Circulating Tumor DNA as a Liquid Biopsy in Cancer

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Abstract

Circulating tumor DNA represents a promising biomarker for non-invasive assessment of cancer progression and evolution. Clinical management of cancer patients could be improved through the development of noninvasive approaches for diagnostic detection, prognosis and recurrent tumors. Biopsies are invasive, costly and provide only a snapshot of the mutations present at a given time and location. For some applications, mutation detection in plasma DNA as a liquid biopsy could potentially replace invasive biopsies as a means to assess tumor genetic characteristics. In this review, we summarize the development and clinical results using circulating tumor DNA and discuss how future studies involving both scientists and clinicians could help to further develop this tool for the benefit of cancer patients.

Introduction

Cancer is a leading cause of death worldwide and requires appropriate diagnostic and prognostic methods [1]. Current tumor diagnosis depends on pathological examination. However, with the continuous emerging of tumor-specific molecules, tissue biopsies cannot always be reflective of the current tumor dynamics or response to therapies. To overcome this problem, the concept of "liquid biopsies" has been proposed, and clinical studies have proven that circulating biomarkers, such as tumor-relevant protein molecules, circulating RNA or microRNAs that can be used to help guide patient management will be available for only a minority of patients [2-4]. However, the sensitivity and specificity of these biomarkers remain suboptimal [5,6]. Therefore, the identification of new, highly sensitive and specific tumor biomarkers is particularly important and will also provide insights into cancer biology that can form the basis of further research.

The history of ctDNA

In 1948, circulating free DNA (cfDNA) and RNA were found in the human blood plasma by Mandel and Métais [7]. The clinical utility of cfDNA in the plasma has been an area of active research in many disciplines of medicine [8]. The most successful area has been the evaluation of fetal DNA in the blood of expecting mothers [9,10]. In patients with cancer, a fraction of the cfDNA is from tumor cells and is referred to as circulating tumor DNA (ctDNA) [11]. Tumor DNA can be released from primary tumors, micrometastasis, or overt metastasis into the blood of patients with cancer[12]. However, the majority of such ctDNA is derived from apoptotic and necrotic tumor cells that release their fragmented DNA into the circulation [13-16]. cfDNA in the circulation is typically fragmented to 160 - 180 bp in length, and this DNA is derived mostly from apoptotic cells [17]. The ladder pattern is frequently considered to be evidence that apoptosis may be the source of the observed DNA fragments in the plasma when the circulating DNA is subjected to electrophoresis [18,19]. In terms of radiotherapy, chemotherapy and other cancer treatments, cell death by apoptosis and less circulating DNA are found in cancer patients after treatment than before treatment, possibly because of the inhibitory effect of treatment on the proliferation of cancer cells [20].

The blood is considered as a "reservoir" where the alterations can be detected in the form of point mutations, copy number variations or chromosomal rearrangements. However, detection of ctDNA derived from tumors carries some challenges, largely because the number of circulating mutant gene fragments is small compared to the number of normal circulating DNA fragments and is sometimes less than 0.01% [21]. The detectable levels of ctDNA are related to the stage of disease and are lower in the localized tumors (49-78%) of patients than in metastatic tumors (86-100%) [6].

Recently, there have been numerous methods reported that involve the quantification of the level of multiple tumor and genetic mutations present in the blood, with several reports suggesting that there are many approaches that have high sensitivity, like droplet digital polymerase chain reaction (ddPCR) [22], beads, emulsion, amplification and magnetics (BEAMing) [23], or

pyrophosphorolysis-activated polymerization (PAP) [24]. Tumor-derived ctDNA reflects the genomic alterations of tumors, but it has a variable half-life in the circulation, ranging from 15 minutes to several hours; consequently, ctDNA can be used as a dynamic biomarker providing an accurate monitoring of tumor masses in real-time [21,25]. Simultaneously, some studies have revealed the correlation of ctDNA with many cancers, such as renal cancer [26], bladder cancer [27], gastric cancer [28], colorectal cancer [15], Esophageal Squamous Cell Carcinoma (ESCC) [29] and non-small cell lung cancer [30].

