# Dietary factors, genetic and epigenetic influences in colorectal cancer (Review)

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**Abstract.** Genetic influences, together with epigenetic components and dietary factors, play a fundamental role in the initiation and progression of cancer by causing a number of deregulations. Colorectal cancer (CRC) is a disease influenced by dietary factors, for which established genetic and epigenetic alterations have been identified. Within CRC, there are hereditary syndromes that present mutations in the germline hMLH1, and also alterations in the methylation of the promoters. Epigenetics has also been established as a pathway of carcinogenesis. In the present review, we analyzed studies conducted to discern the different pathways leading to established CRC, stressing the importance of identifying factors that may predict CRC at an early stage, since it is mostly a silent disease observed at the clinical level in advanced stages.

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## 1. Introduction

Colorectal cancer (CRC) is a disease that involves genetic and epigenetic changes. CRC arises when the epithelial cells of the colon or rectum begin to behave abnormally with an excessive proliferation generally resulting in the formation of an exofitic mass, mainly polyps. A polyp is defined as an abnormal grape-like formation within the inner wall of the colon or rectum. Initially these polyps are benign, but they have the potential to become malignant. Polyps grow slowly over many (3-15) years (1).

Most people do not develop polyps until they are over 50 years of age. Approximately 1 in 20 polyps can become cancerous if not removed. Therefore, prevention can be achieved by detecting the presence of polyps. CRC is one of the most common malignant tumors in humans, and has been observed to occur mainly in two specific patterns: sporadic and hereditary.

Sporadic cases account for 75-80% of the total and are the result of the accumulation of somatic mutations in oncogenes, tumor-suppressor genes and DNA-repair genes. These mutations are likely the result of dietary and environmental factors as well as aging. They tend to develop in individuals 50 years of age or older with no previous family history of the disease.

Hereditary cases account for approximately 10% of the total and include familiar adenomatous polyposis (FAP) and hereditary non-polyposis syndrome (HNPCC, or Lynch syndrome).

FAP is characterized by hundreds of polyps that present from an early age. This syndrome has an autosomal dominant character since it is caused by a dominant mutation in the APC gene (2).

The APC gene encodes a cytoplasmatic protein that regulates β-catenin. β-catenin acts primarily as a transcription activator. Under normal conditions, when the colonic epithelium remains intact and cell proliferation does not occur, most of the β-catenin forms a protein complex. The APC gene induces the phosphorylation and degradation of β-catenin not bound to the complex, generating a decrease in protein concentrations in the cell. When the APC gene is lost, it results in the accumulation of free cytoplasmic β-catenin followed by its translocation to the nucleus, activating several genes involved in cell proliferation. Therefore, the APC gene acts as a tumor-suppressor.

HNPCC is characterized by mutations in specific genes that comprise the machinery of DNA repair (hMSH2, hMSH6, hMLH1, hPMS1 and hPMS2). This is a complex enzymatic machinery that corrects errors during DNA replication. When

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one of the genes is mutated, the repair machinery does not work and results in microsatellite instability (MSI), which is a classic characteristic of this condition. Families with a member presenting HNPCC should not only undergo genetic studies to determine if they have the mutation, but should also be aware of other types of cancer that are associated with the syndrome, such as endometrial and ovarian cancer. Individuals with this mutation have a high risk of developing cancer, but do not have the same number of polyps as patients with FAP; however, the few polyps that do occur are highly likely to become malignant. In addition to the machinery of mismatch repair (MMR) gene mutations, abnormal methylation along the promoters causing gene silencing has been noted (3).

Sporadic cases show such methylation, and hereditary cases can be explained in part by Kundson's theory, which states that a second hit to a normal allele produces a change in phenotype. The first hit is inflicted by a mutation in the germ-line and the second hit by methylation in the promoters (4).

In recent years, epigenetic changes in addition to mutations have been proposed as possible causes of cancer. Cancer does not have a unique origin; on the contrary, many cellular changes occur that together result in the loss of normal behavior. It is therefore important to study the epigenetics of cancer to learn more about this complex disease.

Epigenetics involves changes at the genomic and chromatin levels that do not affect the sequence of nucleotides. At the genomic level, methylation occurs at specific sites, called CpG islands, and results in changes in gene expression. At the chromatin level, epigenetic changes occur through acetylation and deacetylation modifying gene activity (5). A few years ago, it was established that deregulations in the patterns of DNA methylation are a common feature of the neoplastic cell, producing a decrease (hypomethylation) (6) or increase (hypermethylation) (7) in the normal methylation state. These changes result in the activation of oncogenes or the inactivation of tumor-suppressor genes, leading to an imbalance in the cell metabolism. Regarding epigenetics, we also found genes that preferentially express one allele (either paternal or maternal), a feature called genomic imprinting. The differential expression of one allele is the result of methylations at the promoter regions or other regulatory areas. Changes at the chromatin level are also important; they produce a repression or relaxation of heterochromatin, thus regulating transcription. In addition to deregulation in the DNA methylation pattern, variations in the expression of imprinted genes have been found. The problem is accentuated when these genes are part of the proliferation and cell death pathways, altering those processes.

