

Value of methylation markers in colorectal cancer (Review)

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Received March 9, 2021; Accepted June 18, 2021

DOI: 10.3892/or.2021.8128

Abstract. Colorectal cancer (CRC) is a multifactorial and multistage process that occurs due to both genetic and epigenetic variations in normal epithelial cells. Analysis of the CRC epigenome has revealed that almost all CRC types have a large number of abnormally methylated genes. Hypermethylation of cell-free DNA from CRC in the blood or stool is considered as a potential non-invasive cancer biomarker, and various methylation markers have shown high sensitivity and specificity. The aim of the present review was to examine potential methylation markers in CRC that have been used or are expected to be used in the clinical setting, focusing on their screening, predictive, prognostic and therapeutic roles in CRC.

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1. Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-related mortality in the world. The mean 5-year survival rate of CRC is estimated to be <10% if metastasis occurs, and as high as 90% if the cancer is detected at an early stage (1,2). Several methods are currently available for detecting CRC, such as fecal immunochemical testing (FIT), fecal occult blood testing, and the most important measure,

colonoscopy. However, the tolerance of colonoscopy remains low in the general population, due to the troublesome bowel preparation and the risk of complications. Although progress has been achieved in the research of antitumor drugs, the current curative effect for CRC is far from expected due to the late stage of disease at diagnosis and the unselected patients. This highlights the need to identify novel methods or effective biomarkers to diagnose CRC at an early stage and determine the patient's response to individualized treatment.

Definition of epigenetics. Accumulation of genetic and epigenetic changes ultimately lead to the initiation and progression of cancer (2,3). Genetic mutations have long been considered a major cause of cancer, but more recently, epigenetic changes have also been suggested to be important factors in cancer development (4). The unified definition of epigenetics remains ambiguous; however, more researchers support Holliday's definition as it offers two aspects of epigenetics, that is, 'the study of the changes in gene expression, which occurs in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression' and 'nuclear inheritance, which is not based on differences in DNA sequence'. The streamlined definition is 'the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in the DNA sequence' (5).

The study of DNA forms the cornerstone of genetics, while epigenetics is the study of changes in gene expression that do not involve alterations in the underlying DNA sequence. Common epigenetic changes in different cancer types include abnormal DNA methylation, abnormal histone modifications and changes in the expression levels of numerous non-coding RNAs (6). Abnormal DNA methylation was the earliest identified modification and is the most widely studied epigenetic change (7).

Tumor-derived cell-free DNA (cfDNA). For hypermethylated DNA to be a valid biomarker, it should be available via minimally invasive procedures and from tumor-remote media. Moreover, it must be effective in detecting the disease at an early stage. Based on these characteristics, research on cancer-specific hypermethylated genes has focused on tumor-derived cfDNA.

Cancer cells release nucleic acids, proteins, vesicles and other biological components into the blood and other body fluids (8). Among these potential biomarkers, tumor-derived cfDNA has been examined over the past decade as an important tool in oncology precision medicine. Abnormal DNA

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Key words: DNA methylation, colorectal cancer, epigenetics

methylation is one of the characteristics of numerous cancer type, and most importantly, it can be detected in cfDNA in blood, urine, feces and other biological samples. Moreover, cfDNA methylation is used for early detection of cancer, minimal residual disease surveillance, prediction of treatment response and prognosis and tracing of tissue origin.

DNA methylation markers have numerous advantages (9). Firstly, DNA methylation occurs early in tumorigenesis and can be tissue- and cancer-type specific. Secondly, it is consistent across multiple genomic regions and can be detected using numerous CpG dinucleotides. Finally, and most importantly, methylation patterns are often associated with the origin of specific cancer type, and thus, can be used to reveal the tissue origin.

The landmark for cfDNA methylation analysis was the development of a screening test for CRC, based on the methylated septin 9 (mSEPT9), which was approved by the Food and Drug Administration (FDA) in 2016 (10). At the same time, accumulating evidence verified the value of cfDNA methylation as a biomarker for the diagnosis and evaluation of cancer.

2. Characteristics of CRC DNA methylation

Currently, the molecular pathogenesis of CRC mainly includes the following processes: Chromosomal instability, microsatellite instability (MSI), epigenetic instability [such as CpG island methylator phenotype (CIMP)], altered tumor microenvironments and an altered metabolic state (11).

The current level of the understanding of epigenomic alterations in CRC is less than that of gene mutations, but research in this area has advanced rapidly in recent years. Similar to other malignant tumor cells, CRC cells also show two different DNA methylation changes. One is global hypomethylation, and the other is promoter methylation of specific genes (12). Accumulating evidence suggests the existence of a group of CRC subsets with high levels and distinct patterns of DNA methylation, known as CIMP (2). CIMP was defined as a molecular subclass of CRC in 1999, which was a significant advancement in the understanding of the molecular mechanisms of CRC formation. Overall, hypomethylation is associated with increased genomic instability, suggesting that the maintenance of methylation may be chemoprophylaxis (13). Hypermethylation of cfDNA from CRC and other types of cancer in the blood or stool has been considered as a potential non-invasive cancer biomarker, which may exhibit high sensitivity and specificity in some cases (10,14). In addition, with further research on the mechanism of tumor methylation, the reversal of the DNA methylation abnormalities by targeting the maintenance DNA methylation mechanism has become a potential therapeutic method (4).

