

Advances in Brief

Methylenetetrahydrofolate Reductase Polymorphism in Advanced Colorectal Cancer: A Novel Genomic Predictor of Clinical Response to Fluoropyrimidine-based Chemotherapy¹

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Abstract

Purpose: Fluorouracil (5-FU) is widely used in the treatment of colorectal cancer. Methylenetetrahydrofolate reductase (MTHFR) could play an important role in the action of 5-FU, an inhibitor of thymidylate synthetase, by converting 5,10-methylenetetrahydrofolate, a substrate of thymidylate synthetase, to 5-methyltetrahydrofolate. A polymorphism in MTHFR (677 C→T; A222V) reduces enzyme activity and presumably increases the level of 5,10-methylenetetrahydrofolate. This increase would be expected to correlate with an improved response to 5-FU. The aim of the present study was to investigate the association between the MTHFR polymorphism and response to 5-FU and other fluoropyrimidines in patients with metastatic colorectal cancer.

Experimental design: Forty-three patients with metastatic colorectal adenocarcinoma were analyzed. All patients were treated with p.o. or i.v. fluoropyrimidine-based chemotherapy. A comprehensive chart examination was performed to determine tumor response rates. Genomic DNA was extracted from blood, and MTHFR genotypes were determined.

Results: At least one copy of the mutant valine allele was present in 26 patients (21 heterozygotes and 5 homozygotes). The remaining 17 patients carried only the alanine allele. Exploration of the relationship between MTHFR alleles and response rates revealed a statistically significant difference in the frequency of the valine allele among responders versus nonresponders ($P = 0.0351$). This observa-

tion was associated with an odds ratio of 2.86 (95% confidence interval 1.06–7.73) for a response in individuals with a valine allele.

Conclusions: Our results show a link between the MTHFR polymorphism and tumor response to fluoropyrimidine-based chemotherapy and suggest that MTHFR genotyping may be of predictive benefit in selecting treatment regimens.

Introduction

With an estimated 130,200 new cases and 56,300 deaths in 2000, CRC³ is the fourth most common cancer among men and women in the United States and second leading cause of cancer-related death (1). Up to 30% of patients present with metastatic disease and ~50–60% ultimately develop metastatic or advanced disease (2, 3). The prognosis for these patients is grim, with 5-year survival rates of <5% (2–4). There have been novel cytotoxic agents introduced into clinical use that demonstrate statistically significant although clinically small benefits (5, 6). Even these new agents give best results when combined with 5-fluorouracil. Thus, the fluoropyrimidines, in use for >30 years, remain the mainstay of treatment. Modulation of activity of this class of compounds with various agents, including folinic acid, or the use of infusional schedules has resulted in improvement in objective response rates. Survival rates in the metastatic setting, however, have remained modest, and significant benefits in terms of palliation and improved quality of life are not uncommonly obtained with significant disadvantages and drug side effects.

MTHFR is an enzyme that plays a role in the metabolism of folate. The substrate for MTHFR, 5,10-MTHF, is used for conversion of dUMP to dTMP by TS, whereas the enzyme product 5-methyltetrahydrofolate is the methyl donor for synthesis of methionine and S-adenosylmethionine in methylation reactions. A common polymorphism in the *MTHFR* gene has been identified (677 C→T; A222V). This variant, which is present in the homozygous state in 10–15% of many North American and European populations, correlates with reduced enzyme activity and increased thermolability (7). This decrease in MTHFR activity shifts folate derivatives into the nonmethyl forms. Previous studies have shown a possible link between MTHFR genotype and the folate pool in gastrointestinal cancer. It has also been shown that this folate pool in cancer tissue is a critical factor for the effect of fluoropyrimidine-based chemotherapy (8). One postulated consequence of this effect is that

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³ The abbreviations used are: CRC, colorectal cancer; MTHF, methylenetetrahydrofolate; TS, thymidylate synthetase; MTHFR, methylenetetrahydrofolate reductase; LV, leucovorin.

individuals with CRC who have the above sequence variant may respond differently to fluoropyrimidine treatment. In the present study, our aim was to investigate the relationship between the MTHFR polymorphism and response to fluoropyrimidine-based chemotherapy to determine whether the MTHFR genotype is a predictor for the clinical response to therapy. Such an observation could have a significant impact on the choice of drugs and the design of new treatments in advanced CRC, changing the therapeutic approach from a general to an individual treatment strategy.

