Methodologies for ctDNA Detection

Subjects: Otorhinolaryngology

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Circulating extracellular DNA that is tumor-derived, referred to as ctDNA, often adheres to the surfaces of leukocytes and erythrocytes. Circulating tumor DNA (ctDNA) has the potential to improve outcomes by enhancing screening, early diagnosis, and surveillance in head and neck cancer patients.

Keywords: head and neck cancer ; liquid biopsy ; circulating tumor DNA

1. ctDNA Characterization

DNA fragments were first observed in human plasma in 1948 by French scientists Mandel and Métais and described as cell-free nucleic acid [1]. Since then, several studies have attempted to elucidate the mechanisms behind how DNA fragments are released by healthy and tumor cells. It appears this may be carried out through both passive and active processes. The passive process is described as DNA expulsion, resulting from cell death through apoptosis or, as in the case of rapidly proliferating tumor cells, necrosis [2]. Conversely, the active process is not fully understood, but some contend that tumor cells release micro-vesicles that contain DNA fragments [3]. Circulating DNA, although detectable in healthy individuals, is considerably more concentrated in cancer patients [4]. Circulating extracellular DNA that is tumor-derived, referred to as ctDNA, often adheres to the surfaces of leukocytes and erythrocytes [5]. ctDNA that remains unbound is referred to as tumor-derived cell-free DNA (cfDNA).

2. ctDNA Detection Techniques

Detection techniques of ctDNA have evolved considerably since the time of Mandel and Métais in 1948 [1]. Currently, two approaches dominate the detection of ctDNA-targeted and untargeted approaches. Targeted approaches search for tumor-specific genetic sequences that are known in advance, such as driver mutations and integrated viral genes known to induce carcinogenesis (e.g., HPV and Epstein–Barr virus (EBV) genes). This approach includes polymerase chain reaction (PCR)-based techniques such as droplet digital PCR (ddPCR), and BEAMing (beads, emulsion, amplification, and magnetic). ddPCR relies on a water-oil emulsion droplet system that bypasses the limitations of serial dilutions required in other PCR technologies. This contributes to its high sensitivity in samples with low DNA levels with one study demonstrating DNA detection at concentrations as little as 37 copies per 20 µL [6][7][8][9][10][11]. BEAMing is a high-throughput droplet-based PCR that uses magnetized beads to separate DNA molecules which are further analyzed by flow cytometry [12]. BEAMing is notable for its ability to detect very rare genetic events such as mutant and wild type sequences present at ratios greater than 1:10,000 [13]. Both technologies are among the most commonly used assays for ctDNA detection and have been shown to have good agreement in ctDNA detection with limited discordancy [14]. Conversely, untargeted approaches do not require any prior knowledge of genetic alterations but are, consequently, less cost-effective [10]. This approach includes next generation sequencing (NGS)-based technologies including whole genome sequencing (WGS) and whole exome sequencing (WES). While WGS and WES can detect many more types of variants at once, they both also require a larger DNA input in the range of 200–1000 ng compared to 1 ng for BEAMing and ddPCR [15]. WGS and WES produce results as ratios, while BEAMing and ddPCR provide absolute quantification. Additionally, as will be discussed later, ctDNA epigenetic alterations are an active area of interest with gene methylation commonly investigated. ctDNA methylation is often quantified using methylation-specific PCR. This technique relies on purifying and sulphonating DNA and then performing a PCR reaction utilizing two pairs of primers, one specific for methylated DNA and the other for unmethylated DNA [16]. This is also a targeted approach as specific genetic sequences that are to be investigated must be known in advance.

