Applications of Circulating Tumor DNA in Myelodysplastic Syndromes and Acute Myeloid Leukemia: Promises and Challenges

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Abstract

The term ‘liquid biopsy’ has become widely used by clinicians with the development of non-invasive diagnostic and monitoring techniques for malignancies. Liquid biopsy can provide genetic information for early diagnosis, risk stratification, treatment selection and postoperative follow-up. In the era of personalized medicine, liquid biopsy is an important research direction. In recent years, research on circulating tumour DNA (ctDNA) in hematological malignancies has also made great progress. This review provides an overview of the current understanding of circulating tumour DNA in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). Additionally, recent advancements in the monitoring of minimal/measurable residual disease (MRD) through ctDNA are discussed.

Keywords: liquid biopsy; circulating tumor DNA; myelodysplastic syndromes; acute myeloid leukemia; minimal residual disease

1. Introduction

As the world’s population ages and life expectancy increases, the incidence of cancer is rising [1]. Currently, hematological malignancies are commonly diagnosed through invasive methods such as lymph node biopsy, bone marrow aspiration, and bone marrow biopsy. These methods can be painful for patients, making sample collection difficult. Patients without obvious clinical symptoms may be unwilling to undergo invasive diagnostic procedures, which can delay the diagnosis of the disease. In addition, traditional minimal/measurable residual disease (MRD) monitoring requires multiple bone marrow aspirates or biopsies, which can lead to low patient compliance [2]. Circulating tumour DNA (ctDNA) extracted from body fluids offers a high degree of concordance of genetic and epigenetic information with the primary or metastatic tumours. This makes ctDNA a non-invasive and personalised method of diagnosis [3–5]. Due to these advantages, ctDNA has significant potential in hematological malignancies. Myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) are essential components of hematological malignancies [6]. Using ctDNA to aid diagnosis and treatment of these diseases may be a future trend in MDS/AML. In this manuscript we will introduce the application of ctDNA in various cancers with a focus on the literature of ctDNA-mediated liquid biopsy in MDS/AML.

2. Myelodysplastic Syndromes (MDS) and Acute Myeloid Leukemia (AML)

2.1 Characteristics and Incidence

MDS is a complex myeloid clonal hematopoietic disorder that presents with both myeloproliferative and ineffective hematopoietic components, resulting in cytopenia [7]. It has a high risk of transformation to AML [8]. Although not all patients progress to AML, many die of infection, bleeding and/or cardiopulmonary complications.

The pathogenesis of MDS is generally accepted to involve clonal expansion of hematopoietic stem cells caused by somatic mutations. Accumulation of driver gene mutations or chromosomal abnormalities also triggers AML [9]. More than 30 genetic mutations have been associated with MDS, which can be categorized into splicing factors (e.g., SF3B1, SRSF2, U2AF1, ZRSR2), DNA methylation (e.g., DNMT3A, TET2), histone modification (e.g., AXL1, EZH2), cohesion components (e.g., SMC1A, SMC3, RAD21, STAG1 and STAG2), transcription factors (e.g., RUNX1), TP53 pathway and signal transduction (e.g., NRAS, KRAS, FLT3) [10–12].

The 5th edition of the World Health Organization (WHO) classification introduces the term myelodysplastic neoplasms to replace myelodysplastic syndromes. MDS is classified on the basis of genetic abnormalities and morphology. MDS with defining genetic abnormalities includes MDS with low blasts and isolated 5q deletion (MDS-5q), MDS with low blasts and SF3B1 mutation (MDS-SF3B1), and MDS with biallelic TP53 inactivation (MDS-
Peripheral blood is the most common source of liquid biopsies. Tumour cells release ctDNA by apoptosis, necrosis and secretion, which can be isolated and enriched from a blood sample. The information extracted from ctDNA can be broadly divided into quantitative information or genomic information for analysis.

biTP53). Morphologically defined MDS includes MDS with low blasts (MDS-LB), hypoplastic MDS (MDS-h), and MDS with increased blasts (MDS-IB). MDS-IB can be further subdivided according to the number of blasts and whether fibrosis is combined [13].