3. Methylation detection methods

DNA methylation can be detected using a variety of methods, such as methylation-specific polymerase chain reaction (15), DNA sequencing, MethyLight (16), methylation-specific melting curve analysis (17), pyrosequencing (18), microarray analysis (19) and liquid chromatography (LC). Notably, LC was the first platform used to quantify global DNA methylation, which was represented by the total 5-methyldeoxycytidine

content in DNA samples (20). With advances in technology, LC coupled with mass spectrometry provides an accurate and highly sensitive method for overall DNA methylation quantification (21).

Value of methylation markers. Several methylation markers have been found to be associated with the initiation and progression of CRC (2,3,22). Some of these markers show potential for early detection (Table I), prognostic evaluation (Table II), and even prediction of treatment response to CRC. The status of methylation markers is associated with various conditions, such as tumor size, grade or metastasis (Fig. 1). Therefore, methylation markers, such as O⁶-methylguanine-DNA methyltransferase (MGMT) and long interspersed nucleotide element-1 (LINE-1) (23,24), may have a cross-over role in the prognostic assessment of CRC, prediction of treatment response and even early diagnosis, in different conditions. Thus, to achieve the best predictive effect, the examination of multiple methylation markers, multitarget stool DNA (MT-sDNA) and fecal occult blood testing should be combined.

4. Screening and early detection markers

SEPT9. The *SEPT9* gene is a tumor-suppressor gene, which is closely associated with the development of tumors and other types of human diseases. *SEPT9* is involved in a variety of important physiological processes, such as cytokinesis, DNA repair, cell migration and apoptosis (25). Abnormal methylation reduces the activity of *SEPT9* gene transcription, leading to dysregulated gene expression and abnormal physical function, which may eventually lead to the development of cancer (26).

Toth *et al* (27) reported that the protein expression level of SEPT9 in CRC was significantly lower compared with that in normal epithelial cells, and that the mRNA expression level of *SEPT9* was decreased during the transformation from adenoma to CRC. Moreover, Wasserkort *et al* (28) examined the methylation status of SEPT9 in different types of colon lesions and their adjacent tissue, and suggested that hypermethylation of *SEPT9* may be a late event in the progression of adenomas to CRC. This may be the reason why the sensitivity of *SEPT9* gene methylation detection in adenoma is lower compared with that of CRC. A recent meta-analysis revealed that plasma mSEPT9 was of high diagnostic value for CRC and was significantly correlated with CRC stage (29). In addition, survival analysis indicated that there was a negative correlation between SEPT9 methylation levels and disease-free survival (DFS) after CRC surgery (30). Song *et al* (31) also reported that an SEPT9 assay displayed 75.1% sensitivity and 95.1% specificity for CRC detection. Notably, the detection rate for CRC stage 0 was 57%, for stage I it was 64%, and for stage II it was 88%, which was valuable for population screening, alone or combined with other methods (31).

In a previous study that included 1,544 CRC samples (stages I-IV), a prospective evaluation of SEPT9 trial was retrospectively conducted. The sensitivity and specificity of CRC were reported at 68.2 and 78.8%, respectively, while the sensitivity for advanced adenoma was 21.6%. The main performance characteristics of mSEPT9 were demonstrated

Table I. DNA-methylation markers used for the screening and early detection of colorectal cancer.

| Methylated DNA | Authors, year | Country | Sensitivity % | Specificity % | Detection method | (Refs.) |
|--------------------------|-----------------------------------|-------------------|---------------|---------------|------------------|---------|
| <i>SEPT9</i> | Church <i>et al</i> , 2014 | USA | 68.2 | 78.8 | MSP | (30) |
| | Song <i>et al</i> , 2016 | China | 75.1 | 95.1 | MSP | (31) |
| <i>SDC2</i> | Oh <i>et al</i> , 2017 | Republic of Korea | 90.9 | 90 | MSP | (35) |
| <i>MGMT/SEPT9</i> | Freitas <i>et al</i> , 2018 | Portugal | 93.8 | 82 | MSP | (42) |
| <i>NDRG4</i> | Bagheri <i>et al</i> , 2020 | Iran | 86 | 92 | MSP | (46) |
| <i>NDRG4/SNAP91/FIT</i> | Rademakers <i>et al</i> , 2021 | The Netherlands | 86 | 96 | MSP | (47) |
| <i>APC/FOXA1/RASSF1A</i> | Nunes <i>et al</i> , 2018 | Portugal | 78.4 | 69.9 | MSP | (54) |
| <i>BMP3</i> | Loh <i>et al</i> , 2008 | Australia | 56.66 | 93.3 | MSP | (56) |
| | Rokni <i>et al</i> , 2018 | Iran | 40 | 94 | MSP | (58) |
| <i>VIM</i> | Mojtabanezhad | Iran | 52 | 88 | | (48) |
| | Shariatpanahi <i>et al</i> , 2018 | | | | | |
| | Lu <i>et al</i> , 2014 | China | 41.1 | 85 | MSP | (49) |

MSP, methylation specific PCR; SEPT9, septin 9; SDC2, syndecan 2; MGMT, O⁶-methylguanine-DNA methyltransferase; NDRG4, N-Myc downstream regulated gene 4; SNAP91, synaptosome associated protein 91; FIT, Fer-like iron deficiency-induced transcription factor; APC, adenomatous polyposis coli; FOXA1, forkhead box A1; RASSF1A, RAS association domain family protein 1; BMP3, bone morphogenetic protein 3; VIM, vimentin.

