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REVIEW

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DNA methylation biomarkers in colorectal cancer: Clinical applications for precision medicine

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Abstract

Colorectal cancer (CRC) is the second leading cause of cancer death worldwide that is attributed to gradual long-term accumulation of both genetic and epigenetic changes. To reduce the mortality rate of CRC and to improve treatment efficacy, it will be important to develop accurate noninvasive diagnostic tests for screening, acute and personalized diagnosis. Epigenetic changes such as DNA methylation play an important role in the development and progression of CRC. Over the last decade, a panel of DNA methylation markers has been reported showing a high accuracy and reproducibility in various semi-invasive or noninvasive biosamples. Research to obtain comprehensive panels of markers allowing a highly sensitive and differentiating diagnosis of CRC is ongoing. Moreover, the epigenetic alterations for cancer therapy, as a precision medicine strategy will increase their therapeutic potential over time. Here, we discuss the current state of DNA methylation-based biomarkers and their impact on CRC diagnosis. We emphasize the need to further identify and stratify methylation-biomarkers and to develop robust and effective detection methods that are applicable for a routine clinical setting of CRC diagnostics particularly at the early stage of the disease.

KEYWORDS
biomarker, colorectal cancer, diagnosis, DNA methylation, epigenetics, precision medicine

Abbreviations: AA, advanced adenoma; AZA, 5-azacytidine; CAPOX, capecitabine and oxaliplatin; ccfm, circulating cell-free methylated; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CMS, consensus molecular subtypes; CRC, colorectal cancer; DNMT, DNA methyltransferase; DNMTis, DNA methyltransferase inhibitors; FIT, fecal immunochemical test; HRM, high-resolution melting; MMR, DNA mismatch repair; MSI, microsatellite instability; OS, overall survival; PTSD, posttraumatic stress disorder; RECIST, Response Evaluation Criteria in Solid Tumors.

1 | INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy with an increasing incidence in developing countries.[1] CRC screening programs for early detection lead to a considerable decrease in incidence and mortality.[2]

Colonoscopy and the fecal immunochemical test (FIT) are widely used for CRC screening.[3] Both have their limitations. On the molecular side improved screening methods for genetic alterations (gene panels or whole-exome sequencing) involved in CRC, which can be used as diagnostic tests on tumor-derived DNA found in blood and stool have been developed.[4] Gene expression and DNA methylation analyses complement the current route for CRC molecular diagnosis.

CRCs are divided into four consensus molecular subtypes (CMS) that have distinct clinical features. The CMS1 tumors are characterized by a strong antitumor immune response and widespread promoter hypermethylation in tumor suppressor genes such as MutL homolog-1 (*MLH1*). The CMS2 tumors are defined by high activity in Wnt and MYC signaling. CMS3 tumors are identified by *KRAS*-activating mutations and metabolic dysregulation, whereas the hallmark of the CMS4 tumors is upregulation of the TGFβ pathway.[5,6]

Epigenetic modifications play a pivotal role in the pathogenesis of various cancers, including CRC,[7] with DNA methylation being the most broadly considered epigenetic alteration. In general, genome-wide hypomethylation is often observed in cancer cells accompanied by local, specific changes (usually hypermethylation) at regulatory sequences like promoters or enhancers. These changes which often coincide with altered gene expression serve as cancer-specific biomarkers.[8]

A number of studies have elucidated important links between epigenetic abnormalities and genetic alterations in CRC. For example, microsatellite instability (MSI), a hallmark of CRC that arises from a deficiency in the DNA mismatch repair (MMR) system, is caused both by genetic mutations of the MMR genes and by *MLH1* gene silencing due to hypermethylation of their promoter(s). Moreover, global hypomethylation can result in chromosomal instability (CIN) in CRC.[9] Most cases of CRC develop from CIN, whereas only 10% to 15% of CRCs result from defects in the MMR system.[10]

Epigenetic differences among patients can affect the fate of CRCs. A comprehensive understanding of the effects of epigenetic alterations on CRC pathophysiology will open the doors for discovery of new diagnostic and prognostic biomarkers and therapeutic targets.

A simultaneous quantification of both genetic and epigenetic changes in colon epithelial cells can assist to estimate the abundance of CRC subtypes and thus enable clinicians to design an effective personalized therapy.[11,12] For example, coincidence of *KRAS* mutations and p16 promoter methylation are associated with a metastatic phenotype,[11] and tumors with *BRAF* mutations that contain six hypermethylated genes with tumor-suppressive functions may enable patients to use re-expression treatment with demethylating agents such as 5-azacitidine in order to delay cancer progression.[12] However, the majority of demethylating agents is not specific and might result in widespread hypomethylation and production of unwanted effects such as induction of oncogenes.[13] Hence, the drugs that inhibit DNA methylation must be administrated at doses possessing antitumor potential with reduced side effects.

In this review, we first highlight clinical applications of DNA methylation biomarkers that have the greatest potential for improving diagnosis, prognosis and prediction of treatment responses in CRC. Finally, we discuss the expanding evidence that supports DNA methylation changes as a potential therapeutic target for the development of CRC epigenetic therapies, which could form the basis of future precision medicine strategies.

1.1 | DNA methylation in carcinogenesis

Genome-wide hypomethylation is one of the first abnormal methylation events that changes the methylation signature of cells in many tumors including CRC. Global and progressive DNA hypomethylation, especially in repetitive sequences, is frequently observed in the genome of human tumors[14] and has been implicated in different stages, from the adenomatous polyp stage to adenocarcinomas and metastases.[9] Hypomethylation of long interspersed nucleotide element-1 (LINE-1) sequences can lead to reactivation and mobilization of these retrotransposable elements, and thus can be linked to genomic instabilities such as MSI and high CpG island methylator phenotype (CIMP-high) status.[15,16]

Promoter hypermethylation is associated with the silencing of tumor suppressor genes[17] and subsequent oncogenesis by influencing pivotal cellular pathways like DNA repair, programmed cell death, cell cycle regulation, angiogenesis and tumor invasion.[14,18] Some well-known tumor suppressor genes silenced by CpG island promoter hypermethylation are *RB1* in glioma, CRC and head and neck cancer; *hMLH1* in CRC and endometrial carcinomas; *VHL* in renal cancer and *CDKN2A* in CRC, acute leukemia and lung cancer.[19,20]

Some generally well-known methylated and silenced genes in CRC are vimentin (*VIM*), cadherin-1 (*CDH1*), *MLH1*, TIMP metallopeptidase inhibitor-3 (*TIMP3*), secreted frizzled related protein-1 (*SFRP1*) and hypermethylated in cancer-1 (*HIC1*).[17]

