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SYSTEMATIC REVIEW

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Advances in blood DNA methylation-based assay for colorectal cancer early detection: a systematic updated review

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ABSTRACT

Aim: A systematic review was conducted to summarize the methylated circulating tumor DNA (ctDNA) markers reported over the last decade for early detection of colorectal cancer (CRC) and to identify the main technical challenges that are impeding their clinical implementation.

Background: CRC is a major cause of cancer deaths worldwide, but early detection is key for successful treatment. Non-invasive methods such as methylated ctDNA testing show promise for improving detection and monitoring of CRC.

Methods: A comprehensive search was performed using Web of Science, PubMed, and Scopus up to December 30, 2023, limited to articles published in the last 10 years (after 2012), while including advanced adenoma/stage 0 or stage I/II samples in biomarker validation.

Results: After identifying 694 articles, removing duplicates and screening titles, abstracts, and full texts, a total of 62 articles were found to meet the inclusion criteria. Among the single biomarkers, MYO1-G, SEPT9, SDC2, and JAM3 revealed the highest sensitivity for polyps and stage I/II CRC. For multi-biomarkers with suitable sensitivity, combinations of SFRP1, SFRP2, SDC2, PRIMA1, or ALX4, BMP3, NPTX2, RARB, SDC2, SEPT9, VIM or ZFHX4, ZNF334, ELOVL2, UNC5C, LOC146880, SFMBT2, GFRA1 were identified for polyps and stage I/II CRC.

Conclusion: Enhancing sensitivity and specificity of molecular screening methods is crucial for improving CRC detection. Identifying a select few valuable biomarkers is key to reducing costs, despite challenges posed by low ctDNA levels in plasma, particularly in early-stage cancers.

Keywords: Colorectal cancer, Circulating tumor DNA, DNA methylation, Liquid biopsy, Cancer biomarker, Early detection

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Introduction

Despite advances in detection and treatment methods, colorectal cancer (CRC) is the second most deadly cancer and the third most common malignant tumor among men and women worldwide (1, 2). CRC survival rates are closely linked to early detection, which ranges from a 90% five-year survival rate in stage I disease to 10% five-year survival in stage IV disease (3). Approximately 60% of CRC patients at diagnosis present with either locally advanced lesions or distant metastases. This advanced stage unfortunately precludes surgical intervention, significantly impacting treatment options and prognosis (4, 5). Since developing malignant lesions from precancerous lesions (adenomas) is a long process (6), it allows CRC to be detected early by screening (7).

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Early detection of CRC leads to successful treatment, and proactive screening helps reduce its incidence.

Colonoscopy is widely considered the gold standard for diagnosing CRC, but it has limitations such as invasiveness, cost, and patient discomfort, and may not always detect early-stage lesions (8). Molecular biomarkers for CRC detection are currently limited, and non-invasive stool-based tests such as fecal occult blood testing (FOBT) and fecal immunochemical tests (FIT), as well as blood-based protein biomarkers like CEA and CA19, have shown reduced sensitivity and specificity (9). Most patients (83%) prefer blood-based tests over stool-based tests (10). Liquid biopsies, especially blood-based ones, are gaining interest and are seen as the future of cancer screening (11, 12).

The development of CRC is linked to genetic mutations, epigenetic changes, environmental factors, and non-modifiable risk factors such as age, family history, and personal medical history. Liquid biopsies can detect these cancer-related progressive build-up of genetic and epigenetic alterations in plasma DNA (13). However, the identification of mutations for early cancer diagnosis is limited by the frequency and diversity of mutations gained throughout cancer development in CRC patients (14, 15). Epigenetic alterations, particularly DNA methylation, are mostly constant, occur before gene mutations in tumor development (16), with blood/tissue paired studies in CRC showing good tissue and tumor type specificity (17). These aberrant DNA methylations, as a most prevalent epigenetic alteration, can be effectively detected in numerous types of biological samples such as blood, tissue, and stool. Thus, Methylated circulating tumor DNA (mctDNA) can be a valuable source for tumor DNA in the diagnosis, prognosis, and surveillance of tumors (18), with potential applications in therapeutic interventions (19).

Cell-free DNA (cfDNA), generally at approximately 167 bp in length, is fragmented double helix DNA found in the blood circulation. It comprises nucleic acids released into body fluids through active release, apoptosis, necrosis, or other cell death processes. CfDNA in blood circulation has a short half-life of about 15 minutes to 2.5 hours, which allows ongoing surveillance of tumors (20), where the concentration of cfDNA in plasma is 1-10 ng/mL (often <20ng/ml) in healthy individuals, mostly from the hematopoietic system (21, 22). This concentration can be higher in patients with cancer (23) and also in diseases other than cancer (20). CtDNA is a subset of cfDNA that originates from tumor cells and contains unique genetic as well as epigenetic markers of the tumor (24). Compared to cfDNA, which primarily reflects apoptotic cell debris with a characteristic length of ~150-200 bp (coinciding with nucleosome size), ctDNA exhibits substantial heterogeneity. Its fragment size often exceeds 200 bp and can even reach >1000 bp, likely due to impaired apoptotic pathways in cancer cells (25). Further, the ctDNA fraction within cfDNA varies widely between 0.05% and 93% (20, 26).

