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Review

Epigenetic modifications in colorectal cancer: Molecular insights and therapeutic challenges

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ABSTRACT

Colorectal cancer, a leading cause of mortality worldwide, is a multistep disorder that results from the alteration of genetic and epigenetic mechanisms under contextual influence. Epigenetic aberrations, including DNA methylation, histone modifications, chromatin remodeling and non-coding RNAs, affect every aspect of tumor development from initiation to metastasis. Cancer stem cell promotion is also included in the wide spectrum of epigenetic dysregulations. Elucidation of this complex crosstalk network may offer new insights in the molecular interactions involved in the pathogenesis of colorectal carcinogenesis. In the era of translational medicine new horizons are opened for the pursuit of personalized therapeutic approaches and the development of novel and accurate diagnostic, prognostic and therapy-assessment markers. This review discusses the implications of epigenetic mechanisms in tumor biology and their applications "from bench to bedside".

1. Introduction

Colorectal cancer (CRC) is a leading cause of morbidity and mortality worldwide, with more than 600,000 deaths annually [1]. It is a multistep disorder that results from the accumulation of genetic and epigenetic aberrations under microenvironmental influence. Abnormalities in key regulatory genes and pathways, including p53, Wnt, DNA mismatch repair genes and Ras drive the progression of the disease from benign adenoma, to carcinoma and eventually to metastatic disease [2]. Genomic instability is pivotal for CRC development and is attributed to chromosomal instability (CIN) and microsatellite instability (MSI). Such dysregulations may cause sporadic or hereditary syndromes of CRC. CIN is a major defect found in cases of CRC (about 80%), which leads to gain or loss of whole chromosomes or chromosomal parts and the dysfunction of crucial genes such as Ras, adenomatous polyposis coli (APC) and p53. In MSI tumors a defect in mismatch repair genes is observed [3–5].

Epigenetic regulation refers to heritable and possibly reversible changes in the phenotypic expression of the genome, which alter gene expression without affecting DNA sequence [6,7]. In the wide gamut of epigenetic mechanisms are included, DNA methylation, histone modifications, chromatin remodelers and non-coding RNAs, which are implicated in the activation of oncogenes, the loss of function of tumor suppressors and the loss of imprinting [4,7,8]. By fine-tuning the accessibility of DNA, epigenetics orchestrate various physiological procedures (transcription, replication, repair) from developmental to differentiated stages and possess a pivotal role in the process of tumorigenesis. Genetic and epigenetic aberrations are involved in a complex network and can both predispose to or cause the development of each other (Fig. 1) [5,7,9]. The understanding of these mechanisms may contribute to the optimization of diagnostic and prognostic systems as well as the generation of novel and targeted therapeutic approaches.

Abbreviations: 5-FU, fluorouracil; A, acetyl-groups; ALDH1, aldehyde dehydrogenase-1; AP-1, activator protein 1; APC, adenomatous polyposis coli; ASO, anti-sense oligonucleotides; Bmi1, B lymphoma Mo-MLV insertion region 1 homolog; BMP, bone morphogenetic protein; CSC, cancer stem cell; CBP, CREB-binding protein; CDH1, cadherin-1; CDKN2A, cyclin-dependent kinase inhibitor 2A; CDX1, caudal type homeobox-1; CGI, CpG islands; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CpG, cytosine guanine; CRC, colorectal cancer; DNMT, DNA methyltransferase; DNMTi, DNMT inhibitor; EGCG, epigallocatechin 3-gallate; ERK, extracellular signal-regulated kinase; EZH2, histone methyltransferase enhancer of Zeste 2; FDA, Food and Drug Administration; H, histone; HAT, histone acetyltransferases; HDAC, histone deacetylase; HDACi, HDAC inhibitor; HDMT, histone demethylases; HIF, hypoxia inducible factor; HMT, histone methyltransferase; HOTAIR, hox transcript antisense intergenic RNA; IGF2, insulin-like growth factor-2; K, lysine residue; KLF4, kruppel-like factor 4; linc, long intergenic non-coding; LINE, long interspersed nuclear element; LOI, loss of imprinting; me, methylation; LRES, long range epigenetic silencing; LSD1, lysine specific demethylase 1; me, methylation; MGMT, O-6-methylguanine-DNA methyltransferase; MLL4, mixed lineage leukemia-4; miRNA/miR, microRNA; MSI, microsatellite instability; MTD, maximum tolerated dose; NRF1, nuclear respiratory factor 1; NuRD, nucleosome remodeling and histone deacetylase; PCAF, (CBP)/p300 associated factor; PFS, progression-free survival; PI3K, phosphatidylinositide 3-kinase; PPARδ, peroxisome proliferator activated receptor δ; PRC, polycomb repressive complex; PTEN, phosphatase and tensin homolog; RUNX3, runt-related transcription factor 3; SAM, S-adenosyl-methionide; SEPT9, septin 9; VEGF, vascular endothelial growth factor; YY1, yin yang 1; ZEB, zinc finger E-box-binding homeobox

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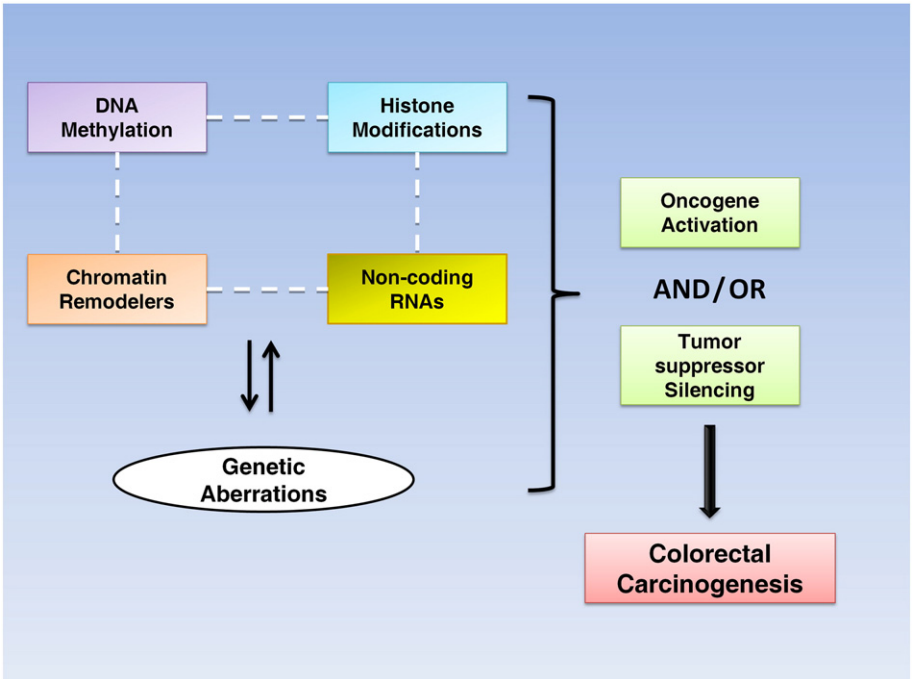

