

Colorectal carcinoma and folate



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More than a million people a year worldwide develops colorectal cancer (CRC), with a mortality rate close to 33%. Most of the CRC cases are sporadic, only 25% of the patients have a family history of the disease, and major genes causing syndromes predisposing to CRC only account for 5-6% of the total cases. The following subtypes can be recognized: MIN (microsatellite instability), CIN (chromosomal instability), and CIMP (CpG island methylator phenotype). CRC arises from an accumulation of genetic and epigenetic alterations such as DNA methylation, which is able to modulate gene expression. Several studies in the literature show a possible correlation between an altered methylation in the promoter of tumor suppressor genes, proto-oncogenes, genes involved in DNA repair and the CRC risk; it has also been observed a global DNA hypomethylation, especially in the presence of a low folate uptake. Epigenetic changes are reversible, then could be interesting to evaluate on their relationship with dietary factors (as well as folates) and the genetic background of the individuals, for the development of novel strategies for cancer prevention.

KEY WORDS: Colorectal cancer, Epigenetic, Folate

Introduction

More than a million people a year worldwide develop colorectal carcinoma (CRC, colorectal cancer), with a mortality rate close to 33% ¹.

In Europe, it is the most frequent tumor of the gastrointestinal tract, with approximately 250,000 new cases per year and represents the 9% of all malignant tumors ².

About 75% of the patients have sporadic forms of the disease. The remaining 25% of the patients have a family history of the disease, suggesting a contribution for shared genes and environment; however, only 5-6% of CRC is due to mutations at high-penetrance of genes that lead to the development of this malignancy (*APC*, *hMLH1*, *MUTYH* etc.) ³. The major CRC genetic syndromes that increase the CRC risk are: Familial Adenomatous Polyposis (FAP), Attenuated FAP (AFAP), *MUTYH*-Associated Polyposis (MAP), and Lynch Syndrome (Hereditary Nonpolyposis Colorectal Cancer HNPCC). FAP and Attenuated FAP result from germline mutations in the tumor suppressor *Adenomatous Polyposis Coli* (*APC*) gene present on chromosome 5 (5q21) (Table I).

The C-terminal region of the protein is responsible for the co-localization of the protein APC with the microtubules of the cytoskeleton and promotes the assembly of microtubules. APC also controls the intracellular levels of β -catenin, a protein involved in gene transcrip-

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TABLE I - Causative genes for familial forms of CRC syndromes ⁴.

Gene(s)	Syndrome	Inheritance	Life time CRC risk
APC	FAP	Autosomal dominant	100%
APC	AFAP	Autosomal dominant	69%
MUTYH	MAP	Autosomal recessive	80%
MLH1, MSH2, MSH6, PMS2, TACSTD1 (EpCAM)	LS	Autosomal dominant	80%
STK11	PJS	Autosomal dominant	39%
SMAD4 (DPC4), BMPR1A	JPS	Autosomal dominant	39%
PTEN	CS	Autosomal dominant	rare

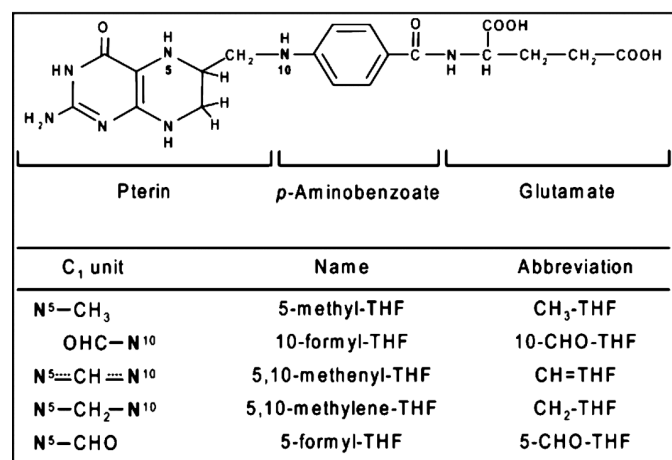


Fig. 1: Folic acid.

tion; the protein Apc regulates the signal transduction through interaction with the β -catenin. The severity of the disease and the presence of extracolonic manifestations seem to correlate with the location of the mutation inside APC ⁴.