In recent years, methylated syndecan-2 (m*SDC2*) and methylated *SEPT9* (m*SEPT9*) have been introduced as potential biomarkers for noninvasive diagnosis of CRC.[21-24]

1.2 | Sources of alterations in DNA methylation

DNA methylation alterations appear to be caused by aging, chronic inflammation and a diet that lacks vitamins and other nutrients.[25] Abnormal DNA methylation occurs in the time of the transformation of chronic inflammation into CRC. These alterations accelerate transformation and furthermore lead to tumor progression and metastasis through activating various signaling pathways that are implicated in carcinogenesis.[26] When chronic inflammation continues, immune surveillance mechanisms fail and then the inhibition of antitumor immune responses leads to tumor development.[27] Patients who suffer from chronic bowel inflammation have a much higher risk for CRC.[26]

There is a correlation between increased age and DNA hypermethylation.[28] DNA methylation is an endogenous generator of

mutations that increases DNA damage, which results in cell apoptosis, aging and death.[29] Several age-related DNA methylation alterations may occur in genes that potentially participate in the transition from adenoma to CRC. Among age-dependent genes, numerous tumor suppressor genes (estrogen receptor-1 [*ESR1*], *SFRP1* and *SYNE1*) appear to be hypermethylated.[10,30]

Besides age, metabolic and nutritional factors are known to influence epigenetic mechanisms. The B-vitamins have a critical role in DNA metabolism and are required for the synthesis of methyl donors, methionine and S-adenosylmethionine, which are used to maintain DNA methylation.[31,32]

One-carbon metabolism is a crucial biochemical pathway that includes several dietary factors such as the B2, B6, B9 and B12 vitamins that influence local and genome-wide DNA methylation levels.[31] Researchers investigated the association between dietary intake of folate, alcohol and B-vitamins with global LINE-1 methylation levels in patients with CRC. Using the genome-wide methylation levels at LINE-1 as a global DNA methylation indicator, they noted that LINE-1 hypomethylation was more common among patients with low folate levels and excessive alcohol consumption.[33] Recently, Boughanem et al reported high methylation of LINE-1 in CRC patients with low B12 vitamin levels.[34] Also, evolutionary younger retroviral sequences like Alu repeats contribute to the epigenetic landscape of colon cancer cells.[35] Alu elements tend to locate near regulatory sequences with a functional impact on chromatin structure and gene regulation.[36]

As an additional environmental factor, stress exposure has been analyzed and some studies suggest an association between behavior problems such as posttraumatic stress disorder (PTSD), depression, posttraumatic growth and resilience with DNA methylation. In these studies, DNA methylation changes were observed in immune, dopamine, hypothalamic-pituitary-adrenal axis and inflammatory genes.[37,38] How and if stress-induced DNA methylation changes also contribute to cancer development remains unclear.

1.3 | Clinical applications of DNA methylation biomarkers in CRC

Common diagnosis, prognosis and therapeutic predictive tests for CRC have many drawbacks and limitations, including low sensitivity and specificity combined with high invasive potential and increased costs. Therefore, the development and detection of novel biomarkers for the management of CRC are of utmost importance.[39,40]

Aberrant methylation of genes can reflect tumor stage and be useful as diagnostic or prognostic biomarker.[41] Below we highlight some of the most extensively studied examples.

1.4 | DNA methylation biomarkers for the diagnosis of CRC

New screening tests with high sensitivity and specificity along with less invasive and cost-effective approaches have good potential for replacing conventional methods for early cancer diagnosis.[40] There is an urgent need to identify reliable noninvasive biomarkers for cancer screening due to the high recurrence rate in CRC patients.[42] Currently, sensitive and semi-invasive tests have emerged to detect aberrant DNA methylation in CRC and adenoma.[40]

DNA methylation modifications mainly occur in the early stages of cancer and may be used as early risk indicators for cancer.[43] Until now, aberrant methylation of various genes has been investigated in the tissues and body fluids of CRC patients that may serve as potential biomarkers in CRC screening (Table 1, Figure 1). The list includes genes related to the Wnt signaling pathway (adenomatous polyposis coli [*APC*], *AXIN2*, Dickkopf Wnt signaling pathway inhibitor-1 [*DKK1*], *SFRP1*, secreted frizzled related protein-2 [*SFRP2*], Wnt family member-5A [*WNT5A*]); DNA repair processes (O6-methylguanine-DNA methyltransferase [*MGMT*], MutS homolog-2 [*MSH2*]); cell cycle regulation (*CDKN2A* and cyclin-dependent kinase inhibitor-2B [*CDKN2B*]); and the RAS signaling cascade (Ras association domain family member-1, isoform-A [*RASSF1A*] and Ras association domain family member-1, isoform-B [*RASSF1B*]).[67] Promoter hypermethylation at *CDH1* is associated with CRC progression and their detection represents a potential diagnostic tool for this malignancy.[66]

DNA methylation biomarkers like N-Myc, downstream-regulated gene-4 (*NDRG4*) and bone morphogenetic protein-3 (*BMP3*), both tumor suppressor genes, can be used for early CRC screening.[68] A positive association of *NDRG4* methylation with CRC and adenoma was reported in several studies, with 27.8% to 81% sensitivity and 78.1% to 91.7% specificity in various sample types.[53,54] Promoter methylation analysis of the *BMP3* gene in blood, stool and tissue samples showed 33.3% to 56.66% sensitivity and 85% to 94% specificity for CRC and advanced adenoma (AA) diagnosis in multiple studies.[53,57,58] In addition, three DNA methylation markers (*NDRG4*, *BMP3* and *SEPT9*) have been included in FDA-approved tests for CRC screening.[69]

One of the most frequently studied noninvasive DNA methylation biomarkers for CRC diagnosis is promoter methylation of the *SEPT9* gene in plasma. SEPT9, a GTP-binding protein, is implicated in actin dynamics, cytoskeletal remodeling, vesicle trafficking and exocytosis. Multiple studies have investigated the diagnostic accuracy of this biomarker in large cohorts of CRC patients, and the results indicated a sensitivity of 48.2% to 95.6% and specificity of 79.1% to 99%.[21,24,61] Epi proColon 2.0 CE, plasma-based test, detects *SEPT9* methylation with a sensitivity and a specificity of 68.2% to 81.0% and 87.4% to 99.0%, respectively.[70,71] The Epi proColon (*SEPT9*) test is approved by the FDA.[69]