Fig. 1. Schematic representation of the mechanisms that lead to colorectal cancer. Genetic and epigenetic aberrations are involved in a complex network, responsible for modulations in gene expression patterns. Activation of oncogenes and/or silencing of tumor suppressors promote colorectal carcinogenesis.

2. DNA methylation

2.1. DNA hypomethylation

In normal cells DNA methylation plays a significant role in securing DNA stability through transcriptional silencing of genetic elements such as repetitive nucleotide sequences and endogenous transponsons. Furthermore DNA methylation reassures gene imprinting, transcriptional blockage of the genes on the inactive X-chromosome and normal growth, development and differentiation, while it also contributes in homeostasis maintenance and genomic adaption in response to environmental stimuli [10]. Global genomic hypomethylation, characterized by the gradual and genome wide depletion of methylated cytosine bases (5-methyl-cytosine) in cancer cells, is observed even in early stages across CRC development and progression [3]. DNA hypomethylation represents an epigenetic alteration that gathers in an age-dependent manner, possibly due to gradual acquisition of errors during DNA methylation mediated by constitutive methyltransferases and correlates to genomic damage. Demethylation of pericentromeric regions seems to induce DNA recombination errors and inaccurate chromosome duplication [11]. More precisely global DNA hypomethylation mainly takes place on cytosine guanine (CpG) dinucleotides within mobile and repetitive genetic elements such as satellite DNA sequences (including the aforementioned centromeres), long interspersed nuclear element (LINE) repeats and retrotransponsons [5]. This pattern of hypomethylation could be involved in tumorigenesis through the increased accumulation of chromosome breakage and the induction of expression of formerly silenced genetic elements (e.g. retrotransponsons) resulting in the disorganization of the normal nucleotide sequence and the impairment of chromosomal stability. The above could explain why DNA hypomethylation is mostly seen in CIN CRCs [3]. LINE-1 is frequently hypomethylated in CRC, exhibits a relatively stable demethylation state across CRC progression, even at early stages. Its enhanced activation through hypomethylation is associated with increased genomic instability and enhanced cancer ability to penetrate surrounding tissues and metastasize. [12,13]. Apart from genomic instability, another mechanism of colorectal carcinogenesis development and promotion, attributed to hypomethylation, is oncogene positive transcriptional regulation [8,10]. The events that propel global demethylation are not yet fully elucidated. APC mutation, which is a driving event in colorectal tumorigenesis, seems however to be able to control DNA methyltransferase (DNMT) expression and activity, which concomitantly results in demethylation of a number of genes and forces intestinal cells to remain in a more undifferentiated and progenitor-like state. Accordingly, genetic and epigenetic interactions constitute a possible mechanism involved in tumor initiation and progression [14].

Loss of imprinting (LOI) is defined as the impairment of the epigenetically regulated in a parent-of-origin manner, monoallelic, selective expression of certain genes, which could lead to developmental abnormalities. Imprinted genes also frequently (about 40% of CRC cases) undergo genome-wide hypomethylation early in the process of colorectal tumorigenesis [8,15]. In CRC tissues and cell lines, LOI of the insulin-like growth factor-2 gene (IGF2), leads to aberrant expression of the normally epigenetically-repressed maternally-inherited copy. Upregulation of the encoded protein levels, enhances the activation of IGF1 receptor (IGF1R) and its downstream signaling pathways, including phosphatidylinositide 3-kinase (PI3K)/Akt and GRB2/Ras/extracellular signal-regulated kinase (ERK) [5,15].

2.2. DNA hypermethylation

DNA methylation in humans takes place on the fifth position carbon of the pyrimidine ring of cytosines located in CpG dinucleotides. This process is mediated by three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B), which require the methyl donor-cofactor S-adenosylmethionide (SAM) in order to add a methyl-group to cytosine residues [13]. CpG dinucleotides are unequally spread throughout the genome, gathered in small DNA stretches called CpG islands (CGIs). These CpG-rich regions are mostly present close to the promoter region of almost 50% of all human genes [3]. While the vast majority of CpG dinucleotides found in DNA sequences between genes are methylated, CpG islands are generally unmethylated in normal colonic epithelial cells [4,13]. There is an established association between aberrant DNA methylation of CGIs located close to gene promoter region and abnormal inhibition of gene

transcription, including the induction of tumor-suppressor, cell-cycle regulator and DNA-repair genes transcriptional silencing (Fig. 2) [4,5]. Aberrant CGI promoter hypermethylation results in gene silencing through the orchestration of the "enrollment" and binding of methyl-CpG binding proteins that in turn affect the recruitment of transcriptional corepressors which confer transcriptional suppression via histone modifications [16,17].

