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Review

DNA methylation and microRNA biomarkers for noninvasive detection of gastric and colorectal cancer

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ABSTRACT

Cancer initiation and progression is controlled by both genetic and epigenetic events. Epigenetics refers to the study of mechanisms that alter gene expression without permanently altering the DNA sequence. Epigenetic alterations are reversible and heritable, and include changes in histone modifications, DNA methylation, and non-coding RNA-mediated gene silencing. Disruption of epigenetic processes can lead to altered gene function and malignant cellular transformation. Aberrant epigenetic modifications occur at the earliest stages of neoplastic transformation and are now believed to be essential players in cancer initiation and progression. Recent advances in epigenetics have not only offered a deeper understanding of the underlying mechanism(s) of carcinogenesis, but have also allowed identification of clinically relevant putative biomarkers for the early detection, disease monitoring, prognosis and risk assessment of cancer patients. At this moment, DNA methylation and non-coding RNA including with microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) represent the largest body of available literature on epigenetic biomarkers with the highest potential for cancer diagnosis. Following identification of cell-free nucleic acids in systematic circulation, increasing evidence has demonstrated the potential of cell-free epigenetic biomarkers in the blood or other body fluids for cancer detection. In this article, we summarize the current state of knowledge on epigenetic biomarkers – primarily DNA methylation and non-coding RNAs – as potential substrates for cancer detection in gastric and colorectal cancer, the two most frequent cancers within the gastrointestinal tract. We also discuss the obstacles that have limited the routine use of epigenetic biomarkers in the clinical settings and provide our perspective on approaches that might help overcome these hurdles, so that these biomarkers can be readily developed for clinical management of cancer patients.

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1. Introduction

Cancer is a major health problem in most developed nations worldwide. Within the gastrointestinal tract, gastric cancer (GC) and colorectal cancer (CRC), represent the two most frequent malignancies. Even though the incidence of GC has declined rapidly in recent decades, GC remains the fourth most common cancer and the second leading cause of cancer-related deaths in the world [1]. Likewise, CRC is the third most common cancer worldwide, with an estimated incidence of more than 1.2 million cases globally [2]. An estimated 608,000 deaths from CRC occur worldwide each year, accounting for 8% of all cancer deaths, making it the fourth most common cause of cancer-related deaths [2]. Both GC and CRC are characterized by relatively rapid progression of the disease and late clinical presentation, which is primarily the underlying reason for increased mortality and morbidity in patients suffering from these malignancies. Both diseases are largely preventable if these can be identified at early stages. However, in order to identify patients with an early stage disease, availability of robust diagnostic biomarkers could tremendously help in reducing overall mortality rates [3]. Additionally, in spite of more frequent use of highly sophisticated and sensitive imaging techniques for the diagnosis of GC and CRC, there is a significant amount of interest in developing inexpensive, highly sensitive and specific, and non-invasive biomarkers that could potentially also be useful for the prognosis and surveillance of these patients.

Cancer, which was earlier thought to be mostly a 'genetic' disease, is now being recognized to involve frequent and widespread 'epigenetic' abnormalities. It is also becoming apparent that micro-environment-mediated epigenetic perturbations play an important role in the development of neoplasia [4]. Epigenetics refers to the study of heritable changes in gene expression that are not attributed to permanent changes in DNA sequence itself, but are sufficiently powerful to regulate the dynamics of gene expression [5]. The principal processes responsible for epigenetic regulation include DNA methylation, histone modifications and posttranscriptional gene regulation through non-coding RNAs (microRNAs, long non-coding RNAs, small nucleolar RNAs etc) [6]. These mechanisms are critical components in the normal development and growth of cells and perturbations in these epigenetic expression patterns contribute to specific and diverse neoplastic phenotypes [7].

Epigenetic alterations are believed to occur early in tumor development and may precede genetic changes, thus providing a rationale for developing molecular biomarkers for the early diagnosis and disease prevention [8]. The emergence of advanced technologies that now allow detection of genome-wide epigenetic changes provide ample promise for advancing our capacity to develop such biomarkers for detecting cancers at an early stage [9]. DNA methylation, which is a result of the covalent addition of a methyl group at the 5′ position of the pyrimidine ring of cytosines within the context of CpG dinucleotides, is important in maintaining the genomic structure and regulation of gene expression [10]. Non-coding RNAs, such as microRNAs (miRNAs), regulate gene expression by inhibiting or inactivating target messenger RNAs (mRNAs). Of interest, a single miRNA can bind to several target mRNAs, making them more attractive and ideal from biomarker development viewpoint. Recent reports have shown that methylated DNA and miRNAs could be readily detected in a wide variety of tissues, as well as various body fluids, indicating that these epigenetic biomarkers could represent the next generation of biomarkers for cancer detection. In this review, we provide an overview of recent advances in epigenetics and discuss the development of DNA methylation and miRNA biomarkers for the identification of patients with GC and CRC (Fig. 1).

2. Potential of methylated DNA in cancer diagnostics

2.1. Overview of DNA methylation

The most widely studied epigenetic alteration in humans is DNA methylation. Aberrant DNA methylation contributes to cancer mainly through DNA *hyper*- or *hypo*-methylation. While DNA hypermethylation refers to the gain of methylation at a locus that was originally not methylated and usually results in stable transcriptional silencing and reduced gene expression [11], DNA hypomethylation represents the loss of DNA methylation, affecting chromosomal stability and enhanced aneuploidy [12]. Global DNA hypomethylation was discovered first, and is usually considered one of the hallmarks of cancer cells, and some of the genes targeted by hypomethylation overlap aberrant hypermethylation-vulnerable genes [13].

During DNA hypermethylation, a methyl group is added to the fifth carbon position of cytosine residues occurring in the context of CpG dinucleotides. This process of cytosine methylation is catalyzed by a group of enzymes, DNA methyl transferases (DNMTs) [14]. There are three established DNMTs: DNMT1, DNMT3a and DNMT3b. DNMT1 is involved in maintaining methylation by methylating newly synthesized DNA strands during DNA replication [15], whereas DNMT3a and DNMT3b are mainly involved in de novo methylation [16]. CpG dinucleotides are scattered throughout the human genome, but are more concentrated either in the CpG-rich regions (often referred to as "CpG islands") that frequently occur in the 5′-flanking promoters of genes, or in regions of large repetitive sequences, such as centromeres and retrotransposon elements [17–19]. Several direct mechanisms have been proposed to explain DNA hypermethylation-induced transcriptional repression. The first mechanism suggests that methylated CpG islands hinder the binding of activating transcription factors to DNA sequences or recruit inhibitory proteins, such as histone deacetylases (HDACs) [20,21]. Another possibility that has garnered acceptance is that a family of proteins that recognize methyl CpGs, known as methyl-CpG proteins (MBPs), could evoke

