Liquid Biopsy: A Future Tool for Posttreatment Surveillance in Head and Neck Cancer?

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Established Facts

- Head and neck cancer prognosis remains poor due to high recurrence rates.
- Current diagnostic testing for posttreatment surveillance of head and neck squamous cell carcinoma (HNSCC) patients is lacking specificity.
- The potential usefulness of ctDNA as a biomarker has already been shown in several applications in cancer management.

Novel Insights

- We urge the potential use of liquid biopsy and genetic biomarkers during posttreatment surveillance of HNSCC patients for the early detection of recurrent disease.
- We presume early driver *TP53* mutations to be highly abundant and concordant in clonal expansions, addressing the challenges of tumor heterogeneity.
- Highly sensitive digital droplet PCR could be used to detect (rare) mutational targets, such as *TP53*, in the blood of HNSCC patients.

Key Words

Head and neck cancer · Posttreatment surveillance · Liquid biopsy · Droplet digital polymerase chain reaction

Abstract

The prognosis of head and neck squamous cell carcinoma (HNSCC) is largely based on disease stage. Despite improvements in treatment, recurrence rates are still considered high. Currently, disease progression or regression after curative treatment is monitored by clinical evaluation com-

bined with flexible endoscopy and/or imaging. However, specificity of imaging is low due to the posttreatment effects. Detection of circulating tumor DNA (ctDNA) from blood samples of HNSCC patients is a minimally invasive technique that could lead to an earlier detection of recurrence. In addition, digital droplet PCR (ddPCR) could be used to sensitively detect these mutational targets. Future study on ctDNA using ddPCR in blood samples of HNSCC patients is recommended during the follow-up stage to detect recurrences in a timely manner.

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Introduction

Head and neck squamous cell carcinomas (HNSCCs) comprise tumors of the oral cavity, oropharynx, hypopharynx, and larynx. The prognosis of HNSCC is largely based on the disease stage at presentation, particularly the presence of lymph node metastases in the neck, and distant metastases. Despite improvements in treatment [1], locoregional recurrence rates after (chemo)radiotherapy range from 25 to 50%, depending on tumor location and stage. In the case of recurrence above the clavicles, salvage surgery remains the only curative option [2]. Therefore, timely diagnosis of locoregional recurrence is crucial to increase the possibility of prompt curative salvage surgery [3]. To date, the standard method for the assessment of locoregional control is clinical evaluation, supported by flexible endoscopy and/or imaging. Therefore, posttreatment imaging such as computed tomography (CT), magnetic resonance imaging (MRI) or F-18-fluorodeoxyglucose positron emission tomography (FDG-PET) in cases where there is a clinical suspicion of recurrence are performed promptly. However, differentiation between posttreatment effects and tumor recurrence in these imaging modalities is difficult, especially after (chemo)radiotherapy. Although very sensitive (94%), the specificity of the FDG-PET for locoregional recurrence is lower (82%), alongside a positive predictive value of 75% as described in a systematic review and meta-analysis of PET trials for detecting residual or recurrent HNSCC treated by (chemo)radiotherapy [4]. This could lead to a considerable amount of false-positive cases, rendering erroneous therapeutic interpretation. As a result, there is a clear need for more specific markers to detect (early) recurrences. With the discovery of genetic aberrations that induce the initiation and progression of malignant tumors, the detection of specific mutations is increasingly being used as a biomarker to predict local recurrence. Moreover, the detection of genetic mutations, either as freely circulating tumor (ctDNA) or in circulating tumor cells (CTCs), has proven to be a promising tool to determine the early spread of cancer cells in the blood [5, 6]. More recently, these so-called liquid biopsies have already shown promising results as genetic biomarkers to predict early recurrences in different cancer types [6–8].

In this paper, we will briefly discuss the various advantages, drawbacks, and current clinical applications of liquid biopsy in oncology, as well as its use as a novel predictive tool in posttreatment surveillance in HNSCC patients. Furthermore, we will delineate the various advantages of a novel PCR technique, droplet digital poly-

merase chain reaction (ddPCR), and the potential implementation of liquid biopsy and ddPCR in daily clinical practice in the future. We will support this by offering a possible workflow of our future strategy.