Currently, several epigenetic in vitro diagnosis (IVD) tests are being added to the market for CRC screening (Table 1). The Cologuard® (27) is the first stool DNA-based test, and the Epi proColon® (28) is the first blood DNA-based test approved by the FDA for early CRC detection. Beyond commercially available kits, researchers have reported a diverse array of methylation-specific PCR assays for CRC detection. Primarily, these encompass potential biomarkers for diagnosis, yet they also include markers aimed at evaluating tumor burden, detecting disease relapse, and estimating patient prognosis. Due to the importance and fast-growing knowledge of blood-based biomarkers, in this article, we review all investigated blood DNA methylation-based biomarkers over the last 10 years to have an updated overview of improvements and directions for research. Although CRC-related epigenetic biomarkers were reviewed in several articles with different points of view (8, 17, 29-31), we aim to gather only blood-based epigenetic biomarkers.

Methods

Search strategy

A comprehensive search up to December 30, 2023, limited to articles published in the last 10 years (after 2012), which included AA/stage 0 or stage I/II samples in biomarker validation, written in English, was performed exploiting three main electronic libraries: Web of Science® Core Collection (Clarivate Analytics, Philadelphia/London, USA/GB), PubMed® (National Library of Medicine's, Bethesda, MD, USA), and Scopus® (Elsevier, Amsterdam, NL, USA). The employed keywords were "colorectal cancer,"

Table 1. Commercially available Epigenetic IVD/RUO tests for CRC

Tests	Sample	Biomarker target	Sensitivity (%)					SPE (%)	Company
			CRC	I/II	III/IV	HGD	AA		
Cologuard (27, 68, 69)	Stool	NDRG4, BMP3, 7 KRAS mutations	92			69	42	87	Exact Sciences
Epi proColon (28, 70)	Blood	SEPT9	75–81	71-77			22	96–99	Epigenomics
Colosafe (63, 71-73)	Stool	SDC2	81- 91	87	95	80	42-58	86 - 98	Creative Biosciences
EarlyTect (74, 75)	Stool	SDC2	90	89			67	90	EarlyTect
Colodefense (45-47)	Blood	SEPT9,	89				48	93	VersaBio
	Stool	SDC2	88-92				55-67	93	
ColoSure™ (76)	Stool	Vimentin	72-77					83-94	Labcorp
ColonSecure (64)	Blood	149 markers	86					90%	-

AA: advanced adenoma, HGD: polyps with high-grade dysplasia, CRC: colorectal cancer

"colorectal neoplasms," "circulating tumor DNA," "cfDNA," "Cell-free DNA," "biomarker," "DNA methylation," "cancer screening," "cancer detection," "diagnosis," "early detection," "adenoma," "plasma," "serum," or "liquid biopsy." The investigation utilized these specific terms to navigate through the keywords, titles, and abstracts of scholarly articles. Additionally, the bibliographies of all accessed full-text articles and significant reviews were meticulously examined to uncover further relevant research. The study was conducted based on Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) (32).

Study selection

The articles were initially screened based on their titles and abstracts to eliminate any irrelevant studies. They were then categorized into three groups: include, exclude, or unclear. For the "unclear" articles, the full-text versions were examined and placed into one of the other two categories: include or exclude. All full-text manuscripts were evaluated to determine if they met the eligibility criteria, which included being written in English, focusing on human subjects, methylation testing at least one ctDNA biomarker in CRC patients, and offering a detailed description of the patients and detection rate of assays. Studies that used animal models, reviews, congress abstracts, or articles in languages other than English were excluded. If needed, the corresponding authors of the selected published reports were contacted.

Data extraction

Two reviewers independently extracted data from the included studies, collecting information such as publication year, number of patients, demographic details, and clinical and laboratory data for methylation evaluation. Duplicate cases were identified and eliminated, and the medical records from all papers were compiled and consolidated. In the event of any disagreements between the two reviewers, a third author was consulted.

Results

Study characteristics

A comprehensive literature search identified 694 articles. Following deduplication, 173 articles were screened by title and abstract, leading to the exclusion of 120. Hand-searching of full-text articles and major reviews yielded an additional 9 articles. As illustrated in Figure 1, 61 articles ultimately met the inclusion criteria and proceeded to full-text assessment.

Based on the nature of the study, the collected studies are presented in three categories. The "Genome-wide methylation studies" part discusses high throughput studies that were centered on biomarker identification, whereas the "Single methylation-based ctDNA biomarker" and "Multi-methylated ctDNA biomarker" sections deal with single or multi-biomarker validation.

