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Review > Oman Med J. 2021 Nov 10;36(6):e315. doi: 10.5001/omj.2020.93. eCollection 2021 Nov.

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Seyedeh Elham Norollahi<sup>1</sup>, Maryam Gholamniya Foumani<sup>2</sup>, Maryam Khoshbakht Pishkhan<sup>3</sup>, Afshin Shafaghi<sup>4</sup>, Majid Alipour<sup>5</sup>, Vida Baloui Jamkhaneh<sup>6</sup>, Mohammad Namayan Marghoob<sup>7 8</sup>, Sogand Vahidi<sup>9</sup>

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PMID: 34804598 PMCID: PMC8581152 DOI: 10.5001/omj.2020.93

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## Abstract

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# DNA Methylation Profiling of MYC, SMAD2/3 and DNMT3A in Colorectal Cancer

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<sup>1</sup>Cancer Research Center and Department of Immunology, Semnan University of Medical Sciences, Semnan, Iran

<sup>2</sup>School of Nursing and Midwifery, Guilan University of Medical Sciences, Rasht, Iran

<sup>3</sup>Department of Nursing, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran

<sup>4</sup>Faculty of Medicine, Department of Gastroenterology, Guilan University of Medical Sciences, Rasht, Iran

<sup>5</sup>Department of Biology, Islamic Azad University of Babol Branch, Babol, Iran

<sup>6</sup>Department of Veterinary Medicine, Islamic Azad University of Babol Branch, Babol, Iran

<sup>7</sup>Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran

<sup>8</sup>Gastroenterology and Liver Disease Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>9</sup>Clinical Research Development Unit of Poursina Hospital, Guilan University of Medical Sciences, Rasht, Iran

## ARTICLE INFO

### Article history:

Received: 3 March 2020

Accepted: 4 June 2020

### Online:

DOI 10.5001/omj.2020.93

### Keywords:

DNA Methylation; Colorectal Neoplasms; Genes, Tumor Suppressor; Genomic Instability.

## ABSTRACT

Epigenetic modifications, particularly DNA methylation, is commonplace and a remarkable factor in carcinogenesis transformation. Conspicuously, previous findings have presented a cluster of irregular promoter methylation alterations related with silencing of tumor suppressor genes, little is accepted regarding their sequential DNA methylation (hypo and hyper) modifications during the cancer progression. In this way, fluctuations of DNA methylation of many genes, especially *MYC*, *SMAD2/3*, and *DNMT3A*, have an impressive central key role in many different cancers, including colorectal cancer (CRC). CRC is distinguished by DNA methylation, which is related to tumorigenesis and also genomic instability. Importantly, molecular heterogeneity between multiple adenomas in different patients with CRC may show diverse developmental phenotypes for these kinds of tumors. Conclusively, studying factors that are involved in CRC carcinogenesis, especially the alterations in epigenetic elements, such as DNA methylation besides RNA remodeling, and histone modification, acetylation and phosphorylation, can be influential to find new therapeutic and diagnostic biomarkers in this type of malignancy. In this account, we discuss and address the potential significant methylated modifications of these genes and their importance during the development of CRC carcinogenesis.

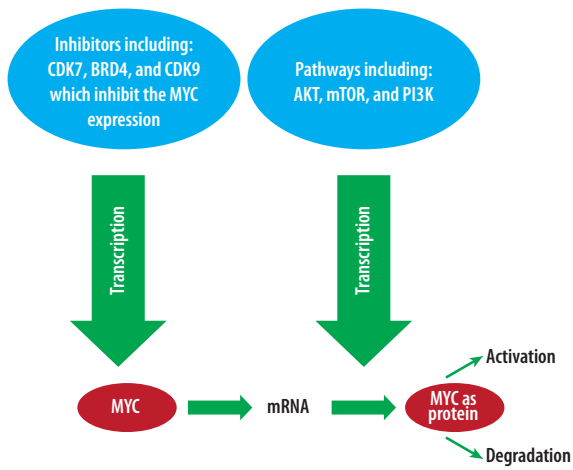
Cancer is a complex cellular mechanism that occurs at least by a mutation of five or six genes, each mutation alone causes changes in the cell.<sup>1,2</sup> Colorectal cancer (CRC) is one of the most common gastrointestinal cancers and has high mortality rates.<sup>3</sup> The formation of tumors in the rectum, colon, and appendix and the extensive and advanced accumulation of genetic and epigenetic changes alter the natural epithelium of the colon to adenoma and ultimately become a malignant tumor.<sup>4</sup> As a result of genetic and epigenetic changes, colon mucosal cells change from normal to cancerous cells.<sup>5</sup> Cancer disrupts the cellular order, and this cellular disturbance directly affects the cell cycle and causes a lack of cell differentiation. An increase in the number of cancer patients and in the average age of the population

