World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i15.4276

World J Gastroenterol 2014 April 21; 20(15): 4276-4287
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Advances in epigenetic biomarker research in colorectal cancer

Xi Wang, Ye-Ye Kuang, Xiao-Tong Hu

Xi Wang, Ye-Ye Kuang, Xiao-Tong Hu, Biomedical Research Center, Sir Run Run Shaw Hospital, Zhejiang University and Key Laboratory of Biotherapy of Zhejiang Province, Hangzhou 310016, Zhejiang Province, China
Author contributions: Wang X and Kuang YY drafted the manuscript; Hu XT approved the final version for publication.
Supported by National Natural Science Foundation of China, No. 81071651 and No. 81372622; the Program for Zhejiang Leading Team of ST innovation, No. 2012R10046-03; Major State Basic Research Development Program, No. 2010CB834303; National High Technology Research and Development Program of China, No. 2012AA02A601; Major Projects in Zhejiang Province, No. 2012C13014-1; and the Fundamental Research Funds for the Central Universities, No. 2012FZA7020
Correspondence to: Xiao-Tong Hu, MD, PhD, Biomedical Research Center, Sir Run Run Shaw Hospital, Zhejiang University and Key Laboratory of Biotherapy of Zhejiang Province, Qing Chun East Road 3, Hangzhou 310016, Zhejiang Province, China. hxt_hangzhou@sina.com
Telephone: +86-571-86006363 Fax: +86-571-86006363
Received: September 23, 2013 Revised: November 5, 2013
Accepted: January 14, 2014
Published online: April 21, 2014

Abstract

Colorectal cancer (CRC) causes approximately 600000 deaths annually and is the third leading cause of cancer mortality worldwide. Despite significant advancements in treatment options, CRC patient survival is still poor owing to a lack of effective tools for early diagnosis and a limited capacity for optimal therapeutic decision making. Since there exists a need to find new biomarkers to improve diagnosis of CRC, the research on epigenetic biomarkers for molecular diagnostics encourages the translation of this field from the bench to clinical practice. Epigenetic alterations are thought to hold great promise as tumor biomarkers. In this review, we will primarily focus on recent advances in the study of epigenetic biomarkers for colorectal cancer and discuss epigenetic biomarkers, including DNA methylation, microRNA expression and histone modification, in cancer tissue, stool, plasma, serum, cell lines and xenografts. These studies have improved the chances that epigenetic biomarkers will find a place in the clinical practices of screening, early diagnosis, prognosis, therapy choice and recurrence surveillance for CRC patients. However, these studies have typically been small in size, and evaluation at a larger scale of well-controlled randomized clinical trials is the next step that is necessary to increase the quality of epigenetic biomarkers and ensure their widespread clinical use.

Key words: Colorectal cancer; Epigenetic biomarker; DNA methylation; MicroRNA biomarker; Histone modification

Core tip: Epigenetic biomarkers, including DNA methylation, microRNA expression and histone modification, may have the potential for the screening, diagnosis, prognosis and recurrence surveillance in colorectal cancer patients.

Wang X, Kuang YY, Hu XT. Advances in epigenetic biomarker research in colorectal cancer. *World J Gastroenterol* 2014; 20(15): 4276-4287 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i15/4276.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i15.4276

INTRODUCTION

Colorectal cancer (CRC) is one of the most widespread cancers in the world, accounting for over 1 million new diagnoses each year and over half of a million deaths[1,2]. Among all CRC cases, approximately 95% are adenocarcinoma. Less common types include lymphoma and

squamous cell carcinoma. CRC patients are characterized by a lack of clinical manifestations until the late stages of cancer, leading to poor prognosis and a high mortality rate. Adenomas are the primary precursor lesion of colon cancer and often develop into colorectal carcinomas, but the process is slow, localized and asymptomatic, which is the primary factor contributing to late diagnosis. At the time of primary diagnosis, 80% of the patients are offered resection and potentially are cured by that. However, 40%-45% of these patients experience a later recurrence and was therefore not cured by resection[3]. Therefore, the identification of useful screening tools for CRC is a high priority. Currently, the detection of trace blood in stool using the fecal occult blood test (FOBT) and subsequent internal imaging of the colon by flexible sigmoidoscopy or colonoscopy represent the gold standard for CRC detection. Although widespread, these techniques suffer from several shortcomings. For example, the FOBT lacks specificity, often needs to be repeated, and is easily interfered by the contents of the bowel. Colonoscopy, on the other hand, is invasive, expensive, and has a high risk of complications, which often leads to poor patient compliance. As a result, the identification of biomarkers that are simple, noninvasive, cost-efficient and reasonably sensitive/specific is urgently needed. Over the past decade, the rapidly expanding field of epigenetics has shown great promise for the detection of CRC at earlier stages and the identification of resectable CRC lesions prior to metastasis, thereby providing patients with the highest chance of survival.

