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Epigenetics of colorectal cancer: biomarker and therapeutic potential

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Abstract

Colorectal cancer (CRC), a leading cause of cancer-related death worldwide, evolves as a result of the stepwise accumulation of a series of genetic and epigenetic alterations in the normal colonic epithelium, leading to the development of colorectal adenomas and invasive adenocarcinomas. Although genetic alterations have a major role in a subset of CRCs, the pathophysiological contribution of epigenetic aberrations in this malignancy has attracted considerable attention. Data from the past couple of decades has unequivocally illustrated that epigenetic marks are important molecular hallmarks of cancer, as they occur very early in disease pathogenesis, involve virtually all key cancer-associated pathways and, most importantly, can be exploited as clinically-relevant

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Author contributions

A.G., L.M. and F.B. made substantial contributions to discussion of the article contents. All authors researched data for the article, wrote the manuscript, and reviewed and/or edited the manuscript before submission.

Competing interests

F.B. declares that he has endoscopic equipment on loan from Fujifilm, and has received an honorarium for consultancy from Sysmex and speaker's fees from Norgine. The other authors declare no competing interests.

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Review criteria

Unbiased PubMed searches were performed for each potential epigenetic biomarker in combination with relevant search terms for colorectal cancer, after which titles and abstracts of studies from the past ten years were screened for relevance. Meta-analyses, reviews and studies evaluating biomarker panels were also included. The most promising diagnostic, prognostic and predictive biomarker candidates were selected and ranked based on the total number of identified publications, the statistical study design and power, independent validation, potential as non-invasive biomarkers, and biological functionality. Detailed Review criteria can be found in Supplementary Box 1.

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disease biomarkers for diagnosis, prognostication and prediction of treatment response. In this Review, we summarize the current knowledge on the best-studied epigenetic modifications in CRC, including DNA methylation and histone modifications, as well as the role of non-coding RNAs as epigenetic regulators. We focus on the emerging potential for the bench-to-bedside translation of some of these epigenetic alterations into clinical practice and discuss the burgeoning evidence supporting the potential of emerging epigenetic therapies in CRC as we usher in the era of precision medicine.

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Epigenetic modifications and regulators, including DNA methylation, histone modifications and non-coding RNA species, have key pathophysiological roles in colorectal cancer (CRC). This Review outlines these epigenetic aberrations in CRC and their potential as diagnostic, prognostic and predictive biomarkers and therapeutic targets.

Introduction

Colorectal cancer (CRC) remains one of the leading causes of cancer-related death worldwide. For 2018, the International Agency for Research on Cancer (IARC) estimates an incidence of ~1.8 million new cases of CRC (≈10% of all cancers) and >860,000 CRC-related deaths worldwide (≈9% of all cancer-related deaths)[1]. In Europe, 388,181 new CRC cases and 174,381 CRC related deaths have been estimated for 2018 (REF.[2]), whereas the American Cancer Society estimates 145,600 new CRC cases (≈12.6% of all cancers) and 174,681 CRC-related deaths (≈8% of all cancer-related deaths) for 2019 in the USA[3]. Accordingly, CRC is the third most frequent cancer worldwide in both sexes, and has high mortality rates of 45%, 35% and 47.8% in Europe, the USA and worldwide, respectively[1–3]. Thus, the development of effective treatments for patients with CRC is an urgent unmet clinical need.

Epigenetics, defined as heritable alterations in gene expression that do not result in permanent changes in the DNA sequence, has a central role in the pathogenesis of various cancers, including CRC[4]. Over the past two decades, the study of epigenetic changes has elucidated the missing link between certain CRC-specific gene expression patterns and the absence of genetic alterations. For instance, microsatellite instability (MSI) — one of the hallmarks of a molecular subgroup of CRC[5] — is the result of a deficiency in the DNA mismatch repair (MMR) system, which cannot only be the consequence of a genetic mutation in one of the MMR genes but also of epigenetic silencing of the *MLH1* gene by hypermethylation of its promoter[6]. Global hypomethylation has also been shown to lead to chromosomal instability in CRC[7]. In addition, microRNAs (miRNAs; also known as miRs) can prevent protein expression and influence many cancer-related pathways at the post-transcriptional level, and have a role in virtually all CRC stages, from initiation to progression and metastasis[8]. For example, miR-143 prevents cell growth by directly targeting the *KRAS* mRNA transcript and was found to be frequently downregulated in CRC[9]. These insights, among others, have not only improved our understanding of CRC pathophysiology but have also opened the door to the discovery of new disease biomarkers and therapeutic targets.

In this Review, we first provide an overview of the basic principles of epigenetic modifications in CRC, including DNA methylation and histone modifications, as well as the role of non-coding RNAs (ncRNAs), such as miRNAs and long non-coding RNAs (lncRNAs), as epigenetic regulators. We then highlight promising epigenetic biomarkers that, after comprehensive appraisal of the literature (see Supplementary Box 1 for detailed Review criteria), we deem to have the greatest potential for rapid bench-to-bedside translation to improve diagnosis, prognostication and prediction of treatment responses in CRC over the next decades. Finally, we discuss the burgeoning evidence supporting some of these epigenetic alterations as putative therapeutic targets for the development of epigenetic therapies, which could form the basis of tailored precision medicine strategies in the future.

Principles of epigenetics

A number of epigenetic modifications, notably DNA methylation and histone modifications, and epigenetic regulators, including ncRNA species such as miRNAs and lncRNAs, have been implicated in the pathogenesis of various cancers, including CRC (FIG. 1).

DNA methylation

DNA methylation is one of the most ubiquitous epigenetic modifications regulating gene expression. The best characterized DNA methylation process involves the addition of a methyl group (CH_3) at the C5 position of the cytosine ring by DNA methyltransferases (DNMTs), yielding 5-methylcytosine[10]. In humans, this modification occurs at CpG dinucleotides, which are regions of DNA where cytosine residues are immediately followed by guanine residues in the 5′ 4to 3′ direction, linked by a C-phosphodiester-G-bond. In the mammalian genome, most of these CpG sites are methylated, including those within the gene bodies. By contrast, CpG islands are CpG-rich sequences that are generally unmethylated in mammals and that usually contain 200–2000 nucleotides, of which >50% are CpGs; approximately 60–70% of gene promoters contain CpG islands[11].

Alterations of normal DNA methylation patterns include DNA hypomethylation, which occurs pathologically in normally unmethylated regions of the genome, and DNA hypermethylation, which usually occurs in the CpG islands of gene promoters[10]. Although most of the hypermethylation events can (randomly) occur within the CpG residues in the promoter regions of already-inactivated genes in normal cells and have, therefore, no consequences on gene expression, robust evidence suggests that promoter CpG hypermethylation is associated with transcriptional suppression of tumour-suppressor genes in cancer cells (FIG. 1)[12]. This evidence is especially strong in the case of CRC, in which such aberrant hypermethylation has been identified in the promoter regions of important tumour-suppressor genes[12–14], including *CDKN2A* (at the promoters of each of its two encoded distinct cell cycle-regulatory proteins, p16INK4A[15,16] and p14ARF[17]), *MLH1*[18] and *APC*[19]. By contrast, hypermethylation outside of the CpG islands, especially in gene bodies, seems to positively correlate with gene expression[20].

Genome-wide hypomethylation was one of the first aberrant methylation events reported in CRC, and constitutes an early event in colorectal carcinogenesis. Indeed, hypomethylation has been observed at different disease stages, from early adenomas to adenocarcinomas and

metastases, with a linear correlation between the grade of demethylation and the disease stage[21–24]. As global loss of DNA methylation has also been described during normal ageing and senescence, its role in carcinogenesis (and, therefore, as an independent risk factor) is the subject of an ongoing debate; however, DNA methylation has been hypothesized to be the missing link explaining why cancer is an age-related disease[25].

Generally, DNA hypomethylation at three sites has been linked to proto-oncogene activation in CRC, including at: promoter regions, which can lead to loss of gene imprinting (for example, *IGF2*)[26] or directly activate proto-oncogenes (for example, *MYC* and *HRAS*)[27]; distant regulatory regions, such as super-enhancers, which have been described for the gene encoding β-catenin[27]; and antisense promoters located downstream in certain repetitive elements (such as long interspersed element-1 (LINE-1) elements), which are evolutionary silenced under normal physiological conditions[23]. As up to 17% of the human genome consists of LINE-1 elements, their hypomethylation has been used as surrogates for global DNA hypomethylation and is associated with early-onset CRC and poor prognosis, making LINE-1 a potentially important biomarker[28,29]. These LINE-1 elements, if activated through hypomethylation, can also function as retrotransposons through a ‘cut-and-paste’ mechanism, inserting themselves in distant fragile sites (unstable genomic regions) and leading to genomic instability. Accordingly, LINE-1 hypomethylation is inversely correlated with microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP)[30].

Histone modifications

In eukaryotic cells that are not dividing, DNA is wrapped around histones — which are protein octamers comprised of a pair of each of the four core histone proteins, histone 2A (H2A), H2B, H3 and H4 — into structural units called nucleosomes, which are packaged with other nuclear proteins to form chromatin. Each histone core protein possesses a characteristic tail that is enriched for lysine and arginine residues, which are subject to post-translational modifications that can influence gene expression either directly, by modifying the histone-DNA interaction, or indirectly, by altering recognition sites for specific binding proteins (FIG. 1)[31]. Several types of histone modifications have been described that are related to many cellular processes during both normal physiological growth and development and the pathogenesis of various diseases, including cancer. Histone modifications can be caused by genetic mutations in various histone modifiers, which are enzymes that catalyze post-translational modifications of the histone tails, such as histone deacetylases (HDACs) and Histone acetyletransferases (HATs)[32]. However, as describing these mutations is beyond the scope of this Review, we focus on the most common and best-studied post-translational histone modifications that alter gene expression in the context of CRC — histone acetylation and methylation[33].

Histone acetylation.—The acetylation and deacetylation of histones is catalyzed by HATs and HDACs, respectively. Histone acetylation neutralizes the positive charge on the histone tails, weakens the electrostatic interaction between the DNA and histones, and, consequently, influences the compaction state of chromatin[34]. Hyper-acetylation, specifically of histones associated with proto-oncogenes, activates gene expression, whereas hypo-acetylation of histones associated with tumour-suppressor genes, often localized within

their promoter regions, silences the respective genes, highlighting the important dual role of histone acetylation status in cancer development and progression[35]. Unsurprisingly, in view of these key cancer-related activities, these enzymes are garnering a lot of interest as potential therapeutic targets in various malignancies, including CRC[36].

Histone methylation.—In contrast to histone acetylation, histone methylation does not only change the compaction status of the DNA, but creates docking sites in the chromatin that can be recognized by various proteins, such as those comprising transcriptional complexes (for example, transcription initiation factor TFIID subunit 3 (TAF3), which can activate WNT-β-catenin target genes)[37,38]. The methyl groups are added to the lysine and arginine residues in the histone tails in a very specific manner, and can lead to the activation or repression of gene expression, depending on the residue that is methylated. Histone methylation regulates many biological functions that are crucial for normal cell differentiation and has a central role in carcinogenesis and tumour progression[39]. Histone methylation and demethylation are catalyzed by histone methyltransferases (HMTs) and histone demethylases (HDMs), respectively, the overexpression or underexpression of which might change the global histone methylation status, alter the expression of hundreds of oncogenes or tumour-suppressor genes and, eventually, promote cancer development or progression[37].

