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DNA methylation detection methods used in colorectal cancer

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Abstract

Colorectal cancer (CRC) remains a major contributor to the number of cancer-related deaths that occur annually worldwide. With the development of molecular biology methods, an increasing number of molecular biomarkers have been identified and investigated. CRC is believed to result from an accumulation of epigenetic changes, and detecting aberrant DNA methylation patterns is useful for both the early diagnosis and prognosis of CRC. Numerous studies are focusing on the development of DNA methylation detection methods or DNA methylation panels. Thus, this review will discuss the commonly used techniques and technologies to evaluate DNA methylation, their merits and deficiencies as well as the prospects for new methods.

Key words: Colorectal cancer; Cancer screening; Marker; DNA methylation detection

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Core tip: We screened the literature on the application of DNA methylation detection technologies as well as colorectal cancer (CRC) associated DNA methylation markers in the diagnosis or prognosis evaluation of CRC. Apart from introducing each method in detail and describing the methylation status of several candidate genes being assayed, we also evaluated the advantages or disadvantages of each method. More importantly, we discuss the new DNA methylation detection methods and their potential use. At last, we prospect on how these methylation markers could be used and what kind of methylation detection techniques might be more practical in clinical work.

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INTRODUCTION

Colorectal cancer (CRC) is a major contributor to cancer-related deaths worldwide^[1]. According to an epidemiological study, the number of newly emerged CRC cases worldwide is predicted to rise to 2.2 million and more and will cause approximately 1.1 million deaths by the year of 2030^[2]. Furthermore, an article published in 2012 reported that there are more than 1 million CRC cases diagnosed per year, and nearly 700000 annual deaths result from this disease^[3]. Genetic analyses of CRC have shown that CRC arises from an accumulation of genetic mutations and epigenetic changes^[4], with CRC typically having 3 to 6 driver mutations and 33 to 66 hitchhiker or passenger mutations that lead to protein expression silencing^[5]. In these instances, molecular data are often used as evidence to support the clinical and pathological changes^[6]. Changes in the methylation levels of specific genes have been shown to be associated with the early stages of CRC or with good or bad patient prognoses^[7]. Epigenetic modifications can be affected by environmental factors, and the identification of site-specific changes in DNA methylation may offer insights into carcinogenetic mechanisms and ultimately aid in uncovering the role of environmental influences in the development of CRC^[8]. Furthermore, colonoscopies and polypectomies are complicated technical procedures that require training and experience to maximize accuracy and safety^[9]. In addition, the procedures for tests such as colonoscopies are not acceptable to all individuals and can have complications^[10,11]. Future research and further investigations aiming to identify predictive biomarkers can be generally useful in developing available, noninvasive, cost-effective diagnostic and prognostic panels that would have immense societal benefits^[12].

For the reasons described above, we believe that further investigations on gene methylation related to CRC and the development of rapid and reliable methylation detection methods for CRC patients are worthwhile. It seems like that CRC epigenetics will be the epitome for multistep tumorigenesis, because new high-throughput methods will make detailed mapping of the CRC chromatin landscape accessible, which is able to offer new vision of CRC etiology and biology, discover more genetic variations in epigenetic regulators linked to CRC, and uncover potential epigenetic biomarkers for CRC diagnosis or prediction of prognosis and response to treatment^[13]. Numerous studies have been undertaken to identify epigenetic biomarkers for diagnosing CRC in early stage or evaluating disease prognosis or to develop brand-new technologies or techniques to assess DNA methylation in CRC, all the results of which may promote a decrease in CRC-associated morbidity and mortality. Dozens of methylation detection methods have been developed, which utilize highly different approaches. Therefore, the intent of this review is to discuss the most widely used methods as well as newly investigated or invented technologies. This review may be useful to researchers or scholars who are interested in the relationship between aberrant DNA methylation and CRC or those who want to investigate new methods for the detection of CRC. We will summarize the techniques used for CRC DNA methylation level analysis, compare different methods, and analyze both the advantages and disadvantages of the most widely used and newly developed methods. Finally, we will make conclusions regarding the preferred techniques and evaluate the value of novel methods that are under development.

STUDY QUESTION

In this review, we focus on the methylation detection methods developed for or implemented in the diagnosis CRC. The advantages and disadvantages of each method are summarized and compared to offer valuable information to researchers who want to undertake investigations in this field.

SEARCH STRATEGY

From January 15 to February 7, 2019, we searched for articles involving the detection of DNA methylation in CRC patients. The key words we used were “gene” OR

“DNA” AND “methylation” AND “detection” AND “colon” OR “intestine” OR “rectal” OR “colorectal” OR “CRC” AND “cancer”.

Electronic databases

We performed literature searches using six online databases, including PubMed, MEDLINE, Ovid, EMBASE, Science Direct, and Web of Science. We only included original research written in English and reported within the past decade.

