EDITORIAL

Circulating cell-free mtDNA as a new biomarker for cancer detection and management

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Cancer is a major cause of death worldwide, and was responsible for 19.29 million new cases and 9.96 million deaths in 2020 alone. The total number of patients with cancer worldwide is estimated to reach 28 million by 2040. The American Association for Cancer Research has also estimated approximately 16.2 million cancer deaths by 20401. Currently, diagnostic and treatment options for cancer rely primarily on imaging, pathology, and serology, and thus are highly limited. Histopathology remains the gold standard in cancer diagnosis, yet is insufficient to detect or sample early tumors, or capture genetic heterogeneity between tumors and metastasis, thus decreasing detection accuracy. Imaging methods have additional limitations, such as insufficient resolution and difficulty in distinguishing benign from malignant lesions, thereby hindering their clinical utility. Serological markers are commonly used for clinical diagnosis, yet their low sensitivity and specificity limit their contribution to early cancer detection. Advances in omics technology and the accumulation of large data sets have enabled the identification of novel tumor biomarkers, which hold great promise for screening more cancer types and developing assays to improve existing screening strategies. Emerging omics technologies have led to the development of the precision oncology field, which has significantly extended the survival times of patients with cancer.

In summary, more sensitive, accurate, and noninvasive novel tumor markers remain necessary.

Recently, liquid biopsy techniques have been used to detect circulating tumor cells (CTC), cell free DNA (cfDNA), circulating tumor DNA (ctDNA), and exosomes released from primary or metastatic tumors, in cancer diagnosis and monitoring applications. Liquid biopsy techniques overcome the limitations of traditional tissue biopsy techniques through non-invasive sampling, real-time dynamic monitoring, and capturing of tumor heterogeneity. Plasma cfDNA originates from damaged cells and is cleaved by nuclease into fragments of approximately 160 bp. Because its production and clearance is dynamic (half-life of 5–150 min), cfDNA is an ideal molecular marker for various diseases2. Previous studies have evaluated changes in cell free nuclear DNA (cf-nDNA), focusing on the detection of tumor-specific mutations, copy number variations, and methylation patterns. Recently, “fragmentomics,” based on the fragmentation patterns of cf-nDNA, including fragment size, end motifs, and nucleosome relationships, has shown great promise as a new tool for cancer diagnosis. In addition to nuclear DNA, mitochondrial DNA (mtDNA) is found in cfDNA. With a size of 16,569 bp, full length mtDNA is much smaller than the nuclear genome. Mutation incidence is much higher in mtDNA than nDNA, owing to the lack of histone protection and an effective DNA damage repair system, in addition to the presence of highly reactive oxygen species in the environment. mtDNA somatic mutations have been reported to be widespread in a variety of cancers3. The accumulation of somatic mutations in mtDNA has also been confirmed lead to mitochondrial dysfunction and to promote the malignant development of tumors in several studies. In contrast to the 2 copies of nuclear DNA, each mitochondrion often contains hundreds to thousands of mtDNA copies.
Moreover, mtDNA copy numbers vary among cell types, and are typically high in metabolically active tissues, such as the skeletal muscle and liver. These characteristics enable easy qualitative and quantitative detection of cell free mtDNA (cf-mtDNA) in peripheral blood from patients. Recent large-scale sequencing studies have highlighted the roles of cf-mtDNA in various diseases including cancer. Advances in accurate measurement have recently enabled more comprehensive and specific analyses of cf-mtDNA features. As a novel tumor biomarker, cf-mtDNA holds great promise in precision oncology. In this editorial, we discuss techniques for cf-mtDNA detection and clinical applications in cancer detection and management.

