Chapter

Epigenetic Biomarkers and Their Therapeutic Applications in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of the most aggressive cancers worldwide and is known to develop through a stepwise process involving the accumulation of several genetic and epigenetic alterations. Furthermore, numerous studies have highlighted the significant role that certain epigenetic enzymes play in CRC pathogenesis, particularly those that govern chromatin components in the promoter regions of tumor suppressors and oncogenes. Here, we delineate the relationship between CRC-associated epigenetic marks, their modifying enzymes, and the classification of CRC into distinct molecular pathways or subtypes. Moreover, we discuss some of the most prominent methyltransferases, demethylases, acetyltransferases, and deacetylases, which have been targeted for preclinical and clinical CRC treatment. Notably, inhibitors against these epigenetic enzymes are a promising new class of anticancer drugs, with several obtaining Food and Drug Administration (FDA) approval for the treatment of blood and solid tumors. By highlighting the epigenetic molecular pathways leading to CRC development as well as providing an update on current CRC epigenetic therapies, this chapter sheds fresh insight into new and emerging avenues for future therapeutics.

Keywords: checkpoint, CIMP, CIN, CRC, demethylase, DNA methylation, DNMT, epigenetics, HAT, HDAC, methyltransferase, MSI

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths globally and is expected to be responsible for an estimated 1.1 million deaths by 2030 [1]. With this growing global burden, prevention and treatment of CRC remains a significant public health challenge. CRC is thought to originate from sequential accumulation of genetic and epigenetic aberrations [2]. Of the identified genetic mutations, approximately 15 have been characterized as "driver mutations" and are thought to be functionally important during CRC initiation and progression [3, 4]. These include genes affecting critical cellular pathways such as those governing proliferation, apoptosis, migration, adhesion, and DNA damage and repair [3]. Importantly, it is now well established that epigenetic alterations can also serve as major driver events in the pathogenesis of CRC [5–7]. However, unlike genetic mutations, epigenetic modifications consist of heritable changes in gene expression

without DNA sequence changes and are intrinsically reversible by nature. These epigenetic events include alterations in DNA methylation, histone modifications, and non-coding RNAs. Moreover, the reversibility of these modifications makes them attractive molecular targets for anticancer therapeutic interventions [3, 8].

CRC is a highly heterogeneous disease and can be classified into molecularly and pathologically distinct pathways and subtypes [9]. Moreover, these classifications have significantly influenced patient stratification, prognosis, and therapeutic response [9, 10]. In this chapter, we focus on three epigenetic-related primary molecular pathways, namely the microsatellite instability (MSI) phenotype, the chromosomal instability (CIN) phenotype, and the CpG island methylator phenotype (CIMP). Importantly, each pathway reflects the underlying mechanisms of carcinogenesis as marked by certain aberrations such as a defective DNA mismatch repair (MMR) system, which is associated with MSI CRCs [11], or by widespread promoter DNA methylation within CpG islands as is the case with CIMP tumors [11, 12]. On the other hand, the CIN pathway, which manifests in majority of CRC cases (~85%), arises through widespread chromosomal imbalances [9, 13, 14]. We also make mention of the relationship between these defined pathways and the four consensus molecular subtyping classifications, with emphasis on the frequent overlap observed between two or more of the aforementioned pathways.

In the past few decades, several studies have analyzed epigenetic marks, the enzymes mediating these marks, and the extent of their active contribution to CRC tumor development and progression [2, 3]. For instance, several methylation-related enzymes have been found to be clinically relevant to CRC [15]. Among these, some of the most prominent histone methyltransferases (HMTs) that have been targeted for preclinical and clinical treatment of CRC are discussed in Section 4 of this chapter [15, 16]. On the other hand, a comparatively less number of histone demethylases (HDMs) have been validated as pertinent to CRC pathogenesis. As important regulators of colon cell transformation, histone deacetylases (HDAC) have also emerged as prominent markers of early carcinogenic events due to their unique role in maintaining higher-order chromatin structure [17].

In this chapter, we also highlight a few chemical inhibitors relevant to epigenetic therapy. However, we also noted that among the CRC-associated epigenetic enzymes, only a few of them have potent inhibitors available [15]. This suggests that the knowledge concerning targeting these enzymes for CRC is still insufficient and needs further evaluation. For example, only a few DNA methyltransferase (DNMT) and HMT inhibitors have been used in CRC cells [2], and a handful of Food and Drug Administration (FDA)-approved HDAC inhibitors are currently being explored for the treatment of solid tumors including CRC [18].

Unfortunately, the use of such epigenetic-based inhibitors has not been without limitations. Major drawbacks, such as adverse side effects and lack of clinical efficacy, have limited their use as single agents. Therefore, many inhibitors show more promise in combination therapy with chemotherapies suggesting that the full therapeutic potential of epigenetic therapy will perhaps be best realized in combination with other anti-cancer agents [19, 20]. This is also complemented by the recent understanding that there is a strong interplay between immune and cancer cells within the tumor microenvironment [21]. Recent studies in CRC cells have shown promising combinations of epigenetic and immunomodulatory drugs. By reversing expression changes of genes involved in immune suppression and thus enhancing expression of tumor-associated antigens, cancer cells potentially become more sensitive to immune checkpoint inhibitors [22]. These and other discoveries have established a highly promising basis for studies using combined epigenetic and immunotherapeutic agents for treating CRC.

2. Epigenetic modifications in CRC

2.1 Histone modifications

Over the past decade, significant advances in our understanding of the CRC "epigenome" have revealed that most CRC cases harbor alterations in their histone modification states, particularly regarding aberrant histone methylation and acetylation [6, 15, 23]. Importantly, these abnormal histone marks are highly recurrent and have recently been used as biomarkers to predict the clinical outcome in CRC patients [2]. These include changes in the global patterns of specific histone modifications. For example, Tamagawa et al. showed that global changes in histone H3K4me2, H3K9ac, and H3K9me2 in metachronous liver metastasis correlated to overall survival of CRC patients [24]. Specifically, low H3K4me2 levels were shown to correlate with overall poor prognosis [24]. Likewise, other studies have identified reduced levels of H3K9me3 and H4K20me3 as diagnostic biomarkers for CRC in circulating nucleosomes which correlated with poor patient outcome [25]. Conversely, high H4K20me3 and H3K9me3, as well as low nuclear expression of H3K4me3, were associated with a better prognosis for early-stage CRC patients [26].

Furthermore, since reduction or enrichment of these marks frequently occurs at the promoters of key CRC-related oncogenes and tumor suppressors, this results in detrimental changes in gene expression that form the basis of tumorigenesis [15, 27]. For instance, H3K4me3, when found to be elevated in CRC primary tumors and cell lines, resulted in activated *Wingless-type* (*WNT*) signaling and target gene expression via interaction between *SET domain-containing protein 1A* (*SETD1A*) and *β-catenin* [28]. Meanwhile, another study revealed that low H3K4me1/2/3 levels were associated with hypoxia-induced silencing of *MLH1* in SW480 cells, which is a key event in the DNA mismatch defects linked to the development of sporadic CRC [29]. Yokoyama's group also demonstrated a role for the well-recognized repressive mark H3K9me3, revealing that its increased levels in metastatic CRC patient-derived cells correlated with enhanced cell motility [30]. Interestingly, this coincided with repression of *Ataxia-telangiectasia mutated* (*ATM*) and *p53-associated KZNF protein* (*APAK*), leading to a defect in p53-dependent apoptosis [30]. Moreover, enrichment of another repressive mark, H3K27me3, was associated with poor CRC patient prognosis while elevated H3K79me2 was shown to enhance interleukin (IL)-22-induced stemness in CRC cells [31, 32]. Intriguingly, more recent studies have also shown that mutations in specific methylation sites could promote CRC development. For instance, the Shah and Lu groups identified histone 3 lysine 36-to-methionine (H3K36M) substitution mutations in CRC patient samples, which promoted more undifferentiated sarcomas *in vivo* [33, 34]. This suggests that H3K36 methylation potentially constitutes a major tumor suppressive epigenetic mark.

