DNA methylation patterns as noninvasive biomarkers and targets of epigenetic therapies in colorectal cancer

Aberrant DNA methylation is frequently detected in gastrointestinal tumors, and can therefore potentially be used to screen, diagnose, prognosticate, and predict colorectal cancers (CRCs). Although colonoscopic screening remains the gold standard for CRC screening, this procedure is invasive, expensive, and suffers from poor patient compliance. Methylated DNA is an attractive choice for a biomarker substrate because CRCs harbor hundreds of aberrantly methylated genes. Furthermore, abundance in extracellular environments and resistance to degradation and enrichment in serum, stool, and other noninvasive bodily fluids, allows quantitative measurements of methylated DNA biomarkers. This article describes the most important studies that investigated the efficacy of serum- or stool-derived methylated DNA as population-based screening biomarkers in CRC, details several mechanisms and factors that control DNA methylation, describes a better use of prevailing technologies that discover novel DNA methylation biomarkers, and illustrates the diversity of demethylating agents and their applicability toward clinical impact.

First draft submitted: 14 December 2015; Accepted for publication: 5 February 2016; Published online: 22 April 2016

Keywords: biomarker • colorectal cancer • DNA • gene silencing • methylation • methylation-specific PCR • microarray • next-generation sequencing • prognosis • pyrosequencing

Colorectal cancer (CRC) affects approximately 5% of the US population, ranking second among cancer-related mortalities [1]. Colonoscopy facilitates diagnosis and management of colonic diseases; however, its effectiveness is inadequate considering low patient compliance and procedural complications that can become serious. Compliance for CRC screening by colonoscopy is very poor: less than 35% in adults aged 50–90 years with normal CRC risk, and less than 50% in those with increased risk, such as family history, adenomatous polyps, or inflammatory bowel disease [2]. The frequency of complications depends on physicians' training and safety precautions: severe complications are not limited to colonic perforations or intraperitoneal hemorrhages, anesthetic events include aspira-

tion pneumonia, diverticulitis, and postpolypectomy [3]. Patients with history of stroke, pulmonary disease, and atrial fibrillation have higher risk for serious events [4]. The lack of a standardized colonoscopic methodology among nonintegrated healthcare systems, such as many in the USA, and difficulty with acquiring life-time patient records from these systems, complicates treatment by allowing various nonstandardized procedures to be performed and limits physicians' coordination with other hospitals and clinics [5]. Therefore, there is a clear and present demand for simplified, safer, and more cost-effective screening methods that detect CRC and profoundly improve early detection of cancer patients.

Decades of accumulated data suggest that colorectal tumorigenesis is influenced

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by a variety of genetic and epigenetic factors. Among epigenetic events, aberrant DNA methylation targets cytosine-guanine (CG)-dense regions of promoters located within the 5' untranslated regulatory sequences of genes, and includes covalent modifications that regulate expression of oncogenes, tumor suppressors, and drug resistance genes [6]. 'Hyper'-methylation of multiple genes in malignancies may also associate with tumor recurrence [7] and drug resistance, where DNA repair [8], cell cycle [9,10], and apoptosis [11,12] mediating genes are transcriptionally silenced resulting in genotoxic resistance. Consequently dysregulated expression of mitogenic and anti-apoptotic genes results in poor prognosis [13]. 'Hypo'-methylation typically occurs at intronic LINE-1 and SINE-1/Alu elements, which serve as surrogate markers for genome-wide demethylation [14] and contribute toward enhanced genomic instability and subsequent accelerated tumor progression [15]. In view of the growing body of literature about the potential clinical use of aberrantly methylated genes in cancer diagnostics, pooling available data and exploiting technologies that interrogate large numbers of methylation sites will improve cancer biomarker based selection by prioritizing DNA markers that allow earlier diagnosis, facilitate identification of high-risk populations, and stratify patients based upon chemoresistance and other aggressive phenotypes.

Over the last two decades, a large number of methylated DNA targets, both gene promoters and other intronic markers, that distinguish between healthy and malignant tissues have been identified (Table 1); however, only a few consistently and accurately predict CRC or have been accepted as blood/stool-based biomarkers for clinical testing. This privation underlines the need for novel biomarker discovery. In this context, DNA-based biomarkers offer certain distinct advantages. DNA is markedly more stable than RNA and protein making molecular testing from collected and handled clinical specimens more practical, reliable, and viable. Combined with these facts, high-throughput technologies, which interrogate large numbers of genomic sites [16–18], can systematically and comprehensively discover the most promising diagnostic, prognostic, and predictive methylated genes/loci (unique to specific diseases and cancers) from large consortiums of existing tumor tissue banks and liquid biopsies (blood and stool). Extrapolating this idea suggests that methylation patterns are specific to subsets within diseases or narrow ranges within spectrum diseases, such as neurological, metabolic, cardiovascular disorders, and autoimmune diseases.

This review describes several mechanisms and factors that control promoter methylation and gene silencing, explains the effects of aberrant methylation on cell

phenotype, evaluates the current state of CRC-specific methylated genes as disease biomarkers, and illustrates the prevailing technologies available for methylated DNA screening and their clinical applications.