Epigenetic alterations are widely known to play an important role in tumorigenesis and are prevalent in CRC. Epigenetic changes in colorectal tumor tissues and CRC cell lines have been widely reported, and a substantial amount of information has been accumulated[4,5]. These alterations include aberrant DNA methylation of promoter CpG islands, changes in microRNA (miRNA) expression profiles and various histone modifications. The exploration of epigenetic biomarkers in cancer for clinical use is a relatively new but rapidly developing field. Applications include screening, diagnosis, classification, surveillance and targeted therapies. If epigenetic factors are to be effective biomarkers in clinical practice, they must be detectable by noninvasive means and outperform the current gold standard. It should be emphasized that sample collection methods are a crucial factor. For example, miRNAs extracted from tissues should be evaluated separately from miRNAs isolated from serum and stool because their clinical potential is quite different. Serum and stool biomarkers are ideal for patient screening, but biomarkers from postoperative tissue may be more effective for prognosis, including the prediction of mean survival, resectability of the primary tumor and the administration of targeted therapies.

DNA METHYLATION

In vitro and preclinical studies

Generally speaking, *in vitro* studies are the first step in the discovery of new epigenetic biomarkers. Researchers often compare profiles of CRC cell lines with normal colorectal cells and then compile a list of candidate biomarkers for further study. Similarly, identifying valuable prediction biomarkers in CRC patients often begins with preclinical studies using xenograft tumors, which allow one to observe tumor growth and how it responds to different therapies. Nevertheless, a significant shortcoming of this approach is that the tumor and vasculature are of mouse origin rather than human. Studying tumors in a different growth environment makes it difficult to explain the results accurately and translate them into clinical application. However, *in vitro* and preclinical studies are still the foundations on which most clinical studies are built.

In the human genome, DNA methylation typically occurs on the cytosine of the sequence 5'-CpG-3', which is found in promoter regions of approximately 70% of genes[6]. In this biochemical process, a methyl group ($-CH_3$) is added to cytosine nucleotides by a DNA methyltransferase (DNMT). A large body of evidence has demonstrated that promoter hypermethylation is associated with gene silencing, while hypomethylation results in gene-product upregulation. In this section, *in vitro* studies will be discussed first, followed by clinical studies that utilize blood or stool to identify DNA methylation in CRC patients.

Khamas *et al*[7] conducted a genome-wide screen of 15 CRC cell lines and 23 paired tumor and normal samples from CRC patients to identify a set of methylation-silenced genes in CRC. Gene expression studies were then used to confirm whether the methylated genes were really regulated by their methylation status. The results of this study revealed that 139 genes showed greater than 1.5-fold up-regulation in at least one 5-aza-2'-deoxycytidine-treated cell line and no less than a 1.2-fold change in other treated CRC cell lines. Among them, eight genes, *DCAF4L1*, *DDX43*, *ICAM1*, *MSX1*, *PGF*, *PTPRO*, *ZFP42* and the cancer-germline antigen families, had previously been reported to be up-regulated by demethylation in CRC and were thus excluded from the analysis. Twenty genes with poor annotation, 20 genes located on the X chromosome, 16 genes with duplicated probes, two genes with no CpG islands, 8 genes with unknown function, 23 without a relevant function in tumorigenesis and 22 genes with potential oncogenic activity were also excluded, leaving 20 candidates (*CAMK2B*, *CHAC1*, *THSD1*, *CSTA*, *COL1A1*, *GADD45B*, *DMRTB1*, *COL6A1*, *GAS5*, *GPRC5A*, *GPSM1*, *KLHL35*, *LTBP2*, *NAA11*, *RBP4*, *SEMA7A*, *SYCP3*, *TBRG1*, *TNFSF9* and *TXNIP*) that had not been previously reported to be affected by epigenetic mechanisms in CRC. Therefore, from the 54613 genes analyzed, a much smaller set of genes was isolated as potential biomarkers for CRC.

In this study, two genes, *THSD1* and *GADD45B*, were selected for further analysis. *THSD1* methylation appeared to have the potential for diagnostic, prognostic or therapeutic use. Thrombospondin type-1 domain-containing protein 1 (THSD1) is located in a region that is strongly associated with the progression of colorectal

Table 1 Biomarkers of DNA methylation in blood of colorectal cancer patients, *n* (%)

Markers	Sensitivity	Specificity	Ref.
APC	3 (6)	0 (100)	[20]
hMLH1	21 (43)	1 (98)	[20]
HLTF	17 (34)	1 (98)	[20]
HLTF	22 (21)	0 (100)	[21]
ALX4	25 (83)	9 (70)	[22]
TMEFF2	87 (65)	56 (69)	[23]
NGFR	68 (51)	29 (84)	[23]
9-Sep	92 (69)	25 (86)	[23]
9-Sep	90 (72)	19 (90)	[24]
9-Sep	24 (72)	3 (90)	[25]
9-Sep	18 (60)	5 (89)	[26]
9-Sep	45 (90)	11 (89)	[27]
NEUROG1	14 (52)-stage Ⅰ 45 (64)-stage Ⅱ	4 (91)	[28]
SFRP2	113 (67)	4 (94)	[29]
CDKN2A/P16	12 (71)	0 (100)	[30]
RUNX3	11 (65)	0 (100)	[30]
TPEF/HPP1	13 (13)	0 (100)	[21]