Genome-wide methylation studies

Genome-wide methylation studies provide the most valuable and straightforward solution for de novo

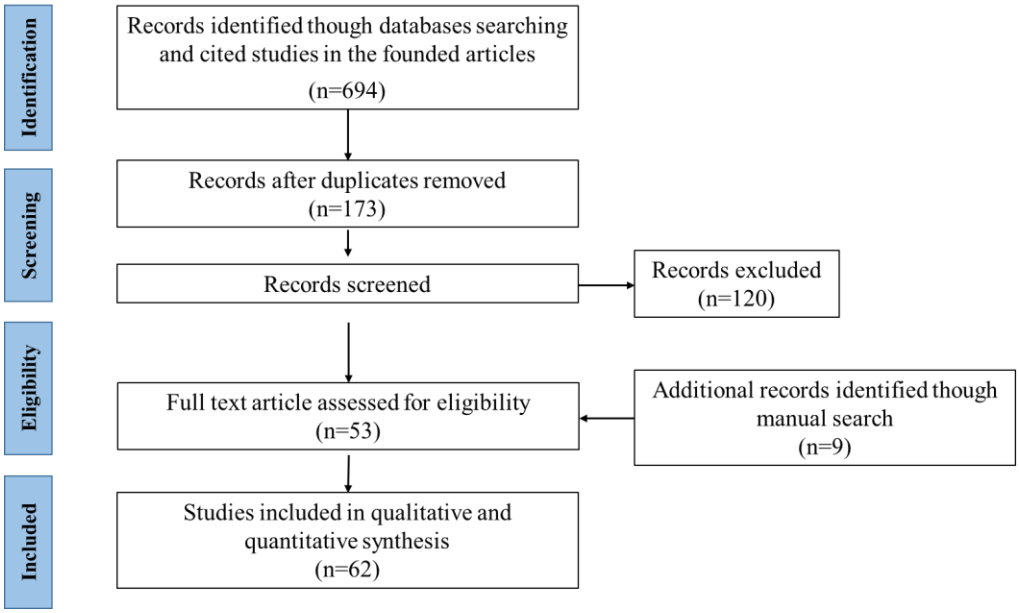

Figure 1. Study selection flow chart.

biomarker discovery. Most de novo biomarker-finding studies were accomplished on tissue, cell lines, or fecal samples (Table 2). Twenty-one studies used different sample sources and methods to discover novel methylated ctDNA (mctDNA) biomarkers in CRC for diagnosis, prognosis, and metastasis or therapy monitoring. Some of them used previously reported data from TCGA and GSE databases (15, 33-36) on cell lines and tissue samples. CpG microarray analysis is the most prevalent method for genome-wide methylation analysis. Nevertheless, recently sequencing-based methods have provided more hope to find suitable biomarkers for CRC. Methylation-specific quantitative PCR (MS-qPCR), digital PCR (dPCR), or droplet digital PCR (ddPCR) mostly have been used for the verification of selected biomarkers. Most of the studies used CRC tissues and paired matched normal tissue adjacent to the tumor (PNAT/MNAT) to discover biomarkers; however, it seems the best strategy is to simultaneously array or sequence both tissue and blood samples. Wang et al. (37) conducted a study on the overlapping markers between tissue DNA and cfDNA in advanced adenoma (AA) and early-stage CRC. They identified seven DNA methylation biomarkers that can be used to monitor the malignant progression from AA to CRC. However, it is important to note that their tissue and blood samples were not matched. Although high-throughput analysis such as simultaneously analyzing fragment length and methylation profiles gives a high AUC (0.989) with good sensitivity (96.8) and specificity (97%) in detecting CRC (38) and could provide high accuracy for early-stage CRC detection, it is expensive and computational analysis is also required. Hence, it is necessary to achieve a cost-effective set of biomarkers that possess the appropriate level of sensitivity and specificity for regular use in CRC screening and monitoring.

Single methylation-based ctDNA biomarker

Totally, 22 studies examined single methylated ctDNA biomarkers in blood-based samples (plasma, serum, or a combination of them) which included AA/stage 0 or stage I/II. SEPT9 biomarker was the most studied biomarker and methylation-specific-qPCR is the method of choice for most of the methylation analysis. Twelve studies included polyp (adenoma or advanced adenoma) samples, 9 studies included polyps and stage I-III samples, and 8 studies included stage I-III without polyps' samples (Table 3). Still, SEPT9 totally had the highest sensitivity and specificity among biomarkers (39) for CRC, while if we consider polyp and stage I/II CRC, MYO1-G (40), SEPT9 (39, 41), JAM3 (42) showed the highest sensitivity. MYO1-G revealed 74-86% sensitivity and 94% specificity in 50 patients with stage I/II CRC

(40). SEPT9 indicated 23-31% (24 patients) sensitivity for advanced adenoma, 40% (12 patients) sensitivity for polyps, and 64-88% (67 patients) sensitivity for stage I/II CRC (39, 41). JAM3 showed AUC=0.8611 (P<0.001) and interestingly methylation rate in stage I/II CRC (81.96%) was greater than in stage III/IV CRC (Table 3).

Multi-methylated ctDNA biomarker

We considered a biomarker panel any evaluation of two or more methylated genes. Totally, 17 studies investigated multitarget methylated ctDNA in blood-based samples (plasma or serum). Two studies used 32 (43) and 191 (44) CpG sites to evaluate CRC samples. The most used method was qMSP; also ddPCR, MS-HRM, and NGS were used to methylation profiles. Most studies have included mSEPT9 and/or mSDC2 markers whose combination has been produced as a ColoDefense® kit. Colodefense is a stool/blood-based DNA hypermethylation screening test for CRC with SEPT9 and SDC2 assessment that has been approved by the China Food and Drug Administration. ColoDefense® showed 89% sensitivity for CRC and 48% sensitivity for advanced adenoma with 93% specificity (45-47).