Folate metabolism, methylation and DNA synthesis

Folic acid or vitamin b9 is composed by the union of three molecules: 6-metilpterina acid, p-aminobenzoic acid (PABA) and glutamic acid.

The first two form a molecule of acid pteroico that, as a result of binding to the glutamate, form the pteroil-glutammico acid or folic acid (Fig. 1) ⁵.

ABSORPTION AND METABOLISM

The absorption of folate occurs at the level of the small intestine 'via' the RFC1 (reduced folate carrier) that

hydrolyzes it to 5-metilTHF, the predominant form in the plasma ⁵, but this process becomes saturated at a dose of 250, μ g; so wherein the remaining portion of the ingested dose, 400 μ g, is transported directly into the cell without being hydrolyzed ⁷.

Folates are both donors that acceptors of methyl groups, used in the synthesis and methylation of DNA, RNA and proteins ⁵. This important biological function is located as a point of convergence of various metabolic processes, whose common denominator is the MTHFR (methylenetetrahydrofolate), an enzyme that converts in an irreversible way, the 5,10-metileneTHF in 5-metilTHF, involved in the synthesis of purines (adenine, guanine), of deoxythymidine (dTTP) and in the formation of methionine from homocysteine.

The 5,10-metileneTHF is the substrate for thymidylate synthase (TS), which transfers a methyl group to a molecule of desossiridinamonofofato (dUMP) to form the deoxythymidine monophosphate (dTTP) and is the only way for its 'de novo' synthesis in mammals ⁸.

The 5,10-metileneTHF is also used for the production of formilTHF, used for the 'de novo' synthesis of purines and as a donor of methyl groups in the remethylation of homocysteine to methionine by the enzyme methionine synthase (MTR). Methionine is converted to S-adenosyl-methionine (SAM), which performs two functions, either as donor of methyl groups in the reactions of DNA methylation both as MTHFR inhibitor ⁸. The changes in the distribution of methyl groups for alteration of folate metabolism have an impact on both the DNA methylation and DNA synthesis, being finally crucial in the cellular processes of neoplastic transformation ⁸.

Normal colon epithelium deficiency of folate appears to increase the risk of colon cancer, while supplementation with folic acid seems to have the opposite effect; on the other hand once foci of aberrant crypts are established, the deficiency of folate could induces regression of these precancerous lesions ⁹⁻¹¹.

An interesting study has suggested that fortification with folic acid in the mother's diet (recommended dose for women of reproductive age) protects against the development of CRC in the offspring. The protective effect could be in part mediated by an increase of the global methylation of the DNA and a decrease of DNA damage in the colon rectum ¹².

It has been noticed the repair of oxidative damage in human lymphocytes and colonocytes is delayed in a growth medium deficient in folic acid. The 8-oxo-dG transitions induces G: C to T: A, lesions repaired by hOGG1; O6-methylguanine can cause equally transitions by G: C to A: T and a decrease of the activity of MGMT (enzyme repairing such lesions) is associated with mutations in the K-ras protooncogene. It has been observed that the folate deficiency increases the activity of these two enzymes involved in DNA damage repair the liver, but not in the mice colon; therefore a deficiency of folic acid might reflect the ability of the liver, but not of the

colon, to up-regulate DNA repair enzymes, in response to a high oxidative damage; the inability of the colon tissue to respond to a folate deficiency may instead increase the risk of malignant transformation¹³. Studies performed in the USA and Chile have observed that a higher intake of folic acid can lead to an increased risk of colon cancer^{14,15}, probably by increasing the levels of methylation of tumor suppressor genes or genes involved in DNA repair. On the other hand a low intake of folate (<200 µg/day) was also associated with an increase of hypomethylation in LINE1 in human tumors of the colon¹⁶. The effect of folic acid on DNA methylation has been estimated than and it is dependent on the initial state of folate, on its amount and duration of administration, on the tissue involved, on the stage and malignant transformation and polymorphisms of folate genes².

