Review

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Circulating tumor DNA methylation: a promising clinical tool for cancer diagnosis and management

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Abstract: Cancer continues to pose significant challenges to the medical community. Early detection, accurate molecular profiling, and adequate assessment of treatment response are critical factors in improving the quality of life and survival of cancer patients. Accumulating evidence shows that circulating tumor DNA (ctDNA) shed by tumors into the peripheral blood preserves the genetic and epigenetic information of primary tumors. Notably, DNA methylation, an essential and stable epigenetic modification, exhibits both cancer- and tissue-specific patterns. As a result, ctDNA methylation has emerged as a promising molecular marker for noninvasive testing in cancer clinics. In this review, we summarize the existing techniques for ctDNA methylation detection, describe the current research status of ctDNA methylation, and present the potential applications of ctDNA-based assays in the clinic. The insights presented in this article could serve as a roadmap for future research and clinical applications of ctDNA methylation.

Keywords: cancer; ctDNA; methylation; detection technology; early diagnosis

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Introduction

Cancer is a significant global public health problem, with an estimated 1,958,310 new cancer cases and 609,820 cancer-related deaths projected in the United States in 2023 [1]. Radiotherapy and chemotherapy remain the mainstay of treatment for patients with advanced cancer [2]. In recent years, targeted therapy and immunotherapy have shown promising results in treating various types of cancer, including melanoma, lung cancer, breast cancer, and others. These treatments are often associated with fewer side effects than traditional chemotherapy and are effective in certain patients with specific genetic or immune system profiles [3, 4]. However, not all patients will respond to these treatments, and the five-year survival rates remain unsatisfactory.

Conversely, early cancer diagnosis, which allows surgical removal of tumors, has significantly improved patient survival and even cured cancer [5]. However, appropriate biomarkers for early screening and diagnosis of tumors are still pretty limited. Therefore, identifying early biomarkers has become a top priority in cancer diagnosis and treatment. Liquid biopsy, a minimally invasive or noninvasive approach to collecting patient samples for testing, has shown great potential for early cancer screening. Most studies related to the early detection of single or multiple cancers based on liquid biopsy have focused on the detection of cancer-related biomarkers in peripheral blood, such as cell-free DNA (cfDNA), circulating tumor cells (CTCs), cell-free RNA (cfRNA), circulating extracellular vesicles (EVs), proteins or metabolites [6, 7]. Among them, cfDNA, approximately 140–1700 base pairs (bp) in length, is mainly derived from apoptotic and necrotic white blood cells in healthy individuals [8–10], whereas in cancer patients, a fraction of cfDNA (ctDNA), less than 145 bp in size and with a short half-life ranging from 15 min to 2.5 h, is shed from tumor cells [11–13] (Figure 1). Several studies have confirmed a high degree of concordance between genetic and epigenetic alterations detected in plasma ctDNA and those found in tumor tissue [14, 15]. Thus, the presence and dynamics of ctDNA have the potential to revolutionize cancer screening, diagnosis, and treatment through a noninvasive approach. Compared to tissue biopsy, ctDNA

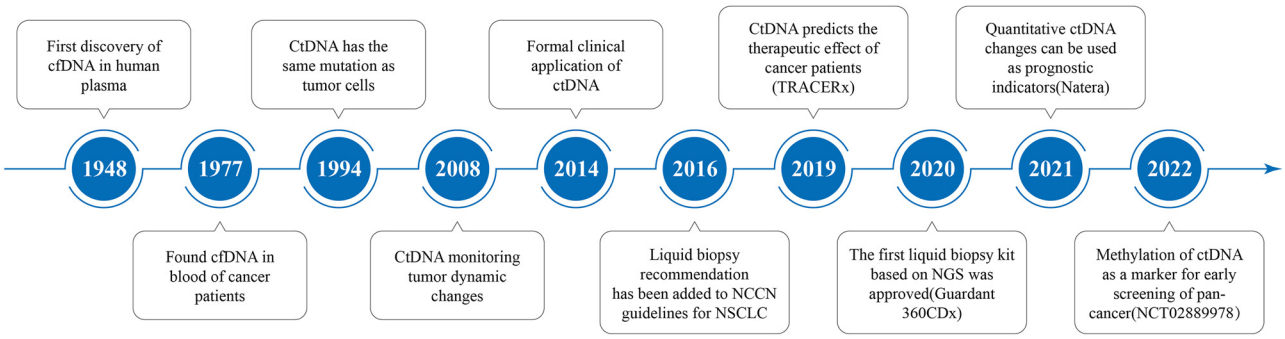

Figure 1: Timeline of ctDNA studies. Several milestones in ctDNA discovery, research, and development.

testing has several distinct advantages. First, it is easier to draw peripheral blood than to biopsy tumor tissue. Second, blood can be drawn at any time during treatment, allowing real-time and dynamic monitoring of molecular changes in the tumor. Finally, ctDNA methylation patterns primarily represent the overall genomic DNA methylation statuses of tumor tissues in patients; whereas those derived from biopsied tumor tissues are impacted by tumor heterogeneity [8, 16].

Early cancer detection based on ctDNA, such as mutation, fragmentation, and methylation, has recently received considerable attention [13]. Compared with methylation detection methods, the first two methods have their own limitations. On the one hand, it is worth noting that ctDNA generally accounts for a tiny fraction of DNA, and mutation loci in ctDNA are also rare. Therefore, ctDNA-based tests often require ultra-high sequencing depths to increase sensitivity, which is often accompanied by problems such as high false-positive rates, increased experimental costs, and interference from clonal hematopoiesis [17]. In addition, the tests generally cover a limited number of gene mutations and cannot be used to trace the origin of tumor tissue. On the other hand, tests based on ctDNA fragmentation rely on a low depth of whole genome sequencing (WGS). However, the low signal intensity due to the low sequencing depth and the high sequencing costs have limited the sensitivity of the assays and their application in clinical settings [18]. In contrast, the characteristic DNA methylation alterations, a globally hypomethylated genome with focal hypermethylation in tumor suppressor genes, make ctDNA methylation-based methods a more promising tool. Surprisingly, methylation alterations usually occur early in cancer development, and ctDNA also carries tissue-specific methylation signals, which in combination attract more investigations on ctDNA methylation and its potential applications for early cancer screening [19, 20].

DNA methylation is one of the most widely studied epigenetic modifications. The process of DNA methylation is catalyzed by a group of DNA methyltransferases (DNMTs), which add the methyl provided by S-adenosylmethionine (SAM) to the 5-position carbon of cytosine to form 5-methylcytosine (5-mC). DNA methylation typically occurs at the cytosine of CpG dinucleotides in mammals. Notably, the frequency of CpGs is low in most genomic regions but tends to accumulate in CpG islands (CGIs), which refer to high-density CpG regions with sizes greater than 500 bp, GC content greater than 55 %, and an observed CpG/expected CpG ratio of not less than 65 % [21]. Accumulating evidence indicates that when exposed to various oncogenic factors, normal cells undergo widespread hypomethylation throughout the genome and localized hypermethylation of many genes, particularly in CpG islands. These epigenetic changes trigger the activation of proto-oncogenes or the silencing of tumor suppressor genes, ultimately leading to cancer initiation and progression [22].