Table II. DNA-methylation markers used for prognostic evaluation in colorectal cancer.

| Methylated DNA | Authors, year | Country | Prognostic value | (Refs.) |
|--------------------|-------------------------------|-----------|--|---------|
| <i>SFPR</i> | Bagci <i>et al</i> , 2016 | Turkey | Associated with lymph node invasion and OS | (72) |
| | Boughanem <i>et al</i> , 2020 | Spain | Increases CRC cell proliferation and tumor growth | (70) |
| <i>p16</i> | Karam <i>et al</i> , 2018 | Egypt | Correlated with Dukes' stage, lymph node metastasis and CEA levels | (74) |
| | Kim <i>et al</i> , 2016 | Korea | Predictive of clinical outcome in metastatic CRC patients | (77) |
| <i>LINE-1</i> | Barchitta <i>et al</i> , 2014 | Italy | Associated with poor prognosis, survival and advanced stage | (82) |
| | Jiang <i>et al</i> , 2021 | USA | Indicative of the CRC tumorigenesis pathway | (81) |
| | Boughanem <i>et al</i> , 2020 | Spain | Associated with survival rates | (84) |
| <i>BCAT1/IKZF1</i> | Pedersen <i>et al</i> , 2015 | Australia | Positively correlated with the degree of invasion | (89) |
| <i>RASSF1A</i> | Sun <i>et al</i> , 2017 | China | Affects the sensitivity of patients with CRC to oxaliplatin-based chemotherapy | (97) |

MSP, methylation specific PCR; OS, overall survival; CEA, carcinoembryonic antigen; SFPR, secreted frizzled-related protein; LINE-1, long interspersed nucleotide element-1; BCAT1, branched chain amino acid transaminase 1; IKZF1, IKAROS family zinc finger 1; RASSF1A, RAS association domain family protein 1.

by this study (30), and mSEPT9 was eventually approved by the FDA for CRC screening (10).

Syndecan 2 (SDC2) precursor. The SDC2 protein is a membrane protein that serves a role in cell proliferation and migration, and helps maintain cell integrity. The SDC2 gene is not expressed in epithelial cells of normal colonic tissues, but is expressed in mesenchymal cells (32).

Important evidence has been discovered using different analyses of methylation conducted in comprehensive experiments. For example, CpG sites of SDC2 abnormal methylation have been observed in the tumor tissues of the majority of patients with CRC, thereby showing the significant potential

of SDC2 methylation in the early detection of CRC. In addition, the level of SDC2 DNA methylation in feces is closely associated with the occurrence of CRC, but not with clinical stage (33,34). Other studies have reported that SDC2 methylation may be highly sensitive in the detection of both advanced adenoma and CRC. Oh *et al* (35) examined the methylation of the SDC2 gene in primary tumors, adenomatous polyps, hyperplastic polyps and normal tissues, and identified that the positive SDC2 methylation was 100, 90.6, 94.1 and 0%, respectively. SDC2 methylation was also significantly elevated depending on the severity of the lesion. The overall sensitivity for CRC and small polyps was 90.0 and 33.3%, respectively, and the specificity was 90.9% for SDC2 methylation in fecal