Genomic and epigenomics instability

The two main forms of genomic instability, which have been identified in colon cancer, are the microsatellite instability (MSI or MIN, Microsatellite Instability) and the chromosomal instability (CIN, Chromosomal Instability); a subset of colorectal cancers show epigenomics instability that results in an altered methylation of tumor suppressor genes (CIMP, CpG Island methylator phenotype)^{17,18}.

The microsatellite instability (MSI) consists in lengthening or foreshortening of a sequence of repeating units of 1-6 base pairs; this represents a characteristic of tumors presenting the inactivation of one of the genes involved in MMR (mismatch repair) such as *MSH2*, *MLH1*, *MSH6*, and *PMS2*. Almost all cancers CRC with Lynch syndrome (Table I) are MIN and constitute about one-third of CRC MIN; the remaining MIN tumors are sporadic and derived from somatic inactivation of the *hMLH1* gene, caused by inactivation of its promoter¹⁹. CIN is the most common type of genomic instability observed in colon cancer and occurs in 80%–85% of colorectal tumors²⁰. It is also known as non-cancer MIN (or MSS, Microsatellite Stable). It is characterized by chromosomal rearrangements and numerical abnormalities, with aneuploid or polyploidy aspect, deletions and allelic loss of heterozygosity (LOH)²¹. The most frequent are located in the chromosomes 18q, 17p, and 8p (Table II). Changes in the number of copies have been found in different types of cancer contributing both to the development and progression of the tumor, through the inactivation of suppressor genes and activation of oncogenes²².

The chromosomal instability is an efficient mechanism for losing the “wild-type copy” of the tumor suppressor gene, as is the case *APC*, *TP53* and *SMAD4*²³. MIN tumors have a better prognosis than tumors CIN. CIMP: The CIMP phenotype is characterized by the

TABLE II - Each row lists the most frequent aberrations found in CRC⁴.

Chromosome loss	Chromosome gain
18, 17p, 1p, 4, 14, 5q, 21	7, 12, X, 5, 8
18q21	–
–	20q13
18q	20q
18p21-pter, 15q11-q21,	8q23-ter, 13p14-31, 20q13
17p12-13, 18q12-21	8q23-ter, 13p14-31, 20q13
4, 18p, 14q	17p, 17q, 1q11, 12p, 19
8p, 18q, 1p22, 4q26, 15q21	20, 8q, 8q28, 16q24.3, 20q13
18q	13q
18, 17p, Y, 1p3, 8p	13, 20, 7, X, 12, 6
8p, 18, 18q	3, 3q, 5, 5p, 5q, 7, 8q, 20, 20q, 13, X
4, 5, 8, 10, 14, 15, 17, 18,	7, 13, 20, X
21, 22, Y, 18q10 [i(8)	7, 13, 20, X
(q10)], 17q10 [i (17) (q10)]	7, 13, 20, X

presence of hypermethylation of CpG islands in the promoter of many genes. CpG islands are small genomic regions (≈ 500bp 1kb) localized at the level of the 5' region of 75% of human genes and are generally unmethylated. However, there is a small subset of highly methylated CpG islands, those ones of genes expressed only in one copy (genes “imprinted”), inactivation genes of the X chromosome in women, tissue-specific genes; additionally various CpG dinucleotides, outside the CpG islands, in particular those ones located within the repeated regions of DNA, are methylated. DNA methylation consists in the addition of a methyl group (CH₃) to a cytosine residue and plays a very important role in the regulation of gene expression.

DNA methylation in colorectal carcinoma

DNA methylation plays therefore a very important role in the regulation of gene expression; in fact the CH₃ group interferes with the binding of transcription factors, but may also acts indirectly by recruiting the methylated DNA binding proteins that in turn call enzymes able to methylate histones and to deacetylate histonic tails: this whole process leads to the configuration of “closed chromatin” and to the “gene silencing”²⁴.

The methylation of cytosine is also an important factor mutation, while in fact the deamination of cytosine produces uracil (base immediately recognized as foreign to the DNA), the deamination of 5-methylcytosine transforms it into thymine, generating a mismatch, that the MMR system is not always able to repair. A more extensive methylation of CpG islands has been associated in many studies to the inactivation of the regulatory regions of several genes, such as tumor suppressor genes.