The highest sensitivity for detection of polyps (adenoma, advanced adenoma) was reported in 4 biomarkers, SFRP1, SFRP2, SDC2, and PRIMA1 (48). Bartak et al, in a small sample size with 37 advanced adenoma tissues, showed 89% sensitivity. Another study with 191 CpG sites (44) in a large sample with 182 AA revealed 79% sensitivity. For the detection of stage I/II CRC, 100% sensitivity and 78% specificity were reported for SEPT9 and SDC2 biomarkers (49). Seven biomarkers (50), ALX4, BMP3, NPTX2, RARB, SDC2, SEPT9, and VIM, showed 89% sensitivity, and another seven DNA-Methylation Marker (37) with unpublished biomarkers indicated 87% sensitivity.

Discussion

CRC has high mortality rates, underscoring the need for effective screening tools to enhance early detection and improve curability. While colonoscopy is the gold standard with sensitivity rates exceeding 95% for CRC and 88-98% for precancerous lesions (AA), its drawbacks, such as invasiveness, discomfort, sedation requirements, risks of bowel damage and infection, and cost contribute to low patient adherence. Sigmoidoscopy and CT colonography offer high sensitivity rates (95% and 90% for CRC and precancerous lesions, respectively) but also require uncomfortable bowel preparation (51, 52). Fecal tests such as FOBT, guaiac FOBT (gFOBT) and FIT have lower sensitivity rates (33–75%) and are more suitable for detecting advanced colorectal abnormalities. In addition, current tumor markers such as CEA and CA19-9 have limitations in diagnosing CRC, highlighting the need for novel, non-invasive, and highly sensitive detection methods (52). Recent progress in circulating DNA methylation analysis shows promise in identifying both CRC and precancerous lesions.

Tumor-specific methylation changes in peripheral blood mononuclear cells (PBMCs), particularly in blood leukocytes, may reflect those seen in cancerous tissues, indicating their potential as novel early cancer biomarkers. However, the clinical significance of PBMC methylation in cancer diagnosis and patient prognosis remains uncertain (53). Studies have shown that a substantial portion of plasma cfDNA comes from hematopoietic lineages, particularly leukocyte genomic DNA, in individuals with or without cancer (22). This poses a challenge as it raises the risk of false-positive results in detecting mutations and methylation markers. The high prevalence of mutations in both normal and cancerous tissue limits their usefulness as early-stage cancer biomarkers due to insufficient specificity (54, 55). However, recently, Chen et al. (2021) demonstrated that the leukocyte's gDNA will not affect the performance of the plasma methylated-ctDNA test, and methylation markers effectively distinguish CRC from benign tumors and healthy controls, while leukocyte levels of these markers lacked discriminatory power (49). They verified the performance of the ColoDefense test, consisting of methylated SEPT9 (mSEPT9) and methylated SDC2 (mSDC2), in plasma and the paired leucocyte fraction of 213 blood sample from CRC patients, adenomatous polyps' patients, hyperplastic polyp patients, and control subjects. It is also worth noting that, when compared to tissue, cfDNA has a lower methylation quantity, which may be useful in their differentiation (56).