As mentioned earlier, m*SDC2* is a potential biomarker under consideration for diagnosis of CRC.[22,23] The results of two studies showed a sensitivity of 87% to 87.2% and 95.2% to 100% specificity for m*SDC2* in screening for CRC in serum or plasma.[55,56] *SDC2* encodes an integral membrane protein that participates in the processes of cell proliferation, migration and cell-matrix interactions via binding of its receptor to extracellular matrix (ECM) proteins.[55] The *SDC2* promoter is hypermethylated in blood and fecal samples of CRC patients.[55,72] Additionally, m*SDC2* showed a higher sensitivity for detecting AA compared to m*SEPT9*.[73]

TABLE 1 Overview of promising epigenetic DNA methylation biomarkers used in diagnosis of CRC and adenomas

Gene	Test	Sample type	Method	% Sensitivity (range)	% Specificity (range)	FDA approval (date)	References
CDKN2A	CRC	Stool	MSP	40	96.8	No	44
		Blood	MSP	27	100	No	45
	Adenoma	Stool	MSP	24-31	84-96.8	No	44,46
MGMT	CRC	Stool	MSP	48.1-51.7	100	No	47,48
	Adenoma	Stool	MSP	28.6-48	73-100	No	46-48
MLH1	CRC	Blood	MSP, Fluorescence based real time PCR assay	18.4-42.9	97.6-100	No	49,50
TFPI2	CRC	Tissue	qMSP	61	84	No	51
		Stool	qMSP	93.4	94.3	No	52
	Adenoma	Stool	qMSP	81.3	94.3	No	52
NDRG4	CRC	Stool	MSP, nested MSP	68.8-76.2	80-89.1	No	53,54
		Blood	Nested MSP	54.8	78.1	No	54
		Tissue	Nested MSP	81	91.7	No	54
		Urine	Nested MSP	76.2	89.1	No	54
	Adenoma	Stool	MSP	27.8	80	No	53
SDC2	CRC	Blood	Pyrosequencing, qMSP, CpG DNA microarray analysis	87-87.2	95.2-100	No	55,56
BMP3	Adenoma	Blood	BS-HRM	40	94	No	57
		Stool	MSP	33.3	85	No	53
	CRC	Blood	MSP, BS-HRM	40	30	No	57
		Stool	MSP	40	85	No	53
		Tissue	MSP	56.66	93.3	No	58
PRIMA1	CRC	Blood	Pyrosequencing, qMSP, BS-PCR	80.9	27	No	56,59
	Adenoma	Blood	Pyrosequencing, qMSP	70.3	27	No	
SEPT9	CRC	Stool	Microarray DNA methylation assay, Pyrosequencing	20	NA	No	60
		Blood	qMSP, Epi proColon® 2.0 CE assay	48.2-95.6	79.1-99	Yes (2016)	24,61
	Adenoma	Stool	qMSP	83.3	92.1	No	62
		Blood	qMSP	11.2-22	78.8-91.5	No	24
SFRP2	CRC	Stool	MSP	60	92	No	63
		Blood	Pyrosequencing, qMSP, MSP	63.8-66-9	97.3-100	No	56
	Adenoma	Stool	MSP	27.8-76	55-100	No	53
		Blood	Pyrosequencing, qMSP, MSP	6.4-81.1	73-100	No	56
Vimentin	CRC	Stool	MSP, qMSP	38.3-81	82-95	No	64
		Blood	MSP	59	93	No	64
	Adenoma	Stool	qMSP, MS-HRM.	33-83	93-100	No	65
CDH1	CRC	Tissue	MSP	87	74.2	No	66

Abbreviations: BS-HRM, bisulfite specific high resolution melting analysis; BS-PCR, bisulfite-sequencing PCR; MS-HRM, methylation-sensitive high-resolution melting; MSP, methylation-specific PCR; qMSP, quantitative methylation-specific PCR.

In several studies, the aberrant methylation of *SEPT9* and *SDC2* was investigated in stool and plasma of CRC patients.[74,75] The ColoDefense test is a new blood-based methylation assay for early CRC screening, which combines methylation of two biomarkers (*SEPT9* and *SDC2*) in a single reaction to improve the detection rate for early-stage CRC and AA. Recent studies show that ColoDefense is a potent, suitable and effective approach with high sensitivity and specificity for early screening of CRC. The combined detection of m*SEPT9* and m*SDC2* in serum has a high potential for semi-invasive screening of CRC. Moreover, the stool ColoDefense test showed higher sensitivity than either m*SEPT9* or m*SDC2* alone in detecting AA and CRC.[22,74,75]