Mechanisms of promoter methylation mediated gene silencing

DNA methylation is a eukaryotic genome modification event, critical for mammalian development, that occurs at the fifth carbon position of cytosine residues within CpG dinucleotides [6]. These dinucleotides cluster into 'CpG islands' (CGIs) and frequently occur in more than 50% of 5'-flanking promoter regions of various tumor suppressor genes [6]. Cytosine methylation is catalyzed by three known DNMTs: DNMT1, DNMT3 and DNMT3B. DNMT1 maintains methylation of newly synthesized DNA strands during replication [50], whereas DNMT3A and DNMT3B involve *de novo* methylation [51].

Illustrated in Figure 1, methylation inhibits binding of transcription factors to promoters in diverse ways: long noncoding RNAs (lncRNAs) can interact with EZH2, which in turn recruits DNMTs to facilitate DNA methylation at target sites [52] and, conversely, some lncRNAs inhibit DNA methylation by inhibiting DNMT binding to CpGs [53]; Piwi-interacting RNAs (piRNAs) can associate with DNMT1 to target methylation at promoter regions [54,55]; methyl-CpG binding proteins (MBPs) contain methyl-CpG binding domains (MBDs), decipher methylated DNA by binding to methylated CpGs, and complex with HDAC and other MBPs that remodel chromatin and repress transcription [56]; and, the transcriptional repressor CCCTC-binding factor (CTCF) manipulates the 3D structure of chromatin to promote and repress gene expression at various sites and is inhibited by CpG methylation [57].

Factors affecting gene methylation in colorectal epithelium

A variety of intrinsic and extrinsic factors may play important roles in mediating aberrant methylation (Figure 2). Environmental and dietary factors that promote cancer can take decades to alter promoter methylation. As a general principle, most cancers experience global DNA hypomethylation, with tumor suppressor genes as frequent targets of site-specific hypermethylation.

Aberrant methylation of cancer-related genes correlates with advanced age, while cancer-related genes are seldom methylated in young people's non-neoplastic colorectal epithelia [58]. CGIs within promoter regions of normal tissues contain unmethylated regions flanked by methylated regions; however, during tumorigenesis,

Table 1. Abnormally methylated genes and predicted clinical outcomes in colorectal cancer.

Function	Marker	Clinical outcome	Sample size	Methylation prevalence (%)	Assay	Ref.
DNA repair	<i>MGMT</i>	Brain metastasis/poor prognosis	885	38	MethyLight	[19]
	<i>MGMT-CDKN2A</i> comethylation	Improved survival	47	43 and 51	MSP	[20]
	<i>MGMT</i>	Improved prognosis	111	34	Pyrosequencing	[21]
Apoptosis	<i>BNIP3</i>	–	61	66	COBRA	[22]
	<i>DAPK</i>	Intraepithelial neoplasia (Met [+])	22	81.2	MSP	[23]
	<i>PCDH10</i>	Stage II, poor prognosis	143	62	MethyLight	[24]
	<i>APARF1, BCL2, and P53</i>	Poor prognosis	137	41	MSP	[11]
Cell adherent/ invasion/ migration	<i>Vimentin</i>	Stage I–IV	46/107	83/53	MSP	[25]
	<i>RET</i>	Stage II, poor prognosis	758	17.6	MSP and pyrosequencing	[26]
	<i>TIMP3</i>	Invasion/metastasis	29	27	MSP	[27]
Cell cycle/ proliferation	<i>CDKN2A</i>	Poor prognosis	582	26.3	MSP	[28]
	<i>CDKN2A (P14ARF, APC1A)</i>	Poor prognosis	111	29	Pyrosequencing	[21]
	<i>IGF2</i>	Poor survival	1033	–	Pyrosequencing	[13]
	<i>P15INK4b</i>	Poor survival	39 (stage I/II)	–	MSP	[29]
	<i>PRDM5</i>	–	61	6.5	COBRA	[30]
	<i>MYOD1</i>	Poor survival	80	–	MethyLight	[31]
	<i>RARβ2</i>	Poor survival	73	80.8	MSP	[32]
Transcriptional regulation	<i>GATA4</i>	Adenoma (Met [+])	90	70	MSP	[33]
	<i>HIC1</i>	CIMP marker	120	67.1	MethyLight	[34]
	<i>TFAP2E</i>	Chemotherapy-resistant/poor prognosis	311	61	qMSP	[35]
	<i>HLTF</i>	Metastasis, size	24	70.8	MethyLight	[36]
Wnt pathway	<i>SFRP1</i>	MSS [+]	1245	95	MethyLight	[37]
	<i>SFRP2</i>	Advanced colorectal tumors	222	61.7	COBRA	[38]
	<i>SFRP2</i>	Poor overall survival	169	–	MSP	[39]
	<i>SOX17</i>	Hyperplasia (Met [+])	126	>90	Pyrosequencing	[40]
	<i>DKK3</i>	–	21	58	MSP	[41]
	<i>WIF1</i>	Poor prognosis	143	33	MethyLight	[40]
	<i>WIF1</i>	–	40	87.95	MSP	[42]
Ras pathway	<i>RASSF1A</i>	Poor prognosis	111	14	Pyrosequencing	[21]
	<i>RASSF2A</i>	Adenoma (Met [+])	243	58	MSP	[43]
Extracellular matrix protein	<i>TFPI2</i>	Lymph node metastasis	50	62	qMSP	[44]

AQAMA: Absolute quantitative assessment of methylated allele; COBRA: Combined bisulfite restriction analysis; MSP: Methylation-specific PCR; qMSP: Qualitative methylation-specific PCR.