APC: Adenomatosis polyposis coli; hMLH1: Homo mutL homolog 1; HLTF: Helicase-like transcription factor; ALX4: ALX homeobox 4; TMEFF2: Transmembrane protein with EGF-like and two follistatin-like domains 2; NGFR: Nerve growth factor receptor; NEUROG1: Neurogenin 1; SFRP2: Secreted frizzled-related protein 2; CDKN2A: Cyclin-dependent kinase inhibitor 2A; RUNX3: Runt-related transcription factor 3; HPP1: Hyperpigmentation, progressive, 1; TPEF: Transmembrane protein endothalial factor.

adenoma to carcinoma and encodes a transmembrane molecule containing a thrombospondin type 1 repeat that might be involved in cell adhesion and angiogenesis. High THSD1 expression positively correlated with better distant metastasis survival in breast cancer. Therefore, its loss may be associated with metastatic tumor spread. Additionally, as one of the consensus radiation-response genes in primary human fibroblasts, THSD1 may play a role in radiation response in cancer stem cell. Moreover, a recent study has shown that THSD1 was expressed in CRC classified as D in Duke's classification scheme for CRC and thus may be relevant to tumor progression[8]. GADD45B functions as a tumor suppressor in many cancers, can inhibit cell proliferation at different stages and induce cell apoptosis, but its function in CRC is unknown.

In addition, Schuebel *et al*[9] described another genome-wide, expression array-based approach for the identification of genes silenced by promoter hypermethylation in human CRC, and approximately 500 hypermethylated genes were identified. They analyzed the top-tier hypermethylome of each cell line (HCT116 and SW480) and then made a comparison of hypermethylation frequencies in cell lines, normal human tissues and human tumor samples. They found that *BOLL*, *DKK3*, *CABYR*, *EFEMP1*, *GNB4*, *GSTM3*, *FOXL2*, *HOXD1*, *JPH3*, *NEF3*, *NEURL*, *PPP1R14A*, *RAB32*, *TLR2*, *SALLA*, *TP53AP1* and *ZFP42* were hypermethylated and underexpressed in both CRC cell lines and in colon cancers, but not in normal tissues. These genes possess great promise as useful biomarkers for molecular diagnostics, early detection and CRC therapy. Recently, Yi *et al*[10] also reported that hypermethylation of promoter DNA in the *FBN2* and *TCERG1L* genes might provide excellent biomarkers for early detection of CRC. Both genes showed a high frequency of methylation in colon cancer cell lines, adenomas and carcinomas.

In addition, methylation of the *hMLH1*, *p16INK4A*, *APC*, *MGMT*, *sFRP1*, *GATA-5*, *sFRP4*, *sFRP5*, *GATA-4*, *B4GALT1 TFPI2*, *SOX17* and *TMEM25* genes has been described in several studies[11-17]. These genes are hypermethylated and downregulated in CRC and thus may serve as excellent candidate biomarkers. In addition, insulin-like growth factor-binding protein 3 and Enah/Vasp-like have been validated as prognostic biomarkers for CRC and found to be useful in stratifying high-risk CRC patients who would benefit from adjuvant chemotherapy[18]. PPP2R2B was also found to be hypermethylated in CRC and was connected to therapeutic resistance[19]. These genes could serve as candidate biomarkers for prognosis. However, clinical studies are required to confirm these results, and it remains to be seen if these alterations can be detected in blood or stool.

Biomarkers of DNA methylation in blood

Biomarkers detected in patient blood samples would provide the most practical screening tool for CRC because of the ease with which these samples can be acquired. It has been well documented that genetic material can shed from tumor cells, and aberrant DNA methylation can be specifically quantified in blood despite the large amounts of normal DNA in circulation. Bisulfite treatment and methylation specific polymerase chain reaction (PCR) are the two most commonly used techniques. A blood biomarker with a high sensitivity and specificity for CRC can not only be used to segregate high-risk patients for further clinical tests but also be an excellent tool for monitoring CRC recurrence in patients who have undergone tumor resection (Table 1)[20-30].

The *SEPT9* gene, encoding a guanosine triphosphate enzyme involved in cytokinesis and cell cycle control, has been reported to be associated with several cancers. The v2 region of the Septin 9 (SEPT9) promoter has been shown to be methylated in CRC tissue compared with normal colonic mucosa. Using highly sensitive real-time PCR assays, methylated SEPT9 was first detected in the plasma of CRC patients with an overall sensitivity of 72% and a specificity of 90%[24]. Significant validation has been performed for this methylation biomarker, and Warren *et al*[27] have confirmed a sensitivity of up to 90% and a specificity of up to 88% for SEPT9. Based on these results, *SEPT9* methylation appears to have the highest probability of correctly distinguishing between the blood of cancerous and non-cancerous persons for CRC detection. Currently, two CRC detection kits using plasma *SEPT9* methylation analysis are marketed for clinical application. Combining SEPT9 with other methylation biomarkers would improve the detection rate[31]. Further studies are needed to compare these panels and kits and discover their advantages and limitations. Ultimately, the

most effective ones should be chosen for clinical use.

Other genes, such as *APC, hMLH1, ALX4, TMEFF2, NGFR, NEUROG1, SFRP2, CDKN2A/P16, TPEF/HPP1* and *RUNX3*, have also emerged as serum methylation markers for CRC, with sensitivities ranging from 6% to 83% and specificities ranging from 69% to 100% (Table 1). Among them, *ALX4, TMEFF2* and *NEUROG1* showed better performance relative to the others, and the use of these markers in combination can improve detection accuracy[25,31].