The correlation between tissues and ctDNA

Although tumor tissue is the gold standard for clinical study, major barriers exist in obtaining tissue biopsies, including the discomfort suffered by the patients, inherent clinical risks to the patient, potential surgical complications and economic considerations [31]. Sampling ctDNA from the blood overcomes the tumor heterogeneity and accessibility problems [32].

Indeed, several reports have shown a high correlation between ctDNA mutations and matched tumor biopsy mutations. The first demonstrations of a correlation between tissue and ctDNA evaluated PIK3CA mutations in tissue biopsies and plasma tumor DNA (ptDNA) from breast cancer patients. This group demonstrated that PIK3CA mutations in the tissue and blood were 100% in agreement [33]. In non-small cell lung cancer (NSCLC), Xu "et al." [34] found that driver mutations in EGFR, KRAS, PIK3CA, and TP53 were correlated between tumor DNA and plasma ctDNA in 32 of 42 (76%) matched samples. In a prospective study, Rothe "et al." [35] sequenced tumor samples and matched the serial plasma samples from metastatic breast cancer patients using NGS from a commercially available 50-gene panel. This study also showed that all mutations detected in a patient's tumor sample were present in the ptDNA, and the ptDNA of 2 of 17 patients contained a mutation that was not detected in their synchronous tumor samples, thereby supporting the concept that ptDNA may provide a more comprehensive mutational profile [36]. Chu "et al." [37] found that in both groups of patients, ESR1 mutations were always detected in the blood if they were present in the tissue, and additional ESR1 mutations that were not detected in the tissue were also present in the ptDNA of some patients. Similar results have been seen in studies involving multiple different cancers [6]. Recently, Schmiegel "et al." [38] found a high concordance of plasma and tissue results. This also demonstrated that blood-based RAS mutation testing is a viable alternative to tissue-based RAS testing using BEAMing. ctDNA is also believed to be released from all the tumor deposits in a given patient and is less impacted by intratumor heterogeneity than a signal tumor specimen [8]. Gerlinger "et al." [39] observed the mutational intratumor heterogeneity in multiple tumor suppressor genes, which can lead to the underestimation of the tumor genomic landscape from a single tumor-biopsy sample. In most cases, only biopsies are available, and treatment decisions depend on the results from a single tumor biopsy [40].

Detection methods of ctDNA

The quantity of ctDNA detectable in the blood is not only good for studying cancer pathogenesis but also beneficial in the clinical management of cancer. Though tumors are rarely observed, the development of non-invasive methods to detect and monitor tumors continues to be a principle challenge in oncology. Recently, PCR-based approaches have reached high levels of sensitivity, ranging from 0.1 to 0.01%, making it possible to detect to 1 mutated allele out

of 10000 normal alleles [41].

Now, there are several methods to detect ctDNA. Originally, researchers used Sanger sequencing to detect plasma ctDNA. However, there are many shortcomings for Sanger-based ctDNA detection, such as low-throughput, laborious protocols, high cost, and the potential bias introduced by PCR methodology [42]. Some studies have developed a technique called BEAMing [23,43] to detect ctDNA in the blood [21]. Using this method, Bettegowda reported a sensitivity of 87.2% and a specificity of 99.2% to detect KRAS mutations in colorectal cancer [6]. Forshew "et al." [44] reported that they applied tagged-amplicon deep sequencing (Tam-Seq) to directly identify mutations in the plasma of cancer patients. The authors conducted a proof-of-concept experiment by tracking the ctDNA from an ovarian patient, while they also re-sequenced the tumor tissue from a right oophorectomy specimen and identified a TP53 mutation [1]. Using this method, they identified cancer mutations present in the circulating DNA at allele frequencies as low as 2%, with sensitivity and specificity of >97% [44]. Newman "et al." [42] developed another new technique called cancer personalized profiling by deep sequencing (CAPP-seq) for quantifying ctDNA. They use a multi-phase bioinformatics approach consisting of biotinylated DNA oligonucleotides for low DNA input masses that target recurrently mutated regions in the cancer of interest. CAPP-Seq achieved a maximum sensitively and specificity of 85% and 96%, respectively.