Hypomethylation at the level of the promoter of the proto-oncogenes or hypermethylation of tumor suppres-

TABLE III - Some of epigenetic alterations found in the CRC ⁴.

Epigenetic alterations in colorectal cancer		
<i>APC</i>	<i>Adenomatosis polyposis coli</i>	Tumour suppressor gene
<i>MGMT</i>	<i>O-6-methylguanine-DNA methyltransferase</i>	Involved in repairing DNA damage; silencing by hypermethylation correlates with G to A mutations in the K-RAS oncogene
<i>hMLH1, hMLH2</i>	<i>MutL homolog 1, 2</i>	DNA repair genes; their silencing, by hypermethylation is associated with MSI CRC
<i>ADAM23</i>	<i>A disintegrin and metalloproteinase domain 23</i>	Members of this family are membrane-anchored proteins and have been implicated in a variety of biological processes involving cell-cell and cell-matrix interactions. <i>ADAM23</i> may be downregulated by aberrant promoter hypermethylation during the progression of colorectal cancer
<i>P15</i>	<i>Cyclin-dependent kinase inhibitor 2B</i>	TIS gene is a tumor suppressor, which encodes a cyclin-dependent kinase inhibitor and it is positively regulated by transforming growth factor- (TGF-) hypermethylation of the <i>P15</i> gene promoter, which should silence gene expression, correlates with CRC risk
<i>P73</i>	<i>Tumor protein p73</i>	Participates in the apoptotic response to DNA damage. May be a tumor suppressor protein, so its silencing by aberrant methylation correlates with CRC risk
<i>CDKN2A/P14</i>	<i>Cyclin-dependent kinase inhibitor 2A, alternated reading frame</i>	Tumour suppressor gene, involved in cell cycle regulation; its silencing by hypermethylation is associated with increased risk of CRC.

sor genes in these regions cause a selective development and transformation of the cells ²⁵. The tumor suppressor genes are in fact involved in a wide range of cellular processes including the regulation of cell cycle, DNA repair, apoptosis, angiogenesis, invasion, migration and cell adhesion ²⁶.

Wasson and collaborators (2006) observed a global hypomethylation in the presence cells deprived of folic acid ²⁷. Hypomethylation of DNA is a frequent phenomenon in many types of tumors and typically occurs in repeated sequences residing in satellite or pericentromeric regions; hypomethylation makes the chromosomes more susceptible to breakage and thus leads to chromosomal instability (CIN) or activation of proto-oncogenes such as *K-RAS*. A study reported an altered methylation of the gene *CDKN2A* (p16), i.e. tumor suppressor, in patients with CRC (mainly at the level of the right colon) and with adenomas of the colon-rectum; patients with absence of methylation of p16, as compared to those ones with its hypermethylation, also exhibited an higher survival ²⁸.

The loss of expression of UNC5C and DCC (deleted in CRC) has been associated with their methylation ²⁹,

³⁰; altered methylation of these genes was observed, respectively, in 68% and in 56% of cases of primitive CRC. Hibi and Nakao (2006) analyzed the methylation status of the promoter of *p16*, *p14*, *HLFT*, *SOCS1*, *CDH13*, *RUNX3* and *CHFR* in 58 CRC primitive patients and its correlation with the disease characteristics of the patients; these Authors observed a significant difference between the number of methylated genes and the stage of colon cancer ³¹.

Germline mutations of the *APC* tumor suppressor gene cause FAP and the same somatic mutations are common in sporadic CRC ³². The promoter hypermethylation 1 A of APC has been reported as an early stage of carcinogenesis in many tumors ³¹. A study enrolling 65 cases of CRC, 8 of adenoma, 5 of patients suffering from cancer but presenting normal mucosa and 1 normal case, compared the methylation profile expressed from the pathologic and not pathologic mucosa, demonstrating an altered level of methylation in the promoter of the *MGMT* gene, *hMLH1*, *p16*, *MINT1*, *MINT31*, *p15*, *RAR-b* ³³. Table III show examples of genes that undergo epigenetic alterations in CRC.