APC, Cadherin-1 (CDH1), runt-related transcription factor 3 (RUNX3), mutL homolog 1 (MLH1), O-6-methylguanine-DNA methyltransferase (MGMT), cyclin-dependent kinase inhibitor 2A (CDKN2A), and RASSF1A are among the genes frequently determined to undergo CGI-promoter hypermethylation-mediated inactivation in CRC [18,19]. APC promoter hypermethylation has been shown to correlate with metastatic CRC, while inactivation due to promoter hypermethylation of the mismatch-repair gene MLH1 is often observed in sporadic CRC with MSI [5,8]. In MSI CRC patients, apart from tumor tissues, MLH1 methylation has been also detected in adjacent morphologically "health-appearing" colonic epithelial tissues. Furthermore, MGMT, another DNA-repair gene, frequently undergoes biallelic promoter-hypermethylation associated silencing in CRC, which in turn seems to favor mutations in cancer-related genes (e.g. p53 and kRAS). MGMT has been also found to exhibit promoter methylation in precancerous polyps [20]. Similarly to hypomethylation, CGI promoter hypermethylation of some genes seems to accumulate in an age-dependent manner in normal colonic epithelium (e.g. MLH1) and in tandem to the "premalignant to malignant" progressive CRC transformation (e.g. MGMT) [3,21]. The above suggest that promoter hypermethylation of certain genes, including DNA-repair genes, may precede tumor formation and its detection in apparently normal colonic epithelium, in premalignant lesions that occur early in CRC development as well as in other body samples (e.g. stool and plasma) could provide promising diagnostic biomarkers. These biomarkers could nominate a subject of patients at great risk for CRC who may benefit from closer follow-up with gold-standard diagnostic methods and more intensive CRC preventive interventions, also by taking advantage of the reversible nature of epigenetic alteration [13]. According to a genome-wide methylation analysis in 24 CRC and matched normal tissues, among the genes whose promoters were found to be hypermethylated several genes did not exhibit any known significant correlation with disease clinicopathological features, suggesting that aberrant gene methylation may not always influence cancer cell fate [22]. Nevertheless this fact does not preclude their usage as diagnostic and prognostic biomarkers [10].

Dependent on the aberrant hypermethylation of a large number of gene promoters CRC can be distinguished into two subclasses, each with distinctive genetic and clinicopathologic characteristics: CpG island methylator phenotype (CIMP) positive (+) and CIMP negative (−) CRCs. More precisely CIMP+ CRCs are mostly met in high-grade mucinous carcinomas, located in proximal colon, with MSI and

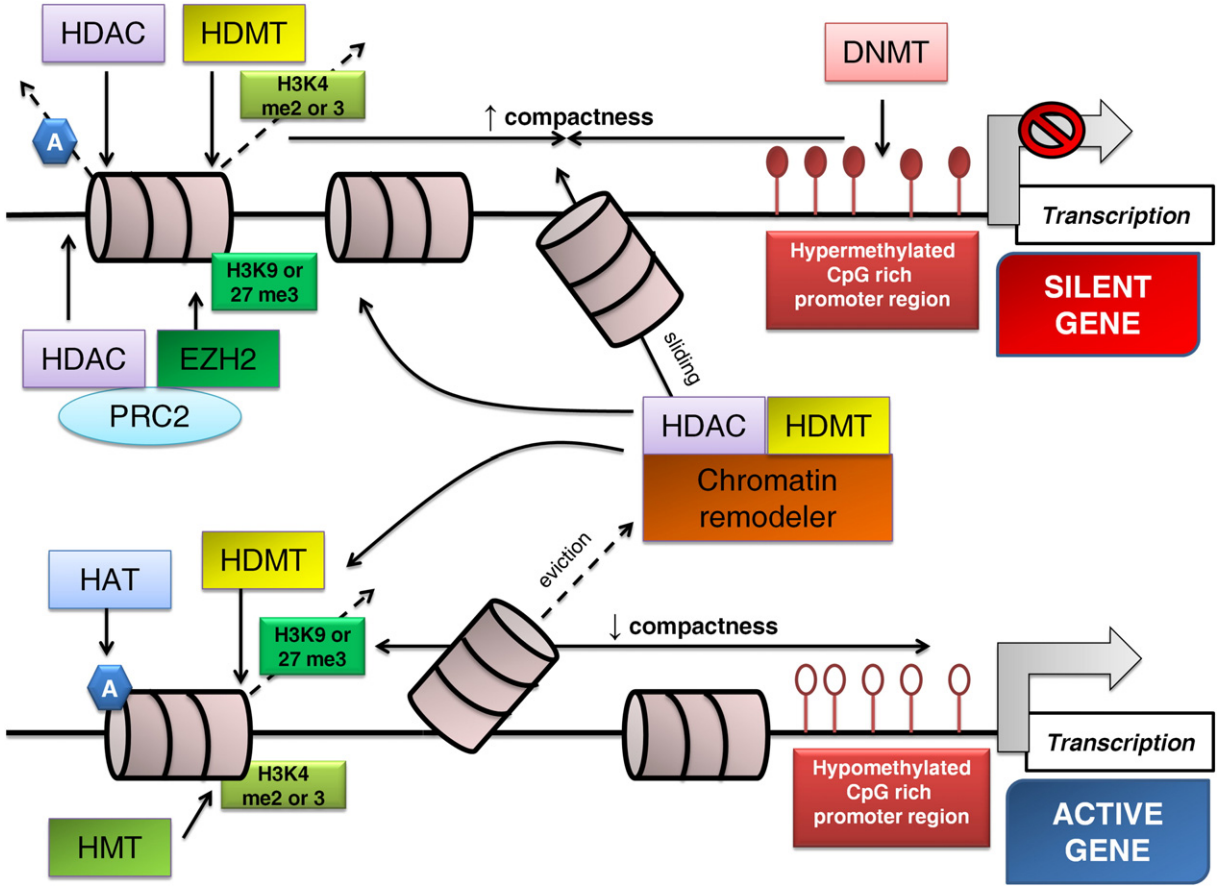

Fig. 2. Proposed model of epigenetic modifications in active and silenced genes. Epigenetics modify gene expression patterns by controlling chromatin condensation. Silent genes are characterized by CpG island hypermethylation, mediated by DNMTs, the removal of acetyl-groups by HDACs and modulations in histone methylation marks by HDMTs and HMTs, such as EZH2 of the PRC2 complex. All these events raise chromatin compactness. Active genes are characterized by the absence of CpG island methylation, the addition of acetyl-groups by HATs and the presence of active histone methylation marks, which promote chromatin loosening. In general, H3K9me3 and H3K27me3 are related with gene repression, while active genes are enriched by H3K4me2 or H3K4me3. Chromatin remodelers can be related with both active and repressed DNA regions through the regulation of nucleosomal architecture and the recruitment of HDACs and HDMTs. Pink cylinders represent nucleosomes, solid arrows represent addition and dashed arrows represent removal. Abbreviations: A, acetyl-groups; DNMT, DNA methyltransferase; EZH2, histone methyltransferase enhancer of Zeste 2; H, histone; HAT, histone acetyltransferases; HDAC, histone deacetylase; HDMT, histone demethylases; HMT, histone methyltransferase; K, lysine residue; me, methylation; PRC, polycomb repressive complex.

