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BASIC SCIENCE REVIEW

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Circulating tumor DNA as a biomarker and liquid biopsy in head and neck squamous cell carcinoma

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Abstract

The use of circulating biochemical molecular markers in head and neck cancer holds the promise of improved diagnostics, treatment planning, and posttreatment surveillance. In this review, we provide an introduction for the head and neck surgeon of the basic science, current evidence, and future applications of circulating tumor DNA (ctDNA) as a biomarker and liquid biopsy to detect tumor genetic heterogeneity in patients with head and neck squamous cell carcinoma (HNSCC).

KEYWORDS

biomarker, circulating tumor DNA (ctDNA), head and neck cancer, liquid biopsy

1 | INTRODUCTION

Head and neck cancer continues to carry a significant global burden of disease.[1,2] Of concern is the increasing incidence and mortality of head and neck cancer, with a greater rise in developing countries and a contrasting increase of oropharyngeal cancer in developed countries.[1,2] Although several cancers have biomarkers that can diagnose disease and monitor pretreatment and posttreatment tumor burden, for example, prostate-specific antigen in prostate cancer or carcinoma antigen (CA19-9) in pancreatic cancer, head and neck cancer has no such test. Thus, head and neck cancer surveillance relies on clinical and radiological findings.[3] Patients often present with advanced-stage disease and the features of early invasion and metastasis create a significant morbidity and impact upon quality of life.[4] For these reasons, despite advances in treatment, head and neck cancer 5-year survival remains in the region of 60%, which is only slightly improved over the past few decades.[5]

Using a blood test, circulating biochemical molecular markers in head and neck cancer hold the promise of being

Paul Nankivell and Hisham Mehanna contributed equally to this study as senior authors.

able to improve diagnosis, planning, treatment monitoring, and surveillance.[6] A blood test carries little morbidity, can be repeated at various time points during treatment, and is cost-effective. One such suggested biomarker is the level of circulating tumor DNA (ctDNA).[7] The discovery that a proportion of circulating DNA in patients with cancer may be tumor-derived has created the potential for a so-called "liquid biopsy," as an alternative to a tissue biopsy, to characterize tumor genetic features.[8,9]

This review provides an introduction to the biological structure and function of circulating DNA. We discuss its use as a biomarker of tumor burden, and the potential utilization of ctDNA as a liquid biopsy in head and neck squamous cell carcinoma (HNSCC) to identify tumor genetic heterogeneity. We deliberately focus on HNSCC and a ctDNA-based liquid biopsy. Although it is beyond the scope of this article, authors should be aware of other circulating components that are under investigation for use as a potential liquid biopsy in head and neck cancer. For example, circulating tumor cells (CTCs)[8] or circulating viral DNA, such as plasma human papillomavirus DNA in oropharyngeal carcinoma[10] and plasma Epstein-Barr virus DNA in nasopharyngeal carcinoma.[11]

1.1 | Circulating tumor DNA

Circulating DNA is extracellular DNA found in circulating blood, which was first identified as early as 1948.[12] This DNA is released into the circulation by both pathogenic and physiological mechanisms, including apoptosis, cellular necrosis, phagocytosis, or exocytosis.[13] Ordinarily, circulating DNA is rapidly degraded by blood nucleases and eliminated by the liver, spleen, and kidneys and has a short half-life of around 10 to 15 minutes.[13] Therefore, systemic illness, such as liver or renal disease, can impact upon ctDNA levels and potentially bias the interpretation of blood results.[14] CtDNA is present in many forms; either free DNA, bound to protein complexes, cell surface bound, or in vesicles (apoptotic bodies, microvesicles, and exosomes).[13]

In 1977, Leon et al[15] were the first to identify that patients with cancer had increased levels of circulating DNA fragments, thus prompting the hypothesis that tumors released DNA into the bloodstream. In 1989, Stroun et al[16] were able to show that a portion of these DNA fragments was in fact of tumor origin due to the presence of genome instability, and then, in 1994, Sorenson et al[17] demonstrated the presence of tumor-specific point mutations in the *KRAS* gene in ctDNA. The presence of cancer-specific genomic alterations (for example, point mutations) allows the differentiation between ctDNA and DNA from normal healthy cells.[9,13] An additional discriminating factor is the difference in DNA fragment base pair length. Cellular apoptosis creates DNA fragments of around 100 to 200 base pair, whereas necrosis, due to more irregular digestion, creates larger fragments sometimes many kilo-base pair in size.[13,18]