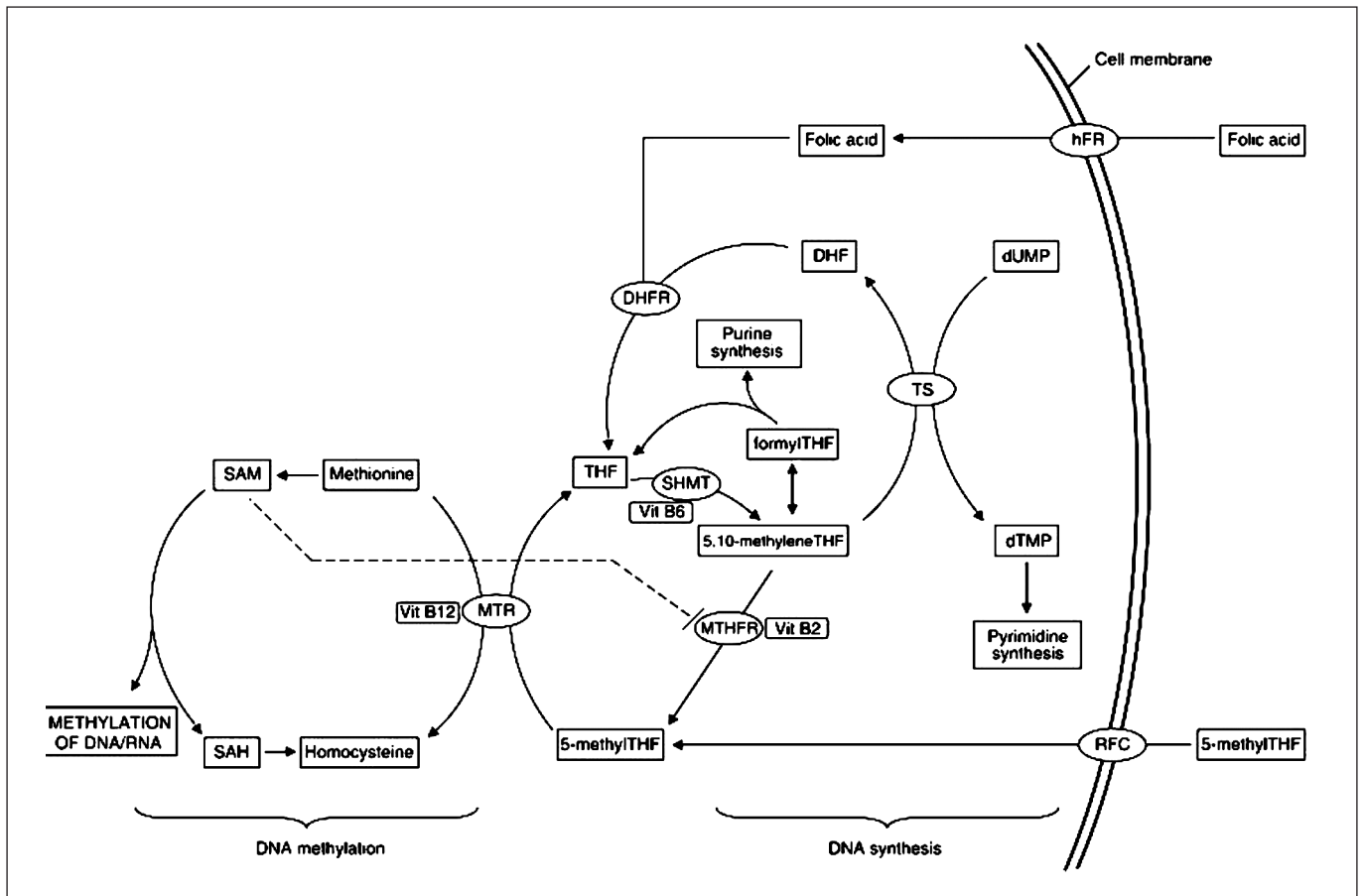


Fig. 2: Schematic representation of folate metabolism, illustrating the entry of folate in the cell and of their metabolism, the processes of methylation and DNA synthesis. RFC1, reduced folate carrier, hFR, folate receptor, MTR, methionine synthase, MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase; THF, tetrahydrofolate; DHF, dihydrofolate; SAM, S-adenosylmethionine, SAH, S-adenosylhomocysteine; dUMP, deoxyuridine monophosphate, dTMP, deoxythymidine monophosphate ⁶.