In addition to abnormal methylation, disruption of histone acetylation patterns also contributes to CRC pathogenesis, particularly relating to transcriptional inactivation of tumor suppressor genes and, sometimes, activation of oncogenes. For example, Richon et al. showed that hypoacetylation at the promoter of the tumor suppressor *p21WAF*[1] led to its repression, an effect that was reversed by inhibition of HDAC activity [35]. Conversely, mass spectrometry-based analyses used to quantify global alterations of histone modifications in CRC samples identified H3K27ac as a modification frequently upregulated in CRC [36]. In fact, one study highlighted the effects of aspirin in reducing the enrichment of H3K27ac in the promoters of *inducible nitric oxide synthase* (*iNOS*), *tumor necrosis factor alpha* (*TNF-α*), and *IL-6* [37]. This in turn corresponded to the dramatic reduction of the mRNA and protein levels of these genes, which suppressed inflammatory colitis symptoms and CRC tumor burden [37]. Taken together, these studies emphasize

the differential abundance of key repressive and activating histone methylation and acetylation marks in CRC and suggests their role in regulating genes associated with CRC development and progression.

2.2 DNA methylation

DNA methylation constitutes the first recognized epigenetic alteration in CRC [38]. Usually, global DNA hypomethylation is frequently seen, which occurs gradually and early in the process of CRC carcinogenesis [38]. More precisely, global DNA hypomethylation mainly takes place on cytosine guanine (CpG) dinucleotides within pericentromeric regions. Initially, this hypomethylation in CRC was hypothesized to be associated mainly with widespread oncogene activation but has now been linked predominantly to increased genomic instability [3]. This increased accumulation of chromosome breakage and overall chromosomal instability contributes to a prevalent subtype of CRCs known as the CIN phenotype as we briefly described in Section 1 [39].

Notably, hypomethylation typically occurs in concert with systematic and discrete DNA hypermethylation events at the promoters of genes involved in DNA repair, apoptosis, proliferation, angiogenesis, adhesion, and invasion [38, 40]. DNA hypermethylation is the most extensively characterized epigenetic alteration in CRC, occurring at CpG dinucleotide-dense regions, called CpG islands, which are present in about 60% of genes [6, 41]. Apart from CpG islands, DNA hypermethylation has also been extensively observed within the first exonic/intronic regions of some genes and generally results in transcriptional silencing [42]. Some of the most frequently hypermethylated genes in CRC include *Adenomatous polyposis coli* (*APC*), *Cyclin-dependent kinase inhibitor 2A* (*p16^{INK4a}*/*CDKN2A*), *Tissue inhibitor of metalloproteinases 3* (*TIMP3*), *O-6-Methylguanine-DNA methyltransferase* (*MGMT*), *Secreted frizzled related protein 1* (*SFRP1*), *Transmembrane protein with epidermal growth factor* (*EGF*) *like and two follistatin like domains 2* (*TMEFF2*), *Heparan sulfate-glucosamine 3-sulfotransferase 2* (*HS3ST2*/*3OST2*), *Ras association domain family member 1* (*RASSF1A*), and *GATA binding protein 4* (*GATA4*) [43].

Another subtype of CRCs with extensive patterns of promoter methylation, known as the CIMP phenotype as described in Section 1, is also characterized by aberrant DNA methylation at genes with roles in CRC initiation or progression [44]. For instance, using a qPCR-based technique, one group identified genes with the highest percentage of methylation in CRC patients including *Runt related transcription factor 3* (*RUNX3*), *Protocadherin 10* (*PCDH10*), *Secreted frizzled related protein 5* (*SFRP5*), *Insulin-like growth factor 2* (*IGF2*), and *Hepatocyte nuclear factor 1 homeobox A* (*HNF1*) *homeobox B* (*Hnf1b*) [45]. Moreover, these genes were observed to have the most promising biomarker potential because of the frequent gene repression patterns [45]. Other commonly hypermethylated genes, such as *Sex-determining region Y* (*SRY*)*-related HMG-box* (*SOX17*) and *Apoptosis-associated speck-like protein containing a CARD* (*ASC*)*/target of methylation-induced silencing* (*TMS1*), were differentially methylated based on the staging of the disease [46–48], whereas *MLH1*, *p16*, *Death-associated protein kinase 1* (*DAP-kinase*), *Ras association domain family member 2* (*RASSF2A*), and *WNT inhibitory factor 1* (*Wif-1*) were regarded as plasma or serum detection markers for CRC [49].

In summary, these data strongly support the promising utility of DNA methylation as a critical diagnostic marker for CRC. Unfortunately, this has not necessarily translated into their prognostic or predictive use in clinical practice [50]. This can be attributed to significant variability in sensitivity, specificity, and reproducibility between diverse patient cohorts and gene expression platforms, which ultimately impacts the prognostic value of many tests. Currently, two FDA-approved

commercial tests, Epi proColon® and Cologuard, have been used for screening alterations in methylation of common genes, such as *SEPT9*, *NDRG4*, and *BMP3*, for early detection of CRC [51]. However, they also generally lack prognostic value and require improvements in terms of sensitivity and specificity. Several other methylation biomarker assays have also been suggested, but validation in independent and large population screening studies is still needed [52].

2.3 Noncoding RNAs

Another epigenetic regulatory mechanism frequently deregulated in CRC involves the role of noncoding RNA (ncRNAs). Specifically, aberrations of microRNAs (miRNAs) expression, a major class of ncRNAs, are often observed in CRC and are considered to play a major role in tumorigenesis and CRC progression [23, 53]. These observations are consistent with the fact that miRNAs tend to exert oncogenic or tumor-suppressive effects. For example, miRNAs, such as miR-141, miR-200c, miR-145, miR-373, miR-520c, miR-135a, and miR-135b, have all been shown to affect CRC by regulating epithelial differentiation, WNT signaling, and CRC cell migratory and invasive potential [54]. Other miRNAs implicated in CRC include miRNA-124a and miRNA-34b/34c, which were shown to regulate the cell cycle and TP53 pathway, respectively [55]. Several miRNAs are also associated with epithelial-to-mesenchymal transition (EMT) in CRC. miR-15/16, miR-140, and miR-200 family members were shown to be associated with suppression of EMT and tumor cell metastatic potential while miR-21 enhanced this process [56].

