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REVIEW

DNA methylation assay for colorectal carcinoma

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ABSTRACT Colorectal carcinoma (CRC) is a common cause of morbidity and mortality worldwide. Two pathogenic pathways are involved in the development of adenoma to CRC. The first pathway involves *APC/β-catenin* characterized by chromosomal instability resulting in the accumulation of mutations. The second pathway is characterized by lesions in *DNA mismatch repair genes*. Aberrant DNA methylation in selected gene promoters has emerged as a new epigenetic pathway in CRC development. CRC screening is the most efficient strategy to reduce death. Specific DNA methylation events occur in multistep carcinogenesis. Epigenetic gene silencing is a causative factor of CRC development. DNA methylations have been extensively examined in stool from CRC and precursor lesions. Many methylated genes have been described in CRC and adenoma, although no definite DNA methylation biomarkers panel has been established. Multiple DNA methylation biomarkers, including secreted frizzled-related protein 2, secreted frizzled-related protein 1, tissue factor pathway inhibitor 2, vimentin, and methylguanine DNA methyltransferase, have been further investigated, and observations have revealed that DNA methylation biomarkers exhibit with high sensitivity and specificity. These markers may also be used to diagnose CRC and adenoma in early stages. Real time polymerase chain reaction (qPCR) is sensitive, scalable, specific, reliable, time saving, and cost effective. Stool exfoliated markers provide advantages, including sensitivity and specificity. A stool qPCR methylation test may also be an enhanced tool for CRC and adenoma screening.

KEYWORDS Biomarker; colorectal carcinoma; DNA methylation; real time PCR; stool

Introduction

Colorectal carcinoma (CRC) is a common cause of morbidity and mortality worldwide. As a major life-threatening malignancy, CRC ranks second to lung carcinoma in men, third to breast cancer and lung cancer in women, and overall second to other cancer types in men and women. Approximately 142,820 new cases were diagnosed and 50,830 individuals died because of CRC in 2013. Clinical and pathological stages at the time of diagnosis largely determine the prognosis of diagnosed patients. The curative rates of CRC in $T_1N_0M_0$, $T_2N_0M_0$, and $T_3N_0M_0$ stages are greater than 90%, 85%, and 70%, respectively. Therefore, CRC screening is an efficient strategy to reduce death caused by this disease. Current screening modalities have resulted in a modest decrease in mortality and failed to achieve high public participation. Among these modalities, highly sensitive colonoscopy is the standard technique used to detect and remove early lesions, but colonoscopy is invasive and costly. For population-wide screening, simple and noninvasive procedures, such as guaiac or immunochemical stool occult blood testing (FOBT), are preferred. However, the performances of these tests are low. As such, molecular tests should be conducted to improve their efficiency. For example, stool aberrant DNA methylation has been developed as a biomarker for CRC initiation and progression. In this article, this technique was discussed to develop real time polymerase chain reaction (qPCR) DNA methylation biomarker test for stool to diagnose CRC. Stool qPCR DNA methylation biomarkers were also examined to predict outcomes and responses to CRC chemotherapy.

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CRC pathogenesis

Approximately 98% of CRC are adenocarcinomas that almost originate from adenomas as generally curable by resection. The peak incidence age of CRC is 60 to 70 years, and less than 20% of cases occur before the age of 50 years. Genetic and epidemiologic studies have linked CRC to several factors, including inherited predisposition, somatic

mutation, dietary influence, and preexisting inflammatory disease[1]. The development of carcinoma from adenoma is referred to as adenoma-carcinoma sequence. Two major distinct pathogenic pathways are involved in CRC development, and stepwise accumulation of multiple mutations is implicated in these pathways[1,2]. The first pathway comprises *APC/β-catenin* characterized by chromosomal instability (CIN) that results in the stepwise accumulation of mutations in a series of oncogenes and tumor suppressor genes. The molecular evolution along this pathway occurs through a series of morphologically identifiable stage. The loss of *APC* tumor suppressor gene is the earliest event in adenoma formation. Both copies of the *APC* gene must be lost to stimulate adenoma development. Normal *APC* promotes β-catenin degradation. With loss of *APC*, the accumulated β-catenin translocates to the nucleus and activates the transcription of several genes, such as *MYC* and cyclin D1. *K-RAS* mutations subsequently occur. Further mutation of a putative cancer suppressor gene on 18q21 and *TP53* leads to the final emergence of carcinoma, and additional mutation ensues. The accumulation of mutations rather than their occurrence in a specific order is essential for colorectal carcinogenesis. This adenoma-carcinoma sequence accounts for approximate 80% of sporadic CRC[2]. The second pathway is characterized by genetic lesions in DNA mismatch repair genes, which are involved in 10% to 15% of sporadic cases. Mutations accumulate, but correlated identifiable morphological characteristics have yet to be determined. Among these DNA mismatch repair genes, *MLH1* is the most commonly involved in carcinogenesis. The loss of *DNA mismatch repair genes* leads to a hypermutable state. In this state, simple repetitive DNA sequences called microsatellites are unstable during DNA replication. This phenomenon causes widespread alterations in these repeats. The resulting microsatellites instability (MSI) is the molecular signature of defective DNA mismatch repair. The loss of mismatch repair then leads to the accumulation of mutations in growth-regulating genes, and thus triggers the emergence of CRC. In addition to these pathways, CpG island methylator phenotype (CIMP) pathway is also involved in CRC development.