According to the new International Consensus Classification (ICC) of myeloid neoplasms, MDS is divided into MDS without excess blasts, MDS with excess blasts and MDS/AML. MDS without excess blasts can be further subdivided into MDS with mutated SF3B1, MDS with del(5q) and MDS, not otherwise specified (NOS). MDS, NOS can be further subdivided according to the number of dysplasias. The subtype of MDS/AML emphasises the biological continuum between MDS and AML [7]. It should be noted that MDS, MDS/AML, and AML with mutated TP53 are grouped together as myeloid neoplasms with mutated TP53 due to similar aggressive behaviour [14].

Due to evolving diagnostic criteria and reclassification of MDS, it is difficult to describe the incidence of the disease in a consistent manner [15]. The incidence of MDS increases with age, and the most recent Surveillance, Epidemiology, and End Results (SEER) data report a median age at diagnosis of 77 [6]. Monitoring for disease relapse after MDS treatment can provide information on the efficacy of treatment and allow individualized adjustment of treatment plans to postpone disease progression, improve quality of life and prolong survival.

AML is a heterogeneous malignant disease characterised by rapid clonal expansion of aberrant myeloid progenitor cells [16,17]. AML is the most common acute leukemia in adults and predominantly affects the elderly population. The WHO classification separates AML with defining genetic abnormalities from AML defined by differentiation [13]. Anderson et al. [18] performed an analysis of the age-standardized incidence of AML in 29 countries on four continents. They reported a median incidence of 2.28 cases per 100,000 people. Compared to patients with MDS, mutations involved in DNA methylation (e.g., DNMT3A), transcriptional dysregulation (e.g., RUNX1, CEBPA) and signalling (e.g., NRAS, KRAS, FLT3) are more common in patients with AML [19,20].

2.2 Diagnosis and Treatment in MDS and AML

Currently, the diagnosis of MDS and AML is mainly based on comprehensive analysis of cell morphology, immunology, cytogenetics and molecular biology (MICM) of samples obtained by bone marrow aspiration and biopsy. Chromosome banding analysis, fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR) and next generation sequencing (NGS) are conventional methods of detecting genomic characterization [21].

DNA hypomethylating agents (HMAs), azacitidine and decitabine, have become the backbone of MDS and AML treatment [22]. Currently, for patients with high-risk or refractory AML and MDS, allogeneic hematopoietic stem cell transplantation (allo-HSCT) may be the only way to potentially achieve long-term survival. Therefore, early diagnosis, early treatment, timely monitoring of treatment efficacy and appropriate adjustment of treatment are particularly important in MDS and AML.
Considering the repetitive nature of bone marrow aspiration and biopsy during treatment and monitoring, pain experienced by the patient and bleeding at the puncture site are unavoidable [23]. Based on the disadvantages of traditional biopsy techniques, we urgently need a convenient and non-invasive means to aid diagnosis and treatment. Thus, the concept of “liquid biopsy” was born.

3. Liquid Biopsy and CtDNA

3.1 Circulating Tumor Cells (CTCs)

In 1869, the pathologist Ashworth discovered a class of cells whose morphology resembled that of tumours in the peripheral blood of patients dying of cancer. He named them circulating tumour cells (CTCs). CTCs are tumour cells that have been shed from a tumour lesion (primary or metastatic) into the bloodstream, and can be present as a single cell or in clusters. In 2010, Pantel et al. [24] first used the term “liquid biopsy” in a review of CTCs to describe the clinical applications of analyzing peripheral blood samples. The analysis of peripheral blood CTCs can screen for early tumours, detect tumour progression, facilitate the monitoring of response to therapy, study mechanisms of drug resistance and even be used to detect the emergence of distinct resistant clones and monitor patterns of early disease recurrence [25–27].

3.2 CfDNA and ctDNA

As the concept of liquid biopsy has been developed, circulating cell-free DNA (cfDNA), RNA and exosomes have become markers analyzed in liquid biopsy, particularly cfDNA. CfDNA refers to extracellular DNA found in body fluids such as plasma, serum and urine [28–31]. In 2019, Alborelli et al. [32] conducted a study of cfDNA in 177 individuals, 63 of whom were diagnosed with lung or breast cancer and 114 of whom were healthy. They reported that the concentration of cfDNA in plasma ranged from 0–100 ng/mL in healthy individuals, which is lower than that in cancer patients. Cell-free plasma DNA is mainly derived from apoptotic or necrotic cells [33]. Normally, DNA from apoptosis is rapidly degraded by DNases [34]; however, in the case of acute injury, stroke, transplantation, infection, tumour, etc., as the uptake of apoptotic bodies is impaired or a large number of apoptotic cells are produced, the concentration of cfDNA in the plasma will increase [35]. In patients with tumors, DNA fragments released from tumour cells within tumour lesions and CTCs are important sources of cfDNA. The type of cfDNA derived from tumours is called circulating tumour DNA (ctDNA) (Fig. 1).

