Circulating tumor DNA: a promising biomarker in the liquid biopsy of cancer

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ABSTRACT

Tissue biopsy is the standard diagnostic procedure for cancers and also provides a material for genotyping, which can assist in the targeted therapies of cancers. However, tissue biopsy-based cancer diagnostic procedures have limitations in their assessment of cancer development, prognosis and genotyping, due to tumor heterogeneity and evolution. Circulating tumor DNA (ctDNA) is single- or double-stranded DNA released by the tumor cells into the blood and it thus harbors the mutations of the original tumor. In recent years, liquid biopsy based on ctDNA analysis has shed a new light on the molecular diagnosis and monitoring of cancer. Studies found that the screening of genetic mutations using ctDNA is highly sensitive and specific, suggesting that ctDNA analysis may significantly improve current systems of tumor diagnosis, even facilitating early-stage detection. Moreover, ctDNA analysis is capable of accurately determining the tumor progression, prognosis and assisting in targeted therapy. Therefore, using ctDNA as a liquid biopsy may herald a revolution for tumor management. Herein, we review the biology of ctDNA, its detection methods and potential applications in tumor diagnosis, treatment and prognosis.

INTRODUCTION

Current tumor diagnosis depends on a variety of pathological examinations, among which, tissue biopsy is considered to be the gold standard. However, tissue biopsy-based tumor diagnosis has many limitations. For instance, the detection of early-stage tumor or residual lesions is unsatisfactory, and its application in the evaluation of treatment efficacy and prognosis is also limited [1-2]. With the continuous emerging of tumor-specific molecules, the interest in molecular diagnosis of tumors is rapidly increasing. Studies have found that tumor-relevant protein molecules and miRNAs as well as circulating tumor cells (CTC) are all suitable tumor biomarkers in the liquid biopsy of cancer [3-9]. However, sensitivity and specificity of these biomarkers remain suboptimal [10, 11], which impede their widespread application to clinical practice. Therefore, the identification of new, highly sensitive and specific tumor biomarkers is particularly important.

Circulating tumor DNA (ctDNA) is released by the tumor cells into the blood and thus harbors the mutations of the original tumor [12]. In the past decade, groundbreaking studies on ctDNA have been carried out, facilitated by advances in the cancer genome project (CGP) and new applications of next generation sequencing (NGS) technology. In addition to being noninvasive, researchers have found that the screening of genetic lesions using ctDNA is highly sensitive and specific [2, 10], suggesting that the use of ctDNA as a liquid biopsy may significantly improve current systems of tumor diagnosis, even facilitating early-stage detection. Moreover, ctDNA analysis is able to accurately determine the tumor progression, prognosis and assist in targeted therapy [13-17]. With the achievements of CGP and wide application of NGS, there is a growing expectation that liquid biopsy based on ctDNA analysis heralds a revolution for cancer diagnosis, prognosis and treatment.
THE RESEARCH HISTORY OF ctDNA

In 1977, researchers made the novel observation that cancer patients carried cell-free DNA in their peripheral blood [18]. Initial progress on further characterization of cell-free DNA was frustratingly slow due to the technological limitations of the pre-genome era. It would be another 17 years until researchers proved unequivocally that this species of nucleic acid was derived from tumor tissues by virtue of the presence of characteristic cancer mutations [12, 19]. Indeed, significant progress was not made until recent 10 years with the advent of NGS technology in combination with the early findings of CGP, which significantly improved the sensitivity and specificity of ctDNA detection [13, 20]. Subsequently, research in this field has entered a “golden age” in which the huge potential of ctDNA investigations in tumor diagnosis and treatment is becoming ever clear [2, 21-24].