Table 1. Abnormally methylated genes and predicted clinical outcomes in colorectal cancer (cont.).

Function	Marker	Clinical outcome	Sample size	Methylation prevalence (%)	Assay	Ref.
Membrane protein	<i>ITGA4</i>	Risk markers for inflammation-associated colon cancer	15	93	MSP	[45]
Signal transduction	<i>IGFBP3</i>	Disease-free survival in stage II and III	452	83	Pyrosequencing	[46]
Cell growth/differentiation	<i>NDRG4</i>	Adenoma (Met [+])	83/184	86/70	qMSP	[47]
Other	<i>MINT2, 3, and 31</i>	Distant recurrence, and poor overall and cancer-specific survival	251	35	AQAMA	[48]
	<i>APC</i>	Improved survival	117	62.4	MSP	[49]

AQAMA: Absolute quantitative assessment of methylated allele; COBRA: Combined bisulfite restriction analysis; MSP: Methylation-specific PCR; qMSP: Qualitative methylation-specific PCR.

flanking methylation encroaches toward unmethylated regions of tumor suppressor genes [59]. Although aggressive research into age-related methylation has identified important cellular–environmental factors that influence methylation, however, the molecular machinery behind this process, *vis-a-vis* cancer-related methylation, remains unclear.

Dietary factors affect genomic methylation. Folate deficiencies, where S-adenosylmethionine (SAM) is intracellularly scarce, restricts the source of methyl donors (methionine and folate) and alters genomic cytosine methylation [60] thereby destabilizing DNA. Folic acid is converted to 5'-methyltetrahydrofolate, which is required for methionine's conversion into SAM. Methionine is an essential amino acid found in poultry, fish, and dairy products, whereas folate is an essential nutrient derived from fruits and vegetables [60]. Folate supplements prevent CRC incidence in patients with ulcerative colitis [61]. Conversely, exposure to arsenic and its derivatives, either through smoking or drinking water, can promote hypermethylation of tumor-suppressor genes [62,63]. Interestingly, serum folate levels failed as CRC biomarkers [64], likely due to variability between individuals. Therefore, biomarkers that 'record' and accumulate aberrant DNA methylation over time will more likely be stable predictors of disease.

The link between cancer and inflammation has been established since the 19th century [65], whereas chronic infection and inflammation has recently been shown to influence methylation. Inflammatory mediators of infection and inflammation, such as TNF- α , IL-1 β , and reactive oxygen species – superoxide (O $_2^{\cdot-}$), alkoxyl (RO), hydroxyl (OH), and peroxide (RO $_2$) – are abundant in colitis and thought to contribute to aberrant DNA methylation during colorectal car-

cinogenesis [66–68]; however, the molecular mechanisms orchestrating specific molecular events leading to altered methylation of specific genes are not fully understood. High-throughput technologies that comprehensively interrogate genome-wide methylation can elucidate the complex mechanism(s) behind environmental and dietary factors that govern methylation patterns of tumor genomes.

Clinical significance of tumor-related gene methylation in CRC

Methylation-mediated silencing of tumor suppressor genes is hypothesized to contribute to CRC initiation and progression [12,69–72]. A large number of aberrantly methylated gene promoters that control a variety of biological functions, such as genomic stability, cell proliferation, and motility, are found in CRC and demonstrate diagnostic, prognostic, and predictive potential in this disease (Table 1).

DNA repair

Disruption of DNA repair mechanisms correlates with CRC survival [73,74]. DNA mismatch repair (MMR) machinery primarily detects and fixes insertions, deletions, and incorrectly incorporated bases within the DNA helix [75]. DNA damage normally activates apoptosis of tumor cells through MMR machinery; however, epigenetic silencing of *MLH1* often occurs during CRC progression and is a major cancer-driving factor as its inactivation permits increased accumulation of spontaneous mutations [76,77]. Recent advances in laser capture micro-dissection indicates that methylation of MMR promoters can be detected in preadenomatous hyperplastic colorectal glands [78]. Another mechanism of DNA repair, MGMT, protects DNA from alkylating agents by removing promutagenic methyl groups

Figure 1. Mechanisms of DNA methylation that regulate gene transcription. (A) Various lncRNAs recruit EZH2 to CpGs, which then recruit DNMTs to promote methylation. Alternatively, lncRNAs can block DNMTs from binding to CpGs and protect promoters from methylated-silencing. (B) PiRNAs facilitate CpG methylation by recruiting DNMT1 and/or EZH2 to promoters. Without piRNA expression and ligation, EZH2 and DNMT1 bind to certain promoters less readily. (C) MBPs bind to methylated DNA and recruit HDAC, which deacetylates histones and facilitates heterochromatin formation and transcriptional repression. (D) CTCF, manipulates the 3D structure of chromatin to promote and repress gene expression at various sites, however, it binds poorly to methylated CpGs.

Figure 2. Factors that cause aberrant DNA methylation. Intrinsic and extrinsic environmental factors affect the epigenome. Smoking, intake of mutagenic chemicals (arsenic), and diet are extrinsic factors that damage DNA and facilitate methylation. Aging leads to natural accumulation of DNA damage and methylation, thereby promoting cancer by unregulating cellular growth, promoting invasion and motility, and suppressing anti-apoptotic signals.

from the O⁶ position of guanines. Promoter methylation mediated silencing of the *MGMT* gene frequently occurs in CRCs [74,79] and increases incidence of G:C A:T transitional mutations in growth determining genes including *p53* [73] and *KRAS* [80].

