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The feasibility of using mutation detection in ctDNA to assess tumor dynamics

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Mini Review

For many decades it has been known that tumor DNA is shed into the blood. As a consequence of technological limitations, researchers were unable to comprehensively characterize circulating DNA. The advent of ultrasensitive and highly specific molecular assays has provided a comprehensive profile of the molecular characteristics and dynamics of circulating DNA in healthy subjects and cancer patients. With these new tools in hand, significant interest has been provoked for an innovative type of tumor biopsy termed a "liquid biopsy". Liquid biopsies are obtained by minimal invasive blood draws from cancer patients. Circulating cancer cells, exosomes and a variety of molecules contained within the liquid biopsy including cell-free circulating tumor DNA (ctDNA) can serve as promising tools to track cancer evolution. Attractive features of ctDNA are that ctDNA isolation is straightforward, ctDNA levels increase or decrease in response to the degree of tumor burden and ctDNA contains DNA mutations found in both primary and metastatic lesions. Consequently, the analysis of circulating DNA for cancer-specific mutations might prove to be a valuable tool for cancer detection. Moreover, the capacity to screen for ctDNA in serial liquid biopsies offers the possibility to monitor tumor progression and responses to therapy and to influence treatment decisions that ultimately may improve patient survival. Here we focus on mutation detection in ctDNA and provide an overview of the characteristics of ctDNA, detection methods for ctDNA and the feasibility of ctDNA to monitor tumor dynamics. Current challenges associate with ctDNA will also be discussed.

The diagnosis, staging and therapeutic management of tumors relies upon cancer imaging systems and histologic or cytologic examination of tumor specimens.[1,2] The widespread integration of biomedical imaging systems with other diagnostic and screening tools has contributed to decreased mortality rates for certain cancer types.[1,2] Although a tumor biopsy is a key diagnostic tool that also guides therapy, tumor biopsies have limitations. For instance, tumor location and size may limit the utility of biopsies. With regard to the molecular diagnosis of DNA mutations, a tumor biopsy may not fully capture the molecular heterogeneity within a tumor.[2–4] Consequently, a tumor may respond poorly when only a portion of tumor cells within a tumor mass harbor genetic mutations that render them susceptible to targeted molecular therapies.[4] Moreover, the molecular differences between primary and metastatic tumor lesions not captured by tumor biopsies may negatively impact overall tumor burden in patients.[3,5] Despite these limitations, tumor biopsies remain a primary diagnostic tool as histopathologic examination of tumor specimens is required to render an accurate medical diagnosis.

Recent technological advancements in next-generation sequencing and quantitative polymerase chain reaction (PCR) have contributed to the growing interest amongst clinicians and researchers in an innovative type of biopsy termed a liquid biopsy.[2,5,6] In a liquid biopsy, blood is drawn, processed and then analyzed for the presence of circulating tumor cells (CTCs),[7] cancer-derived exosomes[8] and cell-free tumor DNA (ctDNA).[4] The major obstacle for liquid biopsies to be effective as diagnostic and screening tools is sensitivity since the majority of cells in the blood are normal cells and blood also contains exosomes and cell-free DNA released by normal cells.[4,9] For example the frequency of cell-free DNA containing tumor-associated mutations might be as low as 0.01%[4,6,9] and between 0 and 6 CTCs have been detected per 7.5 mL of peripheral blood.[7,10] To meet this challenge, researchers have devised a variety of highly sensitive and specific molecular diagnostic techniques.[6,10] An attractive feature of liquid biopsies is the potential to monitor tumor progression and response to therapy in a minimal-invasive manner.[9] In addition, the analysis of

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Abbreviations: cfDNA: cell free DNA; CTC: circulating tumor cell; ctDNA: circulating tumor DNA; dPCR: digital PCR; NGS: next-generation sequencing; NSCLC: Non-small cell lung cancers; PCR: polymerase chain reaction.

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serial liquid biopsies may lead to the identification of newly acquired DNA mutations in therapy resistant tumors that make them vulnerable to other cancer drugs.[11]

Circulating tumor cells (CTCs)[10] and tumor-derived proteins,[12] RNAs.[8] and ctDNA[4] are considered as potential cancer biomarkers contained within a liquid biopsy. The isolation, characterization and analysis of each of them have their unique challenges. The advent of highly sensitivity PCR methods and refinements to next-generation sequencing (NGS) has sparked much interest in ctDNA as a viable tool to provide diagnostic and prognostic information of cancers monitoring.[2] These include DNA mutations, epigenetic alterations and other forms of tumor-specific abnormalities. Here, we provide a discussion on the functionality of ctDNA as a clinically feasible tool to monitor tumor development by detecting tumor specific mutations.