THE BIOLOGY OF ctDNA

The biological characteristics of ctDNA

cDNA is single- or double-stranded DNA, and exists in plasma or serum. Early studies showed that ctDNA possessed many cancer-associated molecular characteristics, such as single-nucleotide mutations [25-29], methylation changes [30-33] and cancer-derived viral sequences [34-36], and therefore were considered to be derived from tumor tissue. These findings were significant for the development of future ctDNA detection technology [37-40]. In recent years, researchers have further demonstrated the potent applications of ctDNA in clinical practice. However, many biological characteristics of ctDNA remain unclear. For instance, the size of ctDNA fragments is still undetermined: some believe that it is longer than corresponding non-tumor cell-free DNA (cfDNA) [41, 42], and others believe the opposite [27, 43]. Such controversy might be explained by differences in the detection methods and sample sources used by different groups. In a recent study, Jiang et al. found that the plasma of liver cancer patients harbored both extremely long and short DNA molecules, and the short fragments tended to contain the tumor-relevant copy number aberrations [44]. Madhavan et al. reported a similar phenomenon in breast cancer patients [45]. These studies suggest that ctDNA is shorter than non-cancer cell-free DNA, and take an important step in solving the controversy. Additionally, whether ctDNA exists as complex is also a current study focus [46].

The mechanism of ctDNA entry into the bloodstream

Though the existence of ctDNA is widely accepted, the mechanisms by which tumor DNA enters the bloodstream remain unclear. It has been suggested that there are three potential origins of ctDNA (Figure 1): 1) apoptotic or necrotic tumor cells, 2) living tumor cells, and 3) circulating tumor cells [46-49]. Actually, it is very likely that there are multiple origins for ctDNA rather than just one.

Several previous studies have reported that fragment sizes of cfDNA are around 166 bp, similar to those released by typical apoptotic cells [46-47]. Consistently, ctDNA display a ladder-like distribution after electrophoresis [48]. It has been widely accepted that the deregulation of proteolytic activities involved in apoptosis can lead to the release of DNA or nucleosomes into the blood circulation [50, 51]. Indeed, Roth C et al. found, the observed changes in apoptosis-related deregulation of proteolytic activities are along with the elevated level of DNA in blood [50]. Intriguingly, circulating nucleosome was significantly associated with DNA concentrations in the blood of patients and healthy subjects [50], which was supported by the previous observations of Holdenrieder S, et al. [52]. Based on the above findings, ctDNA may be released by apoptotic cells in the form of nucleosomes. Although most of the liberated nucleosomes are engulfed and digested by macrophages, this elimination system can be overloaded or impaired in case of tumor progression and enhanced cell death, resulting in high levels of nucleosomes entering into the bloodstream [50, 52]. In addition, cancer patients with a large number of necrotic tumor cells in advanced stage have more plasma ctDNA than the patients in the early stage [10]. These data support the view that apoptotic or necrotic tumor cells are likely to be the main origin of ctDNA.

However, cancer patients in the early stage also contain plasma ctDNA [2, 10], and therefore it is likely that apoptotic or necrotic tumor cells are not the only source. In vitro studies found that living tumor cells, like lymphocytes, can continuously and automatically release DNA [49], which might explain the presence of detectable ctDNA in patients with early-stage cancer. In addition, the amount of ctDNA increases with tumor growth [13], further supporting the hypothesis that ctDNA might be derived from living tumor cells. Evidence also supports a third scenario, in which DNA is released from CTC. Firstly, ctDNA and CTC have been shown to contain identical genetic mutations. Also, CTC can evade the macrophage clearance and easily enter the blood. Moreover, it has also been suggested that blood that contains CTC also contains ctDNA [10]. These findings support the view that CTC might be another source of ctDNA. Since peripheral blood only contains a few of CTC, ctDNA from CTC might not be the main origin.
The biological function of ctDNA

Generally, metastases represent the end-products of the invasion-metastasis cascade, which involves the development of the invasiveness capacity of cells in primary tumors, with subsequent blood dissemination of such cells and extravasation and metastasis to distant sites [53, 54]. However, this theory has been challenged in the last decades owing to the fact that accumulating evidence has indicated ctDNA might play a key role in cancer metastasis through oncogenic transformation of susceptible cells [54-57].