CRITERIA FOR THE SELECTION AND REJECTION OF STUDIES

The selection criteria for our review were as follows: (1) Studies involving DNA methylation detection for CRC; and (2) Studies that implemented or developed a novel method to detect the level of DNA methylation in CRC. The rejection criteria for our review included studies that did not mention the sensitivity of the method or did not report the methods.

QUALITY ASSESSMENT

To evaluate the quality of the included studies, two reviewers were independently chosen to extract the data and assess quality of each study that included DNA methylation detection in CRC patients.

DATA EXTRACTION

All of the data were extracted from 28 carefully selected articles. The selection process used is shown in [Figure 1](#). We focused on the methods and candidate genes the researchers chose, and we collected or calculated the diagnostic sensitivity and specificity of each reported marker in these articles. For those not mentioning the sensitivity and specificity, we used the statistics in the articles for calculation. Generally, the sensitivity for DNA methylation in CRC diagnosis is the proportion of those CRC patients with a positive test. Specificity is negative test results among persons without CRC.

RESULTS

We searched more than 100 articles and summarized all commonly used DNA methylation methods used in these studies ([Figure 2](#)). Among the different studies that focused on DNA methylation in CRC, various types of techniques were used ([Table 1](#)). The diagnostic sensitivity and specificity of the molecular markers obtained using specific methylation detection methods are also provided in [Table 1](#). We observed that most of the methods are PCR-based techniques or technologies, such as methylation-specific multiple ligation-dependent probe amplification (MS-MLPA), direct or nested methylation-specific polymerase chain reaction (MSP), multitarget stool DNA (MT-sDNA) test, methylation-specific high resolution melting curve analysis (MS-HRM), and MethyLight assay. DNA sequencing is also a commonly used technique, including bisulfite sequencing, pyrosequencing, and quantitative allele-specific real-time target and signal amplification (QuARTS) assays. Other methods involving Illumina technologies were typically used for genome profiling.

MT-sDNA test

The development of MT-sDNA test was a remarkable progress that was made on solving problems of CRC screening while keeping high accuracy. It is independent of the CRC lesion site and is believed to be beneficial for ameliorating both the diagnostic yield and quality of the colonoscopy examination^[14]. The MT-sDNA test is a noninvasive technology by detecting CRC-related DNA markers and occult hemoglobin in the feces, including aberrantly methylated promoter regions of BMP3 and NDRG4^[4,15].

Accordingly, recent advances have allowed a combined fecal immunochemical test (FIT) and multitarget DNA stool test to become commercially available. There is high possibility that MT-sDNA test could be an optimal screening choice for asymptomatic people with a moderate risk for CRC, and the early adoption of this assay is

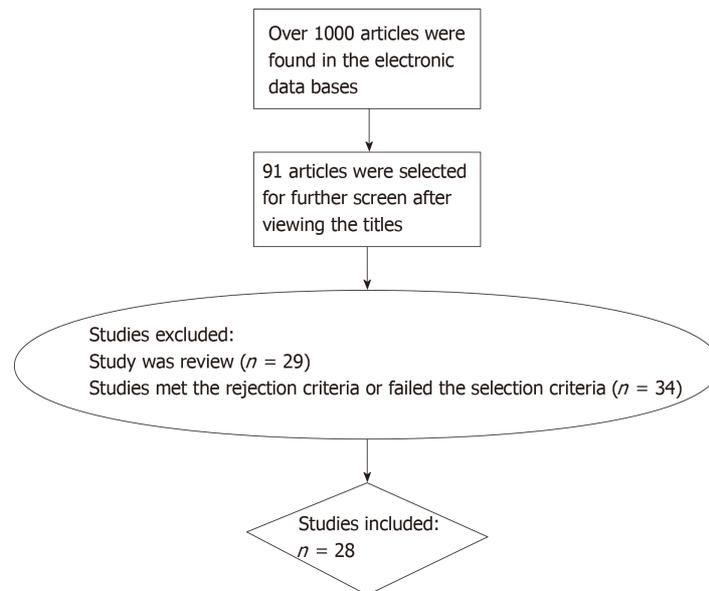


Figure 1 The workflow of the article screening. The simplified article selection procedure is shown in this picture.

recommended^[15,16]. Despite being the most sensitive noninvasive CRC screening test currently available, further investigations on this sDNA test seems to be needed^[17]. What's more, it is reported that the MT-sDNA test is not as effective as FIT and colonoscopy and even more expensive when participation rates of all strategies were identical^[15].

QuARTS assay

The QuARTS is a technology combing polymerase-based target amplification with signal amplification based on invasive cleavage, with the fluorescence-based signal detection resembling real-time PCR. To the best of our knowledge, QuARTS was first used to detect DNA methylation by Zou *et al*^[18]. Kisiel *et al*^[19] measured candidate markers for CRC through QuARTS assays using DNA extracted from normal colon, adenoma, and CRC frozen tissues. An automated sDNA assay developed by Lidgard *et al*^[20] uses the QuARTS method and has been reported to have a 90% specificity and 98% sensitivity when used to test the DNA methylation of stool samples from individuals with CRC^[20]. Ahlquist *et al*^[21] demonstrated that QuARTS is a very sensitive and specific method for detecting CRC in early stage and large adenomas in the colon regardless of the sites, although optimization and standardization of feces collecting methods as well as stool storing protocols are of great importance in improving the performance of QuARTS.