Apoptosis & cell proliferation

The apoptotic pathway normally directs cell death in multicellular organisms, yet it can become defective thereby permitting cancer cells to resist apoptotic signals [12,81]. Interruption at critical junctions of apoptotic pathways, such as with p53 and p21, lead to malignant transformation, tumor metastasis and resistance to anticancer drugs [82]. Hypermethylation of other apoptosis-related genes have been recently reported in CRC. *DAPK* encodes a calcium/calmodulin-dependent serine/threonine kinase that positively mediates apoptosis, and is methylated during early events in colorectal tumorigenesis [23]; *XAF1* methylation correlates with advanced stage and high tumor grade [83]; *BNIP3* is a pro-apoptotic Bcl-2 family member normally expressed under hypoxia and other stress environments, is commonly silenced by methylation, and believed a major determinant in CRC [22]. Patients with *BNIP3* methyla-

tion are resistant to irinotecan chemotherapy and have poorer survival rates [84]. *NDRG2* and *NDRG4* encode for *NDRG* family members, an evolutionarily conserved group of proteins [85] that are putative tumor suppressors, and are downregulated by methylation in CRC [86,87].

Cell cycle is controlled by complex signaling pathways that regulate cell growth, DNA replication, and cell division. Mutation, deletion, or transcriptional silencing of cell cycle genes trigger tumor formation [10]. Tumor-suppressing *CDKN2A* (also known as p16 ARF/INK4A) inhibits CDK4 and CDK6, which bind to cyclin D1 and phosphorylate Rb protein [88,89]. P16 normally dephosphorylates Rb to prevent cell cycle progression. Furthermore, *CDKN2A* is among a panel of surrogate markers used to evaluate global hypermethylation phenotype, defined as CpG island methylator phenotype (CIMP) [28,90]. Levels of methylated *CDKN2A* in primary CRCs associate with advanced Dukes' stages and intimately link with poor clinical outcomes [77,91]. Furthermore, aberrantly methylated *CDKN2A* can be detected in serum, making it a viable noninvasive serum-based biomarker for early and late stage cancers [92,93]. Silencing of *CDKN2A* in CRC more commonly occurs through

hypermethylation than deletions or mutations [94]. The family of transcription factors, PRDMs, contains kruppel-like zinc fingers and broadly regulates cellular functions such as differentiation and malignant transformation. Knock-down of EZH2 in nonmethylated colorectal cell line SW480 induces PRDM5, which is frequently hypermethylated in colorectal and gastric cancers [70], whereas its overexpression inhibits colony formation [30,93].

Cell motility

Invasion involves encroachment into surrounding tissue, and metastasis involves dissemination to distant organs. Loss of cell–cell and cell–matrix interactions causes colon cancer cells to lose intercellular contact and cell adhesion, and gain motility [95]. CDH1 is a transmembrane glycoprotein that localizes to adherens junctions of epithelial cells. The *CDH1* promoter is highly methylated in primary CRCs and cell lines [96]; however, its inactivation also occurs through mutations, chromosomal deletions, and histone deacetylation, resulting in increased proliferation, invasion, metastasis and tumor progression [96–98]. The antagonist of matrix metalloproteinase activity, TIMP3, represses tumor growth, angiogenesis, invasion and metastasis. *TIMP3* is often silenced in CRC cell lines and primary CRC tissues through promoter hypermethylation [27]. PCDH10, a member of the cadherin subfamily, protocadherins, establishes and maintains neuronal connections [99]. Overexpression of *PCDH10* suppresses tumor cell growth, migration, invasion and colony formation [100]; however, its promoter is frequently hypermethylated in CRC and other cancers [101]. Heitzler *et al.* showed that promoter methylation of *PCDH10* has prognostic and predictive value in CRC, and may be important when deciding treatment options for stage II CRCs [24].

Taken together, these studies describe the consequences of methylation at only a few of the many genes that control cell motility and metastasis. Further investigation into tumor genome methylation via array and sequencing-based technologies will yield many more methylation sites that are associated with advanced CRC; therefore, future diagnostic screens will likely include methylated DNAs that represent cell motility and metastasis.

Transcriptional regulation

GATA binding proteins (GATA) are a family of zinc-finger transcription factors, common within promoters, that recognize GATA motifs. Among them, GATA4 and 5 are important in GI tract differentiation and function among many species [102]. Emerging evidence suggests that GATA expression is controlled by epigen-

etic mechanisms. Hypermethylation of *GATA4* and *GATA5* promoters is frequent in CRCs and gastric cancers [103], where *GATA4* hypermethylation is detectable in premalignant tubulovillous adenomas making it an early detection marker of ‘high risk’ for CRC [33].

TFAP2 (AP-2) family of transcription factors consists of five proteins that regulate cell growth, differentiation, and apoptosis in cancer [9,104]. Hypermethylation of *TFAP2E* is observed in 61% of tumor tissues and occurs more often in early stage tumors that lack invasion, lymph node involvement, and high histologic grade, and is associated with favorable outcomes. Approximately 77% of CRC patients with hypermethylated *TFAP2E* survived 5 years after diagnosis compared to less than 42% with unmethylated genes [35].