In the majority of patients diagnosed with CRC, carcinoma cells are no longer confined to the primary sites. Approximately 36% exhibit a locally advanced disease and 19% manifest a metastatic disease. The 5-year survival rate of patients with metastatic diseases is 10.3%. CRC incidence and mortality rates have decreased over the past two decades. These trends are consistent with the effectiveness of CRC screening in detecting and removing adenomatous polyps. While these results are encouraging, CRC screening is underused. In general, screening tests can be classified into two categories: invasive (structural) exams and non-invasive tests. Invasive exams can be subdivided into endoscopic techniques (colonoscopy, flexible sigmoidoscopy, and capsule endoscopy) and radiological exams (barium enema, computer tomography colonography and magnetic resonance colonography). Noninvasive tests can be subdivided into tests that detect blood (FOBT) and tests that detect stool DNA[3,4]. Among these techniques, colonoscopy remains the gold standard, but this technique is invasive, costly, and burdensome. Colonoscopy may also be less protective in the right colon than in the left colon[5]. FOBT is a simple, non-invasive, relatively cheap, and frequently used screening test. However, FOBT is not designed for precursor lesions detection. Adenomas and CRCs usually cause intermittent bleeding. As such, repetitive testing is required. Guaiac FOBT is weakly sensitive, whereas immunochemical FOBT is highly sensitive. The detection of DNA markers in stool specimens is a relatively new noninvasive screening approach. Multi-targeted assays on 21 specific mutations in the *K-RAS*, *TP53*, and *APC*, which is a MSI marker (*BAT-26*), and DNA integrity assay markers has also been developed. Nevertheless, their overall sensitivities in detecting CRCs and adenomas remain suboptimal[6,7], possibly because the accumulation of mutations is essential for colorectal carcinogenesis. The detection of DNA mutation may also fail to reflect the progress of carcinogenesis.

DNA methylation and CRC

Epigenetics refers to heritable alterations in gene activity and expression unlikely caused by changes in DNA sequences and potentially reversible self-propagating molecular signatures[8]. Epigenetics has rapidly expanded as a biological field of study[9]. The mechanisms of epigenetics include DNA methylation, histone modifications, nucleosome positioning, or noncoding RNA[10]. Epigenetic modifications, particularly DNA methylation in selected gene promoters, are recognized as common molecular alternations in human tumors. Among epigenetic markers, DNA methylation is the most widely investigated. In DNA methylation, methyl groups are added to the 5-position of cytosine by DNA methyltransferases (DNMT) to produce 5-methylcytosine, which typically represses gene transcription and modifies DNA functions[11]. Cytosine and adenine in DNA can be methylated. DNA methylation elicits species-specific and tissue-specific effects. For instance, the DNA methylation of cytosine residues in CpG dinucleotides, which are often

clustered in so-called CpG islands, leads to transcriptional silencing of the associated genes. Approximately 60% of all human promoters are associated with CpG islands. In the genome of untransformed cells, approximately 90% of all promoters are unmethylated[12]. The DNA methylation of many genes and their significance in CRC have also been described[13]. The aberrant methylation of CpG islands within gene promoters and first exonic or intronic regions may induce the transcriptional repression of tumor-suppressor genes. Genes are hypomethylated and hypermethylated in CRC[14]. Hypermethylation is a discrete targeted event within tumor cells and thus causes specific loss of gene expression. Hypomethylation usually occurs in advanced stages of tumor development and affects genome to a greater extent than hypermethylation does. The most extensively characterized epigenetic alteration is promoter hypermethylation (**Figure 1**).

