

# MTHFR Polymorphisms as Prognostic Factors in Sporadic Colorectal Cancer

Gelu Osian<sup>1</sup>, Lucia Procopciuc<sup>2</sup>, Liviu Vlad<sup>1</sup>

1) 3<sup>rd</sup> Surgical Clinic. 2) Department of Biochemistry, University of Medicine and Pharmacy, Cluj-Napoca

## Abstract

**Aim.** Theoretically, individuals having at least one mutant allele present a modified activity of the MTHFR enzyme and low methylation, DNA synthesis-repair respectively, which could imply a higher risk of colorectal cancer. The purpose of this study was to investigate the relations of these mutations with the clinico-pathological aspects of colorectal cancer. **Material and method.** The study included 69 patients with sporadic colorectal cancer. The relative risk in homozygous patients with a normal allele and for mutations *C667T* and *A1298C*, in heterozygous patients with one normal and one mutant allele, and for homozygous patients for the mutant allele was calculated. **Results.** *C667T* and *A1298C* mutations represent a risk factor for colorectal cancer with an OR (odds ratio) = 2.13 (CI (0.51-8.91)) and 3 (CI(0.3-29.58)), respectively, in homozygous patients. These mutations are associated with a more frequent location of lesions at the colon level, OR=2.3 and 2.15 respectively. The incidence of the *A1298C* mutation was more frequent in stage N0 than N+ (p<0.05), pT2 vs. pT3 (p<0.05), as well as in Dukes stages B and D vs. A or C (p<0.05). **Conclusions.** The results obtained support the hypothesis of an increased colorectal cancer prevalence in patients with one of the MTHFR gene mutations. These patients develop colon cancer more frequently, they present lymph node invasion more rarely, and develop more often distant metastases.

## Key words

MTHFR polymorphism - colorectal cancer - prognosis

## Introduction

Experimental studies have demonstrated that folate depletion leads to the alteration of the *in vivo* methylation

(1,2). This metabolic route is also essential for the DNA synthesis. At a cellular level the inhibition of the folate metabolism leads to a defective incorporation of uracil into the DNA chain (3), while in man an excessive incorporation of uracil into the DNA was evidenced (4).

At present, it is considered that alteration of DNA methylation is associated with carcinogenesis and is not just an accompanying phenomenon (5), low methylation being evidenced in almost all cancers: colon, stomach, cervix, prostate, thyroid, or breast (6). In colorectal carcinogenesis this event takes place early, long before the mutation of the genes involved in the direct carcinogenic mechanism: *APC*, *DCC*, *ras*, *p53* etc. (7). In a strange way, possibly a compensation mechanism, the methyl deficit at the cellular level leads to the hypermethylation of some genetic loci (8).

Studies of the tumoral DNA have evidenced the presence of phenotypes in which the CpG sequences are selectively and extensively methylated, a phenomenon called "CpG island methylator phenotype" or CIMP (9). Certain genes involved in oncogenesis have CpG areas in their promoting zones, and in a CIMP event they become inactive. Among the genes affected by this hypermethylation the following have been identified: *hMLH1*, *p14*, *p16 (INK4A)*, *p53*, *APC*, *E-caderine*, *THBS* etc. (10-14).

Folate deficit induces double strand breaks, a state associated with increased cancer risk (5). The molecular mechanism consists of the increased uracil incorporation into the DNA, repair being followed by the break of a DNA chain, and if there are two adjacent uracil bases, both chains may be broken (5,4).

Methyltetrahydrofolate reductase (MTHFR) is a key enzyme in the folate metabolism. It catalyzes the irreversible reaction that establishes the balance between 5,10 methyltetrahydrofolate (5,10 methyl THF) and methyltetrahydrofolate (methyl THF) at the cellular level. 5,10 methyl THF is used for the DNA synthesis, while methyl THF for the DNA methylation.

