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Epigenetics of Colorectal Cancer

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Abstract
Colorectal cancer (CRC) has broad geographic distribution and affects millions of people throughout the world. It occurs sporadically in 80% of cases, but the rest have family histories. Epigenetic alterations such as DNA methylation and modification of histones and non-coding RNA are involved in the development of this disease. At present, these alterations have valuable potential as biomarkers for early detection of CRC and could be useful for diagnosis and determination of prognosis for individuals with CRC. The purpose of this review is to describe the main epigenetic mechanisms involved in colorectal cancer and the important roles they have in the development and progression of the disease.

Keywords
Colorectal cancer, epigenetics, DNA methylation, repair genes, tumor suppressor genes.

INTRODUCTION

Colorectal cancer (CRC) is considered to be a public health problem with a wide geographic distribution, according to the GLOBOCAN 2012 registry. (1) Currently, CRC is the third most common cancer in men and women in the world. Its incidence is high in developed countries but low in developing countries. (1, 2) Nevertheless, recent data from the International Agency for Research on Cancer (IARC) show increasing incidence and mortality rates in less developed countries. (3, 4)

Incidence of CRC and CRC mortality rates have increased in Colombia in recent last decades. (5, 6) According to figures from GLOBOCAN 2012 for Colombia, CRC ranks fourth in incidence and mortality for both sexes. In the most recent year, 4,107 new cases were diagnosed, most of which were in advanced stages of the disease. (1)

The CRC occurs sporadically in about 80% of cases, but the remaining 20% have family histories. Germline mutations in the APC and MLH1 genes predispose to hereditary-type CRC. (7-9) Several different genetic alterations are involved in onset and development of this disease. (10) These alterations affect the expression of multiple genes, promoting the transformation of the normal mucosa of the colon into a benign polyp which progresses to early adenoma, then becomes intermediate, and finally progresses to adenocarcinoma. (10)

Several molecular pathways are known to explain the development of CRC. Initially, the classic model of progression from adenoma to carcinoma was proposed by Fearon and Vogelstein. (11) It is called the traditional or suppressor pathway and involves deactivation of the APC and TP53 tumor suppressor genes plus mutations in the KRAS, DCC and SMAD oncogenes. (11) The second pathway is called a mutator and is related to mutations in the MLH1 and MSH2 genes of the MMR repair system which induce microsatellite instability (MSI) in tumor cells. (12) The third pathway is epigenetic and consists of repression of

gene expression by methylation of the promoter region of the tumor suppressor or repair genes. (13)

The term epigenetics was invented in 1942 by Conrad Hal Waddington. It now describes the mechanisms that modify the structure of chromatics and affect levels of gene expression without changes in the sequence of deoxyribonucleic acid (DNA). These mechanisms include methylation, acetylation and hydroxylation of DNA, modification of histone-like proteins, remodeling of the chromatin structure and changes in non-coding ribonucleic acids (RNA). Epigenetic modifications can be induced by external and internal factors that may have effects similar to those of pathogenic mutations, since they can inactivate expression of various genes in a specific tissue. This has been shown in the carcinogenesis of various organs and is of great importance in the development of cancer since these modifications affect expression of tumor suppressor genes of the DNA repair system. (14)

The purpose of this review is to describe the most important epigenetic modifications related to CRC and their possible use in clinical applications as biomarkers for early detection of CRC.