One of the characteristics of epigenetics is that it is influenced by environmental conditions. Factors that can alter the epigenome may appear early in life (in a single occurence) when they are being established, or the alterations may present in a chronic form causing later changes during adulthood. A reduction in certain components of dietary intake may also affect the epigenome. A decrease in folate concentration, the main substrate for the synthesis of amino and nucleic acids, is capable of causing a change in the pattern of DNA methylation.

Genetic mechanisms are therefore not the only pathway leading to cancer. Epigenetic changes – changes in the heritable expression pattern mediated by mechanisms that do not alter the nucleotide sequence of DNA – are being increasingly considered as an alternative to mutations and chromosomal alterations in blocking gene function.

We will now describe in detail concepts and recent advances in colorectal cancer epigenetics.

## 2. Epigenetics

Up until 1950, the term epigenetics was used to refer to all the events comprised in the development of a fertilized zygote to the constitution of a mature organism. The current definition is 'changes heritable and reversible that occur in the DNA resulting in differential expression of alleles of a gene' (8).

Changes that occur at the DNA level include methylation in specific areas of the genome and alterations in chromatin.

In bacteria, methylation is associated with the identification of particular strains, and is also used to differentiate DNA that has been replicated. In eukaryotes, several functions, including transcription regulation, X chromosome inactivation, maintenance of chromosome structure and silencing of parasitic DNA, are carried out.

Among cytosines, 2-7% are methylated in mammalian DNA (9). There are areas where the density of the dinucleotide CGs is low. These regions, representing 80% of CpG sites, are normally methylated and are located in exons, non-coding regions and in areas of repetitive DNA. Methylation of these areas serves to silence the transcription of non-coding regions, which prevents the expression of repetitive elements of DNA and parasitic DNA, which are a threat to the integrity of the genome (7-10).

As previously mentioned, DNA methylation occurs in CG dinucleotide rich areas, called CpG islands, which represent 1% of the genome. These are located around promoters and non-transcribed regions. Generally, CpG islands are not methylated in normal cells, with the exception of the X chromosome and alleles silenced by genomic imprinting. These regions are frequently associated with transcriptional active areas that are not methylated (11).

DNA methylation is catalyzed by methyltransferases which add a methyl group to a cytosine preceded by a guanine. There are two types of methyltransferases: *de novo* methylases and maintenance methylases (Dnmt1, Dnmt2, Dnmt3a and Dnmt3b). *De novo* methylases place a methyl group into a new position and must recognize some specific sequence. The maintenance methylases act constitutively on hemimethylated sites, becoming fully methylated.

Changes occurring on histones that alter genetic expression are acetylations, deacetylations, methylations and phosphorylations. The enzymes involved in expression are histone acetyl transferases (HAT) and those involved in silencing are DNA methyl transferases (Dnmt) and deacetylases (HDAC).

Acetylation occurs on the lysine residues of histones. The acetyl group has a negative charge, so when it associates with lysine its positive charge is neutralized; therefore, histones bind to DNA with less force, and are more accessible to the enzymatic machinery. Deacetylation results in the opposite effect; the union between DNA and histones becomes stronger.

Histone methylation is performed by histone methyltransferase enzymes (HMTs) that methylate in arginine and lysine residues. Unlike acetylation, methylation increases the affinity of basic residues by the DNA. Methylation at lysine 9

of histone H3 is associated with silent DNA and is globally distributed in heterochromatic regions, such as centromeres and telomeres.

Deacetylation is related to methylation, since HDAC is recruited by Dnmt1, 2 and 3. Moreover, in some cases, HDAC activity requires the recognition of previously methylated CpG sites. Since the pattern of acetylation is maintained during mitosis, it is thought that such patterns represent a heritable epigenetic imprinting that may affect genetic transcription.

This finding was confirmed by inhibiting deacetylation with the drug trichostatin (TSA), which resulted in expression of the allele that is normally an imprint of IGF2 in both mouse and human cells. In addition, there was a decrease in DNA methylation in treated cells, indicating that acetylation and DNA methylation are linked in the regulation of the process of imprinting (12).

Histone phosphorylation occurs during cell division and is present in the four histones. In certain phases of the cell cycle, specific phosphorylated histones are found to favor the condensation of chromatin. In addition, phosphorylation is involved in transcription, where histone H3 is phosphorylated, establishing a transcriptional competence in the early response of certain genes, such as FOS or JUN.

A small fraction of acetylated H3 histones are phosphorylated, suggesting that this modification contributes to the activation of genes by the stimulation of HAT activity in the same histone. Therefore, simultaneous phosphorylation and acetylation of H3 at the Fos and Jun loci leads to the activation of transcription. It is generally found to be associated with an increase in transcriptional activity.