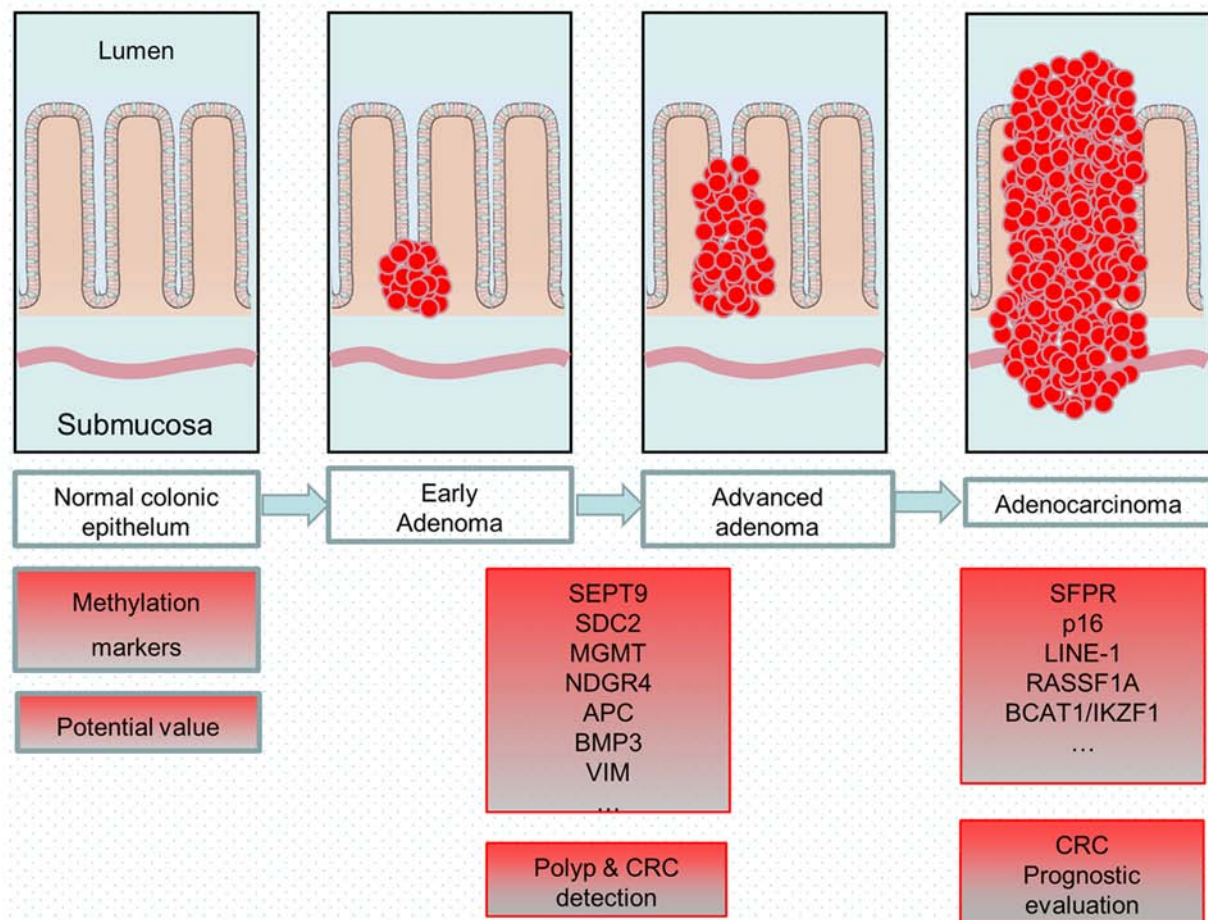


Figure 1. A model of the progression from adenoma to adenocarcinoma and various methylation markers that show potential value in the early detection or prognostic evaluation of CRC. CRC, colorectal cancer; SEPT9, septin 9; SDC2, syndecan 2; MGMT, O⁶-methylguanine-DNA methyltransferase; NDGR4, N-Myc downstream regulated gene 4; APC, adenomatous polyposis coli; BMP3, bone morphogenetic protein 3; VIM, vimentin; SFPR, secreted frizzled-related protein; LINE-1, long interspersed nucleotide element-1; RASSF1A, RAS association domain family protein 1; BCAT1, branched chain amino acid transaminase 1; IKZF1, IKAROS family zinc finger 1.

DNA. Some previous studies indicated that the diagnostic efficacy of *SDC2* methylation had a sensitivity of 77.4-81.1% and a specificity of 88.2-98% in fecal DNA for CRC (36,37). Thus, *SDC2* methylation may be a promising non-invasive biomarker for the early detection of CRC. It has also been reported that the combination of multiple biomarkers may be an effective strategy for improving the sensitivity and specificity of early cancer diagnosis (38).

MGMT. *MGMT*, which encodes five exons and four introns, is located at 10q26 on chromosome 10 and the MGMT protein acts as a DNA repair enzyme (16). Abnormal hypermethylation of the *MGMT* promoter is associated with a lack of mRNA expression, accompanied by the loss of protein content and enzyme activity (16,23). In total, ~40% of metastatic CRCs exhibit silencing of the *MGMT* gene, which results in a corresponding inhibition of protein synthesis (39). Previous studies have reported that *MGMT* promoter methylation was the main cause of *MGMT* gene expression disorder (40).

Shima *et al* (40) evaluated 855 cases of stage I-IV CRC for MGMT using two methods, methylation-specific PCR and immunohistochemistry. The results demonstrated that the methylation rate was 38% and the loss of MGMT expression was 37%, and the consistency of the two methods was 81%.

Sartore-Bianchi *et al* (41) screened the molecular characteristics of 2,044 patients with mCRC and then found that the *MGMT* promoter hypermethylation proportion was 48.7%. In another study that included 70 patients with CRC, *MGMT* hypermethylation was detected in serum-free circulating DNA, and *MGMT* promoter hypermethylation was observed in 90% of CRC cases, while no *MGMT* hypermethylation was found in the serum of healthy subjects (23). Freitas *et al* (42) reported that a combination of methylated *MGMT* and *SEPT9* present a 93.8% sensitivity, 82.0% specificity, respectively, for CRC detection in tissue samples. Therefore, it was suggested that MGMT may be used as a clinical biomarker for early diagnosis of CRC. However, there are some differences in the positive rate of *MGMT* methylation in the aforementioned studies and, thus, future studies are required to verify the findings.

N-Myc downstream regulated gene 4 (NDGR4). *NDRG4*, is involved in a variety of biological activities, such as cell proliferation, differentiation, development and stress. The protein sequences encoded by this family have a 52-65% homology and are highly conserved in the evolution of various species (43), with α/β hydrolase folding. Previously, researchers have identified *NDRG4* promoter CpG island methylation as a potential

and one of the most accurate marker for CRC detection (44), and this has been verified by an independent study (45).

Bagheri *et al* (46) revealed that the sensitivity and specificity of the *NDRG4* gene in the diagnosis of CRC were 86 and 92%, respectively, and the proportion of methylation was proportional to CRC staging. Rademakers *et al* (47) combined types of biomarkers to establish a panel for the highest diagnostic potential, resulting in the combination of SNAP91/*NDRG4*/*FIT* as the best performing, with a sensitivity and specificity of 86.0 and 96.0%, respectively. Moreover, these authors demonstrated that the sensitivity and specificity of the *NDRG4* gene were sufficient to serve as a novel, non-invasive marker for CRC screening. A meta-analysis also showed that *NDRG4* could be considered as an important marker for the diagnosis of CRC (48).

In a previous study, the positive rates of methylated *NDRG4* in cancer tissues, precancerous tissues, blood, urine and feces were shown to be 81, 8.3, 54.8, 72.6 and 76.2%, respectively. It was also confirmed that methylated *NDRG4* in the feces and urine had high sensitivity and specificity. Additionally, this new method is expected to be a promising and potential marker for the early diagnosis of CRC due to the ease of collection of urine or feces samples (49,50).