CIMP is the phenotype of methylated tumor suppressor genes, and tumorigenesis theoretically occurs through progressive genetic silencing even in the absence of genetic mutations[15]. CIMP is also referred to as an epigenetic phenomenon because this occurrence involves a temporary genetic change in the target DNA sequence, that is, CIMP simply triggers a potentially reversible alteration because of methylation. Approximately 30%–40% of proximal CRCs and 3%–12% of distal CRCs were characterized as CIMP[16]. CIMP is regarded as a distinct CRC subgroup, which is fundamentally different from other colon cancers. CIMP tumors exhibit unique pathological features, such as high mutations (KRAS or BRAF) rates, proximal location, wild-type p53, mucinous histological type, poor differentiation, and increased occurrence in female and elderly patients[17]. Primary CRCs can be divided into three subclasses depending on epigenetic and clinical profiles: CIMP1, CIMP2 and CIMP negative. The prognosis of CIMP1 is better than that of CIMP2. CRCs with CIN are likely hypomethylated, whereas CRCs with MSI are hypermethylated. CRCs with neither CIN nor MSI possess a unique methylation pattern and clinical features, including improved prognosis. CIMP mechanisms in the pathogenesis of CRC have yet to be fully elucidated. Hypermethylation secondary to CIMP possibly leads to MSI through MLH1 promoter methylation and subsequent MLH1 mismatch repair gene silencing. DNA methylation has been used as a diagnostic CRC marker because specific methylation events occurring early in multistep carcinogenesis have been identified and epigenetic gene silencing plays a causative role in CRC development. DNA methylation analysis may also provide useful prognostic markers of disease progression and response to traditional chemotherapy.

The majority of CRCs are adenocarcinomas, which almost originate from adenomas. Adenoma is a neoplastic polyp that ranges from small pedunculated tumors to large sessile lesions. The incidence of adenoma is focused on the colon. All adenomatous lesions arise as the result of epithelial proliferation and dysplasia. Adenomatous polyps are classified into three subtypes on the basis of epithelial architecture: (1) tubular adenomas; (2) villous adenomas; (3) tubulovillous adenomas. The risk of CRC increases when polyps are larger than 2 cm, villous than tubular, and sessile rather than pedunculated. Pedunculated lesions are treated through colonoscopic removal with snare electrocautery. Sessile lesions may require surgical excision. Although colorectal adenomatous polyps are recognized as precursor lesions in most CRC cases, only 1%–10% of individuals with resected adenomas likely develop invasive cancer[18]. Thus,

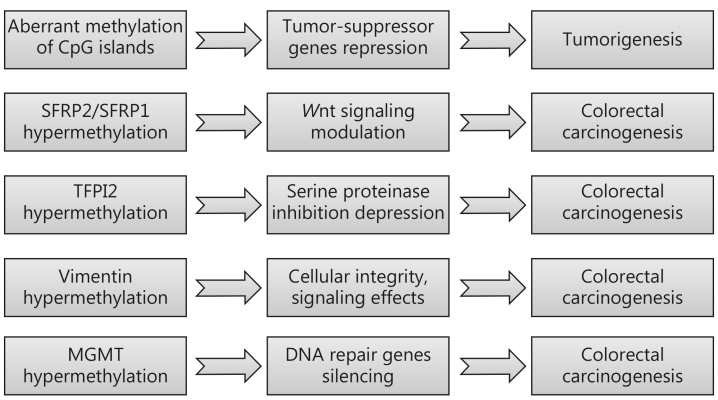

Figure 1 Mechanisms of DNA methylation in the colorectal carcinoma pathogenesis: the roles of multiple DNA methylation biomarkers in colorectal carcinogenesis.

early detection of colorectal adenoma may help prevent CRC. Adenoma is currently diagnosed with colonoscopy and air contrast barium enema. Aberrant DNA methylation occurs in the stool of adenoma patients. Thus, the feasibility of using DNA methylation biomarkers to diagnose colorectal adenoma should also be evaluated.

Multiple DNA methylation biomarkers

DNA methylation biomarkers in stools with CRC and precursor lesions have been extensively examined. Different panels have been reported to improve diagnostic accuracy, although no definite biomarker panel has been established. In our comprehensive analysis of available studies on DNA methylation biomarkers in stools for CRC and adenoma detection, some DNA methylation biomarkers, including secreted frizzled-related protein 2 (SFRP2), secreted frizzled-related protein 1 (SFRP1), tissue factor pathway inhibitor 2 (TFPI2), vimentin, and methylguanine DNA methyltransferase (MGMT), have been investigated. Observations have confirmed that DNA methylation biomarkers are highly sensitive and specific. These markers may also be used to diagnose CRC and adenoma in early stages (**Table 1**).