Various techniques have been developed for the detection of mitochondrial genome copy number, fragmentomics, and mutations (Table 1). Quantitative real-time PCR (qRT-PCR) is the most frequently used method for mtDNA copy number detection. Relative quantification of mtDNA copy number can be achieved by designing primers for amplification of the ND1 gene in mtDNA along with primers for amplification of the HGB-1 gene in nDNA. However, qPCR’s utility is limited in the analysis of DNA with severe fragmentation or of poor quality from plasma, formalin-fixed paraffin-embedded tissues, and paleontological samples. Michelson et al. have reported a simple, high-throughput, cost-effective cf-mtDNA extraction method, MitoQuicLy, which can sensitively detect and quantify cf-mtDNA in copies/μL in a variety of biofluid sample types. Jiang et al. have determined ND4 and ND1 content by using qPCR, and have used their ratio as an indicator of plasma cf-mtDNA integrity. However, primer binding sites in fragmented cf-mtDNA are limited, thus leading to inaccurate results. Advances in sequencing have enabled detection of mtDNA copy number via whole genome sequencing (WGS) for any sample type. WGS can also be used to detect the fragmentomics of cf-mtDNA, including fragment size and end motifs. Zhang et al. have optimized DNA isolation and library preparation protocols to enrich shorter cf-mtDNA fragments in plasma. Similar methods are also available for detection of mtDNA mutations, including droplet digital PCR (ddPCR); beads, emulsion, amplification, magnetics (BEAMing); and next-generation sequencing (NGS) with targeted enrichment technologies, including targeted amplification and targeted hybrid capture. Targeted amplification enriches targeted regions with designed mtDNA-specific primers. However, highly variable sequencing depth within PCR amplicons has been found to severely decrease the coverage uniformity and accuracy of mutation identification. Therefore, researchers have optimized mtDNA sequencing by targeted amplification to achieve uniform coverage of the mitochondrial genome. For this purpose, primer-design methods have been adjusted, including the number of primer pairs, overlap length of amplicons, and primer modifications, thus laying a foundation for accurate mtDNA mutation detection. The quality of DNA also affects the efficiency of PCR amplification. Targeted amplification shows low enrichment efficiency for samples with poor DNA quality. Although targeted amplification is specific, complex primer-design methods, PCR false positives, and an inability to detect cf-mtDNA fragmentomics limit the utility of the method. The targeted hybrid capture technique enriches targeted regions by using biotin-labeled mtDNA-complementary probes. On the basis of long-range PCR amplification and 3 pairs of mtDNA specific primers combined with 6 pairs of nDNA specific primers, we have established a method for simultaneous mtDNA and nDNA capture probe hybridization, as well as a method for simultaneous detection of mtDNA copy number, fragmentomics, and mutation. The presence of circular mtDNA in plasma has recently been reported in several studies. Newell

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Method</th>
<th>References</th>
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<tbody>
<tr>
<td>Copy number</td>
<td>qRT-PCR</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ddPCR</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>WGS</td>
<td>6</td>
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<td>Targeted capture sequencing</td>
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<td></td>
<td>MitoQuicLy</td>
<td>8</td>
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<tr>
<td>Mutations</td>
<td>ddPCR</td>
<td>9</td>
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<tr>
<td></td>
<td>BEAMing</td>
<td>9</td>
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<tr>
<td></td>
<td>SMRT</td>
<td>10</td>
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<tr>
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<td>Targeted capture sequencing</td>
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<tr>
<td></td>
<td>Targeted amplicon sequencing</td>
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<tr>
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<td>qRT-PCR</td>
<td>12</td>
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<tr>
<td></td>
<td>WGS</td>
<td>6</td>
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<td></td>
<td>Targeted capture sequencing</td>
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qRT-PCR, quantitative real-time PCR; ddPCR, droplet digital PCR; WGS, whole genome sequencing; MitoQuicLy, mitochondrial DNA quantification by lysis; BEAMing, beads, emulsion, amplification, magnetics; SMRT, single molecule real time sequencing technology.
et al.\textsuperscript{11} have detected intact mtDNA in plasma by amplifying cfDNA with specific PCR primers for overlapping fragments of mtDNA. Subsequently, Ma et al.\textsuperscript{15} used the BfaI restriction enzyme to cleave mtDNA and distinguish linear from circular mtDNA: mtDNA fragments with BfaI cleavage features at both ends were defined as circular mtDNA, whereas mtDNA fragments without cleavage features or with one cleavage feature were defined as linear mtDNA. Both linear and circular mtDNA were found to be present in plasma. Whether circular cf-mtDNA forms through a different mechanism from that of linear cf-mtDNA remains unclear. The tumor specificity, diagnostic value, and characteristics of circular cf-mtDNA require further exploration and investigation. A stable detection technique for both circular and linear cf-mtDNA is also needed.

