

C677T and A1298C Mutations in the MTHFR Gene and Survival in Colorectal Cancer

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Abstract

Background and aims: Our preliminary results laboratory have shown some association between C677T and A1298C MTHFR mutations and factors influencing survival in colorectal cancer. We studied the survival of patients with colorectal cancer depending on the initial Dukes-MAC stage of the disease at the time of diagnosis and the MTHFR mutation present. **Methods:** We randomly selected 69 patients with sporadic colorectal cancer who underwent surgery at the Surgical Clinic III Cluj between October 2003 and May 2005. The study ended on 15 March 2008. Survival data was verified in 48 cases. Survival analyses were performed using Kaplan-Mayer survival curves and median survival time was calculated. The comparison of two or more categories was performed using the Logrank test, considering the threshold value $p \leq 0.05$. **Results:** In both stage B and C patients with the CT/TT mutation have a poorer survival rate than those with the wild CC genotype ($p < 0.05$). The presence of the C677T mutation (CT or TT genotype) in patients diagnosed in stage D did not result as a significant survival risk factor (HR=0.537, 95% CI 0.128-2.184) $p > 0.05$. Patients diagnosed with stage C colorectal cancer, who have the 1298C allele, have significantly better survival than those without this allele, 60% vs. 15.4%, ($p = 0.0016$). **Conclusions:** In our study in both stage B and C, patients with the CT/TT mutation have poorer survival than the wild CC genotype. In stage B patients, the A1298C mutation is a negative prognostic factor. The presence of the A1298C mutation in a hetero- or homozygous form plays a protective role in stage C.

Key words

MTHFR – colorectal cancer – survival.

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Introduction

The DNA replication error is one of the main mechanisms involved in colorectal carcinogenesis. Proteins that stop DNA synthesis and remove defective sequences play a role in stopping the error transmission. These proteins are the product of MMR (mismatch repair) genes [1]. DNA methylation plays an important role in the repair of replication errors and the regulation of gene expression. Thus, by methylation, DNA is resistant to the action of endonucleases, a control for the maintenance of healthy DNA is obtained.

DNA methylation requires the addition of a methyl group, whose donor is S-adenosyl methionine [2-4]. Its formation depends on the dietary folate intake and on the activity of the enzyme 5,10 methylenetetrahydrofolate reductase (MTHFR). This catalyzes the reduction of 5,10 methylenetetrahydrofolate to 5 methyltetrahydrofolate, which participates in the remethylation of homocysteine to methionine, from which S-adenosyl methionine will be synthesized.

The gene encoding MTHFR is situated on the short arm of chromosome 1 (1p36.3). Of the known MTHFR mutations detected in this gene, two have an effect on the enzymatic activity: the C677T variant, in which the nucleotide at position 677 is modified and leads to the replacement of alanine by valine at that enzyme position, and the A1298C variant, in which the nucleotide at position 1298 is modified and leads to the replacement of glutamate by alanine at that position. The (homozygous) 677TT genotype leads to a decrease in MTHFR activity [5], which causes a decrease in DNA repair in the excision areas of the uracyl group [6, 7]. The A1298CC variant has no major effects on the enzyme activity [8].

Individuals with a homozygous 677TT genotype have an activity of the MTHFR enzyme of 30% compared to the wild variant [9], 677CT heterozygotes have a 65% activity of the enzyme [9]. Individuals homozygous for the 1298CC mutation have a 40% activity [8].

The studies of the MTHFR genotype in patients with colorectal cancer have mainly evaluated the C677T

mutation. The majority report a protective effect of the C677T mutation, with an OR between 0.54-0.80 [10-13], but there are also studies that have evidenced an increase in the risk of colorectal cancer in these patients with an OR between 1.09-1.44 [14-16]. Studies of the A1298C mutation also note its protective effect, with a reduction in the risk of appearance of colorectal cancer for CC homozygotes compared to AA homozygotes, with OR values ranging between 0.6 and 0.8 [17-20].

The folates play the central part in the metabolism of the methyl group, being an important coenzyme for both the DNA methylation and its synthesis.

Genetic studies support the idea that low body folate levels are predisposing to various neoplasms [21], the most frequently reported being colorectal cancer [22]. Moreover, a high supply of folates seems to have a protective effect against cancer. The relationship between the MTHFR mutations and diet is considered to be an example of interaction between environmental and genetic factors in the colorectal tumor genesis [23-25].

Folate deficit induces chromosome ruptures, a condition associated with an increased cancer risk [21]. The molecular mechanism consists of the enhanced incorporation of uracyl into the DNA, the repair being followed by the break of a DNA chain, and if there are two adjacent uracyl bases, ruptures of both chains may occur [21, 26]. This mechanism was confirmed by *in vitro* studies of human colon epithelial cells [27].