Table 2. Biomarker discovery through genome-wide methylation studies

Year/ Ref	Serum/ Plasma Samples		Method of biomarker Discovery / Verification	Selected biomarker	AUC/ SEN, SPE%	AUC/ SEN, SPE% in stages
	CRC/ Adenoma	Normal				
		Tissue				
2013 (77)	S:32 (6I/II, 26III/IV), 26A	161	Illumina Goldengate array (Tissue, stool, Serum, I-IV) / MS-qPCR	NPY, PENK, WIF1	SEN:87, SPE:80	
2013 (78)	S:131(94I, 5II, 17III,15IV)	125	Discovery: MeDIA with microarray (Tissue I-IV, PNAT), Verification: MS-qPCR	SDC2	SEN:87, SPE:95.2	I: SEN:92
2015 (79)	P:353(42I, 140II, 108III, 63IV)		Discovery: HM450 array (Tissue/PNAT, 23 MSI/MSS CRC), Verification: MS-qPCR	AGBL4, FLI1, TWIST1	SEN:93 any gene	SEN for I & II: 90 any gene
2018 (80)	P:45		Discovery: HM450 array of tissue (18 C, 21 A, 7 N), Verification: Pyrosequencing	GRIA4, SLC8A1 and SYN3	-	-
2019 (81)	P: 256 (41I, 143II, 55III, IV17)	178	Discovery: HM450 array (Tissue) Verification: ddPCR	C9orf50, KCNQ5, CLIP4	SEN:85, SPE:99	I:80, II: 85, III: 89, IV: 88
2020 (82)	P:22	20	Discovery: SureSelectXT Methyl-Seq (Tissue/PNAT), Verification: ddPCR	CLDN1, INHBA SLC30A10	SEN:41, SPE:100	
2021 (83)	44 P	44	Discovery: Targeted array (Tissue/PNAT) Verification: MS-qPCR & MSRE-qPCR	WT1, PENK, SPARC, GDNF, TMEFF2, DCC	AUC: >0.80 any gene	-
		Plasma				
2014 (84)	P: 30 (11I, 19II)	30	Discovery: microarray (56 gene, Plasma) Verification: MS-qPCR	CYCD2, HIC, VHL	AUC: 0.93, SEN:83, SPE:94	
2018 (85)	S:20 (7I,13II), 20 AA	20	Discovery: MethylationEPIC (Plasma)	1384 CpG sites	-	-
2021 (86)	13 P	16	Discovery: MBD-seq (Plasma)	CLIP4, LONRF2, RNF217		
2021 (87)	P: 248 (66I, 86II, 62III, 34IV), 40A, 68AA	133	Discovery: Enrichment & HiSeq Sequencing of Plasma	11 markers	AUC:0.92(88-0.96) CRC	0.77 A, 0.85AA, 0.9 I
2022 (88)	P: 5 (I), 5 A		Discovery: MethylationEPIC (HM850)	1865 differently methylated CpG sites	-	-
2022 (89)	4 P (2III, 2IV)	3	Discovery: MeDIP-seq	PRDM14, RALYL, ELMOD1, TMEM132E	-	-
2023 (44)	P: 590, 182 AA	366	Discovery: targeted bisulfite sequencing (ColonES assay)	191 regions	AA SEN: 79, CRC SEN: 87, SPE: 88	0.903AA, 0.937 CRC
		Bioinformatics				
2018 (34)	P: 182	50	Discovery: TCGA and GEO cell/tissue data (HM450 array), Verification: dPCR	EYA4, GRIA4, ITGA4, MAP3K14-AS1, MSC	AUC: 0.86	-
2020 (15)	P: 801	1021	Discovery: TCGA and GSE data Verification: ddPCR	cg10673833	AUC:0.90 SEN:90, SPE: 87	-
2020 (35)	P:117 (17I,24II,33III,23IV)	60	Discovery: TCGA and GEO data Verification: ddPCR	FAM123A, GLI3, PPP1R16B, SLIT3, TMEM90B	SEN:58, SPE:95	SEN:50 I-III, 96 IV, SPE: 95
2022 (36)	47 (7III, 30IV, 6), 41AA	81	Discovery: TCGA and GEO tissue data Verification: MS-qPCR	LINC00473	AUC; CRC:0.88, AA: 0.84	
		Cell line				
2014 (90, 91)	P: 120 (12I, 30II, 12III, 66IV)	96	Discovery: MeDIP (cell lines) Verification: MS-PCR, pyrosequencing	PPP1R3C, EFHD1	SEN:90, SPE:64	
2017 (92)	P: 95 (10I, 22II, 48III, 15IV)	47	Discovery: MeDIP-seq in NCM460 cell line	CBS	-	-
		Tissue, Plasma				
2022 (37)	P: 218 (43I, 56II, 50III, 69IV), 88AA		Discovery: AnchorIRIS™ sequencing (Tissue, Plsama)	ZFHX4, ZNF334, ELOVL2, UNC5C, LOC146880, SFMBT2, GFRA1	AUC: 0.92, SEN:90, SPE:90 CRC VS AA	

CRC: Colorectal cancer; NA: Not ascertained; P/MNAT: paired,matched normal tissue adjacent to the tumor; MS-RE: methylation-sensitive restriction enzyme–based; MeDIP-seq: immunoprecipitation coupled with high-through-put sequencing; P: plasma; S: serum; SEN: sensitivity, MBD-seq: methyl-CpG-binding domain sequencing, MeDIA: methylated DNA isolation assay; SPE: specificity,

