays, other cell-free DNA analyses may be utilized to monitor for MRD, such as viral sequences in the case of HPV-positive oropharyngeal cancer. Tumor-tissue modified viral DNA (TTMV) using a validated digital droplet PCR-based assay, can distinguish tumor-derived viral DNA from non-cancer associated sources of HPV DNA [6]. Methylated cfDNA analysis is another emerging method that does not rely on the detection of specific somatic mutations, but is based on the identification of unique DNA methylation patterns in different tumor types, which are also distinct from those found in normal tissues [7]. Table 18.1 summarizes the different assays that have evaluated MRD in HNSCC and their advantages and disadvantages.

**Table 18.1** Circulating tumor DNA assays applied in the molecular residual disease setting in head and neck squamous cell cancer

Analysis	Assay	Pros	Cons	Evidence
HPV DNA	Quantitative PCR	Low cost and easy logistics High sensitivity in saliva	Low sensitivity in plasma Only for a specific serotype (HPV DNA 16)	Ahn et al. [8]
	Tumor tissue modified viral (TTMV) HPV DNA (digital PCR)	Tumor specific HPV-DNA High specificity High sensitivity Able to detect different strains	Not able to detect <1 copy per mL HPV DNA Only gives quantitative information about HPV DNA	Chera et al. [10], Berger et al. [11]
	HPV sequencing (HPV-seq)	Highly sensitive (limit of detection <1 copy) Provides qualitative information (discriminates serotype, maps location along the HPV genome and provides fragment length)	High cost Further validation in oropharyngeal cancer is needed	Leung et al. [14]
Mutation ctDNA	Bespoke informed ctDNA	Personalized assay for each patient Highly sensitivity and specificity Validated in other tumor types	Requires tumor availability for sequencing High cost Long turnaround time for the first sample	Flach et al. [21], Flach et al. [22]
	Tumor naïve fixed gene panels	No need for tumor sample (naïve) Short turnaround time	Less evidence currently available to confirm clinical utility No consistent data in follow up/surveillance	Burgener et al. [23], Chikuie et al. [24]
Methylated cfDNA	cfMeDIP-seq	Low amount of cfDNA (<10 ng) needed Do not rely on tumor availability Presence of mutations in tumor not needed	Not validated in large cohorts Complex bioinformatic process	Burgener et al. [23]

## Evaluation of MRD in HNSCC

### *HPV DNA*

The evaluation of MRD using HPV DNA in patients with HPV-positive oropharyngeal carcinoma has taken the lead in HNSCC, in the context of malignancies driven by this virus. Different assays have been tested to detect and track viral HPV DNA in plasma and saliva. Quantitative PCR (qPCR) for HPV serotype 16 was initially assessed in a retrospective cohort of 93 patients with locally advanced oropharyngeal carcinoma [8]. The presence of HPV DNA in saliva was predictive of recurrence and survival. However, HPV DNA detection in plasma during follow-up was not a predictive biomarker by itself. The combination of the presence of HPV DNA in both plasma and saliva was 90.7% specific and 69.5% sensitive in predicting recurrence within 3 years. The most contemporary validated assay for HPV DNA detection in HPV-positive HNSCC is TTMV. Sensitivity and specificity of this assay in plasma are 89% and 97% respectively; these values are significantly higher than previously reported qPCR assays [9]. This assay evaluates amplicons within the E6 and E7 genes for HPV strain 16 and E7 gene for strains 18, 31, and 33 using digital droplet PCR. Therefore, it is able to detect different viral genotypes. Moreover, it is considered to be a measurement of tumor specific HPV DNA as there is a high correlation between tumor and plasma HPV DNA as previously mentioned. This assay has prospectively been validated as a biomarker of MRD in a study comprising 115 patients with stage I-III p16-positive oropharyngeal carcinoma treated with definitive (chemo)radiotherapy [10]. Plasma samples were collected at different time points during follow-up starting at 6 months after the end of definitive therapy. The majority of patients (75%) did not demonstrate detectable ctDNA during follow-up and all these patients were free of recurrence after a median follow-up duration of 23 months (negative predictive value (NPV) = 100%). On the other hand, two consecutive positive detections of HPV DNA had a positive predictive value (PPV) of 94% for locoregional or distant recurrence. A transitory spike in HPV DNA was seen in some patients (less than 10% of patients) but those with spontaneous clearance in the next time point were also free of recurrence. Median lead time between HPV DNA detection and biopsy proven recurrence was 3.9 months (0.37–12.9). Therefore, it may be useful in the MRD setting to anticipate clinical progression. This assay has further been validated in a retrospective multicenter cohort of 1076 patients with non-metastatic HPV-driven oropharyngeal cancer treated with any definitive treatment [11]. HPV DNA was detected during surveillance in 80 patients (7.4%), 21 of them have active disease present concurrently at the sample collection time point. Among the remaining 59 patients, 55 patients (93%) were proven later to have recurrent disease. In contrast, only around 5% of patients with no detection of HPV DNA in the follow-up period showed recurrence any time later. Overall, in this cohort the PPV and NPV of a single TTMV test performed 3 months or later after the end of definitive treatment was 95% for both parameters, similar to the previous study. NRG-HN002 study, which evaluated a cisplatin-sparing approach in low risk p16-positive oropharyngeal cancer, has