In addition to the successful identification of DNA methylation-based blood biomarkers, it is important to find genes that have prognostic value in the blood of patients with CRC. Methylation of helicase-like transcription factor (HLTF) has shown a strong correlation with tumor size, metastatic disease and tumor stage and is also associated with an increased risk of disease recurrence in CRC patients. Therefore, the methylation of this gene can serve as an independent biomarker for the identification of CRC with an increased risk of death. These results indicate that detection of HLTF methylation in the blood of CRC patients has the potential as a pretherapeutic predictor of patient outcome[32]. Deafness, autosomal dominant 5 (DFNA5) is another candidate biomarker for the noninvasive screening and monitoring of CRC. *DFNA5* methylation has been observed in DNA from the peripheral blood (PB) of CRC patients at a high frequency (48% or 12/25) relative to healthy controls (only 12% or 3/25). Moreover, the methylation of DFNA5 in PB samples from CRC patients was significantly correlated with lymph node metastasis and distant metastasis ($P = 0.027$)[5], which suggests that DFNA5 could potentially be an independent prognostic serum biomarker for CRC patients. It is clear, however, that further validation in large-scale prospective trials is necessary before these biomarkers are ready for use in the clinic[26].

DNA methylation biomarkers in stool

As a more attractive alternative to tissue sampling, biomarkers from feces could be of great clinical value because sampling is noninvasive and has much higher specificity. These properties offer a distinct advantage over endoscope- and FOBT-based screening strategies for the detection of both CRC and critical precursor lesions. Over the past decade, numerous studies have engaged in the development of methylation-based detection assays for stool biomarkers of CRC (Table 2)[33-66], though the fecal biomarker detection can only be performed in only less than 50% of patients due to very limited compliance. The best-studied and top-performing methylation biomarkers are secreted frizzled-related protein 2 (SFRP2) and vimentin.

SFRP2 was the first reported DNA methylation marker in stool, has shown a sensitivity of 77%-90% and specificity of 77%[66] and has since been studied extensively. *SFRP2* methylation has been shown to be the most sensitive biomarker for CRC, with detection rates ranging from 77% to 94% (Table 2). When *SFRP2* methylation was used in a multigene, fecal methylation panel, detection of CRC and a small number of advanced adenomas reached a sensitivity and specificity of 96%[58]. A follow-up study found that *SFRP2* methylation was detectable in the stool of almost half of all patients with hyperplastic polyps or colorectal adenomas[53], further supporting its use in the detection of premalignant lesions. Fecal *SFRP2* methylation also drops dramatically after surgery [postoperative: 8.7% (6/69) *vs* preoperative: 87% (60/69)][52], suggesting its possible utility as a biomarker for recurrence.

The vimentin gene, which encodes an intermediate filament protein involved in cell attachment, migration, and signaling, was identified in the stool of 83% of CRC patients with a specificity of 90%[62]. Since then, many studies have been devoted to vimentin methylation. Follow-up studies have obtained similar results and thus have reinforced the utility of vimentin as a stand-alone biomarker[47,54,55,59,61]. This has led to the commercialization of a single-gene stool kit for CRC detection based on vimentin methylation. More recently, vimentin methylation has been used in combination with other methylation markers to further increase detection rates, and vimentin has also been found in urine, suggesting an alternative method of detection[34,48,67]. Vimentin has a low detection rate in serum, however, and is thus most likely not suitable for use as a serum biomarker for CRC. Recently, Ahlquist *et al*[26] reported that a panel of methylation markers from stool that includes vimentin has shown a significantly higher sensitivity for CRC, primarily because of higher detection rates in stage I -Ⅲ CRC (91% *vs* 50%).

In addition to SFRP2 and vimentin, several other methylation biomarkers have been identified; these include *GATA4, HIC1, ITG4, NDRG4, OSMR, TFPI2, ESR1, SLIT2, PHACTR3, SPG20, 3OST2* and *MGMT*. These genes have sensitivities for CRC ranging from 38% to 89% and specificities ranging from 79% to 100%. The combination of different methylation biomarkers (combinations of 2 to 7 genes including *APC, ATM, CDKN2A, GSTP1, HLTF, hMLH1, HPP1, MGMT, RASSF1, SFRP2, MAL, P16,* or vimentin) increased sensitivity from 55% to 100% and increased specificity from 87% to 100% (Table 2). However, more clinical studies are required to confirm these results.

MIRNA BIOMARKERS

miRNA and cancer

In recent years, miRNA has been a relatively new but rapidly expanding field, as is evidenced by the increasing number of assays in development. miRNAs are small non-coding RNA molecules that function in transcriptional and post-transcriptional regulation of gene expression and control various cellular functions. Currently, more than 1000 miRNAs have been discovered in the human genome, and their activities and regulatory mechanisms are being intensively investigated. miRNAs typically function *via* base pairing with complementary sequences in mRNA molecules, resulting in gene silencing *via* translational repression or target degradation. It