Table 3. Individual biomarker studies

	Year/ref	marker	Serum/ Plasma Samples			Method	Polyp			Stage I/II			Stage III/IV			Total		
			CRC	Polyp	Normal		SPE %	SEN %	AUC	SPE %	SEN %	AUC	SPE %	SEN%	AUC	SEN %	SPE %	AUC
1	2022 (36)	LINC00473	P:47	AA:41	81	qMSP ddPCR			0,84									
2	2022 (93)	RASSF1A	P: 92 (15I, 24II, 22III, 31IV)	67 CRP	-				0.75			I(0.83) II(0.87)			III(0.87) IV(0.86)			
3	2022 (94)	SEPT9	EORC:27(4I,1II,5III,12IV)		87											90.8	96.3	
4	2021 (95)	SHISA3	P: 30 (8I, 10II, 10III, 2IV)	-	9	BSP	-	-	-	-	I(4.78) II(5.96)	-	-	III(7.02) IV(5.2)	-	-	-	0.50
5	2021 (40)	MYO1-G	P: 305 (3I, 32II, 85III, 185IV)	-	307	ddPCR	-	-	-	95.4	I(85.7) II(74.4)	-	95.4	III(83.2) IV(86.8)	-	84.3	95.4	0.94
6	2021 (41)	SEPT9	P: 53 (14I,16II,9III,14IV)	48AA, 30A	48	qMSP		88.2		83.3	84.9							
7	2020 (39)	SEPT9	P: 90 (18I, 27II, 26III, 10IV)	13AA	81	qMSP	AA (96.3)	AA (30.8)	-	-	I (77.8) II (85.2)	-	-	III(92.3) IV(80.0)	-	85.6	90.1	0.88
8	2020 (96)	NEUROG1	S:16 (6I, 4II, 6III)	89AA, 17A	33	qMSP	-	AA (32.8)	-	-	-	-	-	-	-	33.33	90.6	-
9	2019 (42)	JAM3	P:18 (1I, 4II, 7III, 3IV,3)		18	qMSP				-	I/II (81.96)			III/IV (73.36)		61		
10	2019 (97)	SFRP2	S: 62 (13I,27II,17III,5IV)	7AA	55	qMSP	AA (87.3)	AA (42.9)	-	-	I (46.2) II (74.1)	-	-	III(70.6) IV(100)	-	69.4	87.3	0.82
11	2019 (98)	RUNX3	S:85 (9I, 39II, 34III, 3IV)	40A	40	qMSP	17.5	-	-	-	-	-	-	-	-	-	82.5	60
12	2019 (98)	SFRP1	S:85 (9I, 39II, 34III, 3IV)	40A	40	qMSP	30	-	-	-	-	-	-	-	-	-	70	77.6
13	2018 (99)	BMP3	P:50	40A	50	qMSP										40	94	
14	2018 (100)	MGMT	S: 30 (4I, 17II, 5III, 1IV)		40	MSP											90	
15	2017 (101)	SEPT9	P:85	364A, 216 AA	324	qMSP		A (38.7), AA (47.0-62.5)										
16	2016 (102)	SST	P: 165 (26I, 62II, 62III, 15IV)	-		qMSP												
17	2015 (103)	NDRG4	P: 154 (43I, 44II, 46III, 21IV)	_	444	qMSP	-	-	-	95	I (16) II (11)	-	95	III (35) IV (62)	-	27	95	0.61
18	2015 (103)	FOXE1	P: 154 (43I, 44II, 46III, 21IV)	_	444	qMSP	-	-	-	93	I (35) II (43)	-	93	III (50) IV (67)	-	46	93	0.70
19	2015 (103)	SYNE1	P: 154 (43I, 44II, 46III, 21IV)	_	444	qMSP	-	-	-	96	I (28) II (52)	-	96	III (76) IV (47)	-	47	96	0.72
20	2015 (103)	GATA5	P: 154 (43I, 44II, 46III, 21IV)	_	444	qMSP	-	-	-	99	I (14) II (9)	-	99	III (48) IV (18)	-	18	99	0.59

Continuous																		
21	2014 (104)	SEPT9	P: 34 (6I, 11II, 11III, 5IV)	26A	24	qMSP	-	30.8	-	-	-	-	-	-	-	88.2	-	-
22	2014 (105)	VIM	S: 242 (7 0, 36I, 73II, 74III, 49IV)		25	qMSP	-	0 (57.1)	-	-	I (30.6) II (28.8)	-	-	III(35.1) IV(32.7)	-	32.6	-	-
23	2014 (106)	SEPT9	P:44 (39 I-III, 5 IV)	621A A	444	qMSP	-	AA (22)	-	-	I/II (64)	-	-	III(64) IV(100)	-	68	78.8	-
24	2014 (107)	CAHM	P: 73 (12I, 21II, 23III, 12IV)	73A	74	qMSP	-	4	-	-	I (42) II (52)	-	-	III(52) IV(75)	-	55	93	-
25	2014 (108)	SEPT9	P: 53 (22I, 14II, 12III, 5IV)	209A 314A A	1457	qMSP	-	A(7.7) AA(9.6)	-	-	I (36.4) II (57.1)	-	-	III(58.3) IV(80.0)	-	50.9	91.5	-
26	2013 (109)	PCDH10	S:63(12I,26II, 17III, 8IV)			qMSP	-	-	-	-	62.7	-	-	-	-			

CRC: Colorectal cancer; NA: Not ascertained; P: plasma; S: serum; SEN: sensitivity, SPE: specificity; A: Adenoma; AA: Advanced adenoma; MSP: Methylation-specific PCR; qMSP: Methylation-specific qPCR, CRP: colorectal polyp, BSP: bisulfite sequencing PCR, EOCRC:early-onset colorectal cancer

Table 4. Multi-biomarker studies

	Year/ref	Marker	Serum/ Plasma Samples			Method	Polyp			Stage I/II			Stage III/IV			Total		
			CRC	Polyp	Normal		SEN %	SPE %	AUC	SEN %	SPE %	AUC	SEN %	SPE %	AUC	AUC	SPE%	SEN%
1	2023 (110)	SDC2, NPY, IKZF1, SEPT9	P: 124 (5 0, 36I, 34II, 45III, 3IV)	137AA	164	ddPCR	AA (7)	_	_	_	_	_	_	_	_	_	92	44
2	2023 (111)	GALNT9/UPF3A		27	21	NGS	78.6			I (87.5) II (100)			III(100) IV(33.3)				100	78.8
3	2023 (44)	191 CpG sites	590	182AA	366	NGS	AA (79.0)	_	AA (0.90)	I (85.1) II (79.3)	_	_	IV(91.8)	_	_	0.93	88.1	86.6
4	2022 (37)	Seven DNA-Methylation Marker	P: 218 (43I, 56II, 50III, 69IV)	88AA	_	NGS	_	AA (89.66)		I (87.5) II (93.75)	_	_	III(88) IV(92.8)	_	_	0.86	97	
5	2022 (43)	32 CpG sites	P: 20		4	NGS	_	_	_	_	_	_	_	_	_		92	85
6	2022 (112)	FBN1, SPG20	P:62	8	50	MS-HRM	_	_	_	_	_	_	_	_	_	0.94	97	91.1
7	2021 (83)	35 CpG sites		16	40	MSRE-qPCR	63.0	88.0	0.80									
8	2021 (49)	SEPT9, SDC2	P: 91 (4 0, 9I, 31II, 29III, 4IV)	49A, 27AA	38	qMSP	0 (50)	_		I (100) II (76.9)	_		III(85.7) IV(100)	_		0.97	86.8	85.7
9	2021 (113)	C9orf50, TWIST1, KCNJ12, ZNF132	P:35	2A, 22AA	32	qMSP										0.91	97	80
10	2021 (114)	SEPT9, SDC2, BCAT1	P: 104	83A,47 AA	60	qMSP	Polyps (4.4)	_	_	_	_	_	_	_	_	0.91	96.9	82.7