In 1999, García-Olmo et al. showed that plasma from tumor bearing rats could stably transform normal cells cultured in vitro and for the first time proposed the hypothesis of genometastasis: “metastasis might occur via transfection of susceptible cells, located in distant target organs, with dominant oncogenes that are derived from the primary tumor and are circulating in the plasma” [55]. Consistently, the serum of cancer patients and supernatant of human cancer cells were also able to induce in vitro normal cell transformation and tumorigenesis, while this process did not occur if serum and supernatants were deprived of DNA [57, 58]. In subsequent studies, García-Olmo et al. found in untreated, tumor-bearing rats as well as in surgically treated ones that hematogenous dissemination of tumors appeared to be more closely related to ctDNA than to CTC [59, 60]. It was also showed in rats that not only the infection of tumor cells but also the recruitment of host cells was essential for tumor formation [61]. Furthermore, Roth C et al. found the presence of distant metastasis associated with a significant increase in DNA levels [50]. Their findings also supported the previous observations of Diehl F et al., which suggested that as tumors invaded through the intestine to distant sites, the number of ctDNA molecules progressively increased [27]. Intriguingly, ctDNA could be horizontally transferred between the tumor cells and normal cells via uptake of apoptotic bodies or virtosomes [54, 58, 62], resulting in distant metastasis (Figure 1). Taken together, these data showed that ctDNA might have properties for integrating into susceptible cell genome and transforming these cells oncogenically.

The hypothesis of Genometastasis seems to be a reasonable explanation for cancer metastasis. However, present studies were still not sufficiently potent to prove the pro-metastasis function of ctDNA in consideration of incomplete study design and the absence of trails in vivo, especially in clinic. It is necessary to further verify in the future.

**METHODOLOGIES FOR DETECTION OF ctDNA**

Detection of plasma ctDNA is not only good for the study of cancer pathogenesis, but also beneficial to the clinical management of cancer. Because ctDNA has been shown to possess the characteristic mutations of the corresponding primary tumor, researchers have tried to take advantage of this feature in designing assays that may be used in cancer management. However, ctDNA-based assays are confronted with several challenges, not least that ctDNA accounts for only a small percentage (sometimes < 0.01%) of the total cell-free DNA in the

![Figure 1: The potential origins of ctDNA and the hypothesis of genometastasis.](image)

Three potential origins of ctDNA: (1) apoptotic tumor cells, (2) Living tumor cells, and (3) circulating tumor cells. Additionally, according to the hypothesis of genometastasis, ctDNA may transform normal cells to tumor cells, resulting in distant metastasis.
peripheral blood and that prior knowledge about particular mutations is usually required, which may be hard to obtain [2].

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 potential bias introduced by the PCR methodology [13]. In the last decade, the advances in NGS technology have allowed researchers to develop many effective and convenient alternatives to Sanger sequencing. Diehl et al. developed a technique called BEAMing (beads, emulsion, amplification, and magnetics) to detect ctDNA in blood [20]. In this technique, the object DNA segment is amplified using primers containing known tag sequences, and then covalently bound to magnetic beads. Finally, flow cytometry is used to sort beads containing the mutation. Newman et al. developed another new technique called CAPP-seq (cancer personalized profiling by deep sequencing) for quantifying ctDNA. They designed a probe panel consisting of biotinylated DNA oligonucleotides that target recurrently mutated regions in the cancer of interest. Using this technique, they detected ctDNA in 100% of stage II-IV and 50% of stage I NSCLC patients, with 96% specificity for mutant allele fractions down to ~0.02% [13]. Compared with previous methods, these new techniques provide significantly higher sensitivity for ctDNA detection. They are also high-throughput and less expensive. Such “second generation” sequencing techniques have been essential in fully evaluating the clinical potential of ctDNA analysis. However, there are also limitations to these novel techniques. Firstly, NGS-based methods provide an informative diagnosis in only around 50% of early-stage patients [2, 13], therefore the sensitivity requires further improvement. Additionally, the costs remain relatively high, limiting their application in clinical practice. Fortunately, third-generation sequencing techniques, designed to be highly sensitive and inexpensive, have rapidly advanced and have the potential to expedite extensive application of ctDNA detection for routine patient management [63-69].