Interestingly, the methylation pattern of ctDNA is consistent with that of the tumor cells or tissues of origin, and plasma ctDNA levels correlate with tumor development stages [23]. A study of 640 patients with various cancers and different stages of development found a 100-fold increase in ctDNA levels in stage IV patients compared to early-stage patients. The proportions of patients with stage I, II, III, and IV cancers with detectable ctDNA were 47 , 55, 69, and 82 %, respectively, suggesting that ctDNA methylation may be a viable and reliable cancer biomarker [24].

Technologies for detecting ctDNA methylation

Given the critical role of ctDNA methylation in tumorigenesis and cancer screening, it is critical to accurately and rapidly determine ctDNA methylation status. Several methods for ctDNA methylation detection have been developed recently and are summarized in Table 1 and Figure 2.

Table 1: ctDNA-based methylation detection techniques and the advantages and disadvantages of each technique.

Class	Technology	Genome coverage	Pros	Cons	Cost	Ref
Bisulfite-based	qMSP or ddMSP	One or a few CpGs	Ultra-low DNA input	Loci-specific studies only	Low	[25]
	Microarray	1.7 %	Pre-designed panels for hotspot methylation detection, high degree of automation	Low genome-wide coverage of CpGs	Low	[26]
	TBS-seq	Diverse	Detection of target CpG sites with high coverage	Complicated primer or probe design	Low	[27]
	RRBS	1–3 %	High CGIs coverage	Restriction to regions in proximity to restriction enzyme sites	Moderate	[28]
	WGBS	95 %	The most comprehensive profiling of the whole methylome	Relatively low sequencing depth	High	[29]
Restriction enzyme-based (bisulfite-free method)	qPCR or dPCR	Determined by primer design	Ultra-low DNA input; easy primer design	Limited CpG coverage	Low	[30]
	HELP	~98.5 % CpG islands; >1.32 M CpGs	Low DNA input; easy validation	Constrained by the size of the oligonucleotides	Low	[31]
	MRE-seq	~10 %	Cover a wider genome region than traditional array hybridization	MRE-seq relies on the properties of different restriction enzymes	Low	[32]
Enrichment-based (bisulfite-free method)	MeDIP-seq	<WGBS	The antibody is specific to 5 mC	Low resolution, antibody batch effects	Moderate	[33]
	MBD-seq	17.8 %	MBD-based enrichment outperforms MeDIP in regions with a higher CpG density.	Low resolution, protein batch effects	Moderate	[34]

qMSP, quantitative methylation-specific PCR; ddMSP, methylation-specific droplet digital PCR; TBS-seq, targeted bisulfite sequencing; RRBS, reduced representation bisulfite sequencing; WGBS, whole genome bisulfite sequencing; qPCR, quantitative PCR; dPCR, droplet digital PCR; HELP, HpaII tiny fragment enrichment by ligation-mediated PCR; MRE-seq, methylation-sensitive restriction enzymes sequencing; MeDIP-seq, methylated DNA immunoprecipitation sequencing; MBD-seq, methyl-binding domain sequencing; DMR, differentially methylated regions.

Bisulfite-based methods

Bisulfite conversion-based methods are considered the gold standard for DNA methylation studies. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosine (C) to uracil (U), which is eventually changed to thymine (T) after PCR amplification; while the methylated C remains intact [35]. Finally, methylated and unmethylated C can be inferred by methylation-specific PCR (MSP), DNA methylation microarray, or next-generation sequencing (NGS)-based tests such as whole-genome bisulfite sequencing (WGBS), targeted bisulfite sequencing (TBS-seq), and reduced representation bisulfite sequencing (RRBS).

MSP

MSP is one of the most widely used techniques to study DNA methylation at a specific locus, including quantitative MSP (qMSP) and digital droplet MSP (ddMSP). MSP generally requires two pairs of primers that specifically recognize either the methylated or unmethylated DNA sequence and two separate PCR reactions are performed, one with the methylation-specific primers and the other with the unmethylated primers [36]. Whether the targeted CpG is methylated or unmethylated can be determined by gel electrophoresis to visualize which primer set ends with amplified DNA fragments.

qMSP is a modification of the MSP technique that allows quantitative measurement of DNA methylation levels at specific CpG sites. The assay uses real-time PCR to monitor PCR products during the exponential phase of the reaction, allowing precise quantification of methylated and unmethylated DNA. One modified qMSP method, MethySYBR, is designed to examine DNA methylation and CpG methylation density together. The assay uses multiplex PCR to amplify many target alleles simultaneously with 3 pg of bisulfite-converted DNA. In the second round of PCR, methylation-specific and methylation-independent primer sets are used to identify the specific methylated target from the multiplexed products. This method can identify methylated alleles in the presence of 100,000-fold unmethylated alleles [37]. However, the application of qMSP is limited by the complex primer and probe design, resulting in fewer methylated genetic loci that can be