HISTONE MODIFICATION

On transcriptional control mechanism involves modifications in histone proteins that are associated with DNA. (15, 16) Post-translational covalent modifications in specific regions of histones are known to be an epigenetic mechanism that regulates and modifies the structure of chromatin and are thought to be associated with the origin and progression of cancer. (16)

Current knowledge of histone modifications in the development of cancer in humans is very limited. Histones are organized in a cylindrical structure, and modifications commonly occur in H2A, H2B, H3 and H4 which are part of a histone octamer. (15, 16) The nucleosome is a unit composed of approximately 150 to 200 base pairs of DNA that are arranged around the nucleosomal histones. The N-terminal portion of each histone that emerges from this compact structure is the target at which post-translational modifications occur. The main types of modifications are phosphorylation, glycosylation, ADP-ribosylation, ubiquitination, sumoylation and, most commonly, acetylation and methylation (Figure 1). (15, 16)

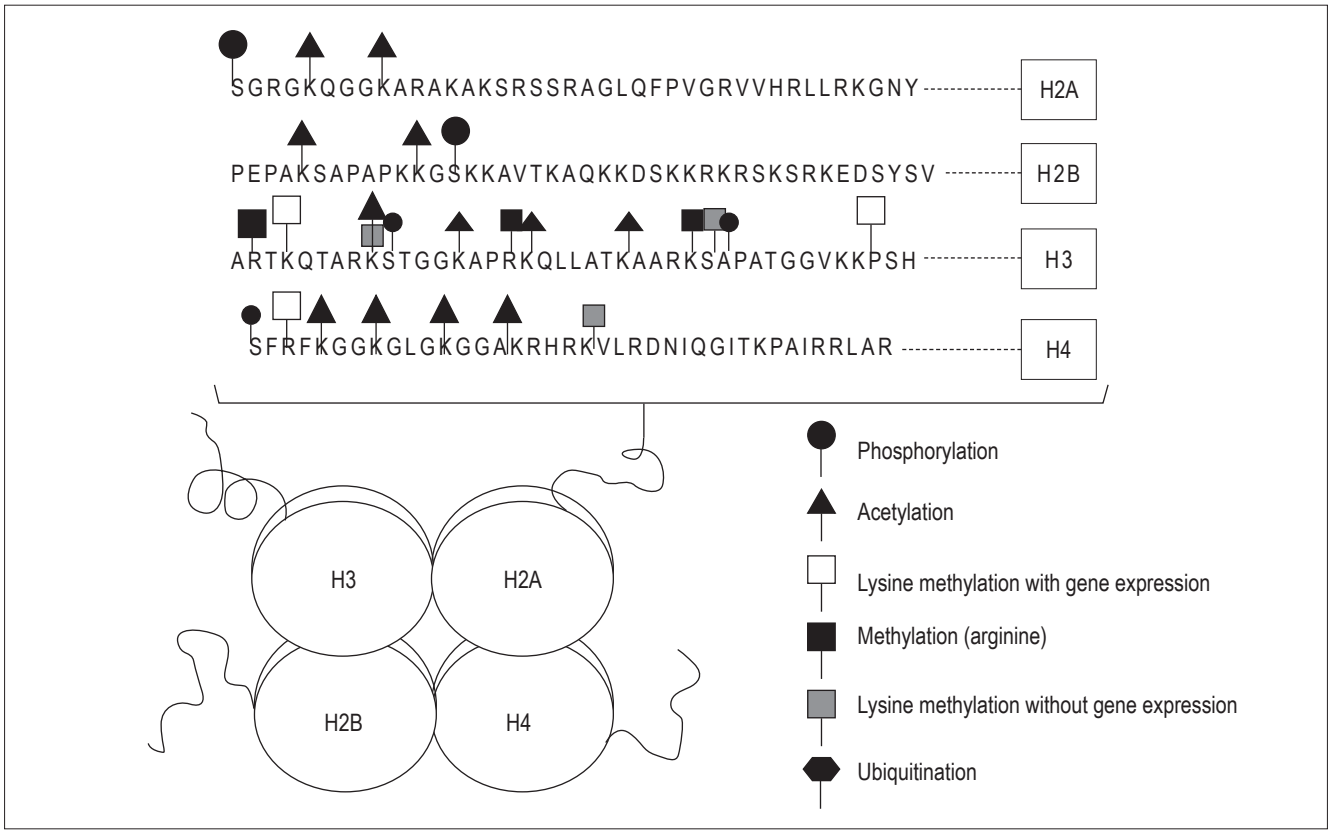

Figure 1. Main post-translational modifications in N-terminal histones residues. The four histone proteins (H2A, H2B, H3 and H4) have a cylindrical structure called the histone nucleus. Main covalent modifications are phosphorylation, acetylation, methylation and ubiquitination. The image shows the sequence of amino acids that make up the N-terminal residue of each histone and the place where each type of modification occurs.