CtDNA is derived from necrosis/apoptosis of tumour cells and CTCs, and from their exosomes. Mouliere et al. [36] reported that the cfDNA fragments at 167 base pairs (bp) can be released by apoptosis, maturation and turnover of blood cells. Mutant ctDNA has a maximum enrichment in fragments between 90 and 150 bp, with additional enrichment in the 250–320 bp size range. Mutated ctDNA is generally more fragmented than non-mutated cfDNA [36]. Peripheral blood from cancer patients contains ctDNA. However, this is diluted by much larger amounts of DNA shed from non-cancerous sources [37]. The half-life of ctDNA is 15 minutes to a few hours and can be cleared rapidly by the kidneys, liver and spleen.

The detection of ctDNA, which is present in very low concentrations in peripheral blood, requires high technology. With the development of PCR and the spread of NGS, highly sensitive detection methods have become feasible, making it possible to detect ctDNA. However, no method yet meets all the requirements for clinical applicability. Here, we list the commonly used methods for ctDNA detection in peripheral blood (Table 1, Ref. [38–42]).

3.3 The Utilization of ctDNA in Solid Cancer

The genetic and methylation information in ctDNA maintains a high degree of correlation with the tumour from which it was derived [32,43,44]. CtDNA can serve as a non-invasive pool for cancer detection and diagnosis by analyzing cancer-specific biomarkers [45–47]. In 2020, GRAIL published the results of three sub-projects of the Circulating Cell-free Genome Atlas (CCGA). Experiments proved that
its methylation sequencing analysis technology of cfDNA can detect more than 50 cancer types simultaneously, with a specificity of >99%, and the prediction accuracy of the tissue of origin (TOO) is >90% [48].

Much research has shown that the detectable levels of ctDNA correlate with cancer stage and burden, and that patients who have undergone cancer resection may have decreased ctDNA levels [49–52]. In addition, it is possible to analyze cancer evolution, genomes and transcriptomes using ctDNA [53]. CtDNA also has the potential to serve as a biomarker for therapy evaluation [54,55]. Research has been conducted in various cancer types on prognostic assessment, MRD surveillance and detection of disease recurrence by serial ctDNA tracking, which has been shown to be more sensitive for detection of recurrence than traditional methods [56–61].

Here we list some of the above references on the use of ctDNA in solid cancers (Table 2, Ref. [46,47,49,53,57,58]).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Disease</th>
<th>N</th>
<th>ctDNA material</th>
<th>ctDNA analysis</th>
<th>Conclusion</th>
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<tr>
<td>Mijnes J [46]</td>
<td>2019</td>
<td>Breast Cancer</td>
<td>125</td>
<td>Plasma</td>
<td>Pyrosequencing</td>
<td>A combination of SPAG6, NXY2-6, ITIH5 and PER1 (SNiPER) may be a promising</td>
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<td>tool for detecting breast cancer detection in peripheral blood.</td>
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<td>Brait M [47]</td>
<td>2017</td>
<td>Prostate Cancer</td>
<td>84</td>
<td>Plasma</td>
<td>QMSP</td>
<td>The detection of MCAM, ERα and ERβ gene promoter methylation in plasma</td>
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<td>ctDNA could be used as a combined biomarker for the early detection of</td>
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<td>Bettegowda C [49]</td>
<td>2014</td>
<td>Multiple cancer types</td>
<td>640</td>
<td>Plasma</td>
<td>WGS</td>
<td>The detectable levels of ctDNA are consistent with cancer stage and ctDNA</td>
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<td>may serve as a viable biomarker for various solid tumour types and clinical</td>
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<td>Herberts C [53]</td>
<td>2022</td>
<td>Prostate Cancer</td>
<td>33</td>
<td>Plasma</td>
<td>DeepWGS</td>
<td>Samples with high levels of ctDNA can be used to analyze the evolution,</td>
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<td>genomes and transcriptomes of aggressive prostate cancers.</td>
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<td>Zhang JT [57]</td>
<td>2022</td>
<td>Non-Small Cell Lung</td>
<td>261</td>
<td>Plasma</td>
<td>NGS</td>
<td>ctDNA has the predictive value of MRD for adjuvant therapy and ctDNA-MRD</td>
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<td>Cancer</td>
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<td>correlates with clinical outcome.</td>
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<td>Henriksen TV [58]</td>
<td>2022</td>
<td>Colorectal Cancer</td>
<td>168</td>
<td>Plasma</td>
<td>NGS</td>
<td>ctDNA-MRD-positive patients have a high risk of relapse after adjuvant</td>
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<td>imaging for detecting recurrence.</td>
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NGS, next generation sequencing; MRD, minimal/measurable residual disease; QMSP, quantitative methylation specific PCR; WGS, whole genome sequencing.