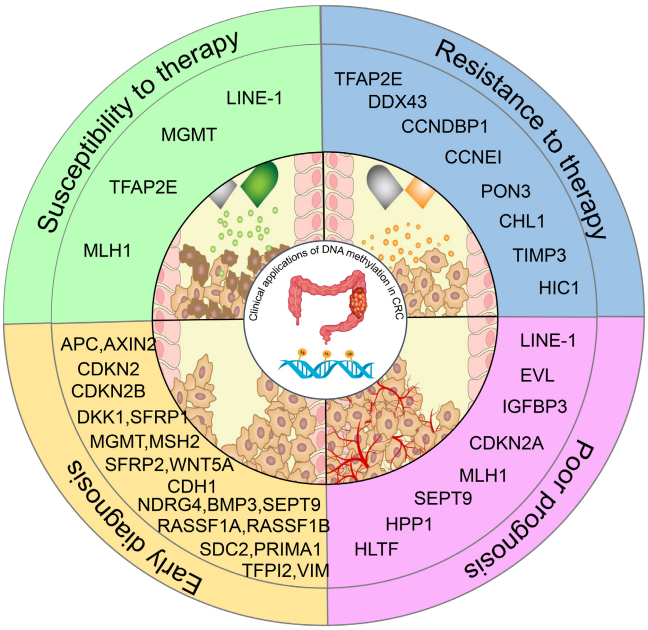

FIGURE 1 DNA methylation-based biomarkers in colorectal carcinogenesis. Aberrant methylation of various genes that have the potential to be biomarkers for early diagnosis, prognosis and prediction of response to therapy in colorectal cancer (CRC). *APC*, adenomatous polyposis coli; *BMP3*, bone morphogenetic protein-3; *CCNDBP1*, cyclin D1 binding protein-1; *CCNE1*, cyclin-E1; *CDH1*, cadherin-1; *CDKN2A*, cyclin-dependent kinase inhibitor-2A; *CDKN2B*, cyclin-dependent kinase inhibitor-2B; *CHL1*, cell adhesion molecule-L1 like; *DDX43*, DEAD-box helicase-43; *DKK1*, Dickkopf Wnt signaling pathway inhibitor-1; *EVL*, Enah/Vasp-like; *HIC1*, hypermethylated in cancer-1; *HLTF*, helicase-like transcription factor; *HPP1*, hyperpigmentation, progressive, 1; *IGFBP3*, insulin-like growth factor binding protein 3; *LINE-1*, long interspersed nucleotide element-1; *MGMT*, methylguanine methyltransferase; *MLH1*, MutL homolog-1; *MSH2*, MutS homolog-2; *NDRG4*, NDRG family member-4; *PON3*, paraoxonase-3; *PRIMA1*, proline rich membrane anchor-1; *RASSF1A*, Ras association domain family member-1, isoform-A; *RASSF1B*, Ras association domain family member-1, isoform-B; *SDC2*, Syndecan-2; *SEPT9*, septin-9; *SFRP1*, secreted frizzled related protein-1; *SFRP2*, secreted frizzled related protein-2; *TFAP2E*, transcription factor AP-2 epsilon; *TFPI2*, tissue factor pathway inhibitor-2; *TIMP3*, TIMP metallopeptidase inhibitor-3; *VIM*, vimentin; *WNT5A*, Wnt family member-5A

Additional hypermethylated biomarkers have been reported in published studies. Proline-rich membrane anchor-1 (*PRIMA1*) hypermethylation encodes a membrane protein that has been reported in plasma samples of colorectal adenoma and cancer patients, especially those with advanced stage disease.[56,59] Tissue factor pathway inhibitor-2 (TFPI2) is a serine proteinase inhibitor that prevents tumor invasion. Hypermethylation of this gene in some studies with high sensitivity and specificity in different samples is associated with the risk of colon cancer and adenoma.[51,52] Another methylation biomarker that has been frequently described for noninvasive diagnosis of CRC is the *VIM* gene. *VIM* encodes an intermediate filament protein that, combined with actin-based microfilaments and microtubules, makes the cytoskeleton. The diagnostic accuracy of this biomarker might be higher in fecal than blood samples. For instance, one study reported 90.7% sensitivity and 72.5% specificity for *VIM* methylation in plasma isolated from 193 CRC patients.[76] By contrast, in stool samples, the sensitivity was 60% with a specificity of 100%, with a high positive predictive value (100%) for 79 patients.[77] According to the evidence-based practical application of *VIM* methylation in stool samples, this biomarker has also been commercialized as the ColoSure test (LabCorp).[78,79] However, according to Table 2, the ColoSure test has not received FDA clearance or approval for CRC screening.

Parallel testing of more than one methylation biomarker (biomarker panels) can lead to higher sensitivity and specificity. Good examples are the combined testing of *IKZF1* and *BCAT1* methylation markers,[80] the TriMeth (*C9orf50*, *KCNQ5* and *CLIP4*) test developed by Jensen et al, and the biomarker panel established by Rademakers et al[81] with the combination of three methylation markers (*GDNF*, *SNAP91* and *NDRG4*) and FIT for early detection of CRC.[82]

As CRC develops from a combination of genetic mutations and epigenetic changes, studies have also investigated to use a combination of types of molecular biomarkers to improve diagnostic accuracy for colorectal polyps and CRC.[9] A number of promising combinations of methylation biomarkers have been recommended for improved diagnostic performance (Table 2). Cologuard is the first multitarget stool DNA test approved by the FDA for CRC screening. This test assesses *KRAS* mutations and *BMP3* and *NDRG4* methylation levels in addition to an immunoassay for human hemoglobin.[68,84] It has a reported sensitivity and specificity of 92.3% and 86.6%, respectively.[68,85] More importantly, the results of one study showed that Cologuard was significantly more sensitive than FIT (42.4% vs 23.8%) for detecting AA or sessile serrated lesions that were ≥1 cm in size.[86] Taken together, using epigenetic or the combination of genetic and epigenetic biomarker panels seems to be a very promising strategy for early CRC diagnosis.

1.5 | DNA methylation biomarkers for prognosis and prediction of CRC

On the genome-wide level, various studies have shown that hypomethylation in retrotransposable elements, such as LINE-1, is associated with poor survival in CRC[87,88] (Table 3, Figure 1). A notable association has been found between hypermethylation of several well-known tumor suppressor genes and poor clinical outcomes. For instance, *CDKN2A* hypermethylation is correlated with poor prognosis and an increased risk of recurrence and metastasis in patients with CRC[90,91] (Table 3, Figure 1).

Simultaneous DNA hypermethylation of a subset of genes that are key components of the ECM remodeling pathway is significantly associated with poor survival in CRC patients. The ECM remodeling pathway is involved in maintenance of the cytoskeleton, cell spreading, lamellipodia formation and regulation of IGF1 activity. Enah/Vasp-like (*EVL*) and insulin like growth factor binding protein-3 (*IGFBP3*) are novel methylation biomarkers and changes in

TABLE 2 Panels of methylation biomarkers for diagnosis and prognosis of colorectal cancer and adenomas

Diagnostic							
Genes	**Test**	**Sample**	**Method**	**% Sensitivity**	**% Specificity**	**FDA approval (date)**	**References**
Cologuard (NDRG4, BMP3, mutation KRAS, hemoglobin)	CRC	Stool	Multitarget stool DNA assay	92.3-98	86.6-90	Yes (2014)	68
	Adenoma		Multitarget stool DNA assay	42.4	86.6		68
ColoSure	CRC	Stool	qMSP	72-77	83-94	No	78
SEPT9, SDC2	CRC	Blood	qMSP	86.5-88.9	92.1-92.8	No	74,75
	Adenoma		qMSP	47.8	92.8		75
IKZF1, BCAT1	CRC	Blood	qMSP	56-79	94-95	No	80
	Adenoma		qMSP	6-7	94-95		
TriMeth (C9orf50, KCNQ5, CLIP4)	CRC	Blood	Methylation-specific ddPCR	85	99	No	81
GDNF, SNAP91, NDRG4 and the fecal immunochemical test (FIT)	CRC	Tissue and stool	qMSP	86	96	No	82

Prognostic						
Genes	**Test**	**Sample**	**Method**	**Finding/management**	**FDA approval (date)**	**Reference**
SEPT9, CEA	CRC	Blood	qMSP	Higher mortality rate	No	83

Abbreviations: ddPCR, droplet digital PCR; qMSP, quantitative methylation-specific PCR.