The amounts of different residues that can be modified in the histones and the combination of these modifications have led to the concept of the histone code (13). The histone code, through the diversity of changes in the amino acids of histones, provides additional information regarding gene expression, creating binding sites for certain proteins that lead to an active or inactive chromatin conformation, depending on the case. For example, methylation at lysine 4 and lysine 14 as well as phosphorylation of serine 10 in histone H3 has been associated with gene activation, while the methylation of lysine 9 in H3 has been associated with gene silencing (14).

## 3. Genomic imprinting

Genomic imprinting is defined as the specific silencing of a parental allele. This silencing is caused by specific methylations on certain areas of the gene, resulting in a mark in the germline that is transmitted to offspring (15). Currently, there are approximately 30 genes known to have genomic imprinting (12).

Efstratiadis (16) initially confirmed that a normal mammalian gene exhibits this characteristic by demonstrating that a mouse with an inherited defective paternal IGF2 allele presented stunting; when the defective allele was maternal, the mouse showed normal growth – assuming that the maternal allele was not involved in the normal growth of the mouse, only the paternal allele.

Genomic imprinting is established during the development of germ cells. After fertilization, during embryo growth, differences in the methylation of alleles are maintained. In an early stage in the development of germ cells, an 'erasure' of methylation occurs. This process is followed by a restoration of methylation at a later stage (17,18).

The expression of genes that have genomic imprinting is constant unless a genetic or epigenetic change occurs. When this occurs, there is an imbalance in monoallelic expression, resulting in biallelic expression or silencing. This state is known as loss of imprinting (LOI).

Many genes present genomic imprinting and are involved in various pathologies, including cancer. Deregulation of imprinting has been observed in various genes and in various types of cancers. Below is a detailed discussion of the literature regarding the most important genes in which LOI has been observed in colon cancer.

## 4. IGF2, loss of imprinting and colon cancer

Insulin-like growth factors (IGFs) are involved in the regulation of cell proliferation. This group is integrated by two factors, IGF1 and IGF2, two surface receptors (IGFIR and IGF2R) and six high-affinity proteins (IGFBP1-6). The growth factor IGF2 acts in an autocrine and paracrine manner and plays an important role in tumor tissues due to its mitogenic and antiapoptotic functions mediated by IGFR (19,20).

IGF2 is located on the short arm of chromosome 11p15.5. The gene has nine exons and the mature peptide consists of exons 7, 8 and 9 (21). IGF2 is transcribed by four different promoters (P1-4), resulting in several proteins of various molecular weights. Promoters P2, 3 and 4 contain CpG islands, and their transcription is subject to imprinting. While the P1 promoter is mainly utilized in the adult liver, it does not present genomic imprinting, resulting in both alleles being active (22,23). It has been observed that the promoters P2-4 undergo methylation with aging (24).

Normal methylation was initially observed in the maternal allele; however, when samples from older individuals were analyzed, methylation was noted on the promoters of the paternal allele. This does not occur in the P1 promoter, which remains active. Since human cancer presents mainly in older populations, it may be caused by methylation spreading from one allele to another. However, this should be used as a targeted age-dependent marker, since the P1 promoter remains unchanged.

The genomic imprinting of IGF2 has been well studied, since it was the first locus found to have this characteristic. It was first identified in mice, and then characterized in humans (25,26). Since it is a locus involved in cell proliferation, several studies have been carried out to elucidate its possible role in the process of carcinogenesis. The focus of the present review is on studies conducted in CRC.

Early studies suggesting that genomic imprinting is a potential mechanism of disease were conducted in patients with the Beckwith-Wiedemann (27) and Prader Willi (28) syndromes. Later, it was confirmed that the LOI of IGF2 contributes to the onset and progression of these syndromes (29). The Beckwith-Wiedemann syndrome showed an inclination to generate embryonic tumors, such as Wilms' tumor, which is in turn characterized by loss of heterozygosity (LOH) of chromosome 11p15.5. LOH involves the loss of genetic material; sometimes these deletions involve the loss of an entire gene and its flanking regions.

When one of the alleles is already deleterious, heterozygosis disappears, resulting in LOH. A total of 69% of Wilms' tumors not showing LOH on chromosome 11p showed biallelic expression of IGF2, suggesting that LOI is a new epigenetic mechanism in the carcinogenesis process (30).

One of the first studies analyzing the status of IGF2 imprinting in CRC revealed that 39% of cases were informative. Among these, 38% were found to have LOI, analyzing a polymorphism in DNA and RNA (22). Other studies have corroborated these results, finding 33% of IGF2 LOI in CRC. These results were confirmed by immunohistochemistry when samples were compared to normal tissue (31).

The deregulation of the genomic imprinting of IGF2 is therefore involved in CRC. LOI was found with high frequency in the normal mucosa of patients with CRC, indicating that it is an alteration that occurs early in carcinogenesis (32). In the same study, IGF2 LOI in the peripheral blood of 4 patients with IGF2 LOI in tumor and normal mucosa was found.

In our studies, we also found LOI in 6% of informative peripheral blood samples. Furthermore, LOI was observed in 36 and 60% of tumor and mucosa cases, respectively. Three cases showed biallelic expression in mucosa and tumors, suggesting that the mucosa was in a period of transition towards a malignant phenotype, and another three showed LOI in tumor tissue but not in normal mucosa, suggesting that they were in a less advanced period (unpublished data).

