Review

Epigenetics of colorectal cancer


Colorectal cancer (CRC) develops through a multistep process that results from the progressive accumulation of mutations and epigenetic alterations in tumor suppressor genes and oncogenes. Epigenetic modifications, that have a fundamental role in the regulation of gene expression, involve DNA methylation, specific histone modifications and non-coding RNAs (ncRNAs) interventions. Many genes have been until now studied to detect their methylation status during CRC carcinogenesis; and the functions of many of these genes in cancer initiation and progression are being clarified. Less is known about the patterns of histone modification alterations in CRC. Epigenetic deregulation of the ncRNAs or the genes involved in their biogenesis have been described in tumor progression and some examples of dysregulated microRNA were found also in CRC cells. Diet has an important role in the etiology of colon cancer. Folate is involved via 5-methyltetrahydrofolate in the conversion of homocysteine to methionine, which is then used to form the main DNA methylating agent S-adenosylmethionine. However, the role of folate in protecting from or in promoting CRC, depending on conditions, is still debated. The study of epigenetic marks to better characterize CRC and to identify new tools for diagnosis and prognosis as well as for therapeutic interventions is extremely promising.

Conflict of interest

We disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning of the submitted work that could inappropriately influence, or be perceived to influence, their work.

Colorectal cancer (CRC) is one of the most aggressive cancers and a common cause of cancer-related deaths in western countries, showing a disease-specific mortality rate of about 33% in the developed world (1). CRC most commonly occurs sporadically and only 25% of the patients have a family history of the disease, suggesting a contribution for shared genes and environment. Major CRC genetic syndromes only account for 5–6% of the total cases. They include familial adenomatous polyposis (FAP), attenuated FAP, MUTYH-associated polyposis, and Lynch syndrome (hereditary non-polyposis colorectal cancer). There are also rare syndromes including hamartomatous polyposis conditions [Peutz–Jeghers syndrome, juvenile polyposis syndrome, and others] and hyperplastic polyposis.

Phenotypically, CRCs are commonly divided in cancers showing microsatellite stability (MSS) or microsatellite instability (MSI). Moreover, chromosomal instability (CIN) and CIMP (CpG island methylator phenotype) subtypes can be recognized. CIN occurs in 80–85% of CRC. CIN proceeds through two major mechanisms: missegregation that results in aneuploidy through the gain or loss of whole-chromosomes, and unbalanced structural rearrangements that lead to the loss and/or gain of chromosomal regions (2). The CIMP phenotype is dependent on promoter hypermethylation of CpG islands and these subtypes of cancers show high, low or negative methylation status (3, 4). In recent years, evidence has accumulated indicating that apart from genetic changes, epigenetic alterations play a major role in the initiation and progression of CRC. Therefore, the development of CRC is now considered a multistep process that involves an accumulation of mutations in tumor suppressor genes and oncogenes.
This CRC-related model can be generalized for understanding of the multistep process of carcinogenesis.

Epigenetic mechanisms

Epigenetics refers to heritable modifications of the genome without change in primary DNA sequence. Epigenetic modifications, that have a fundamental role in the regulation of gene expression, involve DNA methylation, specific histone modifications and non-coding RNAs (notably silencing RNA and microRNA (miRNA)) interventions. In recent years, this field is becoming increasingly attractive as more epigenetic controls of gene activities are being discovered.

One of the best-characterized epigenetic modification is methylation, a covalent addition of a methyl group (CH3) to cytosines that occur within CpG dinucleotides. CpG-rich regions (CpG islands) are associated with promoter regulatory regions of almost all housekeeping genes as well as with half of tissue-specific genes (5). Promoter hypermethylation has been associated with a decreased gene transcription. Global changes in DNA methylation correlate with altered gene expression and genomic instability in cancer. A high density of methylated cytosine in the CpG islands of the tumor suppressor gene promoters can lead to a complete block of transcription, and many types of cancer use this mechanism to inactivate tumor suppressor genes.

