Evaluation of the expression of the *MGMT* gene in normal and neoplastic tissue of patients with colorectal cancer

Avaliação da expressão do gene MGMT *nos tecidos normal e neoplásico de doentes com câncer colorretal*

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A B S T R A C T

Objective: To evaluate the expression of tissue repair gene MGMT by comparing normal and neoplastic colonic mucosa in patients with colorectal cancer (CRC). **Methods**: We studied 44 patients with colorectal cancer confirmed by histopathology. We excluded patients suspected of belonging to families with hereditary colorectal cancer (HNPCC and FAP) and patients with cancer of the lower or medium rectum treated with neoadjuvant chemoradiotherapy. The MGMT gene expression was assessed by the technique of polymerase chain reaction in real time (RT-PCR). The comparison of results for MGMT gene expression between normal and neoplastic tissues was made by paired Student's t test, adopting a significance level of 5% ($p < 0.05$). **Results**: Tissue expression of the MGMT gene in all patients was lower in tumor tissue when compared to normal tissue ($p = 0.002$). **Conclusion**: The repair gene MGMT is less expressed in tumor tissue compared to normal tissues in patients with sporadic CRC.

Key words: Colorectal Neoplasms. Alkylating agents. DNA repair. O(6)-methylguanine-DNA methyltransferase. Polymerase chain reaction.

INTRODUCTION

The development of colorectal cancer (CRC) is a sequential process, which involves the progressive accumulation of mutations that result in the activation of oncogenes and inactivation of tumor suppressor genes. Knowledge of genetic and epigenetic events involved in colorectal carcinogenesis is important both to understand the mechanisms responsible for malignant transformation of normal cells and to the development of new therapeutic strategies[1].

Cholic epithelium is constantly exposed to oxygen free radicals, nitrogen and methyl (CH_3), produced during the metabolism of the epithelial cells or from the external environment. To protect themselves against damage caused by these radicals, the cells of the colonic mucosa have efficient repair mechanisms, mainly represented by the systems BER (Base Excision Repair), NER (Nucleotide Excision Repair) and MMR (Mismatch Repair)[2]. The MGMT gene (Omin: 156569), also known as AGT and AGAT, a component of the DNA repair system, encodes O^6-methylguanine DNA methyltransferase (MGMT), one of the most important DNA repair proteins, specific for correction of the incorporation of CH_3 radicals on guanine base that leads to formation of the methylated base O^6-methylguanine. Removal of the CH_3 radical in the molecule of O^6-methylguanine is of fundamental importance to prevent mutations caused by base transitions arising from the presence of methylated bases.

Studies have shown that the accumulation of mutations in sporadic CRC may be related to reduced tissue expression of the MGMT gene[1,3,4]. As a consequence, the smallest transcript of MGMT protein reduces the ability to correct the errors of base pairing due to hypermethylation. The lower repair capacity increases the possibility of mutations capable of forming clones of cells with proliferative autonomy and resistance to apoptosis, key features of the neoplastic cells. The use of substances with alkylating power (able to transfer CH_3 radicals to DNA bases) to induce apoptosis of cancer cells is one of the oldest strategies

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employed for the chemotherapy of CCR[5]. These drugs incorporate CH_3 radicals in the guanine base, forming large amounts of O^6-methylguanine that induce cell apoptosis by exceeding the capacity of DNA repair[6,7,8]. This possibility was more evident when it was demonstrated that an increased tissue content of MGMT protein, caused by the greater removal of oxidized guanine, was related to lower therapeutic response to chemotherapy[8]. Conversely, the combination of substances that inhibit the MGMT protein significantly increased the response to these drugs.