In contrast to these approaches targeting hotspot mutations, a study used Whole-Genome Sequencing (WGS) analysis from plasma DNA, and this approach suggested that tumor DNA concentrations at levels >10% can be detected with a sensitivity of >80% and specificity of >80% [45]. Murtaza "et al." [46] used Whole-Exome Sequencing (WES) in a proof-of-concept study involving 6 patients with metastatic tumors. Compared to WES, WGS can screen a larger spectrum of the genome. However, it is currently too expensive for routine use to detect SNVs, whereas WES approaches allow more in-depth interrogation of multiple regions [1]. Recently, technology was developed using a high-throughput Droplet Digital PCR (ddPCR) system that can provide absolute quantitation of DNA copy number [22]. A study developed a targeted 23-amplicon Next-Generation Sequencing (NGS) panel for detection of mutations in ESR1, PIK3CA, TP53, FGFR1 and FGFR2 in 48 patients with estrogen receptor-α-positive metastatic breast cancer, and the selected mutations were validated using droplet digital PCR (ddPCR) [47]. As validation, they analyzed the common ESR1 p.D538G mutation in the baseline cfDNA samples by ddPC, confirmed all 3 positive samples and detected 6 additional cfDNA samples with this mutation at <1%. This means that ddPCR is more sensitive than NGS technology.

Comparing ctDNA and CTCs

The first report of Circulating Tumor Cells (CTCs) was shown for the first time in 1869 [48]. CTCs are extremely rare and are lost in a large number of normal blood cells. Only 1.43% of patients with progressive breast cancer had > 500 CTCs per 7.5 ml of blood [49,50]. CTCs may become cloaked by platelets or by coagulation factors, thereby shielding them from the immune system and making it difficult to detect them [51]. The potential clinical value of CTCs is clear in early detection, ultimately targeting the process of blood-borne metastasis and in using CTC analyses as a readout of tumor status therapeutically [52].

Circulating Tumor Cells (CTCs) and circulating tumor DNA (ctDNA) are promising sources for biomarker tests and useful tools

for the management of patients with cancer. Although the current Food and Drug Administration (FDA) has approved liquid biopsy measures for intact CTCs to give a prognosis of overall survival, the potential predictive value of ctDNA is much more exciting. ctDNA allows us to understand specifically what type of molecular changes are happening in the tumor in real time, which is a very big step beyond CTCs in clinical terms [53]. Several investigational studies have shown that ctDNAs could have more sensitivity than CTCs. A study [54] compared the radiographic imaging of tumors with the ctDNA and CTC assay in 30 women with metastatic breast cancer. They found that ctDNA was successfully detected in 29 of the 30 women (97%) in whom somatic genomic alterations were identified; however, CTCs were detected in 26 of 30 women (87%). Their result showed that circulating tumor DNA levels had a large dynamic range and a greater correlation with changes in the tumor burden than circulating tumor cells. Furthermore, another study used droplet digital PCR to assess the BRAF-V600E mutations in both circulating tumor DNA and DNA extracted from CTCs in lung adenocarcinoma [55]. The results showed that ctDNA seemed to be much more sensitive than CTCs. In a comprehensive study by Punnoose "et al." [56], forty-one patients were enrolled in a single-arm clinical trial of erlotinib and pertuzumab, and peripheral blood was analyzed for oncogenic mutations in CTCs and ctDNA. They found greater sensitivity for ctDNA than CTCs in mutation detection and reported that the detected mutations were strongly concordant with mutation status in the matched tumor. This proof-of-concept analysis showed that circulating tumor DNA is a promising, informative, inherently specific, and highly sensitive cancer biomarker [54].

Liquid Biopsies in the Clinic

We have presented the technologic considerations of ctDNA. However, with rapidly evolving sequencing platforms, we can provide only general guidelines about their comparative utility in clinical oncology [52]. For the clinical oncologist, it has become increasingly urgent to have access to accurate and sensitive methods for quantifying the response to cancer therapy, making a prognostic assessment, and predicting recurrence [57,58]. The development of cancer detection biomarkers will be propelled by technological improvements in how biomarkers are objectively measured. These biomarkers should be surrogate indicators for warning about possible recurrence, as well as disease progression or death, and should indicate if a specific treatment will reduce that risk. CtDNA might be a prognosis marker in cancer patients and, in the future, might be especially helpful for the selection of those patients who are at a higher risk of relapse and who might benefit from adjuvant therapies [59]. As such, ctDNA may be rapid, economical, and reliable for clinical applications. The steps required for the use of ctDNA in clinical oncology must be taken with great care, using well-designed, prospective clinical studies to statistically demonstrate clinical validation and clinical utility [36].