Continuous																		
11	2018 (115)	Uc160, Uc283 and Uc346	P: 50	59A	40	qMSP	_	_	_	_	_	_	_	_	_	0.63	74.3	45
12	2017 (50)	ALX4, BMP3, NPTX2, RARB, SDC2, SEPT9, VIM	P:193	33A	102	qMSP				88.7	73.5	0.85	_	_	_	0.86	72.5	90.7
13	2017 (48)	SFRP1, SFRP2, SDC2, PRIMA1	P: 7	37A	47	qMSP	89.2	86.5	0.93	_	_	_	_	_	_	0.97	97.3	91.5
14	2015 (116)	BCAT1, IKZF1	P:129 (29I, 42II, 40III, 16IV)	346A, 338AA	1291	qMSP	AA (6)	_		I /II (56)	_		79	_			94	66
15	2015 (103)	SYNE1 and FOXE1	P:66 (27I, 15II, 20III, 4IV)	_	240	qMSP	_	_	_	I (37) II (87)	_		III (55) IV (100)	_			91	58
16	2015 (117)	GATA5, SFRP2	P: 57	30A	47	MSP	26.77	91.49	_	_	_	_	_	_	_	_	91.49	42.86
17	2013 (118)	TAC1, SEPT9	S:26(I)		26	qMSP	_	_	_	I (73.1)	I (92.3)	I (82.1)	_	_	_	_	_	_

CRC: Colorectal cancer; NA: Not ascertained; P: plasma; S: serum; SEN: sensitivity, SPE: specificity; A: Adenoma; AA: Advanced adenoma; MSP: Methylation-specific PCR; qMSP: Methylation-specific qPCR , MethylLight Droplet Digital PCR (ML-ddPCR), Methylation-Sensitive Restriction Enzyme (MSRE)

Compared to plasma, serum demonstrates a marked increase in the total quantity of cfDNA and displays significantly higher integrity. This observation suggests the presence of contaminating genomic DNA (gDNA) potentially introduced during serum separation via clotting processes. Thus, plasma is favored for cfDNA analyses since serum cfDNA has elevated gDNA (57). Despite variations in ctDNA abundance, several studies demonstrate equivalent sensitivity for detecting KRAS, TP53, BRAF, and SMAD4 mutations in both plasma and serum of breast cancer patients (58).

In ctDNA assays, plasma is the preferred biofluid over serum due to reduced contamination with non-tumor cfDNA. Serum preparation involves blood clotting, which induces leukocyte lysis and release of their cfDNA. This non-tumor cfDNA dilutes ctDNA, particularly those with low allele fraction mutations, potentially hindering their detection and compromising assay sensitivity (59). Therefore, current National Cancer Institute (NCI) Biorepositories and Biospecimen Research Branch Biospecimen Evidence-Based Practices (BEBP) guidelines for cfDNA analysis in biospecimens recommend shorter durations of pre-analytical storage at room temperature. Specifically, they suggest a maximum of 2–4 hours for EDTA tubes and up to 3 days for preservative tubes before initiating plasma isolation and subsequent storage at −80 °C (60).

Bisulfite sequencing (BS-seq) has been widely regarded as the gold standard for quantifying DNA methylation at a base-level resolution. MS-qPCR is the most commonly employed method, followed by MS-PCR (Tables 3 and 4). Methods for profiling methylation at specific genomic locations have progressed to encompass a broader genomic scope through the use of high-throughput sequencing or array-based approaches. This expansion enables a more comprehensive analysis of the genome and the discovery of aberrations that had not been detected before (61) (Table 2). Several detection methods, including ddPCR and next-generation sequencing (NGS), have expanded the options for detecting genetic alterations in trace amounts of cfDNA. However, the sensitivity and specificity of the markers used remain critical factors for accurate detection, particularly in early cancer screening tests. NGS technologies, capable of local deep BS-seq, offer the highest sensitivity for detecting DNA methylation at the single-molecule level; however, when analyzing a limited number of samples, qPCR remains the simplest and most frequently utilized method for DNA methylation detection (20).