Non-coding RNAs

A large body of scientific evidence has unequivocally demonstrated that the ~98% of the non-protein-coding genome does, in fact, participate in the regulation of gene expression, in the context of both normal physiological development and the pathogenesis of virtually all diseases[40]. These transcriptional mediators, which are often referred to as ncRNAs, can be spliced after their transcription but are not translated into proteins, and can have pro-tumorigenic or anti-tumorigenic functions in a tissue-specific manner[41]. Since their discovery in the early 1990s, several thousand ncRNAs have been revealed, which can be broadly categorized according to their size or function (Supplementary Table 1)[42].

miRNAs.—Belonging to the group of small ncRNAs, miRNAs are short (18–25 nucleotides in length), single-stranded RNA species that, given their emerging roles in CRC, are a major focus of this Review[8]. They function as post-transcriptional repressors by binding to complementary sequences in the 3′-untranslated regions (UTRs) of their target mRNAs (FIG. 1), controlling the translation of >60% of protein-coding genes, including those regulating important pro-tumorigenic processes such as cell proliferation, differentiation and apoptosis (FIG. 2). miRNAs function either by regulating specific individual target mRNAs, or by acting as broad regulators of gene expression, whereby they can mediate the expression of hundreds of genes simultaneously. Interestingly, multiple miRNAs have also been shown to regulate the expression of a single target mRNA, illustrating a degree of functional redundancy in miRNA-dependent gene regulation[41].

As miRNAs are often located in fragile sites within the genome, their expression can be dysregulated through a variety of genetic alterations, including point mutations, deletions, amplifications or translocations[43]. In addition, both DNA hyper-methylation and hypo-

methylation can also alter miRNA expression[44]. Many studies have identified divergent miRNA expression levels between neoplastic tissues and tumour-adjacent normal tissues, including CRC[45]. Accordingly, miRNAs can be upregulated or downregulated in tumour tissues, although a greater proportion of miRNAs seem to be overexpressed, rather than underexpressed, in cancer. Finally, miRNAs can either function as oncogenic miRNAs (onco-miRs) by inhibiting the expression of tumour-suppressor genes, or as tumour-suppressive miRNAs (ts-miRs) by inhibiting oncogene expression[46].

LncRNAs.—The lncRNAs represent a diverse group of ncRNAs that are involved in many biological processes, and can be categorized either by their genomic location or by virtue of their function[47]. According to their genomic location and architecture, several types of lncRNAs have been described (Supplementary Table 1). With regard to their function, lncRNAs function as positive or negative regulators of transcription through a multitude of activities, including: interaction with gene promoters or enhancers; modification of chromatin access by acting as guidance molecules for chromatin-modifying protein complexes; regulation of the nuclear architecture; regulation of mRNA stability by direct interaction with target mRNAs and regulatory protein complexes; and by functioning as miRNA sponges that agglutinate miRNAs through multiple specific binding sites within the lncRNA sequences (FIG. 1)[42]. LncRNAs are involved in a broad spectrum of biological processes, including cell proliferation, differentiation, apoptosis and stem cell self-renewal, in a developmental and tissue-specific manner[48]. As a result of their diverse functions, lncRNAs have roles in many cancer-related pathways such as the WNT, EGFR, TGF-β and p53 signalling pathways, and can influence on virtually all pathophysiological steps in CRC carcinogenesis, progression and metastasis[49].

Crosstalk within the epigenetic network

The aforementioned epigenetic modifications are not isolated phenomena that influence gene expression, but rather function in an orchestrated manner to fine-tune a complex regulatory network with important crosstalk occurring between them. In this sense, miRNAs are themselves subject to epigenetic regulation through methylation of their promoter regions. Indeed, this event was shown to occur during colorectal carcinogenesis (examples are described in Supplementary Box 2). Similarly, lncRNA expression can also be regulated by promoter methylation (Supplementary Box 2). In addition, two of the most widely studied lncRNAs in the context of CRC that exhibit important crosstalk to other epigenetic modifications are HOX transcript antisense RNA (*HOTAIR*) and H19 imprinted maternally expressed transcript (*H19*). Overexpression of *HOTAIR* recruits polycomb repressive complex 2 (PRC2), which leads to silencing of its target genes, including *HOX*, through H3K27 trimethylation (H3K27me3)[50]. In CRC, *HOTAIR* overexpression has been associated with an increased capacity for invasion and metastasis in vivo, as well as reduced metastasis-free survival and overall survival[50–52]. Similarly, *H19* can function as an oncogene in CRC by acting as a sponge for various ts-miRs[53]. Crucially, genes that are epigenetically dysregulated in cancer can have roles in different molecular pathways, and crosstalk can also exist within these pathways, adding another layer of complexity[54].

Pathophysiological considerations

Although a detailed description of CRC pathogenesis is beyond the scope of this Review, some pathophysiological aspects, highlighted herein, might be relevant to the role of epigenetic alterations as biomarkers and therapeutic targets in this malignancy.

MSI, CIN and CIMP status

CRC is clearly a very heterogeneous disease in terms of its biological behaviour, prognosis and response to treatment. From a molecular point of view, CRC has been traditionally classified into subgroups on the basis of three pathophysiological pathways of carcinogenesis — chromosomal instability (CIN), MSI and CIMP[55].

The hallmark of the most classical (canonical) pathway is CIN, which accounts for ~80–85% of CRCs and refers to a high load of alterations of whole chromosomes or large portions of chromosomes (duplications or depletions), which results in activation of growth-promoting pathways and/or decreased activity of apoptotic pathways[56]. These tumours develop initially from adenomatous polyps on the basis of a deactivating mutation of the *APC* gene (which encodes an effector in the WNT pathway) and eventually transform into adenocarcinomas through acquisition of further activating mutations in *KRAS* (which is involved in receptor tyrosine kinase signalling) and deactivating mutations in *SMAD4* (which is involved in TGFβ-signalling) and *TP53* (which is involved in cell cycle control)[56,57]. Given the initial event, this pathway is occasionally also called the ‘APC pathway’. MSI, *MLH1* mutations and/or methylation or *BRAF* mutations are not characteristics of the CIN pathway.

MSI, which involves changes in the number of short repeated sequences termed microsatellites that are spread out across the genome, is caused by deficiency in DNA mismatch repair (MMR), specifically mutations in MMR genes such as *MLH1*, *MSH2*, *MSH6* and *PMS2*; germline mutations in these genes cause Lynch Syndrome, the most frequent form of hereditary CRC[58]. However, MMR deficiency can also be caused either by biallelic hypermethylation of the *MLH1* promoter, which leads to the inactivation of *MLH1*[59,60], or double somatic mutations in MMR genes[61]. MSI status has already helped to define a subgroup of patients with MMR-deficient and MSI-high (dMMR-MSI-H) CRC who have a favourable prognosis but who do not benefit from 5-fluorouracil (5-FU) treatment[62,63].

In contrast to CRCs defined by CIN and MSI, epigenetics defines those of the CIMP subgroup, which are characterized by a high level of CpG island hypermethylation at the promoters of several tumour-suppressor genes[64]. CIMP is highly associated with the serrated pathway of colorectal carcinogenesis, is intrinsically associated with *MLH1* promoter hypermethylation[65], and is frequently associated with proximal location, female gender, old age, poor histology and *BRAF* mutations[66]. However, the translation of this pathophysiological aspect into clinical practice is still hampered by the lack of a standardized definition of ‘CIMP-high’. *MLH1*, *MINT1*, *CACNA1G* and *CDKN2A* are the most commonly evaluated genes for CIMP testing, but no consensus currently exists regarding which genes should be included in CIMP testing panels (up to 16 different genes

have been described), which cut-off values should be used to define CIMP-high (or CIMP^{+}) and which laboratory techniques should be used for measurements[67].

Molecular subtypes

To overcome the shortcomings associated with tumour heterogeneity, even within the aforementioned CIN, MSI and CIMP subgroups, Guinney and colleagues[68] used a large-scale data sharing and analytics approach across six international expert teams to identify four gene expression-based consensus molecular subtypes (CMS1–4) of CRC. In this framework, CMS1 tumours were defined by a high degree of promoter hypermethylation in several tumour-suppressor genes, including *MLH1*, leading to MSI tumours with a high mutational loads and, consequently, a strong lymphogenic anti-tumour immune response. This subgroup has important therapeutic implications, given that CMS1 tumours tend to evade this immune response through expression of programmed cell death 1 ligand 1 (PD-L1), which sends an inhibitory signal to T lymphocytes via its receptor programmed cell death 1 (PD-1) pathway[69]. This signal can be suppressed therapeutically using immune checkpoint inhibitors (ICIs) such as the anti-PD-1 antibody pembrolizumab, which gained tissue/site-agnostic accelerated FDA approval in 2017 on the basis of impressive results in phase II studies in dMMR-MSI-H solid tumours, including CRCs[70,71].

By contrast, CMS4 tumours, which account for ~23% of all CRCs, are characterized by a strong EMT gene signature, stromal invasion and intense immunosuppression[68]. At the molecular level, CMS4 tumours are characterized by CIN-low and CIMP-low status, leading to a low mutational burden and, consequently, a low lymphogenic immune response, rendering them resistant to ICIs. Another hallmark of CMS4 tumours is upregulation of the TGF-β pathway, which has been associated with EMT, stromal infiltration, poor prognosis, advanced disease stages and short overall survival and relapse-free survival[68,72]; CMS1 tumours, by contrast, show lower TGFβ signalling and generally have a better overall and relapse-free survival compared to CMS4 tumours, while showing the worst survival after relapse compared to all other CMS subtypes[68]. Briefly, CMS2 tumours are characterized by epithelial differentiation and alterations of the canonical pathway (including overexpression of WNT and MYC downstream targets)[68]. Finally, the most common hallmark of CMS3 tumours is the presence of strong *KRAS*-activating mutations and marked metabolic dysregulation[68].

miRNAs

Guinney and colleagues[68] also analyzed CMS-specific changes in miRNA expression and identified, for example, that downregulation of the miR-200 family was associated with EMT and CMS4 tumours, whereas downregulation of the let-7 family was associated with activation of the RAS pathway in CMS3 tumours. Other examples of downregulated miRNAs that can function as tumour suppressors are miR-143 and the miR-34 family (miR-34a, miR-34b and miR-34c) (FIG. 2).

Both miR-143 and miR-145, which are usually co-expressed, are frequently downregulated in the early phase of adenoma formation rather than during CRC progression; therefore, these miRNAs do not seem to be associated with clinical prognostic factors[73]. In vitro

experiments with synthetic miR-143 have shown its anti-proliferative activity by directly targeting several key molecules of the RAS-RAF-MEK-ERK pathway such as KRAS, AKT, ERK as well as SOS1 (a strong activator of KRAS)[74] (FIG. 2). For this reason, and from a clinical point of view, miR-143 is not only a promising biomarker for early diagnosis but could also be useful as a potential anti-cancer drug, especially in patients with activating-mutations in *KRAS* who are resistant to anti-EGFR therapy. However, clinical studies in humans are still lacking. The in vitro anti-proliferative activity of miR-143 (in human $KRAS^{G12D}$ DLD1 CRC cells) was confirmed by in vivo experiments in immunodeficient mice after xenotransplantation using synthetic miR-143[74]. These results were even more pronounced when combined with EGFR inhibitors in both in vitro and in vivo[74]. Another in vitro study showed that miR-143 replenishment could re-sensitize *KRAS*-mutant LoVo CRC cells to paclitaxel treatment[75].