It is possible that the importance of tissue expression of the MGMT gene in response to chemotherapy may be related to the role of repair genes in the cell cycle. During mitosis if there is an error in base pairing, the cell can follow two distinct paths. When the error is recognized and repaired, the cell division follows its natural course ensuring that the DNA of the new cell formed has not mutated. However, when the pairing defect cannot be repaired, in order to avoid the appearance of mutations in future generations of cells, induction of apoptosis occurs by specific proteins, which form the last line of defense against the formation of mutant cells. Chemotherapeutic agents, such as nitrogen mustard, cyclophosphamide and dacarbazine act forming large amounts of methylated bases that induce cell apoptosis by overwhelming the correction capability of the repair system. These findings suggest that prior knowledge of tissue expression of the MGMT gene in patients with CRC could be considered a useful strategy to separate patients who would benefit from adjuvant chemotherapy or not, improving the cost effectiveness of treatment[9]. However, it is first necessary to assess whether tissue expression of the MGMT gene in tumor tissue is reduced in patients with CRC when compared to normal tissues. The MGMT gene expression comparing normal and neoplastic tissues has so far been little evaluated, which led to the preparation of this study.

METHODS

This study was approved by the Ethics Committee in Research of University of São Francisco (Project No: 0235.0.142.000-07). All patients who provided biological material for the research signed a consent form after being informed of all experimental stages.

We selected 44 individuals (22 women), mean age 62.4 years, with adenocarcinoma of the colon and upper rectum, submitted to colorectal surgery with curative intent by the same surgical team between January 2007 and December 2008. Exclusion criteria were: (1) suspicion of the patient belonging to families with hereditary CRC (familial adenomatous polyposis and hereditary non-polypoid colorectal cancer); (2) patients with CRC associated with inflammatory bowel disease, (3) those operated on an emergency basis; (4) and patients with cancer of medium and lower rectum, they undergo neoadjuvant chemoradiation therapy.

Immediately after removal of the surgical specimen, three fragments were removed from normal colonic mucosa at least 10cm away from the proximal edge of the tumor. Similarly, three fragments were collected from the neoplastic mucosa, always obtained from the periphery of the tumor. The identified fragments were removed, packaged individually in appropriate containers and immediately sent to the Laboratory of Molecular Biology, University of São Francisco, where they were cooled to -80° C until the time of completion of the laboratory tests. The three fragments of each tissue were used to study the expression of the MGMT gene by real time polymerase chain reaction (RT-PCR). MGMT gene amplification by RT-PCR was performed by the same technician, on a single load and in triplicate. The histological diagnosis of colorectal adenocarcinoma was confirmed by a pathologist experienced in colorectal neoplasms, blinded to the objectives of this study.

To evaluate the expression of MGMT genes and the â-actin constitutive gene RNA extraction was performed on three samples obtained from normal colon mucosa and in three from the neoplasm. For the protection and stabilization of RNA all specimens were placed in vials containing RNA-later (QIAGEN, Valencia, CA, USA) after surgical resection and refrigerated at -80° C until RNA extraction. RNA extraction was performed with the use of easy-RN® tissue kit (QIAGEN), following the manufacturer's protocol. After extraction, approximately 100 ìg of RNA were used for the synthesis of cDNA using high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA).

The analysis of the expressions of the MGMT gene and â-actin constitutive gene was performed by RT-PCR using the iCycler IQ equipment (Bio-Rad, USA). The experiments were always done in triplicate, and samples were normalized using one of the constituent controls. Table 1 shows the primers used for amplification of the MGMT and â-actin genes. The relative expression of both genes studied was calculated according to formula $2^{(Rt-Et)/2(Rn-En)}$ previously described[10]. The final value adopted for the expression of repair genes MGMT and â-actin constitutive gene was the average value found in three fragments analyzed for each patient.

The results obtained for the expression of the MGMT gene were expressed according to the average value with its standard error considering the normal and neoplastic tissues. We used the paired Student t test when comparing values by adopting a range of 95%. A significance level of 5% ($p < 0.05$) was established. All data were analyzed using SPSS 13.0 (SPSS Inc., Chicago, USA).

RESULTS

Figure 1 shows the results obtained by comparing normal and neoplastic tissue with regards to amplification

Table 1- Primers used for amplification of MGMT genes and *β*-actin.