TABLE 3 Overview of promising epigenetic DNA methylation biomarkers used in prognosis and therapeutic prediction of CRC

Gene	Test	Sample type	Method	Finding/management	References
MLH1	CRC	Tissue	MSP	Non-LS MLH1 hypermethylated patients are older and female	89
CDKN2A	CRC	Tissue	qMSP, MSP, MethyLight assay, pyrosequencing	Shorter survival increased risk of recurrence and metastasis	90,91
		Blood	RTQ-MSP	Shorter survival	92
EVL	CRC	Tissue	MSP, qMSP	Shorter survival	93
HLTF	CRC	Blood	qMSP	Shorter survival in stage IV and tumor recurrence	94
HPP1	CRC	Blood	qMSP	Shorter survival in stage IV and tumor recurrence	94,95
IGFBP3	CRC	Tissue	MSP, qMSP	Shorter survival	93
SEPT9	CRC	Blood	Epi proColon® 2.0 CE assay, qMSP	Reduced overall survival and disease-free survival	96
LINE-1	CRC	Tissue	Pyrosequencing, MSP, qMSP	Shorter survival	87
		Blood	AQAMA assay	Disease progression	88
LINE-1	CRC	Tissue	qMSP	Response to fluoropyrimidines	97
MGMT	CRC	Tissue	MSP	Response to fluoropyrimidines and dacarbazine	98,99
TFAP2E	CRC	Tissue	MS-HRM, pyrosequencing	Nonresponsiveness to chemotherapy	100,101
			MS-HRM	Good clinical outcomes in stage II/III with 5FU-based chemotherapy	102
HIC1	CRC	Tissue	Sequencing	Responsiveness to therapy	103
TIMP3	CRC	Tissue	MS-MLPA	Responsiveness to therapy	104

Abbreviations: AQAMA, absolute quantitative analysis of methylated alleles; MS-HRM, methylation-sensitive high-resolution melting; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; qMSP, quantitative methylation-specific PCR; RTQ-MSP, real-time quantitative methylation-specific PCR.

methylation levels of their promoters are associated with worse survival in CRC patients[93] (Table 3, Figure 1).

Promoter methylation of the *SEPT9* gene was reported to be one of the best prognostic biomarker candidates in different CRC patient cohorts ($P = .058$-$.036$)[96] (Table 3, Figure 1). It has been conducted a study with 117 patients and reported that m*SEPT9* alone was more sensitive than positive ELISA signaling of carcinoembryonic antigen (CEA) (73.2% vs 48.2%; $P < .001$) for detecting CRC; notably, the combination of m*SEPT9* and CEA was more accurate than either CEA or m*SEPT9* alone ($P = .009$ and $P = .532$, respectively).[83] Detection of increased m*SEPT9* methylation levels may show the strongest association with poor clinical outcomes after curative resection (15.2% vs 1.8%; $P = .024$).[83]

Both the patient population and analyzed tissue type can have a major impact on epigenetic modifications used for prognostic markers, as has been reported for the methylation-induced silencing of the *MLH1* promoter and the concomitant deficient MMR (dMMR).[105]

In the past few years,[94] there was a significant hypermethylation in helicase-like transcription factor (*HLTF*) and hyperpigmentation, progressive, 1 (*HPP1*) observed in serum samples from CRC patients along with elevated lactate dehydrogenase (LDH) levels; therefore, these two genes are considered to be promising candidate biomarkers for noninvasive monitoring of CRC (Table 3, Figure 1). The results of more independent studies showed significant correlations between the methylation status of both biomarkers with advanced cancer stage, poor survival and tumor recurrence.[94,95]

Taken together, testing of more than one biomarker is more beneficial for diagnostic or prognostic analysis.[17] Researchers showed a strong correlation with hypermethylation of *VIM* and intermediate-methylation of *SFRP2* to *BRAF* and *KRAS* mutations, respectively, in patients with CRC.[106] The results of a recent study reported hypermethylation of six genes (*SFRP2*, *DKK2*, *PCDH10*, *TMEFF2*, *SFRP1*, *HS3ST2*) in patients with *BRAF* positive CRC in comparison with *BRAF* negative cases.[12]

Although the above-mentioned DNA methylation biomarkers are associated with advanced disease stages or poor outcomes in CRC patients (Table 3, Figure 1), currently, none have acquired sufficient evidence for use in routine clinical practice for CRC. Nonetheless, the methylation biomarkers presented here appear to be notable for further prospective evaluations because of promising preliminary data. The combination of genetic mutations and epigenetic aberrations, including DNA methylation, may help to identify useful diagnostic biomarkers for designing personalized strategies to improve prognosis in patients with CRC.

1.6 | DNA methylation biomarkers for prediction of CRC response to therapy

DNA methylation is proposed to be a predictive biomarker in cases where the epigenetic status is significantly linked to the probability of response to various therapeutic interventions. Some of the predictive DNA methylation-based biomarkers without clinical approval are worth to be evaluated in clinical trials. An association between LINE-1 methylation, as a therapeutic marker and survival benefit in 155 patients with stage II or III CRC who received oral FP has been reported.[97] Hypermethylation of *MGMT*, a DNA repair enzyme involved in cellular defense against mutagenesis and alkylating agents, is associated with a good prognosis in patients diagnosed with advanced-stage CRC who received 5-FU and dacarbazine, in addition to an improved response to neo-adjuvant chemoradiotherapy in patients with advanced rectal carcinoma[98,107] (Table 3).

It has been documented that *TIMP3* and *HIC1* promoter methylation occurs in several cancers, including CRC, and may predict treatment response.[103,104] It has been shown that hypermethylation of the nuclear transcription factor AP-2 epsilon (TFAP2E) is associated with good clinical outcomes in patients with stage II/III CRCs who received 5-FU chemotherapy.[102] However, a study of two cohorts of 783 CRC patients demonstrated that *TFAP2E* methylation and expression might not play a major role in predicting response to adjuvant chemotherapy in CRC patients[100] (Table 3, Figure 1). This finding shows the need for additional appropriate retrospective or prospective clinical trials to confirm these results.

dMMR is reported to be a predictive marker for the lack of efficacy of 5-FU-based adjuvant chemotherapy in CRC.[108,109] Colorectal tumors with dMMR significantly correlated with CIMP status, which may have different therapeutic implications.[110] Another study evaluated the potential of 5-azacytidine (AZA), a base-analog demethylating agent, to sensitize refractory CIMP-high patients to capecitabine and oxaliplatin (CAPOX)-based chemotherapy.[111] Despite the encouraging preclinical results, 26 patients with CRC who enrolled in the phase I/II trial showed no objective response according to the Response Evaluation Criteria in Solid Tumors (RECIST), and study endpoints were not correlated with CIMP-high status.[111]

