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Perspective

Advance and challenge of DNA methylation as cancer biomarkers for risk stratification, screening and early detection

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1. Introduction

With an estimate of 19,976,499 newly diagnosed cases and 9,743,832 deaths occurred in 2022 worldwide, cancer continues to impose a significant health and economic burden worldwide.[1] The development of cancer is a complex interplay between genetic and environmental factors.[2] In addition to genetic modifications, there is a growing body of evidence suggesting that epigenetic changes, which influence gene expression without modifying the DNA sequence, are playing an increasingly significant role in the development of cancer. DNA methylation, a key epigenetic mechanism, has been notably implicated in the early stages of cancer development, positioning it as a potential biomarker for cancer risk assessment.[3] Studies have identified a diverse array of DNA methylation biomarkers for the early detection and diagnosis of cancer, utilizing DNA extracted from tissues, blood, stool, urine, and bowel lavage fluid.[4] Research of DNA methylation has focused on two primary sources: peripheral blood mononuclear cell or white blood cell (WBC) DNA methylation,[5] linked to cancer susceptibility and tumor-derived cell-free DNA (cfDNA) methylation,[6] which has gained significant attention in recent years as a promising biomarker for cancer screening and diagnosis.

Hence, this article endeavored to consolidate knowledge on the mechanistic role of DNA methylation in cancer etiology, the methodologies for its laboratory detection, and its potential as a biomarker for screening and diagnosis. We envisioned that this post will provide up-to-date evidence, propelling researchers to further investigate and develop effective biomarkers for cancer screening and diagnosis in the era of precision medicine.

2. Mechanism of DNA methylation in cancer etiology

DNA methylation involves adding a methyl group to the 5-carbon position of cytosine rings in CpG dinucleotides, catalyzed by DNA methyltransferases (DNMTs).[7] This modification is critical for gene expression regulation and maintaining genomic stability without altering the DNA sequence itself. In cancer, global hypomethylation and hypermethylation of CpG islands are usually manifested. Typically, CpG islands remain unmethylated even when their related human genes are silenced. However, during development, some CpG islands become methylated and the associated promoters are silenced.[8] This suggests that active methylation of CpG islands may be an important mechanism for gene silencing. The possible mechanism of DNA methylation in cancer etiology is illustrated in the Fig. 1.

Briefly, the canonical cytosine-5 DNMTs, including DNMT1, DNMT3A, and DNMT3B, orchestrate the methylation of genomic DNA. DNMT3A and DNMT3B are *de novo* methyltransferases that independently establish cell-type-specific methylation signatures.[9] However, their dysregulation can lead to cellular dysfunction, oncogenesis, and increased metastatic potential.[10] Moreover, DNMT1 and DNMT3B cooperatively maintain DNA methylation in human cells. They maintain methylation patterns during DNA replication and have a pronounced affinity for hemimethylated DNA.[11] However, inhibition of DNMT1's catalytic activity can silence the activation of oncogenes, contributing to tumor growth and progression.[12]

In the context of oncogenesis, global DNA hypomethylation in cancer cells activates oncogenes and transposable elements, disrupts genomic imprinting, and induce chromosomal instability—all contributors to cancer phenotypes and progression.[13] In general, promoter CpG island hypermethylation typically silences tumor suppressor genes (TSGs) and differentiation genes, impeding transcription factor binding and other transcriptional activities.[14] Additionally, oncogenes, TSGs and differentiation genes represent the oldest human gene classes and evolve concurrently.[15] The inappropriate silencing of differentiation genes can cause loss of cellular identity and acquisition of malignant traits such as unrestrained growth, apoptosis evasion, and invasiveness.[16,17] Subsequently, oncogenes achieve tumor inheritance or evolve into new oncogenes as cells differentiate.[15]