One of the first studies regarding the protective effect of the C677T mutation, which evidenced the decreased risk of colorectal cancer in homozygotes (TT) depending on the exposure to environmental factors, was published by Ma et al in 1997 [10]. The protective effect of the TT mutation is achieved by the increase of the cell content of 5,10-methyl THF that will facilitate the nucleotide synthesis. The protective effect occurs with sufficient folate supply. Subsequent studies have evidenced increased colorectal cancer risk in homozygotes with low folate supply [28].

Contrary to these reports our previously published studies showed an increase in the risk of colorectal cancer in patients homozygous for the two mutations with OR=2.13 for the C677T mutation, OR=3 for the A1298C mutation, respectively [29]. At the same time, we found a statistically significant correlation of the A1298C mutation with a number of factors with a prognostic role in colorectal cancer: lymph node invasion, pT stage, Dukes-MAC stage, results which suggested a possible prognostic value of MTHFR mutations in colorectal cancer.

Working hypothesis

Starting from preliminary results that evidenced the association of mutations with prognostic factors, we may formulate the hypothesis that these mutations influence individually, as prognostic factors, survival in colorectal cancer. We decided to study the survival of patients with colorectal cancer depending on the initial Dukes-MAC stage of the disease at the time of diagnosis and the MTHFR mutation present. These results will show the concrete value,

as prognostic value, of the gene variants in the studied group.

Material and method

Patient selection

We randomly selected 69 patients with sporadic colorectal cancer operated at the Surgical Clinic III Cluj between October 2003 and May 2005. The selection criteria were the histological confirmation of cancer and surgery in the Surgical Clinic III Cluj. The exclusion criteria were a personal or family history of familial adenomatous polyposis or hereditary non-polyposis cancer and a history of inflammatory bowel disease. We closed the study on 15 March 2008. We obtained data on survival only in 48 cases, which are analyzed in what follows.

The colonoscopies were performed in the Medical Clinic III Cluj – Department of Digestive Endoscopy. The histopathological examinations were carried out in the Department of Pathological Anatomy of the “Prof. Dr. Octavian Fodor” Clinical Hospital Cluj-Napoca.

Identification of MTHFR mutations

Peripheral blood samples were taken and DNA was extracted from leukocytes using Lahiri's method (30). The C677T polymorphism located in exon 4 of the MTHFR gene was examined by DNA amplification by PCR (the sense primer had the sequence: 5'-ACCCACAGAAAATGATGCCAG-3'; the antisense primer had the sequence: 5'-TGCCCCATTATTTAGCCAGGAG-3') (Sigma Genosys), and restriction fragment length polymorphism (PCR-RFLP technique). The same technique was used for the identification of the A1298C polymorphism located in exon 7 of the MTHFR gene (the sense primer had the sequence: 5'-CACTTTGTGACCATTCCGGTTT-3'; the antisense primer had the sequence: 5'-CTTTGGGGAGCTGAAGGACTA-3') (Sigma Genosys).

C677T polymorphism was identified by the enzymatic digestion of the fragment obtained by PCR amplification (198pb) with the *HinfI* restriction endonuclease (New England Biolabs). The C677T polymorphism creates a restriction site for the *HinfI* enzyme. The enzymatic digestion was obtained in 10µl reaction mixture and included the following reagents: 1X NE buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9@ 25°C), and 5 units *HinfI*/ 10µl reaction mixture. Enzymatic digestion was performed by incubating the mixture at 37°C for 3 hours. The products of enzymatic digestion were separated by electrophoresis in 3% agarose gel stained with ethidium bromide. By enzymatic digestion, the normal allele with cytosine in position 677 (C677) forms a fragment undigested by 198pb, while the mutant allele with thymine in position 677 (T677) form two fragments of 175 and 23pb.

A1298C polymorphism was identified by enzymatic digestion of the fragment obtained by PCR amplification with *MbolI* restriction endonuclease (New England Biolabs). A1298C polymorphism annuls a restriction site for the *MbolI*

enzyme. Enzymatic digestion was obtained in 10µl reaction mixture and included the following reagents: 1X NE buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9@ 25°C), and 5 units MboII/ 10µl reaction mixture. Enzymatic digestion was performed by incubating the mixture at 37°C for 3 hours. The products were separated by electrophoresis in 3% agarose gel stained with ethidium bromide. The normal allele with adenine in position 1298 (A1298) forms by enzymatic digestion fragments of 84, 31, 30, 28pb, while the mutant allele with cytosine in position 1298 (C1298) forms fragments of 56, 31, 30 and 28pb.