These results indicate that, besides being a change occurring in the initiation of cancer, it can also be a change at the germline level. This suggests that the imprinting of IGF2 is a predictive marker of CRC (33,34).

It has been established that IGF2 LOI occurs in CRC early and frequently in tumors of proximal location (35). No association with environmental changes has been found, suggesting that IGF2 LOI is not an environmental alteration, but rather a hereditary risk factor (36). One recent study assessed the temporal stability of the state of genomic imprinting (37). Although the sample analyzed was small, most individuals were found to have a similar state of genomic imprinting during the reported period, and no significant association was found with age. This suggests that IGF2 LOI is not an acquired phenomenon, but rather a hereditary or congenital one.

The location of the IGF2 gene is unusual, since it is located in a region adjacent to the promoter of the H19 gene. The H19 gene has a monoallelic maternal expression and, with IGF2, was one of the first genes in which genomic imprinting was identified (38). Based on comparative studies of murine and human sequences, the gene does not have an open reading frame. This allows us to conclude that the gene product is an untranslated RNA with an as yet undefined role (39).

It has been shown that a regulatory region is involved in the expression of both genes, which defines the state of genomic imprinting. This zone is called the imprinting control region (ICR) and is divided into areas that are prone to methylation, called differentially methylated regions (DMRs) (19-21). The mouse ICR is divided in four regions, while the human ICR is divided into seven DMRs (22). When DMRs are not methylated, a factor called CTCF binds to the ICR.

The CTCF factor acts as a transcriptional insulator factor which sets boundaries between the enhancer and promoter of specific genes (40). Once it binds to DNA, the CTCF factor act as a repressor or an activator depending on the binding site. When the DMRs of the maternal allele are unmethylated, the CTCF binds and isolates the enhancers of IGF2, resulting in the expression of H19. The opposite occurs in the paternal allele, where expression of IGF2 is exhibited. In this way, the imprinting of both loci is regulated. This has been called 'competition between enhancers'.

The IGF2 gene moreover presents DMRs itself (DMR0, 1 and 2), regulating its expression. DMR0, one of the most studied DMRs, is located upstream of a complex of promoters that are under the control of imprinting. This region is homologous to a mouse region on the maternal allele that has been observed to be methylated (dmr0) (41). Initially, studies in humans found the same pattern of methylation and identified loss of methylation of the DMR0 accompanied by IGF2 LOI, suggesting that there are other areas that regulate gene expression in addition to regulation from the ICR.

Deregulation of genomic imprinting involves the biallelic expression of the three promoters with genomic imprinting. It was noted that these three were unsuppressed simultaneously in the maternal allele (42). Since the difference in methylation is upstream of the first promoter imprinted, it has been suggested that this area is a center that acts in cis, causing silencing of the allele (43).

Corroborating these results, DMR0 hypomethylation has been found in osteosarcome (44), breast (45) and ovarian (46) cancer, but without an association with IGF2 LOI, suggesting that the mechanism for deregulating expression is independent of LOI. Cui *et al* also found IGF2 LOI to be more highly associated with the hypomethylation of DMR0 of IGF2 than the methylation of H19, suggesting that IGF2 DMR is the most important for the maintenance of imprinting in CRC (47). These results imply that the regulatory mechanisms of imprinting are likely to be independent of the enhancer competition model. Possibly, genomic imprinting in this region is regulated by DMRs in IGF2 rather than by those located between both genes.

However, methylation has been found in DMR0 on the paternal allele (48,49). Methylation status in this area has been postulated as a possible predictive indicator of cancer, since a higher prevalence of DMR0 hypomethylation than of LOI was found, and since 36% of CRC cases presented hypomethylation of DMR0 with monoallelic expression (50).

Deregulation of genomic imprinting is a well-established feature not only of human cancer, but also of other pathologies. In CRC, IGF2 LOI constitutes the first step in carcinogenesis, causing uncontrolled cell proliferation. Changes in methylation may be caused by mutations in proteins that maintain the pattern of methylation, or by external factors.

Inactivation of genomic imprinting can occur under conditions of cellular stress. Cell proliferation under stress can lead to permanent genetic and epigenetic changes. It has been shown that in primary cultures exposed to stress, the expression profile of all genes was stable except for the genes that presented genomic imprinting (51).

## 5. IGF2, loss of imprinting and microsatellite instability

Microsatellites are tandem simple sequences that appear throughout the human genome. The repeats involve 2-6

dinucleotides (52). Microsatellites play roles in chromatin organization, the regulation of DNA metabolic processes and gene regulation (53).

Replication machinery is prone to malfunction in regions containing microsatellites, causing mutations when these alterations are not recognized and repaired by the machinery of mismatch repair (MMR). In such cases, additions or deletions of the microsatellite repeats occur, resulting in a change in the size of the microsatellite.