reported recently correlative study results of its HPV DNA analysis. TTMV detection between 2 weeks to 1 month after definitive (chemo)radiation treatment showed a NPV of 95% for 2-year locoregional failure (LRF) and 93.3% for progression-free survival (PFS). When clearance (>95% reduction from baseline) was considered, NPV was 94.3 and 92.7% for 2-year LRF and PFS, respectively [12]. All these results support the potential value of HPV DNA in follow-up of MRD, opening the door to incorporate this biomarker into the surveillance strategy of HPV-positive oropharyngeal tumors.

Other assays to detect HPV DNA using an NGS approach are under evaluation in the MRD setting, one such assay involves viral genome hybrid-capture sequencing [13]. This is the case of HPV sequencing (HPV-seq) that can provide both quantitative and qualitative information regarding the sequenced cfDNA fragments. This assay has been validated in a study involving preclinical models and plasma samples from patients with cervical and oropharynx cancers driven by HPV [14]. The lower limit of detection (LLoD) has been established as less than 1 copy per milliliter enabling the detection of HPV DNA in low burden disease such as MRD. A very high correlation between HPV-seq and digital PCR (gold standard) was seen in patients with detectable HPV DNA. Moreover, some patients with undetectable HPV DNA using digital PCR were found to have detectable viral genome using HPV-seq, due to the lower LLoD of the latter assay. In this study, detection of HPV DNA using HPV-seq at the end of chemoradiation in patients with cervical cancer was associated with shorter PFS. This assay showed a sensitivity of 100% and a specificity of 67% for disease recurrence in cervical cancer. Further validation is being carried out during follow-up in patients with oropharyngeal carcinoma.

### ***Mutation-Based ctDNA***

Mutation-based ctDNA has been widely used in solid tumors from fixed gene panels to personalized (or bespoke) assays. In the MRD setting, bespoke mutation-based ctDNA has emerged as one of the most promising tools in different tumor types such as colorectal [15, 16], breast [17, 18], lung [19] and bladder cancers [20]. There are some recent encouraging findings suggesting that this approach may also be applicable in HNSCC. The LIONESS study evaluated MRD using a bespoke ctDNA assay in p16-negative HNSCC patients who received curative intent surgery [21]. Plasma samples were collected at different time points before and after surgery, adjuvant therapy (if applicable) and during follow-up. MRD in LIONESS was analyzed using the RaDar™ assay which uses multiplexed PCR and targeted NGS to track a median of 48 variants in plasma. These variants are identified in the tumor tissue by whole exome sequencing and are prioritized using an algorithm to build a patient-specific panel. Presence of one variant in plasma was considered as positive for ctDNA detection. Bespoke ctDNA was detected in all 17 patients at baseline. In post-surgery samples, ctDNA could be detected at levels as low as 0.0006% VAF. All patients