increased incidence of kRAS or BRAF mutation, but wild type p53. These tumors are most frequently associated with older ages and the female gender [3,4]. Despite the established relationship between BRAF mutation and CIMP+ occurrence, it should be further enlightened in what way and to what extent this mutation could drive and enhance CIMP+ [4,23]. Apart from the possible implication of BRAF mutation in promoting aberrant hypermethylation of CpG-rich gene promoters, another potent cause that could render CGI promoter regions prone to methylation in CRC cells, is the increased number of polycomb repressive complex (PRC) 2 marks in human embryonic stem cells that is present since the normal early development. It has been shown that in CRC cells, hypermethylated genes, are not only highly pre-marked by PRC2 components, but also exhibit elements within their genome that are able to recruit PRC2. PRC2 could predispose to gene hypermethylation by directly or indirectly interacting with DNMTs [3]. Additionally, these methylation predisposed regions seem to be deprived of some transcription factor binding sites, such as Sp1, yin yang 1 (YY1) and nuclear respiratory factor 1 (NRF1), which are regarded as methylation inhibitors in cancer cells [14].

3. Histone modifications

Histone (H) modifications are included in the spectrum of transcriptional regulation by coordinating DNA accessibility through acetylation, methylation, phosphorylation, ubiquitylation, summoylation, ADP ribosylation, deamination and proline isomerization of specific core histone (H2A, H2B, H3, H4) residues [7,8]. Post-translational modifications of histones are involved in the integration center of transcriptional control by fine-tuning the balance between the active (euchromatin) and the inactive (heterochromatin) states of chromatin [7]. Apart from their contribution in various physiological processes, histone modifications are responsible for the aberrant genetic and epigenetic profiles, which occur in numerous types of solid (prostate, breast, colorectal, lung) and hematological malignancies [7,9]. The established role of epigenetics in CRC pathogenesis offers an alternative pathway to elucidate the deep secrets of cancer development (Fig. 2).

Histone acetylation and methylation are the most widely studied aberrations of histone profiles in CRC. The residue that is modified, the location of the histone and the category of alteration define the outcome [5]. The post-translational addition of acetyl-groups interferes in the electrostatic binding between the negatively charged DNA and histones, thus mediating chromatin loosening and facilitating transition to active states. Acetylation and deacetylation of lysine residues (K) at histone tails is achieved via histone acetyltransferases (HATs) and histone deacetylases (HDACs) respectively [7]. Histone methylation occurs at lysine, arginine and histidine residues, with lysine being the most common site however. Histone methyltransferases (HMTs) and demethylases (HDMTs) specify histone methylation profiles [7,24]. Methylation (me) does not change the electrostatic properties of the structure and is related with both active and silenced DNA region. Lysine residues can be mono-, di- or tri-methylated [6,7]. Active DNA regions are marked with HEK4me2- or me3 and/or H3, H4 acetylation, while H3K9me3 or H3K27me3 and the absence of acetylation characterize repressed regions [5,6].

3.1. Histone acetylation

HATs are divided into two basic categories, namely: the nuclear type A HATs, and the cytoplasmic type B HATs, which acetylate free histones [25]. Their role in CRC is complicated and they are implicated in complex cross-talk networks affecting numerous signaling pathways. CREB-binding protein (CBP)/p300 and their associated factor (PCAF) facilitate the effects of β-catenin through acetylation [26]. Strikingly, the role of p300 in CRC has not yet been elucidated as its overexpression has been associated with advanced tumor stages and poor clinical outcome [27], while other research groups have linked p300 expression with increased survival [28].

The family of HDACs consists of 18 members and it is subdivided into four classes: I (1–3, 8), II (4–7, 9, 10), III (sirtuins 1–7) and IV (11). Classes I, II and IV comprise the classical family, which are Zn-dependent, while class III is NAD^+-dependent [29]. HDACs are members of multiple co-repressor complexes [30]. HDAC2 upregulation is implicated in the early and vital steps of CRC. Additionally, HDAC1–3, 5, and HDAC7 have also been reported to be upregulated in CRC [31]. Evidence from various CRC cell lines implicated class I and III HDACs in the down-regulation of tumor suppressor genes such as caudal type homeobox-1 (CDX1), which belong in the Wnt signaling cascade [32]. Apart from histones, HDACs interact with various other proteins. It has been shown that HDAC3 maintains the DNA-repair gene CtBP-interacting protein (CtIP) in its functional deacetylated state [33]. Sirtuin-3 could act as a tumor suppressor through the reduction of oxidative stress via the regulation of hypoxia inducible factor (HIF) pathway, at least in part. Knock-down of sirtuin 3 in the CRC cell line HCT116 led to increased proliferation, while overexpression inhibited carcinogenesis [34].

3.2. Histone methylation

Histone methylation is a dynamic and flexible procedure. A conserved SET domain, responsible for the enzymatic function can be found in each HMT. In cooperation with HMTs, HDMTs modulate histone methylation profiles [7]. Large co-repressor complexes harbor HDMTs [30].

Lysine specific demethylase 1 (LSD1) is an HDMT, which demethylates H3K4me2 and H3K9 and has been associated with various cellular mechanisms including proliferation and differentiation. Apart from alteration of the histone code, LSD1 interacts with various molecules such as p53 and DNMT1 and is linked with malignant transformation. LSD1 seems to potentiate proliferation, invasiveness and metastatic potential of CRC cells as it has been depicted by knockout experiments on cell-lines and mouse xenograft models [35,36]. LSD1 has been positively correlated with TNM stage, lymph node infiltration and metastatic disease in CRC patients [37].