Acetylation and methylation are the most studied epigenetic modifications in cancer. Amino acid residues, certain types of modifications and the histone domain have been observed to be associated with the development and progression of cancer. (16-18) In general, of all the epigenetic mechanisms, histone modifications are of great importance and are the object of many studies about the epigenetics of cancer.

The acetylation/deacetylation and methylation/demethylation states of lysine and arginine residues among the histones are among the most studied and best understood modifications. In general, it is known that hypoacetylation represses gene expression; while hyperacetylation of histones activates the transcription of genes. Acetylation in specific histone motifs destabilizes chromatin fiber which allows increased mobility of the nucleosomes in the chromosomes. In this state, transcription factors bind to DNA. (19)

Currently, it is known that the deacetylation of histone H3K9 is related to repression of E-cadherin transcription in the cells of colorectal tumors. Other studies report that the loss of E-cadherin expression by methylation and the subsequent loss of cell adhesion appear to be critical steps in the ability of tumor cells to invade adjacent tissues and metastasize. Various studies have shown that expression of this protein is null or very low in poorly differentiated carcinomas, including CRC. (20, 21)

DNA METHYLATION

Regulation of gene expression is the product of interaction between transcription initiation factors and promoter sequences located before the start codon (ATG). In normal cells, 60% of the genes coding in humans, the promoter region is rich in sequences of cytosine-guanine (C-G, called CpG islands). (22)

One mechanism for complete inhibition of gene expression is methylation of the promoter regions which prevents of transcription factors from binding to DNA regulatory sequences. Methylation occurs in the CpG islands by covalent attachment of a methyl group (CH3) to the carbon 5' of the cytosine by means of DNA methyltransferase (DNMT) (Figure 2). CpG islands comprise regions of between 200 and 2000 base pairs with a CG ratio of over 50%. (23) Methylation represses transcription by binding methyl-CpG binding proteins (MBP) that interact with methylated CpG sequences. This prevents transcription factors from binding with DNA sequences and inactivates genes. (24, 25) It is important to note that CpG islands that are outside of an active gene are mostly methylated, whereas CpG islands that are not methylated are part of the promoter region of genes that are constitutively expressed (Figure 3). (10)

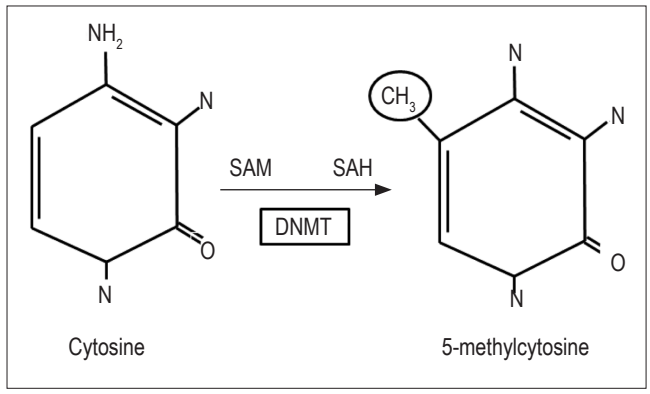

Figure 2. Methylation of cytosine in carbon 5 by means of DNMT. This enzyme catalyzes the union of a methyl group CH3 in the carbon 5 of the cytosine, using the S-adenosyl methionine (SAH) of the CH3 group as a donor molecule. SAH: S-adenosyl L-homocysteine.