We use these methods also used in our previously published study [29].

Collection of data

Data were collected prospectively and retrospectively by specially trained resident doctors, who obtained informed consent of the studied patients.

Data on survival were obtained by the periodic monitoring of patients through the outpatient service of the Surgical Clinic III Cluj, deaths being confirmed over the phone by the patients' families and verified with the official registry of the National Population Evidence Service.

Statistical analysis

We compared the survival of patients with the wild gene variant to the group of patients who presented the mutation in a hetero- or homozygous form.

Analyses were performed using the actuarial method (Kaplan-Mayer survival curves) and including the calculation of the median survival time. The comparison between two or more categories was done using the Logrank test, considering the threshold value $p \leq 0.05$. In the case of the comparison of two groups of patients, the analysis was completed with a proportional hazards model with HR (CI 95%) calculation. Standard HR interpretation, as an estimated relative risk of the event of interest occurring in one group compared to the other group was used. ($HR(CI95\%) > 1$ -risk effect, $HR(CI95\%) < 1$ -protective effect).

Results

The general characteristics of the studied patients are presented in Table I. The genotype distribution was in the Hardy-Weinberg equilibrium.

In relation to Dukes-MAC stages, mortality in the studied interval was as follows: A=0%, B=31.58%, C=68.42% and D=81.81%. Because the mortality in the Dukes-MAC stage A was 0%, in order to avoid any confusion we do not present or discuss any data about patients from this group.

We obtained data regarding survival until the study was closed in 48 cases: 19 cases diagnosed in stage Dukes-Mac B, 18 cases in stage C and 11 cases in stage D.

Our results showed that cancer mortality of the studied group, regardless of stage, was not linked to MTHFR genotypes.

We analyzed survival separately for each stage and each mutation.

Table I. General characteristics of the patients in Dukes-MAC stages B, C and D (48 cases)

Characteristics	Total (48 cases)	Dukes-MAC B (19 cases)	Dukes-MAC C (18 cases)	Dukes-MAC D (11 cases)
Gender		-	-	-
Female (%)	23 (47.91%)			
Male (%)	25 (52.09%)			
Age (years) ± SD	65.86 ± 9.62	-	-	-
Median	64.9			
C677T genotype				
CC	25	11	9	5
CT or TT	23	8	9	6
A1298C genotype				
AA	22	7	13	2
AC or CC	26	12	5	9

Stages B and C

The survival curves of patients with the MTHFR C677T mutation diagnosed in stages B and C are shown in Fig. 1.

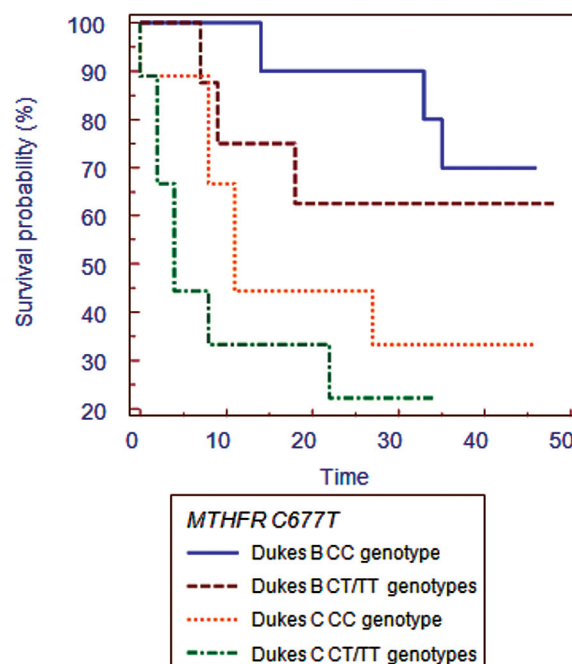


Fig 1. Comparative survival curves between Dukes-MAC B and C stages depending on the C677T genotype.

In stages B and C, patients with the CT/TT mutation had poorer survival than those with the wild CC genotype. ($p=0.0221$) (Table II).

For the A1298C genotype (Fig. 2) we found that survival in stage B was similar to the C677T mutation. The presence of the A1298C mutation in a hetero- or homozygous form played a protective role for patients in stage C ($p=0.0016$) (Table II).

