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Research Progress and Future Prospects of Detection Technology Based on ctDNA methylation in Cancer detection

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Abstract:

CtDNA methylation detection technology, as a novel tumor marker detection method, plays a significant role in cancer screening. This article summarizes the development history of ctDNA methylation detection technology, existing ctDNA methylation detection technology, and practical application cases of ctDNA methylation detection technology in cancer screening. It also analyzes the technical principles of two major existing techniques: affinity enrichment and Em-seq, as well as their respective advantages and disadvantages.

Keywords: Circulating tumor DNA, Cell-free DNA, Tumor, DNA methylation

1. Introduction

Cancer, recognized as one of the primary contributors to elevated mortality rates in both developed and developing nations, has prompted significant interest in treatment research within the medical field. Early detection of cancer plays a crucial role in significantly increasing the cure rate of cancer.

There are currently four traditional methods for cancer detection: imaging, endoscopy, pathological biopsy, and tumor marker detection. Imaging, which uses X-ray, CT, MRI, ultrasound and other techniques to observe structures in the body and detect abnormal masses or tissue lesions, is less sensitive to early micro tumors or cancerous changes without obvious morphological changes and poses a risk of radiation exposure; Endoscopy, which directly examines the surface of hollow organs through spectroscopy, colonoscopy, colonoscopy, etc., and combines biopsy to obtain lesion tissue samples, has the limitations of being highly invasive, having low patient compliance, and relying on operator experience. Pathological biopsy, which involves obtaining tissue samples through surgery or puncture and conducting microscopic morphological analysis, is the "gold standard" for cancer diagnosis. However, invasive procedures may cause complications and cannot dynamically monitor tumor progression. Conventional tumor marker tests include specific protein or metabolite levels such as carcinogenicity antigen (CEA) and alpha-lipoprotein (AFP) in the blood. However, for instance, the AFP test exhibits a sensitivity of 62.4% with a cutoff value of 20 Eng/ml. This level of sensitivity is insufficient and lacks the accuracy required for early detection, potentially leading to false negative results[1].

Liquid biopsy based on circulating DNA methylation, as a novel tumor marker detection method, has the advantages of high sensitivity and specificity and can be used for early screening. Through the utilization of epigenetic features and dynamic molecular-level analyses, non-invasive, highly sensitive, and traceable cancer screening is achieved. This approach is particularly effective for early detection and person-

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alized treatment monitoring in high-risk populations. With the clinical promotion of methylation detection kits (such as SEPT9 detection for colorectal cancer), this technology is gradually becoming an important pillar of cancer detection technology.

This article will provide a brief introduction to ctDNA methylation detection techniques in existing tumor detection, with the aim of providing a reference for the selection of methylation detection techniques.

1.1 Introduction to ctDNA

Circulating tumor DNA (ctDNA) refers to fragments of DNA released into the bloodstream from cancer cells that have undergone apoptosis or necrosis. It is a sub type of circulating cell-free DNA (DNA) that typically originates from pathological processes. In the 1970s, scientists first discovered the presence of circulating tumor DNA (ctDNA) in the blood of cancer patients[2] studies showed that ctDNA has a half-life of less than 2 hours in the bloodstream[3], meaning it is relatively short in the blood. This characteristic makes the detection and analysis of ctDNA somewhat challenging, but also makes it possible to monitor the dynamic changes of tumors in real time.

1.2 Relationship between DNA methylation and cancer

All stages of cancer occurrence and development, including initiation, progression, and invasion, are driven by epigenetic abnormalities and alterations. Geneticist refers to any structural modification in a region of the genome that causes changes in gene expression without altering the DNA sequence. During embryonic development, DNA methyltransferase (DNMT3A and DNMT3B) methyl the C5 position of cytosine in C Pg nucleoside. This methylation marker is maintained by DNA methyltransferase (DNMT1) throughout cell division, thereby establishing an epigenetic marker of the genome[4].Studies have shown that the methylation pattern of tumors remains unchanged and stable throughout the disease progression, making it a reliable biomarker for tumor diagnosis and classification[5].