Other studies have highlighted that alterations in the expression pattern of miRNAs in CRC were considered diagnostic, prognostic, or chemosensitivity markers [57]. For instance, high levels of miR-320 and miR-498 were correlated with progression-free survival in stage II CRC [58], while miR-21 abundance was associated with poor patient response to 5-fluorouracil adjuvant chemotherapy [59]. On the other hand, induced suppression of miR-21 promoted the sensitization of CRC cells to chemotherapeutic regimens [60, 61]. Another study by Toiyama et al. demonstrated a correlation between elevated serum miR-200c levels and stage IV CRC compared to earlier stages. Furthermore, high serum miR-200c showed a significantly positive correlation with lymph node metastasis, distant metastasis, and prognosis [62]. A comprehensive look of miRNAs as CRC biomarkers is reviewed by several other sources [60].

There is also ample evidence of miRNAs being downregulated in CRC and thus playing tumor-suppressive roles [63]. Arndt et al. showed that reduced levels of miR-133a as well as enrichment of miR-224 were associated with CRC initiation [64]. Moreover, this study and others also revealed that CRC patients at the adenomatous and carcinoma stages consistently exhibited reduced steady-state levels of miR-143 and miR-145 [63, 64]. Another classic example includes the miR-34 family. Transfection of miR-34a into CRC cells led to induction of apoptosis and inhibition of cell proliferation in part by amplifying the p53-mediated apoptotic response [65, 66]. Intriguingly, p53 has been shown to regulate miR-34a, suggesting a positive feedback loop between the two in which miR-34a could partly mediate the tumor-suppressive roles of p53 [55].

Interestingly, miRNA deregulation can also induce aberrant activity of many of the components of the epigenetic machinery [67]. Take *DNMT3A* for example, which has been identified as a miR-143 target and is associated with CRC via downregulation of miR-143 and subsequent increase in *DNMT3A* expression levels [68]. Other examples include miR-140 and miR-449, which have been shown to target and downregulate HDAC1 and 4, respectively, thus exerting their tumor-suppressive effects [69, 70]. Taken together, these findings underscore the importance of miRNAs in exerting both oncogenic and antitumor roles in CRC, which may serve as the basis for the development of novel prognostic and therapeutic markers.

3. Classification of CRC pathways and subtypes using epigenetic features

The development of CRC occurs via aberrations in multiple genetic and epigenetic pathways. These pathways can be defined by three principal molecular phenotypes (**Figure 1**). As aforementioned in Section 1, these include the MSI phenotype, which is characterized by mutations in DNA MMR genes; the CIN phenotype characterized by mutations in *APC*/*Wnt*/*β-catenin* pathway; and a third CIMP, defined by global CpG island hypermethylation, which results in widespread silencing of tumor suppressor genes [9, 71]. Notably, each pathway is characterized by distinct epigenetically related pathological features that drive the process of tumor initiation and development. In this section, we will describe various epigenetic aspects of these CRC phenotypes as well as how the molecular aspects of each pathway have been employed as useful diagnostic and prognostic tools to guide the clinical management of CRC patients. Finally, we also briefly acknowledge how these pathways may overlap within broader systems of subtype classification and highlight some of the current challenges in precisely defining CRC subtypes.

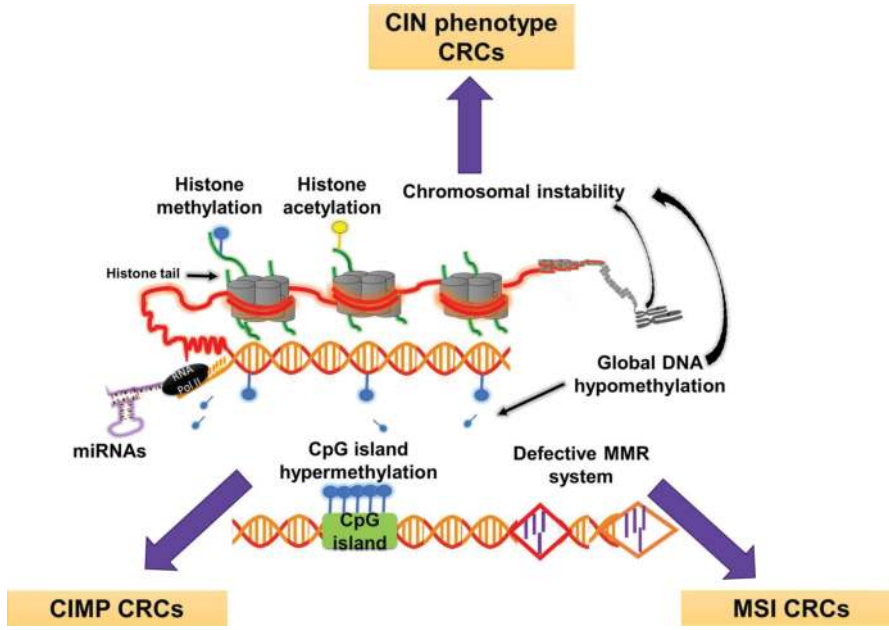

Figure 1.
Schematic depicting epigenetic alterations in CRC and their association with CRC molecular subtypes. The CRC "epigenome" harbors alterations in their histone modification states, particularly regarding aberrant histone methylation (blue dot) and acetylation (yellow dot). These global histone aberrations serve as biomarkers to predict the clinical outcome in CRC patients. DNA methylation is another major epigenetic alteration seen in CRC. Usually, global DNA hypomethylation occurs gradually and early in the process of CRC carcinogenesis. This occurs in concert with systematic and discrete CpG island hypermethylation events at the promoters of genes involved in DNA repair, apoptosis, proliferation, angiogenesis, adhesion, and invasion. This generally results in transcriptional silencing. Aberrations of micro-RNAs (miRNAs) expression, a major class of ncRNAs, are also often observed in CRC and are considered to play a major role in tumorigenesis and CRC progression. This figure also illustrates three main epigenetic-related molecular subtypes of CRC, namely the microsatellite instability (MSI) phenotype, the chromosomal instability (CIN) phenotype, and the CpG island methylator phenotype (CIMP). MSI CRCs arise from a defective DNA mismatch repair (MMR) system, which is associated with frameshift mutations and base pair substitutions in genes. CIMP CRCs are characterized by widespread promoter CpG island hypermethylation, whereas the CIN CRCs arise through widespread imbalances in chromosome number (aneuploidy), global hypomethylation, and loss of heterozygosity (LOH).

3.1 Microsatellite instability phenotype (MSI)

Approximately 15–20% of all CRC cases harbor MSI [72]. Microsatellites are defined as repetitive one to six base pair DNA sequences distributed along coding and noncoding regions of the genome. Importantly, the repetitive nature of these regions makes them particularly susceptible to mismatch errors [72]. Tumors with the MSI phenotype are therefore driven by the inactivation of MMR genes, which are involved in repairing DNA recombination and replication errors as well cellular responses to DNA damage [12]. The net effect of defective MMR machinery is accumulation of single base-pair mismatches, which results in a hypermutable cellular state [72].

MSI tumors are typically classified as MSI-high (MSI-H) or MSI-low (MSI-L). In MSI-H CRCs, usually two or more of the five microsatellite markers show instability, whereas in MSI-L tumors, only one of the five markers shows instability [12]. If none of the markers show instability, however, these are classified as microsatellite stable (MSS) CRC tumors, which account for approximately 80–85% of CRC patients [12]. Although majority of MSI-H tumors sporadically arise, a few are also linked to a familial hereditary syndrome known as Lynch syndrome or hereditary non-polyposis CRC (HNPCC) which account for about 3–5% of all CRC cases [12]. Sporadic MSI tumors are generally affected by epigenetic inactivation at the *MLH1* promoter via CpG island hypermethylation, whereas Lynch syndrome is caused by germline mutations in *MLH1*, *PMS2*, *MSH6*, or *MSH2* [12].