MS-MLPA method

MS-MLPA is a quick, reliable, and cost-effective method derived from MLPA that does not require sodium bisulfite treatment and instead uses the methylation-sensitive endonuclease *HhaI*. If the target DNA is methylated, the *HhaI* enzyme cannot cut its recognition sequence, and the target sequence will then be amplified by PCR. When analyzing the data of MS-MLPA, the percentage of methylated DNA is calculated by comparing the peak patterns of *HhaI*-digested and -undigested products^[22-24]. To perform MS-MLPA, genomic DNA must first be denatured, after which the MS-MLPA probes are added and a 16 h hybridization step is performed^[25]. Furlan *et al*^[26] used this technique to assess CpG methylation of tumor suppressor genes in CRC samples using a well-characterized series of sporadic colorectal adenocarcinoma and neuroendocrine carcinomas with known clinicopathologic and molecular profiles. The results of this study showed that an outstanding merit of MS-MLPA is being able to screen multiple cancer genes in only an assay with better sensitivity and specificity compared with the rest of the DNA methylation detection assays. Their results indicate that the MS-MLPA assay is a simple, cost-effective, reliable method for epigenetic analysis of tumor tissues and can provide innovative aspects for clinical diagnosis and treatment^[27]. In a study by Mäki-Nevala *et al*^[28] focusing on Lynch syndrome-associated adenomas, they employed MS-MLPA to evaluate DNA methylation conditions in the promoters of 8 CIMP-related genes and 7 chosen candidate genes such as TSGs and the gene LINE-1. In an investigation of the use of constitutive MLH1 methylation to diagnose Lynch syndrome in patients with

Table 1 Methods used to study DNA methylation in colorectal cancer cases and controls

| No. [Ref.] | Year | Specimen | Gene | Sample number | | Method | Sensitivity (95%CI) | Specificity (95%CI) | Conclusion and comments |
|-------------------|------|-----------------|---|-------------------|----------------|-------------------------|--|--|--|
| | | | | Case | Control | | | | |
| 1 ^[7] | 2014 | Tissue | 18 genes ¹ | 20 | 20 | Illumina ² | 96% (87%-105%) | 100% | These results suggest that methylation biomarkers in this study may be developed that will, at minimum, serve as useful objective and quantitative diagnostic complements to colonoscopy as a cancer-screening tool. |
| 2 ^[8] | 2015 | Tissue | FAM78A, FSTL1, KCNC1, MYOCD, SLC6A4 | 28 | 97 | Illumina ³ | 100.00% | 100% | Four distinct DNA methylation subgroups of CRC identified can provide novel insight regarding the role of CIMP-specific DNA hypermethylation in gene silencing. |
| 3 ^[9] | 2013 | Stool | NDRG4, BMP3 | 86 | 794 | Automated MT sDNA assay | 98% (95%-101%) ⁴ | 90% (88%-92%) | This automated high-throughput system could be a widely accessible noninvasive approach to general CRC screening. |
| 4 ^[1] | 2014 | Stool | BMP3, NDRG4 | 65 | 9167 | MT sDNA test | 92.3% (83.0%-97.5%) ⁵ | 87% (86%-87%) | This stool test shows a higher single-application sensitivity than a commercial FIT for both colorectal cancer and advanced precancerous lesions, although with lower specificity. |
| 5 ^[1] | 2012 | Stool | Vimentin, NDRG4, BMP3, TFPI2 | 252 | 293 | QuARTIS | 89% (83%-93%) | 90% (85%-94%) | Next-generation sDNA tests appear capable of detecting early-stage CRC and large adenomas with a high sensitivity and specificity at all sites throughout the colon. Validation of optimized next-generation sDNA tests in the screening setting is now needed. |
| 6 ^[9] | 2018 | Tissues | BMP3, NDRG4, SFMBT_895, SFMBT_896, SFMBT_897, CHST2_7890, PDGFD, CHST2_7889, VAV3, DTX1 | 421 | 44 | QuARTIS | 58% (53%-63%) 78% (74%-82%) 90% (86%-92%) 89% (86%-92%) 89% (85%-92%) 82% (77%-85%) 83% (79%-87%) 82% (78%-86%) 82% (78%-85%) 71% (66%-75%) | 100% (90%-102%) 100% (90%-102%) 100% (90%-102%) 100% (90%-102%) 100% (90%-102%) 100% (90%-102%) 100% (90%-102%) 100% (90%-102%) 100% (90%-102%) 100% (90%-102%) | This novel panel is highly specific for colitis-associated neoplasia across geographically diverse patient subsets and among those with varying risk. The marker panel has been optimized by the discovery and incorporation of methylated ZDHHC1, an epithelium-specific marker, as a normalizer for the neoplasm-specific markers, in stool. Further studies are needed to corroborate and extend this finding. Except for DTX1, each of these novel markers is individually more sensitive than the most sensitive marker in the MT-sDNA panel. |
| 7 ^[9] | 2018 | Stool Tissue | BMP3, VAV3, ZDHHC1, BCAT1, IKZF1 | 12 36 | 291 36 | MSP MSP | 92% (60%-100%) 92% (83%-101%) 72% (58%-87%) | 69% (54%-85%) 97% (92%-103%) | This study has shown that highly methylated BCAT1 and IKZF1 exist in all stages of CRC. A positive correlation was observed between hypermethylated IKZF1 and gene activity silencing. |
| 8 ^[6] | 2016 | Plasma | BCAT1/IKZF | 28 | 94 | MSP | 68% (48%-84%) | 87% (79%-95%) | Methylated BCAT1/IKZF1 blood test has a significantly higher sensitivity for recurrent CRC than the CEA test. |
| 9 ^[6] | 2015 | Plasma | BCAT1, IKZF, BCAT1/IKZF | 129 | 450 | MSP | 57% (48%-66%) 48% (39%-57%) 66% (57%-74%) | 95% (93%-97%) 99% (98.1%-100%) 95% (93%-97%) | Accuracy of this two-marker blood test approximates that of gFOBT. This two-marker blood test for screening is likely a rescue strategy for those refusing more sensitive RCT-proven methods such as FIT, flexible sigmoidoscopy, or colonoscopy. |
| 10 ^[6] | 2014 | Plasma | BCAT1, IKZF, BCAT1/IKZF | 74 | 144 | MSP | 65% (54%-76%) 68% (57%-79%) 77% (64%-84%) | 96% (93%-99%) 95% (91%-99%) 92% (88%-96%) | Detection of methylated BCAT1 and/or IKZF1 DNA in plasma may have clinical application as a novel blood test for CRC. Combining the results from the two methylation-specific PCR assays improves CRC detection with a minimal change in specificity. |
| 11 ^[6] | 2018 | Tissue | 9-SEP, MGMT/SEPT9, MGMT/RASSF1A/SEPT9 | 145 145 145 | 50 50 50 | qMSP | 86% (80%-91%) 94% (90%-98%) 97% (94%-100%) | 94% (87%-101%) 82% (71%-93%) 74% (62%-86%) | Results indicate that MGMT/RASSF1A/SEPT9 gene promoter methylation panel accurately identifies CRC, irrespective of molecular subtype and may have a better performance than currently available epigenetic based biomarkers but requiring assessment of its performance in liquid biopsies. |