Members of the miR-34 family, most prominently miR-34a, have also been described as relevant tumour suppressors at different levels of cancer pathophysiology. By targeting NAD-dependent protein deacetylase sirtuin-1 (SIRT1), miR-34a seems to be a crucial part of a positive feedback loop with p53, which itself enhances miR-34a activity and is known to halt the cell cycle at the G1-S checkpoint. This hypothesis originates from a study in which *TP53* wild-type HCT116 CRC cells showed inhibition of cell growth, migration, invasion and metastasis in vitro and in vivo xenografts after ectopic miR-34a-5p expression[76]. In addition, miR-34a has been implicated in regulating the TGF-β-mothers against decapentaplegic homolog 4 (SMAD4) and interleukin-6 receptor (IL-6R)-signal transducer and activator of transcription 3 (STAT3) pathways, both of which are essential for EMT and tumour cell invasion in CRC[77,78]. However, the miR-34 family, especially miR-34b and miR-34c, was also found to be upregulated in CRC and are associated with metastasis and poor prognosis, underlining their dual role in cancer development and progression[79–81].

Many studies have also reported the role of miRNA upregulation in driving several cancer-related cellular processes such as migration, invasion, angiogenesis and metastasis through epithelial-mesenchymal transition (EMT). For example, miR-21 contributes to migration, invasion and metastasis by downregulating expression of phosphatase and tensin homolog (PTEN), therefore activating the AKT pathway, as shown with in vitro and in vivo xenograft experiments using HCT116 CRC cells (FIG. 2)[82]. Furthermore, miR-21 seems to have a key role in inflammation pathways through downregulation of the tumour-suppressor gene *PDCD4*, leading to inhibition of apoptosis in cell lines[83,84]. Thus, miR-21 is an excellent example of how a single miRNA can regulate several pathways that can lead to different cancer-specific phenotypes. Sun and colleagues[85] hypothesized from correlation studies in human CRC samples and bioinformatics approaches that miR-31, another known oncogenic miRNA, might drive CRC progression by repressing expression of RAS p21 GTPase activating protein 1 (RASA1) and, therefore, activating the RAS signalling pathway[85]. In the same study, the authors corroborated this hypothesis in vitro (using transfection experiments and functional assays) and in vivo (using xenograft models) with human CRC cell lines (FIG. 2). This finding might have important clinical implications given that RAS signalling is the main effector pathway for epidermal growth factor (EGF), and that miR-31 has been shown to reduce the response to treatment with EGFR antagonists in CRC[85–87].

As tumours rapidly grow and expand their need for oxygen and nutrients, their survival will depend on the formation of new blood vessels through a process called angiogenesis. These vessels will also favour extravasation and metastasis of tumour cells. A variety of proteins and cytokines are implicated in the regulation of angiogenesis, including vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis that has been implicated in CRC[88]. In this regard, miR-126 — which is frequently found to be downregulated in CRC — seems to be involved in angiogenesis by directly targeting and repressing VEGF, a finding that has been confirmed in functional studies in CRC cell lines[89]. Accordingly, downregulation of miR-126 has been associated with metastasis in both tissue samples and serum from patients with CRC[90,91]. In addition, downregulation of miR-126 was also associated with worse outcomes in patients with metastatic CRC treated with the anti-VEGF-A antibody bevacizumab[92].

Epigenetic alterations as biomarkers

CRC develops through a stepwise accumulation of genetic and epigenetic alterations in precursor lesions (adenomas and serrated lesions) and, therefore, these lesions gain increasingly dysplastic features as they eventually progress to an adenocarcinoma. The detection of precursor lesions and early-onset CRC in average-risk, asymptomatic individuals during screening is essential for the prevention of this disease. Colonoscopy is considered the gold standard for CRC screening because it has the potential to both detect and remove precursor lesions. However, colonoscopy is invasive, expensive, associated with low compliance rates and is hampered by complications such as haemorrhage and perforation[93]. By contrast, the faecal occult blood test (FOBT) and faecal immunochemical test (FIT), the most commonly used non-invasive screening tests in Europe and other Western countries, have a lower sensitivity and specificity than colonoscopy, at least for precursor lesions such as adenomas[93]. These limitations highlight the imperative need for the development of novel and robust non-invasive strategies for the detection of precursor lesions and early-stage CRCs. In addition to these diagnostic challenges, the current tumour-node-metastasis (TNM) classification system for CRC staging is inadequate for prognostication, and clinical decision-making is limited, particularly for intermediate stages[94]. Accordingly, biomarkers that enable the proper selection of patients who have a high probability of recurrence and death (prognostic biomarkers), as well as those who might truly benefit from chemotherapy, immunotherapy and/or targeted therapy (predictive biomarkers), are urgently needed to improve prognostication and reduce the overall (and perhaps unnecessary) therapeutic toxicity and the associated expense of such treatments.

Epigenetic marks and regulators, including DNA methylation, histone modifications, miRNAs and lncRNA, have shown promise as clinically-relevant biomarkers for diagnosis, prognostication and prediction of treatment response in CRC (FIG. 3). Herein, we focus on epigenetic biomarkers that, upon comprehensive review of the literature (see Supplementary Box 1 for detailed Review criteria), we deem to have shown the greatest potential for clinical translation over the past 10 years for diagnostic (BOX 1), prognostic (BOX 2) and predictive (BOX 3) purposes in CRC, some of which have been commercialized or are in currently clinical use and/or guideline recommendations (BOX 4) (see Supplementary Tables 2–11 for an overview of candidates not mentioned herein).

DNA methylation

Evidence for the diagnostic utility of DNA methylation biomarkers is now so convincing that some assays have been recently commercialized, are used in current clinical practice or have even entered clinical guidelines. However, this is still not the case for prognostic and predictive DNA methylation biomarkers, but the evidence supporting their use is expanding continuously in this field.

Diagnostic biomarkers.—One of the most widely studied non-invasive DNA methylation biomarkers for CRC diagnosis is methylation of the *SEPT9* gene in plasma, which encodes septin-9, a GTP-binding protein involved in actin dynamics, cytoskeletal remodelling, vesicle trafficking and exocytosis (Supplementary Table 2). Multiple studies have analyzed the diagnostic accuracy of this methylation biomarker in large cohorts of patients with CRC, with sensitivity and specificity values ranging between 48–90% and 73–97%, respectively[31–42]. This biomarker is commercialized as the Epi proColon test (Epigenomics, Seattle, USA), which was approved by the FDA in 2016 as the first molecular blood-based assay for CRC screening. In one of the largest studies in which *SEPT9* plasma methylation was analyzed using Epi proColon test 2.0 methodology, this biomarker yielded an overall sensitivity of 73.7% and a specificity of 97% in a large cohort (300 patients with CRC and 568 healthy controls individuals)[105]. However, the findings of both this study and of a 2017 meta-analysis are in agreement in that this test performed statistically significantly better in patients with advanced stage (III–IV) CRC than those with early-stage (I–II) CRC[105,107].

In an effort to further improve the diagnostic accuracy of this assay, which is indeed suboptimal for early-stage CRC, other groups have attempted to combine *SEPT9* plasma methylation levels with additional biomarkers, such as FIT or other plasma-based methylated genes (for example, *SHOX2* and *ALX4*)[95,97,108]. In one such opportunistic CRC screening scenario including all CRC stages, Wu and colleagues[97] achieved an overall sensitivity of 97.2% by using a combination of *SEPT9* plasma methylation, FIT and serum carcinoembryonic antigen (CEA) levels. Although the majority of the data for this test has been in case-control or cohort studies, *SEPT9* plasma methylation has been also analyzed in asymptomatic, intermediate-risk populations of healthy individuals to further assess its diagnostic potential as an alternative test to FIT or colonoscopy (PRESEPT Study)[109]. However, when Song and colleagues[107] compared the data from this study with data from a meta-analysis of FIT in the same type of population, they found a lower sensitivity (68% versus 79%) and specificity (80% versus 94%) for *SEPT9* plasma methylation than for FIT.

One of the major limitations of the *SEPT9* plasma methylation biomarker is its poor sensitivity for the identification of precursor lesions (adenomas), ranging from only 7.9–38.7%[97,98,105,106]. *SEPT9* plasma methylation levels exhibited the highest sensitivity (83.3%) in a subgroup of patients with villous adenomas, suggesting the potential of this biomarker for the identification of advanced adenomas[106]. However, the number of patients included in this study was small (n=18), highlighting the need for the development of more powerful approaches for detecting precancerous lesions and early-stage CRC[106].

Another non-invasive methylation biomarker that has been frequently described for CRC diagnosis is methylation of the *VIM* gene encoding the intermediate filament protein vimentin, which, together with microtubules and actin microfilaments, constitutes the cytoskeleton (Supplementary Table 2). The diagnostic accuracy of this biomarker might be greater when using faecal samples than blood samples. For instance, in plasma samples, *VIM* methylation showed a sensitivity and specificity of up to 59% and 93%, respectively, with a major increase in sensitivity in advanced disease stages[110]. By contrast, the sensitivity and specificity were up to 81% and 95%, respectively, in stool samples, with similar values for different CRC stages[110–116]. In light of this decent performance of *VIM* methylation in faecal samples, this biomarker has also been commercialized as the ColoSure test (LabCorp)[117]. However, ColoSure has not yet obtained FDA clearance or approval for its use as a CRC screening test.

In contrast to *VIM*, methylation of *SFRP2* — which encodes secreted frizzled-related protein 2 (SFRP2), a modulator of WNT signaling — has been also studied for detection of precancerous lesions (both adenomas and hyperplastic polyps), with more promising results for adenomas (Supplementary Table 2). The sensitivity of plasma-based *SFRP2* methylation for adenoma detection ranged from 6.4–81.1%, with corresponding specificities of 73–100%[118,119], while the sensitivity and specificity of stool-based *SFRP2* methylation were 27.8–76% and 55–100%, respectively[120–122].