4. Application of CtDNA in MDS and AML

The information extracted from ctDNA can be classified as genomic or epigenetic information. The following references will demonstrate the feasibility of ctDNA for MDS and AML applications at the genetic or epigenetic level (Fig. 2).

4.1 CtDNA and Diagnosis of MDS/AML

In 1994, Vasioukhin et al. [62] detected N-ras gene mutations in ctDNA, blood cells and bone marrow from 10 patients with MDS or AML using PCR. They showed that ras mutation information detected in ctDNA was not always detected in blood cells or bone marrow, suggesting that bone marrow biopsy or aspiration may not necessarily contain all the information about the malignancy. Plasma contains more comprehensive information about the tumour. Taking advantage of its easy availability, plasma can be used to detect and monitor myeloid disorders. Since then, more and more research has been carried out into the use of cfDNA in MDS and AML.

In 2004, Rogers et al. [63] compared the difference in loss of heterozygosity (LOH) between plasma ctDNA
Analysis of ctDNA can focus on multiple mutations, methylation, concentration, copy number variation and loss of heterozygosity. Throughout the evolution of the disease, from diagnosis to treatment and then, in some cases, to progression and relapse, ctDNA has its functions.

and bone marrow in MDS and AML patients with chromosomal abnormalities. They found that LOH was detected in 100% of cases in plasma ctDNA compared to 89% and 70% in bone marrow in MDS and AML, respectively. In the detection of residual disease, fifteen out of sixteen post-therapy samples showed complete concordance between LOH and cytogenetics. These data suggest that peripheral blood plasma is enriched for ctDNA and has the potential to replace bone marrow for the study of genetic abnormalities.

As the technology developed, in 2019, Zhao et al. [64] performed a gene detection study in 26 patients with MDS using a NGS platform with a 127-gene panel. The study strongly suggested high correlations between bone marrow tumour DNA and peripheral blood plasma ctDNA for both variant cells and somatic mutations. In addition, the frequency of gene mutations in ctDNA can, to some extent, predict and reflect disease status and monitor therapeutic response in MDS. Another study examined the molecular and cytogenetic profile of a cohort of 70 patients with MDS by NGS of ctDNA and compared the results with paired bone marrow DNA. It supported that analysis of ctDNA can serve as a promising strategy for performing molecular characterization, detection of chromosomal aberrations and monitoring of patients with MDS [65].

In 2020, Tiong et al. [66] used a single hybridization-based NGS assay of ctDNA and bone marrow to perform low-resolution digital karyotyping. They focused on the detection of sequence variants, copy number variation (CNV) and copy neutral loss of heterozygosity (CN-LOH) in AML patients. Their results showed that there was a high consistency in both sequence variant and CNV detection, supporting ctDNA as an alternative to bone marrow. Ruan et al. [67] detected mutations in ctDNA, bone marrow and peripheral blood mononuclear cells (PBMCs) of pediatric AML patients by NGS. The correlation of clinically significant mutations between ctDNA and bone marrow was 77%. This demonstrated that ctDNA can reflect the genomic information from bone marrow and is a reliable sampling method in pediatric AML. The other study also demonstrated that mutations were missed by both cfDNA and bone marrow analysis, particularly when the variant allele frequencies (VAF) were below 10%, suggesting that cfDNA and bone marrow may be complementary in the assessment and monitoring of patients with AML [68].

All of the above data demonstrate a high correlation between bone marrow and ctDNA results at the genetic level. At the epigenetic level, four CpG regions were identified in a study aimed at establishing a method to estimate MRD based on AML-associated DNA methylation (DNAm). The combinations of four CpG regions could reliably discriminate between healthy and newly diagnosed AML samples [69].