Table II. Survival related to Dukes-MAC stage and MTHFR genotype

Stage	Genotype	Survival at 6 months*	Survival at 12 months*	Survival at 24 months*	Survival at 48 months*	HR	95% CI	P	Chi Square
Dukes B	677CC	1	0.900 (0.095)	0.800 (0.126)	0.700 (0.145)	2.782	0.783 -	0.513	0.427
	677CC/TT	0.875 (0.117)	0.750 (0.153)	0.625 (0.171)	0.625 (0.171)				
Dukes C	677CC	0.667 (0.157)	0.444 (0.166)	0.333 (0.157)	0.333 (0.157)	3.210	1.245 -	0.0221	9.6173
	677CC/TT	0.444 (0.166)	0.333 (0.157)	0.222 (0.139)	0.222 (0.139)				
Dukes B	1298AA	0.833 (0.152)	0.833 (0.152)	0.833 (0.152)	0.833 (0.152)	0.411	0.087 -	0.401	0.704
	1298AC/CC	0.917 (0.080)	0.833 (0.108)	0.667 (0.136)	0.583 (0.142)				
Dukes C	1298AA	0.538 (0.138)	0.231 (0.117)	0.154 (0.100)	0.154 (0.100)	0.411	0.236 -	0.0016	15.2319
	1298AC/CC	1 (0.179)	0.800 (0.179)	0.600 (0.219)	0.600 (0.219)				
Dukes D	677CC	0.800 (0.179)	0.600 (0.219)	0.400 (0.219)	0.400 (0.219)	0.5371	0.1208 -	0.3671	0.8135
	677CT/TT	0.833 (0.152)	0.667 (0.192)	0.333 (0.192)	0.167 (0.152)				
Dukes D	1298AA	0.500 (0.354)	0.500 (0.354)	0.500 (0.354)	0.500 (0.354)	0.6461	0.1035 -	0.6699	0.1817
	1298AC/CC	0.778 (0.139)	0.556 (0.166)	0.222 (0.139)	0.222 (0.139)				

* Values were expressed as survival proportion (standard error)

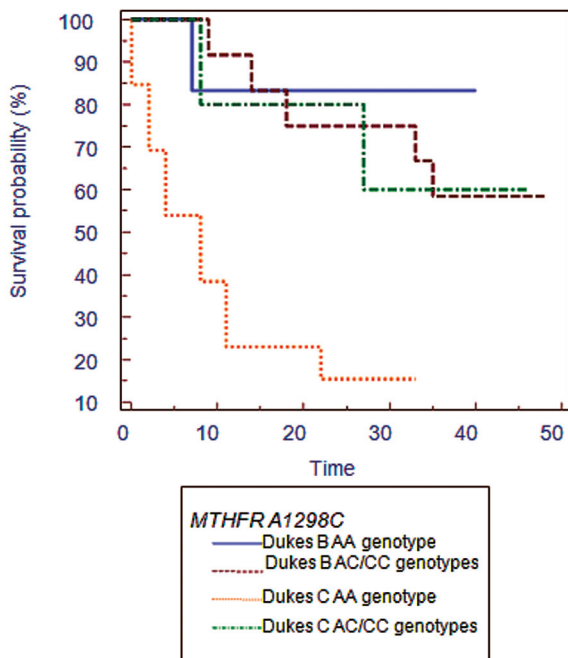


Fig 2. Comparative survival curves between Dukes-MAC B and C stages depending on the A1298C genotype.

Stage D

Figure 3 shows the survival curves for the C677T mutation.

The presence of the C677T mutation had a negative

impact for patients diagnosed in stage D, being associated with poorer survival (Table II).

The analysis of the A1298C mutation evidences a peculiarity: patients with the mutation present (AC/CC)

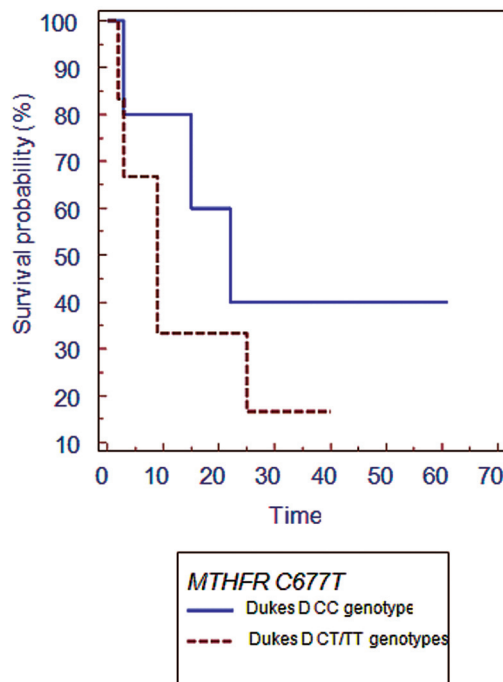


Fig 3. Comparative survival curves between Dukes-MAC D stage depending on the C677T genotype.

initially have better survival, as a percentage of the group, until 3 months postoperatively, but this subsequently decreases and patients with a wild genotype finally have a better survival (Fig. 4, Table II).