Table 2 Biomarkers of DNA methylation in the stool of colorectal cancer patients

Markers	Sample	Sensitivity	Specificity	Ref.
AGTR1/WNT2/ SLIT2/VIM/SEPT9	214 CRC 25 IBD 39 controls	20%-78%	86%-100%	[33]
Vimentin/EYA4/ BMP3/NDRG4	CRC	67%-100%	89%	[34]
ESR1	19 CRC 38 controls	65%	81%	[35]
SLIT2	60 CRC 32 IBD associated CRC 20 HR-IBD 65 LR-IBD 28 controls	 25%	100%	[36]
PHACTR3	64 CRC 71 A 34 controls	66% 32%	100%	[37]
TFPI2	60 CRC 20 A 30 controls	68.3%	100%	[38]
CNRIP1/FBN/INA/ MAL/SNCA/SPG20	78 CRC 61 A 48 controls	65%-94% 35%-91%	95%-100%	[39]
SPG20	9 CRC	67%	Unknown	[40]
MAL/CDKN2A/ MGMT	69 CRC 24 A 19 HP 24 controls	55.1%-78.3% 37.5%-58.3% 10.5%-26.3%	96.2%-100%	[41]
3OST2	21 CRC	72.7%	90%	[42]
ITGA4/SFRP2/p16	30 CRC 25 A 21 controls	70% 72%	96.8%	[43]
RARB2/p16/ INK4a/MGMT/APC	26 CRC 20 A 16 IBD 20 controls	62% 40% 13%	100%	[44]
RASSF1/SFRP2	84 CRC 27 advanced A 29 non-advanced A 12 HP 4 IC 2 UC 113 controls	75% 44% 28% 25% 25% 100%	89%	[45]
OSMR	69 CRC 81 controls	38%	95%	[46]
Vimentin	22 CRC 20 advanced A 38 controls	41% 45%	95%	[47]
MGMT/hMLH1/ Vimentin	60 CRC 22 advanced A 30 non-advanced A 37 controls	75% 46% 70%	87%	[48]
ITGA4	13 A	69%	79%	[49]
NDRG4	75 CRC 75 controls	53%-61%	93%-100%	[50]
GATA4	75 CRC 75 controls	51%-71%	84%-93%	[51]
TFPI2	26 CRC 45 controls	76%-89%	79%-93%	[17]
SFRP2	69 CRC 34 A 26 HP 30 controls	87.0% 61.8% 42.3%	93%	[52]
SFRP2	13 A 6 HP 6 controls	46% 33%	100%	[53]
Vimentin	103 A 75 controls	46%	84%	[54]
Vimentin/DIA	42 CRC 241 controls	86%	73%	[55]
CDKN2A/p16/ MSI/long DNA	25 CRC 20 controls	64%	95%	[56]
SFRP2	52 CRC 10 advanced A 11:00 AM 8 HP 6 UC 24 controls	94% 70% 36% 38% 17%	93%	[57]
SFRP2/HPP1/ MGMT	52 CRC 10 advanced A 11 non-advanced A 8 HP 6 UC 24 controls	96% 80% 64% 38% 17%	96%	[58]
Vimentin/DIA	40 CRC 122 controls	88%	82%	[59]
ATM/APC/ MGMT/hMLH1/ HLTF/SFRP2/GSTP1	20 CRC 30 A 30 controls 241 controls	75% 68%	90%	[60]
Vimentin	74 CRC 62 A 70 controls	72% 84%	89%	[61]
Vimentin	94 CRC 198 controls	46%	90%	[62]
HIC1	26 CRC 13 A 9 HP 41 controls	42% 31% 0%	98%	[63]
CDKN2A/MGMT/ hMLH1	29 A 10 HP 25 controls	55% 40%	72%	[64]
ATM/APC/ MGMT/hMLH1/ HLTF	20 CRC 20 controls	70%	100%	[65]
SFRP2	23 CRC 26 controls 10 HP 25 controls	77%-90% 40%	77%	[66]

A: Adenoma; HP: Hyperplastic polyp; HR: High risk; LR: Low risk; IBD: Inflammatory bowel disease; IC: Ischemic colitis; UC: Ulcerative colitis; CRC: Colorectal cancer; AGTR1: Angiotensin Ⅱ receptor, type 1; WNT2: Wingless-type MMTV integration site family member 2; SLIT2: Slit homolog 2; VIM: Vimentin; EYA4: Eyes absent homolog 4; BMP3: Bone morphogenetic protein 3; NDRG4: NDRG family member 4; ESR1: Estrogen receptor 1; PHACTR3: Phosphatase and actin regulator 3; TFPI2: Tissue factor pathway inhibitor 2; CNRIP1: Cannabinoid receptor interacting protein 1; FBN: Fibrillin; INA: Internexin neuronal intermediate filament protein, alpha; MAL: Mal, T-cell differentiation protein; SNCA: Synuclein, alpha; SPG20: Spastic paraplegia 20; MGMT: O-6-methylguanine-DNA methyltransferase; 3OST2: Heparan sulfate (glucosamine) 3-O-sulfotransferase 2; ITGA4: Integrin, alpha 4; RARB2: Retinoic acid receptor, beta 2; RASSF1: Ras association (RalGDS/AF-6) domain family member 1; OSMR: Oncostatin M receptor; GATA4: GATA binding protein 4; TFPI2: Tissue factor pathway inhibitor 2; CDKN2A: Cyclin-dependent kinase inhibitor 2; ADIA: DNA integrity assay; MSI: Microsatellite instability; GSTP1: Glutathione S-transferase pi 1; HIC1: Hypermethylated in cancer 1; ATM: Ataxia telangiectasia mutated; APC: Adenomatosis polyposis coli; hMLH1: Homo mutL homolog 1; HLTF: Helicase-like transcription factor; SFRP2: Secreted frizzled-related protein 2.