| | | | | | | | | | |
|--------------------|------|--------------------|---|--|--|--|--|--|--|
| 12 ^[51] | 2013 | Serum | NPY, PENK, WIFI | 32 | 161 | qMSP | 94% (86%-102%) ⁶ 59% (42%-76%) ⁷ | 34% (27%-41%) 95% (86%-98%) | This test can be a cost-effective screening tool for detection of asymptomatic cancer patients for colonoscopy, who are at CRC risk that is hard to access and does not necessarily need further examination. The SDC2 DNA methylation in BLF shows a high sensitivity and specificity in patients with CRC and precancerous lesions. Abnormal SDC2 methylation can be a new potential diagnostic biomarker for noninvasive screening of CRC. This study found that aberrant methylation of UNC5C gene exists in CRCs and APs. UNC5C protein expression is negatively correlated with methylation status. MGMT hypermethylation can be used "as a clinical biomarker" for early diagnosis and prognostic assessment of the disease. Methylation of RASSF1A in the promoter region is independently associated with prognosis in CRC patients treated with oxaliplatin-based chemotherapy, and might be a promising target for improving chemotherapeutic effects. |
| 13 ^[56] | 2018 | Bowel lavage fluid | SDC2 | 10 | 54 | qMSP | 80% (44%-97%) | 89% (77%-96%) | The findings suggest that DNA methylation is a useful marker and that promoter methylation in certain genes is associated with more advanced tumor stages, poor differentiation, and metastasis, which can have an application as a risk assessment tool or as a marker of recurrence to help decide on the aggressiveness of the treatment. |
| 14 ^[59] | 2017 | Stool | SDC2 | 50 | 22 | qMSP | 90% (78%-97%) | 91% (71%-99%) | The presence of CDKN2A methylation is associated with poorer overall survival in stages B and C combined. |
| 15 ^[27] | 2017 | Tissue | UNC5C | 73 | 28 | MSP | 78% (69%-88%) | 100% | Methylation of DIRAS1 is a marker of poor prognosis in human colorectal cancer. Methylation of DIRAS1 may promote colorectal carcinogenesis and progression. |
| 16 ^[64] | 2017 | Serum | MGMT | 30 | 40 | MSP | 90% (79%-101%) | 100% | Methylation of ZNF331 is a poor prognostic marker in human colorectal cancer. ZNF331 may serve as a tumor suppressor in human colorectal cancer. |
| 17 ^[57] | 2017 | Plasma | RASSF1A | 108 | 78 | MSP | 44% (35%-54%) ⁸ 21% (14%-29%) ⁹ | 95% (90%-100%) | The data also show that qualitative techniques such as direct MSP and nested MSP (although showing different methylation frequencies) can, when carefully developed, optimized, and interpreted, yield comparable clinical results as pyrosequencing and MS-HRM and could therefore be used for biomarker detection/validation. |
| 18 ^[64] | 2015 | Tissue | <i>hMLH1</i> MGMT APC CDH1 2 OR MORE ALL | 61 61 61 61 61 61 | 14 27 14 31 N/A N/A | MSP | 25% (14%-36%) 66% (54%-78%) 30% (19%-42%) 87% (79%-95%) 71% (60%-82%) 10% (3%-18%) | 57% (31%-83%) 19% (4%-33%) 14% (4%-32%) 25.8% (10%-41%) N/A N/A | The combination of EDNRB locations 1 and 2 can be used as a powerful biomarker, in which the first location is significantly correlated with tumor stage and grade while the second one is aberrantly methylated independent of any clinicopathological features. |
| 19 ^[65] | 2014 | Tissue | CDKN2A | 44 | 155 | MSP | 82% (71%-93%) | 32% (25%-39%) | |
| 20 ^[66] | 2017 | Tissue | DIRAS1 | 146 | 50 | MSP, BS-Seq | 47% (39%-55%) | 100% | |
| 21 ^[67] | 2017 | Tissue | ZNF331 | 146 | 10 | MSP, BS-Seq | 67% (60%-75%) | 100% | |
| 22 ^[34] | 2016 | Tissue | RET ¹⁰ | 25 71 62 41 37 32 25 33 | 184 169 178 199 203 208 215 206 | Direct MSP Nested MSP Pyrosequencing Pyrosequencing Pyrosequencing Pyrosequencing MS-HRM | 11% (-1%-23%) 33% (22%-44%) 23% (12%-33%) 18% (6%-30%) 16% (4%-28%) 14% (2%-27%) 11% (-1%-23%) 15% (3%-28%) | 87% (82%-91%) 74% (67%-80%) 73% (67%-80%) 84% (79%-89%) 85% (80%-90%) 88% (83%-92%) 90% (86%-94%) 87.7% (83.2%-92.2%) | |
| 23 ^[7] | 2017 | Tissue | EDNRB location 1 EDNRB location 2 EDNRB location 3 EDNRB location 4 KISS1 | 45 45 45 45 45 | 45 | MS-HRM | 91% (83%-99%) 91% (83%-99%) 76% (66%-88%) 80% (68%-92%) 58% (43%-72%) | 88.9% (80%-98%) 80% (68%-92%) 78% (66%-90%) 84% (74%-95%) 71% (58%-84%) | |