HNSCC and TP53

The pathogenesis of HNSCC is strongly related to alcohol consumption, tobacco use, and high-risk human papilloma virus (HPV) infection [9]. HPV-negative and HPV-positive tumors have been shown to be (epi)genetically and clinically different entities [10, 11]. In HPV-positive tumors, *PIK3CA* and *PTEN* are found to be the most frequently altered genes [12]. In HPV-negative tumors, the most frequently mutated genes are *TP53*, *CDKN2A*, and *NOTCH* [13, 14]. Moreover, *TP53* mutations appear to play an important role in the early onset of HNSCC [15–17]. Subsequent loss of heterozygosity (LOH) leads to loss of the nonmutated gene [18, 19] and newly formed clonal expansions [20, 21]. The specific *TP53* mutations in the clonal progenitor and the expansion cells could be used as a biomarker to detect the presence of primary and/or metastatic tumor, presuming that these mutations are globally present in the clonal outgrowth of primary tumors.

Liquid Biopsy

Various DNA-sequencing techniques, such as next-generation sequencing (NGS) and Sanger sequencing, are currently applied in clinical practice using tissue biopsy in order to guide cancer therapy [22]. However, major drawbacks of tissue biopsy are the biased acquisition of tissue due to intratumoral heterogeneity and the risk of complications due to the invasive nature of the procedure [23]. Moreover, HNSCC locoregional (micro)metastases are often too small to be sampled and detected by tissue biopsy sampling. Consequently, although tissue biopsies can deliver useful information about the primary tumor genetic profile, there are limitations to its use as a method for monitoring for posttreatment locoregional disease.

Blood samples are another source for the retrieval of DNA (and RNA) to monitor tumor status. It was first identified in 1948 that the blood of healthy individuals contains genetic material in the form of cell-free DNA (cfDNA) [24]. Additionally, in patients with a malignant tumor, blood can contain CTCs and ctDNA [25–27], probably released into the bloodstream by the (metastatic) tumor cells following apoptosis and necrosis, or by the

active release of living cells [28]. cfDNA has already been studied clinically in prenatal testing, and transplant and trauma patients [29, 30], while ctDNA is considered to be a potential prognostic tool in cancer treatment [31, 32].

The clinical application of ctDNA in cancer treatment is roughly divided into 2 categories: the characterization of tumor genetics and the quantitation of ctDNA, representing tumor burden [33]. Applications based on ctDNA for characterizing tumor genetics can be used as a tool to guide targeted drug therapy, particularly in metastatic disease, and as an alternative to conventional tissue biopsy in cases of absolute or relative contraindications to biopsy. The second category of application could be used as a disease-monitoring tool, as there appears to be a relation between ctDNA load and tumor burden [34]. Diehl et al. [35] showed that, in 15 of 16 patients who underwent curative colorectal cancer surgery and developed disease recurrence, plasma ctDNA with tumor-specific genetic alterations was still detectable after surgery. In another pilot study, the possibility of prognostication and the monitoring of oral squamous cell carcinoma were assessed by analyzing the serum-isolated DNA of 64 patients using microsatellite markers to detect allelic imbalances. In >50% of patients, allelic imbalances were identified in serum DNA that corresponded with tumor DNA. In turn, a correlation was found between allelic imbalances in serum DNA and tumor stage [32]. Although the use of microsatellites has been a subject of debate [36], these results are promising. More recently, Bettegowda et al. [37] reported detectable ctDNA levels in 55% of 223 patients with localized tumors of varying origin (i.e. pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers). Also, a directly proportional correlation was found between the fraction of patients with detectable ctDNA levels and tumor stage. Similar to ctDNA, CTCs could be used to genetically analyze tumor DNA for targeted treatment strategies and to detect a recurrence or progression of disease after initial therapy. The main principle of this technique relies on the identification of CTCs by the enrichment of cells expressing epithelial cell adhesion molecules, followed by immunofluorescent staining using different markers.

In a pooled analysis of 1,944 patients with metastatic breast cancer, an independent prognostic effect of CTC enumeration on progression-free and overall survival was confirmed [38]. All patients were analyzed at the start of a new line of therapy. Patients with a CTC count of 5 per 7.5 ml or higher at the start of treatment were associated with shorter progression-free and overall survival ($p < 0.0001$) than patients with a CTC count of <5 per 7.5

ml. Another field of interest in current research is CTC characterization. Several studies investigated protein expression, RNA expression, and DNA aberrations in CTCs in order to help guide drug therapy in different cancer types. In particular, HER2 protein expression in breast cancer patients and AR signaling in castration-resistant prostate cancer are subjects of interest in current studies [39]. There are, however, certain issues pertaining to the use of CTCs before this can be translated into the therapeutic arena, particularly with regard to the detection and characterization of the genetic alterations, because the CTC concentration in the blood circulation is very low. The current estimate of CTC concentration is 1 tumor cell/1 billion normal blood cells. Finding a sensitive way to detect, isolate, and differentiate CTCs remains an underlying challenge. The detection of CTCs is also dependent on the time point of blood sampling. In HNSCC studies, the portion of patients with positive CTC levels varies from 6.5 to 87.5% [26, 40–42].