Gene	Primer	Sequence (5'→ 3')
MGMT	*MGMT*-sense	CGAAACTTGCCCAGGAGCTTTATTT[FAM]G
	MGMT–antisense	CACCACACTGGACAGCCCTTT
***β*-actina**	*β*-actin-sense	CGGCTAATACACACTCCAAGGC[FAM]G
	β-actin-antisense	ACACTGGCTCGTGGACAAGG

of the MGMT gene. In normal tissue the value found for gene amplification was 0.1974 ± 0.02 AU, while in the tumor it was 0.01 ± 0.1100 AU.

Table 2 shows the mean values, with standard error and confidence interval, found by comparing the MGMT gene expression in normal and neoplastic tissues. It was found that in normal tissue the MGMT gene expression was significantly higher when compared to the neoplastic one (p = 0.002).

DISCUSSION

There are two distinct and well-defined pathways in colorectal carcinogenesis, chromosomal instability (CIN) and microsatellite instability (MSI) [11,12]. The first, also known as "classical" via, seems to be the most common and depends on a sequential accumulation of mutations in proto-oncogenes or tumor suppressor genes. The CIN pathway is often related to deletions of large parts of chromosomes. The MIS route, on its turn, is related to genomic instability, where mutations occur in genes of the DNA repair system, allowing rapid accumulation of mutations in genes involved in diverse cellular functions, especially those related to cell cycle control. CIN generally is associated with sporadic CRC that obeys the classical adenoma-carcinoma sequence, while MSI is associated to hereditary non polypoid colorectal cancer (HNPCC) related to deficiencies in DNA repair proteins. Interestingly, these two neoplasm-arising carcinogenic pathways have distinct pathological features when comparing tumors with chromosomal instability with those arising from MSI.

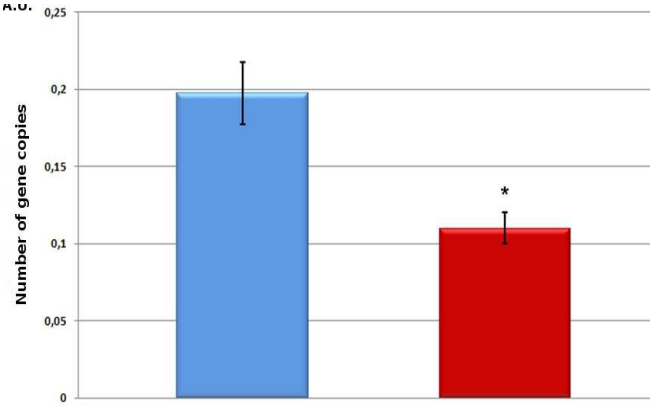

Figure 1 - *Average and standard error of the number of amplified copies of the MGMT gene when comparing normal and neoplastic tissues.*

*A.U. = Arbitrary units, * = significant paired t test.*

Although the majority of colorectal tumors present phenotypes that fall into these two pathways of carcinogenesis, studies have shown some phenotypic characteristics different from those found in the two known pathways[13]. The genetic study of these tumors showed that there was the incorporation of a large amount of CH_3 radicals in "CpG islands" of the genes' promoter region (5' region). The 5' region contains specific sequences of nitrogenous bases that determine where the process of gene transcription should begin,and is therefore known as the promoter region. Should its bases suffer hypermethylation, the transactivation factors encounter difficulties to initiate the transcription process, deeming the gene less expressed. The "CpG islands" are regions formed basically by the bases cytosine and guanine (usually above 50% of the nucleotides of those sequences). The guanine base is the more susceptible to methylation. Genes that have lower expression usually have a greater tendency to increase the incorporation of CH_3 radicals on the bases of "CpG islands".

The hypermethylation of genes, promoter region is currently one of the most studied epigenetic changes in CCR because it can prevent transcription of proteins essential for cell cycle control. When methylation levels are too high there may be even the complete inactivation of the gene,

Table 2 - Comparison of the MGMT gene expression in normal and neoplastic cells of colonic mucosa of patients with colorectal cancer.