Patients and Methods

Patients. The study population consisted of patients with histologically documented metastatic colorectal adenocarcinoma. Only those who had at least one measurable indicator lesion at the time of initial diagnosis or recurrence were included. Ascites and pleural effusions were not considered measurable. All patients were treated with p.o. or i.v. fluoropyrimidine-based chemotherapy. p.o. agents included capecitabine administered at a dose of 1250 mg/m² twice daily as an intermittent regimen in 3-week cycles and tegafur plus uracil (Orzel) administered at 300 mg/m²/day plus LV at 90 mg/day with both drugs given in three daily doses at 8-h intervals for 28 days in a 35-day schedule. i.v. 5-FU was given together with LV according to the Mayo Clinic regimen, consisting of rapid injection of 20 mg/m² LV followed by a bolus injection of 425 mg/m² 5-FU daily, days 1–5 every 4 weeks.

Study Design. A comprehensive chart examination was performed to determine response rates and secondary outcomes. Data were obtained on several pretreatment characteristics, including age, sex and other demographic information, performance status, primary tumor location, involved metastatic sites and objective tumor response, and clinical adverse reactions after three cycles of treatment. Data regarding time to tumor progression and survival were also retrieved when possible. Tumor response classification was based on standard WHO criteria, and adverse events were graded according to the National Cancer Institute CTC grading system (9).

Blood samples were obtained for DNA isolation and determination of genotypes. All procedures were reviewed and approved by accredited ethics review boards, and patients signed informed consent forms. The presence of MTHFR mutations was determined by isolation of genomic DNA from peripheral lymphocytes and amplification of the target sequence by PCR, using our original procedure (7). PCR products were digested with *Hinfl*, because the mutation creates a *Hinfl* recognition sequence. The PCR product of 198 bp is digested into fragments of 175 and 23 bp in the presence of the mutant (valine) allele. The wild-type allele (alanine) remains undigested. PCR products were electrophoresed on 9% polyacrylamide gels and photographed. Genotyping was performed by a technician blinded to the clinical information.

Statistical Analysis. Computer-assisted analyses were carried out using SAS for Windows (Version 8.02). The Fisher's exact test was used to assess the association between MTHFR 677 genotype and clinical response to fluoropyrimidine-based chemotherapy. χ^2 test and calculation of odds ratios with associated confidence limits were used to assess the relationship

Table 1 Patient demographics, disease characteristics, and previous therapy at baseline^a

Gender	
Male	27
Female	16
ECOG performance status	
0	13
1	26
2	3
3	1
Age, years	
Median	59
Range	43–70
Race	
Caucasian	38
Other	5
Primary tumor	
Colon	33
Rectal	10
Prior adjuvant therapy	
Yes	9
No	34
Site of metastasis	
Liver	32
Lung	11
Other	10
Chemotherapy	
5-FU/LV	38
Capecitabine	3
UFT	2

^a ECOG, Eastern Cooperative Oncology Group; UFT, tegafur plus uracil.

between the *MTHFR* 677 alleles and clinical response. Finally, the associations between MTHFR 677 genotype and major toxic events were tested separately using the χ^2 test or Fisher's exact test, where appropriate. Statistical significance was interpreted as $P < 0.05$.

Results

A total of 43 patients was analyzed. Their demographic and disease characteristics together with previous therapy particulars at baseline are shown in Table 1. Seventeen patients (39%) had the homozygous normal or AA genotype. Among the patients with variant forms of the *MTHFR* gene, 21 (49%) were heterozygous (AV variant), and 5 individuals or 12% were homozygous for the mutation (VV variant). Despite the small sample size, the frequency distribution numbers for the normal genotype and variants were in keeping with numbers obtained from previous clinical observations and epidemiological studies (7, 10).