with clinical recurrence were positive for ctDNA during follow-up and before clinical progression with a lead time ranging from 108 to 253 days. An updated analysis with 46 patients presented recently confirmed the potential role of this assay for MRD. All 11 patients who recurred had ctDNA detected in plasma during follow-up [22]. However, there were 5 patients with detectable ctDNA and no recurrence up to the latest follow-up which reduces the specificity of the assay. Median lead time in this updated cohort between ctDNA detection and recurrence was 122 days (ranging from 1 to 260 days) with a median follow-up duration of 307 days. All the patients included in the LIONESS study were surgically treated patients. Therefore, the role of MRD detection in HNSCC patients treated with definitive (chemo)radiation is still unknown. PRE-MERIDIAN study (NCT04599309), conducted at the Princess Margaret Cancer Centre, will hopefully shed more light on the use of bespoke ctDNA and other assays in patients with high risk locally advanced HNSCC treated with this modality.

One of the limitations of bespoke ctDNA is tumor tissue availability to perform whole genome or exome sequencing. This could potentially limit the application of these assays in the MRD setting, especially in those patients without surgical specimen availability (i.e. those undergoing definitive radiation  $\pm$  chemotherapy). While core tumor biopsies may be used, their tumor DNA quantities may limit success for genomic sequencing. One of the potential alternatives for mutation-based targeted ctDNA analysis is the Cancer Personalized Profiling by deep sequencing (CAPP-seq). This assay has been evaluated in 30 patients with LA-HNSCC who were treated with surgery [23]. It was performed using a panel designed to maximize the number of HNSCC-associated mutations, with ctDNA detected in 20 patients (66%) at baseline. However, CAPP-seq was not done in the follow-up samples so the impact of its detection during surveillance and its association with disease recurrence have not yet been studied. A recent study has evaluated a fixed 71-gene panel in 20 patients with LA-HNSCC. This study includes not only surgical patients but also patients who have been treated with definitive chemoradiation [24]. Clearance of ctDNA after treatment was observed in 10 patients, all of them were free of recurrence during follow-up. Similarly, detection of ctDNA post-definitive treatment was associated with shorter relapse free survival (RFS). Indeed, detection of ctDNA was observed in 5 of the 7 recurrent cases (71%). However, in only two of these patients, ctDNA preceded radiological progression thus limiting the application of results from this study in the MRD setting as most patients showed clinical progression at the time of ctDNA detection.

### ***Methylated cfDNA***

Methylated cfDNA analyzes epigenomic changes in cfDNA. Notably, it could potentially be applicable to more patients as it does not depend on HPV status, tissue availability or presence of mutations compared to the abovementioned strategies. However, methylated cfDNA has been challenging to be analyzed in plasma using

standard approaches. Cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq) is a bisulfite-free approach to track aberrant methylation in cfDNA and has been validated in different tumor types [7]. Methylated cfDNA analysis was performed in the same aforementioned cohort of patients with LA-HNSCC who were analyzed with CAPP-seq [23]. Methylated cfDNA was further refined by restriction of cfDNA by fragment size (100–150 bp), which is the usual size range of tumor derived cfDNA. A high correlation between both assays (CAPP-seq and cfMeDIP-seq) was observed in the baseline samples. Interestingly, follow-up samples in that study were also analyzed using cfMeDIP-seq. Patients without clearance of methylated cfDNA during radiation or post-treatment were more likely to show disease recurrence compared to those with a complete or partial (>90%) clearance. Indeed, all patients with increase in methylated cfDNA compared to baseline had disease recurrence or death at the time of the analysis. In contrast, 69% of those patients with no detection of methylated cfDNA by cfMeDIP-seq remained free of recurrence with a median follow-up of 44 months. However, among those with no detection of methylated cfDNA, there were 4 patients with persistent or recurrent disease. Further validation in larger cohorts and prospective studies (such as in the PRE-MERIDIAN study) are also ongoing with this assay.