Importantly, unlike MSS, MSI tumors are poorly differentiated and are more often located in the proximal colon. MSI tumors also harbor a mucinous or signet ring type histology and increased number of tumor-infiltrating lymphocytes [72]. Additionally, when MSI was first identified in CRC patients, it was shown that MSI-H patients tended to have a better patient prognosis compared with MSS tumors and had an overall lower tumor stage at diagnosis [12]. Moreover, randomized phase III clinical trials along with several prospective studies have shown that the MMR status of these patients is also predictive of response to adjuvant 5-FU-based chemotherapy [12, 73]. The consensus was that patients with MSI-H tumors did not benefit from adjuvant 5-FU therapy compared to their non-MSI-H counterparts [73]. Furthermore, these data are consistent with other studies that revealed that human CRC cell lines with MSI-H phenotypes displayed resistance to DNA damaging agents, such as 5-FU, which could be overcome by the restoration of normal MMR function [74, 75].

3.2 Chromosomal instability (CIN) phenotype

The acquisition of genomic or chromosomal instability is a key feature in CRC development [39]. In fact, CIN has been found in approximately 85% of CRC cases and is characterized by increased chromosomal losses and gains as well as increased loss of heterozygosity [13, 14]. Although the exact mechanisms underlying CIN remain incompletely understood, it has been attributed to defects in genes related to the DNA damage response, telomere stability, and chromosomal segregation [39]. Unfortunately, standardizing the precise quantitative criteria that define a "CIN-positive" tumor has been challenging due to difficulties in the detection approaches of chromosomal instability [39]. The approaches currently in use include cytometry, karyotyping, and loss of heterozygosity analyses [76].

Along with the typical chromosomal abnormalities, accumulation of a characteristic set of mutations in specific tumor suppressor genes and oncogenes is

also a prominent feature observed in CIN tumors [39]. These include mutations in *APC*, *p53*, Cyclooxygenase-2 (*COX-2*), and *KRAS* as well as 18q alterations [39]. Interestingly, many studies have sought to determine the prognostic value of *KRAS*, *TP53*, or 18q alterations. So far, evidence of increased risk of relapse or mortality in CRC patients with *KRAS* mutations has been presented, but other studies have failed to confirm this correlation [77]. Consequently, some of these putative individual prognostic markers are still undergoing rigorous study. However, several compelling studies indicate that the overall CIN phenotype is associated with a less favorable outcome in patients than those with the MSI phenotype, and unlike MSI tumors, it is not significantly influenced by adjuvant therapy in patients with stage II–III CRC [78].

Several ongoing phase I and II clinical trials are underway to therapeutically target pathways that directly or indirectly initiate and perpetuate CIN. Some of these include small-molecule inhibitors of *COX-2*, *Polo-Like Kinases* (*Plks*), *Eg5*, and *Centromere protein E* (*CENP-E*) [39]. Swanton et al. also showed that CIN-positive tumors are intrinsically resistant to taxanes due to the similarity between pathways that regulate the chromosomal segregation and those implicated in the taxane response [79]. These and other studies have collectively prompted the Chromosomal Instability and Anti-Tubulin Response Assessment (CINATRA) trial to assess whether patients with MSI-positive solid tumors derive benefit from EPO906 (new microtubule stabilizer) compared to patients with CIN-positive cancers [80]. Overall, phase I trials showed encouraging tumor control and response rates in patients with metastatic CRC (mCRC), although the trial was prematurely closed due to toxicity issues [80]. In summary, these data support the role of the CIN pathway in guiding patient stratification and the clinical management of CRC. However, more studies to better define the mechanisms underlying CIN and determine how CIN influences progression will be critical to advance our understanding of the most common form of genetic instability in CRC. Moreover, the feasibility of standardizing detection of CIN-positive tumors and thus be able to target chromosomally unstable cells, will be critical.

3.3 CpG island methylator phenotype (CIMP)

The term "CpG island methylator phenotype," or CIMP, was coined in 1999 by Toyota and Issa to denote the CRC tumor subtypes characterized by widespread promoter DNA hypermethylation at certain tumor suppressor genes [81]. More than 50% of genes have promoters found within CpG islands [44]. Hence, the frequency of CIMP CRCs depends on which promoters are examined for methylation, with some promoters being more beneficial than others for identifying CIMP. Several studies have revealed that this methylation is common at the promoters of a diverse spectrum of genes, including *Phosphatase and tensin homolog* (*PTEN*), *RUNX3*, and *Unc-5 netrin receptor C* (*UNC5C*), making these key genes part of the expression signature profile in the evolution of CIMP CRCs [44]. Moreover, based on a panel of CIMP-specific markers coupled with the *B-Raf proto-oncogene* (*BRAF*) mutational status, CIMP tumors may be further classified according to the fraction of promoters that exceed a certain threshold of DNA methylation as being CIMP high, low, or negative [44]. Although CIMP-high and CIMP-low CRCs are significantly associated with biological sex as well as BRAF and KRAS mutational status, these classifications need additional refinement [44]. Nonetheless, it is reminiscent of the classification of CRCs based on degrees of MSI [82].

Notably, several of the clinicopathological characteristics of CIMP-high tumors have also been correlated to MSI tumors [82, 83]. For example, like MSI, CIMP tumors also represent a clinically distinct group characterized by epigenetic instability, distinct histological and pathological features, and discrete precursor lesions [84].

Pathologically, CIMP tumors also originate similarly to MSI tumors in the proximal colon, with a mucinous and poorly differentiated histological type and are frequently diagnosed in elderly and female patients [84, 85]. However, the determination of which specific methylated loci should be used to define CIMP remains a major challenge in the evaluation of CIMP tumors. Currently, several studies have proposed the classic panel containing the genes *hMLH1* and *p16* as well as Munc-18-interacting (MINT) proteins *MINT1*, *MINT2*, and *MINT31*. This panel has been further developed to contain the genes *Calcium voltage-gated channel subunit alpha1 G* (*CACNA1G*), *Cellular retinoic acid binding protein 1* (*CRABP1*), *IGF2*, *Neurogenin 1* (*NEUROG1*), *RUNX3*, *Suppressor of cytokine signaling 1* (*SOCS1*), *Hypermethylated in cancer 1* (*HIC1*), *IGF-binding protein 3* (*IGFBP3*), and *Werner syndrome ATP-dependent helicase* (*WRN*) [86].

In recent years, the use of DNA hypermethylation of specific genes to predict CRC patient outcome and therapeutic approach has received much attention. Although further validation is warranted, many studies have found a correlation between MSS CIMP+ CRC patients and poor prognosis [87]. Furthermore, the correlation between CIMP status and response to chemotherapy has been investigated. CIMP status predicts poor survival in metastatic MSS CRC patients treated with chemotherapeutic agent 5-FU [88]. Overall, patients who did not receive chemotherapy treatment but had tumors identified as MSS and CIMP had a worse survival outcome [89]. In contrast, two separate studies reported better outcomes for patients with CIMP tumors, a conflict that could be attributed to differences in the criteria used across the studies to define CIMP status. Additionally, this also suggests that the heterogeneous nature of CIMP tumors may warrant further classification [44].