**THE CRITICAL ROLE OF ctDNA IN CANCER DIAGNOSIS AND PROGNOSIS**

Tissue biopsy is still the gold standard for tumor diagnosis. However, many shortcomings exist. For instance, there are risks associated with invasive sampling, particularly when applied to the fragile organs such as lung, and sensitivity is suboptimal, frequently resulting in an inability to detect early-stage tumors. In addition, because tumors are heterogeneous and constantly evolving [70-75], tissue biopsy-based investigations are often unable to accurately determine tumor progression [2]. Similarly, they also struggle to detect small, residual lesions following therapy. In recent years, it has been suggested that a plasma biomarker-based approach can evaluate the tumor occurrence, progression and recurrence. Such approach is minimally invasive with satisfactory conformity [3]. However, previous tumor biomarkers in plasma only provide limited sensitivity and specificity, and therefore cannot always meet the clinical requirements [2, 10].

cDNA has now been widely evaluated as a novel biomarker for liquid biopsy in cancer diagnosis and prognosis [2, 3]. Liquid biopsy based on ctDNA is superior to that of previous plasma biomarkers in two main areas: 1) sensitivity, and 2) clinical correlations. AFP, CEA, PSA, and CA15-3 are plasma protein biomarkers commonly used in clinical management [76-81]. However, the positive proportion of patients for these biomarkers is usually between 50–70% in cancer patients. Furthermore, they are also found in the serum of individuals without cancer, although in lower concentrations [2, 10, 82, 83]. Dawson et al. assessed ctDNA, CTC, and CA15-3 in 30 metastatic breast cancer patients and found that the detection rate of ctDNA reached 97%, whereas rates for CTC and CA15-3 were only 78% and 87%, respectively [78]. Bettegowda et al., in a study of 206 metastatic cancer patients, found that the sensitivity of ctDNA detection was 87.2% [10]. In their further studies, ctDNA and CTC were tested in 16 cancer patients, revealing that 13 patients who were ctDNA-positive were negative by CTC test. The three patients with both ctDNA and CTC positive contained 50-fold more ctDNA than DNA obtained from CTC [10]. Together, these studies indicate that ctDNA is more sensitive than protein biomarkers and CTC.

Importantly, the half-life of ctDNA is less than 2 hours, whereas the half-life of protein markers in plasma can be several weeks. This means that ctDNA can more accurately reflect the real-time tumor burden in patients receiving therapy. Indeed, ctDNA has been shown to correlate well with tumor load and likelihood of recurrence. Researchers tested blood samples from various cancer patients and found that ctDNA were present at significantly different levels among the patients with different cancer stages. Specifically, patients with advanced-stages of gastro-esophageal, pancreatic, breast and colorectal cancer had a higher level of ctDNA than patients in early stages of those diseases [10, 84]. Additionally, researchers analyzed ctDNA in relapsing and non-relapsing patients, and found that ctDNA could be used to monitor relapse status, resulting a 10-month lead-time on detection of relapse compared with the conventional follow-up [35]. A study by Garcia-Murillas I et al. found similar results [85]. Therefore, ctDNA has potential to be used for the evaluation of tumor progression and prognostic. In summary, liquid biopsy based on ctDNA analysis might represent the next generation of tumor diagnostic and prognostic testing on account of its high accuracy and sensitivity.
THE CRITICAL ROLE OF ctDNA IN CANCER TREATMENT

Genotyping and assistance in personalized, targeted therapy

Genotyping is aimed at the analysis of genetic mutations and has important applications in managing cancer treatment [86-89]. In recent years, it has been suggested that genotyping has the potential to play a crucial role in precision medicine, especially in precision immunotherapy, via facilitating the development of new therapeutic protocols based on known or unknown key genetic lesions [90]. For example, in the exploitation of precision immunotherapy strategies, researchers can potentially screen for the antigens that induce strong immune responses according to the genotyping information and then find and enrich the T cells that can be used in personalized therapy to target those new antigens [90].