Regarding posttreatment tumor monitoring, liquid biopsy could be an interesting tool in the follow-up of HNSCC patients. Firstly, performing NGS is necessary to determine patient-specific genetic mutations in the primary tumor. To date, most NGS testing on HNSCCs is performed within the context of research, and is based on time-consuming whole-exome sequencing [43]. By using HNSCC-specific gene panels instead, a more targeted approach becomes possible, focussing solely on genes of interest. This allows for a faster and more sensitive sequencing method [22, 44] that is clinically pliant. Subsequently, based on these NGS results, early driver gene mutations can be selected and used as templates to create mutation-specific primers (assays). By collecting liquid biopsies from the patient at different time points during follow-up after (chemo)radiotherapy or primary tumor resection, ctDNA can be isolated and used as a biomarker. Performing PCR using the mutation-specific assays enables the detection of tumor-specific mutations in ctDNA. Due to the relatively short half-life of ctDNA of approximately 2 h, tumor changes can be evaluated in hours rather than in weeks to months [23]. This facilitates close monitoring for disease progression or regression and also the early detection of tumor recurrence or metastases after initial treatment with curative intent. This personalized cancer management has the potential to prevent overtreatment and insufficient treatment, thereby possibly avoiding the complications associated with invasive diagnostic techniques and disease progression, respectively. A structured workflow for the use of liquid biopsy in clinical practice is proposed in Figure 1.

Fig. 1. Proposed workflow for the practice of liquid biopsy in HNSCC.

Although earlier data underline the emerging evidence that monitoring genetic alterations in ctDNA is a promising tool for determining disease stage and recurrence, several important technical and biological obstacles have yet to be removed, in order to be able to implement this diagnostic tool in clinical practice [33]. Firstly, analytic sensitivity is necessary, to reliably isolate and detect ctDNA when it is present in the blood. Secondly, a high proportion of patients should carry detectable amounts of ctDNA, because absolute ctDNA levels vary within each subpopulation [37]. Thirdly, a low signal-to-noise ratio due to the presence of high levels of cfDNA (i.e., wildtype DNA) could interfere with the detection of target ctDNA. Lastly, tumor heterogeneity remains an important chal-

lenge. Clonal expansions can arise within the primary tumor, carrying a different mutational profile. During tissue biopsy for initial genetic tumor profiling, a (rare) subclonal mutation could be selected as target mutation for detection in blood. This could possibly lead to inaccurate representation of tumor burden due to inherently low levels of targeted ctDNA [32].

Droplet Digital PCR

ddPCR is a novel tool in genetic diagnostics. Combined with the mutation-specific primers, samples are processed with oil, to create 20,000 water-in-oil droplets

containing the DNA molecules. The readout of the droplets is an end-point PCR based on Poisson statistics, suggesting that target DNA molecules are distributed randomly to the droplets. After actual PCR and amplification has been conducted, some reactions contain target copies while others do not. The yield will be read out as a positive end-point and negative end-point, respectively [45]. This enables the absolute quantitation of nucleic acids in a sample, which will provide great precision due to partition of the sample. This will then facilitate the detection of rare targets of interest. Furthermore, accurate quantification of targets inside the droplets enables the reduction of error rates due to normal PCR efficiency bias. This could accommodate the previously mentioned issue of a low signal-to-noise ratio in ctDNA detection. Several studies have already shown promising results concerning the accuracy of ddPCR during the prospective posttreatment monitoring of various types of cancer patients [46–48]. Although similar prospective data from ddPCR are currently lacking for HNSCC patients, the advantages of ddPCR may contribute efficiently to the diagnostic process in posttreatment monitoring of HNSCC patients.

Future Perspectives

Our future goals are to assess the role of liquid biopsy in the locoregional surveillance of HNSCC patients following curative treatment, and to determine if ddPCR is a sensitive and specific PCR technique for the detection of tumor-specific alterations in ctDNA. In order to do so, pilot experiments will be carried out to see if this technique is feasible for the proposed aim. A prospective, longitudinal study of HNSCC patients will allow for the analysis of blood samples at different time points: before treatment, after treatment, and at recurrence. These future studies will attempt to determine the feasibility of using minimally invasive and sensitive techniques for disease surveillance after the treatment of HNSCC patients.

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Disclosure Statement

The authors declare no conflicts of interest.

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