GRAIL used a targeted methylation- and cfDNA-based technology to develop a custom classification model for hematological malignancies and evaluated its performance for cancer detection and TOO prediction accuracy. They reported that the hematologic-specific classifier achieved the sensitivity of 45.8% and TOO prediction accuracy of 100% for myeloid neoplasms [70].

In summary, ctDNA enrichment can be found in peripheral blood plasma. Many experiments have shown that at the level of genetic and epigenetic analysis, peripheral blood ctDNA and bone marrow cells have a high degree of concordance. The use of ctDNA for genetic and epigenetic analysis is highly reliable. Importantly, ctDNA can be used as a tool to assist in the diagnosis of MDS or AML.

4.2 Assessing Treatment Efficacy and MRD Monitoring of MDS/AML with ctDNA

MRD refers to the small number of cancer cells that do not respond to treatment or are resistant to drugs and therefore remain in the body after cancer treatment. Although the amount of remaining cancer cells may be small, causing no signs or symptoms or even undetectable by conventional methods, they contribute to cancer recurrence. This suggests that for MRD-positive patients, ongoing monitoring for recurrence and early intervention may play a positive role in improving outcomes.

The MRD level of patients with MDS and AML is widely determined by various methods, including multiparameter flow cytometry (MFC), real-time quantitative polymerase chain reaction (qPCR) and, more recently, digital
PCR and NGS. In 2010, Gao et al. [71] analyzed the integrity of cfDNA from 60 acute leukemia patients and 30 healthy controls. They used qPCR to amplify the β-actin gene and showed that DNA concentrations and DNA integrity were significantly higher in acute leukemia patients than in healthy controls. DNA integrity decreased significantly during complete response (CR) and increased during relapse. The data suggest that plasma DNA integrity may be a potential biomarker for monitoring leukemia progression and MRD. This study laid the foundation for ctDNA to monitor MRD in leukemia. Since then, the ultimate goal of researchers has been to use ctDNA-detected genetic mutations for diagnosis, treatment guidance, MRD monitoring and prognosis prediction. However, in Gao’s research [71], the number of samples, particularly those used to infer changes in DNA integrity from diagnosis to relapse, was too small to demonstrate sufficient accuracy. More work is needed.

Yeh et al. [72] collected serial bone marrow and plasma samples from 12 patients with MDS undergoing demethylation therapy in a phase 1 clinical trial. By analyzing serial bone marrow and matched plasma samples using digital PCR (dPCR), they demonstrated that ctDNA is comparable to bone marrow biopsy in the genomic heterogeneity of malignant clones and dynamically reflects tumor burden. CtDNA can be used to track both mutations and karyotypic abnormalities during therapy in MDS. The above conclusions not only prove the feasibility of using ctDNA to reflect the dynamic changes of genetic abnormalities in bone marrow, but also provide strong evidence for the use of ctDNA to assess efficacy.

Monitoring the evolution and type of mutations in ctDNA during and after treatment can be used to effectively monitor treatment response and assess the risk of relapse. Tong et al. [73] conducted a study of 31 patients with MDS and AML. All patients underwent bone marrow NGS and ctDNA testing at baseline. In 27 patients with ctDNA mutations at baseline who received demethylation therapy or chemotherapy, patients who achieved a complete response/complete hematological recovery (CR/CRi) at the end of treatment had a lower mean number of mutant VAF than those who did not achieve CR/CRi. Patients who achieved CR had a greater decrease in mutant VAF. The study also found that post-treatment ctDNA was negatively associated with longer progression-free survival (PFS) and overall survival (OS). Of the 10 patients who progressed from MDS to AML, seven subsequently acquired subclones containing FLT3 or NF1 mutations. In addition, new subcloning was detected in four patients 0.5–4 months prior to AML transformation, suggesting that genetic changes preceded morphological change.

For patients with high-risk and refractory MDS and AML, allo-HSCT is the only treatment. Methods to reduce relapse rates after transplantation are an urgent and important topic in clinical research. Researchers at the University of Tokyo conducted a study to identify patients at high risk of relapse in AML and MDS following allo-HSCT. Their results showed that the cumulative relapse rates of ctDNA-positive patients at 1 month and 3 months after transplantation were 56.6% and 63.0% higher than those of ctDNA-negative patients, and the 3-year OS decreased by 45.9% and 39.1% respectively [74]. This study demonstrated that...
driver mutation persistence in ctDNA could serve as a prognostic biomarker for MRD monitoring in AML/MDS patients undergoing first allo-HSCT.