directly correlates with the increase in cancer in the world. In this regard, the genetic and epigenetic study of different molecular pathways involved in CRC can be beneficial for early diagnosis and treatment.<sup>6-10</sup> Many genes are involved in different molecular mechanisms in the carcinogenic pathway, including *MYC*, *SMAD2/3*, and *DNMT3A*. In this review, we discuss the performance of *MYC*, *SMAD2/3*, and *DNMT3A* and the role they play in carcinogenesis.<sup>11,12</sup>

### Fluctuation of genes

*MYC* is transmitted by the avian myelocytomatosis virus, and viral promoters widely regulate the extent of the gene.<sup>13</sup> Some noticeable changes occur in the expression of *MYC* oncogene and increase the process of cell deformation. At least three different

\*Corresponding author: ✉so.vahidii@gmail.com



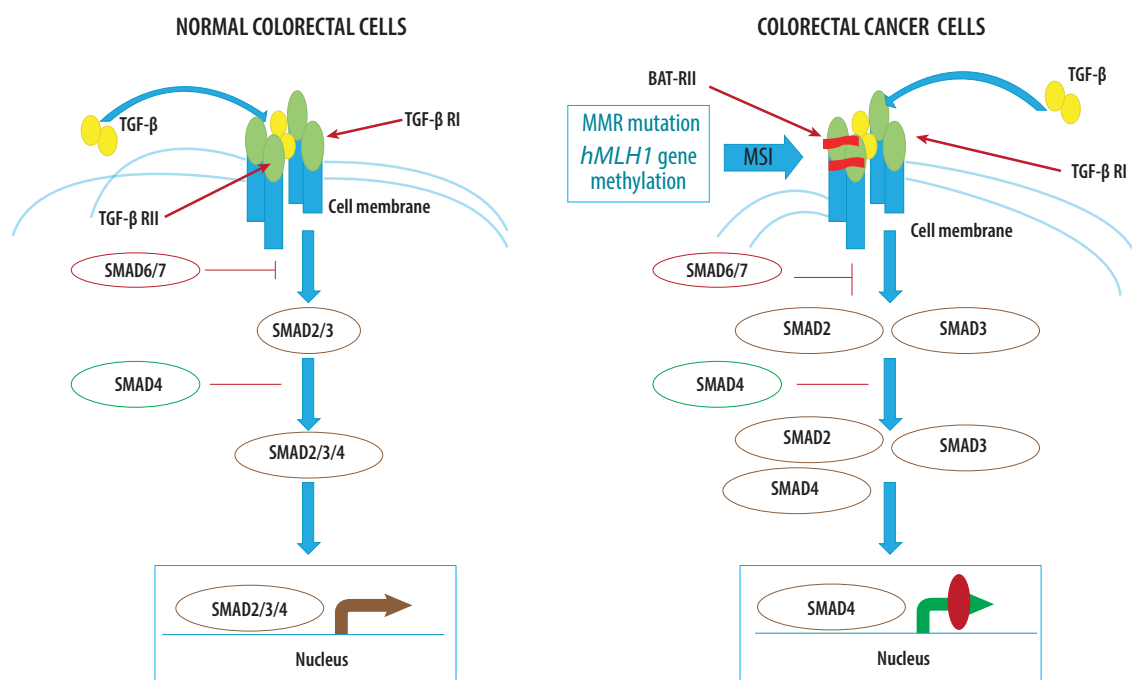
**Figure 1:** Significance of inhibitors like CDK7, BRD4, and CDK9 in pathways including AKT, mTOR, and PI3K, which inhibit MYC expression.