Among the modifications that occur in the genome, methylation has important functions such as regulating processes of replication, transcription, DNA repair and gene expression. In addition, this DNA modification is necessary for permanent deactivation of certain regions with genes that are not expressed after embryonic development and for deactivation of the X chromosome. It is also essential for coordination of transcriptional processes during embryonic development and cell differentiation. (10)

HYPERMETHYLATION OF DNA IN COLORECTAL CANCER

DNA methylation has been studied extensively in various types of cancer. (19, 26, 27). Similarly, the association between hypermethylation of CpG islands located near the gene promoter region and deactivation of gene transcription is well known. This includes deactivation of tumor suppressor gene transcription, and these genes regulate the cell cycle and DNA repair system. (28) They are usually involved in many important biological processes including cell proliferation, apoptosis, angiogenesis, invasion and cell adhesion. (29)

Numerous studies of CRC have reported that hypermethylation of the promoter region of the RB1, APC, MLH1, MGMT, CDH1, CDKN2A, RUNX3, RASSF1A and many other genes occurs frequently (Table 1). (26, 30- 39).

One of the first discoveries of the effect of methylation of CpG islands in a tumor suppressor gene in cancer was that of retinoblastoma (RB1) which is hypermethylated in 10% to 20% of CRC cases of. RB1 codes for a nucleoprotein that has an important regulatory function in the cell cycle. Loss of this gene's functioning causes uncontrolled cell proliferation, and this gene is altered in a wide variety of tumors, including CRC. (30)