Another critical epigenetic mechanism refers to chemical modifications of the histone tails. Histones, besides being DNA-packaging proteins, can regulate the underlying DNA sequences through complex post-translational modifications of their N-terminal tails, such as aminoacid-specific acetylation, methylation, or phosphorylation. Histones are acetylated on lysine residues at their amino termini by histone acetyltransferases (HATs), and deacetylated by HDACs. The opposing effects of HATs and histone deacetylases (HDACs) regulate gene expression through chromatin modification. The HDAC-mediated removal of acetyl groups from lysine residues in the amino termini of histones leads to chromatin condensation and transcriptional inactivation of the involved DNA (6, 7). This transcriptional inactivation can contribute to suppression of tumor suppressor gene expression and enhanced tumorigenesis.

Non-coding RNA (ncRNAs) are involved in the regulation of many important biological processes; the most widely studied class of ncRNAs are miRNAs, which are involved in post-transcriptional gene silencing by controlling mRNA translation into proteins. Alterations (genetic or epigenetic) of genes coding these ncRNAs can modify the expression profile of the ncRNAs and thus alter the mechanism they regulate.

Gene methylation in CRC

Almost 30–40% of proximal site colon tumors and a 3–12% of distal colon and rectal tumors are characterized by a CIMP high, in which numerous CpG islands are methylated and several tumor suppressor genes or ncRNA are inactivated (for a review see 2, 8, 9). According to epigenetic, but also genetic, and clinical profiles, primary CRC is considered to cluster into three distinct subclasses, relatively homogeneous: CIMP1 characterized by intense methylation of multiple genes, MSI and BRAF mutations; CIMP2, which includes methylation of a limited group of genes, increased methylation level for age-related genes, and mutation in KRAS; and CIMP negative, characterized by rare methylation with p53 mutation. CIMP1 and CIMP2 phenotypes are more often expressed in the proximal colon; CIMP1 has a good prognosis, whereas CIMP2 has a poor prognosis (10).

However, colorectal mucosa can show changes in DNA methylation also in non-pathological conditions: CpG island methylation of estrogen receptor alpha (ERα) and secreted frizzled-related protein-1 (SFRP1) genes, in normal colorectal mucosa was found related to advancing age, race, rectal location, and also red blood cell folate levels (11).

In Fig. 1, the most studied genes which have been found methylated in CRC are reported together with their possible functions in cancer initiation and progression.

Recently, many methods have been developed to assess DNA methylation on a genomic scale, including high-throughput techniques, and samples of either colon tumor cells at various stages or colon cancer cell lines have been assayed for detecting differentially DNA-methylated regions (DMRs); they were identified mainly in CpG island shore regions (located within 2kb of a CpG island), gene body regions and intergenic regions (12–15). Many genes with altered levels of methylation were up to now been studied respect to their involvement in CRC initiation or progression. Some genes such as hLMH1, MGMT and TSP1 showed an increase in methylation during all the stages of the disease. Other genes such as RASSF1A or TIMP3 seem more methylated in the last stages or in metastases. However, sometimes conflicting results are found, with the same gene studied. This could be because of the analysis of different CpG sites in the same gene depending on the different methods used to assess methylation [pyrosequencing, vs methylation-specific polymerase chain reaction or vs combined of bisulfite restriction enzyme amplification]. Moreover, differences in age, tumor type or heterogeneity, or different exposure to environmental factors (diet or microorganisms) could also explain the contrasting methylation pattern of specific genes found in various studies.

Leong and co-workers (16) found an inverse relationship between methylation of 10 tumor suppressor genes and chromosomal aberrations; then if the frequency of methylation is less prevalent in advanced disease, it is possible that the advanced rectal cancers are driven via the CIN pathway, whereas the early cancers were driven via the methylation pathway. Some genes could have a dual role to promote or suppress tumor formation depending on tumor type and molecular context; the receptor tyrosine kinase-like orphan receptor 2 (ROR2),
In plasma, cell-free methylated DNA has been reported to be a useful biomarker of noninvasive blood screening for the detection of CRC. Septin 9 (SEPT9) and vimentin (VIM) genes have been analyzed in blood/serum samples and stool samples and reported sensitivity and specificity range of 68–77% and 83–94%, respectively (22, 23). Among genes that have been proven to be promising for an early diagnosis of CRC, besides SEPT9 (24), there are also ALX4 and TMEFF2 (25).