HIC1 is a transcriptional repressor associate with budding epithelia of gastrointestinal tissues [71]. In particular, HIC1 cooperates with p53 to regulate cell growth and suppress cancer [81,105]. Loss of heterozygosity and hypermethylation associate with cancer and diseases related to chromosomal abnormality, such as Miller-Dieker syndrome [71]. Along with *HIC1*, other polycomb group target genes (*SFRP1*, *MYOD1* and *SLIT2*) are hypermethylated in CRC [34] and could be among a panel of novel biomarkers that predict survival.

Signaling pathway-related genes

Wnt/ β -catenin signaling pathway mediates cell growth, proliferation, and stem cell differentiation [106]. APC normally inhibits Wnt signaling in nonproliferating cells by sequestering β -catenin, however, is often silenced by promoter methylation and consequently linked to early CRC development [107]. Meanwhile, epigenetic silencing of Wnt antagonists commonly occurs in human cancers [69,108–109]. Wnt signaling antagonists comprise two functional classes: those that bind to Wnt proteins (WIF1, sFRP and Cerberus) [110] and those that bind to Wnt receptors Dkk family members) [111]. *SFRP1/2* are frequently hypermethylated in CRC [37–38,110], while Dkk family members are suppressed by promoter methylation in various types of human gastrointestinal cancers. Silencing *DKK1* induces over-response of Wnt/ β -catenin activation bolstering tumor promotion through dysregulated apoptosis and differentiation [41]. Recently, Silva *et al.* reported that hypermethylation of *DKK1* significantly correlates with poor prognosis while *SFRP4* hypermethylation improves prognosis; however, survival correlation was confounded once adjusted for microsatellite instability and CIMP, most likely a result of global methylation status [40].

The Ras superfamily of GTP-binding proteins regulates various intrinsic cellular processes, including cellular proliferation and differentiation, intracellular vesicular trafficking, cytoskeletal control, and cell

death. Epigenetic silencing of Ras effector promoters is common in CRC, suggesting these genes are important mediators of colorectal carcinogenesis [112]. *RASSF1A* methylation associates with higher TNM stages and poor prognosis, and *RASSF2A* methylation occurs early in CRC [21,43]. These data strongly suggest that *RASSF1A* and *RASSF2A* can become diagnostic and therapeutic targets for managing CRC.

Other signaling pathways relevant to CRC tumorigenesis are aberrantly regulated by methylation. *TFPI2* is a Kunitz-type serine protease inhibitor that decreases activities of several enzymes such as trypsin, plasmin, and platelet tissue factor VIIa complex. *TFPI2* methylation is common in CRCs, thus silencing obviates tumor suppression; therefore, *TFPI2* methylation is an independent prognostic factor for CRC [44,45]. *IGFBP3* is one of six repressors of IGF-1 and -2 signal transduction [113] and functions to inhibit growth and promote apoptosis [12]. Our laboratory reported that *IGFBP3* methylation levels are elevated in CRCs when compared with normal mucosa. Low methylation levels of *IGFBP3* confer an independent risk factor associated with poor disease-free survival in stage II and III CRC patients. Furthermore, high methylation of *IGFBP3* in patients with stage II and III CRC indicates low benefit from adjuvant 5FU-based chemotherapy [46].

Noninvasive methylation biomarkers in CRC

The ideal methylated DNA biomarker can easily be incorporated into routine noninvasive bloodwork/chemistry screens. Current detection methods include gastrointestinal endoscopy, fluoroscopy and pathological analysis. However, detection rates by these methods are limited by the physicians' technical skill. Moreover, endoscopy is intrusive, dangerous, and repudiated by patients. Thus, development of less-invasive, efficient, accurate, and cost-effective detection methods is urgently needed. Aberrantly methylated DNA is believed to originate from primary CRC cells that are released into the colonic lumen or the blood stream. Aberrantly methylated genes present as attractive biomarkers for cancer detection and diagnosis because of their abundance in noninvasive body fluids (liquid biopsies, such as stool and serum) and have become the focus of much effort to integrate into CRC screening (Table 2).

Methylated DNA biomarkers can be detected in serum/plasma of CRC patients [92,93,115,117,120,123,135]. For example, methylated *MLH1* promoter DNA was detected in serum from microsatellite instable CRC patients [115]. A later study detected methylated *SEPT9* in serum at 72% accuracy [135]. These studies demonstrate that blood-based DNA tests have predictive power and can be adapted into commercially available clinical screening procedures.