1.3 Development of CtDNA methylation detection technology

In 1948, Demand and Meta were the first to identify free nucleic acid molecules within plasma[6]. At the same time, Hotchpotch discovered DNA methylation in calf thymus DNA[7]. It was not until 1988 that the effects of DNA methylation on gene function were gradually recognized, and research in this field began. Leon ET AL. reported that the concentration of plasma free DNA in patients with tumors was significantly elevated compared to that in healthy individuals, and based on this, they speculated that free DNA was associated with tumors. Strong and colleagues subsequently reported that some of the DNA in cancer patients originated from tumor cells[8]. In 1994, M. Imam was the first to identify KRAS mutations in circulating free DNA (DNA) from the blood of pancreatic cancer patients using polymer chain reaction (PCR) methods. These mutations were found to be consistent with those detected in tumor tissues. This groundbreaking discovery enabled the detection of tumor presence by identifying abnormalities in circulating tumor DNA (ctDNA). In 2020, Donning Chen's team achieved the detection of trace tumor methylation signals in blood four years earlier than clinical diagnosis by detecting ctDNA methylation levels in blood samples[9], demonstrating that ctDNA methylation detection technology is more sensitive than traditional methods and plays a significant role in the early diagnosis of tumors.

Table 1 Today's existing ctDNA methylation detection techniques

Year	CtDNA methylation detection technique
1992	BSP cloning Sequencing (Bisulfite Sequencing PCR)
	Proposal of whole genome bisulfite sequencing (WGBS)[10]
2003	Pyrophosphite sequencing
2005	Next generation sequencing (NGS)
	methylation immunosuppression sequencing technology (Me Dip-seq)
	reduced representation bisulfite sequencing (RRBS)
2009	Innovations in whole genome sulfide sequencing (WGBS)[11]
2010-2019	oxidativebisulfite sequencing (obs-seq)[12]
	Single-cell Reduced Representation sulfide Sequencing (scrub)
	High-throughput BSP (N GS-ASP)[13]
	BeadChip (methylation chip)[14]
2020	EM-seq technology

2. Technical principles of two common ctDNA methylation deductions

2.1 Affinity Enrichment (MeDIP-seq and MeDIP-chip)

MeDIP -seq (Methylated DNA immunoprecipitation sequencing) is A method that enriches highly methylated DNA fragments using 5-methylcytosine antibodies and then performs high-throughput sequencing of highly methylated regions in the whole genome (Figure 1A)[15]. This method has the advantages of low cost, convenient data processing, and high sensitivity to highly methylated regions. However, at the operational level, the method also faces several challenges. Specifically, the yield of the MeDip product obtained after co-immunosuppression treatment was low, and it was difficult to meet the quality standards required for sequencing library construction. In addition, during the reaction, double-stranded DNA is converted to single-stranded DNA, which is not suitable for the preparation of sequencing libraries.

MeDIP-chip (Methylated DNA Immunoprecipitation) is currently an accurate and reliable experimental technique for high-throughput analysis of ergonomic DNA methylation changes (Figure 1B)[16], with which methylation sites across the entire genome can be detected; To study the regulation of genes by promoter methylation; Compare methylation profiles of different tissues, cells, tumors, etc. And look for molecular markers for diagnosis and prognosis. The advantage lies in genome-scale detection, which provides a large amount of information; Effective detection of methylation in regions related to biological time in humans; The downside is that the equipment required for chip hybridization is expensive; Chips are limited to human samples; Only a limited number of C Pg sites can be covered.