Environmental risk factors

In the recent years it has been reported that the environment plays a role in epigenetic alterations that are observed in various pathologic conditions, such as cancer of the colon and rectum; in addition to the assumption of folate, there are a number of environmental factors that may contribute to the risk of CRC.

PHYSICAL ACTIVITY AND OBESITY

Numerous epidemiological studies have provided information about the existence of a correlation between physical activity / body mass index and the risk of developing CRC. Overweight people, leading a sedentary lifestyle, are at risk regardless of the type of diet they follow ³⁴.

Tobacco and Alcohol

Tobacco contains many carcinogens that can bind DNA to form adducts, which can cause irreversible genetic

damage to the lining of the colon and rectum. Alcohol intake and smoking habits seem to be involved in the risk of developing colorectal cancer. There is a significant association between colon polyps and consumption of tobacco ³⁵.

In a study conducted by Ashktorab and co-workers (2007), 48% of cancer cases compared with 5.9% of controls were current smokers, indicating that the chance of having a colon polyp was 15.1 times higher in smokers than in nonsmokers ³⁶. The smoking predisposes to 'hypomethylation of numerous genes, with consequent activation of genes, including oncogenes, which contribute to the development of the CRC.

Based on the data available in the literature, which showed that chronic users of alcohol were susceptible to the occurrence of malignancies, Pöschl G. and Seitz H.K. (2004) developed a series of studies to contribute to the understanding how ethanol (made by itself is not carcinogenic) could contribute to the development of tumors ³⁸. It was observed that acetaldehyde, a metabolite obtained by the oxidation of ethanol by cytochrome P4502E1, is responsible for the carcinogenic effects of alcohol ³⁹.

TABLE IV - Genetic and environmental factors associated with epigenetic changes in CRC ⁴.

Genetics and environmental factors linked to epigenetic changes in CRC.

<i>TYMS</i> 28 bp 3R/3R genotype	Associated with CIMP-CRC
<i>MTHFR</i> 677TT + 1298 CC genotypes/adequate folate intake	Reduced MSI CRC risk
<i>MTHFR</i> AC or CC genotype, low folate and methionine, high alcohol	Associated with CIMP+ CRC risk
<i>TCNII</i> 776G variant	Reduced CIMP+ CRC risk
<i>MTHFR</i> 677CT genotype	Decreased <i>MGMT</i> promoter hypermethylation
<i>MTR</i> A2756G	Reduced CIMP CRC risk, among men
<i>MTRR</i> A66G	Reduced <i>MLH1</i> methylation
<i>DNMT</i> -149TT	Reduced risk proximal CRC (principally CIMP+ tumour)
High serum folate/vitB12	Associated with P16 promoter methylation and with <i>MLH1</i> , <i>MLH2</i> promoter methylation(trend)
Low folate/High alcohol	Increased <i>APC</i> 1A, <i>PI4</i> , <i>MLH1</i> , <i>MGMT</i> , and <i>RASSF1A</i> promoter methylation (trend)

The acetaldehyde interferes with the processes responsible for the synthesis and repair of DNA, thus causing the development of tumors, and this was observed in both experiments in-vitro (cell cultures in prokaryotes and eukaryotes) and in-vivo (laboratory animals). One of the enzymes of DNA repair that can be inactivated is O'6 guanine methyl-transferase (*MGMT*, responsible for the removal of adducts from DNA caused by alkylating agents). When the O'6 methyl-guanine is not "repaired" by *MGMT*, during DNA replication, it will be erroneously paired with a thymine, giving rise to a point mutation. Another important aspect due to the consumption of alcohol is the DNA methylation.