| | | | | | | | | | |
|-------------------|------|--------|--|--|--|-------------------------------------|---|---|---|
| 24 ^[8] | 2013 | Tissue | AGTRI, WNT2, SLIT2 SEPT9 VIM | 60 14 26 | 38 | Pyrosequencing | 95% (89%-101%) 93% (50%-100%) 83% (33%-92%) | 89% (79%-99%) 100.00% 86.00% (73%-99%) | This study offers a novel panel of specific methylation markers that can be assessed in stools and may complement currently applied protocols for the early detection of sporadic CRC, which may contribute to improving the follow-up and early diagnosis of high-risk patients with IBD when assessed in non-neoplastic tissues obtained by surveillance colonoscopy. |
| | | Stool | AGTRI, WNT2, SLIT4 SEPT9 VIM | 64 35 33 | N/A N/A N/A | | 78% (68%-88%) 20% (6%-31%) 55% (33%-70%) | N/A N/A N/A | |
| 25 ^[6] | 2016 | Tissue | CMTM3 + SSTR2 + MDF | 42 | 42 | Pyrosequencing | 81% (69%-93%) | 91% (82%-100%) | This study has shown that the combination of CMTM3, SSTR2, and MDFI gene methylation may be an epigenetic biomarker for early stages of CRC but should be studied further to determine their potential role as non-invasive diagnostic biomarkers for CRC. |
| 26 ^[8] | 2013 | Tissue | O ⁶ -MGMT p14 ^{ARF} p16 ^{INK4a} RASSF1A APC1A | 111 | 53 | Pyrosequencing | 34% (25%-43%) 29% (21%-37%) 28% (20%-36%) 14% (8%-21%) 27% (19%-35%) | 100% 100% 100% 100% 100% | p14arf, RASSF1A, APC1A, and O6-MGMT methylation as biomarkers of prognosis in CRC could be utilized as a relevant stratification factor in future prospective and interventional studies on CRC, and might serve as a tool in tailoring treatment for individual patients. |
| 27 ^[6] | 2016 | Plasma | PDX1 SDC2 IKZF1 BCAT1 FGF5 GRASP IRF4 SEPT9 SOX21 | 20 44 74 74 20 44 22 44 20 | 20 44 144 144 40 44 24 44 20 | SYBR Green detection MethylLight | 45% (23%-67%) 59% (45-74%) 68% (57-78%) 65% (54%-76%) 85% (69%-101%) 55% (40%-69%) 59% (39%-80%) 59.1% (45%-74%) 85% (69%-101%) | 70% (50%-90%) 84.1% (73%-95%) 95% (92%-99%) 97% (94%-100%) 83% (71%-94%) 93% (86%-101%) 96% (88%-104%) 96% (89%-102%) 50% (28%-72%) | These data demonstrate the utility of close consideration of the background levels of DNA methylation in WBC DNA as an important step in the selection of biomarkers suitable for development as plasma-based assays. |
| 28 ^[6] | 2017 | Plasma | SFRP1 SFRP2 SDC2 PRIMA1 4 genes as a panel | 47 47 47 47 47 | 37 37 37 37 37 | MethylLight | 85% (75%-95%) 72% (60%-85%) 89% (81%-98%) 81% (70%-92%) 92% (84%-100%) | 81% (69%-94%) 89% (79%-99%) 97% (92%-103%) 73% (59%-87%) 97% (92%-102%) | The present study offers the possibility to measure the hypermethylation of the marker panel in cell-free plasma DNA and provides a potential non-invasive, epigenetic diagnostic test. It also shows that the altered methylation pattern might serve as a key for early diagnosis of precancerous stages. |