As one of the hallmark features of CRC is that it develops in the background of different genetic and epigenetic alterations that co-operate to drive neoplastic transformation in the colon, a few studies have also explored the combination of different types of molecular biomarker for improving the detection accuracy for colorectal polyps and CRC. Indeed, several promising combinations of methylation biomarkers have been proposed in order to improve diagnostic performance (Supplementary Table 3). Cologuard (Exact Sciences, Madison, USA), the first stool-based multi-target panel approved by the FDA for CRC screening, consists of a molecular assay for three biomarkers (seven mutations sites in *KRAS* and the methylation status of *NDRG4* and *BMP3*) combined with an immunohistochemical assay for haemoglobin. In a large study with almost 10,000 intermediate-risk individuals population, Imperiale and colleagues[123] reported a significantly higher sensitivity of Cologuard for CRC detection than FIT (92.3% versus 73.8%; $P<0.002$). More importantly, the sensitivity to detect advanced precancerous lesions, defined as advanced adenomas or sessile serrated lesions ≥1cm in size, was almost two-fold higher for Cologuard than for FIT (42.4% versus 23.8%). However, this increased sensitivity came at the cost of a lower specificity (89.8% versus 96.4%) for patients with a negative colonoscopy[123]. Given these promising results from this prospective study, Cologuard has been proposed for inclusion in the USA National Health Coverage and has been included in the United States Preventive Services Task Force (USPSTF) guidelines as a CRC screening option, with a 3-year interval on equal standing of the other classical screening options (colonoscopy, FIT and FOBT)[124]. In addition, an ongoing prospective Dutch study aiming to include ~4,000 individuals is currently evaluating Cologuard (together with FIT) as a non-invasive alternative surveillance strategy to colonoscopy in symptomatic patients[125].

Finally, the most frequently used epigenetic biomarker in current clinical practice is the analysis of somatic *MLH1* promoter methylation in CRCs that exhibit loss of MLH1 and/or PMS2 protein expression. Universal testing with immunohistochemistry for MMR proteins and/or MSI analysis in patients with CRC is the currently recommended strategy for the identification of patients with Lynch syndrome[126,127]. However, the most frequent cause of *MLH1* inactivation is through somatic inactivation due to bi-allelic promoter hypermethylation[59]. Thus, the use of *MLH1* hypermethylation analysis in patients with CRC with loss of *MLH1* expression is broadly implemented in clinical practice for differentiating between Lynch syndrome and sporadic CRCs with MMR deficiency. *MLH1* hypermethylation, together with *BRAF* mutations, is also considered a hallmark feature of the serrated pathway of colorectal carcinogenesis, and both biomarkers are associated with serrated polyps and serrated adenocarcinomas[128].

Prognostic and predictive biomarkers.—Although several DNA methylation biomarkers detected in tumour tissue or cell-free DNA (cfDNA) from blood have been associated with either advanced disease stages (III and IV) or poor prognosis in CRC (Supplementary Table 4), none of these potential prognostic biomarkers are currently used in clinical practice. To select patients who are candidates for chemotherapy, future predictive biomarkers should ideally be capable of identifying patients with a high risk of recurrence who would benefit from (a perhaps aggressive) adjuvant treatment. Likewise, biomarkers that can identify patients who will not respond to treatment, in whom chemotherapy and associated adverse effects could be avoided, are urgently needed. This need is especially relevant in patients with stage III and high-risk stage II tumours, for whom the actual standard of care is adjuvant chemotherapy with FOLFOX (combined administration of folinic acid, 5-fluorouracil and oxaliplatin). However, very few methylation studies have been conducted in this subset of patients, and studies performed in validation cohorts are lacking. Nevertheless, some interesting results have been reported regarding the prognostic and predictive roles of DNA methylation biomarkers, which could direct future prospective trials (Supplementary Tables 4 and 5).

For instance, hypomethylation of *LINE-1* elements in tumours has been widely studied and is associated with poor survival outcomes in patients with CRC[28,129–131] (Supplementary Table 4). In 2017, *LINE-1* hypomethylation in plasma cfDNA from patients with CRC was also shown to be associated with disease progression, with the strongest associations observed in patients with large tumours (≥6cm), advanced lymph node stages (N≥2) and distant metastasis[22].

In addition, hypermethylation of several known tumour suppressor genes has been reported to correlate with poor outcomes. For instance, hypermethylation of *CDKN2A* (specifically at the p16INK4A promoter), both in tissue and in blood, was shown to be broadly associated with poor prognosis as well as increased risk of recurrence and distant metastasis in patients with CRC[16,132–139] (Supplementary Table 4). In our opinion, the most interesting findings are from two old studies reporting the association between *CDKN2A* hypermethylation and poor survival in patients with T3N0M0 CRC, and poor survival after curative surgery and adjuvant 5-FU chemotherapy in patients with stage II and III rectal cancer[139,140]. By contrast, hypermethylation of *MGMT* — which encodes methylated-DNA--protein-cysteine

methyltransferase (MGMT), a DNA-repair protein involved in defense against mutagenesis and alkylating agents — has been associated with good prognosis in patients with advanced CRC tumours after adjuvant 5-FU treatment and with improved response to preoperative chemoradiotherapy in patients with advanced rectal cancers (particularly to decarbazine)[141–144] (Supplementary Table 5). These tumour-suppressor genes, together with *MLH1*, are commonly included in gene panels for CIMP testing[65].

Hypermethylation of the promoter region of the gene encoding the transcription factor AP-2 epsilon (*TFAP2E*) has been associated with improved clinical outcomes in terms of relapse-free survival and overall survival in patients with stage II or III CRC treated with adjuvant chemotherapy[145] (Supplementary Table 5). Hypermethylation of this gene has also been reported to be associated with chemoresistance in CRC[146]. However, to date, these results have not been confirmed in subsequent validation studies, and the usefulness of *TFAP2E* hypermethylation for predicting response to 5-FU in CRC has been questioned following attempts to validate the previous data in two large, uniformly treated and well characterized CRC cohorts (including a total of 783 patients)[147].

Furthermore, in the past few years, *HPP1* and *HLTF* have emerged as two of the most promising non-invasive methylation biomarkers for disease monitoring in patients with CRC (Supplementary Table 5). Indeed, *HPP1* and *HLTF* methylation status has been associated with advanced disease stages (III and IV), tumour aggressiveness, poor survival and tumour recurrence, both in blood[112,148–152] and stool[111,153]. Moreover, *HPP1* methylation levels in cfDNA could be used to identify patients with metastatic CRC who might respond to the combination of chemotherapy with bevacizumab early after the start of treatment[148].

In summary, DNA methylation biomarkers have been broadly associated with prognosis and survival in unselected cohorts, but evidence on their usefulness in specific clinical settings, which could change current treatment strategies, is still very limited. However, we believe that the biomarkers presented herein are worthy of further evaluation in prospective studies owing to the very promising preliminary data on their utility.

Histone modifications

Although histone modifications have been studied less than DNA methylation for their potential as biomarkers, aberrant patterns of histone marks are well established to have a central role in cancer pathogenesis. Reasons for the lower appeal of histone modifications as biomarkers include the technical limitations associated with their use as quantitative analytes (as most methods used, such as immunofluorescence or chromatin immunoprecipitation, do not allow high-throughput analysis) and their lack of specificity for different cancers. Nonetheless, a number of histone modifications with potential clinical utility as diagnostic and prognostic biomarkers have been identified in CRC (Supplementary Table 6).

With respect to their diagnostic potential, methylation of lysine 9 on H3 (H3K9) is higher in CRC and in adenomas than in normal colonic mucosa, and acetylation of H3K27 and H4K12 is markedly increased in CRC compared with the normal mucosa[154–156]. In an attempt to identify non-invasive biomarkers, some studies have demonstrated the potential of histone modifications in circulating nucleosomes as diagnostic biomarkers for CRC, which

was mostly attributed to their stability in the circulation. Indeed, reduced levels of H3K9 trimethylation (H3K9me3) and H4K20me3 marks in circulating nucleosomes (determined using chromatin immunoprecipitation) were observed in patients with CRC compared with healthy control individuals[157,158]. However, these data are preliminary and further studies are required to determine the feasibility of this novel approach.

In addition, histone acetylation and methylation status has been mainly studied in the context of CRC progression and patient survival. In this sense, expression patterns of some of the histone methylation markers (for example, H3K4, H3K9 and H3K27 methylation status) have been shown to be associated with various clinicopathological features (such as TNM stage and lymph node metastasis); for instance, H3K4 dimethylation (H3K4me2) and H3K9me3 marks have been shown to be independent prognostic markers for metachronous liver metastasis[159,160].

The combination of different histone modifications can potentially enhance their prognostic relevance. An elegant study showed that low nuclear expression of H3K4me3 marks and high nuclear expression of H3K9 methylation (H3K9me) and H4K20me3 marks were associated with improved prognosis, with a hazard ratio (HR) for disease-free survival (DFS) of 3.81 (95% CI 1.72–8.45), a HR for locoregional recurrence-free survival of 2.86 (95% CI 1.59–5.13) and a HR for distant-recurrence-free survival of 2.94 (95% CI 1.66–5.22)[161]. Another study showed that the combination of high nuclear HDAC expression (SIRT1, HDAC1 and HDAC2) with high expression of either H3K56 acetylation (H3K56Ac) or H4K16Ac marks also has clinical prognostic value, with a corresponding HR for overall survival of 0.82 and 0.86, respectively, and a HR for distant-recurrence-free survival of 0.77 and 0.79, respectively[162].

Regarding histone-associated proteins, the polycomb-group proteins have been studied the most for their biomarker potential. Indeed, high expression of histone-lysine N-methyltransferase EZH2 and Polycomb protein SUZ12 — which are both part of Polycomb Repressive Complex 2 (PRC2) - and Polycomb complex protein BMI-1 in association with expression of H3K27me3 marks has been correlated with improved prognosis in patients with CRC[163].

Non-coding RNAs

Given the multiple studies on ncRNAs, miRNAs and lncRNAs are undoubtedly the rising stars on the horizon as future biomarkers for diagnosis, prognostication and response prediction in CRC. However, in contrast to methylation biomarkers, the commercialization and implementation of ncRNA biomarkers into clinical practice still requires large-scale validation studies.

miRNAs.—The potential of miRNAs as biomarker candidates lies in their small size, their limited numbers (relative to protein-coding genes) and their stability in a variety of biological specimens such as tissue, blood and stool[40,164]. In addition, the availability of a variety of routine laboratory techniques that enable their identification and quantification (such as microarrays and quantitative reverse transcription PCR (RT-qPCR)) in virtually all specimen types makes miRNAs attractive biomarker candidates. In the past decade, the

number of studies investigating miRNAs in CRC has increased exponentially, with efforts focusing on evaluating their potential as biomarkers being particularly on the rise. Despite this enthusiasm, only a few well-designed studies have been conducted with large patient cohorts, precisely defined patient populations and independent validation cohorts. For a limited number of miRNAs, for example miR-21[165], miR-224[166], miR-106a[167], miR-29a[168] and miR-92a[169], the first meta-analyses are now available. However, none of these biomarkers have met the key requisites for adoption in the clinical setting at the present time, such as cohorts of >1,000 individuals, inclusion of prospective studies and comparison with established screening or diagnostic methods. Nonetheless, a number of potential miRNA biomarkers have been identified in CRC (Supplementary Table 7–10).

Using a unique approach involving small-RNA sequencing in 48 pairs of frozen CRC tissues and 10 different CRC cell lines, Sun and colleagues[170] identified miR-21, miR-143, miR-148a, miR-194, miR-192, miR-200b, miR-200c, miR-10b, miR-26a and miR-145 as the top 10 differentially dysregulated miRNAs in CRC; miR-21 and miR-143 were the two most abundantly expressed and had key pathophysiological roles in this malignancy. Following our comprehensive literature research on this topic (Supplementary Box 1), which included studies that collectively analysed 15,839 CRC specimens and 453 colorectal adenomas, we noted that six of these miRNAs are highly relevant for biomarker research — miR-21, miR-143, miR-145, miR-194 and the miR-200 family (miR-200b and miR--200c) (Supplementary Tables 7–10).