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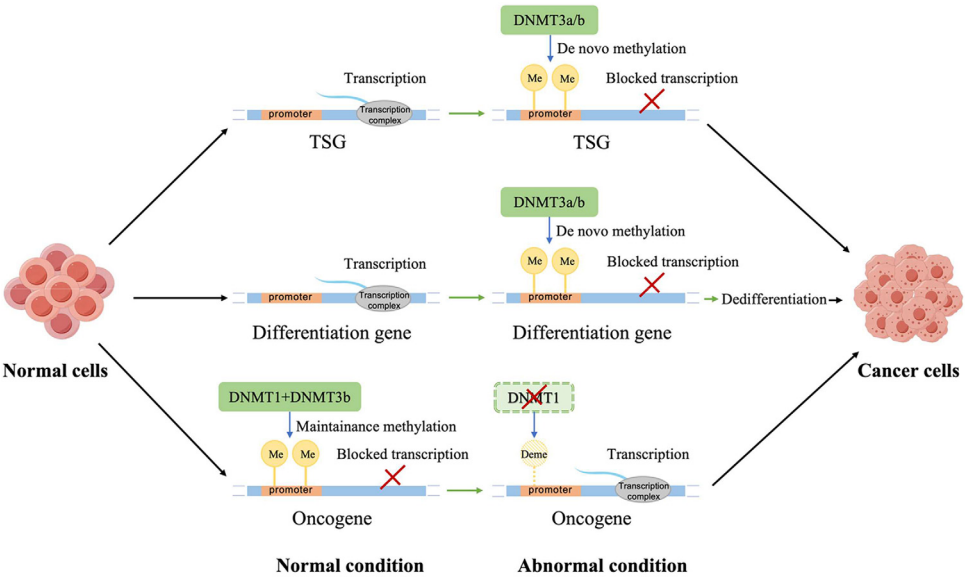

Fig. 1. Mechanism of DNA methylation in cancer etiology. Me, methylation; Deme, demethylation; TSG, tumor suppressor gene; DNMT, DNA methyltransferases.

3. Laboratory detection techniques for DNA methylation

Several laboratory techniques have been developed for the detection of DNA methylation, each with its own strengths and specific applications. We categorized the current established methods according to their purpose and the scale of methylation analysis (more details in the **Supplementary Table 1**). Since there are many available techniques for detecting DNA methylation, the selection of appropriate laboratory methods suitable for study purpose is pivotal. All methods have their inherent advantages and limitations. It is therefore necessary to select the detection methods and analysis strategy based on the specific requirements of the study, including the desired resolution, throughput, coverage, and available resources.

(1) High-throughput methylation analysis: techniques that enable rapid processing of ultra-long read lengths and even whole-genome methylation sequencing offer distinct advantages in resolution, cost, applicability, and sensitivity.[18] For instance, whole genome bisulfite sequencing (WGBS) and nanopore sequencing provide single-base resolution, while WGBS and methylation arrays (such as Infinium Human Methylation family of microarray-based methylation assays: 450K , 850 K, and 935 K) are suitable for whole-genome or large-scale studies. Targeted bisulfite sequencing (TBS) is highly effective for detecting methylation in low-abundance samples, while single-cell sequencing reveals cellular heterogeneity. Additionally, nanopore sequencing enables real-time analysis of long-chain methylation. For studies with budget constraints, reduced representation bisulfite sequencing (RRBS) remains a cost-effective option.

(2) Region- or site-specific methylation analysis**:** techniques that focus on specific regions, such as gene promoters, CpG islands, or specific CpG site, offer high precision, sensitivity, and simplicity.[18] These methods are particularly suited for low-DNA samples, such as liquid biopsies or pathological specimens. The choice of technique depends on experimental goals and sample types. Pyrosequencing and quantitative methylation-specific PCR (qMSP) enable precise quantitative analysis of CpG sites. MSP is a simple and efficient option for initial screening.