As detection technologies advance, cf-mtDNA is increasingly being used as a biomarker for cancer detection and management (Table 2). Yu et al.\textsuperscript{21}, in 2012, proposed that cf-mtDNA is a potential tumor marker presenting both opportunities and challenges. The authors stressed that large prospective cohort studies on various solid tumors would allow for precise definition and evaluation of cf-mtDNA, which are important in the diagnosis and follow-up of malignant diseases. Several studies have further indicated significantly elevated cf-mtDNA in hepatocellular carcinoma (HCC), lung cancer, endometrial cancer, breast cancer, and colorectal cancer. The plasma cf-mtDNA content of patients with papillary thyroid carcinoma is significantly lower than that in healthy individuals, thus aiding in treatment planning\textsuperscript{16}. Longitudinal detection can further confirm the diagnostic performance of cf-mtDNA. Through dynamic monitoring of plasma cf-mtDNA content in patients with advanced ovarian cancer receiving chemotherapy, Kalavska et al.\textsuperscript{19} have found a significant decrease in plasma cf-mtDNA content after chemotherapy, in line with prognosis. By comparing patients with non-small cell lung cancer (NSCLC), patients with chronic obstructive pulmonary disease (COPD), and healthy controls, Bulgakova et al.\textsuperscript{17} have found significant associations of elevated cf-mtDNA copy number with the development of COPD and NSCLC, and poor prognosis, thus suggesting that cf-mtDNA can be considered a biomarker for NSCLC diagnosis and prognosis. The cf-mtDNA integrity (the ratio of ND4 content to ND1 content) calculated by Jiang et al.\textsuperscript{13} also has value in cancer detection, because increased release of intact mtDNA, as detected in plasma, is a tumor-specific process. Defining the mtDNA integrity as the ratio of the number of 230 bp mtDNA fragments to the number of 79 bp mtDNA fragments, Meng et al.\textsuperscript{12} have found lower mtDNA integrity in patients with epithelial ovarian cancer than healthy controls. Elevated cf-mtDNA has also been found to be significantly associated with progression and poor prognosis in ovarian cancer. The difference in fragment characteristics of cf-mtDNA has also been confirmed to be useful for distinguishing between patients with versus without cancer. An et al.\textsuperscript{20} have analyzed plasma cfDNA from patients with HCC and prostate cancer, and found an inverse correlation of cf-mtDNA length with tumor size and ctDNA concentration. Their results have further suggested that monitoring cf-mtDNA fragment size in patients with cancer may be an effective method for estimating tumor burden and cancer progression. Li et al.\textsuperscript{6} have extracted plasma cfDNA and exosome DNA from 15 clinical samples of patients with HCC, patients with hepatitis B, and healthy controls, and analyzed them through WGS and mtDNA capture sequencing technology. Their findings have revealed a relatively longer distribution of exosomes mtDNA fragments in patients with hepatitis B. cf-mtDNA mutations have also been a focus of recent studies. Weerts et al.\textsuperscript{10} have used single molecule real time (SMRT) sequencing technology

\begin{table}[h]
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\caption{Applications of cf-mtDNA as a biomarker for cancer detection and management}
\begin{tabular}{llll}
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Application & Indicator & Cancers & References \\
\hline
Non-invasive early diagnosis & Copy number & HCC, PTC, NSCLC, GBM & \textsuperscript{5,7,10,16-18} \\
& Mutations & HCC, BC, CRC, PDA & \\
& Fragmentomics & GBM & \\
Tumor burden and cancer progression & Copy number & OV & \textsuperscript{12,18-20} \\
& Fragmentomics & NB & \\
& Mutations & OV, HCC, PCa & \\
Design cancer treatments & Copy number & OV, PTC & \textsuperscript{16,19} \\
Clinical efficacy evaluation & Copy number & PTC & \textsuperscript{16} \\
Clinical prognosis indication & Copy number & OV, NSCLC & \textsuperscript{12,17,19} \\
& Fragmentomics & OV & \\
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\end{tabular}
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HCC, hepatocellular carcinoma; PTC, papillary thyroid cancer; NSCLC, non-small cell lung cancer; BC, breast cancer; CRC, colorectal cancer; PDA, pancreatic ductal adenocarcinoma; GBM, glioblastoma; OV, ovarian cancer; PCa, prostate cancer; NB, neuroblastoma.
to detect mtDNA mutations in 19 tissue and 9 plasma samples from 8 patients with cancer (5 with breast cancer and 3 with colon cancer). However, the method was found to have limited value in tracking tumor-specific cf-mtDNA variants in plasma, possibly because of the low mutation detection sensitivity of SMRT, which is better suited for the detection of structural variations. Use of small amounts of tumor tissue may also not reveal a comprehensive tumor profile, owing to intratumor heterogeneity. Tumor-associated somatic mutations of mtDNA in extracellular vesicles of patients with pancreatic ductal adenocarcinoma and the plasma of patients with neuroblastoma have been investigated. By performing ddPCR detection and targeted capture sequencing of circulating nucleic acids in patient-derived orthotopic xenograft models of glioblastoma, Mair et al. have found that the plasma detection rate of tumor-derived cf-mtDNA is higher than that of tumor-derived cf-nDNA, and its detection is also possible in cerebrospinal fluid and urine. Thus, cf-mtDNA is more sensitive than cf-nDNA in detection and monitoring of tumor burden. Campo et al. have used ultra-deep sequencing to detect the genetic heterogeneity of nucleotide positions in individual cf-mtDNA variant populations without detecting specific mutations, and have established a model using machine learning algorithms to effectively distinguish patients with HCC from healthy controls. The final accuracy rate of the developed model was 99.78%. We have also developed a series of precision analysis workflows for cf-mtDNA based cancer detection, including key analysis procedures such as trimming, mapping, and filtering in the detection of cf-mtDNA mutations; we have also established an innovative data analysis platform. To improve the accuracy and sensitivity of mutation detection, we have developed a random forest model to identify cross-contamination, evaluate contamination levels, and detect contamination-derived variants in mtDNA NGS data.