Assessment of Diagnosis and Prognosis

Cancer diagnosis and prognosis, especially through the use of a noninvasive blood test, is of great interest to researchers and patients alike. Obtaining a blood sample is less risky and noninvasive than a tumor or metastatic lesion biopsy [2]. Assessing the prognosis of an individual patient involves a combination of clinical observations and stages and the bimolecular characterization of different tumor types [31]. This information, which is derived from imaging, biopsy specimens and cancer biomarkers, could offer prognostic value for clinical oncology. However, previous tumor biomarkers like

cancer antigen 15-3, CEA, as well as CTCs, provide only limited sensitivity and specificity and therefore cannot always meet the clinical requirements. However, now there is a plasma biomarker-based approach that can evaluate tumor occurrence, progression and recurrence. A number of studies have already shown the ability to use ctDNA as a genetic-based biomarker for cancer detection, though mostly in metastatic disease [60,61].

For example, Shu "et al". [62] implemented NGS with a gene panel of 382 cancer-relevant genes on 605 ctDNA samples, indicating that ctDNA might be a suitable approach to guide treatment decisions in multiple cancers. A study from Lecomte "et al". [63] focused on hotspot KRAS mutations and cyclin-dependent kinase inhibitor 2A (CDKN2A) hypermethylation in patients with CRC. They demonstrated that the 2-year survival rate was 100% in patients with no evidence of ctDNA who possess KRAS mutations or CDKN2A gene promoter hypermethylation, which can be found in 40% or 20%–50% of CRC patients, respectively, suggesting a prognostic value for these markers. Therefore, the presence of ctDNA in plasma seems to be a relevant prognostic marker for patients with CRC and may be used to identify patients with a high risk of recurrence. Furthermore, it was shown that high concentrations of cfDNA and mutant KRAS were clear indicators of a poor outcome for metastatic CRC patients [64]. Therefore, ctDNA has the potential to be used for the evaluation of tumor prognosis. Using ctDNA, the detection rates among patients with stage I, II, III, and IV cancer were 47%, 55%, 69%, and 82%, indicating that ctDNA levels increase with cancer progression[6]. Gene methylation patterns in tumor tissue can be indicative of tumor aggressiveness and likelihood of recurrence [65]. The methylated genes TIMP3 [66], GSTP1, MINT2 [67], SOX17 [68] and RARb2 [69] were present in the serum or plama and are in turn linked to prognosis. Cell immunotherapy is another promising immunotherapeutic approach in cancer. In a study of the response indicators to T-cell transfer immunotherapy in metastatic melanoma using ctDNA, their results show that ctDNA levels can be used to rapidly identify patients who are responding from those that are not [70].

In summary, liquid biopsies based on ctDNA analysis might represent the next generation of tumor prognostic testing on account of their high accuracy and sensitivity [71]. In a multivariate analysis, KRAS mutations present in the plasma of 246 patients with advanced stage Non-Small-Cell Lung Cancer (NSCLC) were shown to predict poor prognosis in patients receiving first-line chemotherapy [72]. However, a parallel study, conducted in 308 patients with advanced-stage NSCLC, showed no correlation between prognosis and KRAS mutations in the plasma [73]. BRAF mutations, as assessed in serum samples, have also been shown to effectively stratify 103 patients with melanoma into both early stage and advanced-stages [74]. Nevertheless, results remain contradictory in these small patient populations. Accurate molecular diagnostics are essential for personalized therapies and precise clinical decisions to assess patient candidacy for other aspects of therapy based on drug-sensitizing genetic tumor alterations [75].

Prediction of Recurrence

CtDNA could be used to monitor relapse status, resulting in a 10-month lead-time on the detection of relapse compared with the conventional follow-up [76]. CtDNA could be a prognosis marker in cancer patients and, in the future, for the patients at a higher risk of recurrence who might benefit from adjuvant therapies [59].