The concentration of circulating DNA in healthy control patients is generally very low, in the region of <5 ng/mL, whereas patients with cancer can have elevated levels of several hundred ng/mL.[13,18] The increase in ctDNA in patients with cancer and the exact origin of ctDNA remains controversial. As tumor size increases, outstripping the metabolic supply, tissue hypoxia causes cellular necrosis, sloughing of tumor cells, and, thus, release of ctDNA.[13,18] The CTCs are not discussed in this review but, in theory, lysis of these cells in the circulation may also contribute to ctDNA, although the evidence is mixed.[10]

It is not clear if ctDNA has an active role in carcinogenesis or whether it is just a byproduct of tumor shedding. García-Olmo et al[19] were one of the first groups to describe the concept that ctDNA could cause cancer metastases by transfecting healthy cells. They were able to induce tumors in healthy rats using plasma from tumor-bearing rats. The same group later demonstrated that the serum from patients with colorectal cancer induced tumor formation in in vitro cultured cells.[20]

The laboratory methods of ctDNA detection and analysis have changed greatly over the past few decades, with the development of next-generation digital sequencing (NGS). The predominant method for the analysis of ctDNA is via polymerase chain reaction (PCR) amplification of ctDNA gene targets followed by downstream analysis. A variety of methods is used for the sensitive detection of point mutations, including real-time PCR, digital droplet PCR, and Sanger-type sequence. For more comprehensive molecular profiling of the circulating tumor genome, whole genome amplification methods (such as multiple displacement amplification, or random hexamer amplification) can be used to amplify limited ctDNA input followed by library preparation and sequencing.[21] As can be seen, the protocol for the collection and analysis of a liquid biopsy assay is both complex and as yet not standardized in its approach, with a major hurdle being the sensitivity and error rate of NGS for ctDNA.[14,21]

1.2 | Clinical applications of circulating tumor DNA

With numerous data end points described in ctDNA studies, it is useful to clarify how each of these would impact upon clinical practice. In previous literature, the terms "biomarker" and "liquid biopsy" have at times been used interchangeably. In this review, we deliberately separate the terms biomarker and liquid biopsy as to avoid confusion. As opposed to a static biopsy that determines tumor characteristics, a biomarker should be an objective and quantitative test of disease progression and outcome.[22] We discuss the clinical application of ctDNA analysis in 2 broad categories: (1) a biomarker

TABLE 1 Clinical applications of circulating tumor DNA analysis as a biomarker and liquid biopsy

Biomarker	Liquid biopsy
Pretreatment	
Diagnostic screening tool Diagnosis in uncertain cases Rationale for treatment protocol (ie, neck dissection in node-negative neck) Baseline evaluation of tumor burden for posttreatment comparison	Tumor genotyping to determine driver mutations and facilitate targeted molecular anticancer therapy
Posttreatment	
Assess residual disease in immediate posttreatment phase Provide risk analysis for decision to give adjuvant chemoradiotherapy Surveillance for locoregional recurrence	Monitor clonal evolution and assess for resistance driver mutations in recurrent cancer

to assess tumor burden; and (2) a liquid biopsy to determine tumor genetic heterogeneity (Table 1).

1.3 | Biomarker of tumor burden

To date, the use of ctDNA to assess HNSCC tumor burden has focused on 2 areas: total ctDNA concentration and the detection of ctDNA as a tool in diagnosis and marker of prognosis. The use of a ctDNA liquid biopsy as a noninvasive screening tool is an interesting proposition and is currently under investigation. As with all screening tools, the technical and clinical demands of creating a sensitive and specific ctDNA-based test are immense. However, the development of low-cost NGS technology and complex bioinformatics data analysis make this concept a potential future reality.[23] The immediate application of ctDNA as a biomarker in the pretreatment phase will likely be for those patients in whom there is high risk or diagnostic uncertainty. For example, the monitoring of premalignant lesions in which the best course of treatment is still debated or when a biopsy may miss potential malignancy in a severely dysplastic lesion.[24] In the posttreatment phase, the high sensitivity of ctDNA poses a real opportunity for the first biomarker in HNSCC to assess for residual disease or locoregional recurrence.

