Research Article

Correlation Between Methylenetetrahydrofolate Reductase (MTHFR) C677T Polymorphism, Fluoropyrimidine Response and Toxicity in Patients Treated for Locally Advanced Rectal Cancer

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ABSTRACT

Preoperative radiation therapy combined with fluoropyrimidine is the standard treatment for locally advanced rectal cancer. However, there is a large individual difference or variation in tolerability and therapeutic efficacy. The genetic polymorphisms represent one of the major causes in this variation. This study was to analyze the relationship between Methylenetetrahydrofolate reductase "MTHFR" (important enzyme in Fluoropyrimidines metabolism) gene polymorphism, tolerability and the therapeutic efficacy of Fluoropyrimidines in patients with locally advanced rectal cancer. Genomic DNA was extracted from 52-blood samples, collected in patients with stage II and III histologically proved rectal cancer. MTHFR gene polymorphism was determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). All patients received Preoperative radiation therapy combined with fluoropyrimidine, followed by surgical resection. The treatment tolerability was evaluated according to the NCI-CTC version 3 toxicity criteria. Therapeutic efficacy was evaluated by histopathological postoperative specimen examination. Kaplan-Meyer survival curves were defined for each polymorphism. The risk of developing severe (grade 3-4) toxicity was observed in 677CC (28%), 677CT (35%) and 677TT (20%). T-level downstaging and complete response after neoadjuvant treatment were demonstrated in 60% of cases in patients with 677TT genotype, 56% in 677CT genotype and 58.8% in 677CC genotype. No association was observed between C677T polymorphism and survival (log rank= 0.02, p = 0.99). In spite of the limited patient number (52 patients), our study shows that the MTHFR 677 TT genotype can have a protective role for fluoropyrimidine toxicity, and it can be a predictive factor in therapeutic efficacy. This study will be continued, in order to include more patients.

Keywords: Rectal cancer, Fluoropyrimidine, MTHFR 677, Polymorphism.

INTRODUCTION

Combined preoperative fluoropyrimidine (5Fluorouracil, capecitabine) and radiation therapy is the standard treatment for locally advanced rectal cancer. Pathologic downstaging (DS) or a pathologic complete response (pCR) after preoperative chemoradiation has been correlated with improved survival, decreased recurrence and a higher rate of sphincter-preserving surgeries.[1-7] However, there is a large individual difference or variation in tolerability and therapeutic efficacy. The genetic polymorphisms represent one of the major causes in this variation.[1,8-10] Factors that cause variations in drug response are multifold and complex. At molecular level, genetic variability of drug metabolizing enzymes has long been recognized as a factor in therapeutic response and drug toxicity.[11-13] Identifying pharmacogenomics factors in drug tolerability and response would develop treatment tailored to each patient.[14-17] Several studies have investigated the impact of genetic polymorphism in enzymes involved in folate and fluoropyrimidines metabolism. Thymidylate synthase (TS), the target enzyme of 5-fluorouracil (5-FU), dihydropyrimidine dehydrogenase (DPD), the main player of 5-FU catabolism in the liver and methylentetrahydrofolate reductase (MTHFR), directly linked to the TS reaction. Two polymorphisms of MTHFR have been reported to determine enzyme activity: MTHFR-C677T and MTHFR-A1298C. The aim of our study is to analyze the relationship between gene polymorphism C677T of the methylenetetrahydrofolate reductase "MTHFR", tolerability and the therapeutic efficacy of fluoropyrimidines in patients with locally advanced rectal cancer. We attempted also to study its impact on the sphincter preservation rate, pelvic local control, survival and established a DNA bank.