Our research also identified upregulated miR-21 expression as perhaps the most promising biomarker for diagnosis, prognostication and predicting treatment response in CRC (Supplementary Table 7–10). For diagnostic purposes, miR-21 has demonstrated a high sensitivity and specificity in both blood and stool, although results from stool samples show a higher degree of variability. In a meta-analysis of 16 studies conducted between 2010 and 2014 that included >1,000 patients, the overall sensitivity and specificity of miR-21 for the early detection of CRC was 64% and 85%[165], respectively, with a 2017 study reporting an even greater sensitivity of 86%, although at the cost of a lower specificity of 73%[171]. For the diagnosis of colorectal adenomas, miR-21 expression in blood showed a sensitivity and specificity of up to 80%[172]. miR-21 also seems to be a good biomarker for prognosis and survival in both tissue and blood samples[165,173]. Additionally, miR-21 is the most frequently reported miRNA predictive biomarker for response to treatment in at least three different clinical settings, including response to neoadjuvant chemoradiotherapy in advanced rectal cancer and response to both neoadjuvant and to adjuvant chemotherapy in advanced CRC[174–176].

Upregulated expression of miR-92a possesses a good discriminatory power for identifying patients with CRC over healthy control individuals using both blood and stool samples, with area under the ROC curve (AUC) values of up to 89% and 78%, respectively[177,178] (Supplementary Table 7); however, the inter-study disparity in sensitivity and specificity data is higher for miR-92a than for miR-21 (Supplementary Table 7). The sensitivity of miR-92a for detection of adenomas is higher in blood than in stool samples, and is markedly high for discrimination of patients with advanced adenomas, but specificities were high (>70%) for both adenomas and advanced adenomas[179]. Upregulation of miR-92a was also associated

with shorter overall survival in at least two studies in both tissue and blood, illustrating its prognostic potential[180,181] (Supplementary Table 9). This miRNA has also been found to be downregulated after chemotherapy (5-FU and oxaliplatin), but, to the best of our knowledge, miR-92a has not been studied as a predictive biomarker for therapeutic response in CRC[182].

In general, sample sizes from studies focusing on miRNA biomarkers for diagnosis of CRC range from 50 to 200 individuals, and are lower for diagnosis of adenomas (n=30–50). Although some of the observed differences in the sensitivities and specificities between studies might be due to varying sample sizes, a clear relationship between sample size and test performance does not seem to exist. For example, using miR-21 in blood for diagnosis of CRC, four studies with sample sizes of 49, 50, 101 and 200 individuals showed sensitivities of 76%, 90%, 61% and 65%, respectively[183–186]. Thus, the underlying disease heterogeneity in the study populations probably has a more important role than sample size alone in explaining these disparities. Regardless, even among studies with comparable samples sizes, the majority of studies lack detailed information on their CRC population, such as tumour stage, grade and location. For these reasons, the comparison and conclusive interpretation of results across different studies is challenging. Although some studies have reported a correlation between miRNA expression and tumour stage, we are not aware of a single well-designed study reporting that a specific miRNA has a better diagnostic accuracy for early versus advanced stages of CRC.

A panel of two or more miRNAs has been suggested as potentially a more accurate and robust diagnostic approach than using a single miRNA biomarker (Supplementary Table 8). However, the additional accuracy of combining multiple miRNAs in a diagnostic panel has shown conflicting results, primarily due the fact that most studies were conducted in relatively small patient cohorts (n≤100). For example, for the non-invasive diagnosis of CRC using blood samples, miR-21 and miR-92a in some studies reached sensitivities of up to 90% and 89%, respectively, and specificities of up 90 and 96%, respectively, whereas a combination panel of these two biomarkers in another cohort only yielded a sensitivity of 68% and a specificity of 91%[186]. By contrast, a 2018 study by Liu and colleagues[187] reported an excellent performance of a serum-based four-miRNA panel (miR-21, miR-29a, miR-92a and miR-125b) to diagnose CRC, with an AUC of 0.95, a sensitivity of 85% and a specificity of 99%. However, this study included only 85 patients and lacked an independent validation cohort. For diagnostic purposes, another two-miRNA panel (miR-223 and miR-92a) analyzed in blood specimens from a cohort of >200 patients showed a good diagnostic accuracy with a sensitivity of 97%, specificity of 75% and an AUC of 0.91 for detection of CRC[188]. Similarly, in a 2019 study with almost 300 individuals, a plasma-based six-miRNA panel (miR-19a, miR-19b, miR-15b, miR-29a, miR-335 and miR-18a) accurately differentiated healthy control individuals from patients with advanced colorectal neoplasms (CRC and advanced adenomas), with an AUC of 0.92 and a sensitivity and specificity of 85% and 90%, respectively[189].

Data is much more limited for studies investigating biomarker panels that focused on other aspects, such as adenoma detection, early disease relapse, survival and treatment response. A four-miRNA panel (miR-19a-3p, miR-223–3p, miR-92a-3p and miR-422a) in serum samples successfully distinguished patients with adenomas from those with CRC, with an

AUC of 0.87[190]. Another two-miRNA panel (miR-29a and miR-92a) achieved similar results with an AUC, sensitivity and specificity of 0.77%, 73% and 80%, respectively[191].

Finally, miR-31, as well as miR-143 and miR-145 (which are usually co-expressed), have been reported as good biomarkers in tissue for prediction of response to different treatments, whereas plasma-based miR-106a was reported to be predictive of response to adjuvant chemotherapy in metastatic CRC[192] (Supplementary Table 10; detailed information on these miRNAs as well as on our Top 10 candidates are given in Supplementary Tables 7–10).

LncRNAs.—Over the past few years, lncRNAs have increasingly gained interest as biomarkers in CRC. In contrast to miRNAs, the precise number of functional lncRNAs remains unclear, primarily for two reasons. First, discovery of new lncRNAs is still ongoing, and second, the number of lncRNAs with relevant functions in cellular physiology remains unclear given that their functions are not fully understood. For these reasons, various online databases exhibit a great degree of disparity in the number of lncRNAs in humans. One of the first such databases, deepBase, was launched in 2010 and currently lists ~14,400 lncRNA transcripts, whereas NONCODE, which launched in 2016, has >170,000 annotations in version 5.0. NONCODE also provides an option to query lncRNAs related to specific diseases.

To date, 47 different lncRNAs specifically related to CRC have been listed in the NONCODE database, while our PubMed search retrieved 116 different CRC-related lncRNAs (but only one single publication exists for most of them). According to our literature research, most of the CRC-related lncRNAs are upregulated and seem to function as miRNA sponges (at least those with already known functions). We have created a list of the Top Ten candidate lncRNAs for CRC (for Review Criteria see Supplementary Box 1; detailed information on lncRNA candidates is given in Supplementary Table 11).

Although tissue-derived lncRNA has been the primary analyte in the studies, a few studies have also analyzed blood-derived lncRNAs to demonstrate their potential as non-invasive biomarkers. One such lncRNA analyzed in both serum and tissue is *HOTAIR*, which was found to be upregulated in early stages of CRC development and was also associated with TNM stage and patient survival[193,194]. Similarly, upregulation of colon cancer associated transcript 1 (*CCAT1*) in both tumour tissue and blood also seems to be an early event in colorectal carcinogenesis and was also associated with TNM stage and both overall survival and recurrence-free survival[195–198]. *CCAT1* has also been studied in two dual lncRNA panels. The combination of upregulated *HOTAIR* and *CCAT1* expression showed a higher sensitivity and specificity for the diagnosis of CRC in plasma samples than *HOTAIR* or *CCAT1* alone[193]. In combination with upregulation of *CCAT2*, another oncogenic lncRNA that blocks miR-145 export to the cytoplasm, *CCAT1* overexpression in tissue specimens predicted poor overall survival and progression-free survival, which was confirmed in an independent validation cohort[198].

In addition, lncRNAs can, albeit infrequently, function as tumour suppressors. Such is the case with growth arrest specific 5 (*GAS5*), which was found to be downregulated in human CRC tissues compared to tumour-adjacent normal tissues[199]. Low levels of *GAS5* were

positively correlated with large tumour size, advanced TNM stage and poor overall survival. In the same study, overexpression of *GAS5* (achieved through transfection) in CRC cell lines confirmed its inhibitory effect on tumour growth[199]. Moreover, in another functional study, knockdown of *GAS5* in CRC cell lines led to increased expression of vascular endothelial growth factor (VEGF), which promotes neoangiogenesis and tumour survival[200], a finding that could be relevant for the treatment with VEGF-targeted agents such as bevacizumab.

Despite the somewhat promising early results, research on lncRNAs still focuses mostly on discovery and functional aspects, and studies exploring their biomarker potential in large patient cohorts are still elusive. For more details on studies with our top 10 lncRNA candidates for CRC, see Supplementary Table 11.

Epigenetic therapy

Research on epigenetic alterations in cancer, has not only provided attractive biomarker candidates but has also opened the door for the development of new anticancer drugs, the so-called epigenetic modifiers. Unlike genetic alterations, which are irreversible and, therefore, challenging from a therapeutic standpoint, epigenetic alterations are essentially reversible, making them attractive therapeutic targets. An increasing number of epigenetic modifiers, which can be grouped into different classes on the basis of their mode of action, have been developed, some of which have received FDA approval for treatment of various diseases, not including CRC (Supplementary Table 12)[201]. These epigenetic modifying drugs include inhibitors of enzymes involved in DNA methylation (such as DNMTs and HDACs) and histone modification (such as HMTs and HDMs), as well as agents that therapeutically modulate miRNA expression, some of which have been tested preclinically or in early-phase clinical trials in CRC. Intriguingly, even some dietary supplements and their metabolites have been known for many years to protect against CRC, and have regained research interest in specific scenarios because of their ability to function as epigenetic modifiers. For example, phenyl-butyrate — a short-chain fatty acid and a fermentation product of dietary fibre — is a known HDAC inhibitor and has been shown to enhance the efficacy of cytotoxic drugs (paclitaxel and doxorubicin) in combination with 13-*cis*-retinoic acid in vitro and in vivo, probably due to a concomitant cell cycle arrest at the G1-S checkpoint[202].

Notably, all clinical studies evaluating epigenetic therapies in CRC have been tested in patients with very advanced stages of disease, in whom other treatments have failed. However, advanced tumours, are by definition more heterogeneous and have accumulated over time not only more epigenetic alterations but also a high load of genetic mutations, leading unsurprisingly to a low efficacy of epigenetic modifiers, if used alone and at late stages. Thus, their use as adjuvant treatment might be more effective at earlier stages of cancer, not only because epigenetic alterations manifest as early events during carcinogenesis, but also because the burden of genomic alterations is lower[203]. In this setting it, is also likely that the treatment needs to be prolonged, because the reprogramming of the cells needs time and is probably not stable after treatment cessation. On the other hand, another potential application of epigenetic modifiers is in advanced stage disease, although they would need to be combined with cytotoxic drugs. At least hypothetically, epigenetic therapies can reprogram tumour cells to re-sensitive them to radiotherapy, cytotoxic therapy

or immunotherapy[203]; herein lies the potential to reduce the doses of cytotoxic drugs, therefore improving patient tolerability.