1.4 | Circulating tumor DNA levels and detection in patients with cancer

As discussed, the finding that total circulating DNA concentration was increased in patients with cancer was the foundation of the hypothesis that part of this DNA may be of tumor origin. However, total ctDNA concentration, regardless of subsequent ctDNA genomic analysis, may also be used as a diagnostic and prognostic tool. Mazurek et al[25] assessed total circulating DNA levels in 200 patients with HNSCC when compared to a control group of 15 patients. Mean total DNA levels were higher in the HNSCC group but not up to significant levels. Of interest, oropharyngeal SCCs had significantly higher levels of ctDNA ($P = .011$) than other HNSCCs (nasopharynx, hypopharynx, and larynx). They also demonstrated a significant relationship among nodal status (N0-1 vs N2-3), stage (I-III vs IV), and age (<63 and >63) with increasing ctDNA concentrations.

To be used as a biomarker, ctDNA detection must be a sensitive test for HNSCC and correlate with severity of disease. In the largest study to date, Bettegowda et al[26] evaluated the detection of ctDNA in 359 patients with 15 various cancer types. They divided the patients into those with localized disease ($n = 136$) and those with metastatic disease ($n = 223$), unfortunately, the numbers of head and neck cancer cases were relatively low ($n = 12$). In the metastatic disease group, ctDNA was detected in 82% of patients, in contrast to 55% in the localized disease group. When evaluating ctDNA as a prognostic tool, by comparing the metastatic and localized groups in cancers with sufficient numbers (colorectal, gastroesophageal, pancreas, and breast), there was a significant relationship ($P < .001$) and also a clear trend with regard to advancing stage of disease and increased ctDNA quantity.

1.5 | Posttreatment surveillance

The ability to use ctDNA as a biomarker of disease recurrence in the posttreatment surveillance phase is arguably more valuable than its diagnostic merits.[27] Given the reliance on poorly sensitive clinical and radiographic tests, a biomarker of posttreatment tumor burden would be a valuable tool.[3] The study by van Ginkel et al[27] discussed the role of ctDNA in head and neck cancer surveillance, and proposed a workflow for how this would be applied to clinical practice. Studies in breast, colorectal, lung, and several other cancers have all demonstrated the clinical application of ctDNA.[8,9,13] In a study of 18 patients with colorectal cancer, Diehl et al[28] were able to directly correlate ctDNA detection and fluctuating levels with recurrence-free survival postsurgical treatment ($P = .006$). All but 1 of the patients who had detectable ctDNA postoperatively experienced recurrence and none of the patients with undetectable ctDNA experienced recurrence. They were able to elegantly plot graphical representations of ctDNA levels over time to provide an accurate representation of "personal tumor dynamic burden." In a similar study, Dawson et al[29] compared ctDNA to CTCs and carcinoma antigen (CA15-3) in 30 patients with

metastatic breast cancer undergoing treatment. The ctDNA was statistically more sensitive than CTCs or carcinoma antigen (CA15-3) to measure treatment response ($P < .002$) and was a significant marker of survival ($P < .001$).

In a study of 47 patients with HNSCC, Wang et al[30] were able to collect postsurgical treatment samples from 9 patients. In 3 patients who developed recurrent disease, the presence of ctDNA in plasma predated the clinical/radiographic evidence of recurrence by 15 months, 9 months, and <1 month. Of the 5 patients with negative ctDNA results, all were recurrence free at a mean follow-up of 12 months.[30] Hamana et al[31] compared the detection of ctDNA in 64 patients with oral SCC in the preoperative and postoperative phase. Forty-four percent of patients (28/64) demonstrated ctDNA with tumor-specific microsatellite alterations preoperatively and this dropped to 20% (13/64) postoperatively. Of the 28 preoperative patients with detectable ctDNA, 20 had no ctDNA detectable postoperatively and all of these patients were disease-free with no recurrence. Four of the patients with detectable ctDNA at 4 weeks in the immediate postoperative phase went on to develop distant metastases. In this study, the presence of cDNA was statistically correlated to early-stage (I/II) versus late-stage (III/IV) disease ($P = .0378$).