There is a limited report of paired evaluation of stool/plasma samples in patients. Meanwhile, patients greatly preferred noninvasive testing, and most of them (83%) preferred a blood-based test over a stool-based test (10). However, normally stool samples showed higher sensitivity in comparison to plasma samples (44). The mSEPT9 showed higher sensitivity in stool samples vs. plasma samples while having a similar specificity (39). Similarly, mSFRP2 demonstrated greater sensitivity in tissue and stool samples compared to plasma samples. However, the methylation of SFRP2 in serum revealed markedly higher specificity for distinguishing CRCs from benign adenomas, as opposed to the methylation levels of SFRP2 found in tumor and fecal DNA (62). The methylation test for SDC2 in stool samples outperformed the mSEPT9 blood test in the detection of nonmetastatic CRC and adenomas (63). Epi proColon, with its improved edition Epi proColon 2.0, was developed using the methylation status of SEPT9 in plasma ctDNA and received FDA approval (28). However, the U.S. Preventive Services Task Force (USPSTF) does not endorse it as an initial screening approach due to its low sensitivity for CRC (48%) including early-stage CRC (35%) and AA (11.2%). Fortunately, a more advanced formulation of this biomarker, Epi proColon 2.0, has been recently introduced, exhibiting enhanced sensitivity and specificity. This development has heightened expectations for the discovery of an optimal blood-based biomarker. Colodefense (45-47) with detection of SEPT9, and SDC2 biomarkers, and ColonSecure (64) with 149 biomarkers are other kits introduced for blood-based CRC screening. Present commercial blood-based tests have a low sensitivity for AA ranging from 22% (Epi proColon) to 48% (Colodefense) and for I/II stage ranging within 71-77% (Epi proColon) (Table 1).

SEPT9 and Syndecan-2 (SDC2) are the two most studied biomarkers in CRC detection. Here we discuss these two biomarkers specifically. MSEPT9 methylation in plasma/serum demonstrated variable sensitivity (47-87%) and high specificity (89-98%) across diverse studies. While sensitivity increased with advanced CRC stages (reaching 100% in some stage IV

reports), it remained suboptimal in the early stages (I-II) (17). Furthermore, mSEPT9's sensitivity for detecting adenomas and polyps is suboptimal in most studies, ranging from 8 to 40%. Notably, combining FOBT and mSEPT9 assessment achieved a remarkable 100% sensitivity for stage I CRC identification (65). While SDC2 methylation shows promise as a biomarker for early CRC detection, its stage specificity appears limited. Assay sensitivity is demonstrably higher for advanced (stage III/IV) disease compared to early (stage I/II) stages, suggesting a potential need for further optimization for improved sensitivity in early detection applications (8). It seems co-analyzing of SEPT9 and SDC2 showed a superior detection rate for stage I/II (49). Another single biomarkers with high sensitivity for polyp and stage I/II CRC include MYO1-G (40), SEPT9 (39, 41), and JAM3 (42) showed the highest sensitivity. Multi-biomarkers with suitable sensitivity for polyp and stage I/II CRC include SFRP1, SFRP2, SDC2, PRIMA1 combination (48), 191 CpG sites (44), ALX4, BMP3, NPTX2, RARB, SDC2, SEPT9, VIM combination (50), and another seven markers (37).

Logically, the detection rate of CRC will grow with increasing the number of biomarkers for analysis. However, there is a discrepancy within studies (Table 1 and 2) which may be related to sample size, heterogeneity of samples, and even the location of CpG island in biomarkers (66). Although the studies and reported biomarkers showed the invaluable potential of blood-based methylation assays for CRC early detection even before dysplasia, identifying potential biomarkers and combinational strategy is an urgent need today.

Conclusion and future perspective

Compared to immunoaffinity tests such as FIT, PCR-based DNA tests offer superior sensitivity for detecting molecular markers, particularly in early-stage CRC. Despite the higher costs associated with these tests, their increased sensitivity may outweigh this factor due to the significant potential for early diagnosis to save lives and reduce costs. In this study, we presented promising biomarkers such as MYO1-G and JAM3, in addition to SEPT9 and SDC2. Multi-biomarker assays, while offering increased sensitivity, may also lead to higher costs. Combinations of biomarkers such as SFRP1, SFRP2, SDC2, PRIMA1, or ALX4, BMP3, NPTX2, RARB, SDC2, SEPT9, VIM, or ZFHX4, ZNF334, ELOVL2, UNC5C, LOC146880, SFMBT2, and GFRA1 have been identified for polyps and stage I/II CRC. However, these molecular screening methods still require enhancements in sensitivity and specificity to maximize their clinical utility.

Early tumor detection using ctDNA methylation faces critical limitations. Low ctDNA abundance in plasma, particularly in early-stage cancers, hinders efficient capture for methylation analysis. Innovations such as Droplet digital PCR or deep sequencing methods utilizing larger volumes of plasma may help overcome this challenge.

Despite numerous studies indicating tumor-specific methylation changes, their validity remains uncertain. Most of these alterations are limited to individual studies lacking independent sample validation. So only a small fraction has advanced through clinical trials and commercialization for CRC detection. Another limitation of current research is the small number of patients included, hindering the discovery of clinically significant biomarker candidates. Genome-wide methylome profiling offers a powerful solution, providing comprehensive and reproducible data to unlock the true potential of methylation biomarkers. Future large-scale studies or the integration of existing methylome-level data will be necessary to identify biomarkers that are reliable enough for clinical application.

Conflict of interests

The authors declare no conflict of interest.

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