DNMT and HDAC inhibitors

Epigenetic modifiers, when used in combination with other therapeutic agents, have demonstrated very promising synergistic effects in preclinical studies. Indeed, the DNMT inhibitors 5-azacitidine, decitabine and zebularine have been demonstrated to function synergistically with classical cytotoxic drugs such as 5-FU, irinotecan or oxaliplatin in CRC cell lines[204,205]. In a phase I/II clinical trial in patients with refractory CIMP-high metastatic CRC, 5-azacitidine combined with CAPOX (capecitabine and oxaliplatin) was very well tolerated with high rates of stable disease, although no objective responses were reported[206]. Similarly, vorinostat and belinostat, two HDAC inhibitors, exhibited synergistic effects in combination with FOLFOX or FOLFIRI (combined administration of folinic acid, 5-fluorouracil and irinotecan) in a preclinical study[207], and vorinostat was shown to be safe in combination with 5-FU and folinic acid in a phase I/II trial in patients with refractory metastatic CRC, but the efficacy was very limited (only 1 of 15 patients showed a partial response, and 8 of 15 had disease stabilization)[208]. In addition, the DNMT inhibitor decitabine was shown to, at least partially, restore sensitivity to 5-FU in nude mice bearing 5-FU-resistant CRC tumours[209], and the second-generation HDAC inhibitor panobinostat was also shown to lower 5-FU resistance in colon cancer cells, illustrating a potential role for these agents as chemo-sensitizers[210]. Emerging preclinical evidence also suggests that epigenetic modifiers might have synergistic activity when combined with other anti-cancer drugs such as epidermal growth factor receptor (EGFR) inhibitors, BRAF inhibitors and proteasome inhibitors[211–214].

Another potential application of epigenetic modifiers might be as enhancers or sensitizers for other anti-cancer therapeutic strategies, such as immunotherapy or radiotherapy. Indeed, the HDAC inhibitor trichostatin A (TSA) was reported to enhance the radiosensitivity of two colon cancer cell lines[215]. Regarding immunotherapy, epigenetic modifiers have been hypothesized to reverse tumour immune escape or enhance the efficacy of immune checkpoint inhibitors, including the anti-programmed cell death 1 (PD-1) antibody pembrolizumab or the anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibody ipilimumab[201,216]. In fact, in a preclinical study using the syngeneic CT26 colorectal tumour-bearing mouse model, low-dose decitabine treatment led to promoter demethylation of many genes involved in antigen processing and presentation, and enhanced the anti-tumour activity of an anti-PD-1 agent[217]. The authors hypothesized that this combination therapy could be beneficial for patients with MSI-low (MSI-L) or microsatellite-stable (MSS) CRC, who respond poorly to ICI monotherapy. Preliminary results from a phase II trial evaluating pembrolizumab in combination with 5-azacitidine for treatment of chemo-refractory metastatic CRC[218] revealed an objective response rate of 3% (1 of 30 patients; 95% CI 1–17%) and a median progression-free survival of 2.1 months (95% CI 1.8–2.8 months); however, serious adverse effects were observed in thirteen patients (43%)[219]. An ongoing phase I study, which is still recruiting patients, is testing the same drug combination in one of its arms in order to decipher optimal dosages[220], whereas another ongoing phase Ib/II study is recruiting patients to assess the safety and tolerability of pembrolizumab

combined with the second-generation HDAC inhibitor entinostat in a dose-expansion cohort of patients with MMR-proficient CRC[221].

However, the majority of CRC tumours have a low immunogenicity and often use different mechanisms to evade the immune response. C-X-C motif chemokine 12 (CXCL12; also known as SDF-1) is a chemokine that is strongly chemotactic for lymphocytes and is often downregulated in MSS CRCs. In three colon cancer cell lines and in a colon cancer xenograft mouse model, treatment with valproic acid — a known HDAC inhibitor — increased the acetylation of H3 in the promoter of CXCL12 and restored CXCL12 expression, which was associated with decreased migration in vitro and reduced tumour growth in vivo[222].

Despite the progress and promising results from preclinical studies, the outcomes — in terms of objective response rates, survival and tolerability — in clinical trials evaluating epigenetic modifiers are still disappointing, and a clear clinical benefit is not evident, at least for single epigenetic modifying drugs. Some epigenetic modifiers, such as 5-azacitidine, have a dual efficacy profile, whereby they have more prominent epigenetic efficacy at lower doses and more cytotoxic effects at higher doses. Many clinical trials evaluating epigenetic modifiers have been terminated early owing to severe adverse effects or to problems with reducing the effective dose to safe levels. The generalized toxicity with some of these drugs is probably due to both a lack of selectivity for cancer cells and a lack of selectivity for gene promoters with aberrant epigenetic modifications. Thus, DNMT inhibitors, for example, not only restore the normal hypo-methylated state of tumour-suppressor gene promoters, but at the same time erroneously diminish the methylation status of normally hyper-methylated regions[36,201].

HMT and HDM inhibitors

DNMTs and HDACs are only two of the several classes of enzymes that regulate gene expression and that can be manipulated pharmacologically. In addition to DNA methylation, specific patterns of histone methylation have also been associated with colorectal neoplasia, but the effects of their pharmacological manipulation with HMT inhibitors or HDM inhibitors are even more difficult to predict because such effects depend not only on the genes involved, but also on the specific histone methylation status. Histone lysine methylation, for example, can either enhance or repress gene expression depending on which lysine residue of a specific histone is methylated[201].

The first identified HDM, lysine-specific histone demethylase 1 (LSD1; also known as KDM1A), which specifically demethylates H3K4 and H3K9, was found to be overexpressed in CRC and to promote cancer progression by activating the WNT-β-catenin pathway[223]. In HCT116 colon cancer cells, treatment with CBB1003, an LSD1 inhibitor, reduced tumour cell growth and colony formation in a dose-dependent manner[224]. In another experiment, treatment with a pan-HDM inhibitor simultaneously targeting Jumonji C and lysine-specific demethylases induced cancer-specific growth inhibition in HCT116 cells, whereas the same treatment did not induce this effect in noncancerous mesenchymal cells in a separate culture, suggesting cancer-cell-selective induction of apoptotis[225]. To the best of our knowledge, no ongoing clinical trials are evaluating the therapeutic potential of HMT inhibitors in CRC.

Targeting miRNAs

Owing to their substantial role in CRC pathogenesis and disease progression, miRNAs have been proposed as potentially attractive therapeutic targets. At least hypothetically, two fundamental treatment approaches exist, including the therapeutic replacement of downregulated ts-miRs using miR-mimics or the inhibition of upregulated onco-miRs. Regarding treatment with miR-mimics, Akao and colleagues[73] proved the feasibility of this concept in a human DLD-1 CRC xenograft mouse model, in which they demonstrated a tumour growth inhibitory effect after both intratumoural and intravenous injection of miR-143-liposome complexes (miR-143 is a well described tumour-suppressor miRNA in CRC). Despite the preclinical evidence, to the best of our knowledge, no clinical trials are currently evaluating miR-mimics specifically in CRC. However, a liposomal miR-34a mimic was investigated in 47 patients with advanced solid tumours, three of whom had CRC, and showed an acceptable tolerability and preliminary evidence of anti-tumour activity in a subset of patients (partial response in 1 and stable disease in 4 patients); one of the three patients with CRC belonged to the group of stable disease and remained stable for 4 treatment cycles[226].

Short interfering RNAs (siRNAs) derived from longer double-stranded RNAs are the exogenous counterparts to miRNAs and have been studied as promising new anti-cancer drugs[227]; siRNAs might also be used to antagonize onco-miRs. An important limitation of the use of miR-mimics or siRNAs is the difficulties associated with their delivery to tumour cells, because they are double-stranded and present heavily hydrated phosphates on the surface of their liposome delivery vectors, which complicates their passage through hydrophobic cell membranes and favours rapid urinary excretion[228]. These difficulties might be overcome in the near future using molecular modifications and the design of vehicles that aid specific delivery[229]. However, as delivery is still an issue, the development of antisense oligonucleotides seems to be an easier approach, because they are single-stranded and pass through the cell membrane more easily. Some preclinical studies have reported promising results regarding the efficacy of antisense oligonucleotides in CRC, especially those targeting miR-21, miR-31, miR-125b and miR-92; however, their clinical application is still elusive[230].

Conclusions

An improved understanding of epigenetic regulatory mechanisms, particularly cancer-specific epigenetic alterations, will enable explorations of their future clinical applications as biomarkers or their potential as therapeutic targets in CRC. In this Review, we described several epigenetic biomarkers that have been studied to date, some of which have promising potential, including methylation of *VIM*, *SFRP2* and *SEPT9*. However, their diagnostic performance for early-stage CRC screening, for prognostication or predicting response to treatment, has yet to be improved, and most of these biomarkers still lack validation in large independent patient cohorts. In our opinion, other cancer-independent factors, such as diet and lifestyle, should also be considered in order to take a meaningful step towards personalized medicine. A combination of epigenetic biomarkers has been postulated to improve performance over single biomarkers, but the evidence supporting or opposing this

hypothesis is still sparse. Nevertheless, the first commercial assays, such as Cologuard, are now available. Non-coding RNAs have also become a powerful field of biomarker research and might find their way into clinical practice in the next decade. For some miRNAs, such as miR-21, well-designed studies with independent validation cohorts, and even meta-analyses, are now available, providing a strong basis for future studies to prove their diagnostic accuracy in specific clinical settings. For lncRNAs, the data is much more limited, but the first preliminary results suggest that they have strong potential as biomarkers and warrant further exploration.

Preclinical studies have reported that epigenetic alterations are potentially reversible through pharmacological manipulation, and the list of available epigenetic modifiers is steadily growing. However, evidence of a clear survival benefit in patients with CRC receiving epigenetic modifying drugs is still scarce. Furthermore, none of the available epigenetic modifiers have entered clinical trials beyond phase II, primarily owing to safety concerns. Nevertheless, we believe that their real potential has not yet been systematically explored. On the one hand, patient selection on the basis of tumour stage and (perhaps new) biomarkers of treatment response could improve response rates. On the other hand, combinatorial strategies with cytotoxic drugs, immunotherapy or radiotherapy should be optimized on the basis of molecular tumour subtypes, pharmacodynamics, pharmacokinetics and expected adverse effects in order to increase efficacy and avoid toxicity.

A huge barrier that has yet to be overcome is how to manage intratumoural heterogeneity. It is well known that tumours do not arise from a single mutated clone, but rather develop in an evolutionary manner and are comprised of multiple cell populations with different biological properties[231]. This intratumoral heterogeneity is also reflected in the diversity of different epigenetic marks and their associated pathways that are altered in cancer[232]. For example, tumour cells at the invasive front might have lost cell adhesion and polarity and have undergone EMT, as reflected by miR-200 downregulation[233], whereas cells at the tumour centre might induce neoangiogenesis to promote survival in the hypoxic environment, as reflected by a completely different epigenetic landscape[234]. Intratumoral heterogeneity has also been reported to have prognostic and therapeutic implications in CRC[232]. Thus, single-cell epigenetics studies will be necessary in the future to deepen our pathophysiological understanding of this malignancy.