mechanisms can produce *MYC* oncogene. In several human tumors, the amount of *MYC* gene is determined by its natural expression promoters. Still, the number of copies of this gene is several times more than the number of copies in the normal human genome.<sup>14</sup> In 30% of children’s neuroblastoma, a similar gene called *N-MYC* is also widely distributed in malignant tumors [Figure 1]. In both cases, the increase in these genetic copies increases the level of the produced gene. Another point is that *MYC* family proteins have a very significant effect on

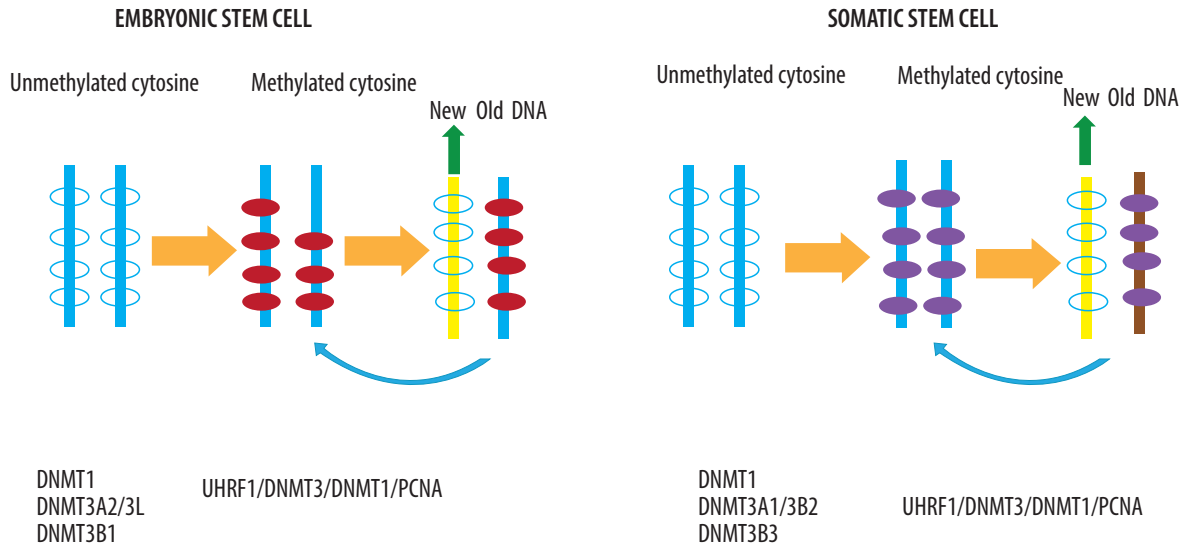
cell growth.<sup>15</sup> Consequently, when they are present in large quantities, they cause uncontrolled cell proliferation. *MYC* proto-oncogenes, commonly referred to as *C-MYC*, are distinct from the two *N-MYC* and *L-MYC* genes. Human *MYC* genes are seen in a variety of human tumors.<sup>16</sup> In addition to genetic and environmental factors, epigenetic factors play a vital role in carcinogenesis. These factors include histone changes, acetylation, phosphorylation, and DNA methylation.<sup>17,18</sup>

DNA methylation, an essential epigenetic agent, is a common feature in vertebrates, and one of the main epigenetic mechanisms is the control of gene expression.<sup>19</sup> Methylation changes can be eliminated or transferred to the next generation without changing the nature of the DNA. Also, CpG methylation is one of the most critical molecular processes in carcinogenesis. The study of promoter hypermethylation can create new hopes and achievements to achieve molecular diagnostic markers of cancer.