		Mean ± SE (AU)			
	n	Normal Tissue	Neoplastic Tissue	95% CI	p
MGMT	44	0,1974 ± 0,02	0,1100 ± 0,01	0,0324 - 0,1424	0,002*

*AU = Arbitrary units; n = number of patients; SE = Standard Error.95% CI = 95% confidence interval; * = significant paired t test.*

rendering it incapable of translating the protein encoded by it[3,4]. The importance of this mechanism in the carcinogenesis of CCR has been confirmed by studies showing that hypermethylation of the promoter region of the hMLH1 repair gene, associated with HNPCC, is found in up to 80% of cases of CRC with replication errors (RER+)[7,14]. This new route of carcinogenesis associated with hypermethylation of the promoter region of genes was named CpG Island Methilated Phenothype (CIMP). Phenotypically, tumors via related to CIMP are located mainly in the proximal colon and usually present themselves associated with MSI[8,15].

Experimental studies have confirmed the relationship between methylation and CRC. The azoxymethane, a carcinogen commonly used in experimental models of CRC, has the increase in the formation of methylated guanine (O^6-methylguanine) as its main mechanism of action [1]. This substance acts by increasing the incorporation of CH_3 radicals in the oxygen at the position '6' of the molecule guanine to form O^6-methylguanine, considered the methylated basis that is most related to the appearance of DNA mutations.

The MGMT gene has been linked to colorectal carcinogenesis for almost two decades[16,17]. It is located on chromosome '10' in the region 10q26, and it transcribes MGMT protein, which acts specifically in the repair of O^6 methylguanine molecules inappropriately paired during DNA replication. The MGMT is one of the few proteins that repair DNA damaged by alkylating agents. Its mechanism of action does not allow it to be considered as a true enzyme, as it receive the CH_3 radical in a stoichiometric reaction, not being regenerated after methylation[18]. The wild protein continuously removes CH_3 radicals from the molecules of O^6-methylguanine produced by cell metabolism or by the consumption of diets rich in fats, red meat and tobacco. The protein is also capable of removing CH_3 radicals provided by alkylating chemotherapy drugs, interfering with the therapeutic response to these substances [9]. The pairing errors during DNA replication resulting in the formation of O^6-methylguanine arise because of the methylated base pairs with thymine instead of cytosine, causing transitions based on the type G:C '! AT[19]. When the MGMT gene has a reduce capacity of expression, usually by hypermethylation of the promoter region, the MGMT protein is not translated[20]. The MGMT protein acts by transferring CH_3 radicals removed from the O^6-methylguanine to a cysteine of a receptor site located in the protein molecular structure[1,21]. After the incorporation of the CH_3 radical The S-methylcysteine formed in the active site of the wild protein prevents it from being converted back to cysteine, so that each molecule of MGMT protein acts only once, probably by a conformational change of the molecule, suffering a rapid degradation by the ubiquitin system[12]. As the inactivation reaction of MGMT is irreversible, the protein must be constantly produced to remove CH_3 radicals from other molecules of O^6-methylguanine. In other words, the cells depend on the continuous synthesis of MGMT protein to remove the errors of matching. In order to assure continuous translation of the protein, the MGMT gene cannot be methylated. Experimental studies have confirmed these facts by showing that transgenic mice with increased expression of the MGMT gene and, consequently, higher transcription of the protein, are more resistant to mutations of the type G:C '! A:T [22].

Hypermethylation of the MGMT promoter gene has been known for several years as a possible cause to explain the lower expression of the gene in cancer cells[23]. In neoplastic tissue, with the loss of gene expression it is possible identify several regulatory genes that have suffered a significant rate of mutations for the transition G:C '! A:T. It has been shown that when the MGMT gene is less expressed, there is an increase of base transversions mutations, both in oncogenes such as K-ras, and in tumor suppressor genes such as p53[24-26]. The sequential accumulation of mutations in these genes is often found in patients with sporadic CRC[24,25,27]. Studies of simultaneous sequencing of both genes showed that the mutation most commonly identified in both types of transversions is G:C '! A:T[24,25].