Supplementary Material

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Key points

- Epigenetic changes, notably DNA methylation and histone modifications, have key pathophysiological roles in the initiation and progression of colorectal cancer (CRC).
- Non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and long ncRNAs are also important regulators of gene expression and are implicated in many CRC-related pathways.
- Epigenetic changes and altered expression of ncRNAs can be exploited as biomarkers for the diagnosis, prognostication and prediction of treatment response in CRC.
- Biomarkers based on DNA methylation have been commercialized and some have already found their way into clinical practice and guidelines in CRC.
- miRNAs are the most promising and fastest-growing group of potential future biomarkers for CRC; their implication in clinical practice is expected within the next decade.
- Epigenetic changes are potentially reversible and are attractive targets for future cancer treatments; epigenetic modifiers have proven their utility in preclinical and phase I/II studies.

Box 1 |

Candidate diagnostic biomarkers in CRC.

Single biomarkers[a]

- Blood-based for adenomas
  - Methylation: *SEPT9, SFRP2*
  - ncRNAs: miR-21, miR-92a, miR-29a
- Stool-based for adenomas
  - Methylation: *SFRP2*, *VIM*
  - ncRNAs: miR-21, miR-92a
- Blood-based for CRC
  - Methylation: *SEPT9, SFRP2*
  - Histones: H3K27me3, H4K20me3, H3K9me3
  - microRNAs: miR-21, miR-92a, miR-29a, miR-20a, miR-223
  - lncRNAs: *HOTAIR*, *CCAT1*, *CRNDE*
- Stool-based for CRC
  - Methylation: *SFRP2*, *VIM*
  - ncRNAs: miR-21, miR-92a, miR-20a, miR-223

Panel biomarkers[a]

- Blood-based for adenomas
  - *APC*, *MGMT*, *RASSF2A* and *WIF1*
  - *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1*
  - miR-19a-3p, miR-223–3p, miR-92a-3p and miR-422a
  - miR-29a and 92a
  - miR-21 and miR-92a
- Stool-based for adenomas
  - *NDRG4*, *BMP3*, *KRAS*^{mut} and haemoglobin (Cologuard)
- Blood-based for CRC
  - *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1*
  - miR-21, miR-29a, miR-92a and miR-125b
  - miR-223 and miR-92a
  - miR-21 and miR-92a

- Stool-based for CRC
  - *NDRG4*, *BMP3*, *KRAS*^{mut} and haemoglobin (Cologuard)
  - miR-21, miR-17–92 and miR-135

[a]The listed biomarkers are those that we deemed to have the greatest potential for rapid bench-to-bedside translation to improve diagnosis in CRC following comprehensive appraisal of the literature (see Supplementary Box 1 for detailed Review criteria). Detailed information on candidate diagnostic biomarkers in CRC can be found in Supplementary Tables 2, 3, 6, 7, 8, 11.

Box 2 |

Candidate prognostic biomarkers in CRC.

Blood-based[a]

- Methylation: *LINE-1*, *CDKN2A(p16)*, *HLTF, HPP1*
- ncRNAs: miR-21, miR-31, miR-34a, miR-92a

Tissue-based[a]

- Methylation: *CDKN2A(p16), LINE-1, TFAP2E, MGMT*
- Histones: H3K4me2, H3K4me3, H3K9me3, H3K20me3, H3K56ac, H4K16ac
- ncRNAs: miR-21, miR-31, miR-34a, miR-224, miR-92a

Biomarker panels[a]

- p14ARF, RASSF1A and APC1A
- miR-21–5p, miR-20a-5p, miR-103a-3p, miR-106b-5p, miR-143–5p, and miR-215

[a]The listed biomarkers are those that we deemed to have the greatest potential for rapid bench-to-bedside translation to improve prognostication in CRC following comprehensive appraisal of the literature (see Supplementary Box 1 for detailed Review criteria). Detailed information on candidate prognostic biomarkers in CRC can be found in Supplementary Tables 4, 6, 9, 11.

Box 3 |

Candidate predictive biomarkers in CRC.

Single biomarkers[a,b]

- Methylation: *HPP1, LINE-1, TFAP2E, MGMT*
- ncRNAs: miR-21, miR-31, miR-143/145 family, miR-106

Panel biomarkers[a]

- *PCDH10, SPARC, and UCHL1*[b]
- miR-99a, miR-Let-7c, miR-125b[b]
- miR-17, miR-21, miR-29a and miR-92[c]
- miR-20a, miR-130, miR-145, miR-216 and miR-372[c]

[a]The listed biomarkers are those that we deemed to have the greatest potential for rapid bench-to-bedside translation to improve prediction of treatment responses in CRC following comprehensive appraisal of the literature (see Supplementary Box 1 for detailed Review criteria). Detailed information on candidate predictive biomarkers in CRC can be found in Supplementary Tables 5, 10, 11. [b]All in tissue. [c]All in blood.

Box 4 |

Commercialized and currently used biomarkers.

Commercialized biomarkers

- Epi proColon (Epigenomics, Seattle, USA), *SEPT9* methylation; FDA approval (2016) for blood-based CRC screening.
- Cologuard (Exact Science, Madison, USA), *NDRG5* and *BMP3* methylation and *KRAS* mutations; FDA approval (2014) for stool-based CRC screening.
- ColoSure (LabCorp, Burlington, USA), stool-based *VIM* methylation; not FDA approved.

Biomarkers in clinical use or in guidelines

- *MLH1* hypermethylation (US Preventive Services Task Force[127], The Mallorca Group[126])
- Cologuard (US Preventive Services Task Force[124])

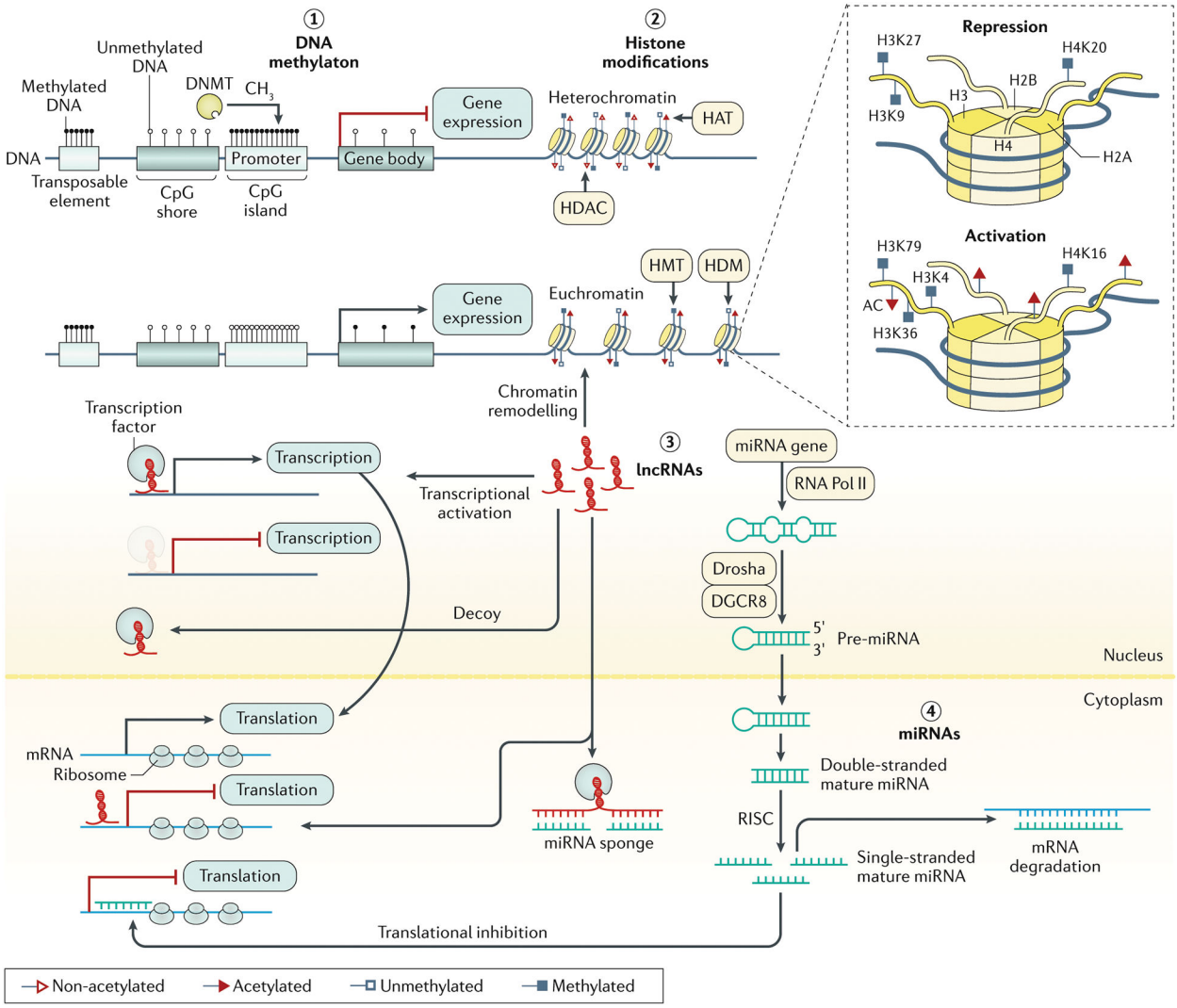

Figure 1 |. Principles of epigenetics.

The main epigenetic modifications implicated in colorectal cancer (CRC) — including DNA methylation (**1**), histone modifications (**2**), long noncoding RNAs (lncRNAs) (**3**) and microRNAs (miRNAs; also known as miRs) (**4**) — are shown. Hypermethylation of CpG islands in promoter regions of tumour-suppressor genes placed by DNA-methyl transferases (DNMTs) inhibits gene expression and can favor tumorigenesis[10,12] (**1**). If hypomethylation occurs in retrotransposable elements (such as LINE-1 elements), these are activated and insert themselves in distant fragile sites, leading to genomic instability[30]. Hypomethylation of promoters or distant super-enhancers can enhance expression of proto-oncogenes[27]. Acetyl-groups are placed by histone acetyl-transferases (HATs) and removed by histone deacetylases (HDACs); acetylation generally weakens the compaction status of the chromatin and makes the DNA accessible to transcription factors[34] (**2**). Histone methylation is regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs); methyl groups on histone tails create docking sites for proteins that can repress or increase gene expression[37] (**2**). lncRNAs influence gene and protein expression through different molecular mechanisms[47] (**3**). They can enhance transcription by recruiting transcription

factors or repress transcription by decoying transcription factors and preventing their recruitment to transcriptional start sites. LncRNAs can also restore translation by 'sponging' miRNAs that would otherwise prevent translation of their corresponding mRNA. LncRNAs can also directly inhibit translation. The biogenesis of miRNAs starts with the transcription of the miRNA gene by RNA polymerase II (RNA Pol II)[8] (**4**). The double-stranded, hairpin-formed pri-miRNA is processed to the pre-miRNA by Drosha/DGCR8 and then translocated to the cytoplasm by exportin-5. The RNAse III enzyme DICER cuts the hairpin loop, resulting in a double-stranded miRNA-miRNA molecule. The RNA-induced silencing complex (RISC) incorporates one of the strands and mediates its interaction with the target mRNA, leading either to translational inhibition or mRNA degradation.