The overall major response rate (complete and partial responses) was 63%. Stable disease was not considered a response. Among homozygous normal patients, 8 of 17 patients (~47%) achieved a major response. In the heterozygous mutant group, 14 of 21 patients (67%) responded. Finally, in the group with patients homozygous for the mutation, all had a major clinical response to therapy (Table 2). The difference in the proportion of objective responses between the three genotype groups did not reach statistical significance, most likely because of the few patients (Table 2; $P = 0.1033$ by Fisher's exact test). However, exploration of the relationship between the *MTHFR*

Table 2 Clinical response rates by MTHFR genotype and MTHFR allele frequencies

Genotype	Total (n)	Response		No response	
		No.	%	No.	%
AA	17	8	47	9	53
AV	21	14	67	7	33
VV	5	5	100	0	0
Allele					
A	55	30	55	25	45
V	31	24	77	7	23

677 allele and response rates revealed a statistically significant difference in the frequency of the allele among responders *versus* nonresponders (Table 2; $X^2 = 4.4398$; degrees of freedom = 1; $P = 0.0351$). This finding was associated with an odds ratio of 2.86 (95% confidence interval 1.06–7.73) for a response in individuals with a valine allele.

Table 3 lists the number of treatment-related adverse reactions at any grade. Overall, patients with the normal genotype were just as likely to experience toxicity as compared with patients heterozygous and homozygous for the mutation. The incidence of myelosuppression or neutropenia was similar in the two groups (Table 3; 76 *versus* 69%, $P = 0.7346$). There were no differences in frequencies of mucositis or stomatitis and hepatic toxic events, including the adverse effects of total bilirubin elevation or abnormalities in alkaline phosphatase and transaminase (Table 3; 59 *versus* 77%, $P = 0.2064$, and 35 *versus* 15%, $P = 0.1578$, respectively).

Discussion

All of the fluoropyrimidines exert their antineoplastic activity in a similar manner. One mechanism is through the direct incorporation of these molecules or their by-products into RNA and DNA (11, 12). A second mechanism, which is likely the dominant one, involves TS inhibition through active metabolites, such as fluorodeoxyuridine monophosphate or 5-fluoro-dUMP. This reaction is facilitated by the formation of a ternary complex consisting of 5-fluoro-dUMP, TS, and the folate derivative 5,10-MTHF.

5,10-MTHF can also be converted to 10-formyltetrahydrofolate for purine synthesis or to 5-methyltetrahydrofolate, the primary circulatory form of folate, by MTHFR. This 5-methyltetrahydrofolate derivative is then used for remethylation of homocysteine to methionine by methionine synthase. Methionine synthase requires activation by methionine synthase reductase. These enzymes divert folates away from thymidine and DNA synthesis into the synthesis of methionine and S-adenosylmethionine. Specific sequence variants of these enzymes have been identified that are common in the general population (7, 10, 13). Several studies have reported that the 677 variant in MTHFR is associated with decreased risk of CRC and some types of leukemia (14). One study with a variant in methionine synthase (2756 A→G; D919G) also reported a trend toward decreased risk of CRC (15).

A postulated mechanism for the protective effect of the MTHFR variant is an increase in the MTHFR substrate, 5,10-

Table 3 Adverse reactions related to treatment

Adverse reaction	Patients			
	AA (n = 17)		AV-VV (n = 26)	
	No.	%	No.	%
Myelosuppression (neutropenia)	13	76	18	69
Diarrhea	9	53	18	69
Nausea/vomiting	10	59	17	65
Mucositis/stomatitis	10	59	20	77
Hepatic Toxicity	6	35	4	15
Hand-foot syndrome	2	12	0	0

MTHF, which might enhance the action of TS and provide adequate amounts of thymidine for appropriate DNA synthesis and repair. In support of this hypothesis is the demonstration that individuals with the MTHFR mutant genotype have increased amounts of formyltetrahydrofolate and decreased amounts of methyltetrahydrofolate in RBCs, suggesting an increased pool of 5,10-MTHF (16).