Table 3 MicroRNAs found in colorectal cancer but not in normal tissue

Up-regulated	miR-7, miR-17, miR-18a, miR-19a, miR-20a, miR-20, miR-21, miR-25, miR-29a, miR-29b, miR-32, miR-33a, miR-34a, miR-34b, miR-92a, miR-93, miR-95, miR-96, miR-101, miR-106a, miR-106b, miR-130, miR-135a, miR-135b, miR-181b, miR-182, miR-183, miR-191, miR-200c, miR-203, miR-222, miR-223, miR-224, miR-378, miR-155
Down-regulated	miR-30a, miR-30c, miR-34a, miR-125a, miR-126, miR-133a, miR-133b, miR-143, miR-145, miR-191, miR-192, miR-195, miR-215, miR-342, miR-497, miR-375, miR-378, miR-1, miR-9, miR-129, miR-137, miR-139

The underlined genes *miR-34a*, *miR-191* and *miR-378* were reported to be up-regulated in some studies[71-73] and down-regulated in others[74-76].

has been well documented that many miRNAs are regulated by the methylation of their promoter region, and some miRNAs target epigenetic activity. For example, miR-29b has been reported to induce DNA hypomethylation and the re-expression of tumor suppressor genes in acute myeloid leukemia by targeting DNMT[68]. These results suggest that there is a strong relationship between miRNA expression and epigenetic mechanisms. Notably, many miRNAs have been found in CRC, and researchers have quantified specific miRNAs for the purpose of CRC diagnosis and prognosis in patient blood, stool and tissue samples. *In vitro* studies have also been conducted to identify any correlation between epigenetic aberrations and therapy response.

In vitro studies

Currently, 54 miRNAs have been identified that are regulated either up or down in CRC cells relative to non-tumor cells (Table 3)[69,70]. Of these, miR-17, miR-20, miR-21, miR-31, miR-92a, miR-93, miR-183 and miR-203 were upregulated in CRC cells, while miR-30a, miR-30c, miR-133a, miR-143, miR-145 were downregulated. These observations have been validated in subsequent studies. The upregulated miRNAs were associated with chromosomal regions that are often amplified in CRC, and the downregulated miRNAs often associated with chromosomal regions that were typically deleted. These changes may be closely related to genetic alterations as well as epigenetic modification.

However, there are some discrepancies between studies. For example, miR-34a, miR-191 and miR-378 were reported to be upregulated in some studies[71-73] and yet were down regulated in others[74-76]. This may have been caused by heterogeneity between the different studies with regards to tumor stage, tumor location, genetic background and technical issues. We believe that the accumulation of further studies will allow us to determine which miRNAs will be the most effective biomarkers and also better understand their role in colorectal cancer.

miRNA biomarkers in blood

It is widely believed that miRNAs can shed from tumor cells *via* exosomes and survive in a stable form in the circulation. Many studies have been performed to quantify miRNAs in the blood for use as a biomarker (Table 4)[77-88]. miR-92a, located on chromosome 13q13, is a member of the *miR-17-92* gene cluster. This cluster promotes cell proliferation, suppresses apoptosis, induces angiogenesis and accelerates tumor progression. miR-92a was first identified by Ng *et al*[84] as a potential noninvasive biomarker for CRC detection with a sensitivity of 89% and specificity of 70%. miR-17-3p, another member of the *miR-17-92* gene cluster, was also evaluated in this study as a detection biomarker. This miRNA produced a sensitivity of 64% and a specificity of 70%.

To follow this study, Huang *et al*[82] performed a receiver-operating characteristic (ROC) analysis on 120 CRC patients, 37 patients with advanced adenomas and 59

Table 4 MicroRNA biomarkers in the blood and stool of colorectal cancer patients

Markers	Sample	Sensitivity	Specificity	Ref.
Blood				
miR-21	186 CRC 43 A 53 controls	76.8%-82.8% 91.9%	81.1%-90.6% 81.1%	[77]
miR-601/ miR-760	90 CRC 48 AA 58 controls	83.3% 72.1%	69.1% 62.1%	[78]
miR-21	30 CRC 30 controls	90.0%	90.0%	[79]
miR-29a	258 plasma sample	75.0%	75.0%	[80]
miR-141	102 CRC	77.1%-90.9%	77.1%-89.7%	[81]
miR-92a	120 CRC 37 AA 59 controls	84.0% 64.9%	71.2% 81.4%	[82]
miR-29a	120 CRC 37 AA 59 controls	69.0% 62.2%	89.1% 84.70%	[82]
miR-221	103 CRC 37 controls	86.0%	41.0%	[83]
miR-17-3P	90 CRC 50 controls	64.0%	70.0%	[84]
miR-92a	90 CRC 50 controls	89.0%	70.0%	[84]
Stool				
miR-21	88 CRC 57 polyps 101 controls	55.7% 43.9%	73.3%	[85]
miR-92a	88 CRC 57 polyps 101 controls	71.6% 56.1%	73.3%	[85]
miR-144*	75 CRC	74.0%	87.0%	[86]
miR 17-92 cluster	197 CRC 119 controls	69.5%	81.5%	[87]
miR-135	197CRC 119 controls	46.2%	95.0%	[87]
miR-21	197 CRC 119controls	14.7%	91.6%	[87]
miR-92a	59 CRC 74 controls	50.0%	80.0%	[88]
miR-21	59 CRC 74 controls	50.0%	83.0%	[88]

A: Adenoma; CRC: Colorectal cancer.