Adenomatous polyposis coli (APC). APC is a tumor-suppressor gene that was discovered during the research of familial adenomatous polyposis. Furthermore, hypermethylation of APC promoters has been frequently observed in sporadic and familial CRC types over the past two decades (51).

A previous study of patients with CRC examined the methylation status of APC and found that APC methylation occurred 33% of the time in patients with CRC (52). And recent research also reported that in CRC patient under 50 years of age, the rate of APC promoter methylation was 40% (53). Nunes *et al* reported the methylation levels of APC combined with FOXA1 and RASSF1A to diagnose CRC in stages 0, I and II with 78.4% sensitivity, 69.9% specificity, respectively, but the result needs further validation because the CRC samples were only 37 (54). Moreover, methylated APC promoters were found to be significantly associated with later stages and an older age. The incidence of APC methylation in patients with mCRC was 53.7%. That study demonstrated that APC promoter hypermethylation is a common epigenetic event in patients with both early and metastatic CRC, and it serves an important role in the development of CRC (52). A meta-analysis by Liang *et al* (55) investigated APC hypermethylation in the early diagnosis of CRC. This study included 24 articles and 2,025 patients with CRC. The analysis demonstrated that APC promoter hypermethylation was an early cancerous event of CRC and, thus, may be a noteworthy diagnostic indicator of early CRC.

Bone morphogenetic protein 3 (BMP3). The first important evidence of BMP3 inactivation in early polyp formation and colon tumor development was reported in 2008 by Loh *et al* (56). These authors reported that the percentage of aberrant BMP3 hypermethylation in colorectal tumors was 55% (33/60). This result suggested that BMP3 may be a potential marker for the early detection of neoplastic lesions. In subsequent studies, researchers have focused on the diagnostic

value, such as the sensitivity and specificity, of methylated *BMP3* in CRC. For example, Houshmand *et al* (57) examined the methylation status of the *BMP3* gene in CRC tissue samples, and detected a sensitivity of 56.66% and specificity of 93.3%. In addition, Rokni *et al* (58) detected the methylation status of the *BMP3* gene in plasma DNA samples from 50 patients with histologically diagnosed polyps or tumors and 50 healthy individuals. Their results showed that the frequency of BMP3 methylated DNA was significantly higher in patients with polyps compared with that in healthy controls, with a sensitivity of 40% and specificity of 94%. In a study by Kisiel *et al* (59), the combination of abnormal methylated DNA markers with *BMP3* and *NDRG4* had a sensitivity of 100% and a specificity of 89% for CRC and high-grade dysplasia. These findings suggest that methylated *BMP3* may be a useful biomarker for CRC detection, but that it should be combined with other biomarkers due to the lack of specificity. A typical example of a combination method is the application of MT-sDNA test, which consists of fecal hemoglobin, quantitative detection of methylation *BMP3* and *NDRG4*, β mutant *KRAS* and β -actin tests. The sensitivity for curable stage CRC ranges from 93 to 100%, while its specificity ranges from 87 to 93%. Due to this reliable performance, it was approved by the US FDA (60).

Vimentin (VIM). *VIM* encodes a cytoskeletal protein that is considered to be involved in cancer invasion and metastasis. *VIM* promoter methylation has been observed in CRC (61), such as in the fecal DNA of patients with CRC (62). *VIM* promoter methylation has been detected in stool at a sensitivity of 52% and specificity of 88% (61). Lu *et al* (49) reported that the promoter methylation levels of *VIM* in patients with CRC were 41.1% and the specificity was 85.0%. These results suggest that promoter methylated *VIM* in fecal samples was sensitive, specific and non-invasive for CRC screening and, therefore, it has been commercialized as a non-invasive method for early CRC detection (63).

In addition, previous studies have suggested that the hypermethylation of the following genes, including ALX homeobox 4 (*ALX4*), neurogenin 1 (*NeuroG1*), helicase like transcription factor (*HLTF*), hyperpigmentation progressive 1 (*HPPI*), WNT inhibitory factor 1 (*WIF1*), Ras association domain family member 2a (*RASSF2a*), GATA binding protein 4 (*GATA4*), β -1,4-glucuronyltransferase 1 (*B4GAT1*), proline rich membrane anchor 1 (*PRIMA1*), APC, ATM serine/threonine kinase, glutathione S-transferase π 1 (*GSTP1*) and tissue factor pathway inhibitor 2 (*TFPI2*) (63,64), have been analyzed in fecal, blood and urine samples from patients with CRC and used for the early detection of CRC. From the potential methylation markers, methylated *VIM*, *SEPT9*, *BMP3*, *SDC2*, *NDRG4* and a combination of methylated branched chain amino acid transaminase 1 (*BCAT1*)/IKAROS family zinc finger 1 (*IKZF1*) have been proven to be reliable and accurate, and have been approved for clinical use (10,60,63,65).

As shown by a recent meta-analysis (66), the value of a single hypermethylated DNA promoter region as a marker for CRC screening is limited, but the combination of indicators demonstrates significant potential. Therefore, the combination of different detection indicators must be utilized to balance their respective diagnostic performance, thus achieving the best diagnostic effect with regards to specificity and sensitivity.

5. Prognostic biomarkers

At present, the most effective method for evaluating the prognosis of patients with CRC is based on the histological characteristics of the tumor, such as pathological type and stage. However, the survival time of patients with CRC at the same stage is heterogeneous; thus, a more accurate method is required to determine the prognosis of these patients. In recent years, numerous clinical studies have investigated the feasibility of using specific methylated DNA markers to evaluate the prognosis of patients with CRC.