Intriguingly, many studies have also found an association between CIMP status and other important epidemiological factors [90]. For instance, reports of an association between cigarette smoking, obesity, and CIMP showed that the number of cigarettes smoked as well as body mass index (BMI) had a significant relationship to CIMP tumor development [90, 91]. Furthermore, associations of CIMP status with smoking and obesity were evident only for females [90]. Taken together, the above evidences support a critical role of the CIMP pathway in the pathogenesis of CRC, which has also become a significant part of the current management of CRC. In the future, it will also be essential to have a consensus on a standardized panel of loci to define CIMP, similar to that utilized to identify MSI CRCs.

3.4 Consensus molecular subtypes

The three molecular pathways described so far also fall within several consensus classification systems for CRC. These systems vary in terms of the number of proposed subtypes, which can range from three to six depending on the combinations of genetic, epigenetic, clinical, and histopathological parameters used as well as the extent of the overlap between the three molecular pathways. For instance, the Consensus Molecular Subtypes (CMS) consortium has been suggested as one of the most robust classification systems and describes four groups (CMS1–4) based on expression profiling data from multiple studies [92]. While the majority of MSI-H CRCs fall into the CMS1 category, CMS2–4 display higher CIN. However, CMS3 samples have a distinctive profile compared with other CIN tumors. They tend to have lower CIN, higher prevalence of CIMP and close to 30% of the tumors are hypermutated which confers significant overlap with MSI status tumors [93]. Additionally, the Cancer Genome Atlas study also demonstrated that CIMP overlaps with the MSI pathway because of the fact that sporadic MSI-H CRCs usually harbor CIMP-high clinicopathological features [94]. Meanwhile, CMS4 are defined as CIN-heterogeneous tumors with mesenchymal characteristics that occur in later disease stages [93].

It is also noteworthy that many CRC tumors demonstrate mixed characteristics compatible with two or more of these subtypes, which may represent a transition phenotype or intratumoral heterogeneity, while others cannot be precisely classified into any of these pre-defined subgroups [95]. Furthermore, these classifications often lack incorporation of the molecular markers used for traditional TNM staging of CRC [96]. Taken together, these challenges as well as the existing incongruity between the various systems illustrate the need to further refine these consensus classifications by developing more progressive and integrated approaches.

4. The role of major epigenetic enzymes in CRC and therapeutic strategies for targeting them

4.1 Histone methyltransferases and demethylases

As discussed so far, aberrant changes in epigenetic modifications can significantly contribute to CRC progression. It is therefore unsurprising that many of the epigenetic enzymes mediating these modifications are themselves deregulated during the initiation and progression of CRC. Here, we describe the significance of changes in the expression levels of two such families of enzymes that oppose each other in terms of function, namely histone methyltransferases (HMTs) and demethylases (HDMs). Although changes in the expression or activity levels of several methylation-related enzymes have been linked to CRC, in most cases only a limited knowledge regarding the molecular mechanisms by which these enzymes contribute to disease development exists [15]. We summarize current knowledge regarding some of the preclinical validated implications of these enzymes as proof of principle for the employment of epigenetic agents in CRC. We also briefly discuss potential mechanisms of action of these enzymes as well as the advantages of targeting them using combinatorial over monotherapy approaches.

Histone lysine methyltransferases (HKMTs) have been widely studied across multiple solid tumor types including CRC [97]. For instance, studies in a preclinical model of CRC found that increased expression and activity of *SET and MYND domain containing 3* (*SMYD3*), a well-known HKMT, was strongly correlated with tumorigenesis. Moreover, RNAi-mediated depletion of *SMYD3* significantly impaired CRC cell proliferation, indicating a crucial role of *SMYD3* in maintaining CRC malignancy [98]. More recent studies suggest a putative mechanism by which this overexpression might occur by demonstrating that hypomethylation of the *SMYD3* promoter was observed in CRC tumor tissues compared to adjacent normal tissues. Further subgroup clinicopathological analyses showed that this hypomethylation was observed with stage III and IV tumors as defined by moderate to well-differentiated histology and positive lymph node metastasis [99].

Another well-studied HKMT, *enhancer of zeste 2* (*EZH2*), is also frequently deregulated in CRC. Both mRNA and protein levels of *EZH2* were found to be significantly increased in CRC tissues compared to non-cancerous counterparts [16]. Additionally, increased *EZH2* expression was directly correlated with tumor size, metastases, and overall worse disease-free survival of CRC patients [100]. He et al. also showed that siRNA-mediated depletion of *EZH2* inhibited the proliferation and migration of SW620 CRC cells, while inducing apoptosis and G0/G1 cell cycle arrest [101]. Another mechanistic study also revealed that knockdown of *EZH2* significantly reduced CRC cell invasion and *matrix metalloproteinases 2/9* (*MMP2/9*) secretion *in vitro* while promoting increased overall survival and decreased lung metastasis *in vivo* [102]. Furthermore, this *EZH2*-induced CRC cell invasion was mediated by direct binding of *Signal transducer and activator of transcription 3*

(*STAT3*) to the *EZH2* promoter, resulting in downregulation of the vitamin D receptor (VDR) [102]. Interestingly, an association between a missense variant in *EZH2* and risk of CRC was discovered by the Li group. They identified that the presence of the rs2302427 variant showed a significant association with increased CRC susceptibility [103]. Recent studies point to other mechanistic roles of HKMTs in CRC. For example, depletion of *SETD1A*, a member of the trithorax (TrxG) family of HMTs, inhibited CRC cell growth and colony formation in part by decreasing expression of approximately 50% of *Wnt/β-catenin* target genes [28]. Finally, in a mouse model, IL-22-mediated activation of *disruptor of telomeric silencing 1-like* (*DOT1L*) promoted CRC stemness and tumorigenic potential and was considered a predictor of poor survival outcome in CRC patients [32].

Protein arginine methyltransferases (PRMTs), although studied to a lesser extent, have also been shown to play critical roles in CRC malignancy via activation of *Wnt/β-catenin* and NF-κB signaling [104]. *CARM1*, for example, is an important positive modulator of *Wnt/β-catenin* transcription and was found to promote survival and anchorage-independent growth of CRC cells with aberrantly activated *Wnt/β-catenin* signaling [105]. Meanwhile, our lab and others have shown that *protein arginine methyltransferase 5* (*PRMT5*) was overexpressed in CRC cells and patient-derived primary tumors, which correlated with increased cell growth, migration, invasion, and NF-κB activation as well decreased overall patient survival [106–109]. The enzymes catalyzing removal of methylation marks, HDMs, are perhaps the least studied among the enzymes mentioned thus far and only a few have been implicated as playing tumor suppressive or oncogenic roles in CRC. *LSD1*, *KDM4B*, *KDM4C*, and *KDM5B* have all been shown to play pro-tumorigenic roles by promoting CRC cell growth and metastasis, whereas HDMs, such as *JMJD3* and *JMJD1B*, have been implicated as tumor suppressors [15]. Taken together, these data provide strong support for the continued development of selective and potent small-molecule inhibitors against these methylation-modifying enzymes as promising therapeutic agents for CRC.