## MRD Clinical Trials Design

There are no prospective clinical trials focusing on cancer interception in the MRD setting of LA-HNSCC. As such, it seems reasonable to draw reference from reports in other malignancies whereby therapeutic intervention in MRD has led to improved clinical outcome. In the IMvigor010 phase III study (NCT02450331), 809 patients with high-risk, resectable, muscle invasive urothelial carcinoma were randomized to the anti-Programmed Death-Ligand 1 (anti-PD-L1) antibody atezolizumab versus observation. Based on the intention-to-treat analysis in unselected patients, the study did not meet its primary endpoint of improved disease-free survival (DFS) in the atezolizumab group compared to the observation group, nor in the secondary endpoint of OS [25]. However, in a follow-up report which focused on 581 patients from IMvigor010 who were ctDNA evaluable using the Signatera assay (a bespoke ctDNA assay), atezolizumab was found to improve DFS and OS compared to observation in those with detectable ctDNA post-surgery. No difference in these two clinical endpoints were observed in patients whose post-operative ctDNA levels were undetectable [26]. This biomarker-based evaluation suggests that ctDNA analysis is able to identify a molecularly high-risk group post-surgery who may benefit from additional therapeutic intervention to improve clinical outcome. The recently published DYNAMIC study in stage II colon cancer randomized patients in a 2 to 1 ratio to a prospective ctDNA-guided approach (Safe-Sequencing System tumor-informed ctDNA assays) versus treating physician decision based on standard clinicopathological features to determine the administration of adjuvant chemotherapy [16]. The primary efficacy endpoint of RFS at 2 years using a ctDNA-guided strategy was

noninferior to standard management. This de-escalation approach proved to spare some patients from the toxicity of adjuvant chemotherapy without compromising RFS. Both IMvigor010 and DYNAMIC studies provide evidence that ctDNA results in the MRD setting are informative to guide treatment escalation in patients who are at high risk for clinical relapse, or treatment de-escalation in those who are at low risk for disease recurrence. Treatment escalation strategies may be considered in the MRD setting of LA-HNSCC post-curative therapy (e.g., surgery followed by post-operative [chemo]radiotherapy, or upfront definitive [chemo]radiotherapy), to compare additional investigational treatment versus standard observation in patients with detectable ctDNA. At the Princess Margaret Cancer Centre, such a study (MERIDIAN, NCT05414032) is about to be launched using the RaDaR™ assay to determine MRD in patients with high-risk HPV-positive and HPV-negative LA-HNSCC; patients who have MRD will be randomized to receive a novel immunotherapeutic agent versus observation. The primary endpoint of MERIDIAN is to assess the clearance of bespoke ctDNA at different time points (week 2 and week 10) after the end of MRD interception, which will be correlated with longer term clinical outcomes such as DFS and OS. Various ways to ascertain MRD status and follow-up of ctDNA kinetics will be applied using bespoke DNA, HPV DNA and methylated DNA assays.

## Conclusions

Advances in ctDNA technology have led to the definition of MRD as a disease status not previously identifiable in solid tumors, since microscopic circulating quantities of nucleic acids such as DNA shed by tumor cells cannot be readily detected by conventional investigations such as radiological imaging. Various ctDNA assays currently exist in different stages of clinical development, such as TTMV to measure viral genomes in the case of HPV-positive malignancies, bespoke and other mutation-based assays to track variants in plasma, and methylated assays to evaluate differential methylated cfDNA patterns in HNSCC compared to normal states. These have been applied in retrospective and prospective studies and some assays have demonstrated clinical utility in predicting clinical outcome. Clinical trials in the MRD setting are beginning to accumulate evidence in multiple cancers. Such studies are being actively designed to investigate the impact of cancer interception of MRD in LA-HNSCC.



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