In addition, we have developed a package to identify tumor-specific mtDNA mutations in mtDNA NGS data, applicable to both tumor and nontumor tissues without paired controls. Moreover, we have found that the copy number of mtDNA in the plasma of patients with HCC is significantly lower than that in tissue, and the cf-mtDNA fragments are shorter than those of cf-nDNA. Many potential cancer-specific mtDNA mutations have been demonstrated in plasma samples from patients with HCC. Subsequent studies have shown that urine cf-mtDNA also has the potential to detect tumors. The proportion of short fragments of cf-mtDNA in the urine in patients with tumors is significantly greater than observed in healthy controls and patients with benign disease. Enrichment analysis of urine cf-mtDNA short fragments (< 150 bp) significantly enhances the detection of mtDNA somatic mutation. Nevertheless, more studies are needed to demonstrate the clinical utility of cf-mtDNA content, fragmentomics, and mutation characteristics in cancer detection and management.

**Perspective**

Compelling evidence indicates cf-mtDNA's substantial value as a novel biomarker for cancer prediction and screening, guiding treatment, assessing prognosis, and detecting drug resistance, all of which are crucial for prolonging patient survival. However, no cf-mtDNA based liquid biopsy technology has been approved for clinical application, possibly because of (i) limited understanding of cf-mtDNA biology and release mechanisms, (ii) masking of tumor-derived cf-mtDNA by non-informative cf-mtDNA from other cells at low concentrations, (iii) high cost, difficulty, and time requirements of liquid biopsy detection techniques, and (iv) a paucity of excellent strategies for identifying and eliminating nuclear mitochondrial sequences, thus substantially influencing the precision of cf-mtDNA mutation analysis. Nevertheless, the potential of cf-mtDNA as a novel biomarker for providing tumor information is undeniable. Additionally, attention to the preanalytical handling of biospecimens is crucial for the successful implementation of cfDNA analysis in clinical use. Hence, a thorough evaluation and resolution of the problem of false positives resulting from factors including sample collection, processing, storage, and other variables is imperative before analysis of the characteristics of cf-mtDNA. With further advances in precision detection technology and clinical research, such as the development of single cell sequencing technology, transcriptome analysis, and long read sequencing, cf-mtDNA characteristics can be detected more accurately and comprehensively at lower cost. Cf-mtDNA multi-omics is expected to soon be routinely applied in cancer management. The application of multi-omics base detection procedures may further enable feature integration analysis, thus yielding more comprehensive tumor profiles, and potentially improving the sensitivity and clinical application of cancer detection and management. The development of machine learning models has also provided excellent opportunities for precise diagnosis and treatment based on cf-mtDNA detection technology. Machine learning automatically induces logic or rules from data by selecting appropriate algorithms, and constructs predictive models according to the induction
results. In recent years, machine learning algorithms have been widely used to identify tumor origin. Machine learning greatly improves the sensitivity and specificity of detection, and provides strong support for the precise diagnosis and management of tumors. Further research should be focused on clinical practice to demonstrate the practical value of cf-mtDNA in cancer management and to pave the way for its use in personalized medicine.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conceived and designed the analysis: Yang Liu, Jinliang Xing. Collected the data: Fan Peng, Siyuan Wang, Zehui Feng, Kaixiang Zhou, Huanqin Zhang, Xu Guo. Conceived and designed the analysis: Yang Liu, Jinliang Xing. Author contributions

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