MTHFR is encoded by the *MTHFR* gene situated on the short branch of chromosome 1- 1p36.3. Multiple mutations of the *MTHFR* gene have been described, two of

them being more important, with effect on enzymatic activities. These are the C677T variants, in which the nucleotide in 677 position, codon 222 is changed, leading to the replacement of alanine by valine in that position, and variant A1298C, in which the nucleotide in position 1298, codon 429, is changed, leading to the replacement of glutamate by alanine in that position. The wild-type 677CC genotype is associated with defective DNA repair in the areas of uracil group excision (15,16). The A1298C variant has lesser effects on the MTHFR activity (17).

While recent published studies suggest that the C677T polymorphism is likely to modify the enzyme stability rather than enzyme activity (18), classically it is considered that individuals with homozygous genotype 677TT have 30% of the MTHFR enzymatic activity of the wild variant (19), while the homozygous one for the 1298CC mutation 40% (20). The 677TT heterozygotes have 65% of enzymatic activity (19). The relationship between the *MTHFR* mutation and diet is considered to be an example of interaction between environmental and genetic factors in colorectal tumor genesis (21,22).

Due to the variability of the biochemical tests assessing the intermediate level in the folate metabolism chain (23), the identification of the genotype represents the most accurate method of evaluating the relationship with colorectal cancer.

Studies performed until now have evidenced the protective effect of the 677CC variant T-allele against colorectal cancer, the lack of this effect being found in patients with insufficient folate intake. The A1298C mutation has similar but lesser effects.

We intended to study the relations between MTHFR genotype, tumor site and prognostic factors in a population with low folate intake.

#### *Working hypothesis*

Theoretically, individuals presenting at least one mutant allele have a reduced activity of the MTHFR enzyme and low methylation (TT genotype) and altered DNA synthesis-repair (CC genotype) and therefore are at a higher risk of colorectal cancer. We aimed to investigate this relationship. The population studied, ethnic Caucasians from the region of Transylvania, is homogeneous and has a diet poor in folates.

## **Material and method**

### **Patient selection**

We studied 69 patients with sporadic colorectal cancer, randomly selected, operated in the 3rd Surgical Clinic Cluj (October 2003 – May 2005).

The selection criterium was: histologically confirmed colorectal cancer. The exclusion criteria were the following: personal or family history of familial adenomatous polyposis or hereditary non-polyposis cancer, or a history of inflammatory bowel disease.

The controls were 67 patients matched for age and sex, admitted to the 3rd Surgical Clinic or 3rd Medical Clinic

Cluj-Napoca, for symptoms requiring the performance of colonoscopy (haematochezia, intestinal transit disorders), also randomly selected. All patients with colorectal diseases were excluded, except those having hemorrhoids and diverticula.

The laboratory staff were blinded for the case/control provenience of the analyzed samples.

### **Sample harvesting. DNA extraction. Identification of the MTHFR gene variations**

Peripheral blood samples were collected from all the patients in vacutainer tubes with EDTA and stored at  $-20^{\circ}\text{C}$ .

DNA was extracted from peripheral blood leukocytes by using Lahiry's method. The DNA concentration and purity were determined by measuring the optical density at 260 nm and 280 nm. All the samples with the ratio  $\text{DO}_{260}/\text{DO}_{280} < 1.8$  were considered pure.

The C677T polymorphism located in exon 4 of the MTHFR gene was examined by the genomic DNA amplification by PCR and enzymatic digestion with the restriction endonuclease of the amplified fragment (the PCR-RFLP technique). Sense primer has the sequence: 5'-ACCCACAGAAAATGATGCCAG-3'; antisense primer has the sequence: 5'-TGCCCCATTATTTAGCCAGGAG-3' (Sigma Genosys).

The A1298C polymorphism located in exon 7 of the MTHFR gene was examined by the genomic DNA amplification by PCR and enzymatic digestion with the restriction endonuclease of the amplified fragment (the PCR-RFLP technique). Sense primer has the sequence: 5'-CACTTTGTGACCATTCCGGTTT-3'; antisense primer has the sequence: 5'-CTTTGGGGAGCTGAAGGACTA-3' (Sigma Genosys).

### **Data collection**

Data collection was prospective-retrospective. We drew up a follow-up record of these cases which included data related to the underlying disease. The data were collected by specially trained resident doctors who, at the time of biological sampling, asked the written consent of the patients.