Origins and Molecular Characteristics of ctDNA

In healthy individuals, cell free DNA (cfDNA) is released into the circulation by cells undergoing apoptosis.[13,14] Apoptotic cells shed DNA fragments approximately 185 to 200 bp in length.[14,15] In contrast, cfDNA of cancer patients is derived from both non-malignant and malignant cells. Interestingly, the percentage of circulating DNA derived from cancer cells ranges from 3% to as much as 93%.[16] Since cancer cells die through multiple mechanisms that ultimately lead to DNA cleavage such as apoptosis, necrosis and autophagy,[17] ctDNA displays less uniformity in size and integrity relative to cfDNA in healthy individuals.[14,15,17] Some researcher groups report that ctDNA has less integrity and is smaller relative to cfDNA[13–15] while others report the opposite.[18,19] These conflicting results may be a result of the different DNA isolation methods used and the sample sources tested. More recently, one study showed that decreased cfDNA integrity and increased cfDNA concentrations distinguish normal individuals from patients with primary and metastatic breast cancer.[20] A second study used single-base pair resolution sequencing to demonstrate that the shorter DNA fragments in cancer patient plasma preferentially contained tumor-associated copy number alterations.[21] Collectively, these two reports suggest that the isolation and analysis of short DNA fragments may improve the sensitivity of detection of ctDNA. Irrespective of DNA fragment size, the ability to detect and quantify ctDNA fragments carrying tumor-specific mutations has maintained interest in ctDNA as a useful tool to monitor tumor growth.

Detection of ctDNA

Given that the frequency of mutant DNA alleles in cfDNA is as low as 0.01%,[4] highly sensitive and specific detection methods are required for ctDNA to serve as a clinically feasible approach. Here we provide a brief description of the ctDNA detection methods capable of such high specificity and sensitivity. Initial studies on ctDNA relied upon real-time allele-specific PCR to detect mutations of interest.[22] However, primarily patients with high tumor burden, and thus higher ctDNA levels in the plasma,[23] were chosen for these studies as the sensitivity and specificity of real-time PCR approaches is limited.[22] Digital PCR (dPCR) has increased sensitivity and specificity relative to real-time PCR.[22] With dPCR, a DNA sample is segregated using limiting dilution such that individual DNA molecules are captured within water-oil emulsion droplets or chambers.[24] A portion of the droplets or chambers will contain no DNA while others will contain individual DNA molecules. The segregated individual DNA molecules may be wild type or mutant in nucleotide sequence. Using unique sets of primers and probes, mutant and normal DNA sequences are amplified, quantified and the percent mutant allele frequency determined. For liquid biopsy samples, dPCR is capable of detecting mutant alleles with a fractional abundance of 0.005%[25] to 0.04%.[26] There are several advantageous features to dPCR including precision, sensitivity and increased resistance to PCR inhibitors.[24,26,27] In addition, dPCR is capable of absolute measurements of rare DNA alleles without the need for a standard curve.[24,28,29] Recently novel modifications to dPCR have been introduced with the intention of detecting highly infrequent mutant DNA molecules in plasma.[9,30–32] The ability to detect these extremely rare mutant DNA molecules might allow for efficient screening and diagnosis of cancers at earlier stages with liquid biopsies.

The analytical sensitivity of dPCR is one advantageous feature that is counterbalanced by the limited capacity of dPCR to interrogate multiple genomic alterations simultaneously.[33] Without prior and precise knowledge of the DNA rearrangements, translocations and mutations of interest, dPCR is compromised in its utility to detect cancer and monitor tumor growth. Massive parallel sequencing, also known as next-generation sequencing (NGS), offers to circumvent this limitation as NGS has the ability of detect multiple somatic DNA alterations simultaneously.[33–35] Early ctDNA studies with NGS demonstrated the ability of NGS to detect chromosomal rearrangements and chromosomal copy number changes.[35,36] In the setting of metastatic prostate cancer, Heitzer and colleagues[36] devised a genome-wide NGS approach capable of identifying novel genomic alterations in ctDNA associated with the emergence of metastatic tumor clones. Impressively, this approach provided genomic cancer profiles in 2 days thus making it a more feasible test in a clinical setting.[36] A shortcoming of most NGS-based approaches is the low sensitivity that renders them unfit to be reliable molecular diagnostic tests.[34,35,37] More recently, Newman and colleagues devised an ultrasensitive NGS-based approached termed CAncer Personalized Profiling by deep Sequencing (CAPP-Seq) with the ability to detect mutant allele fractions as low as 0.02% and with 96% specificity.[38] Importantly, CAPP-Seq reduces cost to approximately $200–300 US dollars per test.[33] Further refinements to both dPCR and NGS may ultimately make ctDNA cancer screening a reality.