¹18 genes include: ANKRD15, CASP8, ED42R, ENPEP, GRB10, IGFBP5, INS, ITGB4, LGALS2, MGC9712, NMURI, RASSF5, SLC16A3, SULFITC2, TIMP4, VAV1, VHL, and VMD2;

²Illumina high throughput "Veracode" array;

³Illumina Infinium HM27 DNA methylation assay;

⁴The data were analyzed by using logistic regression algorithm;

⁵The data were analyzed by using marker-specific cutoff and scoring;

⁶The researchers used the cumulative methylation index (CMI) as a threshold. The first sensitivity was calculated when the CMI was 0.05;

⁷The sensitivity was calculated when the CMI was 2.1;

⁸The plasma samples used were collected before chemotherapy;

⁹The plasma samples used were collected after chemotherapy;

¹⁰To compare the clinical sensitivity and specificity of direct MSP, nested MSP, pyrosequencing, and MS HRM, in this study, they computed clinical sensitivities and specificities among deceased patients and subjects still alive, respectively, using overall mortality for the total follow-up period of up to 8 years as the standard. When using the pyrosequencing, the researchers compared different thresholds for positivity which RETI promoter CpG island methylation was dependent on. From the up to down, the thresholds for positivity were, respectively, 5%, 10%, 15%, 20%, and 25%. In this table, we summarize the methods used in the 28 selected articles. The characteristics of each marker or method are described.

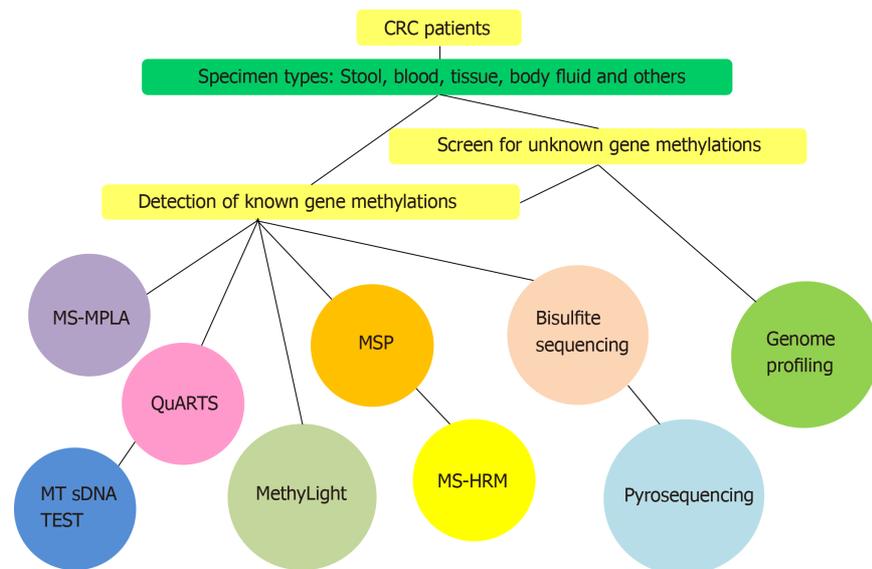


Figure 2 Diagram of the basic choices for methylation detection methods to study gene methylation in colorectal cancer. In this diagram, we show the currently available methods and their different requirements for colorectal cancer assays. Researchers typically use two or more methods to investigate gene methylation. CRC: Colorectal cancer; MS-MPLA: Methylation-specific multiple ligation-dependent probe amplification; MS-HRM: Methylation-specific high resolution melting curve analysis; MSP: Methylation-specific polymerase chain reaction; MT-sDNA: Multitarget stool DNA test; QuARTS: Quantitative allele-specific real-time target and signal amplification assays.

tumor MLH1 downregulation, MS-MPLA was used to analyze MLH1 promoter methylation in peripheral blood leukocyte DNA from the index patients^[29].