(3) Direct methylation analysis**:** techniques that enable direct detection of DNA methylation, without any chemical or enzymatic conversions of DNA and PCR amplification prior to sequencing, avoid biases and preserve the integrity of original DNA information.[19] Emerging techniques in this field, such as nanopore sequencing and SMRT sequencing, allow the analysis of long DNA strands and provide comprehensive methylation profiles. Recent advancements in SMRT sequencing, including the Holistic Kinetic model and Cabernet method, enable simultaneous detection of DNA sequences and methylation states at single-molecule resolution, while also identifying various epigenetic modifications, such as 6 mA and 5mC.

4. DNA methylation as biomarkers for cancer risk stratification

Previous studies have suggested that peripheral blood cell or WBC DNA methylation is associated with cancer susceptibility, highlighting its potential for identifying high-risk populations.[20] A series of DNA methylation biomarkers have been identified; however, current risk assessment studies remain inconclusive regarding which methylation markers are promising for cancer risk stratification.

For example, Sturgeon et al.[21] conducted a nested case-control study within the Prostate, Lung, Colorectal, and Ovarian Cohort, finding no significant association between WBC DNA methylation and breast cancer risk. In contrast, a recent study by Wang et al. suggested that DNA methylation alterations in peripheral blood mononuclear cells (PBMCs) could serve as markers for early-stage breast cancer detection through a 4-hypermethylated-marker BC-mqmsPCR assay, though its utility for breast cancer risk assessment remains uncertain.[22] Similarly, a prospective nested case-control study by Dugué et al.[23] reported that higher genome-wide DNA methylation levels in peripheral blood were linked to a reduced risk of superficial urothelial cell carcinoma, but this finding requires external validation. Furthermore, an analysis of open-source databases stratified cervical cancer patients into high- and low-risk groups based on human papillomavirus-related methylation signatures, demonstrating potential for prognostic predictions but necessitating further research to confirm its role in risk stratification.[24]

In summary, all of the above studies suffer from a lack of external independent validation of the reported DNA methylation markers, which limited their broader applications. In addition to this, several limitations should be considered when interpreting the utility of DNA methylation markers for risk stratification. First, most studies were conducted in clinical cross-sectional settings, with cases typically collected shortly before or at the time of clinical diagnosis. Consequently, the identified biomarkers may primarily reflect symptomatic disease rather than function as reliable predictors of long-term risk or tool for early detection in asymptomatic individuals.[25] Second, blood processing methods and leukocyte subtypes distribution significantly influence the identi-

fied biomarkers, as different leukocyte subtypes exhibit unique DNA-methylation profiles.[26] A summary of studies focusing on DNA methylation as biomarkers for cancer risk stratification is provided in **Supplementary Table 2**.

5. Carriers of genetic information from tumor cells: cfDNA/ctDNA

The knowledge of liquid biopsies has grown exponentially in recent years, encompassing nucleic acids, extracellular vesicles, proteins, and other biological components released into body fluids by cancer cells. Among these analyses, cfDNA in the circulating blood plasma of cancer patients may originate from tumor cells, normal cells, or other tissues. However, several methodological and pre-analytical factors limit the clinical sensitivity of the cfDNA-based detection of cancers from liquid biopsies.[27] ctDNA, a fraction of cfDNA that contains tumor-derived DNA sequences and carries cancer-specific genetic and epigenetic aberrations, is a promising biomarker.[28] In recent years, with the development of laboratory techniques, candidate ctDNA methylation markers have expanded from single markers, or multi-marker panels, to whole genome algorithms by machine learning methods. We summarized the current advances in cfDNA/ctDNA methylation as biomarkers for cancer screening and early diagnosis.