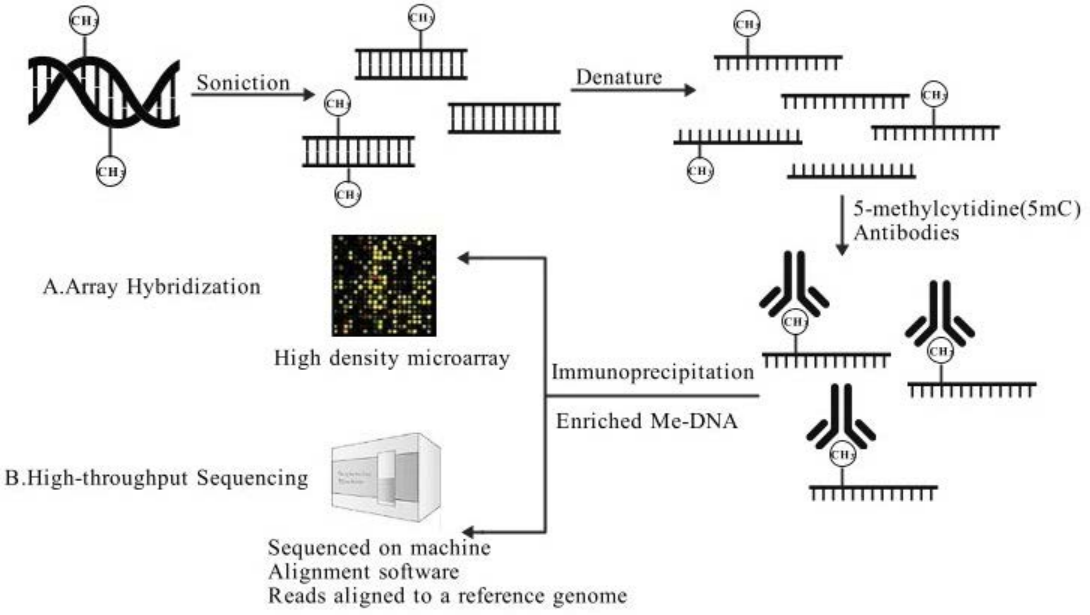

Figure 1 Pattern diagram of affinity enrichment (MeDIP-seq and MeDIP-chip)

A whole-genome methylation detection technique based on antiantibody enrichment sequencing, using methylated DNA co-immunoprecipitation technology, which specifically enriches methylated DNA fragments on the genome through 5mC antibodies, and then high-throughput sequencing can be used to study CPG-dense hypermethylated regions with high precision at the whole-genome level.

2.2 EM-seq technology

EM-seq (Enzymatic Methyl-sequencing, Enzymatic methylation sequencing) is an enzymatic reaction-based DNA methylation detection technique that distinguishes methylated cytosine (C) from methylated cytosine (5mC/5hmC) through mild enzymatic reactions (as shown in figure 2), It has shown significant advantages in epigenetic studies.

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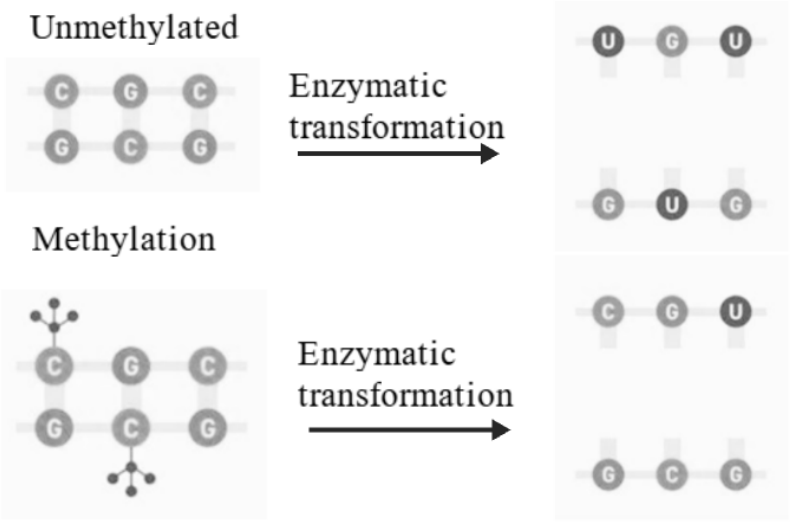

Figure 2 EM-seq technology distinguishes methylated cytosine from methylated cytosine by enzymatic reaction and the principle

TET2 catalyzes the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) through a series of three consecutive steps (Figure 3A). T4-β-glucosyltransferase (T4-BGT) subsequently converts 5hmC, which is generated by the action of TET2 on the original genome, into glucosylated methylcytosine (5gmC) (Figure 3B). Additionally, APOBEC3A deaminates cytosine (C), as well as both 5mC and, to a lesser extent, 5hmC (Figure 3C).