Recent experimental data reveal novel wirings between HMTs and key regulatory signaling pathways. A specific HMT-complex mediates the suppressive effects of Notch on genes downstream of Wnt, whereas mixed lineage leukemia-4 (MLL4) regulates the expression of cell cycle and angiogenesis factors in CRC cells [38,39]. Immunohistochemical studies have correlated the expression patterns of certain histone methylation marks (e.g. H3K9me2, H3K9m3, H3K27me2) with various clinicopathological parameters in CRC patient, including lymph node infiltration and distant metastasis [40–42].

Moreover, the Polycomb-group protein family, which consists of the complexes PRC1 and PRC2, mediates epigenetic silencing via histone methylation. PRC1 binds at H3K27me3 and possesses histone-ubiquitylation abilities. The histone methyltransferase enhancer of Zeste 2 (EZH2) is the central unit of PRC2, responsible for H3K27me3. PRC1 and PRC2 may cooperate for gene repression or act in an independent manner [43,44]. Polycomb proteins coordinate crucial developmental steps and their malfunction is often observed in various malignancies. In vitro evidence from CRC cells indicate the involvement of EZH2 in tumor proliferation and growth [45]. PRC1 and PRC2 complexes are speculated in metastasis through their ability to repress E-cadherin via B lymphoma Mo-MLV insertion region 1 homolog (Bmi1) and EZH2 respectively [46]. Alternatively, ERK, Akt and activator protein 1 (AP-1) seem to enable EZH2 expression, which in turn in facilitates EMT through the down-regulation of integrin alpha2 [47]. Members of the polycomb group family, such as Bmi1, have been found elevated in CRC patients and have been correlated with advanced stages and aggressive types of carcinomas [48].

3.3. Histone ubiquitination

Apart from the widely studied histone modifications, ubiquitination may serve as an alternative mechanism of gene regulation in CRC. Histone ubiquitination can be associated with both gene silencing and activation. It has been shown that an H2B ubiquitin ligase is mutated in various solid tumors including CRC, whereas an H2B deubiquitinase has been reported to be overexpressed in colorectal and breast cancer [49]. Recently, loss of H2B monoubiquitination has been linked with the abnormal glucose metabolism of cancer cells, which permits survival in adverse conditions [50]. In vitro and in vivo data point out that histone 2A ubiquitination, mediated by the PRC1 complex member Bmi1, is actively involved in the proliferation of CRC cells [51].

4. Chromatin remodeling complexes

Chromatin-remodeling complexes, by forming a dynamic equilibrium (activation/repression), orchestrate a wide range of physiological processes including, proliferation, self-renewal and differentiation [30]. By consuming ATP-derived energy, these complexes manipulate nucleosomal architecture through the dynamic mobilization (insertion/eviction) of nucleosomes as well as the configuration of nucleosomal DNA and histone-octamers [7,9,52]. Additionally, chromatin-remodelers, which can function as tumor suppressors or promoters depending on contextual signaling, recruit HDACs, HDMTs and numerous auxiliary proteins in order to construct large scaffolds, and participate in complex cross-talk networks with numerous transcription factors [30]. Overexpression or mutated forms of several members of the remodeling machinery have been identified in a broad range of hematological and solid malignancies (Fig. 2) [7,9,30,52].

Nucleosomal insertion in the promoter CpG island region may facilitate epigenetic gene silencing through heritable modifications in chromatin conformation, as in the case of MLH1 [53]. Similarly, distant Wnt-related responsive enhancers may reach and up-regulate c-Myc, a pivotal coordinator of proliferation and apoptosis in CRC, through the generation of large chromosomal loops [54]. Recent experimental data link the upregulation of vascular endothelial growth factor (VEGF) by peroxisome proliferator activated receptor δ (PPAR-δ) through a mechanism that involves β-catenin-mediated chromatin looping [55].

SWI/SNF complex seems to be involved in multiple aspects of CRC development and progression. For instance, BRG1 is implicated in the promotion of metastasis through a mechanism involving the downregulation of E-cadherin [56]. Inactivating mutations of several SWI/SNF members is a frequent event in CRC patients (especially with MSI), including ARID1A and SMARCC2 [57,58].

Furthermore, another chromatin-remodeler, nucleosome remodeling and histone deacetylase (NuRD), determines the fate of various signaling cataracts [30]. NuRD is responsible, at least in part, for the abrogation of the transcriptional activity of AP-1 transcription factor. AP-1 possesses a pivotal role in the orchestration of intestinal proliferation and the promotion of tumorigenesis [59]. NuRD however is also involved in the silencing of tumor suppressor genes, such as negative regulators of Wnt, often in synergy with DNMTs [60]. In accordance to this data, immunohistochemical analysis of surgically resected colorectal tumors correlated the positive expression of NuRD members with decreased overall survival and poor clinical outcome [61].

Recent data from mouse models reveal that members of the chromatin-remodeling machinery, such as the HAT Tip60 and the ATPase p400, modulate the actions of Wnt-cascade. Tip60 is implicated in DNA-damage response, exerts anti-proliferative effects and represses the Wnt-pathway. On the other hand p400 exerts counteracting effects by up-regulating Wnt and inhibiting Tip60. In CRC, the ratio of Tip60/p400 is unbalanced in favor of p400 and promotes Wnt activity and carcinogenesis [26].