Aside from differences in cost and technology, detection techniques may have varying performances in different body fluids. This was examined by Mattox et al., where plasma samples and oral rinses from 66 patients with HPV-positive oropharyngeal squamous cell cancer (OPSCC) were collected and analyzed for HPV ctDNA [17]. In plasma samples, NGS and ddPCR had similar sensitivities (68.3% and 69.8%, respectively), and both outperformed qPCR, which had a

sensitivity of 20.6%. In oral rinses, NGS demonstrated significantly greater sensitivity at 75% compared to 8.3% and 2.1% for ddPCR and qPCR, respectively, indicating varying fluids may necessitate differing techniques to optimize detection. Notably, a key limitation in this study is that it examines isolated DNA previously extracted from banked plasma samples and oral rinses. DNA may have degraded over time, which may account for why this study found poorer performance by NGS, ddPCR, and qPCR than in other studies [18][19][20].

3. ctDNA Fluid Sources

ctDNA is a versatile biomarker and can be sourced from many fluid types, including pleural fluid for pulmonary adenocarcinomas and urine in urologic malignancies [21][22]. Studies examining ctDNA in HNC have largely focused on blood (plasma and serum) and saliva samples. Understandably, the anatomic location of the tumor can play a role in directing researchers on which bodily fluid is optimal for detecting ctDNA. This aspect was examined by Wang et al. in a study that enrolled 93 patients with head and neck squamous cell carcinoma (HNSCC) [18]. Saliva and plasma samples were collected from patients prior to a definitive treatment for primary HNSCC or salvage treatment for recurrent HNSCC. Digital PCR was used to detect tumor DNA with HPV sequences and Safe-SeqS (Safe-Sequencing System) for detecting low-frequency somatic mutations. For oral cavity SCC, saliva samples detected tumor DNA in 100% of patients versus only 80% of plasma samples. Conversely, plasma samples performed better in OPSCC patients by detecting tumor DNA in 91% compared to 47% of saliva. Similarly, plasma samples performed better than saliva samples in laryngeal (86% vs. 70%) and hypopharyngeal (100% to 67%) SCCs.

Sampling multiple fluid types in combination may further enhance detection. In a subset of patients (n = 43), Wang et al. demonstrated increased sensitivity for each tumor site when assays of plasma and saliva samples were combined [18]. Additionally, Ahn et al. also illustrated increased screening performance by combining analysis of ctDNA samples from multiple fluid types [23]. Here, 93 patients with OPSCC were enrolled and plasma samples and saliva rinses were collected. Quantitative PCR (qPCR) was conducted on samples to detect HPV E6 and E7 genes. Pretreatment saliva and plasma sources were found to have sensitivities of 52.8% and 67.3%, respectively, for detecting HPV-positive OPSCC. However, when both detection sources were combined, the sensitivity increased to 76.1%.

4. Assessing Limitations and Optimizing ctDNA

Preliminary work in the liquid biopsy area has shown promise in translating this science into clinically useful measures and possible screening tools. Despite these advances, significant challenges remain. ctDNA and other liquid biomarker techniques will benefit from improved sensitivity, specificity, and overall accuracy required for widespread, meaningful clinical use. While solid tissue biopsies presently remain the gold standard and reference for most ctDNA analyses, as demonstrated by the above studies, even this presents controversy. ctDNA may be derived from areas of tumors that were not biopsied from the index lesion and may contain a divergent genomic composition limiting targeted approaches [24]. Questions remain how representative liquid biopsies can be of solid tumors with respect to intra-tumor heterogeneity. This may not present an issue in virally mediated HNC as HPV and EBV ctDNA are specific biomarkers that can reliably be detected. However, this does pose a challenge in non-viral HNC, as driver mutations may be heterogeneously distributed throughout a primary tumor. Liebs et al. illustrated the concordance of the mutational profiles between tumor tissue and cfDNA in HNC patients to only be 11%, highlighting the significant heterogeneity of HNC [25]. While CTCs did not perform better, they were interestingly able to detect specific mutations not detected in the tissue samples, suggesting a potential advantage of liquid biopsies. While they may not fully capture the intra-tumor heterogeneity and tumor microenvironments of primary tumors, liquid biopsies may have the potential to detect genomic data from micrometastases and other subclones that contribute to treatment resistance [26]. For example, in patients with metastatic breast cancer, Chu et al. utilized ctDNA sequencing to detect ESR1 mutations, which would suggest tumor resistance to endocrine therapy, which was not identified in the corresponding solid biopsies of metastatic lesions [27]. This highlights a similar limitation of spot core biopsies and fine-needle aspirations of solid tumors, as they also do not capture tumor heterogeneity or specific niches of tumor microenvironment. Nonetheless, the pursuit of capturing tumor heterogeneity is an important field of research, and tumor heterogeneity likely contributes to why reliable assessable mutations in non-virally associated HNC have not yet been identified. Thus, further studies are needed to clarify these associations and identify mutations, cell surface markers, or other metabolites which may increase the ability to more accurately isolate liquid biopsy targets among various HNC tumors and subsites. Potential opportunities include ctDNA mutational panels, or expression signatures.