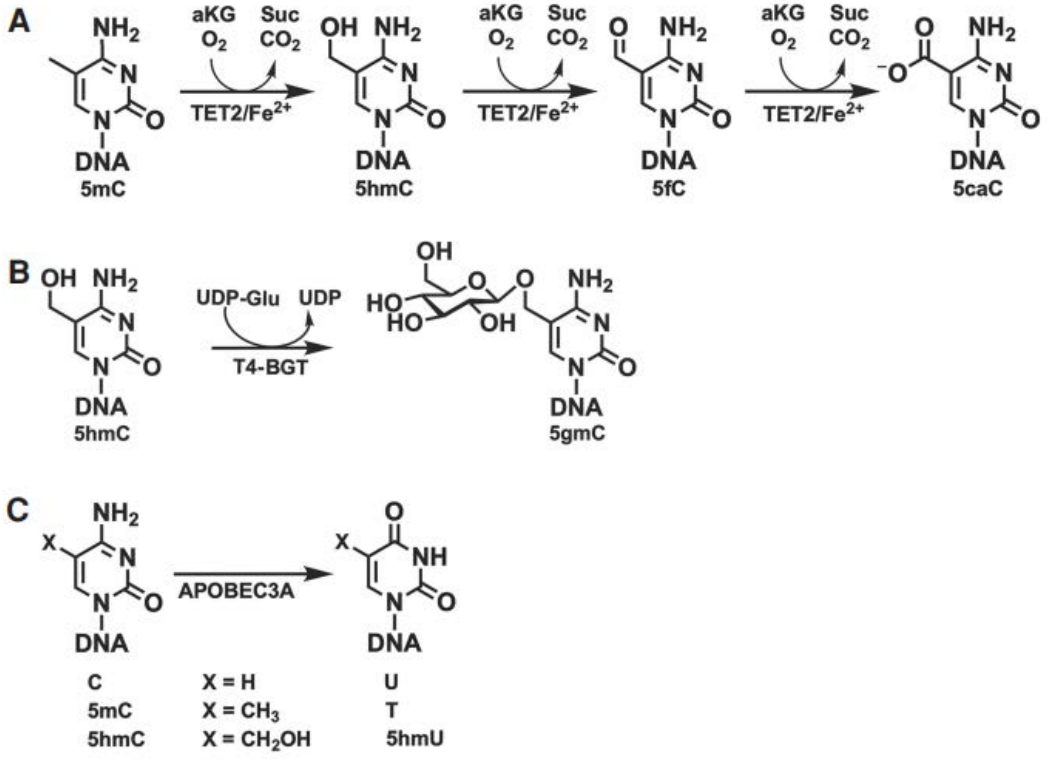

Figure 3 Mechanism of action of Em-seq technology enzyme[39]

The method uses two sets of enzymatic reactions to detect 5mC and 5hmC. During the first reaction, translocation dioxygenase 2 (TET2) and T4-phage -β -glucosyltransferase (T4-BGT) convert 5mC and 5hmC to 5-carboxylcytosine (5caC) and 5-(β -glucoxymethyl) cytosine (5ghmC), These reactions protect 5mC and 5hmC from downstream

deamination, and the apolipoprotein B mRNA editing enzyme catalyzes subunit 3A (APOBEC3A) to deamen C to U. Subsequent polymerase chain reaction (PCR) amplification converts the modified 5mC or 5hmC to C and converts U to T. Libraries were constructed using genomic DNA from NA12878, cfDNA, and FFPE DNA, respectively. The levels of 5mC and 5hmC detected in the EM-seq library were comparable to those observed in the bisulfite library; however, EM-seq demonstrated superior performance across specific metrics such as coverage, repeatability, and sensitivity. Notably, EM-seq remained effective and maintained its advantages even when the initial amount of DNA was as low as 100 pg.