1.6 | Liquid biopsy to assess tumor genetic heterogeneity

The ability to identify driver mutations and epigenetic modifications of a tumor is an important step in the implementation of targeted therapy and improving outcomes in patients with head and neck cancer. Because HNSCC shows considerable tumor genetic heterogeneity, this means that different parts of the tumor may have different mutations.[32] Knowledge of all the important "driver" mutations of a tumor are necessary to be able to provide targeted treatment for that tumor. The current use of tissue biopsy as a diagnostic technique is a major deficiency in this regard. A tissue biopsy captures 1 or 2 parts of a tumor, and, therefore, is at high risk of bias and missing important driver mutations due to intratumoral heterogeneity, together with the invasiveness nature and morbidity of the procedure itself.[27] In contrast to the "static biopsy" obtained from tissue samples, the ability to collect multiple liquid biopsies at different time points during treatment creates the reality of a "dynamic biopsy" to detect tumor clonal evolution and identify recurrence or treatment resistance. This would allow the potential for real-time monitoring of cancer genetic mutational progression and the tailoring of personalized targeted molecular therapy. Recent studies in head and neck cancer have focused on the identification of tumor-specific genomic alterations in ctDNA. We discuss each applicable genomic alteration in turn.

1.7 | Mutations

Lebofsky et al[33] investigated tumor-specific mutations in the ctDNA of 34 patients with 18 various types of metastatic cancer (head and neck cancer = 5). In 27 patients, ctDNA mutations matched those from solid tumor biopsies. In the aforementioned study of 47 patients with HNSCC by Wang et al,[30] they were able to detect plasma ctDNA with tumor-specific point mutations in 87% of cases. They assessed the presence of mutations in 6 genes (*TP53*, *PIK3CA*, *CDKN2A*, *FBXW7*, *HRAS*, *NRAS*, and *E7* [human papillomavirus] DNA) frequently associated with HNSCC (>85% had TP53 mutations). The majority of patients had advanced (stage III or IV) disease. When they combined findings from plasma with sloughed DNA fragment detection in saliva this increased the diagnostic sensitivity to 96%. Of note, there was little variation in plasma detection rates among sites (80% oral cavity, 91% oropharynx, 86% larynx, and 100% hypopharynx). As expected, salivary DNA detection was significantly higher in oral cavity tumors (100%) when compared with other sites (47%-70%). Although mutation frequency was higher with advanced-stage disease (70% vs 92%) this was not to statistical significance. They concluded that the combination of DNA detection in 2 compartments (plasma and saliva) was a valuable tool to increase sensitivity.

1.8 | Microsatellite alterations

Microsatellite alterations include microsatellite instability (MSI) and loss of heterozygosity (LOH). Microsatellites are sections of DNA with short base pair motifs (usually 1-6 base pairs in length) repeated 5 to 100 times. In brief, they signify a defective mismatch repair system, which in turn is a marker of mutations in DNA repair genes.[34] There is strong evidence for the role of MSI in colorectal cancer as a marker of prognosis and survival, but the role of MSI in head and neck cancer is unclear.[34] Some studies report no relationship, whereas others reported a positive MSI conferring a better prognosis. The LOH is a result of loss of a copy of a diploid gene and is a common mechanism of inactivation of tumor suppressor genes. In a recent review by De Schutter et al,[34] they highlighted LOH as a more useful prognostic predictive marker than MSI, due in part to an increased frequency of LOH compared to MSI in HNSCC. The LOH is associated with advanced high-grade disease and is a negative prognostic indicator of survival,[35] with suggested evidence of a correlation with chemotherapy resistance.[36]

Some of the earliest work to identify HNSCC tumor-specific genomic alterations in ctDNA was performed by Nawroz et al.[37] In a cohort of 21 patients, they identified microsatellite alterations in the ctDNA of 6 patients with HNSCC. All 6 patients had advanced (stage III-IV) disease

and 5 had nodal metastases. The same group followed up these preliminary findings with a larger study of 152 patients with HNSCC.[38] Forty-five percent of patients (68/152) had tumor-specific microsatellite alterations in ctDNA, with 84% of the cohort having advanced-stage or recurrent disease (127/152). Those with advanced-stage disease and nodal metastases had a higher positive rate of microsatellite alterations than those with early-stage cancer. Of note, there was an obvious jump in detection from stage I to II disease (17% vs 47%) when compared to stage III and IV disease (52% and 44%). With a mean follow-up period of 27 months, disease-free survival was decreased in the positive ctDNA detection group but not to statistical significance.

1.9 | Tumor suppressor gene hypermethylation

The silencing of tumor suppressor genes via the hypermethylation of their promoter regions is one mechanism of gene suppression involved in carcinogenesis.[39] This epigenetic phenomenon of hypermethylation has been investigated and validated in HNSCC, with a host of tumor suppressor genes implicated as potential targets.[39] The detection of hypermethylation of ctDNA is, therefore, another potential prognostic application in HNSCC.