SFRPs are a family of secreted proteins that can bind to *Wnt* ligands and frizzled receptors and thus modulate the *Wnt* signaling cascades. *Wnt* signaling cascades play an important role in colorectal carcinogenesis and progression[19]. SFRPs are initially and independently identified as soluble factors implicated in early embryonic development and modulators of apoptotic events. Alterations in SFRP expression levels have been associated with tumor formation and bone and myocardial disorders[20]. SFRP1 and SFRP2 hypermethylation likely occurs at the onset of all tumor types, including colon carcinomas[21-24]. SFRP1 hypermethylation may reduce gene expression and contribute to CRC formation[25]. Epigenetic SFRP1 inactivation is linked to the upregulation of *Wnt/β-catenin cascade* in CRC; *Wnt/β-catenin* repression has also been considered a mechanism that inhibits tumor cell growth and prevents metastatic invasion (**Figure 1**)[22]. SFPR2 and SFRP1 methylation in stool also exnibits high sensitivity and specificity for CRC detection[26-40]. SFRP2 methylation for CRC identification in stool samples reaches a sensitivity of 90% and specificity of 77%[31]. A systematic meta-analysis has revealed that the pooled sensitivity and specificity of methylated SFRP2 are 0.71 and 0.94, respectively[41]. SFRP2 methylation is therefore a promising biomarker for CRC screen[42]. The DNA stool test of SFRP1hypermethylation also achieves a sensitivity of 89% and specificity of 86% in colorectal neoplasia detection[35].

TFPI2, a member of the Kunitz-type serine proteinase inhibitor family, inhibits the tissue factor/factor VIIa complex and various serine proteinases. The aberrant methylation of TFPI2 promoter CpG islands in human cancer is responsible for the decreased TFPI2 expression during cancer progression. TFPI2 also maintains the stability of tumor environment and inhibits neoplasm invasiveness and growth and metastasis formation (**Figure 1**)[43]. TFPI2 methylation in stools also demonstrates high sensitivity and specificity among CRC patients[44-46]. TFPI2 gene promoter methylation is detected in the stool of CRC patients with a sensitivity of 86.7% and a specificity of 100%[46]. The sensitivity and specificity of fecal TFPI2 methylation assay for CRC detection range from 76% to 89% and from 79% to 93%, respectively[44].

Vimentin, a major constituent of the intermediate filament family of proteins, is ubiquitously expressed in normal mesenchymal cells and known to maintain cellular integrity. Vimentin has been considered as a marker for epithelial-mesenchymal transition (EMT), although the molecular aspects of vimentin in the function of tumorigenesis remain unknown. The aberrant promoter methylation of the vimentin gene may contribute to colorectal carcinogenesis

Table 1 Major stool DNA methylation biomarkers for colorectal cancer

Markers	Source	Epigenetics	Pathway
SFRP2	Stool	Hypermethylation	Wnt signaling
SFRP1	Stool	Hypermethylation	Wnt signaling
TFPI2	Stool	Hypermethylation	Serine proteinase inhibitor
Vimentin	Stool	Hypermethylation	Cellular integrity
MGMT	Stool	Hypermethylation	DNA damage repair

SFRP2, SFRP1, TFPI2, vimentin, and MGMT are investigated, and findings confirm that stool methylation biomarkers exhibit high sensitivity and specificity.

(**Figure 1**)[47]. The promoter hypermethylation of vimentin in stool is also a sensitive, specific alternative for CRC screening[38,45,48-52]. Aberrant vimentin methylation is detected in fecal DNA from CRC patients, with a sensitivity of 46% and a specificity of 90%[51].

MGMT is a suicide enzyme that repairs the pre-mutagenic, pre-carcinogenic and pre-toxic DNA damage O6-methylguanine. MGMT also repairs large adducts, which are formed in response to pollutants, carcinogens, methylating agent, and chloroethylating anticancer drugs, on the O6-position of guanine. Therefore MGMT is a key node in the defense against commonly found carcinogens and marker of resistance of normal and cancer cells exposed to alkylating therapeutics (**Figure 1**)[53,54]. A meta-analysis suggested that the frequency of MGMT hypermethylation is significantly higher than that in CRC, and MGMT gene promoter methylation involved a stepwise carcinogenesis of CRC development[55]. The loss of MGMT expression, which is secondary to MGMT gene promoter methylation, may increase the responses to alkylating agents[56]. The methylation stool testing of MGMT is a promising, sensitive, and specific method for early CRC detection[30,49,57]. Stool-methylated MGMT is detected in 48.1% of CRC patients and 28.6% of adenoma patients[30].