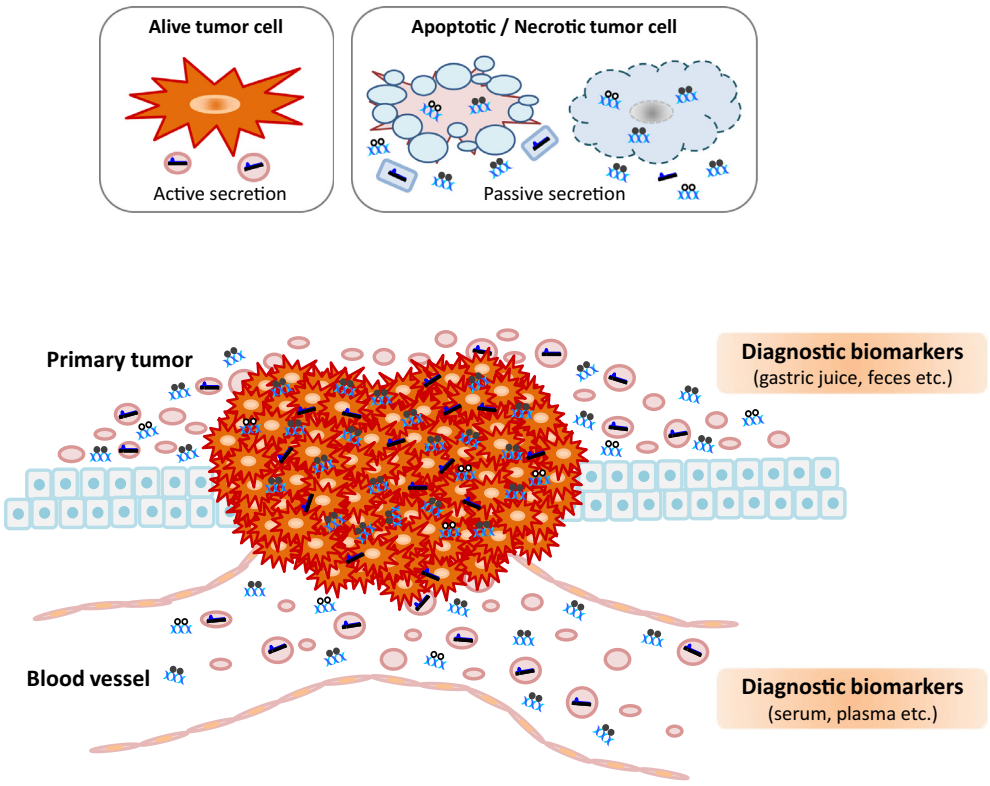

Fig. 1. Sources of non-invasive DNA methylation and miRNA biomarkers in gastric and colorectal cancer. Cell free nucleic acids are released from primary tumor cells into body fluids by multiple mechanisms, such as active secretion from tumor cell and passive secretion from apoptotic and necrotic tumor cells. Active secretion; tumor derived miRNAs can circulate in secreted extracellular vesicles (e.g. apoptotic bodies and exosomes: red circles) Passive secretion; miRNAs from apoptotic and necrotic tumor cells form complexes with specific RNA binding proteins (e.g. miRNAs bind to the AGO2 protein: blue square). Tumor cells release small fragments of cell-free DNA including methylated and unmethylated DNA by passive secretion. Cancer associated epigenetic changes represent available non-invasive sources of diagnostic biomarkers of gastric and colorectal cancer, such as the analysis of methylation status and miRNA expression. Abbreviations: AGO, Argonaute; miRNAs, microRNAs.

the repressive potential of methylated DNA [22]. X-chromosome inactivation and genomic imprinting are classical examples of such an epigenetic regulatory mechanism [15]. Over the past decade we have learnt that during the initiation and tumorigenesis of human cancer, a large number of critical tumor suppressor genes undergo methylation-induced transcriptional silencing, which leads to altered cellular signaling that manifests in carcinogenesis [23]. Although from a biomarker perspective hypermethylated genes may be more relevant, from a molecular standpoint hypomethylation of repetitive sequences is essential in preventing chromosomal instability and maintaining chromosomal integrity [12,24–27]. At an individual gene level, DNA hypomethylation is often associated with activation of proto-oncogenes and various retrotransposon elements.

Recent evidence suggests that epigenetic deregulation may actually even precede classical genetic changes, such as mutations and deletions in various tumor suppressors or oncogenes. These data highlight that presence of DNA methylation may be a more ideal representation and reflection of early molecular alterations that take place in human cancers.

2.2. DNA methylation in gastric carcinogenesis

Histologically, GCs are divided into intestinal and diffuse types, according to the Lauren classification [28]. Intestinal GC mostly progresses through successive steps of normal gastric mucosa, leading to acute and chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and finally GC [29]. In contrast, the sequence of events in the development of diffuse GC is poorly understood, although a subset of diffuse type GC appears to develop independently of atrophic gastritis or intestinal metaplasia [30]. Each histological type is a consequence of a progressive accumulation of different genetic and epigenetic alterations, which play a central role in gastric carcinogenesis [31].

Recent reports indicate that infections with *Helicobacter pylori* (*H. pylori*) or Epstein-Barr virus (EBV), pathogens with a substantial role in development of GC, are associated with elevated levels of aberrant DNA methylation in GC [32]. Compare et al. suggested that global DNA hypomethylation might be implicated in GC associated with *H. pylori* infection at an early stage [33]. In contrast, most reports have favored the school of thought that *H. pylori* infection induces aberrant methylation in a number of gene promoters in the gastric mucosa, including cell growth-related genes p16 (INK4a), p14 (ARF) and APC; DNA-repair genes, MLH1, BRCA1 and MGMT; the cell adherence gene E-cadherin; as well as LOX, FLNC, HRASLS, HAND1, and THBD, which are methylated in gastric cancer patients [34–36]. The epithelial cadherin gene, CDH1, which is a well-studied gene involved in cancer, is downregulated in gastric tumors and is hypermethylated more frequently in the diffuse type than in the intestinal type of GC. Loss of CDH1 expression during tumor progression has led to the hypothesis that it is a tumor suppressor gene [37]. Unlike the CDH1 gene, the p16 gene is hypermethylated, mainly in the intestinal type of GC [11,38]. This epigenetic marker was associated recently with tumor location and *H. pylori* infection in GC [39].

Other studies have described a number of other genes that are silenced by hypermethylation in association with *H. pylori* or EBV infection. According to Chan et al. [40], the eradication of *H. pylori* infection significantly reduces the methylation index of the CDH1 promoter. In contrast, some studies have shown that aberrant DNA methylation induced by *H. pylori* infection may persist even after the infection has disappeared [41,42]. Shin et al. [43]

observed that the methylation levels in MOS remained significantly increased in patients with previous *H. pylori* infection compared with *H. pylori*-negative subjects.

Moreover, hypermethylation of several gene promoters has also been observed in the premalignant stages of GC, suggesting that aberrant methylation occurs early during gastric carcinogenesis [44–46]. For example, the methylation levels of the catalytic subunit of the telomerase gene (hTERT) promoter were increased during gastric carcinogenesis. Wang et al. [47] reported that the hTERT promoter was more methylated in GC than in precancerous lesions and non-neoplastic gastric tissues, highlighting that the degree of methylation of the hTERT promoter may be useful for the early diagnosis of GC and/or may have an impact on the anti-telomerase strategy for cancer therapy. Recently, aberrant hypermethylation of the newly associated metastatic suppressor gene RECK was found to be associated with GC development, which may also serve as a useful biomarker for early diagnosis and treatment [48]. These findings delineate that specific epigenetic alterations occur during different stages of gastric tumorigenesis.

2.3. DNA methylation in colonic carcinogenesis

Almost all CRCs progress from benign neoplasms into adenocarcinoma through a stepwise sequence characterized by histological changes that start with aberrant crypt focus (ACF) and benign tubular adenomas, which can progress to advanced adenomas with a high risk of malignant progression, and can further proceed to invasive adenocarcinoma [49]. Epigenetic alterations have now been linked to specific steps in the adenoma-carcinoma sequence, and are believed to play a central role in the initiation and progression of CRC [50,51].

In 1983, global DNA hypomethylation and deficiency of overall 5-methylcytosine content in CRC tissues was first observed by Feinberg and Vogelstein [52]. These investigators demonstrated that such loss of methylation events were predominantly observed at CpG dinucleotides within the repetitive sequences, occurred early and enhanced gradually in an age-dependent manner in CRC patients. Global DNA hypomethylation in CRC tissues was simultaneously accompanied by hypermethylation and transcriptional silencing of tumor suppressor or DNA repair genes [53]. Hundreds of genes are believed to be aberrantly methylated in the genome from CRC patients, but only a subset of these are likely to be critical for the pathogenesis of this malignancy. Recent reviews have revealed that several genes are frequently methylated in the multiple-step process from normal colonic epithelium to adenocarcinoma [54–56]. For instance, six genes (CDH13, CRBP1, SFRP1, SFRP2, SLC5A8, and RUNX3) and two loci (MINT1, MINT31) are consistently methylated in during transformation of normal colonic mucosa to ACF [54,57–60]. Additionally, other genes (CDH1, CDKN2A/p16, ESR1, HLTF, ITGA4, and p14) are methylated during progression of ACF to adenoma/polyp. Furthermore, four genes (CXCL12, ID4, IRF8, and TIMP3) are frequently methylated in the late stages of the adenoma-carcinoma sequence and could have a role in CRC progression and metastasis [54,61–63].