The results of this study confirm that the tissue expression of the MGMT gene is reduced in neoplastic tissues of patients with sporadic CRC when compared to normal tissues. The findings suggest that in tumor tissue there is less capacity to repair DNA O^6-methylguanine, which increases the possibility of mutations for transitions G:C '! A:T [26]. Moreover, the higher gene expression in normal tissues confirms the importance of MGMT protein production in the prevention of mutations in colic epithelial cells, constantly exposed to CH_3 radicals. The loss of this ability in tumor tissue suggests that, at some point, reducing the cellular content of MGMT protein caused a deficiency in the repair mechanism, allowing the appearance of mutant cells.

Theories try to explain the lower expression of repair genes in tumor tissue of patients with sporadic CRC. One proposes that methylation of the promoter region of the gene repair is the main mechanism responsible for its lower expression in tumor tissue[28]. Another one suggests that the methylation process can occur simultaneously in different repair systems[3,14,15]. In a previous study, evaluating the same group of patients, we found that the hMLH1 tgene had lower expression in tumor tissue when compared to normal tissue[29]. It is possible that the hMLH1 gene, one of the main components of the MMR DNA repair system, was also less expressed due to hypermethylation of its promoter region. This possibility was not assessed at that time, though. However, previous studies evaluating this possibility confirmed that there is lower expression of repair genes in patients with sporadic CRC, which is related to an increase in the incorporation of CH_3 radicals in their promoter region[24,25,26].

There are still needed studies with more patients,

to simultaneously assess the presence of basic transitions of the type G:C '! A:T in genes related to cell cycle control, such as k-ras and p53, and the expression of MGMT, in order to better understand the role played by the development of gene mutations responsible for the breakdown of homeostasis between controlled cell proliferation and programmed apoptosis. Although in recent years there has been an exponential increase in knowledge of the epigenetic events related to the MGMT gene, research is needed to validate the routine search of MGMT gene expression before proposing a specific regimen. Only studies with sequencing related eventual MGMT gene polymorphisms, response to chemotherapeutic agents and survival in large series can answer these questions[30].

The results of this study showed that, in tumor tissue, there is reduced expression of the MGMT gene, rendering it potentially sensitive to chemotherapy protocols using alkylating agents.

R E S U M O

Objetivo: *Avaliar a expressão tecidual do gene de reparo* MGMT *comparando a mucosa cólica normal e neoplásica em doentes com câncer colorretal.* ***Métodos:*** *Foram estudados 44 portadores de adenocarcinoma colorretal confirmado por estudo histopatológico. Foram excluídos doentes suspeitos de pertencerem a famílias com câncer colorretal hereditário (HNPCC e PAF) e os portadores de câncer do reto médio e inferior submetidos a tratamento quimioradioterápico neoadjuvante. A expressão do gene* MGMT *foi avaliada pela técnica da reação de polimerase em cadeia em tempo real (RT-PCR). A comparação dos resultados encontrados para expressão do gene* MGMT *entre tecidos normais e neoplásicos foi feita pelo teste t de Student pareado, adotando-se nível de significância de 5% (p <0,05).* ***Resultados:*** *A expressão tecidual do gene* MGMT *em todos os doentes foi menor no tecido neoplásico quando comparada a do tecido normal (p=0,002).* ***Conclusão:*** *O gene de reparo* MGMT *encontra-se menos expresso no tecido neoplásico quando comparados aos tecidos normais em portadores de CCR esporádico.*

Descritores: *Neoplasias Colorretais. Alquilantes. Reparo do DNA. O(6)-metilguanina-DNA metiltransferase. Reação em cadeia da polimerase.*

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Received on
Accepted for publication
Conflict of interest: none
Source of funding: Research Foundation of the State of São Paulo. (Case No 2008-51499-7)

How to cite this article:
Cordeiro AT, Silva CMG, Bartchewsky Júnior W, Ribeiro ML, Martinez CAR. Evaluation of the expression of the mgmt gene in normal and neoplastic tissue of patients with colorectal cancer. Rev Col Bras Cir. [periódico na Internet] 2012; 39(1). Disponível em URL: http://www.scielo.br/rcbc

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