Secreted frizzled-related protein (SFRP). Researchers have observed abnormal methylation of SFRPs in various types of human cancer. The activation of the Wnt pathway induced by the loss of *SFRP* gene expression is one of the most important mechanisms for tumorigenesis and cancer development (67). In humans, there are five types of SFRPs (SFRP1, 2, 3, 4 and 5). Among the SFRP family members, SFRP1 and SFRP2 have been the most extensively researched in human cancer, and studies have shown that *SFRP1* and *SFRP2* promoter methylation may contribute to the risk of CRC (68).

The loss of SFRP expression induced by DNA methylation is the primary mechanism causing the silencing of SFRP and it is associated with tumor formation in CRC (69,70). Kumar *et al* (71) evaluated the promoter methylation status of the *SFRP1* gene in 54 cases with stage II-III CRC. It was found that *SFRP1* gene methylation was associated with lymph node invasion ($P=0.05$) and a poorer mean overall survival (OS). Moreover, these authors indicated that SFRP1 gene methylation was a prognostic marker in CRC (71). It has also been shown that the hypermethylation rate of the *SFRP2* promoter in patients with CRC was 66.7% (72). In addition, a recent meta-analysis of the SFRP family revealed a significant association between hypermethylation of SFRP1 and cancer risk (67). A review by van Loom *et al* (73) revealed that numerous studies have reported promoter hypermethylation downregulated *SFRP2* gene expression in several types of cancer. These authors discussed the role of SFRP2 in tumor angiogenesis and noncanonical Wnt signaling, and suggested its potential as an anti-angiogenic therapeutic target or an effective prognostic marker in cancer.

p16. p16 is a tumor-suppressor gene frequently studied due to its significant function in the cell cycle. There are three main mechanisms of p16 inactivation: Gene mutation, homozygous deletion and 5'-CpG island methylation, which is one of the major causative factors of various human cancers (74).

Ye *et al* (75) detected p16 methylation in CRC tissues and adjacent normal tissues, and the results demonstrated that p16 methylation was higher in CRC tissues compared with adjacent normal tissues. Moreover, Lee *et al* (76) found that hypermethylation of the *p16* gene was significantly associated with lymph node metastasis in CRC, and reported that the hypermethylation frequency of the *p16* gene was 32.3%. However, p16 gene promoter hypermethylation frequency was detected at 15.1% in CRC, and this rate was lower than that previously determined (72). This variation may be caused by the different sensitivity of detection methods, which can be further verified in future studies. Karam *et al* (74) reported a sensitivity

of 55.38%, specificity of 98.5% and diagnostic accuracy of 77.7% in patients with CRC. Furthermore, p16 methylation was significantly correlated with age, sex, Dukes' stage, lymph node metastasis, carcinoembryonic antigen levels, a shortened time to progression and overall survival (75,77). Therefore, it may be associated with prognosis and may thus serve as a prognostic biomarker.

Long interspersed nucleotide element-1 (LINE-1). LINEs are retrotransposable elements identified in numerous eukaryotic genomes. These are highly methylated in normal somatic cells and, therefore, are mostly inhibited, thus preventing their potential to cause genomic instability (78). Full-length LINE-1 can lead to adenoma formation and cancer progression, and the activity of LINE-1 is dependent on the epigenetic regulation of its promoter (79). Hypomethylation of the LINE-1 promoter has been observed in gastrointestinal cancer as well as other types of cancer (79,80).

Jiang *et al* (81) examined promoter methylation of *LINE-1* in adenomas and sessile serrated lesions (SSLS); the results showed that high-grade dysplasia (HGD) and increasing size of adenoma were associated with decreased *LINE-1* methylation. Shademan *et al* (78) quantified promoter methylation and LINE-1 transcripts in three stages of CRC, non-advanced adenoma, advanced adenoma and adenocarcinoma. Their results demonstrated that the methylation of the *LINE-1* promoter in non-advanced adenoma was significantly higher compared with that in advanced adenoma and adenocarcinoma. The correlation analysis also revealed a decrease in *LINE-1* promoter methylation, genomic polymorphism insertion and an increase in LINE-1 transcription in advanced adenomas, suggesting that early and late polyps may involve some main pathogenetic mechanisms that ultimately lead to cancer.

Bachitta *et al* (82) conducted a meta-analysis in 2014, which described the role of *LINE-1* hypomethylation in human cancer. It has also been shown that LINE-1 hypomethylation in CRC was associated with poor prognosis, survival and advanced stage, and that it could be a prognosis predictor of CRC (83,84). In addition to its prognosis value, a recent study reported that *LINE-1* hypomethylation was effective in the early detection of CRC (85). Hence, *LINE-1* hypomethylation may be a promising marker for the prognostic and early detection of CRC.

BCAT1/IKZF1. BCAT1 controls the metabolism of branched-chain amino acids, which are essential nutrients for growth. Abnormal methylation of the *BCAT1* gene locus has been observed in CRC and various pathologic conditions (86). *IKZF1* encodes a DNA-binding protein, which regulates the cell cycle. In CRC, *IKZF1* promoter methylation is associated with the loss of regulation of cell proliferation and differentiation (87).