This change is called microsatellite instability (MSI). MSI is a situation in which a microsatellite allele in the germline wins or loses repeat units and changes its somatic length. MSI is one of the pathways in which the genome is destabilized, and this destabilization has been suggested to be an early prerequisite for carcinogenesis (54).

MSI is an indicator of the typical clonal expansion observed in cancer, since the alteration can be detected only when many cells are affected by the same change. MSI is produced by the loss of function of MMR genes, mainly hMLH1 or hMSH2.

The loss of function of MMR proteins results in the widespread accumulation of somatic mutations throughout the genome. These mutations sometimes occur in oncogenes and tumor-suppressor genes that play a key role in the initiation and progression of cancer (55,56). In Lynch syndrome, the MSI phenotype requires the double biallelic inactivation of the MMR genes. The first inactivation involves a mutation in the germline. The second inactivation is due to three possible mechanisms: LOH, somatic mutations or epigenetic alterations. Changes at the epigenomic level occur through the methylation of the hMLH1 promoter, and lead to its silencing (57). MSI in sporadic CRC is present in 15% of cases and occurs through the methylation of the promoter of both alleles of hMLH1 (7).

In 1997, the United States Cancer Institute established a panel of five microsatellite markers (Bat 26, Bat 25, D2S123, D5S346 and D17S250) to determine the stage of MSI in tumors (58,59). When instability is found in two or more microsatellites it is called MSI-H, when it is observed in one microsatellite it is called MSI-L, and when there is no observed instability it is termed microsatellite stable (MSS). Analysis of Bat-26 or Bat-25 is sufficient for detecting most MSI-H cases (60).

It has been established in several studies that tumors presenting MSI-H have a higher prevalence in the proximal colon and present a rapid alteration from benign polyps to malignant ones; however, these tumors are associated with increased patient survival as compared with MSS or MSI-L tumors (61,62). It has been suggested that this more favorable prognosis is due to the fact that MSI-H tumors are more sensitive to chemotherapy.

MSI is therefore directly related to epigenetic alterations, since methylation of the gene promoter of MMR genes is an established feature of CRC. It is possible that, in addition to the occurrence of aberrant methylation in the MMR genes, abnormal methylation also occurs at the sites of regulation of imprinting. In such cases, we propose that LOI occurs before MSI. Otherwise, the MSI state occurs first, with the subsequent disruption of the regulation of imprinting. Significant associations have been demonstrated between LOI and MSI. Nishihara et al (63) found that a high frequency of cases presenting MSI also presented IGF2 LOI as compared with MSS cases.

Cui et al (32) found IGF2 LOI in 91% of MSI cases and in 12% of MSS cases. Since MSI is present in 16-37% of sporadic cases while somatic mutations occur in only 16%, they proposed that other genetic or epigenetic factors affect different loci, promoting a state of instability. For example, alterations in genes that encode chromatin factors can affect DNA replication, and hence the fidelity of the imprinting causing MSI and LOI.

Nakagawa et al (64) confirmed previous results and noted that the majority of sporadic MSI(+) CRC with IGF2 LOI showed hypermethylation of the hMLH1 and p16 promoters. A minority of MSI(-) CRC cases exhibited IGF2 LOI (4/37, 11%) and methylation of p16, indicating that p16 methylation is strongly associated with LOI even in MSI(-) cases. Thus, the CpG island methylator phenotype (CIMP) affects not only p16 and hMLH1, but also the imprinting control region, resulting in LOI.

However, Ohta et al (35) found that several CRC cases with IGF2 MSI were located in the distal colon, while CRC cases with IGF2 LOI were located in the proximal colon (22.7 vs. 56.6%), corroborating previous findings. However, they did not find a significant association between MSI and IGF2 LOI, possibly because they analyzed a very small number of MSI(+) CRC cases.

Finally, Sasaki et al (65) found IGF2 LOI with a significant predominance in the right side and in poorly differentiated carcinomas. Since MSI is present in cases in the same location, it would be expected that cases with LOI also showed MSI. However, they did not find any association, and so suggested that IGF2 LOI is involved in the development of CRC but must belong to a different pathway.

Therefore, more research is needed to determine whether an association exists between LOI and MSI. Although IGF2 is not part of the group of genes which have the characteristic CIMP(+), this methylated locus is found with biallelic expression. This methylation must be produced through a different pathway, rather than the already described CIMP.

## 6. p57$^{Kip2}$ (CDKN1C) and colorectal cancer

The cell cycle is regulated by a series of proteins known as cyclins, cyclin-dependent kinases (CDK) and cyclin-dependent kinase inhibitors (CDKI). Together, these proteins coordinate the sequence of transitions of the cell cycle.

The activity of kinases depends upon their union with the appropriate cyclin; therefore, kinases act as positive regulators of cyclin activity. CDKIs act as negative regulators and play an important role in cell cycle progression.