4.2 Targeting HMTs and HDMs in CRC

Disruption of epigenetic regulation in CRC mediated by deregulated HMTs, DNMTs, and HDMs has garnered increasing interest in recent years. In this section, we aim to review the current status on the development of therapeutic strategies to modulate histone methylation for CRC treatment. The current therapeutics including pre-clinical and clinical agents that target epigenetic enzymes in CRC are listed in **Table 1**. Thus far, more than 20 histone-methylation enzymes have been found to be clinically relevant to CRC, including 17 oncoproteins and 8 tumor suppressors, although their exact mechanisms of action are not fully understood [15]. Furthermore, more than 20 small-molecule inhibitors targeting HMTs, DMNTs, and HDMs have been employed for preclinical or clinical studies. For example, treatment of DLD1 colon cell line and primary CRC cells with a potent HKMT inhibitor EPZ004777 (anti-*DOT1L*) resulted in significant reduction in sphere formation *in vitro*, thus inhibiting cell growth [32]. Other HKMT inhibitors, such as BCI-121 and Chaetocin, have significantly suppressed CRC cell growth and migration by inhibiting *SMYD3* and *SUV39H1*, respectively [98]. Notably, inhibitors against the HKMT *EZH2* have yielded some of the most promising results for treating CRC. DZNep, an indirect *EZH2* inhibitor, induced apoptosis in CRC cell lines and stem cells, while GSK346 impaired the migratory potential of CRC cells and reduced H3K27me3 levels in Colo205 and HT-29 cells (**Table 1**) [110].

Unlike HKMTs, the development of inhibitors against PRMTs has only recently gained prominence in the cancer field, and only a couple of these have made it

Epigenetic drug class	Drug & Target	Description of study design	Agent used in combination with epigenetic drug
DNMT inhibitors	5-Azacitidine: DNMT1	Phase I in solid tumors including mCRC	Erlotinib [20]
		Phase I/II in mCRC	Capecitabine [20]
			Oxaliplatin [20]
	Decitabine: DNMT1	Preclinical and Phase I in solid tumors including mCRC	Carboplatin [20]
			Gefitinib [115]
			Panitumumab [20]
	5-fluoro-2'-deoxycytidine: DNMTs	Phase I in solid tumors including mCRC	Tetra-hydrouridine (THU)[20]
	Guadecitabine: DNMTs	Ongoing Phase II in mCRC [NCT01896856]	Irinotecan [20]
HMT inhibitors	EPZ004777: DOT1L	Preclinical in CRC cells and mouse xenografts	Unknown [32]
	DZNep: EZH2	Preclinical in CRC cells and mouse xenografts	Unknown [101]
	GSK346: EZH2	Preclinical in CRC cells and mouse xenografts	Unknown [110]
HDAC inhibitors	CI-994: HDAC 1, 2, 3, and 8	Phase I in advanced solid tumors including mCRC	Capecitabine [20]
	Entinostat: HDAC1 and HDAC2	Phase I in advanced solid tumors including mCRC	13-cis retinoic acid [20]
			Sorafenib [20]
	Panobinostat: HDAC	Phase I in advanced solid tumors including mCRC	Bevacizumab [20]
	Pheniylbutyrate: HDAC	Phase I in mCRC	5-FU [20]
	Vorinostat: HDAC, HDAC1, HDAC3	Phase I/II in solid tumors including mCRC	Modified FOLFOX6 or Bortezomib [20, 135]
			Pazopanib [20]
			FOLFOX [135]
			Doxorubicin [134, 135]
			Hydroxychloroquine or Regorafenib [134, 135]
	ACY-1215: HDAC6	Preclinical in CRC cells and mouse xenografts	Oxaliplatin [129]
	CG2: HDAC	Preclinical in CRC cells and mouse xenografts	Oxaliplatin 5-FU Irinotecan [128]

Table 1.
Overview of pre-clinical and clinical drugs that target epigenetic enzymes in CRC.

to the clinical trial phase thus far. AMI-1, which inhibits *PRMT1* and *PRMT5*, demonstrated antiproliferative activity in CRC cells and xenograft mouse models [106]. However, further *in vivo* validation studies are needed, and it has not

entered clinical trial yet. Another promising *PRMT5* inhibitor that recently made it to Phase I clinical trials is GSK3326595, which potently inhibited tumor growth *in vitro* and *in vivo* [111]. Trials with GSK3326595 are currently being conducted in adult subjects with relapsed and/or refractory solid tumors (NCT02783300). Additionally, inhibitors targeting HDMs are even fewer in number and have shown limited efficacy in suppressing CRC cell growth. For example, *KDM4A*/*C* inhibitors were ineffective in blocking HCT116 CRC cell growth when used in isolation [112]. However, they exhibited potent antiproliferative effects in combination with another HDM inhibitor, NCL-2, which targets *LSD1* [113]. These data suggest a potential for synergy between the two classes of HDM inhibitors.

Finally, the use of DNMT inhibitors for CRC treatment has also shown some exciting promise. In studies using CRC cell lines, suppression of *DNMT1* and *DNMT3B* resulted in significant reduction in methylation, which correlated with the re-expression of tumor suppressor genes. This also resulted in induction of apoptosis as well as reduced cell proliferation and stemness [114]. Notably, studies with the *DNMT1* inhibitor, 5-aza-2′-deoxycytidine (decitabine), exhibited its ability to re-sensitize colorectal tumors to both irinotecan and 5-FU, thus becoming a major component of the treatment regimen for CRC in the clinic [19]. Another recent preclinical study showed that combination of the anti-*EGFR* inhibitor, gefitinib and decitabine showed highly synergistic inhibition of CRC cell proliferation and migration [115]. Additional combination regimens are outlined in **Table 1**.

4.3 Acetyltransferases and deacetylases

Acetylation of histones by acetyltransferases (HATs) and removal of these acetyl marks by HDACs are essential events for the maintenance of normal chromatin organization and function [116]. However, as is often the case in cancer, these enzymes are dysregulated, leading to increased chromosomal instability and aberrant gene expression changes [117]. To date, only a handful of HATs have been reported as contributing to the pathogenesis of CRC. Here, we describe the role of a few of these HATs namely *p300*/*CREB-binding protein* (*p300*/*CBP*), *GCN5*, *N-Acetyltransferase 10* (*Nat10*), and *Human males absent on the first* (*hMOF*). Assessment of 262 CRC samples from patients receiving 5-FU treatment demonstrated that low expression of *p300*/*CBP* in CRC tissue was closely associated with poor clinical response to 5-FU based-chemotherapy [118]. Furthermore, low *p300*/*CBP* expression also correlated with poor disease-free survival and increased early disease progression in the same patients [118]. Mechanistic studies also uncovered that 5-FU induced degradation of *p300*/*CBP* which was dependent on chaperone-mediated autophagy involving *heat-shock cognate protein 70 kDa* (*hsc70*) and *lysosomal-associated membrane protein 2A* (*LAMP2A*). In short, degradation of *p300*/*CBP* was found to be relevant to chemoresistance to 5-FU, since blocking this degradation also enhanced 5-FU's cytotoxicity in CRC cells [118].