3 Specific cases of ctDNA methylation detection technology in tumor detection

3.1 Using circulating DNA methylation detection techniques for hematological malignancies

Hematological malignancies are malignant tumors that originate in the blood or lymphatic system, including leukemia, lymphoma, multiple myeloma, etc. These tumors are distinguished by the uncontrolled proliferation of atypical cells within the bone marrow, blood, or lymphoid tissue, with the potential for dissemination throughout the body. Unlike solid tumors, the cells of hematological malignancies are typically present in the circulatory system, making it easier to obtain tumor-related markers through liquid biopsies such as blood tests[17].

Leukemia (malignant clonal disease of hematopoietic stem cells), commonly known as blood cancer, is a type of malignant clonal disease that originates from hematopoietic stem cells. The core feature is the uncontrolled proliferation of abnormal white blood cells in the bone marrow or other hematopoietic tissue, which inhibits normal hematopoietic function and may infiltrate other organs and tissues. In 2023, Freemasons Koowattanasuchat used the methylation pattern of the C Pg site to distinguish normal DNA from ctDNA and successfully differentiated normal and cancer DNA in real blood samples, demonstrating that circulating DNA methylation detection technology can assist in early screening[18]. Jun-Ding Li's team used methylation digital PCR (decrypt) to dynamically monitor treatment responses in AML patients and predict the risk of recurrence[19]. In addition, Floridan Cheerer used DNA methylation-specific PCR for detecting DNMT3A biallelic mutations in acute myeloid leukemia (AML), revealing the hypermethylation driver mechanism[20].

multiple myeloma (MM) is a malignant tumor caused by the growth of plasma cell clones. It is an incurable disease mainly characterized by the proliferation and extensive infiltration of monoclonal malignant plasma cells in the bone marrow. Currently, bone marrow biopsy remains the gold standard for the diagnosis of MM This method requires a high level of skill from the sampler and causes some trauma to the patient[21].Xiang Tan determined in 2021 that the presence of ctDNA was associated with a less favorable prognosis observed in patients diagnosed with hematological malignancies, including lymphoma, multiple myeloma, thermoplastic syndromes, and leukemia after screening data from 996 articles. Subsequently, Li JY conducted targeted deep sequencing and decrypt tests on plasma ctDNA in 2023, and compared the consistency with BM puncture and time-matched extracellular cytoplasm biopsy showed very good results for ctDNA[22].

3.2 Use of circulating DNA methylation detection techniques for solid tumor detection

Solid tumors refer to tangible tumors, which are tumors originating from solid organs and include both benign and malignant tumors. Cancer is a general term for malignant tumors, including lung cancer, breast cancer, stomach cancer, liver cancer, rectal cancer, colon cancer, pharyngeal cancer, thyroid cancer, cervical cancer, endometrial cancer, pancreatic cancer, prostate cancer, bladder cancer, etc. Compared with the mature application in hematological malignancies, CtDNA-based methylation detection technology shows a rapidly developing potential area in the detection of solid tumors. Methods such as WGBS (whole-genome sulfide sequencing), RRBS (reduced representation sulfide sequencing) and MSP (methylation-specific PCR) have emerged[23], and methylation sites related to solid tumors such as SPG20,SFRP2 genes have also been discovered[24].

Colon cancer is among the most prevalent tumors, frequently arising from the malignant transformation of colon polyps. With the accumulation of gene mutations and epigenetic changes, colon cells within the polyps show malignant proliferation, leading to an increasing size of the polyps[25]. Over time, the increase in DNA damage within the cells raises the risk of developing invasive cancer[26]. If not removed in time, the tumor cells may invade the surrounding tissues and beyond the collector wall. This malignant growth promotes tumor particularization, which is easy to enter the lymphatic and circulatory systems and ultimately promotes distant metastasis of cancer cells[27]. In Chinese cancer statistics, colon cancer ranks fifth in incidence among tumors in both men and women, but fourth in mortality[28]. Therefore, the

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early detection and removal of tumors are crucial for the treatment of colon cancer. In 2024, Ciao Taiping's team discovered through peripheral blood ctDNA methylation detection that ctDNA methylation detection and analysis play a crucial role in the diagnosis and treatment of collector cancer, and changes in ctDNA methylation levels can also reflect changes in tumor burden, providing a sensitive and dynamic approach for efficacy evaluation and recurrence monitoring[29]. Tan Ciao constructs methylation detection biosensors to detect the methylation of the Septin9 gene in blood and the SDC2 gene in feces for the initial screening of colon cancer[30]. Chaos Jinny excavated key DNA methylation genes in the progression of colon cancer and predicted the biological functions of DNA methylation marker genes, establishing a data model related to normal and disease of colon cancer[31].