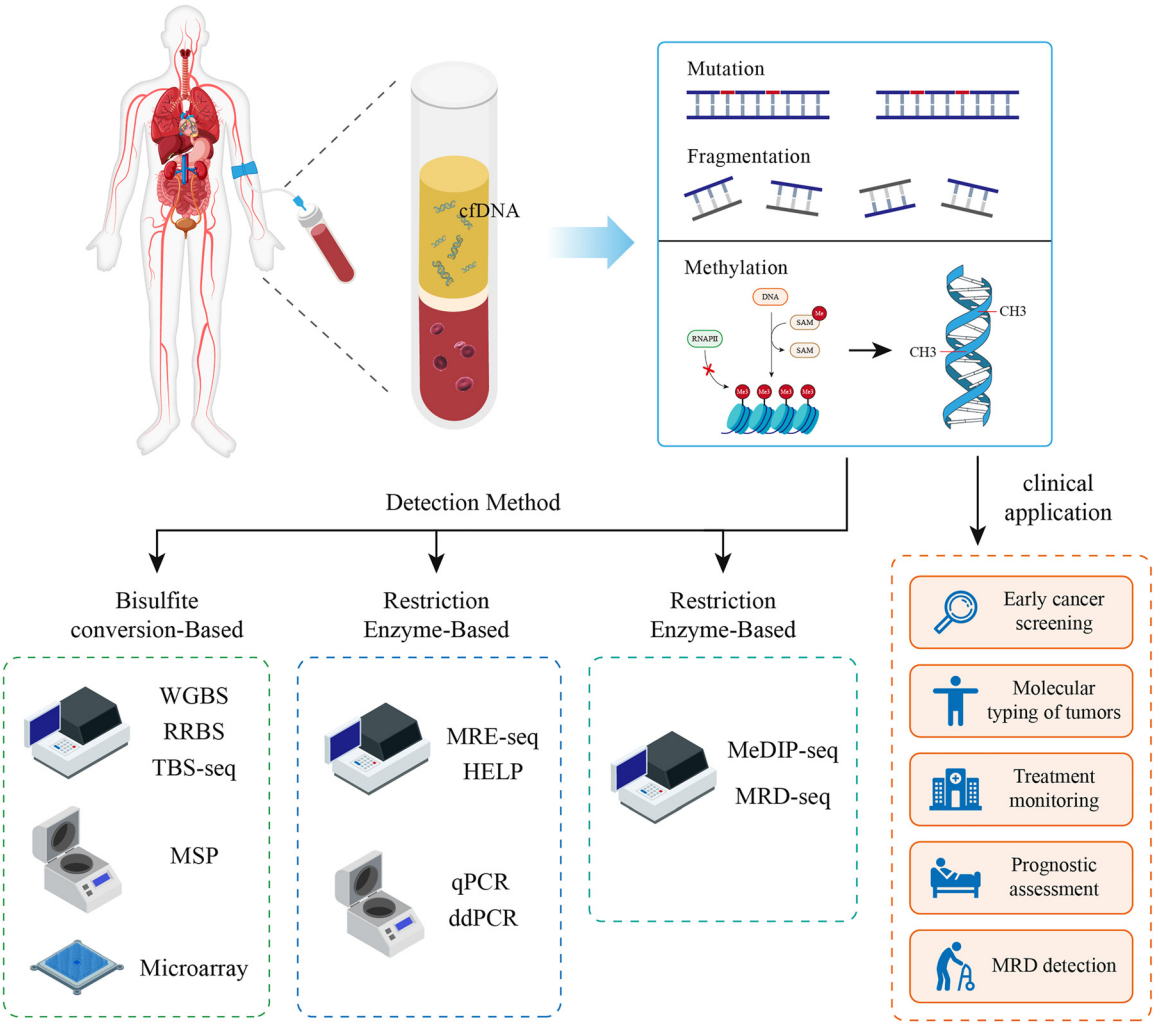

Figure 2: Sources of ctDNA and the main techniques for ctDNA methylation detection. Top: the ctDNA is isolated from plasma and can be analyzed for mutation, fragmentation and methylation. Bottom left: several ctDNA methylation analysis methods have been developed. Bottom right: possible applications of ctDNA in cancer prevention, treatment and prognosis in clinical settings. ctDNA, circulating tumor DNA; WGBS, whole genome bisulfite sequencing; RRBS, reduced representation bisulfite sequencing; TBS-seq, targeted bisulfite sequencing; MSP, methylation-specific PCR; MRE-seq, methylation-sensitive restriction enzymes sequencing; HELP, HpaII tiny fragment enrichment by ligation-mediated PCR; dPCR, droplet digital PCR; qPCR, quantitative PCR; MBD-seq, methyl-binding domain sequencing; MeDIP-seq, methylated DNA immunoprecipitation sequencing.

detected and interference from non-methylation-specific amplification [38].

ddMSP is another MSP variant that can quantify DNA methylation levels at specific loci with high sensitivity. It involves partitioning a sample into thousands of droplets, each containing a single DNA template, and using fluorescent probes specific for methylated or unmethylated DNA to monitor the PCR amplification in real time. The resulting data is then analyzed to determine the proportion of methylated and unmethylated DNA in the original sample [39]. Horakova and coworkers have used ddMSP for cfDNA-based breast cancer screening with a sensitivity and specificity of 86.2 and 82.7 %, respectively [40].

DNA methylation microarray

DNA methylation microarrays are specifically designed to measure DNA methylation at many CpGs across the genome. The assay uses probes that selectively bind to either methylated or unmethylated CpG sites. These probes can be labeled with fluorescent dyes or other markers, and the intensity of the fluorescent signal is typically used to determine the methylation status of each CpG site. The advantages of DNA methylation arrays include rapid and efficient detection, a high degree of automation, and comprehensive coverage [41]. Several commercial microarray-based platforms are currently available, with the Illumina Human

Methylation 450 K BeadChip (HM 450 K) being the most widely used in clinical trials. The HM 450 K consists of predefined probes covering approximately 450 K CpGs and 96 % of CGIs and was the primary method for methylation studies before the advent of NGS [42, 43]. A more advanced version, the Infinium Methylation EPIC Bead Chip, covers over 850 K CpGs, including nearly all sites in the HM450K array and extra CpG sites in enhancer regions [44].

The use of the HM450K array to profile colorectal cancer (CRC) cell lines (n=149) resulted in the identification of five cancer-specific methylation genes (EYA4, GRIA4, ITGA4, MAP3K14-AS1, and MSC). These five biomarkers were further validated using ddMSP in CRC tumor tissue (n=82) and ctDNA (n=182) from patients with metastatic CRC (mCRC), suggesting that methylated ctDNA biomarkers may serve as potential indicators to monitor treatment outcomes in mCRC [45]. In another study, ctDNA was extracted from the serum of patients with advanced colorectal cancer, and methylation levels of approximately 850 K CpG loci in the genome were measured using methylation microarrays. The analysis revealed at least a 10 % difference in methylation levels between controls and advanced tumors, with most of the differences being hypermethylation patterns. This finding demonstrates the ability to discriminate between advanced tumors and healthy controls based on methylation levels [46].

Microarray hybridization has become one of the most widely used techniques for DNA methylation detection due to its excellent cost-effectiveness. However, compared to WGBS and RRBS, microarrays detect relatively fewer methylation sites and offer lower genomic coverage, which may result in the loss of other methylation patterns in the genome.

RRBS

Reduced representation bisulfite sequencing (RRBS) is a targeted and cost-effective method for genome-wide DNA methylation profiling that focuses on specific CpG-rich regions. The assay is based on restriction endonucleases such as MspI, which recognize CCGG regardless of the methylation status of the binding sites. After size selection and bisulfite treatment, which converts unmethylated cytosines to uracils while leaving methylated cytosines unchanged, high-throughput sequencing is performed to assess the methylation status of CpG sites at a single base resolution. RRBS typically sequences about 1–3% of the human genome but is capable of enriching coverage of CpG-rich regions, including 60–80 % of promoters, 80–90 % of CGIs, and a fair representation of enhancers and CpG island shores [47–49]. RRBS is finding broad applications in a variety of cancer research. For example, Widschwendter et al. analyzed 31 breast cancer (BC) tissues and detected 18 BC-specific ctDNA methylation patterns, six of which were validated in independent serogroups (n=110). One biomarker, EFC#93, emerged as an independent poor prognostic marker for pre-chemotherapy BC patients with a specificity of 88 % [50]. Marinelli discovered DNA methylation-based biomarkers in ovarian cancer (OC) patient tissues using the RRBS technique. In particular, 33 biomarkers showed significant methylation ploidy changes (ranging from 10 to >1,000) in all OC subtypes compared to normal tissues. Subsequently, 11 (GPRIN1, CDO1, SRC, SIM2, AGRN, FAIM2, CELF2, RIPPLY3, GYPC, CAPN2, BCAT1) were tested in plasma of 91 OC patients and 91 healthy individuals. The cross-validated 11 methylated DNA marker (MDM) panels demonstrated high accuracy in distinguishing OC from controls with a specificity of 96 %, a sensitivity of 79 %, and an AUC of 0.91 [51]. In contrast to WGBS, RRBS exhibits substantial sequencing cost reduction, yet its expense remains notable. Thus, the ongoing challenge for this technology is to further reduce sequencing costs in the future [22].