Stool is commonly used to screen for gastrointestinal ailments, including cancer, in the form of fecal occult blood test [136]. A large number of studies verified the efficacy of detecting methylated DNA in stool to screen for early CRC [33,47,125–126,128–129,132–134]. Our laboratory completed a proof-of-concept study that detected hypermethylated *RASSF2* and *SFRP2* in CRC and gastric cancer patients' stool. The method successfully detected methylated markers in stool DNA from over half of gastric cancer patients and 75% of CRC patients [132], which is a major improvement compared with an earlier stool-based DNA study that detected methylated vimentin – an intermediate filament protein that comprises microtubules and actin microfilaments and controls cell shape, cytoplasmic integrity, and cytoskeletal stability [137] – in less than half of colon cancer patients [25]. These studies demonstrated the plausibility of using stool-based methylated DNA as noninvasive biomarkers and led to FDA approval of the multitargeted stool DNA test, ColoGuard [130]. Although these studies demonstrate the usefulness of stool-based DNA detection assays by detecting fully established cancers through noninvasive means (Figure 3), the goal of detecting precancerous polyps is, however, not achieved. Continued effort is needed to find and optimize methylated genes that can be incorporated into tests like ColoGuard that identify patients who are precancerous. Systematic and comprehensive high-throughput technologies that screen genome-wide methylation will identify which of the above-mentioned methylation markers (as well as those still unidentified) can be combined to depict a tumor's methylation fingerprint that detects and predicts invasiveness and chemotherapeutic resistance early in the disease.

Current technologies for DNA methylation analysis

Cancer screening for a single locus of methylated DNA in serum or stool may not be optimal for CRC detection. Genome-wide epigenetic technologies enable screening of large panels of methylated DNA targets where methylation profiles may distinguish which organ the primary tumor resides. Identifying the tumor origin via liquid biopsy will greatly expedite detection and grant favorable prognosis (Figure 3).

Whole-genome bisulfite sequencing (WGBS) is performed on a next-generation sequencing (NGS) platform, and evaluates CpG methylation by converting unmethylated cytosines into thymidines through bisulfite treatment followed by realignment of the sequences with a reference genome to determine methylation at single CpG resolution. To date, only a few studies have used this technology to study cancer. In medulloblastoma, novel cancer candidate genes were

Table 2. Potential noninvasive diagnostic markers for colorectal cancer.

Marker gene	Type	Source	Detected stage	Assay	Ref.
<i>ALX4</i>	Liquid biopsy	Serum	CRC	MethylLight	[114]
<i>MLH1</i>		Serum	CRC/AD	qMSP	[36]
<i>MLH1</i>		Serum	Colon	MSP	[115]
<i>HLTF, MLH1</i>		Serum	CRC	MethylLight	[116]
<i>HPP1, HPP1/TPEF, MLH1</i>		Serum	CRC	MethylLight	[36]
<i>HPP1/TPEF</i>		Serum	CRC	MethylLight	[117]
<i>HPP1, HLTF, CEA</i>		Serum	CRC	MethylLight	[118]
<i>HPP1, HLTF</i>		Serum	CRC	MethylLight	[119]
<i>NEUROG1</i>		Serum	CRC	MethylLight	[120]
<i>SEPT9</i>		Plasma	CRC	MethylLight	[121]
<i>SEPT9</i>		Plasma	CRC/polyp	MethylLight	[122]
<i>SEPT9, ALX4</i>		Plasma	CRC/polyp	MethylLight	[123]
<i>Vimentin</i>		Plasma	CRC	Methyl-BEAMing	[124]
<i>ALX4</i>	Solid biopsy	Serum/feces	CRC/AD	qMSP	[125]
<i>CDKN2A</i>		Feces	CRC	MSP	[126]
<i>CDH4, GATA5</i>		Feces	CRC	MSP	[127]
<i>GATA4</i>		Feces	CRC	MSP	[33]
<i>ITGA4</i>		Feces	AD	MSP	[128]
<i>MGMT</i>		Feces	CRC, AD	MSP	[129]
<i>NDRG4</i>		Feces	CRC	qMSP	[47]
<i>NDRG4 and BMP3</i>		Feces	CRC	ColoGuard	[130]
<i>RARB2, p16INK4a, MGMT, APC</i>		Feces	CRC	MS-MCA	[131]
<i>RASSF2 and SFRP2</i>		Feces	CRC/AD	qMSP	[132]
<i>TFPI2</i>		Feces	CRC/AD	qMSP	[133]
<i>Vimentin</i>		Feces	CRC	MSP	[134]
<i>WIF1</i>		Feces	CRC/AD	qMSP	[108]

AD: Adenoma; BEAMing: Beads, emulsion, amplification and magnetics; CRC: Colorectal cancer; MS-MCA: Methylation-specific melting curve analysis; MSP: Methylation specific PCR; qMSP: Qualitative methylation specific PCR.

identified and both messenger RNA/microRNA expressions correlated with methylation [138], and in breast cancer, methylation patterns were derived from patient blood prediagnosis to predict cancer risk [139]. However, no study has produced a comprehensive biomarker panel that both predicts and evaluates cancer. Also, to date no study has applied this technology to discover novel serum/stool biomarker panels for CRC screening. Although WGBS comprehensively analyzes all CpGs, it is not cost effective at interrogating large numbers of samples and the massive amount of data yielded is unwieldy and requires extensive expertise to interpret results.

Another high-throughput methylation screening technology that should be mentioned here, but to date has not been used for CRC biomarker discovery, couples bisulfite sequencing with restriction

enzymes to identify CpG dense regions. Reduced representation bisulfite sequencing (RRBS) interrogates the majority of promoters and repeat sequences that are normally analyzed by WGBS [18]. Therefore, the amount of sequencing required is significantly decreased, making the procedure more cost effective, requiring less expertise to interpret data, and optimal for biomarker discovery among a large number of samples. However, restriction enzyme digestion does not allow all CpGs to be analyzed, and therefore, is not fully genome-wide and comprehensive. Besides genome-wide technologies, locus-specific applications are attractive because single gene analysis is cost effective and several candidate sequences have been identified.