Curiously, some of genes that are aberrantly methylation in CRC, including ESR1, IGF2 and TUSC3, are also methylated in histologically normal colonic mucosa. Aberrant methylation of these genes is thought to increase in an age-dependent manner, and some of them are also implicated in the pathogenesis of CRC [64–66]. The concept of "field cancerization or field defect" was proposed to account for the multiple primary lesions within the same organ, local recurrence or increased susceptibility of normal tissue to malignant transformation [67]. Such field alterations are subtle and may occur in normal epithelial cells, but these abnormal modifications of the normal colonic mucosa could serve as potential biomarkers for assessing the risk for developing CRC later in life. For example, it was reported that the SFRP gene, which is a negative regulator of the WNT pathway, is hypermethylated, and consequently downregulated in normal colonic mucosa from patients with CRC [68]. Additionally, Kawakami et al. [69] demonstrated that higher methylation levels of age-associated markers, such as ESR1 and MYOD, occurred in the normal colonic mucosa from patients with CpG Island Methylator Phenotype (CIMP)-positive CRCs. Recently, genome-wide DNA methylation analysis revealed that the methylation levels of genes implicated in lipid, carbohydrate and amino acid metabolic pathways are directly influenced by diets, and were significantly different between the normal colonic mucosa from the patients with and without CRC [70]. Although accumulation of DNA methylation is now expected to contribute to "field cancerization" in the colonic mucosa, further research is required to establish useful surrogate markers for CRC surveillance.

3. Methylated DNA in body fluids

3.1. Methods to detect DNA methylation in body fluids

The collection of body fluids is a relatively noninvasive procedure, which enables early cancer detection or monitoring of minimally recurrent disease after treatment. For gastrointestinal cancers, typical body fluids include plasma or serum from blood, as well as organ-specific fluids, such as gastric juice and fecal specimens. In contrast to rare genetic mutations, analysis of DNA methylation biomarkers in bodily fluids is a far more compelling strategy as it allows early detection of disease, and permits simultaneous measurement of frequently methylated genes/loci that adequately represent tumor heterogeneity [71].

However, methodologies with high enough sensitivity and specificity suitable to investigate bodily fluids are particularly challenging. For any diagnostic approach, candidate biomarkers and methods with high sensitivity and specificity must be adopted to allow the detection of rare cancer-specific methylation events in the large background of normal DNA. Consequently, the choice of optimal biomarkers with extremely low levels of background methylation is preferable to avoid high false-positive rates and decreased specificity of the assay.

Conventional methylation-specific PCR (MSP) is a very sensitive method for methylation detection, and is frequently used for the detection of low levels of DNA methylation [72]. MSP utilizes PCR primers that contain multiple CpG dinucleotides, which allow selection and amplification of methylated alleles. However, MSP is unsuitable for use in a clinical setting for a variety of reasons. First, conventional MSP is strictly a qualitative approach and indicates just a 'positive' or 'negative' result for the methylation status of a given gene/target. Second, conventional MSP generates high rates of false-positive as well as false-negative results, particularly when DNA is extracted from formalin fixed paraffin embedded samples [73], making it an arduous task to standardize these assays between independent laboratories [74]. In contrast, Quantitative MSP-based approaches, such as MethyLight [75] or SMART-MSP [76], offer a more suitable choice for use in molecular diagnostics, as these allow a better handle on the selection of methylation thresholds. In addition, SMART-MSP identifies incomplete bisulfite conversion, which can be an important source of false positives. As described above, the majority of diagnostic tests based on DNA methylation levels have employed bisulfite conversion of cytosine to uracil residues. However, in many important diagnostic scenarios, DNA from the cancer cells represents only a small fraction of the total DNA in the clinical samples from plasma, serum, feces and other body fluids. For such purposes, there are several advantages of using digital approaches. Li et al. [77] recently

Table 1
Aberrantly methylated genes as diagnostic biomarkers in body fluids, including serum, plasma and gastric juice in patients with gastric cancer.

Methylated DNA	Source	Sensitivity	Specificity	Methods	Refs.
HLTF	Plasma	20.8%	100%	MSP	[87]
Reprimo	Plasma	95.3%	90.3%	MSP	[86]
SLC19A3	Plasma	85%	85%	qMSP	[88]
APC	Serum	17%	100%	qMSP	[81]
CDH1	Serum	57.4%	100%	MSP	[80]
CYP26B1	Serum	73.9%	93.4%	qMSP	[85]
DAPK	Serum	48.1%	100%	MSP	[80]
DLEC1	Serum	33.8%	NA	MSP	[84]
E-cadherin	Serum	13%	100%	qMSP	[81]
GSTP1	Serum	14.8%	100%	MSP	[80]
MLH1	Serum	41%	92%	qMSP	[81]
KCNA4	Serum	67.4%	97.4%	qMSP	[85]
p15	Serum	55.6%	100%	MSP	[80]
p16	Serum	51.9%	100%	MSP	[80]
p16	Serum	26%	100%	MSP	[185]
p16	Serum	26.9%	100%	MSP	[95]
RASSF1A	Serum	34%	100%	MSP	[92]
RUNX3	Serum	95.5%	62.5%	qMSP	[91]
RUNX3	Serum	45.2%	NA	MSP	[90]
SFRP2	Serum	66.7%	100%	MSP	[82]
SULF1	Serum	55%	81%	MSP	[83]
TFPI2	Serum	10%	100%	qMSP	[186]
TIMP3	Serum	17%	100%	qMSP	[81]
ADAM23	Stomach juice	70%	83.3%	Pyrosequencing	[96]
CDH1	Stomach juice	Median levels of methylation (65%)	Median levels of methylation (0%)	qMSP	[187]
GDNF	Stomach juice	65%	89.6%	Pyrosequencing	[96]
MINT25	Stomach juice	90%	95.8%	Pyrosequencing	[96]
MLF1	Stomach juice	60%	85.4%	Pyrosequencing	[96]
PRDM5	Stomach juice	65%	93.7%	Pyrosequencing	[96]
RORAa	Stomach juice	60%	85.4%	Pyrosequencing	[96]

MSP, methylation-specific PCR; qMSP, quantitative methylation-specific PCR.

demonstrated a technology, called "Methyl-BEAMing", for direct digital quantification of cancer-derived Vimentin DNA in plasma and fecal DNA from CRC that is readily applicable to clinical samples from body fluids.

The way it stands currently, several challenges must be overcome before DNA methylation-based biomarkers can be adopted in the clinics. First, previous studies have failed to demonstrate reproducibility of results using different analytical methods. The absence of standard, reproducible methods have negatively affected the credibility of DNA methylation assays as potential diagnostic tools for cancer detection. Second, when considering non-invasive approaches, the most important determinant could be the experimental conditions under which plasma/serum, stool or gastric juice was collected. Third, another critical problem remains with regards to the efficiency of DNA extraction, quantification of DNA and efficiency of bisulfite conversion. Collectively, these limitations reinforce the notion that all these challenges must be overcome and DNA methylation detection must be standardized to be permit efficient development of these biomarkers for cancer detection [78]. Guidelines, including universal individual laboratory instructions, should be encouraged for the standardization of methylated DNA analysis in body fluids.