CDKIs are grouped according to their structure and their various targets. There are two classes, the INK4 class (p16$^{INK4a}$, p15$^{INK4b}$, p18$^{INK4c}$ and p19$^{INK4d}$), which inhibits only the catalytic subunits of CDK4 and CDK6, and the second class, Cip/Kip, which is capable of inhibiting cell cycle progression by binding and inhibiting CDK complexes in the G1 phase, the cyclins D, E and A. The Cip/Kip family is composed of the following inhibitors: p21$^{Cip1}$, p27$^{Kip1}$ and p57$^{Kip2}$ (66). Due to the role of p57$^{Kip2}$, its eventual loss contributes to accelerated cell proliferation.

The p57$^{Kip2}$ protein is localized in the nucleus and has been found to have tissue-specific expression, unlike p21$^{Cip1}$ and p27$^{Kip1}$ (67). The locus is located on chromosome 11p15.5, and there have been no reports of any somatic mutations except normal genetic variations (68).

Significant differences in the expression of p57$^{Kip2}$ compared with normal tissue have been found. Since no mutations were found in this gene, it was suggested that the low expression was likely due to post-transcriptional and post-transductional modifications. It was also proposed that the gene had genomic imprinting (69).

The genomic imprinting of p57$^{Kip2}$ was first confirmed in mice, where it was found that the paternal allele was methylated and repressed. In mice, the gene is located in the distal region of chromosome 7, in a cluster of genes including IGF2, H19 and Mash2 that show genomic imprinting (70).

The same locus has been characterized in humans. Genomic imprinting was also found to be present, expressing the maternal allele preferentially, but it was noted that the imprinting was not absolute; the paternal allele is expressed at low levels in some tissues and at comparable levels with the maternal allele in fetal brain tissue and in some embryonic tissues (71).

Of the CDKIs, p57$^{Kip2}$ is the only one to show genomic imprinting (72). After confirming that the p57$^{Kip2}$ gene is a genomic imprinted locus located in an area frequently disrupted in cancer, studies involving various malignancies were conducted, some of them focused on CRC (73).

In Wilms' tumors, there was an absence of this protein in all the samples analyzed as compared with normal tissue (74). In hepatic cancer, a significant decrease in the expression of p57$^{Kip2}$ was also found. This decrease was associated with high biological aggressiveness (75).

In breast and stomach cancer, mutations were not observed, but the levels of mRNA were significantly decreased (76). These results suggest that epigenetic alterations rather than mutations are more important in the inactivation of p57$^{Kip2}$.

LOI of p57$^{Kip2}$ was observed in head and neck cancer in 13% of cases, while monoallelic expression was observed in the normal mucosa associated with each tumor (77).

In CRC, few studies have been carried out. A significant correlation was found between the low expression of p57$^{Kip2}$ and tumor size (78). In addition, it has been shown that the protein expression of p57$^{Kip2}$ is increased in samples from normal mucosa compared to adenomas, but decreases when it moves from adenoma to primary carcinoma samples. This suggests that the loss of p57$^{Kip2}$ occurs late in colorectal carcinogenesis (79). These results have also been observed in thyroid and ovarian cancer (80,81).

All mechanisms involved in the inactivation observed for the loss of protein expression involved changes in the methylation of the genome. In gastric cell lines treated with HDAC inhibitors (TSA or n-butyric), the activation of mRNA expression was noted and, upon treatment with demethylating agents (5-aza-2'-deoxycytidine), an increase in expression was observed. These results suggest that p57$^{Kip2}$ is inactivated by the formation of heterochromatin with HDAC, and that the methylation of the promoter may also be involved (82). In addition, CpG island methylation was observed in a region of initiation of transcription in CRC cell lines; when these were treated with 5 azacitidine, expression was restored. In the same study, it was determined by immunoprecipitation with anti-AC H3 and H4 that histone deacetylation was present in the methylated promoter region, corroborating the hypothesis that genomic methylation and histone deacetylation play an important role in p57$^{Kip2}$ silencing (55).

## 7. hMLH1 epimutations

Epimutations are defined as hemiallelic methylations that occur in the germline of a gene. Mutations of the hMLH1 gene are involved in hereditary non-polyposis syndrome (HNPCC). In patients with HNPCC, either both alleles are mutated or only one. In the latter case, the remaining normal allele may be subjected to a second inactivation produced by another point mutation or by an epigenetic alteration at the promoter level.

The term epimutation was first coined in a study in which the authors found a case with hMLH1 hypermethylation in peripheral blood among a pool of 14 cases suspected to have HNPCC. This case was MSI(+) and did not have any mutations in the MMR genes (83). When the authors examined the tumor tissue of this case, they found hypermethylation in one allele, while the other had a methylated promoter but showed LOH. Since they did not have samples from the patient's relatives, they were unable to determine the inheritance of the methylated allele, but demonstrated that methylation occurred in other tissues that were not neoplastic.

Vertical transmission was demonstrated when an epimutation was found in the sperm of a patient with multiple tumors, all with weaknesses in the MMR system but without mutations at that level (84).

In one study, hemiallelic methylation was found in peripheral blood, in hair follicles and in oral mucosa, and was assumed to be a somatic alteration. Although the methylated allele found was maternal, when mothers and other relatives were studied, the same variation was not found, suggesting that the epimutation appeared *de novo* (85).