Loss of Smad4, a tumor suppressor frequently inactivated in pancreatic and CRCs, correlates significantly with decreased survival in colon cancer patients. High Smad4 expression, however, is significantly associated with increased survival, especially in colon cancer patients who has undergone potential curative surgery (26). Methylation of IGFBP3, EVL, FLNC, and CD109 genes is associated with an eightfold increase in mortality risk relative to that of patients with no DNA methylation of these genes (8). Also, hypermethylation of CDH13 and FLNB3 genes is associated with poor prognosis in CRC (27). Looking for novel prognostic biomarkers for CRC, recent studies showed that tumors that have silenced genes in the extracellular matrix remodeling pathway show worse survival (8, 28).

**Histone modifications in CRC**

Little is known about the patterns of histone modification alteration in human tumors and even less in CRC. Chromatin remodeling is, together with methylation, a key mechanism for gene regulation and consists of modifications at conserved lysine residues on the tails of histone proteins; lysine acetylation consents the transcription by weakening the association of the histone with DNA and allows transcription factor binding. Lysine methylation can be associated with both active and repressed regions of DNA; trimethylation of
Histone H3 lysine 4 active transcription, instead methylation of H3K9 and H3K27 appears at transcriptionally silent gene promoters (29). Hypomethylation alone cannot turn on silenced genes, instead increased histone H3 acetylation with localized hypomethylation allows long-term reversion of epigenetically silenced genes; a study showed as CD01, HSPC105 and MAGEA3 were still expressed 10 days post 5-aza-dC treatment, in fact they had localized hypomethylation at the transcriptional start site and an increased histone H3 acetylation (30). Enzymes for chromatin remodeling can alter chromatin by covalent modification of histone or by using the energy from ATP hydrolysis. Included in the ATP-dependent chromatin remodeling enzyme family is the chromodomain helicase DNA-binding protein (CHD) family, which consists of nine proteins (CHD1–9) in humans. With regard to cancer, CHD5 controls proliferation and apoptosis. In humans, CHD5 is inactivated not only by deletion but also by hypermethylation. These alterations might contribute to cancer pathogenesis by deregulating CHD-mediated chromatin remodeling (31). Socs and Shp1 genes seem to have a role as tumor suppressor and DNA methylation as well as histone acetylation/deacetylation could control their transcriptional regulation in CRC cells. Xiong and colleagues (32) showed trichostatin A (TSA), an histone deacetylase inhibitor (HDACi), increased the mRNA levels of SOCS1 and SOCS3. The induction of SOCS1 and SOCS3 expression by TSA in human CRC cells was because of an increase in the acetylation of H3 and H4 histone proteins associated with their promoter regions. However, they did not observe significant changes in histone acetylation of Shp1 promoter regions. Two different studies reported that the global levels of H4K12ac and H3K18ac increased in adenocarcinomas respect to the normal tissue or adenomas and that the H3K9me2 expression was also associated with the progression adenoma–adenocarcinoma (33, 34).

**NcRNA alterations in CRC**

Chromosome anomalies (deletions, translocations, copy-number alterations), DNA mutations and epigenetic deregulation of the ncRNAs or the genes involved in their biogenesis have been described in tumor progression and the best-characterized miRNAs dysregulated by DNA hypermethylation in tumors, including CRC and the functional consequences in tumoral cells, have been reviewed recently (35).

In Table 1, we report some recent examples of ncRNAs found dysregulated in CRC cells. For instance, epigenetic alteration of mir-143 which targets Kras can interfere with its expression and induce cell proliferation (36); epigenetic alteration of mir-148b which targets cholecystokinin-2 receptor gene (CCK2R) can lead to cell proliferation (37).