Mitchell *et al* (88) demonstrated that the expression levels of the two genes, *BCAT1* and *IKZF1*, were low in healthy human plasma (3.5 and 4.9%, respectively) but were significantly increased in patients with CRC; therefore, these two genes were selected as biomarkers for CRC detection. In a previous study that included 2,127 samples, 85/129 cases of CRC had a sensitivity of 66%. For stages I, II, III and IV, the positive rates were 38, 69, 73 and 94%, respectively. It was also found

that the positive rate was positively correlated with the degree of invasion (89). In a trial involving 1,381 volunteers, which examined FIT and BCAT1/IKZF1 DNA methylation, the results demonstrated that the sensitivity of the BCAT1/IKZF1 methylation to CRC was 62%, with a specificity of 92%, which was higher compared with the commonly used positive threshold of FIT. When combining FIT (cut-off 10 μg Hb/g) with the BCAT1/IKZF1 blood test, the sensitivity for cancer was 89%, with an improved specificity of 74% (90). From research on recurrent CRC, postoperative patients who were BCAT1/IKZF1-positive were found to have an increased risk of residual disease and subsequent recurrence. It has been shown that the BCAT1/IKZF1 test was more sensitive to recurrence compared with the conventional CEA test, and in the case of positive test, the recurrence rate was twice as high as that of CEA (91,92). The BCAT1/IKZF1 test is a novel blood test that have been approved under the commercial name 'COLVERA' for CRC (65), and could be used for monitoring recurrence and as a non-invasive diagnostic method.

RAS association domain family protein 1 (RASSF1A). *RASSF1A* is a tumor-suppressor gene that serves a role in regulating cell proliferation and apoptosis, and is involved in numerous types of cancer, including CRC. More importantly, it can be detected early in liquid biopsies (93). *RASSF1A* is subject to epigenetic regulation and suppression, the main regulatory mechanism being via DNA methylation (94). It has been reported that the development of adenomatous polyps and colorectal laterally spreading tumors (LSTs) are precursor lesions of CRC, and 80-90% of CRCs develop via these factors (95). In a previous study, Ni *et al* (96) detected the degree of *RASSF1A* methylation in patients with LSTs. The results indicated that *RASSF1A* methylation was lower in patients with LSTs compared with CRC cases, but was higher than that in polypoid adenomas. Thus, it was suggested that *RASSF1A* has the potential to be used for the early diagnosis of CRC and may enable timely intervention.

Promoter methylation of *RASSF1A* can affect the sensitivity of patients with CRC to oxaliplatin-based chemotherapy. In a study by Sun *et al* (97), it was identified that *RASSF1A* methylation was independently correlated with the prognosis of patients treated with oxaliplatin-based chemotherapy, while another meta-analysis revealed that *RASSF1A* hypermethylation was a risk factor and a potential prognostic biomarker in CRC (98). Moreover, it was indicated that the methylation of *RASSF1A* may be a prognostic marker for stage II and III CRC cases. In addition, with further research on the mechanism of methylation, aberrant methylation may be a promising and novel target to improve chemotherapy efficacy.

6. Predictive biomarkers for response to treatment

Due to the existence of chemotherapy resistance, patients show different responses to chemotherapy; thus, their prognosis also varies. Therefore, it is important to identify an effective prognostic evaluation index. In recent years, numerous studies have been conducted to evaluate the biomarkers of the 5-fluorouracil (5-FU)-based chemotherapy response in patients with CRC, but most have not completed the 1/2 discovery

phase and will not be discussed in this study (63). However, some methylation markers that may be useful for predicting the response to treatment of CRC have been suggested in previous studies.

Human mutL homolog 1 (hMLH1). *hMLH1* is considered to be an important member of the mismatch repair gene, and it encodes a variety of DNA repair enzymes to cooperate in the recognition and repair of DNA mismatches (99). Abnormal hypermethylation of the *hMLH1* promoter CpG island has been shown to be closely associated with the occurrence of CRC, and its epigenetic changes may affect DNA stability (100). In total, ~15% of CRC cases exhibit high levels of MSI, reflecting the dysfunction of the post-replication DNA mismatch repair system, which mainly occurs via *HMLH1* gene silencing mediated by CpG methylation (101). It has been reported that a *MLH1* promoter hypermethylation assay is a cost-effective method for identifying microsatellite-high (MSI-H) in patients with sporadic CRC (102).

Fu *et al* (103) evaluated 115 patients with stage II CRC who were assigned to four groups: CIMP⁺/*MLH1*-unmethylated, CIMP⁺/*MLH1*-methylated, CIMP⁻/*MLH1*-unmethylated or CIMP⁻/*MLH1*-methylated. The results demonstrated that the CIMP⁺/*MLH1*-unmethylated group was associated with higher aggressiveness and poorer prognosis. DFS and OS were predicted by CIMP/*MLH1* methylation status, with the shortest DFS and OS observed in the unmethylated CIMP⁺/*MLH1* group. This study revealed that the CIMP combined with *MLH1* methylation status was meaningful for tumor subtype classification in patients with stage II CRC. In 2018, a meta-analysis, which that included 47 studies with 4,296 cases and 2,827 controls, suggested that *hMLH1* methylation was associated with an increase in CRC risk (99).

Furthermore, Kuan *et al* (104) demonstrated that patients with CRC at an advanced stage who exhibited *hMLH1* methylation in tumor tissue had a higher risk of CRC recurrence compared with patients with local invasion who had an unmethylated status. It has also been reported that the methylation of *hMLH1* has significant potential in predicting treatment response, such as that to 5-FU-based chemotherapy, and survival (105,106). It was also observed in an *in vitro* study that *hMLH1* might play a key role in cancer stem cells response to 5-FU (107).