3.2. Blood-based DNA methylation diagnostic biomarkers in gastric cancer

The prognosis of GC patients is largely dependent on clinical or/and pathological staging at diagnosis and treatment [79]. Diagnostic tools, such as gastrointestinal endoscopy (GIS) followed by pathological analysis or fluoroscopy are used clinically, but their diagnostic capacity heavily relies upon the technical skills of the endoscopist. In addition, endoscopy is neither comfortable nor free from the risk of morbidity. Thus, there is an urgent need for the development of less-invasive and more efficient strategies to detect early GCs. DNA methylation is a major mechanism of silencing tumor-related genes, such as tumor suppressor genes, in neoplastic cells [14]. The advantages of gene methylation biomarkers for the detection and diagnosis of cancer in biopsy samples or body fluids, such as serum/plasma or gastric washes, have persuaded several investigators to undertake studies to determine the diagnostic usefulness of these molecular signatures in gastric cancer (Table 1).

In one of the first studies on analysis of plasma/sera-based DNA methylation in patients with GC, Lee and colleagues observed high prevalence of hypermethylation of various genes such as DAPK, CDH1, GTSP1, p15 and p16, in the serum of GC patients [80]. Thereafter, research on plasma/serum have continued, demonstrating that methylation of APC, Reprimo, MLH1, TIMP3, SFRP2, HSurf-1, DLEC1, CYP26B1, KCNA4, HLTF and SLC19A3 could be easily detected in blood circulation; and that their methylation in circulating nucleic acids released by GC cells significantly correlated with methylation levels of these genes in GC tissues [81–88]. RUNX3, one of the RUNX family members that plays important roles in both normal developmental processes and carcinogenesis, is frequently inactivated by methylation-induced silencing [89]. In addition, hypermethylation of RUNX3 was detected in 45.2% GC patients, which was significantly higher than that in healthy donors [90]. The quantification of serum RUNX3 methylation has a great potential to detect and diagnose GC, as well as use this biomarker for postoperative monitoring of tumor recurrence in these patients [91]. In addition, serum RASSAF1A methylation in GC patients was significantly higher (34%) than those with benign disease (3.3%) or in healthy donors (0%). Importantly, even though the sensitivity of serum RASSAF1A methylation in detecting GC patients was relatively low, the specificity of this biomarker was extremely high [92]. Furthermore, a recent meta-analysis revealed that the frequency of p16 promoter methylation in GC tissues was higher than those of normal and adjacent tissues. Based on these results, several studies have focused on developing p16 methylation assay in cell-free DNA from serum and plasma, and have

concluded that detection of p16 DNA methylation is serum may be an important biomarker for the early detection of GC [93–95].

3.3. Gastric juice-based DNA methylation diagnostic biomarkers in gastric cancer

The use of gastric juice as a molecular diagnostic or predictive tool has been attempted, but shown to be not practical due to denaturation of DNA in the high acidity in the stomach. However, since free circulating mucosal cells can be frequently found in the stomach juice, detection of DNA methylation in these cells presents a plausible platform for the development of a noninvasive diagnostic approach for the detection of GC. Bisulfite pyrosequencing is a quantitative approach for measuring DNA methylation, which has recently been used to detect DNA methylation in gastric washes, including methylation of MINT25, RORA, GDNF, ADAM23, PRDM5 and MLF1 [96]. Among these targets, MINT25 methylation demonstrated the highest sensitivity and specificity to differentiate GC patients from normal healthy controls. These findings suggest that DNA from gastric washes could be an appropriate alternative to DNA from biopsied tissues for GC screening.

3.4. Blood-based DNA methylation diagnostic biomarkers in colorectal cancer

CRC is a preventable disease, and can be easily cured by surgical procedures if the cancer is diagnosed early before metastasis is established. Early detection of CRC is thus a decisive step in the successful and complete cure of the disease. Among currently available screening tests for CRC, colonoscopy and fecal occult blood tests are most frequently used tests [97]. While colonoscopy is highly sensitive, it often requires hospitalization of the patient, is invasive and expensive: in contrast, fecal-occult tests are relatively simple to use, but have a low positive predictive value [98,99].

For the diagnosis of colon cancer, biomarkers that have high sensitivity and specificity are paramount. Conventional cancer markers, such as carcino-embryonic antigen (CEA), were developed by quantifying a small amount of circulating proteins. These markers are specific for certain types of cancer, permitting early detection of cancers and monitoring cancer relapse and cancer prognosis. However, this approach suffers from well-documented limited sensitivity and specificity [100]. Thus, the potential utility of nucleic acid markers in body fluids, such as serum and plasma, has been an area of intense investigation in recent years. PCR-based assay of small amounts of nucleic acids can detect and quantify genetic and epigenetic alterations in circulating tumor DNA [101]. Using the same principle, recent research has focused on the detection of methylated DNA in serum and plasma of patients with cancer vs. its absence in healthy controls [102]. In CRC, promoter hypermethylation analysis of blood and fecal DNA also has the potential to be used as a noninvasive test for the early diagnosis of CRC, and efforts are underway to develop these molecular markers for clinical applications. In Table 2, we summarize some of the key data on aberrantly methylated genes in stool and serum/plasma samples of patients with CRC.

Lofton-Day et al. recently described a sieving strategy for identifying high-performing markers that detect CRC-specific methylated DNA in plasma [103]. Three methylated genes, TMEF2, NGFR and SEPT9, selected from a list of 56 candidates were selected for further validation. All three genes demonstrated over 50% sensitivity in plasma DNA from CRC patients and over 69% specificity in controls. In particular, SEPT9 methylation had the highest probability of correctly distinguishing CRC patients from healthy controls, with 69% sensitivity and 86% specificity. Other reports concluded that the presence of aberrantly methylated SEPT9 in plasma is a valuable and non-invasive blood-based PCR test, showing a sensitivity of almost 70% and a specificity of 90% in detecting CRC patients [104–107]. Researchers have evaluated the possibility

Table 2
Aberrantly methylated genes as diagnostic biomarkers in body fluids, including serum, plasma and feces as in patients with colorectal cancer.

Methylated DNA	Source	Sensitivity	Specificity	Methods	Refs.
ALX4	Plasma	40%	82%	qMSP	[106]
MGMT	Plasma	39%	96%	MSP	[188]
NGFR	Plasma	51%	84%	qMSP	[103]
RARβ2	Plasma	24%	100%	MSP	[188]
RASSF2A	Plasma	58%	100%	MSP	[188]
SEPT9	Plasma	69%	86%	qMSP	[103]
SEPT9	Plasma	72% (training set), 68% (validation)	93% (screening), 89% (validation)	qMSP	[104]
SEPT9	Plasma	48% (training set), 58% (validation)	93% (screening), 90% (validation)	qMSP	[105]
SEPT9	Plasma	73%	91%	qMSP	[106]
SEPT9	Plasma	48%	92%	qMSP	[115]
TMEFF2	Plasma	65%	69%	qMSP	[103]
Vimentin	Plasma	59%	93%	Methyl-BEAMing	[77]
Wif-1	Plasma	74%	98%	MSP	[188]
ALX4	Serum	83%	70%	qMSP	[108]
NEUROG1	Serum	61%	91%	qMSP	[109]
CDKN2A/P16	Stool	31% (adenoma)	84%	MSP	[50]
FBN1	Stool	72%	93%	MSP	[119]
GATA4	Stool	71% (training set), 51% (validation)	84% (screening), 93% (validation)	qMSP	[116]
HIC1	Stool	42% (CRC), 31% (adenoma)	97%	MSP	[189]
ITGA4	Stool	69% (adenoma)	79%	qMSP	[189,190]
MGMT	Stool	48% (adenoma)	73%	MSP	[50]
NDRG4	Stool	61% (training set), 53% (validation)	93% (screening), 100% (validation)	qMSP	[117]
OSMR	Stool	38%	95%	qMSP	[118]
PGR	Stool	78%	69%	qMSP	[191]
SFRP2	Stool	83%	74%	qMSP	[191]
SFRP5	Stool	78%	65%	qMSP	[191]
Vimentin	Stool	46%	90%	MSP	[192]
Vimentin	Stool	72.5%	86.9%	qMSP	[123]
Vimentin	Stool	41% (CRC), 45% (adenoma)	95%	Methyl-BEAMing	[77]
SFRP2	Stool	63.1% (CRC), 32.1% (adenoma)	92%	COBRA	[120]
RASSF2	Stool	45.3% (CRC), 12.6% (adenoma)	94.7%	COBRA	[120]

MSP, methylation-specific PCR; qMSP, quantitative methylation-specific PCR; COBRA, Combined bisulfite restriction analysis.

of including the methylation analysis of additional genes, such as ALX4 [108] and HLTF [109], which were known to be potential diagnostic markers of CRC, respectively, to further enhance the sensitivity of this serum/plasma based test [106,107].