In the case of the *SMAD* gene, the proteins encoded by these genes belonging to the *SMAD* group of proteins are similar to those of *Drosophila melanogaster* genes (*Elegans SMAD* gene).<sup>20</sup> The *SMAD* proteins are a signal transducer and transcription modulator that interfaces multiple signaling pathways. These proteins are the main



**Figure 2:** Fluctuations of *SMADs* (*SMAD2/3/4/6* and *7*) alongside other related genes in normal and colon cancer cells.



**Figure 3:** Role of DNA methyltransferases in embryonic and somatic stem cells.

signal transducers for the transforming growth factor-beta (TGF- $\beta$ ) superfamily receptors, which are critical for regulating cell proliferation, apoptosis, and differentiation.<sup>21</sup> In response to the signal, TGF- $\beta$  superfamily ligands bind to a type II receptor, which recruits and phosphorylates a type I receptor. The type I receptor then phosphorylates receptor-regulated *SMADs* (R-*SMADs*), which can now bind co*SMAD* and *SMAD4*. R-*SMAD*/co*SMAD* complexes accumulate in the nucleus, where they act as transcription factors and participate in regulating target gene expression.<sup>22</sup> Besides *SMAD2*, which is a protein-encoding gene, major diseases associated with *SMAD2* include urogenital disease and many common cancers.<sup>23</sup> The pivotal paralog of the *SMAD2* is the *SMAD3* [Figure 2]. Human immunohistochemistry assessment of *SMAD3*/*SMAD2* phosphorylation and p300 activator showed association with human glomerulonephritis and renal injury.<sup>24</sup> Also, *SMAD2* and *SMAD4* mutations in the TGF- $\beta$ -*SMAD* signaling pathway have been proven in head and neck carcinoma.<sup>25</sup> In addition, the *SMAD* pathway is also active in scleroderma fibroblast, and the level of *SMAD2/3* phosphorylation and the site of the *SMAD2/3* phosphorylation was increased.<sup>26</sup> *SMAD2/3/4* heterodimers correspondingly regulate *SMAD2/3/4* transcriptional activity. The *SMAD3* gene produces a protein involved in transmitting chemical signals from the cell surface to the nucleus.<sup>27</sup> This signaling process begins when TGF- $\beta$  protein binding to a receptor on the cell surface and activates a group of

*SMAD* proteins such as *SMAD2* and *SMAD3*.<sup>28</sup> These *SMAD* proteins form a complex with *SMAD4* and then accumulate in the nucleus and binds to specific regions of DNA to control the activity of particular genes. Through the TGF- $\beta$  signaling pathway, the *SMAD2/3* proteins also affect many aspects of cellular processes, including growth and division (proliferation), cell movement (migration), and cell death (apoptosis).<sup>29</sup>

Another enzyme is the DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*) that is encoded in humans by the *DNMT3A* gene [Figure 3].<sup>30</sup> *DNMT3A* catalyzes the transfer of methyl groups to specific CpG structures in DNA, a process called DNA methylation.<sup>31</sup> DNA methylation plays a role in many cellular functions, such as gene expression regulation, protein and lipid reaction regulation, and chemical processing control in nervous system signaling that by *DNMT3A* occurs through methylation during evolution.<sup>32</sup> This enzyme can also lead to the formation of more mature cell types in the early cells. In early blood stem cells called hematopoietic stem cells, methylation patterns are generated by the *DNMT3A*, which develops the differentiation to different types of blood cells.<sup>33-36</sup>

#### **Induction of epigenetics elements in carcinogenesis and tumorigenesis**

Epigenetic elements include DNA methylation, histone modification, histone acetylation, histone phosphorylation, and RNA remodeling. The most important is DNA methylation.<sup>37</sup> Additionally,