The data related to histopathological results, staging, postoperative evolution, genetic typing results etc. were registered as they became available. We investigated the link between the genotype and the following pathological parameters: tumor grading, T stage, lymph node invasion and Dukes-MAC stage, in order to establish a relationship with the prognostic factors.

### **Statistical analysis**

For the analysis of the relationship between the MTHFR genotypes and colorectal cancer risk the  $\chi^2$  test was used. We calculated the relative risk in the homozygous patients for the normal allele and for the C677T and A1298C mutations, in the heterozygous patients with one normal and one mutant allele, and in the homozygous patients for the mutant allele. The relative risk was expressed as odds ratio (OR) with 95% CI.

**Results**

The characteristics of the groups studied are presented in Table I. The most frequent finding was left colon cancer (39.13% of the cases), followed by rectal cancer (34.78%). The demographic features of the two groups were similar except the age (median 64.5 vs. 61 years).

**Table I** Characteristics of the studied groups

Characteristics	Cases (n=69)	Controls (n=67)
<b>Tumor location</b>		
Right colon, no. (%)	13 (18.84%)	-
Left colon, no. (%)	27 (39.13%)	-
Transversus colon (%)	5 (7.24%)	-
Rectum, no. (%)	24 (34.78%)	-
<b>Age (years)</b>		
50- 59, no. (%)	25 (34.28%)	31 (46.26%)
60- 69, no. (%)	21 (30%)	24 (35.82%)
> 70 years, no. (%)	25 (35.71)	12 (17.91%)
Age (years) ±SD	65.78 ± 9.55	59.86 ± 11.91
Median age	(64.5)	61
<b>Gender</b>		
Women, no. (%)	34 (49.27%)	36 (53.73%)
Men, no. (%)	35 (50.72%)	31 (46.28%)
<b>Smoking</b>		
Current smokers, no.(%)	16 (23.18%)	16 (23.88%)
Smoking in the past, no.(%)	3 (4.34%)	3 (4.47%)
Non-smokers, no.(%)	50 (72.46%)	48 (71.64%)
<b>Alcohol consumption</b>		
Yes, no. (%)	38 (55.07%)	42 (62.68%)
No, no. (%)	31 (44.92%)	25 (37.31%)

The frequencies of the mutations were compared in the two groups for the C677T and A1298C mutations, both in negative homozygotes and positive heterozygotes and homozygotes (Table II). The analysis of the study groups revealed 42 CT heterozygous patients and 9 TT homozygous from a total of 136 patients, prevalence of 30.88% and 6.62%, respectively.

The patients with positive C677T and A1298C polymorphisms had a higher frequency of left as compared with right colon cancer (18.84% vs. 4.34%, p<0.01), and 23.18% vs. 10.14%, p=0.03, respectively). These patients developed colon cancer more frequently than rectal cancer (34.78% vs. 18.84%, p=0.03, and 28.98% vs. 15.94%, p=0.06 respectively) (Table I).

Regarding the link between the genotype and the pathological parameters, we found no relation between the genotype and the tumor grading (Table III). Table IV presents the relation with pT stage. We found a significant association

**Table II** Distribution of C677T and A1298C mutations and the risk of sporadic colorectal cancer

Genotype	Patients no (%)	Control no (%)	OR, 95%IC	p
<b>CC (677)</b>	38(55.07%)	47(70.15%)	-	-
CT	25(36.23%)	17(25.37%)	1.57(0.74-3.28)	0.2
TT	6(8.7%)	3(4.47%)	2.13(0.51-8.91)	0.4
CT+TT	31(44.93%)	20(29.85%)	1.81(0.89-3.66)	0.09
<b>AA</b>	33(47.82%)	41(61.19%)	-	-
AC(1298)	32(46.37%)	25(37.31%)	1.63(0.82-3.23)	0.1
CC	4(5.81%)	1(1.50%)	3.00(0.3-29.58)	0.6
AC+CC	36(52.18%)	26(38.81%)	1.82(0.92-3.6)	0.08