In addition to gene-based MRD monitoring, ctDNA methylation-based MRD monitoring is also emerging. Iriyama et al. [75] used bisulfite pyrosequencing to perform a global methylation analysis based on the specific CpG sites of the LINE-1 promoter. They found that the methylation level of peripheral blood ctDNA decreased rapidly after azacitidine treatment. However, the study mentioned above, which proposed a method to estimate MRD based on AML-associated DNA methylation, showed poor accuracy in separating MRD-positive from MRD-negative samples by identifying four CpG regions [69]. This may be because the four AML-associated methylation regions selected in this study were from a small sample and were not highly representative of abnormal methylation regions. Increasing the sample size and the range of methylation detection regions, combined with genetic mutations, will make it possible to detect MRD-positive samples earlier and more accurately.

Although ctDNA cannot replace the gold standard of bone marrow sampling, it can be used as a complementary diagnostic tool for those MDS/AML patients without obvious clinical symptoms or who are unable to undergo traditional diagnostic procedures. It also has potential in the assessment of prognosis and monitoring of MRD.

5. Challenges of CtDNA in Application

Current ctDNA detection technology still faces some challenges.

In the last decade, with the application of second generation gene sequencing technology in MDS, some potential pre-MDS conditions that do not meet the minimum diagnostic criteria for MDS have been defined. Clonal hematopoiesis of indeterminate potential (CHIP) is used to define the acquisition of somatic mutations that drive clonal expansion without cytopenia and dysplastic hematopoiesis in healthy individuals (Fig. 3) [76].

In 2019, Razavi et al. [77] demonstrated that CHIP can be detected in ctDNA sequencing analysis. More than half of the mutations in the blood of cancer patients come from white blood cells. When analyzing ctDNA, interference from CHIP must be excluded. The reliability of the analysis is reduced if the CHIP present in the ctDNA of healthy individuals is considered as a mutation in the ctDNA. However, existing technology cannot exclude CHIP interference.

In addition, most current ctDNA studies focus on advanced cancers with relatively high levels of ctDNA and lack detailed experience with early cancers and low levels of ctDNA. Importantly, due to its short half-life, ctDNA must be processed as soon as possible after collection. However, there is no standardized procedure for ctDNA collection, extraction and amplification. Analysis of the specificity and sensitivity of ctDNA testing will not be accurate unless these issues are addressed.

6. Conclusions

CtDNA and the genetic and epigenetic information of tumours are highly correlated. Compared to traditional bone marrow aspiration or biopsy, ctDNA has the advantage of requiring only a small sample size and is relatively easy to obtain, while overcoming potential sampling error and providing a means for tumor gene detection. It also provides a convenient method for post-operative monitoring of tumour recurrence. At present, there is a need to improve the standard of ctDNA detection. More experimental data are needed to improve the reliability of ctDNA as an adjunctive diagnostic tool, as a tool for disease diagnosis, evaluation of treatment efficacy and monitoring of MRD in MDS/AML. It is believed that with further development and resolution of the above issues, ctDNA may become an indispensable tool in the diagnosis and treatment of tumours in the future.

Availability of Data and Materials

Data availability is not applicable to this article as no new data were created or analyzed in this study.

Author Contributions

YHX and XXW initiated and conceptualized the main points of the manuscript. YHX screened the literature and worked with XJX on the writing of the review. XJX and HCG participate in the design of the tables. XL and YHZ contributed to the figure design. HCG reviewed and edited the draft. XXW oversaw the entire review process and provided guidance and direction. All authors contributed substantially to the editorial revisions and jointly reviewed and approved the final manuscript and agreed to take responsibility for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Thanks to Xuerong Zhou for her help and encouragement. And thanks to the understanding and help of all the medical staff at the Department of Hematology, the First Hospital of China Medical University.

Funding

This research was funded by the financial supported by the General Program of Basic Scientific Research Project of Liaoning Provincial Department of Education (No.LJKMZ20221177), the Natural Science Foundation of Liaoning Province (No.2022-YGJC-62) and Project on the xinrui cancer support therapy (No.cphcf-2022-102).
Conflict of Interest
The authors declare no conflict of interest.

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