Quantitative methylation-specific PCR (qMSP) or MethylLight and other methods that require direct

Figure 3. Noninvasive screening for methylated DNA biomarkers. Effective methylated biomarkers will be discovered by interrogating primary cancer tissues and liquid biopsies (blood and stool) with high-throughput screening technologies.

bisulfite conversion, such as pyrosequencing, are reliable for determining DNA methylation levels and predicting CRC survival. To date, much of what is known about methylated CRC biomarkers was determined using these methods, unfortunately progress has been slow, laborious, and requiring lots of luck, while yielding only a few candidate biomarkers that still cannot detect precancerous polyps. High-throughput technologies can potentially discover thousands of candidate biomarkers that more accurately identify patients with CRC as well as those who are precancerous.

Compromising between the cost of WGBS and the limited scope of locus-specific analysis, oligonucleotide microarrays combined with CpG-specific bisulfite sequencing allow comprehensive genome-wide assessment of selected CpG dinucleotides using designed probes that target CpGs of

interest. The HumanMethylation 450 bead chip assay (Illumina) assesses 99% of all RefSeq genes and 450,000 CpGs [140,141]. This technology has the breadth and sensitivity to identify tumor type specific methylation patterns [142]. Fernandez *et al.* identified 1505 CpG sites to create cancer-specific DNA methylation fingerprints that predict tumor origin with 69% accuracy [143]. These sites included enhancers, gene promoters, and intragenic regions. Contrasting with whole genome sequencing analysis, DNA methylation arrays are low cost and time effective and allow high-throughput sampling at high resolution. The preceding techniques are ideal for profiling epigenetic fingerprints from primary tissues or biological fluids, such as blood and stool, and potentially saliva and urine. Continued research can optimize methylated DNA fingerprints to detect, predict, locate, and characterize cancers.

Chemotherapeutic demethylation & re-expression of epigenetically silenced oncogenes

After complete tumor resection of late stage tumors, CRC prognosis is poor despite recent advances in diagnostics and treatment technologies; therefore, novel avenues of anti-cancer strategies should be investigated. Epigenetic modifications are potentially reversible; therefore, methylation inhibitors are potential anticancer agents [144,145] and promoters of tumor suppressor genes are promising targets of epigenetic drug therapies.

Theoretically, re-expression of tumor suppressor genes will cause cell cycle inhibition and apoptosis in cancer cells.

DNMT inhibitors are classified into two groups: nucleoside analogs and non-nucleoside. Nucleoside analogs, such as azacytidine and decitabine (5-aza-2'-deoxycytidine), are antimetabolites that demethylate DNA, noncytotoxic at low doses, and FDA approved to treat MDS and AML [146]. The drawbacks of these compounds, including most chemotherapeutic agents, are harsh side effects and transient benefits, meaning DNA

Figure 4. DNA methylation pathways. DNMT proteins regulate cytosine methylation. DNMT3A/B are important regulators of *de novo* methylation. TET proteins demethylate DNA by converting 5-mC into 5-hmC, 5-fC, and 5-caC through a pathway of three consecutive oxidation reactions. Subsequently, 5-fC and 5-caC are recognized by TDG which activates the base excision repair pathway. 5-caC: 5-carboxycytosine; 5-fC: 5-formylcytosine; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; TDG: Thymine-DNA glycosylase.

re-methylates after drug removal. Therefore, sustaining therapeutically relevant levels of the drugs necessary for clinical benefit is difficult. For example, azacytidine is cleaved and deaminated by cytidine deaminase; however, covalent ligation of phosphate-containing dinucleotides grants aqueous solubility and resistance from degradation [145]. Yet, in either form, these drugs have not demonstrated clinical efficacy against solid tumors.

Non-nucleoside molecules have gained interest for preventing cancer because they are naturally occurring. Flavonoids are organic molecules derived from vegetables, green tea, and fruits. Green tea derived epigallocatechin-3-gallate (EGCG) is an antioxidant with preventive anticancerous properties when taken daily [147]. EGCG is one of several polyphenolic compounds that inhibit DNMT activity [148] leading to demethylation of multiple gene promoters. Methylation of *RARB* [149], *CDKN2A*, *MGMT*, and *MLH1* are significantly lower in colorectal and gastric cancer patients who routinely consume green tea [150]. Other polyphenols and isoflavones demonstrate subtle DNMT inhibition: genistein, myricetin, quercetin, hesperetin, naringenin, apigenin, luteolin, garcinol, curcumin and hydroxycinnamic acid [151]. Curcumin is an anti-inflammatory dietary polyphenol that inhibits cyclooxygenase and epigenetic enzymes such as HDAC and acetyltransferases [152,153]. Our lab identified that curcumin also modulates genome-wide DNA methylation in colon cancer cells [154]. Therefore, the chemopreventive effects of this natural compound likely function through epigenetic modifications. Non-nucleoside type inhibitors have not yet entered clinical trials, therefore further investigation is needed.