5.1. cfDNA/ctDNA methylation for cancer screening

Cancer screening aims to identify individuals at risk of or suffering from cancer before symptoms appear. The alteration of DNA methylation occurs early in carcinogenesis, making it an attractive approach for cancer screening. Over the past decades, several well-established markers have shown promising potential in cancer screening, such as plasma *SEPT9* methylation for colorectal cancer,[29] and *PTGER4/SHOX2* for lung cancer.[30] The plasma-based *SEPT9* gene methylation assay (also known as Epi proColon test) was the first US Food and Drug Administration (FDA) approved blood-based test for colorectal cancer screening. In a meta-analysis including results of 25 studies, the pooled sensitivity, specificity, and the area under the curve (AUC) for colorectal cancer (CRC) screening were 0.71, 0.92 and 0.88, respectively. However, this marker has limited ability to identify precancerous lesions.[31] Considering its poor performance in detecting precancerous lesions compared to other stool-based screening methods and the lack of long-term evidence in reducing CRC mortality and incidence, current CRC screening guidelines recommend against the use of blood *SEPT9* methylation for CRC screening.

To date, although a number of methylation-based blood biomarkers derived from cfDNA/ctDNA have been suggested to have good potential in cancer screening, only a few have been prospectively validated in a true screening setting, which typically requires very high financial and labor investment. This is similar to the case of peripheral blood cell or WBC DNA methylation, which has been linked to cancer susceptibility. A good example of such a study is the large-scale population-based trial conducted to evaluate a multitarget stool-based DNA test for CRC (also known as Cologuard). This trial, conducted in North America, involved a head-to-head comparison of this new test with the fecal immunochemical test (FIT) in 9989 participants at average risk for CRC.[32] Adopting a similar study design, a novel multitarget stool RNA (mt-sRNA) test, also known as ColoSense, was also prospectively evaluated in 8920 participants in the US.[33] Due to the good screening yield in average-risk population in a true screening setting, both tests have been approved for screening purposes by the US FDA. In the future, large-scale prospective evaluations of promising cfDNA/ctDNA methylation biomarkers in the target screening population will be essential.

5.2. cfDNA/ctDNA methylation for early cancer diagnosis

Early detection and treatment of cancer remain the most effective ways to improve patient survival rates. Currently, widely used early diagnosis techniques include serology (such as cancer biomarkers), imaging examinations, and invasive endoscopic examination (for digestive tumors).[34,35] Non-invasive methods for early tumor diagnosis still pose significant challenges. In recent years, the development of liquid biopsy technology has opened up new possibilities for achieving non-invasive early diagnosis.[36] Since the methylation of TSGs increases in many early tumor changes, it may be the first detectable alteration in the tumorigenic process. By capturing altered methylation of genes released into the bloodstream, cfDNA and ctDNA have the potential to detect cancer in its early stages, enabling timely intervention and improving patient prognosis. We summarized some studies focusing on cfDNA/ctDNA methylation as biomarkers for early detection of cancer, as listed in the **Supplementary Table 3**.

6. Challenges and possible solutions

Despite the immense promise of DNA methylation-based biomarkers in guidance of cancer prevention, diagnosis and therapy, they also face several challenges. Here, we listed some key aspects that needed to be considered.

(1) Laboratory detection techniques: cfDNA/ctDNA is highly fragmented (as short as 50 bp in length), highly sensitive techniques are required to detect and quantify tumor-specific genomic alterations in the presence of background ctDNA released by non-cancerous cells. In addition, the variations in sample collection, storage and processing can strongly impact the results. Furthermore, the lack of standardization and reproducibility across different platforms and methodologies poses a significant challenge to the adoption of DNA methylation-based biomarkers in clinical settings, as highlighted in multiple studies and reviews in the field.

(2) PBMC DNA methylation: Since PBMCs are a diverse population of cells, including lymphocytes (T cells, B cells, natural killer cells) and monocytes, each of these cell types has a distinct DNA methylation profile. However, selecting specific cell types for analysis in a sample is challenging. Moreover, methylation patterns can change with age and environmental factors, leading to variations that are not necessarily related to the condition being studied. This drift can confound the interpretation of methylation data.