MLH1 methylation may also predict therapeutic efficacy in different cancers because of its association with MMR status.[112,113]

Since DNA methylation-mediated silencing of genes has a fundamental role in CRC etiology, DNA methyltransferase inhibitors (DNMTis) have been recently proposed to treat patients with CRC.[114] The results of preclinical studies show that inhibition of DNMTs with two synthetic cytosine analogs, AZA and 5-aza-2′-deoxycytidine (decitabine, DAC) reduced cancer cell growth and enhanced cytotoxic chemotherapy-induced apoptosis in CRC.[115] Thus, DNMTis (5-azacitidine, DAC and zebularine) combined with standard chemotherapeutics (5-FU, irinotecan or oxaliplatin) might improve the treatment of patients with CRC.[116,117]

Baharudin et al identified several potentially important genes (DEAD-box helicase-43 [*DDX43*], cyclin-D1 binding protein-1 [*CCNDBP1*], *CCNEI*, paraoxonase-3 [*PON3*] and cell adhesion molecule-L1 like [*CHL1*]) by DNA methylation profiling in patients with recurrent CRC that could be used as potential novel therapeutic targets in chemoresistant patients.[118]

Overall, the identification of methylation biomarkers in CRC could be useful for monitoring response to therapy and for switching current therapy protocols based on the methylation phenotype of patients.

2 | ROLE OF EPIGENETIC BIOMARKERS IN PRECISION MEDICINE

The growth in our knowledge of cancer biology has shown that many malignancies, including CRC, are composed of several different molecular subtypes that may show diverse responses to therapeutic intervention. Identification of reliable and powerful molecular biomarkers that can differentiate between these different subtypes would be helpful for clinical decision-making and enable clinicians to select the most effective treatment based on a patient's molecular profile. This personalized medicine approach has the potential to improve therapeutic efficacy and minimize treatment-related toxicity.[119] From a molecular point of view, CRC can be classified into subgroups based on its global genomic status, including MSI, CIN, epigenomic status and CIMP. Both genetic and epigenetic alterations dysregulate cancer-related signaling pathways and transform normal colorectal epithelium into benign adenomas and ultimately into adenocarcinomas.[120]

The four CMS subgroups classification may assist for a subtype-based therapeutic intervention, especially in patients with metastatic CRC. The goal of a large number of studies is the identification of CMS-dependent prognostic factors that are potentially involved in CMS-based therapeutic strategies.[120] For example, Okita et al[121] reported that irinotecan-based chemotherapy was more effective in CMS4 patients (n = 50) compared to oxaliplatin. Mooi et al[122] noted that CMS2 (n = 113, HR 0.50; 95% CI 0.33-0.76) and possibly CMS3 (n = 28, HR 0.31; 95% CI 0.13-0.75) tumors preferentially benefited from the addition of bevacizumab to first-line capecitabine-based chemotherapy in patients with mCRC compared to other CMS groups.

CIMP-high colorectal tumors have distinct clinical and molecular features, including an association with female sex, proximal tumor location, poor differentiation and dMMR and *BRAF* mutations. Patients who have stage III adenocarcinoma, CIMP positive, MMR-intact tumors benefited most from the addition of irinotecan ($P = .01$) to combined fluorouracil and leucovorin chemotherapy.[123]

Two retrospective studies and a post hoc analysis of the CALGB 89803 prospective trial assessed the prognostic value of CIMP.[123-125] All three studies demonstrated that CIMP-high tumors have worse survival outcomes compared to CIMP-low tumors. However, further studies are needed because of the potential interactions between CIMP and *BRAF* mutations and MMR status. For example, the results of one study have shown that tumors with the *BRAF* V600E mutation, and are CIMP-high and MSI-positive, have a good prognosis[126] whereas MSI-negative tumors that are CIMP positive and contain the *BRAF* mutation have a poor prognosis.[127] Therefore, it is necessary to validate the independent value of CIMP before determining its benefit in the clinic setting.

Another study demonstrated that CIMP status can be considered as a useful predictive marker of survival after surgery for patients with CRC, particularly stage I/II disease ($P = .006$).[128]

DNA aneuploidy, as a marker for CIN, is associated with poor prognosis in most cases of sporadic CRC.[129] The results of two meta-analyses showed worse overall survival (OS) for patients with stages-II and III CRC that had CIN.[130,131] Furthermore, CIN can act as an independent predictor of early relapse and death in patients with stage-II CRC.[132] These findings suggest that DNA aneuploidy may be a potential predictive biomarker; nevertheless, further studies are required to validate the prognostic value of DNA aneuploidy.

Emerging data that describe the role of epigenetic changes in cancer development and progression provide the justification for pharmacological targeting or new therapeutic interventions. Although the focus on epigenetic modifications in cancer therapy, as a precision medicine strategy, is complex and requires comprehensive prospective clinical evaluations, cumulative research results in this area will increase their therapeutic potential over time.[133]

3 | SENSITIVE DETECTION OF DNA METHYLATION IN CRC

A crucial point in routine diagnostics and personalized treatment is early and sensitive detection of DNA methylation changes in tissue and, less invasive, in blood or stool. Several techniques were developed and applied in routine testing since they proved to be fast, sensitive and cost-effective. Stool is the easiest object for analysis that can be obtained. Single-, dual- and multiple-target methylation-specific qPCR approaches show high sensitivity (>95%) and specificity (>88%).[134,135] Detection of circulating cell-free methylated (ccfm) DNA in blood plasma with qPCR was shown to be highly sensitive when *SEPT9* and *SDC2* promoters were analyzed.[75,136] Coupled with single-nucleotide primer extension, the sensitivity of mSEPT9 detection can even be enhanced to the detection of two to three methylated molecules.[137] Multipanel methylation qPCR assays on ccfm DNA revealed *BCAT1*, *IKZF1* and *IRF4* as suitable and flexible in the detection of advanced adenomas and neoplasia.[138] Recently, multiplexed methylation-specific qPCR on 13 candidate loci in fresh-frozen tissue yielded 95% to 100% sensitivity and specificity, respectively.[139] When single or few samples have to be analyzed, qPCR is one of the easiest and most applied techniques for DNA methylation detection. Alternatively, methylation-specific high-resolution melting (HRM) analysis has the capacity to detect cancer-related de novo methylation down to 0.1%.[140] Besides the use of HRM for the detection of microsatellite instability,[141] it has been applied to screen genetic variations[142,143] and multiple DNA methylation markers in CRC[144,145] with similar sensitivity and specificity compared to qPCR. NGS technologies, however, offer the highest sensitivity since local deep bisulfite sequencing is able to detect DNA methylation on the single-molecule level.[146] Several potential marker genes have already been screened in different body fluids, formalin-fixed, paraffin-embedded (FFPE) and tissue.[52,147] The challenges of highly sensitive methylation detection assays lie in the avoidance of false positives due to the technical variance of bisulfite conversion[148,149] and detection of sparse methylation that can originate from sequencing background noise without relevance for the development of CRC tumors.[53]