PATIENTS AND METHODS

Eligibility Criteria

The study was conducted between December 2011 and November 2013. Population consisted of patients with histologically documented rectal adenocarcinoma. The patients that were eligible to participate in this study, were 18 to 70 years old, had tumor extension through the bowel wall (T3-T4) or pelvic lymph-node involvement (according to the TNM classification 7th edition 2009)[18] and lower pole of the primary tumor was less than 15 cm from anal verge margin (as determined by clinical work-ups, including computed tomography, pelvic magnetic resonance imaging and/or endoscopic ultrasound), with a

good performance status OMS (PS= 0 or 1). However, those with history of malignancy treated with pelvic radiotherapy, or prior chemotherapy, synchronous metastases, and patients rescued from surgery (for other serious medical condition), were not included in this study. Patients who fulfilled the above eligibility criteria were made aware of the study aim and were required to sign the informed consent.

Genotyping of C677T SNP

Blood samples (6 to 10 ml) were collected on Ethylene Diamine Tetraacetic Acid Tube (EDTA) and used to extract genomic DNA by a salting-out method.[19] The genotyping of C677T MTHFR polymorphism (rs1801133) located in exon 4 of the MTHFR gene was carried out using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). PCR was performed with 200 ng of genomic DNA in a final volume of 50 µl. The PCR mixture contained 5µl of 10 X Gold Taq buffer, 1.5 mM of MgCl2, 2.0 mM of dNTPs, 10 µM of each primer and O.4 U of Taq DNA Polymerase. PCR was done using the primers: Oligo F (forward primer): 5'TGA AGG AGA AGG TGT CTG CGG GA 3', OligoR (reverse primer): 5'AGG ACG GTG CGG TGA GAG TG 3' as described.[20] Every PCR was accompanied with a negative control without any genomic DNA. PCR cycling conditions included initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 65°C, and 30 sec at 72°C, and a final extension step at 72°C for 10 min. Restriction enzyme digestion, for the PCR products of C677T was carried out by adding 1X Buffer, 0.2 µl of bovine serum albumin, and 10U of *Hinf I* to a PCR tube containing 30 µl of PCR product. The mixture was then spun down and incubated overnight at 37°C. Fragments were separated by electrophoresis in a 3% agarose gel and subsequently visualized with ethidium bromide under UV light and then photographed. There were three genotypes of MTHFR C677T: C/C (198 bp); C/T (198 bp/175 bp); T/T (175 bp).

Treatment

All patients received preoperative concurrent pelvic radiation therapy associated with fluoropyrimidine. The total irradiation dose of 45 Gy was delivered using conventional fractionation (daily fractions of 1.8 Gy/five days per week over 5 weeks). Concurrent fluoropyrimidine were administered as 5 Fluorouracil (750mg/m^2, day 1-5 in IVI/22 hours) combined with folic acid (20 mg/m²/d over 30 minutes), in two cycles; the first cycle was administered during days 1 to 5 of the external-beam radiotherapy and the second cycle was administered on days 29 to 33. In some patients treatment was administered as capecitabine continuously throughout the 5 weeks of radiotherapy course at 825 mg/m^2 given twice daily 5 days per week during the days when radiotherapy was delivered. Surgery was planned approximately 6-8 weeks after the completion of chemoradiotherapy.

Treatment Evaluation

We assessed patients by clinical and hematological examination weekly during treatment. Toxicities were evaluated using the National Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTC v 3.0).[21] Therapeutic efficacy was evaluated by histopathological postoperative specimen examination according to the pTNM staging system. A tumor and/or nodal down-staging was considered when pathological T (pT) and/or pathological N (pN) was lower than clinical T and/or N as defined by computed tomography and/or magnetic resonance imaging obtained after preoperative concurrent chemoradiation. Evaluation of histological regression was carried out according to rectal cancer regression grading established by Wheeler and al[22] grade 1, sterilization or only microscopic foci of adenocarcinoma remaining with marked fibrosis; grade 2, marked fibrosis but macroscopic disease; and grade 3, little or no fibrosis with abundant macrocopic disease. Kaplan-Meyer survival curves were defined for each polymorphism. Relationships between MTHFR C677T polymorphism variants and the incidence of grade 3–4 toxicity, tumor response (measured by DS and ypT0 rates) and overall survival were assessed. Overall survival was calculated in weeks between the start of treatment and death of 50% of patients. The SPSS software package (version 22.0J; SPSS Inc.; Chicago, IL, USA) was used for statistical analyses.