(3) Organ specific variability: Various studies have demonstrated that different tissue types have distinct DNA methylation profiles, highlighting the importance of considering tissue specificity when identifying and validating DNA methylation biomarkers for cancer.[37] This is essential for the development of pan-cancer early detection panels, with one key point being the precise prediction of the tissue of origin when specific cfDNA markers detected.

(4) Interindividual variability: Interindividual variability in DNA methylation profiles presents a significant challenge in identifying consistent biomarkers for cancer prognosis. Studies have shown that individuals with the same cancer type can exhibit diverse DNA methylation profiles, highlighting the complexity and individuality of the epigenetic changes associated with cancer.[38]

(5) Clinical validation and long-term follow-up evaluation: Many DNA methylation biomarkers lack robust clinical validation. Ideally, the developed biomarkers should be prospectively validated in representative samples from the target population, such as an asymptomatic population, to validate screening and early detection biomarkers. The mentioned Cologuard and ColoSense are examples of tests that have been approved for CRC screening after external validation.[32,33] However, such rigorous evaluation have rarely been conducted for most of the proposed markers. Moreover, the long-term effectiveness and cost-effectiveness should also be comprehensively evaluated and compared with the currently established screening modalities and strategies.

(6) Clinical adoption and integration: Integrating the biomarkers into routine clinical practice requires addressing logistical, financial, and educational barriers. Healthcare systems must adapt to incorporate new biomarkers into existing screening, diagnostic and prognostic pathways. Additionally, long-term follow-up evaluations are needed to iteratively update the strategies.

To address the challenges in adopting DNA methylation-based biomarkers for cancer, several strategic solutions can be implemented. First, it is crucial to develop and implement highly sensitive and specific detection methods to manage the fragmented nature of cfDNA/ctDNA, and to standardize protocols for sample collection, storage, and processing. Adopting standardized methodologies and rigorous validation processes can mitigate reproducibility issues across various platforms.

For PBMC DNA methylation, cell-type-specific analysis techniques, such as fluorescence-activated cell sorting (FACS) or single-cell methylation profiling, are essential due to the diverse cell populations and their distinct methylation profiles. Conducting longitudinal studies is critical to differentiate between stable, condition-related methylation changes and those resulting from epigenetic drift.

In terms of organ-specific variability, identifying and validating tissue-specific DNA methylation profiles is key to developing pan-cancer early detection panels capable of accurately predicting the tissue of origin for cfDNA markers. Addressing interindividual variability requires large, diverse cohorts to identify consistent biomarkers, focusing on common epigenetic patterns.

Rigorous prospective studies in representative samples from the target population are necessary for clinical validation, along with the evaluation of the long-term effectiveness and cost-effectiveness of DNA methylation biomarkers compared to established screening methods.

For successful clinical adoption, it is essential to overcome logistical, financial, and educational barriers. Healthcare systems must adapt to incorporate new biomarkers into existing screening, diagnostic, and prognostic pathways. Ongoing long-term follow-up evaluations are needed to continuously update and refine these strategies. By implementing these solutions, DNA methylation-based biomarkers can be effectively used in cancer prevention, diagnosis, and therapy, ultimately improving patient outcomes.

7. Summary

In summary, DNA methylation is a promising biomarker for cancer screening and early detection due to its crucial role in carcinogenesis. It is important to emphasize that different types of DNA methylation detection techniques exist, each with its advantages, disadvantages, and clinical indications. The interpretation and application of DNA methylation results should not be done in isolation; the clinical context must be considered alongside technical issues. Despite significant efforts to identify effective DNA methylation markers, only a few have successfully been translated into clinical use. Further prospective validation of promising biomarkers in ongoing clinical trials will be crucial to advancing the field.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

H.C. conceptualized the study. N.L. and K.S. contributed literature review and data extraction. N.L. and K.S. wrote the first draft of the paper. All authors approved the final version of the paper. N.L. and K.S. are the guarantors of the manuscript, and all the authors have full access to the data.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jncc.2024.12.007.

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