MSP technique

MSP is probably the most ubiquitously used technique to study DNA methylation and can rapidly detect the methylation status of DNA without the use of methylation-sensitive restriction enzymes^[30]. Despite only requiring minute amounts of DNA, MSP can be utilized to detect less than 0.1% of allele methylation in a specific locus and could be applied to analyzing DNA methylation in various types of specimens like body fluids and paraffin-embedded specimens^[31]. To perform MSP, primers are needed for amplifying the genomic DNA that has been treated with sodium bisulfite^[27,31,32]. Normally, based on the amplification results, MSP can produce quantitative or qualitative (using agarose gels) results to assess changes in DNA methylation^[33]. Draht *et al.*^[34] tested a number of tissue samples using variations of this method, including MSP, direct MSP, and nested MSP. As expected, the results showed that direct MSP is less sensitive than nested MSP. The reason is that the sensitivity of nested MSP in detecting decreased CpG island methylation of the RET promoter was higher compared with direct MSP. In a study in which a panel of tumor-specific methylation genes (NPY, PENK, and WIF1) was evaluated, a quantitative multiplex methylation-specific PCR (QM-MSP) assay was shown to be not as sensitive and specific as a direct assay of the tissue samples, but it was suggested to be an efficient test to assess serum DNA methylation^[35]. To assess SDC2 methylation in DNA from bowel lavage fluid, Park *et al.*^[36] used a two-step fluorescence-based quantitative methylation-specific PCR (qMSP) method to measure SDC2 methylation of DNA from tissues and bowel lavage fluid. Sun *et al.*^[37] used MSP to detect the methylation status of RASSF1A in blood samples from patients before and after chemotherapy. Exner *et al.*^[38] used a microfluidic high-throughput and methylation-sensitive qPCR approach to assess methylated sites of DNA in freshly frozen and formalin-fixed paraffin-embedded samples. With the intension of determining whether SDC2 methylation detection in stool DNA could be used to screen CRC and adenoma, Oh *et al.*^[39] performed qMSP to analyze stool DNA.

As an endpoint analysis technique^[40], the primary limitation of MSP is that it yields qualitative results, meaning that well-standardized MSP assays provide information that is restricted to the presence of methylated, unmethylated, or both methylated and unmethylated alleles^[41].

MethyLight

MethyLight is a quantitative, fluorescence-based, real-time PCR method^[42] that is

capable of detecting and quantifying DNA. It is especially suitable for detecting hypomethylated DNA regions when the ratio of unmethylated DNA is relatively high because of the combined use of methylation-specific priming and methylation-specific fluorescent probing^[43]. The remarkable advantages of this technique, which was proposed by Eads *et al.*^[44], are its high throughput and great sensitivity other than the ability of yielding high-resolution information of methylation. The MethyLight technique is able to show the methylation level of a specific DNA region without requiring a significant amount of DNA, and it differs from MSP by being able to quantify the relative amounts of a particular methylation pattern. The way of MethyLight quantifying products is through recording the cycle number right when the fluorescent signal goes across a threshold during the exponential amplification phase of PCR^[44,45]. Barták *et al.*^[46] successfully measured the methylation level of four chosen DNA patterns in two types of specimens, colon tissues and plasma, *via* MethyLight assay. As reported by Li *et al.*^[47], the detection limit for SFRP2 methylation using the MethyLight assay was approximately 200 pg (approximately 60 copies of human genome) per reaction. In addition, as a PCR-based method, the throughput of MethyLight can be of great use. For example, He *et al.*^[48] reported on a multiplex MethyLight assay for the detection of methylated genes in CRC.

MS-HRM technique

MS-HRM can detect DNA methylation status with a relatively good sensitivity and specificity. In MS-HRM, the difference between methylated and unmethylated DNA PCR products will be shown as different high-resolution melting curves. By comparing the results of samples with reference amplicons, the extent of methylation of the unknown samples can be estimated^[49,50]. Compared to MSP and pyrosequencing, it has been reported that the MS-HRM method is an inexpensive and simple technique with high accuracy and its methylation level quantification function works as well as or somehow even better than pyrosequencing^[51]. MS-HRM has a higher sensitivity than MSP, is high throughput, and is performed in a closed tube^[52]. Kidambi *et al.*^[53] applied MS-HRM to detecting MLH1 intron 1 methylation not only in cancer samples, but also in peripheral white blood cells as well as buccal cells. However, the accuracy of the HRM method can be affected by factors such as the location or length of the amplicon and the DNA extraction method used^[54,55]. Thus, the use of MS-HRM is typically restricted due to the fact that it could only provide the approximate range of methylation level^[56].