RESULTS

Patients and Tumors Characteristics

Between December 2011 and November 2013 a total of 52 patients, 31 men and 21 women, with locally advanced rectal adenocarcinoma were enrolled in this study. Patient characteristics are summarized in Table 1. The median age was 50.8 years (ranging from 23-70 years). Family history of cancer was found in 5 cases (four colorectal cancer and one breast cancer). Average time between first symptoms and diagnosis was 7.4 months (1-24 months). Tumor was located in the lower rectum in 29 cases (55.7%); 58% of the patients (30 pts) had stage III rectal cancer.

Genotyping of MTHFR C677T

The genotyping results are presented in Table 2. Twenty-five patients (48%) had homozygous normal or CC genotype. 17 patients were heterozygous (CT) and 10 individuals (19,2%) were homozygous for the mutation (TT).

Table 1: Patients and tumor characteristics

Patients and tumor characteristics	N of patients (N=52)
Median age, years	50.8 (23-70)
Gender	
Men	31
Women	21
Pathological history	
Family history of cancer	5
Familial adenomatous polyposis	2
Diabetes	3
High blood pressure	3
Average time: first symptom - diagnosis (month)	7.4 months (1-24 months)
Clinical symptoms	
Rectal bleeding	35
Change in bowel habits	15
Mucous discharge	9
Pain	4
Tumor site	
Low rectum	29
Mid rectum	23
TNM clinical stage	
T3N0M0: stage II	22
T3N1M0: stage III	27
T3N2M0: stage III	1
T4N1M0: stage III	1
T4N2M0: stage III	1
Chemotherapy	
5Fluorouracil+ Folic Acid	30
Capecitabine	22

Table 2: MTHFR genotypes distribution

MTHFR 677 C >T Genotype	No of patients	Percentage (%)
CC	**25**	**48**
CT	**17**	**32,6**
TT	**10**	**19,2**

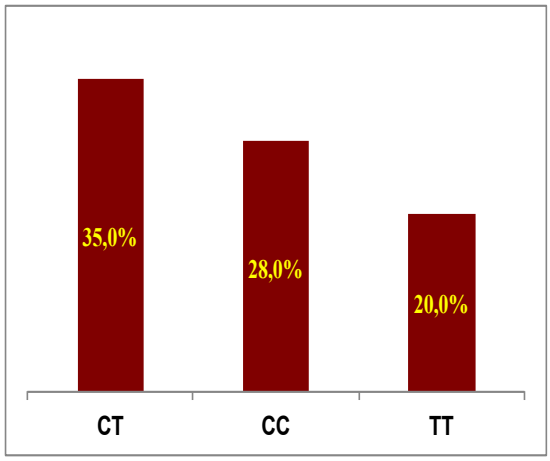

Figure 1: C677T polymorphism and fluoropyrimidine toxicity

Impact of C677T Polymorphism and Fluoropyrimidines Toxicity

Tolerability was satisfactory, with grade 3-4 toxicity observed in 13.4% of patients for diarrhea and 15.3% for radiation dermatitis. 677 (CC–CT) genotypes were related to a higher rate of grade 3–4 toxic events respectively (35%, 28%) (Figure 1). Grade 3-4 diarrhea was found in 4CC genotype and 2CT genotype respectively (16% and 11.7%), 8 patients presented grade 3-4 radiation dermatitis (3CC, 4CT et 1TT), only 2 patients with 677TT have grade 3-4 toxicity (20%) (Table 3).

Impact of C677T Polymorphism and Treatment Response

After completion of concurrent chemoradiation and before surgery, evaluation of response was defined clinically. Tumor response was assessed by computerized tomography and/or magnetic resonance imaging. Down-staging (DS) and pathological complete response (pCR) or (ypT0N0M0) were evaluated by histopathological postoperative specimen examination according to the pTNM staging system and regression grading established by Wheeler and al.[22] Down-staging and pCR were obtained respectively in 23 patients (44.2%) and 4 patients (7.6%) (Table 4).