Theoretically, the tumor burden is reflected by the ctDNA changes and should correspond with the stage of the tumor. This was also confirmed by a study from Diehl "et al." [21], in which patients who had detectable ctDNA after surgery generally relapsed within 1 year. Garcia-Murillas "et al." [77] found that noninvasive mutation tracking in plasma DNA can detect Minimal Residual Disease (MRD), which the standard treatment has failed to eradicate, and thus identify patients at high risk of recurrence. Schiavon provided two controls to suggest that this is not the main explanation. The subset of patients on Aromatase Inhibitors (AIs) with a ctDNA sample taken at the time of relapse had a very low level of ESR1 mutation detection, and they observed no mutation in the independent series of tumor biopsies taken at relapse on AI therapy [78]. Additionally, Roschewski "et al." [79] found that the surveillance of circulating tumor DNA identified a risk of recurrence before clinical evidence of disease in most patients, with a higher sensitivity than CT imaging, resulting in a reduced disease burden at relapse. Another study found similar results [80]. Similarly, Forshew "et al." [44] found that targeted deep sequencing of cancer-related genes was carried out on cfDNA in a patient who had previously undergone surgery to resect synchronous cancers of the bowel and ovary. It was shown that, on relapse, the metastasis was derived from the original ovarian cancer. Additionally, Diehl "et al." [21] found that the majority of patients had significantly decreased or absent ctDNA levels after surgery. Further follow-up studies suggested that the patients with detectable ctDNA after surgery all relapsed, while those without detectable ctDNA after surgery remained in remission. Without elevated CEA, Sarah found that ctDNA could detect recurrence prior to clinical detection [81].

Thus, there is great interest in whether the use of ctDNA for monitoring disease can reliably predict patients that have responded to therapies and were cured versus those that will ultimately have recurrence [36]. Recently, a study reporting a better selection of patients for whom liquid biopsy could be a good surrogate for a tumor biopsy and for whom the cfDNA NGS analysis will contribute to the sensitivity prediction score [82].

Monitoring Tumor Burden

Measuring the treatment response in patients with cancer is usually done by serial clinical evaluation of symptoms and estimates of tumor burden. However, serial radiographic imaging and tumor biopsy are expensive and may fail to detect changes in tumor burden [83]. Given these challenges, tumor biopsy may not represent an ideal source for the genetic characterization of the cancer. Circulating tumor DNA offers a "real time" tool for serial monitoring of cancer tumor genomes in a non-invasive manner that provides accessible genetics biomarkers [84].

Quantifying the disease burden to monitor the response to cancer therapy has a direct intuitive appeal, with many potential applications for individualizing treatment choices [57]. Circulating tumor DNA (ctDNA) may also represent a promising biomarker for noninvasive assessment of cancer burden, especially in circumstances where imaging delivers indeterminate results [8]. Indeed, the specific detection of tumor-derived cfDNA has been shown to correlate with a change in the tumor burdens in response to treatment or surgery [85]. Serum markers, such as PSA for prostate cancer, Cancer Antigen (CA) 19-9 and Carcinoembryonic Antigen (CEA), can be helpful but are not available for many tumor types; they frequently lack specificity and may be elevated as a result of clinical situations not related to tumor growth or progression [8]. Immunological

detection of circulating tumor cells identifies only cells present in the blood and can result in false positive results due to nonmalignant cells expressing the marker of interest [86]. Furthermore, the half-life of ctDNA is less than 2 hours [21], whereas most protein biomarkers persist in circulation for several weeks, thereby only allowing accurate assessment over weeks to months [8]. The investigators observed that mutant ctDNA concentrations showed a greater dynamic range and greater correlation with changes in tumor burden than did CA15–3 [54]. A study used the known tumor burdens and pre-treatment ctDNA levels measured in patients who harbored KRAS mutations in their tumors prior to therapy [85], as well as data obtained in patients with previous metastatic disease [21]. At a median follow-up of 507 days in patients with detectable ctDNA (5 of 6, 88%) compared with undetectable ctDNA (5 of 72, 7%), recurrence rates were >10-fold higher than the postoperative rates [87]. Tumor heterogeneity might be limited in advanced disease, in which case radiographic imaging should be involved [88].

However, compared with the tissue-based approach, prospective studies will be needed to illustrate that treatment strategies guided by the unique information from ctDNA yield superior clinical outcomes [87].