Conversely, another HAT *GCN5* has been implicated in promoting CRC cell growth via its upregulation rather than downregulation. One study found that *GCN5* overexpression in human colon adenocarcinoma tissues was attributed to the activities of the transcription factors, *c-Myc* and *E2F transcription factor 1* (*E2F1*) [119]. Depletion of *c-Myc* inhibited CRC cell proliferation mainly by downregulating GCN5 transcription, an effect that was rescued by ectopic expression of *GCN*5. However, ectopic overexpression of *E2F1* had the opposite effect by suppressing *GCN5* levels, thus inducing cell death. Furthermore, inhibition of *GCN5* with CPTH2, a HAT inhibitor, also suppressed CRC cell growth, revealing an avenue of great therapeutic potential [119]. Other HATs implicated in CRC include *Nat10* and *hMOF*, which were downregulated in CRC tissues. Particularly, recent studies

showed that *Nat10* downregulation and subcellular redistribution were associated with increased cellular motility and invasion in CRC cells [120]. Meanwhile, low expression of *hMOF* correlated with clinicopathological features of CRC such as lymph node metastasis and advanced tumor stage [121].

In CRC, HDACs are also frequently overexpressed and represent another attractive class of targets for anticancer therapy. *HDAC1–3* and *HDAC5–8* have emerged as some of the most relevant deacetylases in CRC. Although all are highly overexpressed in CRC, only few studies have explored the relevance of this overexpression to disease [23]. For example, knockdown of *HDAC1*, *2*, and *3* reduced the growth of several CRC cells by largely unknown mechanisms [122]. Interestingly however, a mechanistic link between *HDAC2* expression and sensitivity of CRC cells to other anticancer agents was recently established. Alzoubi et al. demonstrated that depletion of *HDAC2* specifically enhanced the combined anti-tumor effect of the pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and the DNA-damaging agents, 5-FU and oxaliplatin, in SW480 and HT29 cells. On the other hand, overexpression of *HDAC2* conferred resistance to these agents, which were independent of the p53 mutational status [123]. In summary, these findings strongly suggest that HATs and HDACs are critical biomarkers for CRC and influence the sensitivity of CRC cells to certain therapeutics as evidenced by their frequent combination with other anticancer agents (**Table 1**).

4.4 Targeting HATs and HDACs in CRC

Like methylation, several studies have demonstrated that inhibitors targeting HATs and HDACs also induce epigenetic alterations that modulate the expression of genes or pathways critical for CRC treatment. One study showed that direct inhibition of *p300/KAT3B* histone acetyltransferase, a coactivator of *β-Catenin* with rimonabant, induced downregulation of *Wnt/β-catenin* target genes in HCT116 cells [124]. Furthermore, rimonabant also significantly reduced tumor growth in HCT116 xenografts [124]. The general HAT inhibitors such as garcinol and anacardic acid have also been shown to inhibit CRC tumor growth in mice as well as sensitize cancer cells to irradiation [125].

Compared to HATs, a far greater number of studies have been dedicated to investigating the efficacy of HDAC inhibitors at both the preclinical and clinical level. Overall, the use of HDAC inhibitors in preclinical solid tumor models has shown some early promise albeit their progress to the clinic has been hindered by serious limitations including ineffective concentrations and cardiac toxicity [126]. In CRC specifically, these inhibitors are mainly being administered as combination therapy with conventional chemotherapy or other agents [127]. In pre-clinical models for instance, treatment of irinotecan-resistant CRC cells with HDAC inhibitors conferred sensitization of these cells to irinotecan, whereas HDAC inhibitor CG2 showed an additive effect when used with irinotecan, 5-FU, or oxaliplatin in HCT116 xenografts [128]. Meanwhile, a small molecular inhibitor of *HDAC6*, ACY-1215, was able to enhance the anticancer activity of oxaliplatin by promoting apoptosis and blocking cell proliferation in CRC cells and xenograft models [129].

4.5 Benefits and pitfalls of epigenetic enzyme inhibitors

Despite their potential, a large gap still remains between the biological activity of epigenetic enzyme inhibitors in preclinical studies and their potential clinical utility. For example, the development of HAT inhibitors poses several challenges [130]. Because of their function in complexes consisting of many proteins which play multiple roles in HAT target specificity, this significantly limits inhibitor use *in vivo* [130].

Additionally, many undesired effects such as reactivity or lack of selectivity between different HAT subtypes are often associated with HAT inhibitors [130]. Hence, studies geared towards the development of more potent and selective inhibitors by carefully studying the catalytic mechanism and enzyme kinetics of various HATs are needed. As far as HDAC inhibitors are concerned, they have shown preferential efficacy against hematological malignancies, and therefore, drugs such as vorinostat (SAHA) and romidepsin (FK228) have achieved FDA approval for the treatment of cutaneous T cell lymphoma [131, 132]. Unfortunately, the evidence regarding HDAC inhibitors efficacy for solid tumors has not been as convincing and encouraging although they are well tolerated at low but not high doses. Currently, adverse side effects and inadequate clinical efficacy are the major limitations to their use, and more efforts are underway to generate specific HDAC inhibitors for solid tumors such as CRC [133]. Nonetheless, a few early phase clinical trials using vorinostat in combination with other chemotherapeutic agents have shown some early promise for mCRC patients. These include combinatorial regimens of vorinostat with 5-FU, leucovorin, and oxaliplatin (FOLFOX) as well as randomized phase II trial studies investigating the efficacy of vorinostat and hydroxychloroquine or regorafenib in refractory mCRC patients [134, 135]. Other regimens are outlined in **Table 1**. Similarly, while DNMT inhibitors have also met with some degree of success for treating blood cancers such as myelodysplastic syndrome (e.g., decitabine and 5-azacitidine), the major drawbacks of these compounds in solid tumors are harsh side effects and transient demethylation, which revert after drug removal [136, 137]. Interestingly, however, some studies have suggested that this transient demethylation that occurs with DNMT inhibition (e.g., 5-azadeoxycytidine) potentially creates a therapeutic window that can be leveraged for epigenetic reprogramming and/or combinatorial therapies with cytotoxic drugs [138].

Other general limitations regarding the use of epigenetic therapy in solid tumors deal with the unfavorable pharmacokinetic properties of these drugs, including instability, toxicity, and short half-life [137]. Some of these invariably contribute in some way to common toxicities associated with HDAC and DNMT inhibitors in CRC including thrombocytopenia, neutropenia, diarrhea, nausea, vomiting, and fatigue [139]. Furthermore, maintaining therapeutically relevant levels of the drugs necessary for clinical benefit is particularly difficult, and as of yet, no FDA-approved epigenetic treatments exist for CRC despite promising preclinical studies. This signifies the overall marginal clinically compelling responses to these agents in CRC patients. To overcome some of these limitations, newer formulations have been made to render these inhibitors more bioavailable, stable, and ultimately usable at lower doses with less toxicity and greater therapeutic efficacy. Examples of these include the oral HDAC inhibitor, entinostat, used in *in vitro* and *in vivo* models of CRC and an orally active formulation of 5-azacitidine, cc-486 [140].

4.6 Emerging immunomodulatory and epigenetic combinatorial therapies

Compared to MSS tumors, there are an exponentially higher number of mutations acquired in MSI-H CRCs. Interestingly, these mutations have the potential to elevate the production of neo-antigens [141]. The result is increased tumor immunogenicity, which is further complemented by the fact that these tumors also harbor a high number of tumor-infiltrating lymphocytes. Within this context, CRC patients with MSI-H represent a subgroup more likely to benefit from immune checkpoint inhibitors compared to those with MSS tumors. Immune checkpoint inhibitors have shown unprecedented benefit across multiple tumor types. These agents specifically target the proteins *programmed death ligand-1* (e.g., durvalumab) and *programmed death-1* (e.g., nivolumab, pembrolizumab) and are administered

as monotherapies or in combination with other anticancer agents. At the present time, several ongoing early and late-phase II and III clinical trials investigating the efficacy of immune checkpoint inhibitors in MSI-H and MSS CRC patients are being extensively explored including pembrolizumab (Keytruda), which recently obtained FDA approval (e.g., NCT01876511 and NCT02060188) [142].