Currently, numerous assays to detect ctDNA exist; however, no single ideal technique or assay has been universally adopted. Concerns for inter-laboratory variability in accuracy and interpretation have hobbled mainstream clinical adoption. Further studies evaluating agreement of different assays in ctDNA detection are needed, similar to how O'Leary

et al. demonstrated good agreement between BEAMing and ddPCR [14]. This will clarify whether there is sufficient reproducibility for clinical use and if analyses among different studies can appropriately be made. Additionally, by improving the associated sensitivity and specificity of these techniques and further understanding ctDNA biology, more accurate detection of HNC, disease burden, surveillance, and prognostication may be possible [28].

Through combinatorial approaches and future technological refinements, integration of ctDNA data with various other circulating biomarkers may ultimately broaden the scope of ctDNA applications in HNC and improve its utility as a clinical tool. Several studies have investigated the combined role of ctDNA and CTC detection in solid tumors, but to date, none have been conducted in a solely HNC sample [29][30]. Furthermore, advancements in platforms similar to Bu et al. that can simultaneously capture ctDNA, exosomes, and CTCs and provide combinatorial analysis of their expression profiles through machine learning will ease this path of investigation [31].

ctDNA has been increasingly studied as a biomarker for HNSCC post-treatment surveillance and used to assess residual disease, monitor for recurrence, and stratify patients at increased risk for relapse of disease [20][32][33][34]. Despite the early integration of ctDNA and similar biomarkers in treatment algorithms, further large-scale prospective and randomized clinical trials in HNC cohorts are needed to fully integrate ctDNA data into treatment stratification paradigms. Indeed, several clinical trials are underway including a phase II trial using ctDNA testing to determine the optimal time to begin routine treatment in patients with HPV-positive HNC [35][36][37]. While clinical validation of ctDNA use in non-viral HNC is continuing [38], clinical validation in virally mediated HNC cohorts have demonstrated excellent detection of viral ctDNA as biomarkers for screening, treatment response and surveillance (as noted above by Chera et al. in HPV-related HNC and Lo et al. in EBV-related HNC, among others) [20][39]. Additionally, although not yet standard of care, HPV ctDNA is already being used in patient care as a biomarker [40]. ctDNA assays have also already been clinically validated in several cancer types, including non-small-cell lung cancer, colorectal cancer, and pancreatic cancer, representing the robustness of the technology and ctDNA as a biomarker [41][42][43]. The studies presented in above contents do reveal areas in need of improvement in the field of ctDNA usage for HNC, including more cost-effective detection options, enhanced accuracy, expanded gene targets, and broadened combinatorial approaches with other liquid biopsy targets. Yet, with the break-neck speed of research in this field, these areas are to be strengthened quickly. While the American Society of Clinical Oncology (ASCO) and College of American Pathologists published a joint review in 2018, concluding at the time that ctDNA assays did not possess the evidence to suggest clinical utility outside of clinical trials [44], a re-evaluation of new literature is warranted and may soon suggest otherwise.

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