Monitoring of Molecular Resistance

Serial analysis of ctDNA during treatment can provide a dynamic picture of molecular disease changes, suggesting that this non-invasive method could also be used to monitor the development of secondary resistance of tumor cells that develop during the course of treatment [53]. One major barrier to testing any hypothesis about the nature of acquired resistance to anti-EGFR antibodies is the limited access to post-treatment tumor tissue [85]. Repeated biopsies to study genomic evolution as a result of therapy are difficult, invasive and may be confounded by intra-tumor heterogeneity [39,89]. Molecular tools, such as WES, can be implemented to find genetic differences between the tissue collected before and after therapy. This will offer a snapshot of the predominant resistant clones in a portion of the lesion under examination [8]. Recently, a cancer exomes sequencing report of the serial plasma samples was created to track the genomic evolution of metastatic cancers in response to therapy [46]. Their results showed that the CNVs and somatic mutations identified in the ctDNA are widely representative of the tumor genome and provide an alternative method of tumor sampling that can overcome the limitations of repeated biopsies [46]. A case reported that ctDNA can be used to dynamically monitor the onset of secondary resistance to anti-EGFR therapy [90].

Acquisition of the T790M substitution in the membrane receptor EGFR confers resistance to gefitinib and erlotinib in lung cancer in approximately 50% of patients [91-93]. The T790M mutation was first observed in relapsed patients and later confirmed through the non-invasive analysis of plasma samples, suggesting that resistance to targeted therapies can be monitored in the blood [31]. More recently genomic heterogeneity of T790M-mediated resistance may explain the reduced specificity observed for plasma-based detection of T790M mutations versus tissue[94]. Analogously, secondary resistance to anti-EGFR antibodies cetuximab and panitumumab is associated with the emergence of KRAS mutations in colorectal cancer [95]. The detection of KRAS variants in the blood during treatment with cetuximab or panitumumab demonstrated that it is possible to detect the emergence of resistant alleles up to several months before radiologic examination [85]. For metastatic breast cancer, targeted

NGS of cfDNA has potential clinical utility to detect biomarkers, which are corrective with HER2-targeted therapies [96].

Parallel analyses of tumor biopsies and serial ctDNA monitoring showed that lesion-specific radiographic responses to subsequent targeted therapies can be driven by distinct resistance mechanisms arising within separate tumor lesions in the same patient [97]. At present, this more sensitive approach for detecting cancer exomes in plasma is readily applicable to patients with high systemic tumor burden, enabling detailed and comprehensive evaluation of the clonal genomic evolution associated with treatment response and resistance [46].

Discussion

To date, the prospects for using ctDNA as a liquid biopsy for tumor management has generated a lot of excitement as this strategy provides the opportunity for the noninvasive detection of human cancers. As discussed, the study provides proof of the concept that ctDNA represents a sensitive biomarker for tumor burden, diagnosis and monitoring of drug resistance. Additionally, ctDNA may have broad clinical applications because it is non-invasive and convenient, and these assays can be repeatedly performed [98]. However, some studies miss the majority of somatic alterations in cancer that would require sufficient genomic coverage to identify in a tumor with multiple molecular markers, rather than simply recognizing the existing alterations [52].

The US Food and Drug Administration (FDA) has approved the cobas® EGFR mutation Test v2, which is the first "liquid biopsy" blood test for detecting epidermal growth factor receptor (EGFR) gene mutations in Non-Small Cell Lung Cancer (NSCLC). The ability to both isolate and genetically interrogate tumor DNA from a simple, minimally invasive test that can subsequently inform treatment decisions is a win for both the physician and patient. Currently, there are no consistent guidelines regarding the choice of analyte, the standardization of platforms or how the results are reported [36]. Likewise, the development of more accurate detection methods based on ctDNA could benefit the cancer patients and may, as a result, improve the clinical outcome in the near future [1]. There has been a study on the application of sequencing, liquid biopsies, and patient-derived xenografts for personalized medicine in melanoma [99]. Together, different technologies are likely to be synergistic, rather than strictly competitive in their clinical oncology applications [52]. Only in this way will their results optimize care for the majority of patients.

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Statement of Conflict of Interest

The authors have declared that no conflict of interest exists.

Authors' Contributions

Conceived and designed the paper: WY and RZ. Wrote the paper: RZ and WY.

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