Wnt5A. Wnt signaling is a term used to describe a group of signaling pathways that regulate processes essential to physiological functions, including cell proliferation and differentiation. It is also closely associated with CRC (108). Wnt5a/b expression is associated with epithelial-to-mesenchymal transformation (EMT), and an increase in its gene expression has been observed in numerous malignancies, including CRC (109). EMT is a process that enhances the migration of epithelial cells (110). In cancer, EMT increases the ability of cancer cells to escape from the primary tumor, allowing them to infiltrate into neighboring tissues.

In most patients with CRC, genes that regulate the Wnt pathway display genomic and epigenomic abnormalities. The decrease of Wnt pathway inhibitor protein can facilitate the growth and metastasis of CRC, which is an important factor in determining patient prognosis (111). Wnt5A is one of the

negative regulators of the Wnt pathway and is often inhibited by promoter methylation in CRC (112). Hibi *et al* (113) reported that the *Wnt5A* gene was detected in 35% of primary CRC cases and that it was methylated from the early stages of CRC. Furthermore, Kim *et al* (112) collected tissues from 194 patients with metastatic or recurrent CRC and detected the methylation status of *Wnt5A*. The results indicated that the methylation frequency of *Wnt5A* was 32.0%, which was similar to those of a previous study (113). Jiang *et al* (114) also found that, in 5-FU-treated CRC cases, the *Wnt5A* methylation status was significantly associated with longer progression-free survival and improved drug response, suggesting that 5-FU was more effective in CRC cases with *Wnt5A* hypermethylation. Therefore, the *Wnt5A* methylation status may predict the 5-FU treatment response in patients with CRC.

7. Methylation-based therapies in CRC

In recent years, the comprehensive treatment of CRC has shown progress, but the patient response to treatment remains limited. The benefits that patients derive from systemic treatment are often compromised by resistance to conventional chemotherapy agents, such as 5-FU, oxaliplatin and irinotecan (115). CRC occurs as a result of gradual genetic and epigenetic changes and their long-term accumulation. These epigenetic modifications are reversible. It has been suggested that combined traditional treatments with epigenetic agents may represent novel relevant therapeutic targets and may help to reverse drug resistance (116,117).

DNA methylation is considered to be an epigenetic change that is closely associated with the occurrence and development of CRC (118). It is catalyzed by DNA methyltransferases (DNMTs), which can be divided into three groups according to their function: DNMT1, which is the most abundant and important isoenzyme (119), DNMT2 and DNMT3 (DNMT3A and DNMT3B). DNMTs regulate gene expression and the deregulation of DNA methylation. On that basis, DNMT inhibitors (DNMTi) have been widely researched in the prevention and treatment of CRC (115). In CRC cell lines and animal models, inhibition of DNMT1 and DNMT3B reduced the overall genomic methylation rates by 95%, leading to re-expression of tumor-suppressor genes, and was associated with the induction of apoptosis, decreased cell proliferation and reduced stem cell function, and helped to overcome oxaliplatin resistance to enhance the effectiveness of anti-CRC therapy (120,121). In several clinical trials, DNMTi have been used as a novel treatment for patients with CRC, particularly those with high levels of DNA methylation (118,122). The combined use of methylation-based therapy and standard chemotherapeutics showed improved treatment efficacy, without increasing toxicity (122). The latest research has also shown that the detection of tumor DNA methylation and the timely use of epigenetic therapies, including DNMTi, may help expand the therapeutic armamentarium (4). However, additional complete results are required and should be validated by further trials.

8. Current situation and difficulties

Although DNA methylation biomarkers show potential in contributing to the early diagnosis of cancer, and while some

have already been approved for detecting CRC, such as the methylated SEPT9 DNA plasma assay and MT-sDNA (123), with large numbers of trials having demonstrated their effectiveness in CRC screening (10,14,60,124), there remain multiple issues to be overcome before these can be widely used in the clinical setting. For example, potential candidate methylation markers should be validated using a large number of high-quality studies and epigenomic analysis. Furthermore, standardized methods are required, particularly in quantifying methylation data in clinical trials. In addition, clinical trials must aim to detect the true significance of the methylation markers.

Chemotherapy and radiotherapy may also cause epigenetic changes in patients with CRC, and the clinical implications of this observation are unknown and should be further evaluated (125). Although some of the underlying mechanisms have not yet been fully determined, researchers have begun to study the clinical use of epigenetic drugs, such as radiosensitizer therapy or antitumor therapy. However, the clinical use of epigenetic drugs may not ultimately produce the desired results.

9. Conclusion

Research on DNA methylation is promising. The application of DNA methylation biomarkers is expected to serve an important role in non-invasive testing in order to improve the acceptability of CRC screening in the general population. Moreover, these DNA methylation changes may contribute to the assessment of treatment response and adjustment of therapeutic strategies in CRC. It has been shown that the identification of these biomarkers may help stratify patients and, potentially, facilitate the development of precision medicine. However, although methylation markers have great potential, some limitations must be overcome and the true significance of biomarkers should be further validated before their wide use in the clinical setting.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Clinical Research Fund project of Zhejiang Medical Association (grant no. 2019ZYC-A182) and the Science and Technology Plan Project of Taizhou (grant no. 1902ky37).

Availability of data and materials

All information included in this review is documented by relevant references.

Authors' contributions

TF designed the review and collected the literature data; CK drafted the manuscript. TF and CK reviewed the literature findings and results. TF and CK read and approved the manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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