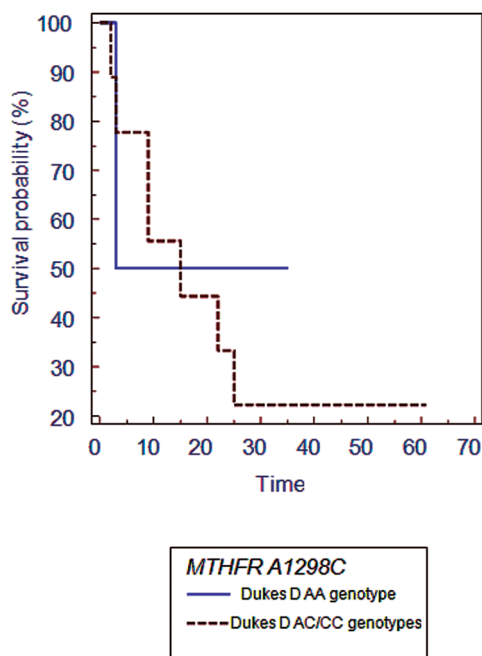


Fig 4. Comparative survival curves between Dukes-MAC D stage depending on the A1298C genotype.

Discussion

The analysis of the correlation of the MTHFR genotype with mortality and survival represents the main element of evaluation of their prognostic significance in colorectal cancer.

Regarding overall mortality, we found no statistically significant correlations. Patients with the A1298C mutation have better survival than patients with a wild genotype, the risk of death being $OR=0.721$, but statistical significance cannot be confirmed.

In both stage B and C, patients with the CT/TT mutation present have poorer survival than those with the wild CC genotype ($p<0.05$). The presence of the C677T mutation has a negative impact in patients diagnosed in stage D, its presence being associated with poorer survival-17.7% vs. 40% at 25 months but this was not statistically significant ($HR=0.5371$, 95%IC 0.1208- 2.1846, $p>0.05$).

In stage B, the survival of patients with the A1298C mutation (AC/CC) is similar to the C677T mutation, so patients diagnosed in this stage have poorer survival; consequently the mutation is a negative prognostic factor for this group. Patients diagnosed with stage C colorectal cancer, who present the 1298C allele, have better survival than those without this allele, 60% vs. 15.4% at 27 months, statistically significant $p=0.0016$. So, the A1298C mutation may be a positive prognostic factor in stage C. In stage D, during

the first 3 months, the A1298C mutation brings a survival benefit (77% compared to 50%); subsequently the patients of this group have a much lower survival rate compared to those with the wild genotype. Overall, the influence of the A1298C mutation is unfavorable in stage D.

A number of recent studies have evaluated the correlation of the MTHFR polymorphism with survival in colorectal cancer. Derwinger et al [31], by studying the C677T mutation in 544 cases of colorectal cancer, reported that its presence may affect sensitivity to 5-FU chemotherapy and the risk of side effects, so that survival in stage III, possibly in stage IV is affected. In as much as we can associate stage Dukes-MAC C with stage TNM III and stage D with stage IV, our results appear similar. However, it should be mentioned we evidenced no relationship between the sensitivity to 5-FU chemotherapy and MTHFR polymorphism. Capitain et al [32], by evaluating 76 cases of colorectal cancer, shows an increase in side effects and a shorter survival time in patients with the A1298C mutation. Yoshiya et al [33] found no correlations between the C677T mutation and survival in 114 cases of colorectal cancer.

We are, however, aware of our study limits, especially regarding the number of cases. Chosen sample size can induce testing power decrease for part of the analyses. For this reason analyses were not further adjusted to prevent even more restrictions of sample size per subgroups. Further research is needed, with extended sample size, to validate/invalidate present results.

Conclusions

We report that MTHFR C677T and A1298C mutations are significantly associated with survival in our small study.

For patients diagnosed with Dukes stage B and C, the CT/TT mutations are associated with a significantly poorer survival than those with the wild CC genotype. The presence of this mutation may be a negative prognostic factor.

In stage B patients, the A1298C mutation may be a negative prognostic factor. The presence of the A1298C mutation in a hetero- or homozygous form plays a protective role in stage C patients.

Overall, the influence of the A1298C mutation is unfavorable in stage D.

Conflicts of interest

Nothing to declare.

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