TBS-seq

Targeted bisulfite sequencing (TBS-seq) is another commonly used approach for methylation analysis, providing methylation status at the level of individual CpGs for specific genes and gene regulatory regions [52]. The test can be categorized based on how the target regions are enriched: by PCR-based amplification or probe hybridization [53]. Both categories have been used in preclinical cancer studies. For example, one study used TBS-seq to examine 94 pairs of tissues: esophageal squamous cell carcinoma (ESCC) and adjacent normal tissues from the Chinese Han population. The study validated specific hypermethylated CpG loci as candidate biomarkers and subsequently integrated them into a diagnostic model. The resulting model showed consistent detection performance parameters (sensitivity=75 %, specificity=88 %, AUC=0.85) [54]. Zhang et al. assessed the methylation status of the QKI (RNA-binding protein Quaking) gene by merging methylation data across different cancer types. The authors confirmed QKI methylation levels by TBS-seq in an independent dataset (n=388) and observed CRC-specific hypermethylation at all CpG sites detected within the QKI promoter in 31 tumor tissues [27]. Despite its potential for cancer diagnosis and therapeutic assessment, the application of TBS-seq is limited in how to select appropriate cancer-associated DNA methylation sites [55].

WGBS

WGBS is the most comprehensive and informative DNA methylation sequencing technology that can detect the methylation status of almost all cytosines genome-wide, including low CpG density regions and non-CpG sites (CpA, CpT, and CpC). Like other bisulfite sequencing assays, such as RRBS and TBS-seq, WGBS involves the treatment of DNA with sodium bisulfite, which converts unmethylated cytosines to uracil, allowing differentiation between methylated and unmethylated cytosines upon sequencing. Continuous improvements in library construction and sequencing techniques allow WGBS libraries to be generated from nanograms or even single-cell DNA [56]. This makes it a common method suitable for methylation profiling using cfDNA [57]. For example, Zhang and colleagues employed low-pass WGBS to detect cfDNA in a cohort of 51 patients with hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). The authors identified hypomethylation near HBV integration sites in HCC patients but not in patients with hepatitis and cirrhosis, enabling noninvasive HCC detection and oncovirus surveillance [58].

Early detection of breast cancer is essential, and conventional mammography and ultrasound have high false positive rates, especially in Breast Imaging Reporting and Database System (BI-RADS) category four patients. In a multicenter study with 203 patients, the authors profiled DNA methylation alterations using cfDNA-based WGBS libraries and identified hypomethylated regions. The integration of DNA methylation with mammography and ultrasound data demonstrates the utility of cfDNA for early breast cancer detection. It suggests the potential for clinical improvement through a combined liquid biopsy and imaging approach to reduce false-positive results and unnecessary procedures [59]. However, the disadvantages of WGBS-based testing are apparent, including a high sequencing cost and a significant number of sequencing reads that do not contain CpGs [52].

Restriction enzyme-based methods

Selective cleavage of specific nucleotide sequences using methylation-sensitive or methylation-insensitive restriction endonucleases is a classical approach for methylation studies [55]. These methods rely on targeted recognition and cleavage by restriction endonucleases to enrich CpG-containing DNA fragments. Among these restriction endonucleases, HpaII is commonly used as a methylation-sensitive restriction endonuclease that binds selectively to unmethylated and subsequently cleaves at C|CGG, whereas this cleavage is blocked if the C in the CpG dinucleotides is methylated [60]. Digested DNA product is subsequently assayed to clarify the methylation status of the target fragment. Based on this principle, many restriction endonuclease-based methods have been developed and utilized for DNA methylation analysis. For example, Ko and colleagues used a plasma HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP) assay to measure HpaII digested small fragments and qPCR to detect LINE-1 fragments. This study of 99 gastric cancer patients found that low methylation levels before treatment were associated with poorer overall survival. In patients undergoing curative surgery, low pre-surgical methylation correlated with worse recurrence-free and overall survival [31].

Notably, the use of restriction enzyme-based approaches for cfDNA methylation assessment is moderately limited by low genomic coverage and restriction site depletion caused by the highly fragmented nature of ctDNA [61]. In addition, these methods typically require extensive large-scale experimental validation to achieve clinical utility.

Enrichment-based methods

The approach taken by enrichment-based methylation detection methods is to use methyl CpG-binding proteins or antibodies directed against 5 mC to selectively capture methylated regions of the genome while effectively eliminating unmethylated DNA fragments through stringent wash steps [62]. Methyl-CpG-binding proteins include members of the MBD family, such as MBD2 and MBD3L1 [62, 63]. The methyl-binding protein MECP2 was first demonstrated for the affinity purification of methylated DNA [64]. Another common approach involves using antibodies that target 5 mC in single-stranded DNA, resulting in the efficient enrichment of methylated DNA fragments [65]. Consequently, two methods, methyl CpG binding domain protein capture sequencing (MBD-seq or MBDCap-seq) and methylated DNA immunoprecipitation sequencing (MeDIP-seq), have been developed [65, 66].

MBD-seq

MBD-seq combines the use of methyl-CpG binding domain proteins, such as Methyl-CpG Binding Domain 2 (MBD2) or Methyl-CpG Binding Protein 2 (MeCP2), with NGS to profile DNA methylation levels across the genome. The MBD-seq procedure consists of several sequential steps. First, genomic DNA is fragmented, followed by the application of MBD proteins conjugated to magnetic beads for selective capture of methylated DNA fragments by stepwise elution to eliminate unmethylated DNA fragments. The enriched DNA

fragments are then used for sequencing library generation and subsequent DNA methylation profiling [41]. This technique has two advantages: 1) the MBD protein recognizes the natural double-stranded form of methylated DNA; 2) the MBD protein binds only to methylated CpG to ensure the enrichment of methylated DNA [60].

Schabort et al. performed an analysis of MBD-seq data obtained from canine mammary tumors (CMT) and identified the intron regions of canine ANK2 and EPAS1 as differentially methylated regions associated with CMT. Notably, ANK2 exhibited significant hypermethylation in ctDNA isolated from CMT, suggesting its potential as a liquid biopsy biomarker. Similarly, in human breast cancer, the ANK2 gene in the human genome showed a similar trend of hypermethylation at specific CpG sites [67]. In a separate study, Dallol and coworkers identified KLOTHO as a tumor-specific methylation gene by using the MBD-seq and ctDNA isolated from patients with primary breast tumors [68]. It is important to note that while MBD-seq has proven valuable, the method has limitations. Specifically, it cannot effectively capture DNA fragments lacking unmethylated CpG sites, and the assay has a bias toward CpG-rich regions and provides information at a regional level rather than a single base resolution [69, 70].