Although many efforts have been made to discover novel methylated DNA markers for CRC detection, combined analysis of several genes rather than a single gene has shown to improve the clinical efficacy. Array-based methylation profiling implicated in carcinogenesis demonstrated that methylation analysis of a gene panel containing CYCD2, HIC1, PAX5, RASSF1A, RB1 and SRBC could differentiate CRC patients and controls with 84% sensitivity and 68% specificity [110]. Moreover, a genome-scale marker discovery approach revealed that analysis of methylated THBD and C9orf50 outperformed CEA serum measurement and resulted in a highly sensitive and specific test for the early detection of colorectal cancer [111].

During clinical validation, parameters such as area under ROC curve (AUC) are common indicators used for evaluating efficiency of a diagnostic assay. In addition, the sample size used in each study directly affects the significance of the results. However, most of the biomarkers identified till date lack convincing ROC analysis, primarily because of the limited number of cases enrolled in various reports. Moreover, most studies have only included a small number of healthy controls; leading to poor characterization of normal patterns of DNA methylation. Importantly, some methylation markers did not yield positive results, even in benign diseases or heavy smokers [112,113]. These results indicate the relatively poor specificity of these potential biomarkers [103,114], and highlight the need for validation of such methylation biomarkers in large-scale clinical trials, preferably prospective trials, for the determination of their true specificity and sensitivity. The first commercially available DNA methylation test for diagnosis of early CRC is the detection of SEPT9 methylation. In a prospective trial with 7941 asymptomatic average risk individuals undergoing screening, the first generation of the SEPT9 methylation test detected up to 48.2% of cancer cases, with a specificity as 91.5%. However, the low sensitivity (11.2%) for advanced adenoma hindered its clinical utility for cancer screening [115]. Currently, blood-based assays that detect methylated SEPT9 are being marketed as colorectal cancer screening tests under names of Epi proColon 1.0 (Epigenomics), ColoVantage™ (Quest Diagnostic) and RealTime ms9 (Abbott).

3.5. Fecal-based DNA methylation diagnostic biomarkers in colorectal cancer

Identification of tumor-derived DNA alterations in stool is also an approach with high potential as a noninvasive detection tool for CRC. Recently, increasing numbers of genes methylated in tissue and stool from CRC patients have been identified. High incidence of NDRG4 and GATA4/5 methylation was found in colorectal adenomas and cancers [116,117]. Based on these observations, the potential value of NDRG4 and GATA4/5 as early diagnostic markers was evaluated by two cohorts of stool specimens from CRC patients and healthy controls. The results demonstrated that aberrant methylation of NDRG4 was detected in more than 50% of fecal DNA from CRC patients with 100% specificity [117]. In addition, GATA4 methylation in stool also differentiated CRC patients from healthy controls with high sensitivity (>50%) and specificity (>84%) [116]. Methylation of Oncostatin M receptor-B (OSMR), which is an intelukin-6 cytokine family member, was also detected in the stool of 38% of CRC patients with 95% specificity [118]. Collectively, the high specificities of NDRG4, GATA4 and OSMR emphasize the value of methylation markers for stool-based CRC detection. A more recent study revealed that hyper-methylated FBN1 was found in stool DNA from CRC patients and showed 72% sensitivity and >90% specificity for detecting CRC [119]. Another study from our laboratory demonstrated that methylation of RASSF2 and SFRP2 promoters in fecal DNA from patients with gastric and colorectal cancer had a very high sensitivity and specificity in identifying cancer patients, as well as allowed discriminating between patients with GC vs. CRC [120]. Several other hypermethylated genes isolated from stool samples have been identified as biomarkers for the detection of CRC or colorectal adenoma, including APC, p16, hMLH1, MGMT, SFRP1 and VIM [121], however, the sensitivity and specificity reported for these DNA methylation markers was highly variable. A recent meta-analysis showed that the mean sensitivity for CRC and adenoma were 64% and 62%, with mean specificities of 90% and 89%. Collectively, these findings are encouraging, and build the promise that the performance of methylation testing in fecal DNA is reasonably accurate and perhaps better than conventional FOBT, which has a sensitivity of less than 15% for the detection of CRC or adenoma and a specificity of over 90% [121,122]. As a testament to this approach, at this time, stool-based methylated VIM is already clinically validated as a marker for early detection of CRC, with a sensitivity and specificity of almost 80%, and is now commercially available in the United States under the name ColoGuard assay (LabCorp) [123,124].

4. Potential of non-coding RNAs in cancer diagnostics

4.1. Overview of non-coding RNAs

The central dogma of gene expression is that DNA is transcribed into mRNA, which then serves as the template for protein synthesis. [125,126]. Extensive research over the last few decades has focused on the role of protein-coding genes in the pathogenesis of human cancer. However, recent technological advances, such as tiling arrays and RNA sequencing (RNA-seq), have made it possible to survey the transcriptomes of many organisms to an unprecedented degree, the results of which have led to both great insights and unexpected conundrums. In fact, a minority of the transcripts are protein coding genes (1.5%), and the rest, which used to be referred as "dark matter", are now known to be the non-coding RNA (ncRNA) transcripts [127], which have significant gene regulatory function in complex organisms.

Based on their transcript size, ncRNAs can be grouped into two major classes. Those transcripts that are shorter than 200 nucleotides (nt) are usually recognized as small ncRNAs, which include Piwi-interacting RNAs, small-interfering RNAs, miRNAs and some bacterial-regulatory RNAs. Among these, miRNAs have been most widely explored and cited in the present literature. The miRNAs are about 19–24 nt long and serve as major regulators of gene expression through their ability to bind and post-transcriptionally mediate the expression of targeted mRNAs [128]. MiRNAs play a central role in cellular differentiation, development, proliferation and apoptosis, in a variety of cell types, including cancer cells. In cancer, all of these processes are deregulated through altered expression of miRNAs, suggesting that miRNAs are involved in carcinogenesis and could contribute to the initiation and progression of cancer [129]. In addition to their biological role in cancer, tumor-specific miRNA expression profiles have been found to be more informative and discriminatory compared with mRNA profiles between healthy and malignant cells, which provides the basis of their exploitation as potential for development as cancer biomarkers [130].

In addition to the relatively well-described miRNAs, increasing knowledge of the mammalian non-coding transcriptome has revealed that the genome is also replete with long ncRNAs (lncRNAs). LncRNA are classified as over 200 nt long transcripts that lack

a functional open reading frame. These lncRNAs are also involved in cellular differentiation and proliferation. The mechanisms through which they act are as molecular scaffolds, which are associated with the transcriptional machinery as post-transcriptional regulators of splicing or as molecular decoys for miRNAs [131,132]. LncRNA research is a relatively young field that is emerging in molecular genetics; and thus far only a small number of lncRNAs have been partially characterized. Compared to miRNAs, there is a very small body of literature on lncRNAs at this time. Nonetheless this limited evidence still touts the promise that lncRNAs may also have the potential for development as biomarkers for diagnosis and prognosis in cancer patients. In this section, we will highlight the potential of miRNAs in cancer diagnosis, and will focus this article specifically on miRNAs that have been studied as noninvasive biomarkers in body fluids.