Bisulfite sequencing

Bisulfite sequencing (BS-Seq) is an available option for profiling methylated cytosine in DNA at the genome-wide level at single nucleotide resolution. BS-Seq is regarded as the “gold standard” for single-base resolution measurements of DNA methylation levels^[57]. The basic methylation detection principle of BS-Seq is that unmethylated cytosine can be deaminated to uracil under sodium bisulfite treatment but methylated cytosine cannot. The converted DNA can be amplified using a gene-specific primer^[58], and methylation status of mappable cytosine in genome was measured by means of deep sequencing of the bisulfite-treated genomic DNA^[59,60]. Like all sequencing technologies, BS-Seq involves sophisticated procedures, and problems remain in the sequencing of short amplicons amplified from a bisulfite-modified template^[61].

Pyrosequencing

Pyrosequencing is a DNA sequencing technique that is based on the “sequencing by synthesis” method. By examining the activity of both DNA polymerase and another chemiluminescent enzyme, a single chain of DNA is sequenced using this technique by synthesizing the complementary strand and detecting which base is added at each step^[62]. This method has high throughput after years of investigations^[63,64]. When using this technique, DNA samples are typically treated with bisulfite to detect DNA methylation. Pyrosequencing can be employed to directly detect DNA methylation or to identify the products from MSP.

Genome profiling

When screening for abnormal methylation patterns of genes, genome profiling is the first option used, and the Illumina BeadChip is a popular platform for profiling DNA methylation. The Illumina HumanMethylation450 BeadChip can profile more than 450000 CpGs across the human genome in a cost-effective, comprehensive manner^[65,66]. Lin *et al.*^[67] used an Illumina HumanMethylation450 array while studying the clinical relevance of plasma DNA methylation in CRC patients. Sung-Eun Kim and his team used the Illumina Infinium HumanMethylation27 (HM27) BeadChip to analyze the methylation status of more than 27 thousand individual CpG sites located at promoter regions of over 14 thousand genes in cultivated cells genes in cultivated

cells^[68]. Pekow *et al*^[6] utilized an Illumina Infinium HumanMethylation450 array for the analysis of around half a million methylation sites in each specimen at single-nucleotide resolution. Despite its popularity, this array-based measurement of DNA methylation status has issues with measurement variation. For instance, the negative values or truncation of low intensity signals produced by background subtraction methods can potentially introduce additional bias^[69]. One study showed that part of the probes used in genome profiling could potentially cause false signals due to unspecific hybridization with unintended genomic sequences that are highly homologous to the target genes. Additionally, probes targeting polymorphism of CpGs which have overlapping SNPs have been discovered^[70]. Furthermore, this technique requires the use of complicated procedures to perform DNA profiling^[66,71].

CONCLUSION

In this review, we summarize and discuss all of the techniques or technologies used in scientific studies or in clinical settings. Despite being time-consuming and involving the use of sophisticated procedures, the use of DNA sequencing techniques is needed to identify genes that are aberrantly methylated for use as disease markers. When the candidate genes are identified, it is obviously unwise to use DNA sequencing to screen for them in many types of specimens. In these cases, PCR-based methods have the advantages of being generally more sensitive, scalable, specific, reliable, time-saving, and cost-effective than other methods^[72]. For this reason, we believe that MS-PCR is a preferred technology, as methylation-specific PCR alone can be used to determine the methylation status of a gene. Developing methods for only one specific gene^[73], in our point of view, will limit the use of PCR technology. Combined with various types of methods, the use of methylation-specific PCR can be promising to assess the methylation status of multiple genes, which can be genuinely valuable. Furthermore, not all PCR-based methods require extra equipment to further confirm multiple products. Techniques such as MS-HRM have been successfully developed for the closed-tube screening of aberrant DNA methylation with satisfactory throughput, although we have concerns regarding the deficiencies of this technique. For example, ambiguous results obtained for several samples in an HRM-based screening experiment did not allow for the straightforward classification of the methylation status of the sample^[61]. Thus, we believe that the development of new high-throughput technologies is still urgently needed to allow for faster and cheaper DNA methylation detection in CRC and other diseases.

As shown in [Table 1](#), although only a portion of the investigated markers of CRC are presented, we can see that the number of useful methylation markers is considerable, which we should make good use of. We think individuals with positive FIT results should first have the CRC DNA methylation marker screening test done instead of going straight for tests like colonoscopy. If the DNA markers show that the individual is at the average or high risk of CRC, colonoscopy and other invasive tests are recommended. However, we believe that more studies are needed for selecting the methylation markers that could indicate the precancerous changes in colon. We also suggest that combined methylation marker screening should be a routine test of regular physical examination. What's more, in terms of detection methods, if 10 or more of these markers could be simultaneously assayed in just one closed tube using PCR-based method, the societal benefits would be immense. If the SNPs of multiple genes could be detected and identified in just one tube^[74,75], there could be a means of developing a PCR-based method to detect the methylation status of multiple genes. With the identification of Hachimoji DNA^[76], faster, simpler, and more cost-effective technologies for methylation analysis are not far off.

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