Cancer Surveillance With ctDNA

A small number of studies have examined the feasibility of ctDNA in detecting early stage cancers and fewer still have considered ctDNA for screening pre-symptomatic cancer

patients.[39–43] Since effective screening of asymptomatic patients requires prior knowledge of mutations in genes commonly altered in low grade cancers, ctDNA is most suited for the detection of advanced cancers with well-known mutations. Complicating matters further is the observation that healthy subjects have detectable mutations in their circulating DNA. For instance, during minimal-invasive prenatal testing, Amant and coworkers detected genomic alterations in 3 of 4,000 asymptomatic pregnant women that were later confirmed to have malignancies by magnetic resonance imaging and biopsy.[40] In addition, the percentage of individuals with mutations in the blood increases with age[44,45] at a rate that exceeds the clinical incidence of hematological malignancies in the general population.[44] Moreover, independent studies reported that mutations in *KRAS* and *TP53* in the circulating DNA of non-cancer control subjects range from 1 to 3.6%.[46] Collectively these studies suggest that cfDNA of healthy subjects contains mutations that might pose a significant challenge in accurately diagnosing and screening patients for cancer.[47] On the other hand, presymptomatic testing for cancer might be more feasible with ctDNA than originally thought. Interestingly, Sausen and coworkers demonstrated that pancreatic cancer recurrence was predicted 6.5 months earlier with ctDNA detection relative to CT scanning[48]; thus supporting the notion that ctDNA might be useful in screening and diagnosing asymptomatic patients. Additional studies combining ctDNA analysis with biomedical imaging will ultimately determine the benefits of ctDNA in cancer surveillance as an adjunct diagnostic test.

Monitoring tumor burden and responses to therapy using ctDNA

Studies in multiple cancers have shown a pattern where ctDNA levels buildup as tumors progress and then decline following surgery or drug treatment.[23,32,37,49,50] Thus it seems logical that ctDNA levels might be used as a surrogate marker of tumor burden and therapeutic responses. Conceptually, ctDNA has clear advantages to monitor tumor burden and therapeutic response compared with protein cancer markers and biomedical imaging. For instance, ctDNA has a half-life of approximately 2 h[9] while proteins persist in the blood for weeks to months,[51,52] thus analysis of ctDNA should permit a more rapid assessment of tumor changes within hours rather than weeks to months.[53] Tumor specific protein markers have the limitation of being elevated in circumstances not related to tumor growth[54] while elevated ctDNA levels are associated with tumor changes. Moreover, a direct comparison of ctDNA, CTC and cancer antigen 15–3 levels in the same patients revealed that ctDNA levels showed a greater correlation with tumor burden and the earliest measure of response to therapy,[23] suggesting that ctDNA levels more accurately predict tumor changes relative to protein markers[55] (Fig. 1). With regard to imaging techniques, increases in ctDNA levels predicted tumor recurrence much earlier than conventional tumor imaging techniques.[38,48,56]

The genetic basis of resistance by tumors to targeted molecular therapies has recently been determined.[57–60] Non-small cell lung cancers (NSCLC) bearing activating mutations in the EGFR gene are extremely sensitive to the small molecule inhibitors erlotinib and gefitinib.[59] In approximately 50% of NSCLC patients resistance to EGFR inhibition is driven by a gatekeeper mutation in the EGFR kinase domain that changes threonine 790 to a methionine (T790M). The T790M mutation increases the affinity of the EGFR kinase domain for ATP while decreasing its affinity to erlotinib and gefitinib. In the case of colorectal cancers, mutations in KRAS (G12R and G13D) activate survival pathways that drive resistance to EGFR monoclonal antibodies.[56] With such precise knowledge of the molecular basis of response to targeted therapies, the possibility of detecting resistance in a minimal-invasive manner in the blood has been examined.[61,62] As might be predicted, detection of the T790M gatekeeper mutation was shown to correlate with therapy resistance and disease progression for NSCLC patients treated with EGFR inhibitors.[11,63] Impressively, in some cases, T790M-driven resistance was detected 16 weeks before tumor progression was detectable with radiographic imaging.[63] In colon cancer, ctDNA analysis revealed that the presence of mutant KRAS was associated either primary or acquired resistance to EGFR inhibition.[62,64] Upon withdraw of anti-EGFR antibodies, mutant Ras levels dropped and relatively novel mutations associated with either primary or acquired resistance were found in *NRAS, MET, ERBB2, FLT3, EGFR and MAPK21*.[62]

Thus far, several studies show that the analysis of ctDNA levels is a powerful tool to monitor tumor dynamics and response to therapy. In analyzing ctDNA, novel mutations associated with resistance to conventional and targeted therapy have been identified.[62,65] These newly identified mutations might offer new avenues of treatment for cancer patients with advanced disease.