Hitchins *et al* also demonstrated vertical transmission in a family with a mother who presented the epimutation in all somatic cells (86). Two of the sons had the epimutation with a maternal origin. This had reverted, resulting in the biallelic expression of hMLH1. In the third son, who had also inherited the epimutation from his mother, there was no reversal, leaving him at high risk of losing monoallelic expression. Researchers studied his sperm without finding the epimutation at that level. Apparently, the normal process of gametogenesis allows the correction of the epimutation by erasing the methylation of the imprinted genes in the primordial germ cells. This is observed in sons who do not show the hemimethylation, while having inherited the maternal allele. Therefore, it reflects a resistance to reprogramming through an incomplete erase or retention of an epigenetic memory. Epimutations appear to be reversible between generations, presenting a non-Mendelian inheritance.

## 8. Folate metabolism and colorectal cancer

Epigenetics, through the methylation of DNA, is part of the initiation and progression of CRC. The folate metabolism is

the main pathway for obtaining the substrate for DNA methylation, and is restricted mostly to components of the diet. Migration studies have shown that people who migrate from countries with low risk of CRC to countries with higher risk adopt the CRC risk rate of the country to which they emigrated within one or two generations. In these studies, the main focus was alterations in dietary intake, though environmental conditions may also confer different activities to the same genes. Therefore, we can infer a relationship between nutritional and epigenetic factors (87,88).

The monocarbon reserve of the cell is very important, since it depends on the synthesis of amino acids, purines, pyrimidines and the generation of methionine. All of these molecules are vital for most metabolic pathways, and their production depends entirely on what the cell obtains from the external environment.

In order to transform the carbon unit into molecules, it must be activated by a carrier. Therefore, the monocarbon reserve includes carbon units attached to carriers. The major carriers are carbon folate and S-adenosylmethyonine (SAM) (89).

SAM is a high energy compound formed by the condensation of methionine with ATP. SAM represents the principal methyl donor to DNA, RNA, hormones and neurotransmitters. However, the active form of folate is 5,6,7,8 tetrahydrofolate (THF).

Folate and methionine must be obtained from the diet. Therefore, a balanced diet is important to incorporate these compounds for a correct metabolic function. The richest sources of folate are leafy vegetables, fruit, yeast and liver (90).

Based on our observation of methylation variations (hypo- and hypermethylations) in cancer, it is extremely important to study the folate metabolism in CRC. It has been hypothesized that alterations in the methylation of the genome can be produced, in some cases, by a decrease in these nutrients in the diet (53,91,92).

Although the results of several studies are contradictory, it is essential to ascertain whether there is an association between folate metabolism and cancer, as environmental factors in primary tumors are central to sporadic carcinogenesis.

The first step in folate metabolism is the reduction of folate to THF through dihidrofolate reductase. Then, one unit of carbon from serine or glycine is transferred to THF to form 5,10-methylenetetrahydrofolate.

5,10-Methylenetetrahydrofolate is used for the synthesis of thymidine and purines, or is reduced to 5-methyl-THF by the enzyme methylenetetrahydrofolate reductase (MTHFR), which is used to methylate the homocysteine to form methionine via methionine synthase. It then forms SAM and is used for DNA methylation.

The folate metabolism can be disrupted by various factors, such as a reduction in substrates, mutations in the enzymes involved in the process, or a decrease in the enzyme activities due to polymorphisms.

It is known that certain gene polymorphisms produce a reduction in enzyme activity that is encoded. For example, the MTHFR has a germline variant which includes a substitution of a cytosine for a thymidine at position 677, converting an alanine to valine in the protein sequence. This change results in a thermolabile enzyme with a decrease in their activity (93).

It has been reported that individuals homozygous for the TT variant have an enzyme activity of 30%, while that of CT heterozygote individuals is 65% (94). A reduction in activity leads to a decrease in the substrate used for the methylation of the homocysteine, thus generating SAM. Theoretically, a reduction in the substrate for the methylation of DNA would be observed, hence hypomethylation of the genome would be seen; an effect observed in CRC.

There are several studies which have confirmed an association between a reduced risk of CRC and the MTHFR TT genotype, suggesting a protective effect of the T allele (86,89,95,96). In a study conducted with individuals with Lynch syndrome who had a confirmed mutation of the MMR gene and were homozygous for the wild-type allele of MTHFR, CRC developed earlier than in individuals with one or two copies of the T allele, corroborating results regarding its protective effect (97). However, contrary finding were found in studies where the TT genotype was determined to be a risk factor (98).

Methylated phenotype (CIMP), a feature of CRC, involves the hypermethylation of specific CpG islands of specific promoters, leading to the silencing of gene transcription. Abnormal methylation of the hMLH1 gene promoter is an example of CIMP, resulting in the majority of sporadic CRC cases with a MSI(+) phenotype. It has been observed in various studies that individuals with the MTHFR 677TT genotype are more susceptible to developing CRC by the microsatellite instability pathway (99,100). This association is probably due to an alteration in DNA methylation resulting in the abnormal methylation of specific promoters, such as hMLH1. Confirming these results, promoter hypermethylation of hMLH1 in patients with the 677TT genotype was observed (101,102).