MeDIP-seq

Similar to MBD-seq, MeDIP-seq combines immunoprecipitation with NGS to analyze DNA methylation. The MeDIP-seq workflow consists of several steps. First, genomic DNA is fragmented, typically by sonication, to obtain smaller DNA fragments. Sequencing adapters are then ligated to the fragmented DNA, which is then denatured to convert double-stranded DNA to single-stranded DNA. Next, an antibody specific for 5 mC is used to immunoprecipitate methylated DNA fragments. After immunoprecipitation, the enriched methylated DNA fragments are purified and subjected to next-generation sequencing [65]. MeDIP-seq is a powerful method that enables the analysis of DNA methylation even in trace amounts of circulating tumor DNA (ctDNA) ranging from 1 to 10 ng [34]. An enhanced version, cfMeDIP-seq, exhibits high sensitivity and specificity utilizing cfDNA, striking a balance between detection cost and accuracy, thus holding potential for early cancer screening [71].

There has been a surge of interest in utilizing MeDIP-seq-based cfDNA methylation analysis for tumor screening in recent years. For instance, Xu et al. conducted a study employing MeDIP-seq to explore ctDNA methylation patterns in lung cancer patients. By comparing data from healthy individuals to those with lung cancer, the researchers identified 330 DMRs, consisting of 33 hypermethylated and 297 hypomethylated regions, located at gene promoters, suggesting their potential utility as early diagnostic markers for lung cancer [72]. Similarly, Li et al. employed MeDIP-seq for genome-wide ctDNA methylation analysis in patients diagnosed with pancreatic ductal adenocarcinoma (PDAC). Their investigation led to the identification of 775 DMRs within gene promoter regions and an additional 761 DMRs within CpG islands. Notably, eight DMRs were found to effectively discriminate PDAC patients from healthy individuals, providing a promising avenue for noninvasive early diagnosis of pancreatic cancer [73].

Thus, MeDIP-seq, coupled with cfMeDIP-seq, has emerged as a valuable tool for tumor screening, enabling the identification of potential biomarkers that can aid in the early detection and diagnosis of cancer. However, the challenges and limitations remain, such as bias towards regions of higher CpG density, resolution limitations to fragment sizes rather than single bases as in NGS-based assays, and false-positive results due to non-specific binding [70, 74].

Current status of ctDNA applications in cancer research

Early cancer screening

A large number of studies have consistently shown that patients diagnosed with cancer at early and intermediate stages have a significantly better prognosis than those diagnosed at advanced stages. Therefore, early detection plays a critical role in improving the survival of these patients. In particular, collecting screening samples from large populations and the need for regular testing pose challenges for invasive testing methods. As a result, noninvasive testing is emerging as the preferred choice for early diagnosis [26].

The development of effective noninvasive pan-cancer screening has had limited success in the clinical setting [75]. A comprehensive study, the Circulating Cell-free Genome Atlas (CCGA, NCT04820868) study, using WGS, WGBS, and TBS, evaluated potential blood-based screening methods for multiple cancers in a cohort of 2,800 individuals. The results showed that classifiers using whole-genome methylation had the highest detection sensitivity (39 %) with a specificity of 98 %, thus offering a promising avenue for early cancer screening [76]. In the following study, Liu and coworkers recruited 6,689 participants, including 2,482 patients with more than 50 cancer types and 4,207 non-cancer controls, and performed TBS using plasma cfDNA. The authors developed a classifier that showed consistent performance with a specificity of 99.3 % and a false positive rate of 0.7 %.

Sensitivity for stages I–III varied between cancer types but increased with higher stages, reaching 18 % for stage I, 43 % for stage II, 81 % for stage III, and 93 % for stage IV. The study concludes that cfDNA sequencing using informative methylation patterns has the potential to detect multiple types of cancer and warrants further evaluation in population-level studies [26]. The third stage of study using a distinct cohort showed a specificity of 99.5 % and an overall sensitivity of 51.5 %, ranging from 16.8 % for stage I, 40.4 % for stage II, 77.0 % for stage III, and 90.1 % for stage IV across 50 cancer types [77].

In the THUNDER study (NCT04820868), Gao and colleagues developed a cfDNA methylation-based test called enhanced linear array sequencing (ELSA-seq) for the early detection and localization of colorectal, esophageal, liver, lung, ovarian, and pancreatic cancers [78]. Using retrospective and prospective cohorts, the authors constructed two Multi-Cancer Detection Blood Test (MCDBT-1/2) models. The validation test showed that MCDBT-1 achieved 69.1 % sensitivity, 98.9 % specificity, and 83.2 % tissue-based accuracy, while MCDBT-2 showed a slightly lower specificity of 95.1 % but a higher sensitivity of 75.1 % for high-cancer-risk populations. Overall, these models showed promising results for the detection of the six cancers [78]. Similarly, the preliminary results of the PanSeer study on the Taizhou Longitudinal Study (TZL) cohort showed that a noninvasive blood test based on ctDNA methylation, called PanSeer, detected five common types of cancer in 88 % of post-diagnosis patients with 96 % specificity. The test further demonstrated the ability to detect cancer in 95 % of asymptomatic individuals who were later diagnosed, suggesting its potential for early noninvasive detection up to four years before the current standard of care [79].

In addition to being validated for multi-cancer screening, cfDNA methylation testing has also been extensively evaluated for the screening and diagnosis of single cancers. For example, the Epi proColon® (mSEPT9 assay) is an FDA-cleared test for colorectal cancer screening in adults ≥50 years of age. The test uses qualitative real-time PCR to access the methylation status of the SEPT9 gene using cfDNA. In early retrospective case-control studies, the test demonstrated promising sensitivity (approximately 70 %) and specificity (90 %) for the detection of colorectal cancer [80, 81]. A study evaluated the efficacy of integrating blood-based noninvasive tests, including cfDNA-based methylation and mutation profiling and blood-based protein biomarker testing, to assist clinicians in diagnosing pulmonary nodules (PNs) and to address the high false-positive rate of conventional low-dose computed tomography (LDCT) for lung cancer. The integrative multi-analyte model, based on statistical and machine learning methods, achieved an area under the receiver operating characteristic curve (AUC) of 0.85 in a discovery cohort. The performance of the model was confirmed in an independent validation cohort, which reproduced an AUC of 0.86 with 80 % sensitivity and 85.7 % specificity [82]. Taken together, these results show great promise for the application of ctDNA methylation in early cancer detection (Tables 2–5).

Molecular typing of tumors

Histomorphology has traditionally served as the basis for cancer diagnosis, and now the emerging NGS-based molecular profiling, which includes gene mutations, gene expression, and epigenetic alterations, is emerging as a complementary approach to improve diagnostic accuracy. By analyzing genome-wide DNA methylation patterns in tumor tissue using machine learning, robust diagnostic classifiers have been developed, leading to the discovery of new cancer subtypes and unifying morphologically diverse cancers into coherent biological categories [84–87].