5. Non-coding RNAs

MicroRNAs (miRNA, miR) constitute a complementary mechanism of gene expression control involved in a multi-level interplay with the epigenetic machinery by both regulating epigenetic alterations and being regulated by them [62]. This subset of short endogenous non-coding RNAs (≈22 nucleotides) post transcriptionally downregulates gene expression through binding to a complementary site that resides on the 3′-untranslated region of target mRNAs. Upon binding, miRNAs incorporated into an RNA-induced silencing complex induce cleavage or translational repression of target-mRNAs [8,63]. Aberrations of miRNA expression are often observed in CRC and seem to play a significant role in tumor development and progression, which comes in accordance with the suggestion that miRNAs exert oncogenic or tumor-suppressor effect. It has been shown their behavioral pattern could sometimes be modulated dependent on tumor-derived microenvironmental stimuli. For example, miR-145 displayed anti-tumor promoting activity in primary CRC, while in advanced disseminated disease enhanced tumor promotion [64,65]. However, miRNA implication in virtually every important aspect of the multistep colorectal carcinogenesis and their clinicopathological impact have already been proved and several examples are available. Tumor suppressor genes, such as phosphatase and tensin homolog (PTEN) and topomyosin 1 have been identified as targets of miR-21 and thus its upregulation in CRC results in reduced expression levels and activity of those genes. It has also been shown that miR-21 promoter region carries a binding loci for STAT3 transcription factor, whose upregulation has been suggested to occur in an age-related manner and in carcinogenesis, triggered by sustained IL6 signaling [8,66]. Furthermore, miR-21 is found abundantly expressed in chemo-resistant CRC cells. Induced suppression of miR-21 promoted both differentiation, as indicated by cytokeratin 20 upregulation, and sensitization to chemotherapeutic regimens [67]. miR-135 has been also considered to exert oncopromoting activity in colorectal pre-malignant and malignant lesions, by downregulating expression levels and activity of APC. On the contrary, induced activation of APC in CRC cells led to miR-122a positive-regulation, which has been shown to mediate APC anti-tumor effects. Thus the presence of a “functional intersection” between miR-135, APC and miR-122a in tumor cell fate control could be hypothesized [66].

On the other hand, miR-34 family members and miR-137 act as tumor-suppressors and are often downregulated in CRC [68,69]. miR-34 family members (miR-34a, b, c) are positively regulated by p53. Transfection of miR-34a into CRC cells led to inhibition of cell proliferation-rate and apoptosis induction, at least partially by amplifying p53 signaling cascade. It has also been reported that miR-34a targets Sirtuin-1, which in turn induces p53 transcriptional upregulation. The above suggest the existence of a positive feedback loop between p53 and miR-34a, and that miR-34a could partly mediate p53 tumor-suppressive function [8,66,69]. Additionally, it seems that miR-34 family members are part of the “reciprocal” constitutive interplay between miRNAs and the enzymatic components of the epigenetic machinery, as CGI promoter hypermethylation of miR-34b/c takes place in the vast majority of primary CRCs [69,70]. In the same context, miRNAs with tumor-suppressing functions such as miR-1-1, miR-9-1, miR-129-2 and miR-124 family members have been reported to undergo CGI promoter hypermethylation-induced silencing and this irregular methylation often occurs at an early stage during CRC development [69,71]. What is more, miRNA dysregulation can further induce aberrant activity of the enzymatic components of the epigenetic machinery. As an example, DNMT3A has been identified as miR-143 target, and thus miR-143 suppression in CRC results in DNMT3A increased expression levels [66,69]. miR-140 and miR-449 have been shown to exert their tumor-suppressive effects by targeting and downregulating HDAC4 and HDAC1 respectively. Thus miR-140 and miR-449 downregulation in CRC may be responsible for aberrant histone acetylation induction [64,70]. Furthermore, miR-627 enforces the anti-tumor function of vitamin D by directly downregulating, an histone demethylase, levels

[72]. Last but not least, miRNAs in CRC are implicated in cancer invasion and migration. miR-200 family members for example, suppress EMT and tumor cell metastatic potential by directly downregulating E-cadherin repressors, such as zinc finger E-box-binding homeobox (ZEB) 1 and 2. ZEB1 seems also to negatively regulate the expression levels of certain miR-200 family members, indicating the existence of an interactive network between ZEB1 and miR-200 family, in the control of CRC dissemination [64,66]. Apart from miRNAs, increased expression of long intergenic non-coding (linc) RNAs, such as lincRNA-p21 and hox transcript antisense intergenic RNA (HOTAIR), has been correlated to advanced CRC stage and enhanced metastatic potential of CRC cells. Linc-RNAs probably also interact with the epigenetic machinery, namely HOTAIR, has been shown to exert its oncogenic activity in association with PRC2 [6,73].

Taken together, these findings highlight the importance of miRNAs in association with epigenetics in CRC carcinogenesis.

6. Long range epigenetic silencing

Epigenetic modifications do not refer only in single and local gene control, but may also affect larger chromosomal regions, where numerous genes are embedded. The term long range epigenetic silencing (LRES) was established after the discovery of a silenced large region at chromosome 2q14.2, which contained multiple tumor-suppressor genes with promoter CpG hypermethylation and H3K9me2. Similarly, in MSI-H CRCs LRES has been observed for a 3p22 locus, where most of the harbored genes, including MLH1, were reported to undergo transcriptional repression mainly due to promoter hypermethylation [5]. Recent studies suggest that LRES may span in the deregulation of multiple genes that control crucial cellular processes. The transcription factor Ikaros, which controls differentiation, as well as a large region at 5q31, which contains several members of the tumor suppressor family of protocadherins are under LRES [74,75]. The exact mechanism and the sequence of events that drive LRES remain to be elucidated [5].

7. Colon cancer stem cells and epigenetics

The newly introduced cancer stem cell (CSC) model approaches carcinogenesis from a different point of view. Notably, CSCs are regarded as the "top-notch" players in cancer, responsible for its propagation, heterogeneity and metastasis [8]. The characteristic feature of CSCs to switch various genes between "on and off" positions according to the need for proliferation or differentiation suggest the existence of an epigenetic mechanism [76,77]. Indeed, a wide gamut of proposed CSC markers such as CD133, CD44 and aldehyde dehydrogenase-1 (ALDH1) have been reported to be epigenetically reprogrammed in hematologic and solid malignancies, including CRC, whereas Bmi1, a member of the PRC1 complex, is a putative marker of both normal intestinal and cancer stem cells. Moreover epigenetics modulate the activity of signaling circuitries (Wnt, Hedgehog and Notch), which coordinate CSC maintenance and amplification [76,77]. The Wnt pathway seems to function as an indirect activator of Bmi1, possibly through the c-myc oncogene. Additionally, kruppel-like factor 4 (KLF4), which regulates the proliferation and differentiation of intestinal SCs, directly blocks Bmi1. The exact role of KLF4 in CSC biology however needs to be elucidated [51].

8. Epigenetic modifications: biomarker potential

CRC remains a global burden in world health and economy despite having a 90% of 5 year survival when detected and treated early [1,4]. It is therefore essential to develop robust techniques, which will not only aid current diagnostic procedures but will also permit non-invasive and more accurate screening, diagnosis, follow-up and response to therapy of cancer patients [4,5]. In this direction, epigenetics seem to be very promising candidates.