Table 5 Relationship between microRNAs and screening, diagnosis and prognosis in colorectal cancer

Screening	miR-17-92, miR-20a, miR-21, miR-92, miR-96, miR-106a, miR-135, miR-144, miR-203, miR-326, miR-181b, miR-601, miR-760
Diagnosis	miR-133b, miR-143, miR-145, miR-17-92, miR-18a, miR-20a, miR-21, miR-31, miR-92, miR-96, miR-135b, miR-183
Prognosis	miR-18a, miR-21, miR-20a, miR-31, miR-143, miR-145, miR-155, miR-181b, miR-200c, miR-203, miR-106a, miR-17-92, miR-135a, miR-335, miR-206, miR-10b, miR-146a/b, let7a/b
Treatment	miR-21, miR-17, miR-215, miR-125b, miR-137, miR-143, miR-145, miR-192, miR-622, miR-630

healthy controls. In this analysis, the researchers found that they could not only discriminate CRC from controls (miR-29a yielded an area under the curve (AUC) of 0.844, and miR-92a yielded an AUC of 0.838), but also discriminate advanced adenomas from controls (the AUC was 0.769 for miR-29a and 0.749 for miR-92a). Furthermore, combined ROC analyses using these two miRNAs revealed an increased AUC with an 83.0% sensitivity and 84.7% specificity in discriminating CRC, and an AUC demonstrating 73.0% sensitivity and 79.7% specificity in discriminating advanced adenomas. These results suggested that plasma miR-29a and miR-92a have potential as novel noninvasive biomarkers for CRC detection and that a combination of different miRNAs may provide a higher sensitivity and specificity than a single miRNA.

More recently, miR-21, miR-601, miR-760 and miR-221 from plasma were also reported to be potential CRC biomarkers. In these studies, miR-221 and miR-21 were up-regulated in the plasma of CRC patients compared to healthy controls[79,83], while miR-601 and miR-760 were down-regulated[78]. Moreover, a study conducted in two independent CRC cohorts suggested that high levels of plasma miR-141 could predict poor survival, and thus miR-141 may serve as an independent prognostic factor for advanced CRC patients[81].

miRNA biomarkers in stool

Stool-based miRNA detection has been widely studied as a noninvasive screening method for CRC (Table 4). Koga *et al*[87] conducted an miRNA expression analysis of exfoliated colonocytes isolated from the feces of 197 CRC patients and 119 healthy controls. They analyzed the miRNA expression of the miR-17-92 cluster (including miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a), miR-21, and miR-135 by quantitative real-time PCR and found that expression of the miR-17-92 cluster and miR-135 was much higher in CRC patients than in healthy controls ($P < 0.0001$). miR-21, on the other hand, could not discriminate between the two groups. The miR-17-92 cluster detected distal tumors better than proximal tumors, as the sensitivity of miRNA expression for these tumors was 81.5% and 52.9%, respectively.

In another study, Wu *et al*[85] evaluated the feasibility of miR-21 and miR-92a detection in stool samples from 88 patients with CRC, 57 patients with colorectal polyps and 101 healthy controls. These results showed that patients with CRC had significantly higher levels of miR-21 ($P < 0.01$) and miR-92a ($P < 0.0001$) in their stool compared with normal controls. miR-92a levels provided a higher sensitivity for distal rather than proximal CRC ($P < 0.05$). In addition, stool miR-21 and miR-92a levels decreased significantly ($P < 0.01$) after surgical resection of tumor, which suggests that miR-92a and miR-21 from stool samples could serve as screening biomarkers for colorectal cancer.

In addition, miR-144* and miR-106a were found to be significantly overexpressed in adenomas and in the stool of CRC patients compared with healthy individuals[86,89]. These studies have confirmed that miRNAs from stool samples require validation as diagnostic biomarkers for CRC.

Brief summary

miRNAs have been closely linked to colorectal cancer development. They can serve as screening and diagnosis markers for CRC and also as potential prognostic and predictive markers. As a rough outline for the reader, we provide here a table to display the relationship between currently identified miRNAs and screening, diagnosis, prognosis and treatment in colorectal cancer (Table 5). As research continues, more miRNAs correlated with CRC will be discovered, and the mechanism of miRNA regulation will be deciphered. Therefore, it is highly likely that more effective miRNA biomarkers for CRC patients will be found in the future.