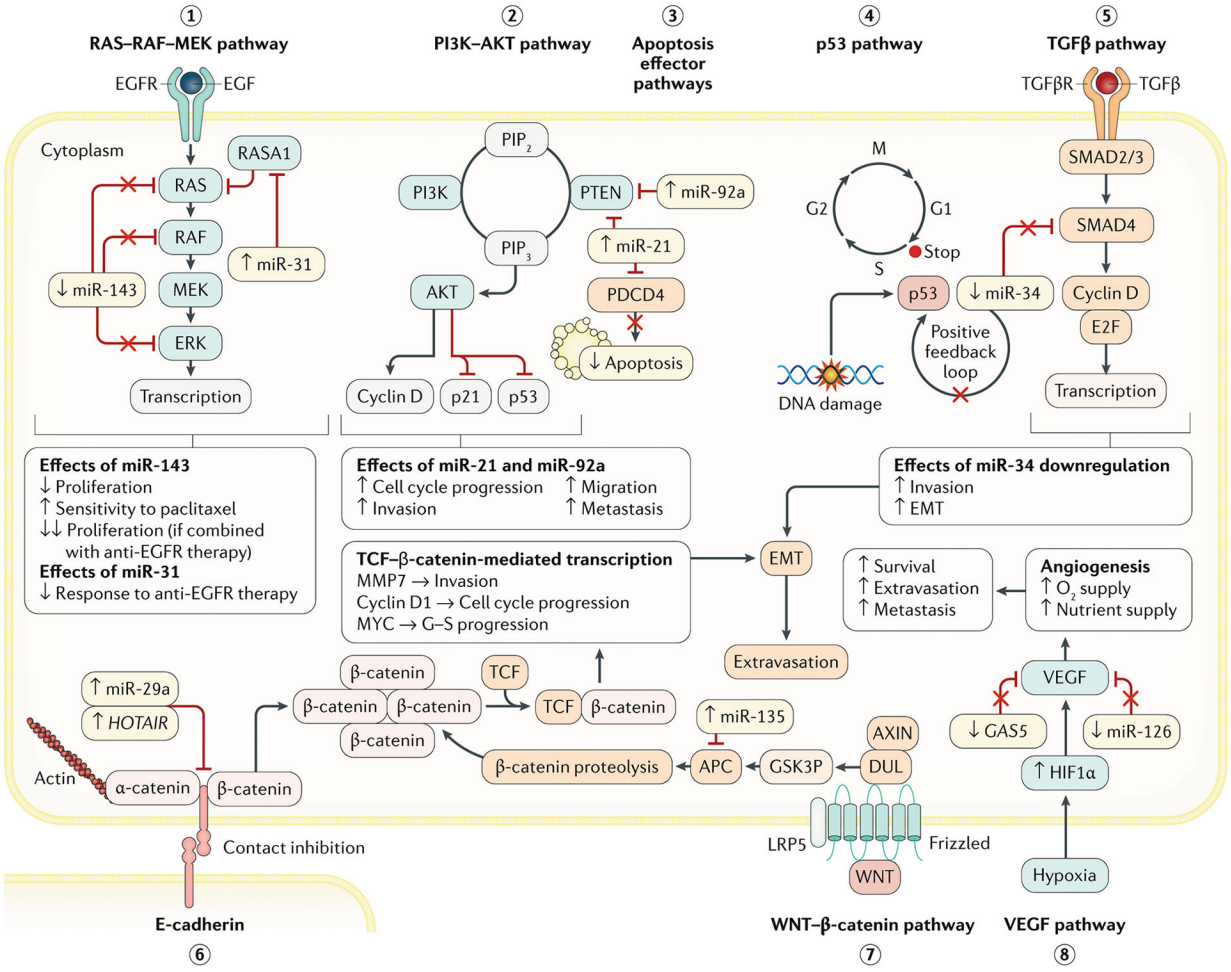

Figure 2 |. Role of miRNAs and lncRNAs in CRC.

The roles of selected long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) in regulating virtually all important signalling pathways relevant to colorectal cancer (CRC) are shown. Activation of the RAS-RAF-MEK pathway, which leads to enhanced proliferation and can modulate treatment responses, can occur via downregulation of miR-143[74] and/or upregulation of miR-31[85] (**1**). miR-21, the most frequently overexpressed miRNA in CRC, can favour cancer progression by targeting PTEN, preventing PIP3 dephosphorylation and resulting in hyperactivation of the PI3K-AKT pathway[82] (**2**). In addition, miR-21 can target PDCD4, which is thought to be an important mediator in apoptosis effector pathways[83]. (**3**) In the presence of DNA damage, p53 holds the cell cycle at the G1-S checkpoint to allow DNA repair or to induce apoptosis if repair is not possible. In a positive feedback loop, p53 increases the expression of miR-34a (which is frequently downregulated in CRC), resulting in enhanced p53 activity[76] (**4**). Moreover, miR-34a directly targets SMAD4, a key effector in TGFβ signalling. Thus, downregulation of miR-34a enhances TGFβ signalling and results in enhanced epithelial-mesenchymal transition (EMT) and tumour cell invasion[77,78] (**5**). Loss of E-cadherin in epithelial cells leads to a loss of contact inhibition, which favours cell growth, migration and invasion via β-catenin-TCF signalling (**6**). In CRC cells, miR-29a[235] and the lncRNA *HOTAIR*[52] have been described to decrease the expression of E-cadherin.

miR-135 directly targets and downregulates APC, which, under normal conditions, degrades β-catenin, resulting in downstream activation of the WNT-β-catenin pathway[236] (**7**). When tumours grow rapidly, hypoxia stimulates the formation of new blood vessels through angiogenesis, which is crucial for tumour survival and is regulated by the VEGF pathway. VEGF is a direct target of miR-126 and is also repressed by the lncRNA *GAS5*, and both are frequently downregulated in CRC[89,200] (**8**).

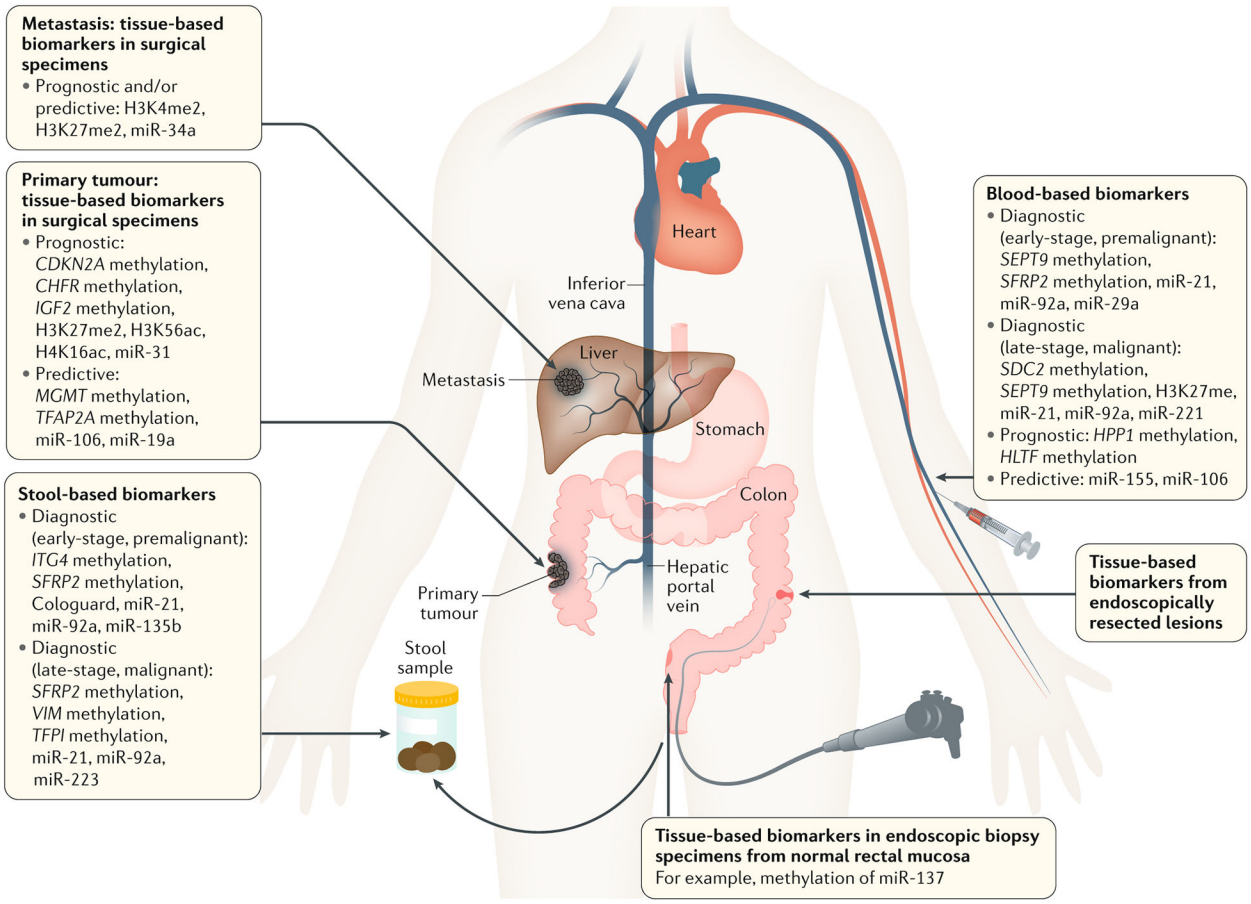

Figure 3 |. Epigenetic biomarkers in CRC.

Given their emerging roles in colorectal cancer (CRC), epigenetic marks or regulators can be exploited as clinically relevant disease biomarkers for diagnosis, prognostication and prediction of treatment response. Blood-based biomarkers (serum or plasma) might have diagnostic, prognostic or predictive value in CRC. Tissue-based biomarkers in endoscopically resected lesions might also be used clinically to improve prediction of the risk of invasion of early lesions (carcinoma in situ or pT1) and guide treatment options and surveillance strategies. Stool-based biomarkers (from abraded carcinoma cells) have a number of potential diagnostic applications; for example, Cologuard (*NDRG4* methylation, *BMP3* methylation and *KRAS* mutation) for screening of adenomas and early-stage CRC. Endoscopic biopsies of normal rectal mucosa (which is less invasive than endoscopic resection) could be used to identify patients at a high risk of metachronous or synchronous lesions owing to ‘field defect’ in normal rectal mucosa; for example, methylation of miR-137 in endoscopic biopsy tissue is an independent risk factor for ulcerative colitis-associated CRC[237]. Finally, tissue-based biomarkers in surgical specimens (primary metastatic or tumours) might also have prognostic or predictive roles.