<table>
<thead>
<tr>
<th>ncRNA</th>
<th>Comments</th>
<th>References</th>
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<tr>
<td>mir-34b/c</td>
<td>Silencing of these genes was observed in normal CRC cell lines and R-137 tumors</td>
<td>36</td>
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<tr>
<td>mir-9-1, 5-aza-dC</td>
<td>in primary CRC tumor respect and normal mucosa</td>
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<tr>
<td>mir-143</td>
<td>Regulates KRAS expression and cell proliferation</td>
<td>36</td>
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<tr>
<td>mir135</td>
<td>Suppression of APC expression and Wnt pathway activity</td>
<td>36</td>
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<tr>
<td>mir-34a</td>
<td>Act as a tumor suppressor by blocking SIRT1; regulates cell proliferation</td>
<td>60</td>
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<tr>
<td>mir-345</td>
<td>Downregulation of BAG3; involved in cell proliferation and invasion in human CRC</td>
<td>38</td>
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<td>HOTAIR</td>
<td>Regulates expression of multiple genes in cooperation with PRC2 Associated with CRC metastasis</td>
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<td>let-7c</td>
<td>Destabilizes KRAS, MMP11 and PBX3 mRNAs; associated with CRC metastasis</td>
<td>61</td>
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<tr>
<td>mir-148b</td>
<td>Regulates cholecystokinin-2 receptor gene (CCK2R) and cell proliferation</td>
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<tr>
<td>let-7a</td>
<td>Regulates Np95 ICBP90 RING finger and cell proliferation</td>
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<tr>
<td>mir-499-5p</td>
<td>Suppression of FOXP4 and PDCD4; associated with CRC metastasis</td>
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CRC, colorectal cancer; ncRNA, non-coding RNA.

Methylation analyses showed that the promoter region of the mir-345 gene was heavily methylated in HT29 cells (64.73%) and it was decreased to 35.78% after 5-aza-dC treatment, which results in a major mir-345 expression; overexpression of mir-345 may suppress colon cancer cell invasiveness in vitro. mir-345 might be involved in pathogenesis of CRC through downregulation the expression of BAG3, one of the molecules that regulates apoptosis (38). Hox transcript antisense intergenic RNA (HOTAIR) is a long ncRNA that regulates expression of multiple genes in cooperation with PRC2; HOTAIR expression levels in CRC tissues were higher than those in corresponding non-cancerous tissues; its overexpression increased the invasiveness of CRC cells, then this ncRNA might play a role in promoting metastasis of CRC. Moreover, patients with high HOTAIR expression had a significantly poorer prognosis than those with low HOTAIR expression (39).

**Folate, methylation and CRC**

Lifestyle factors including diet are associated with cancer risk. Diet has an important role in the etiology of colon cancer and the evidence linking low
Folate status with an increased risk of CRC is increasingly evident (40). Folate metabolism, also known as one-carbon metabolism, is fundamental for the synthesis of DNA and RNA precursors or for the conversion of homocysteine (Hcy) to methionine, which is then used to form the main DNA methylating agent S-adenosylmethionine.