4 | CONCLUSION

In summary, the past decade has witnessed a steep rise in interest in epigenetic research for CRC. Epigenetic changes notably play a fundamental role in all aspects of colorectal tumorigenesis, from tumor initiation to progression. Each stage of different cancer types generates a unique epigenetic signature, which makes them favorable additions to the current set of clinical detection methods. Univariate or multivariate regression analyses show that indeed numerous DNA methylation markers have significant relations to AA and CRC stages I-IV (Figure 2A,B).[69]

The use of sensitive and combined marker panels, including genetic tests, offers great new opportunities for screening and prognostic testing. However, the road from biomarker identification to clinical use is long and there are many challenges that have to be overcome.[150] At first, the confirmation of clinical outcomes in prospective trials is critical for medical professionals and regulatory agencies. In addition, it is essential to develop a commercial product that has good efficiency, reasonable cost and ease of use. One big challenge is the need to extend our knowledge on the biological context, that is, the relation of the epigenetic change to the tumor biology and hence a qualified interpretation of the results using computational support. The second challenge is the development of robust and easy to use technology platforms. Here technical barriers preclude the use of some of the epigenetic techniques, particularly advanced detection methods, including mass spectrometry or NGS. All of these issues will be addressed in the near future because of the direction taken by modern medicine toward a more personalized or precise practice. Finally, global harmonization should be established for the harmonization and regulation of medical diagnostic technologies, which would greatly support the translation of epigenetic assays into the clinic.

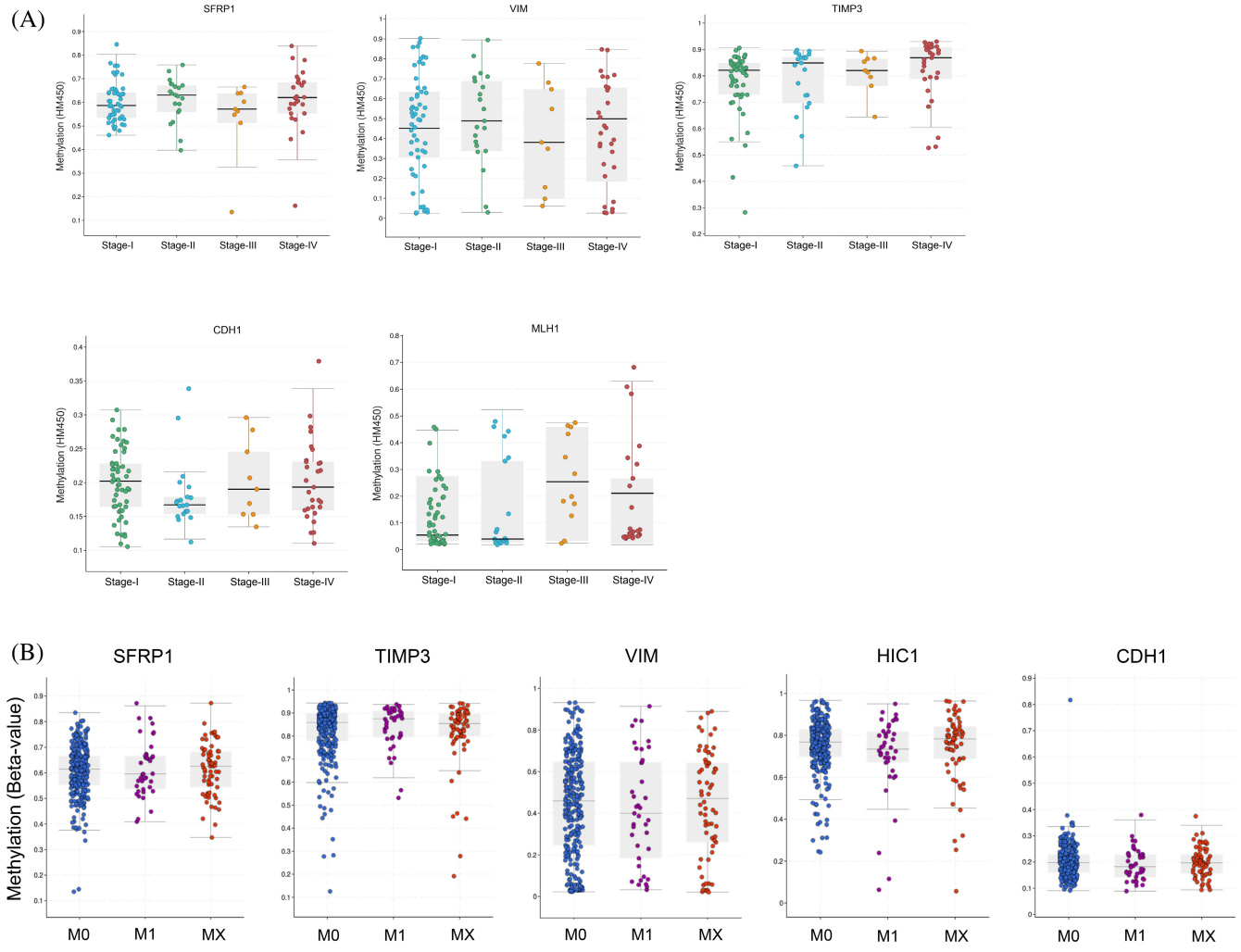

FIGURE 2 Association between hypermethylation of *VIM*, *CDH1*, *MLH1*, *TIMP3*, *SFRP1* and *HIC1* with tumor progression stages and metastasis across 640 colorectal cancer (CRC) patients from The Cancer Genome Atlas (TCGA). (A) Association between methylation of six selected genes with various stages of CRC. (B) Association between methylation and metastasis stages of CRC. *CDH1*, cadherin-1; *HIC1*, hypermethylated in cancer 1; M0, cancer has not spread to other parts of the body; M1, cancer has spread to other parts of the body; *MLH1*, MutL homolog-1; MX, metastasis occurred but cannot be measured; *SFRP1*, secreted frizzled related protein-1; *TIMP3*, TIMP metallopeptidase inhibitor-3; *VIM*, vimentin