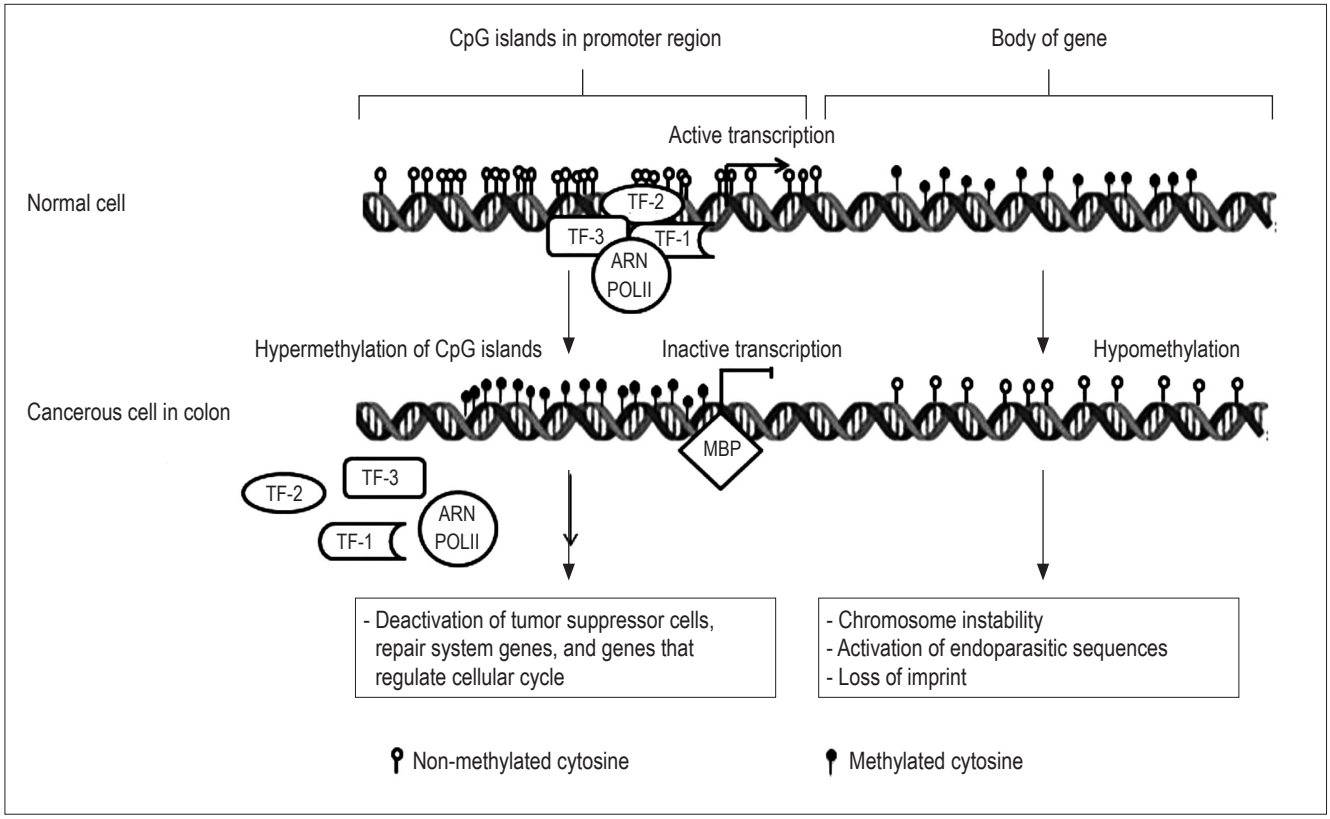

Figure 3. DNA methylation status. In the promoter region of a normal colon cell, CpG islands within the promoter are not hypermethylated (blank circles) which allows RNA polymerase II (RNA POLII) and transcription factors such as FT-2, FT-3 and FT-1 to bind. In this way, the normal transcription of the gene occurs. In contrast, in a colon adenocarcinoma cell, the CpG islands of the promoter region are hypermethylated (black circles) which represses transcription by binding MBP proteins that interact with methylated CpG sequences. This prevents binding of transcription factors with DNA causing gene deactivation.

Table 1. Most important hypermethylated genes in CRC. A list of frequently methylated genes in CRC including gene function and the technique used for epigenetic analysis.

Gene	Name	Function	Methylation (%)	Technique	Reference
RB1	Retinoblastoma	Tumor suppressor	10-20	MSP (PCR)	(30)
MLH1	*MutL Homolog 1*	DNA repair system	60	MSP (PCR)	(26, 32)
MGMT	06-methylguanine-DNA-methyltransferase	DNA repair system	38	MethyLight	(34)
CDH1	E-cadherin	Tumor suppressor	46	MSP (PCR)	(35)
CDKN2A	Cyclin 2A-dependent kinase inhibitor	Tumor suppressor	30	Pyrosequencing, MSP (PCR)	(36)
RUNX3	Runt-related transcription factor	Tumor suppressor	29	MSP (PCR)	(37, 38)
RASSF1A	*Ras association domain family 1 isoform A*	Tumor suppressor	47	MSP (PCR)	(39)

MSP: methylation-specific PCR; PCR: polymerase chain reaction.

On the other hand, hypermethylation of the promoter of the MLH1 gene is related to the development of sporadic and hereditary CRC in which MSI occurs. (26) The MLH1 gene is part of the mismatch repair system (MMR). Defects in these genes due to point mutations or epigenetic silencing are the cause of MSI in the development of CRC. (33) This gene is methylated in up to 60% of the sporadic cases of CRC. (32) In some cases, it has been observed that the MLH1 gene is methylated in tissue with normal morphological appearance adjacent to the tumor, so it has

been proposed as a biomarker for early detection of CRC. Nevertheless, more studies are required before this is established. (31, 32)

Similarly, MGMT is hypermethylated in 38% of colorectal polyps. This gene is part of the DNA repair system and is responsible for removing DNA adducts and preventing the formation of MMR. In these cases, deactivation of this gene is associated with development of CRC and with mutations in other genes such as APC, KRAS and TP53 that are involved in this type of cancer. (34)