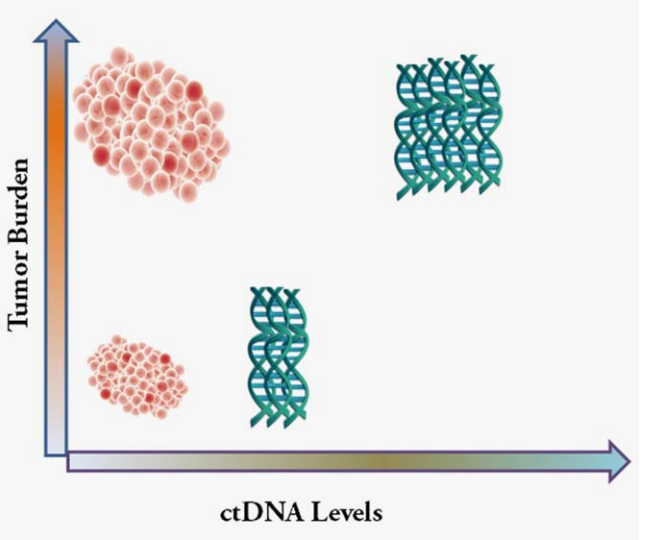

Figure 1. Tumor burden and plasma ctDNA levels show a direct correlation. As tumor burden increases, ctDNA accumulates in the plasma. With therapeutic intervention, tumor burden and ctDNA levels decrease. Thus, ctDNA can serve as a surrogate marker of tumor progression and regression. [Color figure can be viewed at wileyonlinelibrary.com]