Numerous mechanisms have been hypothesized by which ethanol may interact with folate metabolism and DNA methylation, thus causing cancer. Alcohol affects the absorption, consumption and metabolism of B vitamins (folic acid and pyridoxal-phosphate) involved in the reactions of trans-methylation in the liver, thus decreasing the synthesis of the methyl groups ^{40,41}; additionally the ethanol reduces the activity of methionine synthase, which (under physiological conditions) adds the methyls to homocysteine forming methionine, using the methyl-tetrahydrofolate as a donor of methyl groups on ^{42, 43}. Hamid and colleagues (2009) observed in vivo (in male rats) a decrease in the production of mRNA and of protein expression of RFC1 (Reduced Folate Carrier) after the daily administration of alcohol to guinea pigs ⁴⁴. This study suggests that, during the intake of alcohol, there is a decrease in the function of the protein RFC1 and this can then induce the hypomethylation of DNA ⁴⁵.

Fibres

A high fiber consumption may decrease the likelihood of developing CRC. The mechanism of action by which this effect is obtained has been attributed to the action of dilution of potentially carcinogenic substances within the intestine by the fibers, thereby decreasing the time

of contact with the walls. The data in support of this thesis, however, are still controversial ^{46,47}.

Genetic susceptibility to CRC

Several polymorphisms have been studied as possible risk factors in the development of CRC; polymorphisms in the genes of glutathione S-transferase (*GSTs*), which produce enzymes involved in the metabolism of potentially carcinogenic substances, have been associated with an increased risk of developing CRC, especially in the Caucasian population ⁴⁸.

Various studies report conflicting results concerning the polymorphisms in DNA repair genes and their possible involvement in the risk of CRC ⁴⁹. In literature, there are many studies regarding genes involved in the metabolism of folic acid coding for enzymes which have a key role in DNA synthesis and in the processes of methylation; polymorphisms in these genes may be associated with altered methylation of genes involved in development of CRC.

In particular the polymorphisms *MTHFR* C677T - A1298C, *MTR* A2756G *MTRR* A66G, *TYMS* 28 bp repeats, *DNMT* 3b (DNA methyltransferase)-149C> T, *TCNII* (transcobalamin II) variant 776G, often in combination with the intake of folate, have been associated with the risk of CRC, CIMP and MSI ⁵⁰⁻⁵⁷.

Conclusions

A restricted folate diet and / or polymorphisms in genes that regulate metabolism, either through mechanisms of global hypomethylation of DNA, or through promoter hypermethylation of tumor suppressor genes, appear to be important factors in the promotion and progression of cancer colorectal cancer.

However many aspects of this topic are still under investigation, particularly concerning the dose and the time

of intake of folate, necessary for ensuring a protection. A more large casuistry is necessary, particularly including patients in the preclinical phase and in stage of overt disease, in order to investigate not only the methylation status of the patient suffering from carcinoma of the colon and rectum, but especially that one of the subjects in which the mucosa is still normal or expressing benign adenomatous. This last cohort of patients could benefit of resolute medical and surgical therapies. To date this is still a working-progress aimed to understand the controversial role of folates in the development of colorectal cancer.

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Riassunto

Più di un milione di individui all'anno in tutto il mondo sviluppa il carcinoma del colon-retto (CRC), con un tasso di mortalità prossimo al 33%. Circa il 75% dei pazienti presenta una forma sporadica del tumore; il rimanente 25% dei pazienti ha una storia familiare della malattia, suggerendo un contributo genetico ed ambientale condiviso; tuttavia solo il 5-6% di CRC è dovuto a mutazioni ad alta penetranza di geni che portano allo sviluppo di tale neoplasia (*APC*, *hMLH1*, *MUTYH* etc). Esistono tre principali sottotipi di CRC: MIN (microsatellite instability), CIN (chromosomal instability), and CIMP (CpG island methylator phenotype). Il CRC risulta dunque da un accumulo di alterazioni genetiche ed epigenetiche come la metilazione del DNA, la quale è in grado di modulare l'espressione genica. Diversi studi in letteratura riportano una possibile correlazione tra un alterato grado di metilazione nel promotore di geni soppressori tumorali, proto-oncogeni, geni coinvolti nella riparazione del DNA e il rischio di CRC; inoltre, è stata osservata un'ipometilazione globale del DNA soprattutto in presenza di una dieta povera di folati. I cambiamenti epigenetici sono potenzialmente reversibili; risulta dunque importante studiare una loro correlazione con i fattori dietetici (come ad esempio i folati) e genetici della popolazione, per lo sviluppo di nuove strategie nella prevenzione del CRC.

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