Similarly, methylation studies of CDH1, a tumor suppressor gene, have shown that this gene is hypermethylated in 46% of cases of CRC. (35) As a consequence of this epigenetic modification, expression and functioning of the E-cadherin protein decrease which favors increased cell proliferation, invasion and metastasis of the colon tumor cells. Also deactivation of this gene may be associated with alterations of other genes such as APC that are important in the development of CRC. (35)

The CDKN2A (p16) gene, another tumor suppressor, is hypermethylated in 30% of cases of CRC, and transcriptional repression of this gene also favors development, progression and invasion of transformed cells in this type of cancer. In addition, this modification is frequently found in the ascending colon and is associated with a poor prognoses of patients with this disease. (36)

Epigenetic deactivation in CRC also occurs in the case of RUNX3 which acts as a tumor suppressor in the epithelium of the colon. It becomes deactivated in approximately 20% of cases of CRC. (37) From these results, a significant association has been proposed for deactivation of RUNX3 and CRC with MSI, and it has been concluded that the RUNX3 gene is important for the development of CRC. (37, 38)

RASSF1A is another gene that is commonly hypermethylated in CRC. This tumor suppressor gene acts in the progression of the G1 phase to the S of the cell cycle. (39, 40) Methylation of this gene is observed in 47% of CRC cases and is related to advanced stages of the disease, metastasis and lymphatic invasion. Similarly, a significant correlation has been proposed between cases of CRC and mutations in codon 12 of the KRAS gene and the hypermethylated state of this gene. It has been suggested that, in addition to being important in the development of the CRC, this could also be useful as a methylation biomarker in this type of cancer. (39)

DNA HYPERMETHYLATION AS A DIAGNOSTIC MARKER IN COLORECTAL CANCER

The hypermethylation mechanisms described above suggest that methylation of certain genes may occur prior to the appearance of a tumor. For this reason methylation status in the epithelium, in premalignant lesions, and in other samples including blood and/or feces might serve as a biomarker for early detection and preventive diagnosis for patients with CRC.

Hypermethylation has been studied as an epigenetic marker in many types of neoplasms. (27, 41, 42). Techniques for analysis of DNA methylation for diagnostic purposes may have several advantages compared to other known biomarkers such as point mutations and gene expression profiles. First, alterations in the patterns of DNA methylation are found in well-defined genome regions: the CpG islands, and they can be detected by several very specific and sensitive techniques. In addition, it is important to mention that some of these techniques are inexpensive. (39) Second, the possibility analysis for these biomarkers of methylation in peripheral blood and feces allows evaluation of individuals by less invasive methods. On the other hand, DNA stability makes it possible to analyze these biomarkers in paraffin-embedded tissues, which allows us to perform retrospective studies from pathological tumor files. (41)

Methylation of the SEPT9 gene has been observed in more than 90% of patients with CRC and is the only epigenetic biomarker approved by the Food and Drug Administration (FDA) as a diagnostic test for early detection of CRC. (43, 44) Detection of methylated SEPT9 in blood is associated with colorectal adenocarcinoma. In individuals who test positive, colonoscopy should be recommended to confirm a diagnosis of CRC. The SEPT9 methylation test's sensitivity and specificity are both high which is a great advantage for early detection of CRC. (45). This test is routinely offered to individuals who are at high risk of CRC in some developed countries. (45)

In addition, it has also been suggested that the analysis of the methylation status of genes such as APC, MLH1, MGMT, CDKN2A and RASSF2A in peripheral blood samples could be important for early detection of CRC. (46)

The epigenetic biomarkers described are not only very useful for early detection of CRC, they may also be very beneficial for studying individuals with high risks of developing CRC. Results could be used to recommend early diagnostic testing, surgical intervention or more specific pharmacological therapies targeting the reversible nature of epigenetic modifications. (47) However, more studies are required to validate the testing of epigenetic markers for diagnosing patients with CRC.