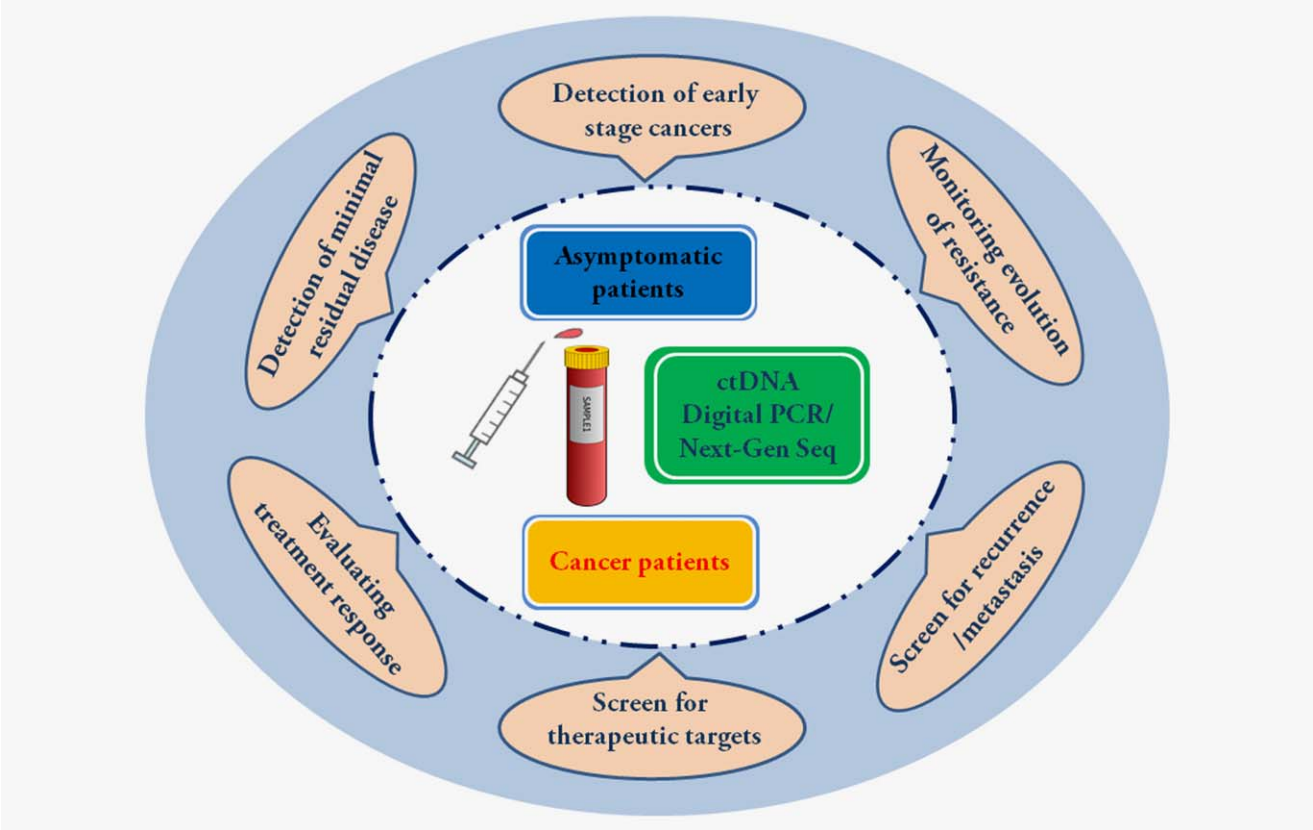

Figure 2. Clinical application of ctDNA as a tool for cancer monitoring. ctDNA can be obtained from plasma and in combination with digital PCR and next-generation sequencing (Next-Gen Seq) might allow for detection of cancers at their earliest stages. In some reports, ctDNA detection predicts cancer recurrence or progression months earlier than conventional cancer imaging methods. ctDNA could be applied in cancer diagnosis, prognosis and therapy monitoring. [Color figure can be viewed at wileyonlinelibrary.com]

Current Challenges and Limitations

Although ctDNA represents a promising "real time" tool for tumor characterization and is now being extensively studied, there are still many challenges that need to be overcome before it's routinely used by clinicians. One major challenge with analysis of ctDNA is sensitivity and specificity. ctDNA is not always detectable in peripheral blood due to the extremely low level presented in the blood. In addition, current digital PCR method preferably measures relatively longer fragments, *i.e.*, >120 bp, while smaller fragments derived from tumors are not detected efficiently.[66] Therefore, the results from ctDNA analysis might be false negative. Many studies have shown an average of 70–80% concordance between tumor somatic mutations and the presence of ctDNA.[67,68] This could be improved by the advances in genomic approaches that have higher sensitivity to identify all ctDNA in the blood. Additionally, we do not have a standard and validated procedure for samples handling and liquid biopsy analysis, both aspects would affect the final results. A standard operation procedure should be established and be strictly followed to ensure we get the right information from ctDNA analysis.

In addition to ctDNA, CTCs, cell free RNAs and exosomes also attract extensive attention for their potential clinical application. Compared to these measurements, which can address tumor status at DNA, RNA and protein levels, ctDNA results are restricted to genomic alterations. Some drug resistance can only be reflected by mRNA or protein expression analysis, such as AR-V7.[69] In this situation, ctDNA analysis may not be a good choice to monitor tumor development. In contrast to tumor tissues, ctDNA represents mainly the genome of dying tumor cells, or the cells that respond to therapy, one cannot ruled the exist of resistant cells or subpopulations that contain few or no dying cells based on ctDNA analysis. Selection of the appropriate time points for ctDNA screening will be very important in some situation. Also, results from ctDNA analysis cannot determine the exact origin of a mutation, *i.e.*, whether it is somatic or germline. The discovery of germline mutations might be an important indication for genetic screening for the patients' family members as well.

Conclusion and perspectives

The shedding of ctDNA into the circulation offers researchers and clinicians a novel target that carries the same DNA mutations found in primary and metastatic cancer lesions. Access to ctDNA is minimal-invasive and simple as it involves a standard blood draw. With newly developed ultrasensitive and specific methods to detect and characterize genomic alteration present in ctDNA the opportunity exists to monitor tumor progression

and recurrence. Tumor burden and recurrence have been shown to directly correlate to ctDNA levels in patients and the frequency of ctDNA molecules carrying mutations that confer resistance to cancer therapies. In some studies, detection of ctDNA is superior in predicting tumor recurrence relative to conventional tumor imaging techniques. With further refinements to the methods to detect and quantify ctDNA, it may be possible to screen asymptomatic patients with ctDNA to detect cancers at their earliest stages (Fig. 2). As discussed above, many limitations associated with ctDNA still exist. Further studies should be launched to validate the potential of ctDNA for routine clinical use. However, based on the large amounts of promising data and with the rapid advances and the decreased cost of next-generation sequencing, we anticipate there is much promise in influencing cancer patient treatment and survival with ctDNA. In addition to ctDNA, cfRNA, CTCs, circulating exosomes or blood platelets are also promising as novel liquid biopsy candidates.[70–72] The advantages and disadvantages of these technologies are worth to be further explored.

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