Recent studies have shown that cfDNA methylation profiles also have the potential to further classify a pathologic tumor type into distinct subtypes and to integrate morphologically diverse cancers into cohesive biological

Table 2: Application of cfDNA methylation test in early tumor screening.

Trial name	Method	Applied cancer	Cases	Sensitivity; specificity	Ref
CCGA	WGBS	Pan-cancer	2,800	51.5 %; 99.5 %	[76]
ELSA-seq	–	COAD/READ, ESCA, LIHC, LUAD/LUSC, OV, PAAD	1,693	69.1 %; 98.9 %	[78]
PanSeer	PCR+WGBS+RRBS	Multi-cancer	123,115	88 %; 96 %	[79]
Epi proColon	Real time PCR	Colorectal cancer	354 + 514	70 %; 90 %	[80, 81]
–	NGS	Lung cancer	99	80 %; 85.7 %	[82]
PulmoSeek	TBS-seq	Lung cancer	389	93.3 %; 60.0 %	[83]

CCGA, circulating cell-free genome atlas; WGBS, whole genome bisulfite sequencing; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; ESCA, esophageal carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian cancer; PAAD, pancreatic adenocarcinoma; NGS, next-generation sequencing; TBS-seq, targeted bisulfite sequencing.

Table 3: Application of ctDNA methylation assay in tumor therapy monitoring.

Cancer type	Method	Therapy	Cases	Conclusion	Ref
CRC	qMSP	Surgery	120	A higher risk of death after surgery correlated with positive mSEPT9 detection before surgery.	[92]
CRC, OC, NSCLC	dPCR	Chemotherapy	420	A high correlation between ctDNA response and median survival was found across all tumor types and treatments, surpassing the initial assessment and the ORR.	[93]
NSCLC	–	Neoadjuvant therapy	167	The percentage of patients with MPR or pCR was higher among those with ctDNA responder than those without ctDNA responder (33–86 % vs. 0–17 %).	[94]
PC	Infinium human methylation 450 K BeadChip	Abiraterone acetate (AA)	108	Thirty cytosines showed significant modification differences between AA-sensitive and AA-resistant patients during the treatment.	[95]
LC	qMSP	Chemotherapy	316	When at least one gene exhibited methylation levels greater at 24 h compared to 0 h, it resulted in a correct prediction rate of 82.4 % for tumor response.	[96]
CRC	–	Radiotherapy and chemotherapy	175	Assaying blood for ctDNA methylated in BCAT1/IKZF1 can identify residual disease due to treatment failure.	[97]

CRC, colorectal cancer; qMSP, quantitative methylation-specific PCR; OC, ovarian cancer; NSCLC, non-small cell lung cancer; dPCR, droplet digital PCR; PC, prostate cancer.

Table 4: ctDNA methylation assay in tumor prognosis assessment.

Cancer	Method	Sample	Cases	Stage	Conclusion	Ref
Ovarian cancer	qMSP	Plasma FFPE	250	I–IV	The DNA methylation of SLFN11 in plasma cfDNA is significantly correlated with worse PFS.	[98]
Colorectal cancer	TBS-seq	Plasma	1,138	I–III	RFS: 76.9 %, OS: 72.6 %.	[99]
Ovarian cancer	dPCR	Plasma	32	–	Patients with HOXA9 meth-ctDNA exhibited a median PFS of 5.1 months, whereas patients lacking HOXA9 meth-ctDNA showed a PFS of 8.3 months.	[100]
Gastric cancer	dPCR	Plasma	148	III–IV	The top 50 % methylated SFRP2 has shorter PFS and OS than those with bottom 50 % in patients.	[101]
Lung squamous cell carcinoma	TBS-seq	Plasma	26	Various stages	Higher methylation levels are also associated with poorer OS.	[102]
Colorectal cancer	ddMSP	Plasma	499	–	Patients with ctDNA postoperative or post adjuvant chemotherapy experienced a significant lower recurrence-free survival than patients without ctDNA.	[103]
Pancreatic adenocarcinoma	dPCR	Plasma	372	–	Median PFS and OS are 5.3 and 8.2 months in ctDNA positive and 6.2 and 12.6 months in ctDNA negative patients, respectively.	[104]
Lung cancer	qMSP	Plasma	163	I–IV	Sharp decrease of plasma mSHOX2 level observed in patients with partial response (PR) while not in those with stable disease (SD).	[105]

TBS-seq, targeted bisulfite sequencing; dPCR, droplet digital PCR; qMSP, quantitative methylation-specific PCR; FFPE, formalin-fixed paraffin-embedding.

groups. An illustrative example of this approach can be found in the work of Francesca et al. where the authors investigated the utility of cfDNA methylation profiling for subtyping small cell lung cancer (SCLC) samples and reflecting molecular subtypes found by other methods. Focusing on the achaete–scute complex homolog-like (ASCL1), neurogenic differentiation factor 1 (NEUROD), and double-negative subtypes prevalent in clinical samples (n=174), the investigation used principal-component analysis (PCA) to classify these categories based on methylation analysis and successfully segregated them using the top 50,000 variable methylated regions. This confirms the presence of methylation differences among SCLC subtypes [88]. Similarly, Mathios and colleagues used a machine learning approach to identify tumor-specific cfDNA methylation. The authors performed genome-wide methylation analysis in 365 individuals at risk for lung cancer and then validated the cancer detection model in an independent cohort of 385 non-cancer individuals and 46 lung cancer patients. The model achieved a remarkable 94 % detection rate across all cancer

Table 5: Application of ctDNA MRD in clinical trials.

Cancer	Stage	Case	Therapy	Primary outcome	Conclusion	Ref
Non-small cell lung cancer	IA–III	76	Curative-intent lung resection	–	There is significant consistency and correlation between mutation-based and methylation-based MRD detection, and methylation-based MRD shows a wider dynamic range of risk classification.	[112]
Colorectal cancer	I–III	350	Surgery and chemotherapy	RFS	The recurrence-free survival rate of patients with positive ctDNA is lower than that of patients with negative ctDNA.	[113]
Breast cancer	–	235	Neoadjuvant chemotherapy	Sensitivity and specificity	During neoadjuvant chemotherapy, breast ctDNA levels decreased dramatically.	[114]
Rectal cancer	II/III	303	Neoadjuvant chemo-radiation therapy	ctDNA concentration	Compared with patients with MRD, patients with cancer eradication have significantly lower ctDNA.	[115]
Colorectal cancer	–	172	Surgery	RFS	Post-surgery ctDNA positive was independently associated with an increased risk of recurrence.	[116]
Colorectal cancer	II/III	184	Surgery	TTR	The TTR was significantly shorter in patients with detectable ctDNA during the early postoperative follow-up.	[117]
Colorectal cancer	–	187	Surgery	ctDNA methylated in BCAT1 or IKZF1	Forty-seven patients were ctDNA-positive at diagnosis, and 35 (74.5 %) became negative after tumor resection.	[118]

DFS, disease free survival; OS, overall survival; RFS, recurrence free survival; TTS, time to recurrence.

stages and subtypes, effectively discriminating between SCLC and NSCLC patients with high accuracy (AUC=0.98) [89].