DNA methylation biomarkers, collectively referring to genes that are abnormally repressed due to promoter methylation in CRC, are appealing targets as they appear early in carcinogenesis. Recently, novel DNA methylation biomarker assays passed clinical trials and became commercially available. ColoVantage® is a blood based test, which detects the presence of methylated septin 9 (SEPT9). The assay achieves an overall sensitivity of 90%. Other clinically available methods utilizing SEPT9 methylation are Epi proColon® 1.0 and Abbott RealTime mS9. Additionally, ColoSure™ is a commercially available fecal-based assay that operates based on the methylation of the vimentin gene. This test should be used in combination with colonoscopy and has a reported sensitivity ranging from 38 to 88% [78,79]. The majority of DNA methylation biomarkers are not yet clinically available. Numerous fecal-based (e.g. *NDRG4*, *GATA4*) and blood-based (e.g. *RUNX3*, *SDC2*, *NEUROG1*) candidate biomarkers are being extensively tested in laboratory and clinical trials with encouraging results [13,78,79].

miRs have been also characterized as promising putative biomarkers. A wide variety of miRs seems to function as tumor-suppressors such as miR-149, which are epigenetically silenced due to hypermethylation in CRC [78]. Oncogenic miRs carry also useful clinical information, as in the case of miR21 which has been associated with metastasis and poor survival [13]. On the contrary, histone modifications are not in the spotlight of the research community, regarding their utility as biomarkers. Several studies have correlated the presence of histone marks or histone modifying enzymes with various clinicopathological characteristics in patients with CRC [13]. Future studies are needed to elucidate the biomarker potential of histone modification.

9. Therapeutic implications

The era of translational medicine dictates the future of therapeutic approaches in cancer. Treatment modalities should be tailored according to the concept of personalized medicine. Targeting of epigenetic modifications offers an additional weapon to our therapeutic arsenal in combination with conventional measures. The plethora of in vitro and in vivo evidence suggesting that epigenetics exert a fundamental role in CRC, as well as their reversible nature determine the rationale behind this approach. Additionally, epigenetics are involved in the acquisition of drug resistance to conventional agents such as 5-fluorouracil, irinotecan and oxaliplatin. Therefore combined therapies may reverse resistance and sensitize previously refractory tumors, whereas the simultaneous administration of agents modulating DNA methylation and histone acetylation patterns may produce enhanced synergistic anti-cancer efficacy [80–82]. DNMT and HDAC inhibitors (DNMTi, HDACi) administration has been extensively investigated in both preclinical and clinical level. Most clinical studies were performed on heavily pretreated advanced stage patients and sought to determine maximum tolerated dose, pharmacokinetics, pharmacodynamics, possible adverse reactions and anti-cancer potential. Data from studies however are not so encouraging [80]. Major clinical studies that have been conducted so far are summarized in Table 1.

9.1. Synthetic compounds

9.1.1. DNA methylation inhibitors

DNA methylation inhibitors are categorized into nucleoside analogs, non-nucleoside analogs and anti-sense oligonucleotides (ASO). The family of nucleoside analogs consists of members with a modified cytidine, namely: 5-aza-cytidine, 5-aza-2-deoxycytidine, 5-fluoro-2-deoxycytidine and zebularine. These drugs, when incorporated into DNA, formulate covalent bonds with DNMTs and block their enzymatic activity. 5-aza-cytidine and 5-aza-2-deoxycytidine have been approved by the Food and Drug Administration (FDA) for use in myelodysplastic syndromes [80–82]. Non-nucleoside analogs, such as the small molecular inhibitor RG108 and ASO, such as MG98 inhibit DNMTs without incorporation into DNA. 5-aza-2-deoxycytidine seems to function as a

Table 1
Clinical trials of agents that target epigenetic modifications.

Agent	Disease	Phase	Result	References
Nucleoside analog DNMTi				
5-aza-cytidine in combination with sodium phenylbutyrate	Advanced solid tumors	I	34 enrolled patients 7 CRC patients No objective response Well-tolerated	[85]
5-aza-2-deoxycytidine (decitabine) in combination with carboplatin	Advanced solid tumors (pre-treated)	I	35 enrolled patients 7 CRC patients 1 partial response and 1 stable disease but not in CRC Establishment of dose that can be safely combined with carboplatin	[86]
Anti-sense oligonucleotide DNMTi				
MG98	Advanced solid tumors	I	34 enrolled patients 7 CRC patients 1 partial response and 1 prolonged stable disease but not in CRC Establishment of MTD Well-tolerated	[87]
HDACi—alphatic acid				
Vorinostat (suberylanilide hydroxamic acid, SAHA) in combination with bortezomib	Advanced solid tumors	I	29 enrolled patients 3 CRC patients Disease stabilization in one patient with CRC Establishment of MTD Well-tolerated	[89]
Vorinostat in combination with 5-FU	Refractory metastatic CRC	II′	58 enrolled patients 1 partial response Some disease stabilizations Failure of reaching the prespecified 2 month PFS rate Well-tolerated	[90]
Vorinostat in combination with 5-FU	Pre-treated metastatic CRC	I/II	10 patients enrolled 2 stable disease Failure of MTD establishment Poor tolerability	[91]
Phenylbutyrate in combination with 5-FU	Pre-treated metastatic CRC	I	9 enrolled patients 3 stable disease Fairly tolerated	[92]
Valproic acid in combination with epirubicin	Advanced solid malignancies	I	48 enrolled patients 2 CRC patients 9 partial responses 16 stable disease/minor responses Well-tolerated	[93]
HDACi—cylic peptide				
Romidepsin (Depsipeptide, FK228)	Pre-treated advanced CRC	II	28 enrolled patients 4 stable disease Ineffective at this dose for metastatic CRC No significant side-effects	[94]
HDACi—hydroxamic acids				
Panobinostat (LBH589) in combination with bevacizumab and everolimus	Advanced solid tumors heavily pre-treated	I	12 enrolled patients 9 CRC patients 1 partial response (breast) 3 disease stabilization (CRC) No proof of HDAC inhibition Not acceptable tolerability profile	[95]
Belinostat (PXD101) in combination with carboplatin and/or paclitaxel	Advanced solid malignancies	I	23 enrolled patients 2 rectal patients 2 partial responses (1 rectal) Well-tolerated	[96]
HDACi—benzenamides				
Mocetinostat (MGCD0103)	Advanced solid tumors	I	38 enrolled patients 9 CRC patients 5 patients (including CRC) with stable disease Favorable pharmacodynamics/kinetics profile Well-tolerated	[97]