Although a strong association was found between the TT genotype and MSI(+) cases, such a relationship was independent of the methylation status of hMLH1 (103). Furthermore, it was demonstrated that the relationship between MSI and 677TT is influenced by the intake of folate.

Among individuals with an adequate intake of folate, combination of the two MTHFR polymorphisms (C677T and A1298C) has been associated with a decreased risk of MSI(+) CRC. This protective effect was not observed in individuals with low folate intake (104).

The current hypothesis asserts that when there is a good intake of folate, although the activity of MTHFR is low, enough folate is converted to 5-methylenetetrahydrofolate to cover the needs of methyl groups in metabolism. However, an association was not found between the MTHFR polymorphism and MSI status. It was proposed that the reduced activity of MTHFR favors the synthesis of DNA and the MMR systems, reducing the erroneous incorporation of uracil and resulting in stable (MSS) tumors.

The methionine synthase (MS) gene also presents polymorphisms (85). This gene has a polymorphism (A919G) at position 2756, resulting in a change of asparagine to glycine at position 919 of the transcript (105).

The amino acid sequence is therefore altered in a functional site, causing the functional alteration of the enzyme (106). MS is part of the folate metabolic pathway, methylating the homocysteine to methionine using a methyl group donated by 5-methylenetetrahydrofolate.

The A919G polymorphism has been associated with an increase in homocysteine levels. It has been noted that individuals with cancer who have the GG genotype have a significant reduction in the methylation of CpG islands in tumor-suppressor genes (107).

There are no direct associations between homozygous genotypes for the polymorphism and the risk of CRC (108,109). However, when a polymorphism in MS reductase was analyzed, a correlation was observed.

The MS gene may be inactive due to the oxidation of its cofactor, vitamin B12. In order to function again, when oxidation has occurred, the gene depends on the remethylation of vitamin B12 via methionine synthase reductase (MSR) (110). It has been observed that allelic variants of the MSR gene (A66G) generate an enzyme with reduced affinity for MS, associated with a decreased risk of CRC (111).

Therefore, the activity of the MS gene may be altered not only by a polymorphism within the gene, but also by a polymorphism in an enzyme that regulates the activity of the protein. In another study, researchers found no association between the A919G polymorphism and the risk of CRC, but did detect a decreased risk of CRC in the presence of polymorphisms that resulted in a decrease in the activity of MTHFR with low expression of thymidine synthase (TS). The decrease in enzyme TS expression involved a decrease in DNA synthesis, and thus a reduction in cell replication (112).

It can therefore be concluded that, when the deregulation of the folate metabolism is one of the causes of abnormal methylation observed in neoplastic cells, this abnormal methylation is not only due to one polymorphism in the enzymes involved in the pathway, but may rather be caused by an interaction between several gene polymorphisms or the imbalance of several enzyme activities involved in the process.

## 9. Conclusions

Epigenetics is increasingly considered a cause of cancer since it involves changes that do not alter the sequence of the DNA and that are potentially reversible. It is vital to identify alterations which precede the onset of cancer, thus allowing us to eliminate the suffering related to cancer.

The first step towards achieving this goal was the discovery of the loss of IGF2 imprinting in normal tissue adjacent to tumors, suggesting that this change occurs at the onset of carcinogenesis. Next, loss of IGF2 imprinting in peripheral blood was demonstrated, indicative of change at the germline level. Loss of imprinting may therefore act as a biological marker for the early detection of cancer.

The second step should be to clarify the mechanisms by which epigenetic patterns are established, to determine which areas are actually involved in the process of silencing of an allele. In the case of IGF2, it was initially thought that the regulation only occurred in the area shared with H19. However, it has now been established that there are other areas in this gene that are differentially methylated, that regulate its expression independent of the theory of competition between enhancers.

The reversal of methylations through the use of certain drugs is a potential solution in need of further study. Researchers are likely capable of changing the disturbance that occurs and results in pathology, but can also produce the deregulation of other areas causing other problems. Thus, initially, epigenetics may provide us with answers related to the diagnosis and prognosis of cancer patients, even if it cannot yet be applied in the treatment of cancer.

Dietary factors are also related to epigenetics, and are perhaps the most important aspects of the regulation system. Changes in diet affect the pool of substrates for methylation. It has been shown that a good folate intake prevents CRC.

It is very important to continue studies along these lines, since the discovery of a predictive feature using a simple blood test would enormously facilitate patient diagnosis. Understanding the properties of tumors, such as imprinting status, microsatellite instability and the presence of epimutations, would provide information regarding the prognosis of the disease as well as tailored treatments according to the epigenetic status of the tumor. In the future, epigenetic alterations observed in pathologies will provide us with a new framework within which to work for the benefit of the patient.

## Acknowledgements

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