Primary liver cancer is the fourth most common malignant tumor and the second leading cause of tumor-related deaths in China, with high morbidity and mortality rates worldwide[28]. Primary liver cancer includes hepatocellular carcinoma (HCC) and a intrahepatic cholangiocarcinoma. Among ICC and combined hepato-cellular-cholangiocarcinoma (cHCC-CCA), HCC accounts for 75% to 85%[32] and is the dominant pathological type of liver cancer. In 2017, Lamina A Man determined that hypermethylation of RASSF1A in serum could be used as a tool for early screening of HCC by detecting features such as point mutations, amplifications, rearrangements, and DNA methylation in ctDNA, and had a higher detection rate than the traditional liver cancer marker AFP [33]. In 2021, WANG J used abnormal DNA methylation to detect ctDNA. The experiment showed that the sensitivity of the circulating DNA methylation detection technique was 78.57%, the specificity was 89.38%, and the accuracy was 85.27%, indicating that the detection of methylation may serve as a viable liquid biopsy approach for the diagnosis of extracellular carcinoma (HCC)[34].

Conclusion

Background interference has always been a major challenge in liquid biopsies of tumors. Free DNA (DNA) released by normal cells in the blood dominates, greatly diluting tumor-derived ctDNA, making the testing process as challenging as looking for a needle in a haystack. In addition, there is heterogeneity in the methylation patterns among individuals, and this difference may further reduce the specificity of the test, making it even more difficult to accurately identify tumor-derived ctDNA.

In terms of tumor localization, while methylation detection can suggest the presence of a tumor and predict its possible primary organ, it is significantly inadequate in providing detailed information such as the exact location, shape, or depth of invasion of the tumor within the organ. For example, in endometrial cancer screening, methylation detection can only be used as an adjunct, and it still needs to be combined with other examination methods such as B-ultrasound and CT to ultimately confirm the lesion area.

Dynamic monitoring of tumor changes is an important part of tumor treatment, but ctDNA has a short half-life and a single test may not accurately reflect the real-time state of the tumor. Therefore, multiple samplings are needed to obtain more comprehensive information. However, the limited amount of DNA obtained from a single blood draw greatly limits the possibility of combined multi-access analysis, such as simultaneous detection of gene mutations and methylation, making dynamic monitoring more difficult.

The risk of false positives is also a problem that cannot be ignored in tumor liquid biopsies. Certain benign diseases or inflammatory states may cause abnormal methylation, which can lead to false positive results. This can not only cause unnecessary panic and anxiety in patients, but also affect doctors' accurate judgment of the condition and the formulation of treatment plans. Therefore, when performing a liquid biopsy of a tumor, these potential risk factors must be fully considered and corresponding measures taken to deal with them.

Finally, liquid biopsy techniques based on circulating DNA methylation will have a broader direction of development. From the perspective of detection techniques, there are certain deficiencies in various current detection techniques: DNA methylation analysis techniques pre-treated with bisulfite have strict requirements for transformation conditions and may be affected by methylation factors to cause non-specific amplification; The DNA methylation analysis technique pre-treated with restriction endonucleases has a significant impact on the analysis results due to incomplete transformation; The second reason is that restriction endonucleases have the characteristic of specifically recognizing DNA sequences. The problem that the recognition sequence of this enzyme is limited in whole-genome DNA, and the detection range of DNA methylation sites is also limited; The shortcomings of DNA methylation analysis based on single-molecule real-time sequencing technology lie in its high sequencing cost, high requirements for instruments, equipment and personnel qualifications, and the problem of random misjudgment of bases[35-38], fortunately, through the combination of multiple technologies and high-throughput sequencing, It greatly compensates for the shortcomings of the single method.

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