CPG ISLAND METHYLATOR PHENOTYPE (CIMP) IN COLORECTAL CANCER

The concept of CIMP (CpG island methylator phenotype) was proposed by Toyota et al. who suggested that CRC could be divided into two categories: one with less than

two methylated genes called CIMP-, and another with more than two methylated genes called CIMP + . (48)

These authors selected five genes to use for evaluating CIMP status in CRC samples: CDKN2A, MINT1, MINT2, MINT31 and MLH1. (49) Other authors have proposed different panels with a greater number of markers to determine CIMP status including CACNA1G, CRABP1, IGF2, NEUROG1, RUNX3, SOCS1, HIC1, IGFBP and WR. However, there is no consensus on which and how many genes can be used for adequate classification of CIMP in patients with CRC. (49-53)

On the basis of analysis of a large panel of methylation markers in CRC, it has been proposed that CIMP be divided into CIMP1 and CIMP2. (51) CIMP1 tumors present test positive for MSI and correlate with mutations in the BRAF gene whereas CIMP2 tumors test negative for MSI present mutations in the KRAS gene and infrequently in TP53, BRAF. (52) For this reason, it is very important to highlight the usefulness of methylation analysis in patients with CRC, since a more precise molecular classification of these patients can be achieved by using their methylation profiles.

EPIGENETIC THERAPY FOR COLORECTAL CANCER

Analyses of methylation in promoter regions of several genes could become prognostic markers of disease progression as well as indicators of likely responses to certain antineoplastic treatments. It has been observed that specific epigenetic markers in CRC are associated with a poor prognosis. Similarly, the clinical utility of epigenetic markers for predicting patient responses to certain antineoplastic drugs has been under constant investigation. (10, 53)

Unlike molecular alterations involved in initiation and progression of CRC such as mutations and allelic loss, epigenetic alterations are potentially reversible. This characteristic has allowed development of new therapies based on restoring the activity of silenced genes epigenetically inhibiting DNA methylation. Currently, 5-azacitidine and 5-azadeoxicitidine are widely under investigation, and the FDA has approved methylation inhibitor drugs for use in patients with myelodysplastic syndrome and in patients with chronic myelocytic leukemia. (54, 55)

Altered methylation of promoter regions of several genes is considered to be one reason that tumors develop resistance to certain antineoplastic therapies. One of the advantages of demethylation drugs for epigenetic therapy for colorectal tumors is that treatment can act simultaneously on several genes that become hypermethylated simultaneously. In addition, the effect of epigenetic therapy can be predicted in advance by analyzing hypermethylated genes directly in the tumor using currently available massively parallel sequencing platforms. (54, 55)

It has been observed that patients with CRC who have a hypermethylated MLH1 gene also have MSI resistant to 5-fluorouracil (5-FU) therapy. (53) For this reason, these patients should be given another type of chemotherapy. This indicates the importance of determining the methylation status of MLH1 in patients with CRC for guiding not only the choice of the most appropriate therapy, but also the type of surgery required by patients.

In addition, in-vitro cellular models have been used to test whether demethylation of the promoter region of MLH1 in CRC cell lines can restore sensitivity to treatment with 5-FU. CRC cell lines such as SW48 (methylated MLH1), HCT116 and HCT116 + chr2 (mutated MLH1), and HCT116 + chr3 (normal MLH1) have been used. It was observed that treatment with 5-azacytidine (5-AZA) induces demethylation of the MLH1 gene and, consequently, reestablishment of gene expression and messenger RNA (mRNA) in SW48 cells. Also, treatment with 5-FU alone reduces cell growth in the HCT116 + chr3 line but was less effective for the other cell lines. (56) These observations corroborate the fact that resistance to 5-FU can be counteracted by reestablishment of MLH1 gene expression through treatment with 5-AZA. (56) These findings are having a great impact on the development of new antineoplastic epigenetic therapies.

Finally, the advance of the new massive genomic technologies will allow analysis of the entire epigenome which will lead to better epigenetic characterization of colorectal tumors and will advance the search for new epigenetic biomarkers for early detection of CRC.

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