HISTONE MODIFICATION

Although DNA methylation has been the most extensively studied epigenetic alteration in CRC, increasing numbers of studies have also explored how histone modifications in tumor cells compared to normal colorectal cells. Only tissue samples can be used for histone profiling, so these biomarkers are most useful for the postoperative prognosis of CRC patients. Thus far, the best studies on histone modification have addressed post-translational methylation and acetylation by multiple enzymes. Tamagawa *et al*[90] created duplicate 2-mm-core tissue microarrays from 54 paraffin-embedded samples of primary colorectal adenocarcinomas and corresponding liver metastases to evaluate the methylation patterns of histone H3 lysine 27 (H3K27), H3 lysine 36 (H3K36) and the expression of H3K27 methylase EZH2. These microarrays were then probed in immunohistochemical assays to search for biomarkers that could identify these patients. These results revealed that H3K27me2 levels were lower in liver metastases than in the corresponding primary tumors, and these levels correlated with tumor size and poorer survival rates. H3K36me2 levels were higher in liver metastases than in the corresponding primary tumors and correlated with histological type and lymph node metastasis. In addition, this study conducted a multivariate survival analysis and suggested that the methylation level of H3K27me2 detected by immuno-

histochemistry may be an independent prognostic factor for metachronous liver metastasis in colorectal cancer patients. In fact, prior to this study, this group used the same method to validate other histone patterns, including histone H3 lysine 4 (H3K4) dimethylation, histone H3 lysine 9 (H3K9) dimethylation and histone H3 lysine 9 (H3K9) acetylation. They found that dimethylation of H3K4 and acetylation of H3K9 correlated with tumor histological type, and lower levels of H3K4 dimethylation correlated with a poor survival rate. Multivariate survival analysis showed that H3K4 dimethylation status is an independent prognostic factor for colorectal cancer patients[91].

Using chromatin immunoprecipitation (ChIP) coupled with quantitative PCR and high-throughput sequencing, Gezer *et al*[92] observed reduced plasma levels for two histone methylation biomarkers, H3K9me3 and H4K20me3, in patients with CRC and characterized these modifications in the circulation. They found that lower H3K9me3 levels had potential as biomarkers for CRC. These studies have provided a good start for the examination of histone modification for the prognosis of CRC. Research is ongoing to find histone biomarkers useful for colorectal cancer patients.

DISCUSSION

As we have discussed above, a variety of DNA methylation, miRNA and histone biomarkers from stool, blood and tissue have been reported for CRC detection. Some of the markers identified are derived from tumor cells and others are derived from non-tumor cells in the tumor microenvironment or blood. DNA and miRNA biomarkers mostly shed from tumor cells, and so, theoretically, these molecules should be more specific than protein biomarkers such as carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, CA242 and CA724, which are currently applied widely in the clinic. This is because nucleic acid-based markers can be amplified and thus produce a stronger signal, thereby permitting a greater sensitivity. In addition to the superior assay performance of DNA and miRNA, these samples are easier to store because effective preservation buffers that can prevent DNA and miRNA degradation in stool samples are available. In contrast, no preservation buffer for proteins in stool samples has been reported so far. In comparison with DNA and miRNA, protein biomarkers show lower specificity because tumors often induce inflammatory reactions, and some of the biomarkers that initially showed promise for cancer detection now appear to also detect a wide range of bowel diseases, such as ulcerative colitis and Crohn's disease. Moreover, protein biomarkers have often been altered in more than one type of cancer. For example, CEA has been reported as a biomarker for various malignancies, including colorectal, pancreatic, lung, renal and breast cancers[93-96]. In spite of these issues, protein biomarkers may still be useful for large-scale screening for CRC because proteins can be observed through assays in small sample volumes with relatively simple and cheap assays.

Regarding the comparison between stool and blood biomarkers, we know that both of these sample types have been under investigation and improvements continue to be made. In a biomarker search, sample collection, storage and handling have a significant impact on the performance of a specific test. Indeed, using stool samples to detect new biomarkers is not standardized; for example, the buffers used to collect and store stool samples were different in each study, and the methods of DNA or RNA isolation also varied. Therefore, it is difficult to compare the performance of different biomarkers based on the current research. By contrast, blood detection is more standardized and readily accepted by the general population. Moreover, biomarkers in blood are more stable than in stool because of the absence of microflora. DNA, miRNA and proteins have all been shown to be stable in unprocessed EDTA tubes or non-centrifuged clotted blood for 24 h or longer at room temperature. This is particularly true for miRNA, which, as a result of its short length, is more stable in blood than other types of nucleic acids. So, at a practical level, degradation problems for biomarkers during storage and transport should be taken into account, and it is important to standardize detection procedures. For example, the bias will be enormous and the results will not be interpretable if comparing the data from newly collected samples of CRC patients with those from archived samples of adenoma patients or healthy persons. Thus, various samples should be collected in the very same manner at the very same time according to the REMARK guidelines to improve the comparability between various results. Studies specifically addressing these questions are highly desirable

CONCLUSION

Epigenetic biomarkers and the use of blood and stool samples each have their own advantages and disadvantages for clinical screening, diagnosis and prognosis. Although many studies on these biomarkers are preliminary, some markers have demonstrated better performance than the current FOBT test. No biomarker-based assay is ready for large-scale population screening, however.

The standardization of sample preparation and testing protocols is very important for the widespread deployment of techniques and the comparison of results from different studies. Moreover, large well-controlled studies are urgently needed to identify the accuracy of epigenetic biomarkers for CRC detection in asymptomatic populations. Much work remains before such observations can be translated into routine clinical practice.

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ISSN 1007-9327

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