**Table 1:** Main models of involved genomic uncertainty in colon cancer.

Main disorders	Main genes	Phenotypic characteristics	Type of defect
Sporadic colorectal cancer with mismatch repair deficiency	MLH1 somatic methylation	Colorectal cancer with increased risk of poor differentiation, more commonly located in the right colon, less aggressive clinical behavior than tumors without mismatch repair deficiency	Somatic
Base excision repair defect MYH-associated polyposis	MYH	Development of 15 or more colorectal adenomas with increased risk of colorectal cancer	Germline
Chromosomal instability- loss of heterozygosity at multiple loci	Loss of heterozygosity at APC, TP53, and SMAD4	Characteristic of 80% to 85% of sporadic colorectal cancers, depending on stage	Somatic
CpG island methylator phenotype-methylation target loci	Target loci MLH1, MINT1 MINT2, and MINT3	Characteristic of 15% of colorectal cancers, with most showing mismatch repair deficiency from loss of tumor MLH1 expression	Somatic
DNA mismatch repair defects. Hereditary nonpolyposis colorectal cancer	MLH1, MSH2, and MSH6 germline gene mutations	Multiple primary colorectal cancers, accelerated tumor progression, and increased risk of endometrial, gastric, and urothelial tumors	Germline

all these elements indicated their remarkable and impressive role in carcinogenesis and tumorogenesis.<sup>38</sup> Unlike changes related to the main DNA sequence, such as mutations, most epigenetic changes are reversible. Naturally, phenomena such as genomic imprinting, inactivation of the X chromosome, and the expression of gene sets that are important in the process of embryonic development are controlled by epigenetic mechanisms.<sup>39,40</sup> In recent decades, studies have shown that epigenetic alteration patterns and genetic changes in some genes play an important role in tumorigenesis. These changes include abnormal methylation patterns in gene regulation, histone modification, and alterations in miRNA expression.<sup>41</sup> Recent studies suggest that the abnormal methylation pattern on CpG islands is effective in tumor cell proliferation. The increase in the methylation of the regulatory regions of tumor suppressor genes and DNA repair leads to the extinction of these genes and the development of cancer. On the other hand, the reduction of methylation in the regulatory regions of oncogenes increases their expression and leads to the conduction of cells to tumors.<sup>42</sup> This mechanism is involved in the development of cancer cells by activating the enzymes involved in cell growth and survival of apoptosis and the cell cycle.<sup>43</sup>

Because DNA has an important role in replication and transcription and ultimately cell proliferation, the most important targets are regulatory molecules and anticancer drugs.<sup>44</sup>

DNA methylation is regulated by the DNA methyltransferase enzyme. Increased expression of DNA methyltransferase appears to be a common feature in a variety of cancers. Methylation patterns are inherited through mitosis. These normal patterns are disrupted in the DNA of the cancer cell; CpG islands are prone to methyltransferase activity and other areas of DNA are hypotensive.<sup>45</sup> The hypermethylation profile of CpG islands varies in different genes for each type of cancer. In general, hypermethylation of CpG islands occurs in tumor suppressor genes, and genes involved in the cell cycle, DNA repair, carcinogenic metabolism, intercellular interactions, cell death and regression, and promotes cancer progression.<sup>46</sup>

#### **Performance of DNA methylation in CRC**

Investigating gene expression in gastrointestinal cancers to evaluate their fluctuations, alongside any epigenetics alterations, is of great importance [Table 1].<sup>47-51</sup> Remarkably, the mechanisms underlying CRC pathobiology remain subjects of wide study in the pathogenesis of cancer. Genetic and epigenetic modifications have resulted in CRC and also, the cellular genome that transforms normal glandular epithelium into adenocarcinoma is involved in this process.<sup>52,53</sup> Conspicuously, the evaluation of methylated genes in CRC has also revealed a unique molecular subgroup of CRCs called CpG island methylator phenotype cancers; these tumors have a high frequency of methylated genes. In addition to DNA hypermethylation that



often takes place in the promoter region of tumor suppressor genes, epigenetic regulation of CRC epigenome also includes histone post-translational modifications, primarily histone acetylation and methylation that also play critical roles in the regulation of expression of oncogenes and tumor suppressor genes.<sup>52,54,55</sup> In this way, epigenetics alludes to heritable gene expression modifications that are not mediated by alterations in the DNA sequence. The epigenetic regulation of gene expression happens in normal tissue and plays a key role in many cellular activities, including tissue differentiation, embryonic development, and imprinting.<sup>56</sup>