In current clinical practice, genotyping is achieved using DNA obtained from the tissue biopsy. However, tissue biopsy can only obtain local and static tumor information, and is unable to reflect the real-time tumor genotyping due to heterogeneity and constant evolution of tumors [1]. ctDNA analysis overcomes these problems by reflecting the genetic mutations of the whole tumor tissue. Additionally, ctDNA from the same patients at different stages can be used to dynamically monitor the genetic mutations during the cancer progression [14, 91]. Therefore, Liquid biopsy based on ctDNA analysis might improve tumor genotyping and targeted cancer therapy, which would be of significant benefit to the field of personalized medicine.

Disease monitoring and treatment evaluation

Cancer often relapses and evolves during the treatment. Disease monitoring and treatment evaluation are important for clinician to determine subsequent treatment protocols. It has been shown that serial ctDNA detection can be used to evaluate treatment efficacy by assessing remission status and detecting relapse and progression [10, 20, 92-95]. Using ctDNA, the detection rates among the patients with stage I, II, III, and IV cancer were 47, 55, 69, and 82%, indicating that ctDNA levels increase with cancer progression [10]. Additionally, Diehl et al. found that majority of the 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 [20]. Reinert et al. found that there was 10 months’ lead-time on detection of relapse using ctDNA detection compared with conventional follow-up [92]. These studies indicate that ctDNA detection can quickly predict the therapy outcomes, and provide an important reference for clinicians in determining the next treatment protocols.

Illustration of the mechanisms of therapy resistance

Therapy resistance is the major cause of cancer treatment failure. However, so far appropriate methods of investigating tumor resistance have not yet been established [37, 96]. Recently, studies on ctDNA have provided new insight into this area [96-101]. Diaz et al. analyzed the ctDNA of lung cancer patients who were subject to EGFR inhibitor treatment and found 42 KRAS gene mutations, which are known to be markers of therapy resistance. Further studies showed that this method could provide 5 months’ lead-time on detection of tumor evolution and resistance compared with the conventional method [97, 98]. In addition, Murtaza M et al. collected blood samples from six patients with advanced-stage breast cancer, lung cancer or ovarian cancer, and performed whole-exome sequencing. They found frequent mutations in genes involved in pathways relevant to the development of cancer resistance. For instance, they found the mutations that blocked the binding of drug to its biological targets [96]. Therefore, ctDNA detection can be used to monitor cancer evolution, illustrate the mechanisms of the tumor resistance, and guide drug selection for the clinicians.

IMPLICATIONS AND FUTURE DIRECTIONS

Recent advances in our understanding of the biology and clinical application of ctDNA have provided evidence that the use of ctDNA as a liquid biopsy can improve cancer diagnosis and treatment via genotyping, disease monitoring, treatment evaluation, and so on. However, there are still some challenges that should be addressed so that this technique may eventually be implemented into routine clinical practice. Firstly, to use ctDNA as a diagnostic marker, it will be important to gain a better understanding of the biological characteristics of ctDNA, including its size, existing form, and the mechanisms by which ctDNA is released into the bloodstream. Additionally, though the clinical correlations of ctDNA analysis have been verified, adoption of this technique into routine clinical practice still need to further demonstrate its analytic validity, clinical validity, and, most importantly, clinical utility in consideration of the suboptimal sensitivity of the methods for ctDNA detection and the limited sample quantity. These challenges indicate that it will take some time to introduce ctDNA analysis
into clinical practice [2, 102]. However, as sequencing technologies quickly develop, and the understanding of ctDNA biology and clinical potential deepens, the eventual use of ctDNA in clinical practice seems to be assured.

**CONFLICTS OF INTEREST**

The authors declare no conflict interest.

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