In an independent study, Gao et al. optimized a ctDNA-based methylation assay and identified 12 ctDNA differentially methylated regions (DMRs) as potential biomarkers for distinguishing different clinical subtypes of breast cancer. The authors performed validation using a training set of 38 breast cancer patients and a validation set of 123 patients. These 12 biomarkers showed strong discriminatory ability between ER(+) and ER(−) breast cancer patients, yielding AUC values of 0.984 and 0.780, sensitivity of 93 and 73 %, and specificity of 93 and 87 %, respectively [90].

Treatment monitoring

The short half-life of ctDNA and its correlation with tumor burden in patient plasma facilitates monitoring of tumor progression and treatment efficacy [91]. For example, Song et al. observed a significant 57.6-fold and 131.1-fold reduction in mean plasma mSEPT9 levels at 1 and 7 days postoperatively, respectively, in 120 colorectal cancer patients, allowing treatment evaluation in 86.7 % of them [92]. A retrospective study compared ctDNA response rate and objective response rate (ORR) as predictors of overall survival (OS) in metastatic cancer patients receiving chemotherapy in 420 patients from different cancer cohorts. The ctDNA response rate, determined by measuring tumor-specific methylation using cfDNA, showed moderate correlations with objective response (R^2=0.68) and ORR (R^2=0.57) at first analysis. Notably, ctDNA response significantly correlated with median survival (R^2=0.99), suggesting its potential as a surrogate OS marker [93]. Shen's study demonstrates a robust association between the pathological response and ctDNA response in patients with non-small cell lung cancer (NSCLC). Following neoadjuvant therapy, patients who achieved a pathological complete response (pCR) and clearance of ctDNA exhibited a significantly enhanced long-term survival rate [94].

Liquid biopsy also holds promise for monitoring the effects of drug therapy and detecting DNA methylation alternations associated with drug resistance. Infinium Human Methylation 450 K microarrays were used by Juozas et al. to identify 30 CpGs associated with abiraterone acetate (AA)-sensitive and -resistant prostate cancer patients, providing potential markers for assessing AA treatment response [95]. Another study by Wang et al. quantified plasma APC and RASSFlA gene methylation levels in lung cancer patients before and 24 h after chemotherapy to predict treatment efficacy. Elevated plasma methylation levels were found to predict sensitivity to cisplatin chemotherapy, with significant tumor DNA release and higher ctDNA levels after drug administration indicating drug sensitivity. Notably, Kaplan-Meier analysis and dynamic methylation monitoring indicated that patients with elevated plasma APC or RASSFlA methylation levels after cisplatin chemotherapy had improved outcomes [96]. Following that, the investigators observed a correlation between the concentration of ctDNA and the advancement of colorectal cancer in patients who tested positive for ctDNA. The ctDNA level was found to be associated with tumor diameter and volume. After receiving treatment, which included surgery and adjuvant

chemotherapy, 98 % (47/48) of the patients experienced a decrease in their ctDNA levels, and in 88 % (42/48) of the patients, ctDNA could no longer be detected in their blood samples. However, among those patients who did not complete the full course of adjuvant chemotherapy following surgery, approximately half of them continued to exhibit ctDNA positivity [97].

In conclusion, cfDNA methylation patterns exhibit significant clinical potential for monitoring treatment response and predicting outcomes in cancer patients, surpassing other methods in efficacy and applicability.

Prognostic assessment

Continuous monitoring of disease response to treatment, progression, or metastasis is essential during tumor therapy, and regular follow-up is critical to prevent recurrence during asymptomatic survival. Epigenetic alterations in ctDNA have emerged as potential liquid biopsy biomarkers for ovarian cancer diagnosis, prognosis, and treatment response. Specifically, the methylation status of six gene promoters, including BRCA1, CST6, MGMT, RASSF10, SLFN11, and USP44, was investigated for their utility as liquid biopsy biomarkers in ovarian cancer prognosis, and revealed a significant correlation between aberrant SLFN11 methylation in cfDNA and PFS in advanced-stage high-grade serous ovarian cancer. This suggests that epigenetic inactivation of SLFN11 may predict resistance to platinum-based chemotherapy [98]. Mo et al. then conducted a multicenter cohort study and developed a diagnostic model for advanced adenoma and colorectal cancer based on 191 blood ctDNA methylation haplotype markers. In addition, patients with elevated preoperative methylation levels had a worse prognosis than those with lower levels [99]. Furthermore, results from a phase II trial evaluating veliparib in ovarian cancer patients with platinum-resistant BRCA mutations showed different outcomes based on HOXA9 methylation status. Patients with detectable HOXA9 methylation after three cycles of treatment had a median PFS of 5.1 months and a median OS of 9.5 months, compared to patients without HOXA9 methylation who had a median PFS of 8.3 months and a median OS of 19.4 months [100]. Subsequently, Yan discovered in their investigation involving gastric cancer patients that individuals with a high level of SFRP2 methylation exhibited decreased PFS and OS compared to those with low methylation levels. Specifically, patients at stage III with high methylation had a median PFS of 11.0 months (vs. NR for those with low methylation) and a median OS of 17.0 months (vs. NR for those with low methylation). Similarly, stage IV patients with high methylation experienced a median PFS of 4.0 months (vs. 7.0 months for those with low methylation) and a median OS of 12.0 months (vs. 16.0 months for those with low methylation) [101]. Moreover, several studies all reveal the same conclusion, that is, patients with high methylation level of ctDNA have poorer prognosis than patients with low methylation level [102–105]. Taken together, these findings highlight the potential of altered ctDNA methylation as a robust prognostic indicator with promising clinical applications.

Minimal or measurable residual disease (MRD) detection

MRD refers to the small number of cancer cells that may remain in the body after initial treatment, even when conventional diagnostic methods, such as imaging, fail to detect disease [106]. Numerous studies have shown that MRD is a major cause of cancer recurrence. Although radiologic imaging is commonly used to monitor patients for disease recurrence after surgery, its cost, limited frequency due to radiation concerns, and low sensitivity for detecting MRD make it inadequate [107]. In light of this challenge, emerging ctDNA detection technology offers a potential solution. For example, in a prospective cohort of stage II colon cancer patients without adjuvant therapy, postoperative aberrant ctDNA methylation was detected in 7.9 % of patients (14 of 178), and 79 % (11/14) of them subsequently experienced recurrence at a median follow-up of 27 months, while only 9.8 % (16) of the 164 ctDNA-test negative patients recurred. In patients treated with chemotherapy, post-treatment ctDNA also indicated a higher risk of recurrence [108]. Detection of MRD based on ctDNA mutations is currently the most widely used method, but the limited availability of ctDNA and the relatively small number of tumor-associated mutation loci make detection accuracy extremely challenging [109–111]. The results highlight that aberrant ctDNA methylation in CRC patients can be used as a tool for MRD detection and identification of high-risk patients.