5. Potential microRNAs in cancer diagnosis

5.1. MiRNA stability and function in body fluids

The development of any biological molecules as biomarkers that can be potentially evaluated in human specimens depends on their stability and resistance to storage and handling. RNase, present in body fluids such as blood, rapidly degrades RNA molecules, particular mRNA, and thus affects their stability [133]. As a result, the development of RNA-based molecular biomarkers has been challenging in the last couple of decades. In contrast, endogenous miRNAs in serum or plasma have been shown to remain stable, even when subjected to extreme conditions such as boiling, very low or high pH levels, extended storage time and multiple freeze-thaw cycles [133,134]. In addition, miRNAs have been easily detectable in archival tissues, and human serum specimens stored for more than a decade, highlighting their stability and resistance to degradation [135]. These unique features of miRNAs in body fluids provide a compelling advantage of these new and more useful biomarkers for cancer diagnosis [134,136]. In general, cancer-related miRNAs function as oncogenes (oncomiRs), tumor suppressor genes (tsmiRs) or both, in a tissue-specific context [3]. In addition, circulating miRNAs have a large number of biological effects proximally or distally to various types of cells, and could be delivered independent of cell contact or adhesion. Additionally, circulating miRNAs could also deliver multiple messages on each occasion and control numerous target genes simultaneously [137].

5.2. Methods to detect miRNAs in body fluids

The main techniques to detect miRNA expression in biological specimens include microarrays, deep sequencing or next-generation sequencing (NGS), and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Microarrays have long been used to determine miRNA expression profiles and identify cancer-related miRNAs. However, this technique can only screen expression of previously known miRNAs only. In contrast, NGS has several advantages over conventional microarrays in characterizing the miRNA transcriptome including its ability to identify novel previously unknown miRNAs, as well as detection of low abundance miRNAs exhibiting expression differences between distinct samples [138,139]. Importantly, NGS offers a highly robust, accurate and scalable system for rapid and productive investigation of miRNAs expression on a genome-wide scale [138,139]. However, both microarrays and NGS systems are expensive and labor-intensive techniques, requiring a large amount of RNA sample and need complex bioinformatics data analysis. Furthermore, analysis of the large number of genes, including those whose changes are not associated with cancer, in clinical settings is not ordinarily necessary. In contrast, qRT-PCR can be more easily performed and is a cost-effective technique. Importantly, this technique has already been widely used in clinical settings for analysis of other disease-biomarkers. Therefore, qRT-PCR could be applied in laboratory settings to examine the expression of miRNA-based biomarkers.

Although qRT-PCR demonstrates promising performance in the determination of miRNA expression in clinical samples, several obstacles still must be overcome before this technique can be routinely employed in clinical settings. For example, miRNAs exist as single stranded molecules; therefore, methods that amplify these molecules usually use one specific and one universal primer, which results in somewhat lower specificity compared to the use of two strand-specific primers [140]. The other limitation is that different sample preparation methods make it difficult to compare miRNA expression profiles among experiments carried out at different times. Extraction of miRNAs from serum and plasma is relatively easy. However, some miRNAs are highly expressed in blood cells, and the levels of circulating miRNA biomarkers can be altered significantly by extent of hemolysis [141]. Thus, detection approaches that combine measurement of hemoglobin levels and expression of hemolysis-related miRNAs, like miR-15b and miR-16, may be required to determine whether such samples are suitable for further quantification of target miRNAs [142,143]. Another key hurdle in the analysis of circulating miRNAs is the lack of consensus on suitable reference genes to normalize the expression of unknown target miRNA in body fluids. The most commonly used internal controls or reference genes are miR-16 or the small nucleolar RNA RNU62 and SNORD43. Furthermore, synthetic versions of specific C. elegans miRNAs, like cel-miR-39, cel-miR-54 and cel-miR-238, have also been used as internal control genes. However, as described above, miR-16 is highly expressed in erythrocytes and its levels in circulation can be affected by hemolysis [141]. In addition, RNU62 is a poor representative because it is not synthesized by the same polymerases that synthesize precursor miRNAs [144]. Therefore, at present, the standardization of sample processing and normalization of miRNAs analysis methodology are the most urgent requirements for preclinical screening and validation, which once resolved will promote the development of miRNA biomarkers in body fluids for clinical applications.

5.3. MiRNAs as diagnostic biomarkers in body fluids

Detecting the pre-malignant or early stage cancers currently represents one of the major challenges in modern medicine. The ability to identify cancers in their earliest stages of growth would allow for rapid intervention and, consequently, improve patient outcomes while reducing the necessity for invasive procedures. In this section, we summarize the recent findings with regards to cancer-related circulating miRNAs in GC and CRC. We also present a comprehensive summary of cell-free miRNAs present in circulation, gastric juice or feces, which are associated with cancer diagnosis.

5.3.1. Blood-based miRNA biomarkers in gastric cancer

Despite promising developments in treatment, the overall outcome of patients suffering from GC remains poor. Availability of suitable biomarkers that can facilitate early detection and clinical management of GC are highly desirable [145]. Table 3 summarizes key studies that have explored the diagnostic significance of miRNA biomarkers in body fluids of patients with gastric neoplasia. In order to develop circulating miRNA biomarkers for early detection of GC, Tsujiura et al. first determined expression of five miRNAs in plasma, based on their previous findings in GC tissues, in 69 GC patients and 30 healthy controls [146]. Plasma miR-17-5p, -21, -106a and -106b were observed to be at significantly higher levels, whereas let-7a expression was lower in GC patients compared to

Table 3
Circulating miRNAs as diagnostic biomarkers in patients with gastric cancer.

miRNA	Sample	Type of biomarker	miRNA levels and clinical significance	Refs.
let-7a	Plasma	Diagnosis	Decreased	[146]
miR-1	Serum	Diagnosis	Increased; correlated to TNM stage	[147]
miR-17-5p	Plasma	Diagnosis	Increased	[146]
miR-17-5p	Plasma	Diagnosis/prognosis	Increased; correlated to TNM stage, a poor OS	[154]
miR-20a	Serum	Diagnosis	Increased; correlated to TNM stage	[147]
miR-20a	Plasma	Diagnosis/prognosis	Increased; correlated to TNM stage, a poor OS; independent risk predictor for prognosis	[154]
miR-20a	Plasma	Diagnosis	Increased	[152]
miR-21	Plasma	Diagnosis	Increased; decrease in post-operative samples	[146]
miR-21	Plasma	Diagnosis	Increased	[151]
miR-27a	Serum	Diagnosis	Increased; correlated to TNM stage	[147]
miR-34	Serum	Diagnosis	Increased; correlated to TNM stage	[147]
miR-106a	Plasma	Diagnosis	Increased; decrease in post-operative samples	[146]
miR-106b	Plasma	Diagnosis	Increased	[146]
miR-106b	Plasma	Diagnosis	Increased	[152]
miR-151-5p	Plasma	Diagnosis	Increased; decrease in post-operative samples	[153]
miR-187*	Serum	Diagnosis	Increased	[148]
miR-199a-3p	Plasma	Diagnosis	Increased; decrease in post-operative samples; associated with lymph node metastasis and TNM staging	[153]
miR-218	Plasma	Diagnosis	Decreased	[151]
miR-221	Serum	Diagnosis	Increased in cancer and dysplasia compared to controls; correlated with poor differentiation of cancer; showed retrospective correlation in pre-diagnostic samples	[149]
miR-221	Plasma	Diagnosis	Increased	[152]
miR-223	Plasma	Diagnosis	Increased; associated with helicobacter pylori infection	[151]
miR-371-5p	Serum	Diagnosis	Increased	[148]
miR-376c	Serum	Diagnosis	Increased; correlated with poor differentiation of cancer; showed retrospective correlation in pre-diagnostic samples	[149]
miR-378	Serum	Diagnosis	Increased	[148]
miR-423-5p	Serum	Diagnosis	Increased; correlated to TNM stage	[147]
miR-451	Plasma	Diagnosis	Increased; decrease in post-operative samples	[150]
miR-486	Plasma	Diagnosis	Increased; decrease in post-operative samples	[150]
miR-744	Serum	Diagnosis	Increased; showed retrospective correlation in pre-diagnostic samples	[149]
miR-21	Gastric juice	Diagnosis	Decreased in GC; correlated with TNM stage. High expression in intestinal type compared to diffuse type.	[159]
miR-106a	Gastric juice	Diagnosis	Decreased in GC; correlated with TNM stage.	[159]
miR-129-1-3p	Gastric juice	Diagnosis	Decreased in GC	[158]
miR-129-2-3p	Gastric juice	Diagnosis	Decreased in GC	[158]
miR-421	Gastric juice	Diagnosis	Decreased in GC	[157]