The best overall response was obtained in 677TT; DS was achieved in 4/10 patients (40%)(Table 4) with pathological complete response pCR in two cases (20%) (Table 5). In those with 677CT and 677CC, DS and pCR were obtained respectively in 6CT, 1CT (35.2%, 5.8%) and 13CC, 1CC (52%, 4%).

Table 3: C677T polymorphism and fluoropyrimidines toxicity

MTHFR 677 C >T Genotype	Leukopenia G3-4	Diarrhea G3-4	Radiation dermatitis G3-4	%
CC	0	4	3	28
CT	0	2	4	35
TT	0	1	1	20

Table 4: ypTNM classification

TNM	N patients	ypTNM	N patients (ypTNM)	N patients (stage)
T3N0M0 = stage II T3N1M0 = stage III T3N2M0 = stage III T4N1M0 = stage III T4N2M0 = stage III	22 27 1 1 1	T0N0M0 T1N0M0 T2N0M0 T2N1M0 T3N0M0 T3N1M0 T4N1M0 T4N2M0	4 9 7 1 18 12 1 1	4 = stage 0 15 = stage I 1 = stage III 18 = stage II 12 = stage III 1 = stage III 1 = stage III
Stage II: 22 Stage III: 30				Stage 0: 4 Stage I: 15 Stage II:18 Stage III:15

Table 5: Impact of MTHFR gene polymorphism C667T on response

MTHFR C677T genotype	N patients	Downstaging (DS)	Complete response (pCR)
CC	25	13	1
CT	17	6	1
TT	10	4	2

Table 6: Impact of MTHFR gene polymorphism C667T on sphincter-preserving surgery

MTHFR C677T genotype	Anterior resection	Abdominal-perineal resection
CC	**14**	**5**
CT	**11**	**10**
TT	**5**	**7**
	30	**22**

All patients underwent curative surgical resection. 30 patients (57.7%) underwent low anterior resection (sphincter-preserving surgery). 22 patients (42%) patients underwent abdominal-perineal resection in low rectal tumor (distance from the lower pole of the primary tumor to the anal verge was less than 4 cm).

In 29 tumors localized in low rectum, 7 patients underwent low anterior resection (n=4; 677CC, n=2:677CT and n=1; 677TT) (Table 6).

Impact of C677T Polymorphism on Survival

The overall survival of all patients has been followed up every 3 months for the first 2 years after the end of treatment.

Overall survival at 2 years was 88% (Figure 2). In this study overall survival was not significantly different among patients with 677 (TT, CT, and CC) genotypes; this was respectively (88.9%, 88.2%, 87.5%; p=0.9) (Figure 3).