In 1982, aberrant epigenetic modifications were first explored in CRC. Noticeably, the epigenetic study has indicated an epigenetic landscape comprising an elaborate array of epigenetic regulatory mechanisms that control gene expression in tumor and non-tumor tissues.<sup>57,58</sup> The epigenetic landscape is largely a reflection of agents that ascertain the condensation state of the chromatin, which identifies whether the DNA is related to proteins that manage the gene transcription. A relaxed or open chromatin state permits gene transcription, whereas a condensed chromatin state prevents gene transcription.<sup>56</sup> Evidently, it is confirmed that the DNA methylation seen in cancer and aging may stem from a small population of cells. Notably, not only are the target sites found partially methylated in normal tissues but are also highly altered in polyps,<sup>59</sup> a very early stage in the generation of colon cancer in men. It reveals that a subpopulation of stem cells in the colon undergoes de novo methylation of target CpG islands during aging, which presumably generates small patches of tissue that carry an aberrant DNA methylation profile.<sup>60-62</sup> This alteration probably induces a state of constitutive heterochromatin, which is not easily reversible. Thus, proliferative cells in the crypt that transform this sign may have the ability to skip the polycomb structure itself, but would not be capable of activating the critical differentiation genes, thereby inhibiting these cells from undergoing a transition to the epithelium, thus leaving them in a relatively proliferative state. Although this might not be sufficient for generating a tumor, it could very well provide the necessary background for cells that have transformed either through prior genetic predisposition or spontaneous mutation. These particular cells collect and organize DNA methylation during aging and then perform

as preferred targets for the transformation process, which is protected by the observation that both polyps and normal tissue surrounding the tumor are highly methylated and by the experimental evidence showing that 5-azacytidine is only capable of preventing the accumulation of intestinal tumors in mice if it originates from early in life.<sup>59,63,64</sup> As DNA methylation plays a significant role in CRC formation, it can be used as a potential diagnostic biomarker in cancer detection.<sup>65</sup> Considerably, some gene promoter methylation in the plasma or serum of patients with CRC has shown great promise as a potential diagnostic indicator of CRC.

To date, a lot of hypermethylated genes have been reported in CRC, but only a few have been included in commercial blood-based tests. Studies are needed to find new practical biomarkers for prognosis that would aid researchers and practitioners in decision-making. High-throughput technologies, such as methylation microarrays and next generation sequencing, have helped advance our understanding of epigenetic events at the genomic level.<sup>66-68</sup>

#### ***Role of DNA methylation of SMAD2/3, MYC, and DNMT3A in CRC***

Aberrant de novo methylation of DNA is considered a remarkable mediator of tumorigenesis. The processes that mediate aberrant DNA hyper- and hypo-methylation are under study. Although certain mechanisms have yet to be identified, it is now clear that DNA methylation is regulated through reciprocal interactions with histones. Modifications in the post-translational state of histones are closely related to cancer-related alterations in DNA methylation.<sup>69</sup> The enzymes that mediate DNA methylation, *DNMT1*, *DNMT3A*, and *DNMT3B*, are overexpressed, hyperactive, or misdirected. Increased DNMT expression has been proposed as a mechanism for the increased methylation seen in the promoter region of tumors. Compared to normal tissues, the increased expression and increased function of the DNA methyltransferases (DNMTs) have been reported in human cancers, including colon cancer.<sup>70</sup> DNMTs catalyze the addition of a methyl group to the 5-cytosine residue of CpG dinucleotides. This family of enzymes comprises *DNMT1* that performs as a DNA maintenance methyltransferase, and *DNMT3A* and *DNMT3B* that methylate previously unmethylated regions of DNA and are required for genome-wide de novo