Moreover, the possibility of combining epigenetic therapy and immunotherapy has also been recently explored, and several ongoing clinical trials in CRC investigating the combination strategies of HDACi and DNMTi with checkpoint inhibitors have been undertaken. Specifically, these epigenetic therapies have been shown to augment the effect of checkpoint inhibitors and are currently in early and late phase clinical trials [143]. However, since MSS subtypes represent the larger fraction of CRC cases, the marginal activity displayed by drugs such as pembrolizumab for treating MSS CRCs has been less than encouraging [144]. Hence, overcoming the clinical ineffectiveness of this class of drugs for this subtype remains an important need. Intriguingly, however, recent studies showed that treatment with 5-azacitidine and entinostat in CRC cell lines conferred a shift towards a CIMP+ signature, which would predictively convert them into a more immunogenic state [145]. This increased sensitivity to immunotherapy has prompted a clinical trial evaluating this strategy, with the combination regimen of romidepsin (HDAC inhibitor) and cc-486 with pembrolizumab in MSS-CRC patients (NCT02512172) [145]. Finally, romidepsin was also found to potentiate 5-FU cytotoxicity in HCT-116, HT29, and SW48 cells by inducing apoptosis and cell cycle arrest [146]. Interestingly, MHC class II gene expression was also induced with this combination, once again supporting the possible cooperation of epigenetic therapy with immunomodulatory agents [146]. In summary, the above evidences support a cooperative role between epigenetic and immune therapies, although further efforts to optimize the epigenetic control of immune-related gene expression will be necessary to successfully translate these notions to the clinic.

5. Conclusion and perspectives

In this chapter, we have highlighted the pivotal contribution of epigenetic deregulation, specifically, DNA methylation, histone alterations, and miRNAs to the initiation, progression, and prognosis of CRC. We also underscored the relevance of these epigenetic mechanisms in terms of classifying CRC subtypes as well as their importance in guiding strategies for therapeutic intervention. Moreover, we emphasized the epigenetic enzymes that are involved in these aberrant pathways and presented some up-to-date findings on pre-clinical and clinical trials of epigenetic drugs used as single agents or in combination with conventional anticancer agents in CRC. Furthermore, mounting evidence demonstrates that epigenetic drugs are also capable of altering the immunogenicity of the CRC microenvironment and creating opportunities for potentiating the effects of immune checkpoint inhibitors.

Understandably, drugs targeting the cancer epigenome are also plagued with major challenges including lack of specificity, toxicity, and short half-life. Fortunately, these challenges have facilitated re-evaluation of the dosing and formulation strategies for epigenetic drugs, leading to superior therapeutic drugs with lower toxic profiles. Another underexplored avenue includes targeting less commonly manipulated epigenetic mechanisms such as the use of miRNA mimics [147]. Furthermore, in light of the advent of personalized therapies, more intricate studies are also needed to elucidate the relationship between individual driver genetic mutations and epigenetic alterations, thus providing a pathway-driven basis for developing selective therapeutic strategies. This may call for a more stringent control of gene expression in CRC cells via selective targeting of epigenetic regulatory

enzymes. This includes the prospects of CRISPR/Cas9/Cas13-based genome and RNA editing, which may provide validated starting points for further development towards novel CRC therapeutic agents [148].

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Abbreviations

APAK	ATM and p53-associated KZNF protein
APC	adenomatous polyposis coli
ASC/TMS1 or PYCARD	apoptosis-associated speck-like protein containing a CARD
BET	bromodomain and extra-terminal motif
BMI	body mass index
BRAF	B-Raf proto-oncogene, serine/threonine kinase
CACNA1G	calcium voltage-gated channel subunit alpha1 G
CARM1	coactivator-associated arginine methyltransferase 1
CDKN2A/p16^{INK4a}	cyclin-dependent kinase inhibitor 2A
CENP-E	centromere protein E
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
COX-2	cyclooxygenase-2
CRABP1	cellular retinoic acid binding protein 1
CRC	colorectal cancer
DAP-kinase	death-associated protein kinase 1
DNMT	DNA methyltransferase
DNMT3A	DNA methyltransferase 3A
DOT1L	disruptor of telomeric silencing 1-like
E2F1	E2F transcription factor 1
Eg5	kinesin 5 family member
EMT	epithelial-to-mesenchymal transition
EZH2	enhancer of zeste 2
FDA	Food and Drug Administration
5-FU	5-fluorouracil
GATA4	GATA-binding protein 4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
HIC1	hypermethylated in cancer 1
HKMT	histone lysine methyltransferase
HKMTs	histone lysine methyltransferases
hMLH1	human mutL homolog 1
hMOF	human males absent on the first
HMT	histone methyltransferase

Hnf1b	HNF1 homeobox B
HNPCC	hereditary non-polyposis colon cancer
HS3ST2 (3OST2)	heparan sulfate-glucosamine 3-sulfotransferase 2
hsc70	heat-shock cognate protein 70 kDa
IGF2	insulin-like growth factor 2
IGFBP3	insulin-like growth factor binding protein 3
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
KDM	lysine demethylase
KRAS	Kirsten rat sarcoma 2 viral oncogene
LAMP2A	lysosomal-associated membrane protein 2A
LOH	loss of heterozygosity
mCRC	metastatic colorectal cancer
MGMT	O-6-methylguanine-DNA methyltransferase
MINT	Munc-18-interacting
miRNA	microRNA
MLH1	mutL homolog 1
MSH2, 6	MutS protein homolog 2, 6
MSI	microsatellite instability
MSS	microsatellite stable
Nat10	N-acetyltransferase 10
NEUROG1	neurogenin 1
nRNA	noncoding RNA
p300/CBP	p300/CREB-binding protein
p53 or TP53	tumor protein 53
PCDH10	protocadherin 10
PD-1	programmed death-1
PDL-1	programmed death ligand-1
Plks	polo-like kinases
PMS2	PMS1 homolog 2, mismatch repair system component
PRMT	protein arginine methyltransferase
PRMT5	protein arginine methyltransferase 5
PTEN	phosphatase and tensin homolog
RASSF1A	ras association domain family member 1
RASSF2A	ras association domain family member 2
RUNX3	runt-related transcription factor 3
SAHA	suberoylanilide hydroxamic acid
SETD1A	SET domain-containing protein 1A
SFRP1	secreted frizzled related protein 1
SFRP5	secreted frizzled related protein 5
SMYD3	SET and MYND domain containing 3
SOCS1	suppressor of cytokine signaling 1
SOX17	SRY-Box 17
STAT3	signal transducer and activator of transcription 3
SUV39H1	suppressor of variegation 3–9 homolog 1
TIMP3	tissue inhibitor of metalloproteinases 3
TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2
TNF-α	tumor necrosis factor alpha
UNC5C	Unc-5 netrin receptor C
Wif-1	WNT inhibitory factor 1
WNT	wingless type
WRN	Werner syndrome ATP-dependent helicase

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