Aberrant alterations in methylation at the genomic level of tumor cells are prevalent in tumors compared to mutations, and thus ctDNA methylation-based detection of MRD is increasingly favored by researchers. A study called MEthylation-based Dynamic Analysis for Lung cancer (MEDAL) demonstrated significant concordance and correlation between mutation-based and methylation-based MRD testing in NSCLC patients, with methylation-based MRD showing a broader dynamic range for risk classification [112]. In a multicenter prospective cohort study of 299 colorectal cancer patients, ctDNA methylation-based MRD

testing identified positive results in 232 patients (78.4 %), with ctDNA-positive patients having a 17.5-fold higher risk of recurrence in the first month after surgery and shorter recurrence-free survival even after adjuvant chemotherapy [113]. MOSS analyzed the methylation profiles of ctDNA in blood samples collected from 235 individuals diagnosed with breast cancer both before and after neoadjuvant treatment and chemotherapy. Moss et al. revealed a substantial correlation between elevated ctDNA levels in the samples and the range of invasive molecular tumors as well as the disease's metabolic activity [114]. Then, some findings were subsequently replicated in colorectal cancer studies, yielding similar outcomes [115–118]. The results highlight that aberrant ctDNA methylation in cancer patients can be used as a tool for MRD detection and identification of high-risk patients.

While several commercial MRD assays for solid tumors exist, there are currently no FDA/NMPA approved products on the market, underscoring the need for extensive multicenter prospective clinical trials to establish the clinical utility of these products [119].

Pre-analytical factors

The pre-analytical factors could influence the precision of results within a clinical setting. Sample integrity, volume, and the timing of blood sampling were the general pre-analytical variables. Cell preservation tubes or cfDNA collection tubes are recommended to prevent the lysis of white blood cells and other cells even nucleic acid degradation. To ensure sample integrity, several sampling tubes are available, including PAXgene® Blood cfDNA Tube (Qiagen), Cell-Free DNA Collection Tube (Roche), cf-DNA/cf-RNA Preservative Tube (Norgen Biotek), and Cell-Free DNA BCT Tubes (Streck) [120, 121]. The input quantity of cfDNA that can be tested is directly related to the volume of plasma extracted. Table 6 presents the required volume of plasma or blood for cfDNA methylation tests currently available on the market. The level of ctDNA or cfDNA may be influenced by the patient's condition, including surgery, treatment (chemotherapy, targeted therapy, immunotherapy, and radiation therapy), and inflammation. As a result, the timing of blood collection for ctDNA analysis should be carefully chosen based on the purpose of the test. It is recommended to collect blood samples before surgery, radiotherapy, or chemotherapy when the clinical application scenario involves early cancer screening and molecular typing of tumors. However, for advanced cancer genotyping, it is recommended to refrain from drawing blood samples during active therapy of responding or non-progressing tumors to reduce the risk of false-negative results [17]. After surgery or chemotherapy, cfDNA concentrations may increase due to tissue injury [122]. Therefore, blood collection should be performed at least 1–2 weeks after surgery depending on the extent of tissue damage and healing time to treatment monitoring, prognostic assessment, and MRD detection [17]. There were several consortia developed the guidance or workflows for pre-analytical conditions of cfDNA methylation assays [121, 123].

Summary

Circulating tumor DNA (ctDNA) has emerged as a valuable tool in cancer clinics, offering non-invasive diagnostics, monitoring, and treatment assessment [128]. Various methods providing access to ctDNA methylation have paved

Table 6: Current accredited blood cfDNA methylation tests in the market for cancer detection.

Company	Disease	Tests	Technology (biomarkers)	Biospecimen	Status	Cost
Epigenomics	Colon cancer	Epi proColon	MethyLight (SEPT9)	Plasma, 3.5 mL	FDA approval 2016	~$112–$182 [124]
Epigenomics	Liver cancer	HCCBloodTest	MethyLight (SEPT9)	Plasma, 3.5 mL	CE-IVD mark 2019 in Europe	~$112–$182
Epigenomics	Lung cancer	Epi proLung	MethyLight (SHOX2 and PTGER4)	Plasma, 3.5 mL	CE-IVD mark 2017 in Europe	$192 [125]
GRAIL	Multicancer	Galleri	NGS (no disclosed markers)	Whole blood>3 mL	FDA breakthrough device designation 13 May 2019	$949 [126]
Laboratory for advanced medicine	Liver breast, colon, lung	IvyGene liver Dx	ddPCR/NGS (no disclosed markers)	Blood 40 mL (10 mL in each of the four tubes)	FDA breakthrough device designation 3 September 2019	$400
Clinical genomics	Colorectal cancer	COLVERA™	MSP (IKZF1, BCAT1)	Plasma, 3.9 mL	2016 CLIA LDT	$449 [127]

the way for the identification of tumor-specific epigenetic alterations, resulting in benefits such as early cancer detection, molecular typing, real-time monitoring of treatment response, and tracking of minimal residual disease (Figure 2). The discriminatory power of ctDNA methylation patterns within or across cancer types and their ability to provide insight into tumor heterogeneity contribute to our broader understanding of cancer epigenetics and treatment dynamics. To improve clinical utility, integration of ctDNA methylation with other molecular markers could enhance diagnostic precision and predictive ability [129, 130]. At the same time, optimization and validation of detection methods across cancer types and stages remains critical to facilitate broader clinical adoption. Future research should prioritize the establishment of standardized protocols, robust validation studies, and comprehensive databases of ctDNA methylation profiles, ultimately enabling seamless integration into routine cancer care.

Nevertheless, there are challenges to the clinical application of ctDNA methylation, particularly in cancer screening. The scarcity of ctDNA in blood at early stages of cancer, coupled with variations in methylation site enrichment between detection technologies, directly affects detection efficacy [131, 132]. The ctDNA has a short half-life of 16 min to 2.5 h in cancer patients [133]. Cell preservation tubes can be used to ensure the ctDNA quality. Blood can be stored at room temperature for 5–7 days if collected in cell preservation tubes [134]. Ensuring high-quality ctDNA and comprehensive methylation site information becomes paramount for reliable analysis. The possibility to use the ctDNA methylation ranges from single-gene methods (with PCR-based methods, such as dMSP and qMSP) to multigene panel analysis such as NGS methods [135]. Several registered blood cfDNA methylation tests in the market for cancer detection have been summarized in Table 6. Furthermore, ctDNA methylation research is still in its infancy, with a limited number of tumor-related methylation biomarkers hindering translation into clinical practice. Despite these hurdles, ctDNA methylation has significant clinical potential. Continued advances are expected to pave the way for widespread adoption of ctDNA methylation-based liquid biopsy, extending its benefits to a larger patient population.

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