TNM, primary tumor (T), regional nodes (N), and metastasis (M); OS, overall survival.

healthy controls. In addition, the investigators observed a significant drop in the levels of miR-21 and -106b in postoperative plasma samples, as well as a correlation between tissue and plasma levels, suggesting that the levels of plasma miRNAs reflected the expression levels of matched primary GC [146]. In contrast, Liu et al. utilized Solexa sequencing to carry out global miRNA screening using pooled serum samples from patients with GC and healthy individuals. A biomarker signature comprising of 5 miRNAs (miR-1, -20a, -27a, -34a and -423-5p) was identified for the diagnosis of GC with high sensitivity and specificity. The discriminatory ability of this biomarker panel was claimed to be even higher than the currently established tumor marker (CEA or CA19-9), highlighting that the use of multiple miRNAs related to tumorigenesis is a more comprehensive approach to diagnosing GC than a conventional single protein marker [147]. With respect to serum miR-187, -371-5p and -378, identified by genome-wide miRNA expression profiles followed by qRT-PCR, further validation indicated that miR-378 by itself had a strong potential in detecting patients with GC [148]. Another study using miRNA profiling demonstrated that analysis of a three-miRNA panel (miR-221, -376c and -744) in serum could distinguish GC patients from healthy controls. Moreover, this combination of miRNAs also accurately classified early GC patients, indicating that it represents a biomarker for early detection of GC [149]. An interesting approach, comparing pre- and post- operative matched serum samples, identified candidate GC-associated miRNAs (miR-451 and -486) that were expressed significantly lower in post-operative serum. Validation data confirmed that both miRNAs were present in higher amounts in GC patients compared with controls [150]. Another study assessing the diagnostic significance in plasma samples from GC patients demonstrated that while miR-21 and -223 were higher, miR-218 was significantly lower in cancer patients compared to healthy controls. An interesting finding of this study was that plasma levels of miR-223 associated with *H. pylori* infection, which were correlated significantly with gastric carcinogenesis etiologically [151]. Recently, Cai et al. re-assessed the clinical utility of 15 miRNAs that were previously identified as potential diagnostic biomarkers for GC in various independent studies [146–150]. Expression of three of the 15 miRNAs (miR-106b, -20a and -221) in serum was confirmed as potential biomarkers for the early detection of GC [152]. Other miRNAs proposed as diagnostic markers for GC include miR-151-5p and -199a-3p, which are expressed at very high levels in the plasma of GC patients. Of these, miR-199a-3p was significantly associated with lymph node metastasis and progression of TNM [primary tumor (T), regional nodes (N), and metastasis (M)] staging, thus emphasizing the diagnostic significance of these miRNA biomarkers [153].

Of the above-described miRNAs, miR-17-5p, miR-20a and miR-21 were tested in an independent study and shown to be elevated in GC patients, and correlate with tumor progression and poor prognosis [154,155]. Cox regression analysis in these two articles revealed that the elevated levels of circulating miR-20a and miR-21 significantly correlated with overall survival, indicating poor prognosis. On the other hand, high levels of circulating miR-196a,

which is associated with epithelial-to-mesenchymal transition closely correlated with tumor recurrence, suggesting its potential utility as a disease monitoring biomarker following surgery [156].

5.3.2. Gastric juice-based miRNA biomarkers in gastric cancer

As shown above, several independent research groups have now demonstrated that circulating miRNAs in serum or plasma can be potential diagnostic biomarkers for GC. However, an important issue that needs to be addressed is that several types of cancers share most of these miRNAs, when measured in systemic circulation. Therefore, for the diagnosis of GC, perhaps the analysis of miRNAs in gastric juice has obvious advantages because gastric juice is present only in the stomach. Recently, few studies have provided proof-of-principle data in which it was demonstrated that the expression of several miRNAs were stable in gastric juice. In these reports, the expression of miR-21, miR-106a, miR-129-1-3p, miR-129-2-3p and miR-421 was analyzed in gastric juice, and all of these miRNAs were expressed at higher levels in GC tissues, suggesting their potential usefulness as biomarkers for screening patients with GC [157–159].

5.3.3. Blood-based miRNA biomarkers in colorectal cancer

Several studies have specifically addressed the role of circulating miRNAs in patients with CRC (Table 4). These reports revealed that blood-based (serum or plasma) miRNA biomarkers might constitute an accurate diagnostic method for CRC [160]. The first study on the diagnostic usefulness of serum-based miRNA biomarkers in CRC reported that a subset of 69 miRNAs was highly expressed in CRC patients compared to healthy controls. Additionally, 14 of these serum-based miRNAs from CRC patients were not detectable in those with lung cancer, highlighting the CRC-specificity of these miRNAs [134]. In a follow-up study, Ng and colleagues performed a more systematic and comprehensive analysis by profiling miRNAs in matched tissue and plasma specimens, and discovered 95 CRC-specific miRNAs in plasma samples [161]. Ultimately, miR-17-3p and miR-92, both belonging to the same miRNA gene cluster and known to be oncogenic, were confirmed to be elevated in both CRC tissues and plasma. In addition, these two miRNAs were significantly reduced following surgical resection of the tumors in patients with CRC, reinforcing their potential role as disease monitoring biomarkers for patients that undergo curative surgeries. More interestingly, plasma miR-92 has the potential to differentiates CRC from other diseases, such as GC and inflammatory bowel disease [161]. The diagnostic ability of miR-92a was further verified by another study in plasma, which also illustrated that miR-29a was increased in CRC, and was reduced following removal of tumors by surgery. A more important finding of this study was that miR-92a and miR-29a could even discriminate patients with

Table 4
Circulating miRNAs as diagnostic biomarkers in patients with colorectal cancer.

miRNA	Sample	Type of biomarker	miRNA levels and clinical significance	Refs.
miR-15b	Plasma	Diagnosis	Increased	[164]
miR-17-3p	Plasma	Diagnosis	Increase; decrease in post-operative samples	[161]
miR-18a	Plasma	Diagnosis	Increased in CRC and advanced adenomas compared with controls	[164]
miR-18a	Serum	Diagnosis	Increase in stage III CRC compared with controls	[193]
miR-19a	Plasma	Diagnosis	Increased	[164]
miR-21	Plasma	Diagnosis	Increased	[168]
miR-21	Serum	Diagnosis	Increased in CRC and advanced adenomas compared with controls	[169]
miR-21	Serum	Diagnosis/ prognosis	Increased in CRC and advanced adenomas compared with controls	[170]
miR-21	Serum	Prognosis	Correlates with the recurrence and mortality of CRC patients	[174]
miR-29a	Plasma	Diagnosis	Increased in CRC and advanced adenomas compared with controls	[162]
miR-29a	Plasma	Diagnosis	Increased	[164]
miR-29a	Serum	Diagnosis	Increased in CRC with liver metastasis compared with non-metastatic CRC	[163]
miR-29a	Serum	Diagnosis	Increase in stage III CRC compared with controls	[193]
miR-92	Plasma	Diagnosis	Increase; decrease in post-operative samples; can differentiate CRC from gastric cancer, IBD and controls	[161]
miR-92a	Plasma	Diagnosis	Increased in CRC and advanced adenomas compared with controls	[162]
miR-92a	Serum	Diagnosis/ prognosis	Increased in CRC and advanced adenomas compared with controls	[169]
miR-141	Plasma	Prognosis	Increased; correlated to OS	[172]
miR-200c	Serum	Prognosis	Increased; correlated to OS, DFS and metastatic phenotype (lymph node and liver metastasis)	[173]
miR-221	Plasma	Diagnosis/ prognosis	Increased; correlated to OS and p53 score	[175]
miR-335	Plasma	Diagnosis	Increased	[164]
miR-601	Plasma	Diagnosis	Decreased in CRC and advanced adenomas compared with controls	[165]
miR-760	Plasma	Diagnosis	Decreased in CRC and advanced adenomas compared with controls	[165]
miR-1246	Plasma/ serum	Diagnosis	A pseudo-microRNA representing an RNU2-1 fragment	[171]
miR-17	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[178]
miR-18a	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[178]
miR-19a	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[178]
miR-19b	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[178]
miR-21	Stool	Diagnosis	Upregulated in stool of CRC and adenoma patients compared with controls	[177]
miR-21	Stool	Diagnosis	Upregulated in stool of CRC and adenoma patients compared with controls. Downregulated in stool after resection of CRC	[179]
miR-20a	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[178]
miR-92a	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[178]
miR-92a	Stool	Diagnosis	Upregulated in stool of CRC and adenoma patients compared with controls. Downregulated in stool after resection of CRC	[179]
miR-106a	Stool	Diagnosis	Upregulated in stool of CRC and adenoma patients compared with controls	[177]
miR-106a	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[194]
miR-135a	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[178]
miR-135b	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[178]
miR-144	Stool	Diagnosis	Upregulated in stool of CRC patients compared with controls	[180]