(continued on next page)

Table 1 (*continued*)

Agent	Disease	Phase	Result	References
HDACi—benzenamides				
Entinostat (MS275,SNDX275) in combination with 13-cis retinoic acid	Advanced solid tumors	I	19 enrolled patients 9 CRC patients 7 stable disease MTD establishment Fairly tolerated	[98]
Pracinostat (SB939)	Advanced solid tumors	I	39 enrolled patients 15 CRC 10 stable disease 3 patients with stable CRC > 6 months Well-tolerated	[99]

Abbreviations: 5-FU, fluorouracil; CRC, colorectal cancer; DNMT, DNA methyltransferase; DNMTi, DNMT inhibitor; HDAC, histone deacetylase; HDACi, HDAC inhibitor; MTD, maximum tolerated dose; PFS, progression-free survival.

global DNMTi, which not only re-activates epigenetically silenced genes due to promoter hypermethylation but also facilitates the expression of unmethylated genes, including those involved in apoptotic pathways [83]. Recent experimental data demonstrated that the sole application of DNMTi may be inadequate to reactivate gene expression. Instead, the concomitant use of HDACi may function synergistically and re-enable aberrantly silenced tumor suppressors [84]. Clinical application of DNMTi in patients with various solid tumors has not yielded very encouraging results. Most studies did not meet pre-specified endpoints and the best clinical outcome was usually disease stabilization (Table 1) [85–87].

9.1.2. HDAC inhibitors

Due to their involvement in multiple aspects of carcinogenesis, HDACs reasonably are regarded as appealing targets in the fight against cancer. HDACi have shown a preferential efficacy against hematological malignancies and therefore vorinostat (SAHA) and romidepsin (FK228) have gained FDA approval. Data regarding solid tumors however are not so convincing and encouraging [30,80,88]. Results from most clinical trials indicate that these agents are not particularly efficient against solid tumors, whereas their tolerability profile is fairly favorable (Table 1) [89–99]. Possible side-effects and the limited clinical efficacy may result from the inability of HDACi to act as selective inhibitors [88]. In this direction, various groups attempt to generate specific inhibitors. In vitro data demonstrated that a small molecular inhibitor of HDAC6 was able to promote apoptosis and block cell proliferation in CRC xenograft models [100].

9.1.3. Other therapeutic approaches

In contrast to DNMTi and HDACi, the application of agents intervening in histone methylation, HATs, chromatin remodelers and miR is still at primitive level [81]. Targeting of HMTs has been reported to inhibit the migrating potential of CRC cells and to facilitate apoptosis in colon CSCs [41,101]. Intriguingly, statins, a class of cholesterol-reducing drugs, seem to lower CRC risk and could be used as putative anti-cancer agents. Novel findings have shed light on their mechanism of action, suggesting an interaction with the epigenetic machinery. It has been demonstrated that statins, possibly through EZH2 inhibition, suppress proliferation and induce growth arrest of CRC cells, while their anti-cancer efficacy was further sustained when co-administered with HDACi [102]. Recent experimental data also suggest that statins exert their tumor-protecting effects by forcing CRC cells to shift into a mature phenotype and thus facilitating chemo-sensitization through demethylation of the promoter of the gene encoding bone morphogenetic protein (BMP) 2 [103]. The application of LSD1 inhibitors in CRC cell lines has produced encouraging results as it reactivated commonly silenced tumor suppressor genes [104]. Small molecular HAT inhibitors have been shown to ask cytotoxic effects upon CRC cells, with a favorable pharmacokinetic profile [105]. Additionally, modulation of chromatin remodeling may be achieved via pharmacological inhibition of each individual members of a given complex [30].

Radiation therapy, which has been used for the treatment of rectal cancers, seems to affect epigenetic patterns. Radiation could cause a state of genetic instability and promote apoptosis in cancer cells through inhibition of DNMTs and alterations in histone modifications and miR expression profiles [19].

Finally, miRs are regarded as alternative rational targets. In general re-activation of miRs bearing tumor-suppressive potential can be achieved through treatment with a DNMTi, an oligonucleotide mimic or a miR expression vector [69,106]. On the other hand silencing of oncogenic miR is achieved via the application of ASOs [67]. For instance, restoration of miR-212, miR-145 and miR-33a in CRC cell lines and xenograft models has been shown to induce growth arrest [69,106], whereas silencing of miR-21 in CRC cell lines induced differentiation and chemosensitization [67].

9.2. Natural compounds

Natural compounds modulate epigenetic modifications in a multi-level manner. Numerous dietary factors including, folate, vitamins, epigallocatechin 3-gallate (EGCG) from green tea, genistein from soybean, catechol-containing coffee polyphenols and flavonoids alter the availability of methyl groups, influence the activity of DNMTs and control DNA methylation and histone acetylation [82]. Evidence from a recent study indicates that genistein could ask its protective effects by altering DNA methylation and histone modifications. Indeed genistein is able to keep Wnt pathway members in normal levels in colonic epithelia of rat models after exposure to carcinogens [107]. Curcumin, the biologically active ingredient of the spice turmeric, is another putative chemopreventive agent due to its modulatory actions in numerous cellular mechanisms, including DNA methylation. Moreover it seems that curcumin functions in vitro as a specific DNMTi [108]. In a phase I study, the administration of freeze-dried black raspberries in CRC patients altered the expression of genes involved in cell proliferation, angiogenesis and apoptosis through the inhibition inhibition of DNMT1, which led to the hypomethylation of various tumor-suppressor genes [109]. Green tea extracts are regarded as promising cancer preventing agents. Currently another clinical study has focused on determining the beneficial effects of green tea (EGCG) on minimizing the risk of metachronous adenomas and thus CRC [110].

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