The working hypothesis for this study was that individuals with the MTHFR sequence variant might respond differently to 5-FU or other fluoropyrimidine agents. If these variants decrease enzyme activity and reduce the flux of folate derivatives into methionine synthesis, they might increase the amount of folates (specifically 5,10-MTHF) available for the TS reaction. Consequently, the effect of 5-FU or other fluoropyrimidines, inhibitors of TS, might be enhanced in these individuals. The combination of folate (LV) with 5-FU results in higher responses in cell lines (17) and clinical trials of cancer patients (18), compared with 5-FU alone. This is likely attributable to the formation of a ternary complex between 5,10-MTHF (the substrate for TS and MTHFR), the fluoropyrimidine, and TS (19). Observations in animal experiments have suggested that the efficacy of 5-FU is enhanced when 5,10-MTHF is administered, in a rodent colon carcinoma model (20). In resected gastrointestinal cancer tissues, the level of 5,10-MTHF was higher in individuals who were mutant for MTHFR (VV genotype), although this observation did not reach statistical significance (8).

Our data in the present study demonstrated that the MTHFR 677 C→T allele correlated with the clinical response to fluoropyrimidine-based chemotherapy. Response rates were significantly higher for those with at least one mutant allele compared with patients carrying only the wild-type allele. This association was most striking among the homozygotes. The response rate for those individuals was 100%. Although the presence of the mutant allele predicted response, its presence did not necessarily result in a clinical response; a subset of individuals with the allele did not respond to treatment. These patients may have had a variety of mechanisms of resistance to 5-FU, which the MTHFR polymorphism could not overcome. A number of these mechanisms has already been described, including those related to decreased accumulation of activated metabolites, target-associated resistance, and pharmacokinetic resistance. It is also possible that the VV genotype has an intrinsic negative effect on tumor growth, through a mechanism that is independent of the change in 5,10-MTHF levels; this effect may be synergistic with 5-FU therapy. In other studies, we demon-

strated that inhibition of MTHFR by antisense administration *in vitro* reduced the growth of several transformed lines; these findings are consistent with the concept that reduction of MTHFR activity, through antisense or mutation, may inhibit tumor growth (21).

Analyses of adverse events showed no impact of the polymorphism on major toxic occurrences, including myelosuppression, mucositis, and hepatic toxicity. However, the few patients in this study precluded a thorough evaluation of toxic effects. It is therefore possible that a larger study might have identified an increase in toxicity.

Although the MTHFR mutant allele appears promising in predicting response to fluoropyrimidine compounds, several other molecular markers have been investigated. These include intratumoral gene or protein expression of TS, the pyrimidine catabolism enzyme dihydropyrimidine dehydrogenase, thymidylate phosphorylase, p53, BCL-2, and microsatellite instability (22–31). The most predictive data have been reported for TS expression. High TS expression in CRCs would be expected to predict a poor response to the fluoropyrimidines. This hypothesis was confirmed in a study by Leichman *et al.* using reverse transcription-PCR quantification methods to measure the ratio of mRNA expression of TS in relation to that of the housekeeping gene β -actin (22). In that study, no patient with a TS: β -actin mRNA ratio $>4.1 \times 10(-3)$ responded. More recent studies have shown that the use of more than one independent determinant of response permits a considerably better response prediction than that with just one determinant.

In conclusion, these results suggest that the *MTHFR 677* allele could identify CRC patients that would be responsive to fluoropyrimidine-based chemotherapy. Our study, however, had some important limitations. It looked only at a small series of arbitrarily selected cases rendering it open to biases, and it was statistically underpowered to give reliable results; this limitation was in part attributable to the study design, because we specifically excluded patients receiving other medications, such as Irinotecan or Oxaliplatin, which work through different pathways. In addition, our study was mainly a retrospective assessment of this molecular marker, in which patients had undergone treatment, and the outcomes were already known. Future studies, preferably within the context of large randomized control trials, should attempt to determine how accurately this marker will predict response prospectively, and, if found to be truly an independent determinant of response, trials should be developed in which treatment is assigned on the basis of marker status. In the aforementioned studies using antisense oligonucleotide inhibition of MTHFR *in vitro*, we suggested that MTHFR might be a novel target for cancer therapy (21). The results from this study support MTHFR-based targeting in conjunction with fluoropyrimidine-based therapy to treat CRC.

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