OS, overall survival; DFS, disease free survival; IBD, inflammatory bowel disease.

premalignant colorectal adenomas with high accuracy, which might further influence their diagnostic utility [162]. Another study indicated that miR-29a levels in serum could differentiate metastatic and non-metastatic patients with CRC, especially hepatic metastases [163]. Furthermore, by undertaking miRNA profiling followed by analytical validation of results, miR-601 and miR-760 were found to be decreased in patients with colorectal adenomas and cancers, indicating the promise of these as screening biomarkers for colorectal neoplasia [164]. In addition, a combination of miR-760, miR-29a and miR-92a improved the detection sensitivity of early stages of CRC, when complemented with CEA [165]. Additionally, a genome-wide miRNA profiling of plasma identified miR-15b, -19a, -19b, -29a and -335 expression levels to discriminate CRC patients from healthy controls, whereas miR-18a was able to also discriminate patients with advanced adenomas from healthy controls [164].

One of the most commonly studied miRNAs in cancer, which is also upregulated in CRC compared to normal colonic mucosa, is miR-21. The miR-21 is known to be an onco-miR that functions by increasing cell proliferation and invasion, and by inhibiting cellular apoptosis [166,167]. Recently, using a strategy that profiled miRNAs between CRC and normal tissues followed by validation of the candidate miRNAs in plasma, miR-21 was identified to be a biomarker for the early detection of CRC [168]. The diagnostic potential of circulating miR-21 was further confirmed in other studies. Liu and coworkers demonstrated that serum miR-21 and miR-92a levels could be promising diagnostic markers of advanced adenomas and CRC [169]. Likewise, work from our laboratory has also revealed that tumor-derived serum miR-21 could differentiate patients with CRC and advanced adenoma from healthy controls, and its levels dramatically were reduced after curative resection, indicating that circulating miR-21 is a promising diagnostic marker of CRC incidence and recurrence after curative surgery [170]. In spite of being designated as a pseudo-miRNA, miR-1246 has also been proposed as a possible candidate biomarker for CRC in both serum and plasma, as well as an early predictive marker for response to chemo- and radiation therapy because of its plummeted levels following surgical resection [171].

Although all the studies described above have introduced the diagnostic potential of circulating miRNAs in CRC, to date, limited effort has been made with regard to their prognostic usefulness. Circulating miR-141 and miR-200c, which belong to the miR-200 family that is associated with epithelial-to-mesenchymal transition, were significantly higher in CRC patients with metastases than in those without, and were proposed as useful prognostic biomarkers in CRC [172,173]. In addition, oncogenic miR-21 and miR-221 in circulation were identified to have prognostic utility in addition to being diagnostic biomarkers for CRC [170,174,175].

5.3.4. Fecal-based miRNA biomarkers in colorectal cancer

Although a blood test appears to be easier to perform because of its higher compliance and acceptance, it is likely that the earliest detectable changes in the expression pattern of specific miRNAs may be in feces rather than blood. Furthermore, because of their stability and ease of detection, fecal miRNAs also represent a novel and promising noninvasive approach for the early detection of colorectal neoplasia. Thus far, only a handful of studies have reported the potential of fecal miRNA biomarkers, a concept that requires validation in large prospective, or even retrospective, trials. Initial evidence for the feasibility of fecal miRNAs as screening markers for CRC was presented by Ahmed et al. [176]. These researchers reported that the expression levels of several miRNAs are disease-specific, and can distinguish healthy controls from patients with inflammatory bowel disease, including ulcerative colitis, Crohns' disease and CRC; and could differentiate among different Dukes' stages in CRC patients.

We reported the feasibility of direct miRNA detection in fecal specimens from healthy subjects and patients with colorectal adenomas and cancers [177]. In this study, we developed a one-step fecal miRNA extraction and amplification method (called direct miRNA analysis or DMA), and demonstrated that the levels of fecal miR-21 and miR-106a were significantly increased in patients with colorectal adenomas and cancers compared to healthy controls.

In the largest cohort study to date, Koga and colleagues [178] used immune-magnetic beads conjugated with an EpCAM monoclonal antibody to isolate colonocytes from stools. Using the isolated colonocytes, the authors reported that fecal miR-17-92 cluster and miR-135 were significantly overexpressed in CRC patients, with very high specificity and specificity, respectively. Although the expression levels of miR-21 were increased in cancer tissues, in fecal samples, this difference was not significantly different between CRC patients and healthy volunteers [178]. In contrast, a subsequent study demonstrated that patients with CRC had significantly higher fecal miR-21 and miR-92a levels compared with normal controls, and the expression levels of these miRNAs decreased significantly following tumor resection [179]. Likewise, it was recently shown that miR-144 was overexpressed in fecal specimens, and that it could be a viable candidate diagnostic marker for CRC detection, with a sensitivity of 74% and a specificity of 87% [180]. Taken together, there is lot of excitement and promise that both circulating miRNAs in blood and fecal miRNAs have the potential for development as noninvasive diagnostic and prognostic biomarkers in patients with CRC.

6. Conclusions and perspectives

In recent years, the role of epigenetic alterations in carcinogenesis has received greater attention than before. After the initial elucidation of the fundamental role of epigenetic changes in human carcinogenesis, considerable efforts have been dedicated to the identification and development of epigenetic biomarkers for cancer detection, disease monitoring for tumor recurrence and prognostic outcomes [9,181]. The presence of cell-free methylated-DNA and miRNAs in circulation provide a paradigm-shifting perspective in the development of cancer biomarkers for non-invasive early detection of these two malignancies. Promising data have been gathered using advanced, state-of-the-art technologies to measure levels of circulating nucleic acids with a very high degree of sensitivity and specificity. Undoubtedly, the clinical significance of epigenetic markers individually, or as biomarker panels, is poised to lead the way for the development of actionable, highly promising assays that will play a pivotal role in the personalized care of patients suffering from this highly morbid cancers [110,182]. However, before we get to that stage, we must act collaboratively and overcome some of the limitations discussed previously, and develop standardized methodologies, including sample storage, DNA or RNA extraction and quantification of circulating epigenetic biomarkers. To genuinely reap the benefits of all the fascinating research that has been conducted in the recent years, we must conduct carefully designed, sufficiently powered, prospective clinical trials to validate the true significance of these biomarkers – a step that would be truly clinically transformative, in routine diagnosis and management of patients afflicted with gastrointestinal and other cancers [183,184].

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