Sulforaphane is an isothiocyanate obtained by eating cruciferous vegetables such as broccoli and cabbage, and has been shown to provide anticancer benefits through regulation of epigenetic mechanisms [155]. Sulforaphane reduces global methylation by inhibiting *DNMT1* and *DNMT3A* mRNA expression [156] resulting in demethylation of CpGs in CTCF binding regions [155]. An alternative epigenetic regulating mechanism of sulforaphane is HDAC inhibition. HDACs are important for regulating protein acetylation and responding to DNA damage, where inhibition by sulforaphane induces cell cycle arrest, autophagy and apoptosis in colon cancer cells [157].

Demethylating compounds can inhibit methylating enzymes and function at various steps in the methylation process (Figure 4). Vitamin C potentially decreases CRC incidence by restricting methylation through an alternative methylation regulating mechanism. Vitamin C facilitates the enzymatic activity of TET1 [158]. Tet1–3 proteins are DNA hydroxylases that prevent DNA methylation [159] by converting 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) [160–162].

TET regulation of 5-hmC formation is critical for mesenchymal–epithelial transition [163]. A recent study showed that TET1 suppressed colon and prostate cancer invasion [72,164]. Exploitation of TET proteins to prevent tumorigenesis is emerging; targeted therapies that restore 5-hmC and protect cytosine from aberrant methylation could be new strategies for cancer treatment.

Current challenges to chemotherapeutic demethylation include nonspecificity to cancerous tissues, which become aberrantly methylated at tumor-suppressor genes through *de novo* methylation. Nonspecificity may cause adverse effects on patients' health by affecting vital cellular pathways that maintain homeostasis. However, inhibition of DNMTs and other epigenetic regulators, either by nucleoside analogs or polyphenols and other natural compounds, may be powerful cancer-preventative methods that disrupt multistage carcinogenesis by regulating multiple cellular functions, thereby mediating many anticancer pathways simultaneously.

Conclusion & future perspective

Numerous reports concerning epigenetic alterations in CRC suggest that aberrant methylation of DNA provides compelling and promising avenues that improve the sensitivity and specificity of future diagnostics and therapies. Although several studies report that solitary methylated genes are potential diagnostic and prognostic biomarkers, these findings rarely extend across cohorts of the same cancer type, and more rarely extend across different cancer types. A panel of hypermethylated DNA biomarkers potentially increases the sensitivity to detect cancer, decipher between multiple types of cancer, and better predicts tumor progression. Liquid biopsies are readily available and contain stable methylated DNA targets. Further applications of high-throughput epigenetic-screening approaches can discover appropriate combinations of blood/stool biomarkers that detect both common and less-characterized cancer types in the next 5–10 years only if certain hurdles are overcome. The major drawbacks with using these complex technologies are increased costs of high-resolution screening and limited expertise of the informatics needed to decipher data, which are difficult hurdles for a single laboratory to overcome. Until then, low-cost single loci approaches will continue to be explored by solitary laboratories or small collaborations and will unlikely yield competent biomarkers that detect and describe various malignancies from liquid biopsies. We are on the cusp of major breakthroughs, however, as this review has implied, the current paradigm of single labs attempting to identify a single or several methylated DNAs in serum or stool that discerns multiple aspects of cancer will likely be unsuccessful. A better approach is to form consortiums of laboratories so that dozens of patient cohorts can be

interrogated using high-throughput technologies, such as microarrays, RRBS and WGBS, to identify a panel of biomarkers that represent a comprehensive epigenetic profile that is interpretable by practitioners and facilitates personalized care and effective treatments.

Financial & competing interests disclosure

The present work was supported by grants R01 CA72851, CA18172, CA184792, and U01 187956 from the National

Cancer Institute, NIH, funds from the Baylor Research Institute and a pilot grant from Charles A Sammons Cancer Center. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Mechanisms of promoter methylation-mediated gene silencing

- DNA methylation is a classical mechanism of gene regulation controlled by methyltransferases and transcription factors.
- Noncoding RNAs, as well as many unknown methylating mechanisms, may be exploited for their therapeutic potential.
- *TET* genes are novel mechanisms that mediate methylation.

Factors affecting gene methylation in colorectal epithelium

- Aging, lifestyle, and inflammation alter the human methylome.
- Changes in lifestyle may reverse aberrant methylation.

Clinical significance of tumor-related gene methylation in colorectal cancer

- Abnormal DNA methylation causes tumor-suppressor gene dysregulation.
- Methylation profiling of primary cancer tissues provides information for prognosis and diagnosis.

Noninvasive methylated DNA biomarkers in colorectal cancer

- All patients benefit from simple, accurate, and painless biomarkers. Several studies report the usefulness of noninvasive cancer biomarkers.
- Noninvasive sources have not been fully explored for candidate cancer biomarkers.

Current technologies for DNA methylation analysis

- Next-generation sequencing of genome-wide methylation can identify many biomarkers that can be incorporated into cancer screening practices.
- Locus-specific analysis is an available cost-effective strategy that interrogates methylation of single genes.
- Microarray-based DNA methylation profiling is a commercially available technology that compromises between complex genome-wide next-generation sequencing and limited single-loci analysis.

Chemotherapeutic demethylation & re-expression of epigenetically silenced oncogenes

- Methyltransferase inhibiting chemicals target cancerous lesions.
- Natural compounds safely demethylate DNA through various mechanisms.

Future perspective

- Stability in tissues, blood, and stool promises methylated DNAs as effective biomarkers for colorectal cancer screening.
- Methylated DNA profiling can be integrated into conventional diagnostics to predict and personalize therapies.

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