DNA methylation. *DNMT3A* protein expression in human tissue samples and its potential inhibition by DNMT inhibitors remains unclear.<sup>71</sup> MYC protein has been implicated in development through the cell cycle and in differentiation-related regulation of transcription. Additionally, overexpressed MYC protein in dysplastic and tumor cells, accumulating in the cytoplasm and transferring persistently to the nucleus, may modify the cellular response to growth factors and abrogate normal growth control mechanisms by controlling cells from escaping from the proliferation cycle.<sup>72</sup> Conspicuously, C-MYC protein may manage its expression fluctuation via binding process, directly or indirectly, to the *C-MYC* gene. If this interaction is influenced by DNA methylation,<sup>73,74</sup> there may be a feedback effect between hypomethylation of the third exon of MYC and deregulation of expression. One study indicated that a 34-base pair sequence spanning the CCGG site of the C-MYC third exon exhibits methylation-dependent binding of particular protein species from normal colonic epithelium; dysplastic tissue yields a modified binding pattern.<sup>75</sup> Changes in the downstream methylation model may influence MYC expression by binding trans-acting agents, either directly or via induction of long range conformational alterations. The TGF- $\beta$  pathway plays a key role in embryonic development, organ homeostasis, tissue repair, and disease.<sup>76,77</sup> This diversity of tasks is achieved through the intracellular effector *SMAD2/3*, whose canonical function is to control the activity of target genes by interacting with transcriptional regulators.<sup>78</sup> Despite that, a complete description of the factors interacting with *SMAD2/3* in any given cell type is still lacking. *SMAD2/3* could act as a hub coordinating several proteins known to have a role in mRNA processing and alteration, apoptosis, DNA repair, and transcriptional regulation.<sup>79</sup> Remarkably, in the therapeutic approach, a probiotic strategy like using gut microbiota is of great importance.<sup>80</sup>

#### ***The key role of microRNAs in carcinogenesis***

Based on the chemical structure of RNA, which is made up of only four flat bases and the nucleotides have a negative charge, it seems that the drug target is not promising. However, RNA molecules can bind to small molecules. The binding of small ligands to RNA by blocking macromolecule binding

changes the active RNA configuration, induces a sub-configuration on the RNA, and inhibits the RNA catalytic activity, affecting its biological activity. Some herbal anticancer compounds, such as curcumin in turmeric, interact with RNA. More than 80% of the genome is actively grouped with RNA transcripts, referred to as non-coding RNAs. This group includes tRNA, rRNA, small nuclear RNA involved in splicing, and microRNA.<sup>81</sup>

MicroRNAs are a type of non-coding RNA that is fully protected nucleotides during evolution. These molecules are induced by binding to 3'UTR inhibiting translation or induction. Epigenetic factors reduce the expression of microRNAs by over-methylation of gene promoters or histone modifications. Increased expression of microRNAs in cancer cells could be due to the proliferation and lack of control of a transcription factor or demethylation of CpG islands in gene promoter areas.<sup>82</sup> It is not yet clear whether the change in microRNA expression is the result of a pathological state of cancer or whether cancer is the main cause of these changes. However, many microRNAs, especially the two groups of oncogenic microRNAs and tumor inhibitors, are abnormally expressed in cancer cells.<sup>83</sup> Epigenetic factors reduce the expression of microRNAs by overmethylation of gene promoters or histone modifications.

## **CONCLUSION**

Cancer is a genetic disease that occurs due to sequential mutations in human genes and genetic and environmental factors. CRC is one of the most prevalent lethal forms of cancer worldwide. Several mechanisms are involved in cancer development, which play major roles in altering cellular signaling and cancer formation. Oncogenes, tumor suppressor genes, apoptosis genes, and restorative genes are among the major factors in cancerous cells. These genes are responsible for controlling the differentiation and growth of the cells. Consequently, the mutation in these genes causes the normal process of the cell to become cancerous. Oncogenes are also activated by mutation in the original genes and converted to proto-oncogenes. Mutations in tumor suppressor genes also cause abnormal cell division and transform healthy cells into cancerous ones. Another factor is programmed cell death (apoptosis), which is the last cellular escape from

the cancerous process. Therefore, studying all factors involved in carcinogenesis, particularly the changes in epigenetic factors, such as DNA methylation, is useful in identifying diagnostic and therapeutic biomarkers in CRC.

#### Disclosure

The authors declared no conflicts of interest. No funding was received for this study.

#### Acknowledgements

The authors express their appreciation and gratitude to all people who were involved in this review.

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