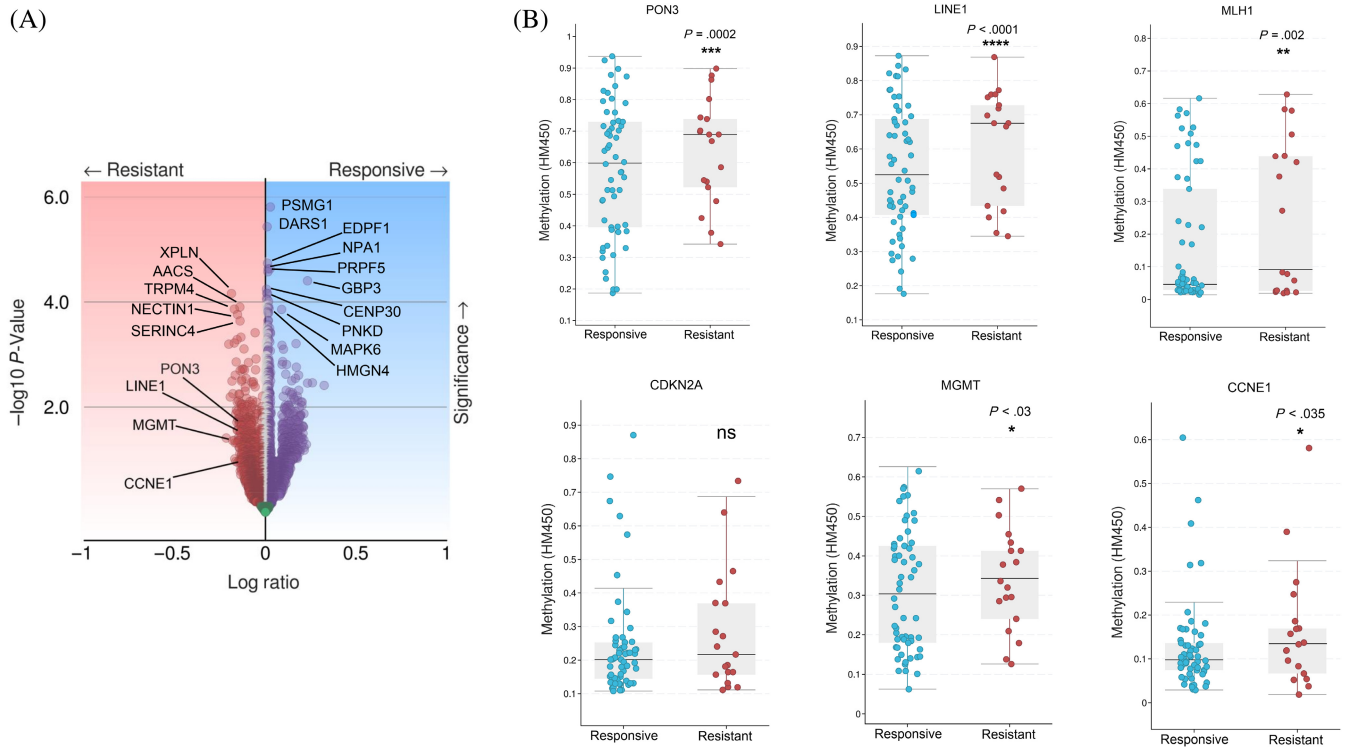

FIGURE 3 The association and enrichment analysis of methylation with drug response (n = 66) or resistance (n = 25) colorectal cancer (CRC) patients from TCGA. (A) Volcano plot presents enrichment of gene methylations in two groups, of responsive and resistant. Genes with the highest correlation are shown in the plot. (B) The box plots show a comparison between methylation of these genes across the responsive and resistant groups. *CCNE1*, cyclin-E1; *CDKN2A*, cyclin-dependent kinase inhibitor-2A; *LINE-1*, long interspersed nucleotide element-1; *MGMT*, methylguanine methyltransferase; *MLH1*, MutL homolog-1; *PON3*, paraoxonase-3

In order to facilitate widespread clinical use of epigenetic biomarkers, both biomarkers and detection methods should be standardized according to tumor type. Predictive biomarkers can reduce resistance to therapies and evaluate patient suitability for targeted therapy; in other words, "individualized biomarker-driven cancer therapy" or "personalized medicine" (Figure 3).

Therefore, it is important that these biomarkers must be noninvasive in order to increase screening acceptability and have the capability to differentiate between responders and nonresponders with relative ease and at a decreased cost. To the best of our knowledge, only m*SEPT9* made it to an FDA-approved blood-based biomarker for the diagnosis of CRC.[69] Cologuard, as the first multitarget stool DNA test, has received FDA approval for CRC screening.[68,84]

Until now, many drugs used for personalized medicine have been approved by the FDA.[151] Numerous other drugs and drug combinations that target the epigenome are currently under investigation in clinical trials. Future clinical trials should determine which medications are beneficial for patients with known tumor defects (eg, gene expression changes that block cellular differentiation).

Without doubt, a better understanding of epigenetic mechanisms and their interrelationships in terms of classifying CRC subtypes is essential for the development of novel approaches for targeted cancer therapy. Here, we emphasized the critical role of DNA methylation for CRC development, progression and prognosis. Due to the advent of personalized therapies, more intricate research is necessary to clarify the relationship of individual genetic and epigenetic alterations and consequently, provide a pathway-driven basis to select the best therapeutic strategies.

AUTHOR CONTRIBUTIONS

Conceptualization: Nayeralsadat Fatemi, Sascha Tierling, Hamid Asadzadeh Aghdaei, Mehdi Totonchi; *Investigation*: Hamidreza Aboulkheyr Es, Ehsan Nazemalhosseini Mojarad; *Supervision*: Jörn Walter, Mehdi Totonchi; *Visualization*: Nayeralsadat Fatemi, Maryam Varkiani; *Roles/Writing - original draft*: Nayeralsadat Fatemi, Sascha Tierling, Hamidreza Aboulkheyr Es, Maryam Varkiani, Ehsan Nazemalhosseini Mojarad; *Writing - review & editing*: Hamid Asadzadeh Aghdaei, Jörn Walter, Mehdi Totonchi. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding authors upon reasonable request and with the permission of the respondents.

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