In the normal colorectum, folate deficiency appears to enhance, whereas folic acid supplementation suppresses, the development of CRC. In contrast, once aberrant crypt foci are established, folate deficiency inhibits the progression and induces regression of these established pre-neoplastic foci (41, 42). Several studies, using rat models of CRC, showed that the maternal folic acid supplementation significantly reduced the CRC risk by 64% in the offspring, whereas post weaning folic acid supplementation had no effect; moreover, tumor multiplicity were significantly higher in the pups from the dams fed the control diet and with post weaning folic acid supplementation than those without post weaning supplementation; it has been hypothesized that the pups from the dams with control diet have developed precancerous lesions in the colorectum respect to those from the folic acid-supplemented dams; then post weanig folic acid supplementation might have promoted the progression of these pre-neoplastic lesions (43, 44). Folic acid supplementation might protect the developing colorectum because of its critical role in maintaining DNA stability; in vitro animal and human studies show that folate deficiency induces epigenetic changes leading to global DNA hypomethylation, protooncogene activation and CIN; moreover, folate provide the nucleotide precursors for DNA synthesis and replication, ensuring DNA fidelity, maintenance of DNA integrity, and optimal DNA repair. Furthermore, when folate is deficient, base excision repair is unable to have an adequate response to process the DNA damage induced by folate deficiency (45). No significant effect of post weaning folic acid supplementation on DNA damage was observed at 34 weeks of age in the study reported above; the effect could be primarily on the developing colorectum, in fact a new DNA methylation pattern during embryogenesis is established and DNA methylation of the developing fetus seems susceptible to environmental factors such as maternal supplementation of methyl group donors (40, 44). Studies carried out in United States and Chile suggest that increased intake of synthetic folic acid has increased colon cancer risk (46, 47). However, low dietary folate intake (<200 µg/day) was also associated with an increased frequency of hypomethylated long-interspersed nucleotide element (a marker of genome-wide DNA methylation) repeats in human colon tumors (48). The effect of intervention with folic acid on DNA methylation is thereby conflicting and highly dependent on initial folate status, level and duration of supplementation, tissues examined, stage of malignant transformation, and polymorphisms in folate metabolizing genes (44). However, carcinogenesis seems to accelerate if folic acid is given after the emergence of lesions, presumably through provision of DNA precursors for cancer cell growth (49) or by hypermethylation of several tumor suppressor genes. The involvement of polymorphisms in folate metabolizing genes is observed in several studies; in fact the availability of methyl donors depends also by enzymatic activity of these genes. Lower activity of MTR and MTHFR is inversely associated with either CIMP or MLH1 hypermethylation; however, DNA methyltransferase 3b (DNMT3b) overexpression is associated with increased promoter hypermethylation in different types of cancers (50). In a large family-based case-control study of CRC risk, variants in DHFR and MTR genes were associated with a decrease in CRC risk in non-users of multivitamin supplements, while there was no association in multivitamin users. For the MTHFR C677T genotype, several studies reported that the TT genotype is more protective in those with a diet high in folate or low in alcohol (51–53).

Folate depletion downregulates, whereas replenishment or supplementation upregulates pathways related to inflammation and immune response. These findings provide novel support to the concept that excessive folate supplementation might promote colorectal carcinogenesis by enhancing proinflammatory and immune response pathways (54). A summary of these last acquisitions is shown in Fig. 2.

Concluding remarks

Definitely the study of epigenetic marks to better characterize CRC and to identify new tools for diagnosis and prognosis is extremely promising. For instance, an interesting digital quantification of rare methylation events has been proposed for diagnostic evaluations of preclinical samples, this approach was shown to detect cancer-derived methylation of vimentin gene DNA in plasma and fecal DNA from colon cancer patients (22, 55).

But there is another area where epigenetic studies are proving to be hopeful: the translation to therapy, as epigenetic modifications are reversible. To date, a few pharmacological compounds directed toward epigenetic enzymes have shown promise in treating leukemias and lymphoma. These include DNA demethylating agents or (DNMT) inhibitors such as 5-azacytidine and 5-aza-20-deoxycytidine and HDACi such as vorinostat suberylanilide hydroxamic acid (SAHA). It would be very important to identify new anticancer therapy based on the epigenetic regulation of miRNA; the identification of a greater number of epigenetically silenced tumor suppressor miRNA genes in human cancer, targeting these miRNAs will become a potentially powerful approach to the development of novel epigenetic drugs. Despite some discouraging results, DNMT and HDACi are still being tested in metastatic CRC patients to improve the quality of life and survival of patients in the near future (56). A study showed an increase in the expression of DNMT1 in colon tumor group as compared to the normal control; moreover, the animals treated with 5-azacytidine alone or with combination of 5-azacytidine and cisplatin showed significant reduction...
in the expression of DNMT1, resulting in a protection against tumor growth (57). Treating pancreatic and colon cancer cells with valproic acid, an HDACi, leads to upregulation of GRP78, an endoplasmic reticulum chaperone immunoglobulin-binding protein. GRP78 is involved in APP maturation and inhibition of tumor cell growth by downregulation of APP and secreted soluble APPα. HDAC and epidermal growth factor receptor (EGFR) are highly expressed in CRC with high risk of metastasis and recurrence. Thereby targeting EGFR could be a promising approach for the CRC treatment; particularly HDACi were able to disrupt the EGF-signaling in colon cancer cells (58, 59).

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References
Migliani and Migliani