Mydlarz et al[40] evaluated the methylation status of 100 patients with HNSCC when compared to a control group of 50 patients. They specifically examined hypermethylation of the *EDNRB*, *DCC*, and p16 (*CDKN2A*) genes. Ten patients (10%) exhibited *EDNRB* hypermethylation, 2 of these patients had *DCC* hypermethylation, and 1 of these 2 patients also had p16 hypermethylation. It was statistically significant that HNSCC samples had *EDNRB* amplification when compared to the control group ($P = .02$) but not with the *DCC* or p16 genes. It is difficult to ascertain the clinical utility of this data. Detecting hypermethylated regions in ctDNA is highly specific for a diagnosis of HNSCC but the sensitivity is poor. Moreover, with tens of tumor suppressor genes implicated in HNSCC,[39] an assay with the diagnostic specificity to be used as a diagnostic screening tool would need to evaluate each of these genes individually. One solution is to perform genomewide analysis of DNA methylation, which is a technique under investigation.[41]

1.10 | Future questions to answer about circulating tumor DNA

For ctDNA to be used as a biomarker and liquid biopsy the quantitation of ctDNA levels via the detection of genomic alterations must be standardized and validated in HNSCC. A gold standard investigation would need to take into account patient, tumor, procedural, and treatment factors to produce a risk-adjusted determination of prognosis and tumor heterogeneity (Figure 1). Unfortunately, each of these factors has unanswered research questions.

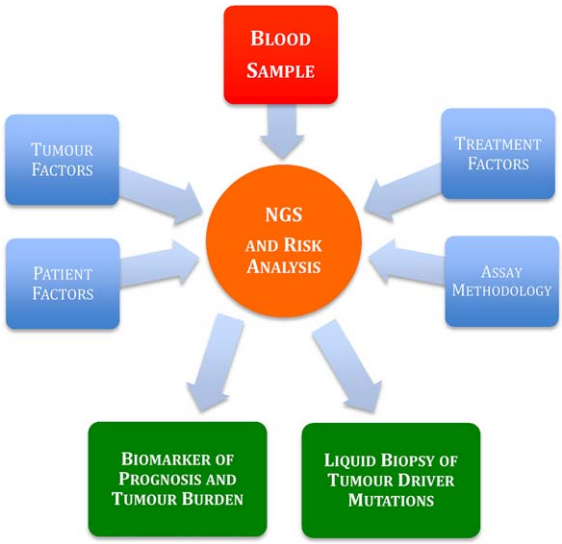

FIGURE 1 Factors influencing the application of circulating tumor DNA as a biomarker and liquid biopsy. NGS, next-generation sequencing [Color figure can be viewed at wileyonlinelibrary.com]

A significant challenge is the harmonization of the methodology used to detect and analyze ctDNA.[8,9] Herein lies the problem of creating a validated and universally accepted biomarker/liquid biopsy assay with set parameters that can be reproduced by different institutions. Furthermore, the mutational ctDNA load seems to vary greatly between tumor type and site,[18,26,33] and stage of disease or therapy may not always correlate with ctDNA levels.[18] For example, Mazurek et al[25] noted that oropharyngeal SCC had significantly greater levels of ctDNA than other HNSCC sites, the cause for this is unknown but presumably individual tumor biology has an impact. Thierry et al[13] noted that tumor growth kinetics and variance in cell proliferation and cell loss factors will impact upon ctDNA levels. In addition, the impact of systemic factors (comorbid disease, age, and smoking) on the levels and clearance of circulating DNA is not fully understood. The ability to quantify the above factors and apply these calculations to individual patient samples remains an unanswered task.

2 | CONCLUSION

As evidenced by this review, there are limited data relating to HNSCC and ctDNA. The presence of ctDNA in HNSCC seems to correlate with early versus late-stage disease and in the postoperative phase may predict recurrence or metastasis. The majority of studies provide proof-of-principle data that lay the foundation for future clinical trials. This review serves as an introduction for the head and neck surgeon into this flourishing field of research and, in time, a systematic review is required to further quantify all available data.

Although other specialties have made great strides toward the application of ctDNA analysis into clinical practice, progress has not been as rapid in head and neck oncology. Despite this, the evidence is encouraging that ctDNA as a biomarker and liquid biopsy holds great promise to provide a noninvasive, cost-effective, and tumor-specific test in HNSCC. The advent and further development of NGS technology is a turning point in this regard and the need for robust clinical trials of ctDNA in HNSCC is paramount.

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