**Table III** Genotype C677T, A1298C – tumor grading relations

Genotype	G1	G2	G3	G4	p (G2 vs. G3)
<b>CC(677)</b>	5(50%)	17(54.84%)	16(61.54%)	0	0.8096
CT	4(40%)	14(45.16%)	6(23.07%)	1 (50%)	0.1437
TT	1(10%)	0	4(15.39%)	1 (50%)	-
CT+TT	5(50%)	14(45.16%)	10(38.46%)	2 (100%)	0.8096
<b>AA(1298)</b>	4(40%)	15(48.38%)	12(46.15%)	2 (100%)	-
AC	6 (60%)	15(48.38%)	11(42.30%)	0	0.8477
CC	0	1(3.24%)	3(11.55%)	0	-
AC+CC	6(60%)	16(51.26%)	14(53.85%)	0	0.7594

between the A1298 mutation and the pT<sub>2</sub> vs. pT<sub>3</sub> stage. The relationship with the lymph node invasion was studied by comparing the N0 group with the N+ group (Table V).

For the analysis of the mutations in relation to the Dukes-MAC stage, the patients were assigned to four main stages of disease in order to maximize the size of subgroups in the analysis (Table VI).

**Discussion**

The statistical methods used to analyze our results were those especially used for small sized groups.

In our study, we found 42 CT heterozygous patients and 9 TT homozygous from a total of 136 patients, prevalence of 30.8% and 6.62%, respectively. This prevalence was below that reported for TT homozygotes. The presence of the mutation (CT or TT) was found in 36.77% of the patients, a value correlated with data in literature (24).

The studies reported on the MTHFR genotype have mainly assessed the C677T mutation, most of them evidencing its protective effect (25-28). However, some

**Table IV** Genotype C677T, A1298C – pTstage relationship

Genotype	T 1	T 2	T 3	T 4	p (T2 vs. T3)	p (T3 vs. T4)
<b>CC(677)</b>	3(60%)	5 (83.30%)	25(54.34%)	5(41.66%)	-	-
CT	2(40%)	1 (16.70%)	17(36.95%)	5(41.66%)	-	0.9724
TT	0	0	4(8.71%)	2(16.66%)	-	-
CT+TT	2(40%)	1 (16.70%)	21(45.66%)	7(58.72%)	-	0.6299
<b>AA(1298)</b>	1(20%)	0	24(52.18%)	8(66.66%)	-	-
AC	4(80%)	6 (100%)	19(41.30%)	3(25%)	0.0231	0.4823
CC	0	0	3(6.52%)	1(8.34%)	-	-
AC+CC	4(80%)	6 (100%)	22(47.82%)	4(33.34%)	0.0482	0.566

studies have found an increased risk of colorectal adenomas in these patients (29-31).

**Table V** Genotype C677T, A1298C – N stage relationship

Genotype	N0	N+	p
CC(677)	19(47.5%)	19(65.51%)	0.215
CT	18(45%)	7(24.14%)	0.127
TT	3(7.5%)	3(10.35%)	-
CT+TT	21(52.5%)	10(34.49%)	0.215
AA(1298)	14(35%)	18(62.06%)	0.0477
AC	22(55%)	9(31.04%)	0.0837
CC	4(10%)	2(6.90%)	-
AC+CC	26(65%)	11(37.94%)	0.0477

The A1298C mutation was also found to have a protective effect, with a decreased risk of colorectal cancer in CC homozygotes as compared to AA, with OR values between 0.6 – 0.8 (32-35). The results reported in literature for these mutations do not reveal a deviation of the distribution of genotypes from the Hardy-Weinberg balance (24).

Slattery et al (27) and Marugame et al (31) evaluated the relationship between the C677T mutation and the tumor location, the risk of tumour development at each site being less or unchanged, with the protective effect being more marked for the location in the proximal colon than the rectum.

Regarding tumor location (Table VII), most studies have evidenced an increased risk of tumor development in the terminal colon or rectum in patients with C677T mutation (28,31). LeMarchand et al evidenced an association with the location in the colon and not with the rectum, especially at more advanced stages (36). Also, Kim et al (37) found a decrease of the rectal cancer risk in these patients. Our findings were similar, a higher frequency of the left colon tumors as compared with right colon in patients with mutations, as well as a more frequent location in the colon than in the rectum.