Other stool methylation biomarkers, such as oncostatin M receptor-β, human Mut L homolog-1, hyperplastic polyposis protein gene, SFRP5, GATA4, β-1, 4-galactosyltransferase-1, N-myc downstream-regulated gene 4, 2qI4.2, hypermethylated in cancer 1, ESR1, phosphatase and actin regulator 3, spastic paraplegia-20 and RASSF2, have been proposed to diagnose CRC.

Perspectives of DNA methylation assay for CRC

Definite DNA methylation biomarker panels haven't yet to be established. Unlike DNA mutation assay, DNA methylation can be measured quantitatively through qPCR, which may be used to monitor disease progression. Purified stool DNA can be chemically modified by sodium bisulfate to convert all unmethylated cytosine to uracils while leaving methylcytosines unaltered. Treated DNA retains methylated cytosines, and shows specific changes in the DNA sequence. Colonocyte shedding from colorectal neoplasm differs quantitatively and qualitatively from that of normal epithelium. Cellular efflux toward the lumen is much greater from CRC than from normal epithelium. Although CRC typically occupies less than 1% of the intestinal surface, tumor-derived DNA in stool from patients with CRC may account for as much as 14% or 24% of the total recovered DNA[58,59]. Exfoliated coloncytes and colonocyte constituents provide a diverse class of candidate stool markers. Unlike occult bleeding, which occurs as intermittent leaking into the lumen, exfoliated markers are sensitive because of their continuous release. Furthermore, exfoliated markers are potentially specific because they originate from neoplasm per se, where occult blood emanates from circulation. Since the earliest study of abnormal methylation in CRC was reported by Feinberg and Vogelstein[60], hundreds of methylated genes have been described in CRC and adenoma. The correct genes to be analyzed should be accurately selected to improve the sensitivity and specificity of DNA methylation tests.

DNA methylation may provide useful prognostic markers of disease progression and response to therapy. Specific methylation events have been associated with different tumor stages and poor prognoses[61]. As the first chemotherapeutic agent, 5-fluorouracil (5-FU) has been combined with folinic acid and has been used for CRC chemotherapy. Hence, 5-FU-based therapies remain the standard regimens against advanced CRC. 5-FU inhibits thymidylate synthase, which is a key enzyme in pyrimidines synthesis. Thus, pyrimidines available for DNA replication are reduced. Newly available 5-FU-based oral formulations, such as capecitabine, offer maximum antitumor properties. CIMP status can be assessed as a predictive marker for 5-FU responsiveness[62]. Cancer-specific DNA methylation events may also be involved in different stages of colorectal carcinogenesis. Unlike gene mutations, DNA methylation may also be reversible. DNA methylation can be affected by single demethylating drug. Chemotherapeutic effect may be predictable prior to drug administration through DNA methylation analysis. Aberrant genes methylation may be essential for the chemosensitivity of CRC to various drugs. The response and chemosensitivity of patients to chemotherapy may also be predicted by determining the status of DNA methylation biomarkers in the stool of CRC patients.

Recent advancements in engineered DNA-binding molecules, such as transcription activator-like effector (TAL or TALE) proteins, clustered regularly interspaced short palindromic repeats (CRISPR), and CRISPR-associated proteins (Cas) system, have been applied in genome editing in cells[63,64]. These engineered DNA-binding molecules can bind to a specific DNA sequence and be applied to other purposes. Engineered DNA-binding molecule-mediated chromatin immunoprecipitation has been developed by utilizing a TAL or CRISPR/Cas system to target specific genomic regions and to investigate associated DNA-binding interactions[65,66].

Conclusions

The development of a sensitive stool qPCR DNA methylation assay for CRC and adenoma can provide a non-invasive, scalable, specific, safe, convenient, and widespread accessible screening tool, which is more acceptable to patients than current commonly used screening methods. This assay may help decrease the morbidity and mortality of CRC. Genomic markers for survival in patients with CRC have yet to be developed. DNA methylation status may be a prognostic factor in patients treated with standard chemotherapy. Patients with high methylation levels may be recommended with alternative treatments.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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