Kawakami et al (38) evidenced an association between DNA hypermethylation and the absence of lymph node invasion and the tumoral leukocytic infiltrate, without pinpointing to relations with the genes involved in the DNA methylation, including *MTHFR*.

LeMarchand et al (36), analyzed the relationship with

the tumoral stage and evidenced a protective association of the TT genotype with regard to advanced stages only for colic tumors.

The majority of the studies in patients presenting liver metastases of colorectal cancer found a high response rate to 5-fluorouracil (5FU) of TT homozygotes, without establishing a relationship with the drug toxicity or the survival rate (39,40). Etienne et al (40) found no link between the A1298C mutation and the 5FU response, but found that the presence of this mutation represented a negative prognostic factor, being associated with a lower survival rate. Our results also evidenced a significant association of the A1298C mutation with the prognostic factors: N0 stage, pT2 vs. pT3 stage, Dukes-MAC B and D respectively.

This last association in particular stimulates comments. Practically, the AC or CC mutation or both, AC+CC, represent a protective factor, being more often associated with stage B, while the prevalence of the mutation in stage D is higher than in stage C. Is it possible that these patients develop metastases in time, more frequently in the liver, if they have an N0 status? The question opens a path in the investigation and follow-up of these patients. This finding might explain the sudden evolution towards the onset of distant metastases in patients that were initially at stage B, and could suggest the necessity for a more aggressive follow-up for metastases from the time of the diagnosis, or after surgery, as well as for chemotherapy at this stage in patients with positive A1298C mutation.

## Conclusions

Even if the study group is small and does not always allow statements with statistical value, our preliminary results support the idea of an increased colorectal cancer risk in subjects with *MTHFR* gene mutations. However, a statistical significance was present in relation to the tumor location, positive patients presenting a higher risk of developing a distal rather than a proximal colon cancer, as well as colon rather than rectal cancer. The C677T mutation seems to be more favorable.

**Table VI** Genotype C677T, A1298C – Dukes-MAC stage relationship

Genotype	A	B	C	D	p
CC(677)	3 (50%)	16 (53.33%)	15 (65.21%)	4 (40%)	-
CT	3 (50%)	12 (40%)	7 (30.43%)	3 (30%)	B vs. C=0.0666
TT	0	2 (6.66%)	1 (4.35%)	3 (30%)	-
CT+TT	3 (50%)	14 (46.66%)	8 (34.78%)	6 (60%)	B vs. C=0.5559 D vs. C=0.3351 D vs. B=0.7147
AA(1298)	1 (16.66%)	12 (40%)	17 (73.91%)	3 (30%)	C vs. B= 0.0293
AC	5 (83.33%)	15 (50%)	5 (21.74%)	7 (70%)	B vs. C= 0.0491 D vs. C= 0.0241 D vs. B= 0.4630
CC	0	3 (10%)	1 (4.35%)	0	-
AC+CC	5 (83.33%)	18 (60%)	6 (26.09%)	(70%)	B vs. C=0.01387 D vs. C=0.0472 D vs. B=0.8504

**Table VII** The risk of colorectal cancer at the patients with MTHFR 677TT genotype - relations with tumor site

Author	Site	CRC cases	Control	OR
Slattery et al (57)	Proximal	722	1816	0.75
	Distal	718		0.92
Marugame et al* (60)	Proximal	114	220	1.04
	Distal	95		1.63
Le Marchand et al (36)	Colon	56	225	0.88
	Rectum	31		0.52
Kim et al ** (37)	Colon	111	225	2.01
	Rectum	132		0.67

\*investigate adenoma incidence; \*\*CT+TT combined

The A1298C mutation is associated with positive factors, stage pT2 vs. pT3 and stage N0 vs. N+, while its relationship with the Dukes-MAC stages should be elucidated.

Further investigation might allow to individualize each case of colorectal cancer based on the genotype, and this might eventually lead to individualized therapy. A mid-way assessment of the evolution and survival of the patients studied would be necessary, with the evaluation of the patients' response to 5-FU.

### Conflicts of interest

None to declare.

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