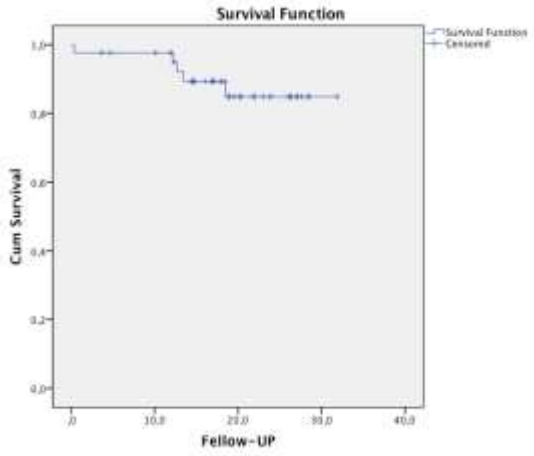

Figure 2: Overall survival

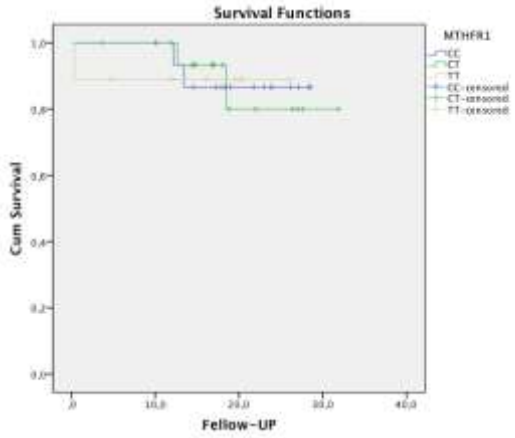

Figure 3: impact of C677T polymorphism on survival

DISCUSSION

Fluoropyrimidine remain the drugs of choice for preoperative chemoradiation therapy of locally advanced rectal cancer. The present pharmacogenetic study icluded 52 patients with stage II and III rectal cancer, all receiving Fluoropyrimidine-based preoperative chemoradiation. Continuous infusion (CI) of 5-FU has the biological advantage of prolonging the exposure of cells to 5-FU and improving anti-tumor activity. Capecitabine is an orally administered fluoropyrimidine carbamate, which is preferentially converted to active 5-FU in the liver and in the tumor cells. Treatment tolerability, Tumor regression after properative chemoradiation varies substantially among individuals and pathological complete response is recognized as an important prognostic factor for locally advanced rectal cancer. Genetic variability of drug metabolizing enzymes is recognized as a factor in therapeutic response and drug toxicity. MTHFR is an enzyme that plays a role in the metabolism of folate. Alterations in MTHFR enzyme metabolism can affect the activity and function of this enzyme and could also possibly alter the treatment effect. Polymorphisms in the MTHFR gene represent one of these alterations. The aim of the present study was to examine the role of C677T polymorphism on toxicity and response of Fluoropyrimidines in patients with locally advanced rectal cancer. The prevalence of the different MTHFR genotypes 677CC, CT, and TT varies among studies and geographical regions. In this study 25 patients (48%) had homozygous normal or CC genotype. 17 patients were heterozygous (CT) and 10 individuals (19,2%) were homozygous for the mutation (TT). In the prospective French study published in the British Journal of Clinical Pharmacology (BJCP), to evaluate the predictive value of gene polymorphism potentially related to 5FU and oxaliplatin pharmacodynamics in advanced colorectal cancer treated with FOLFOX (enrolled 117 patients), MTHFR genotypes distribution (CC, CT and TT) was shown respectively in 44, 58 and 14 cases (37.6%, 49, 5% and 11.9%).[23] In the American study published in 2011, concerning MTHFR gene polymorphism and 5FU toxicity combined with preoperative concurrent radiotherapy in 131 patients with locally advanced rectal cancer[24], MTHFR genotypes (CC, CT and TT) was found in 60, 59 and 12 cases respectively (45.8%, 45.03% and 9.16%). The TT genotype frequency observed in Salvatore Terrazzino study included 125 patients with rectal adenocarcinoma treated with concurrent preoperative chemoradiation therapy using 5FU was lower: 677CC (n=41, 33%), 677CT (n=57, 46%) and 677TT (n=27, 22%)[25] In this study, Treatment tolerability was, satisfactory, with grade 3-4 toxicity observed in eight patients (13.4%). We found that patients with the MTHFR TT genotype had a significantly better tolerability than those with the CC/CT genotypes. 677TT genotype seems to have a protective role in the toxicity of grade 3 and 4. Patients with CT MTHFR genotype have an increased risk of developing severe acute toxicity due to fluoropyrimidine treatment.

In Etienne-Grimaldi study published in the British Journal of Clinical Pharmacology[23], grade 3 and 4 toxicity occurred in 22.9% of cases. None of the analyzed gene polymorphisms were predictive of toxicity considered either as the maximum observed grade, or as the toxicity score. The study of S. Afzal showed that grade 3-4 toxicity was overrepresented among patients with genotype CC 677 with an odds ratio (OR) confidence interval [95% (CI); 1.13- 2.96, p= 0.01] 1.83. 26

The best overall response was obtained in 677TT, in 60% of cases; DS was achieved in 4/10 patients 40%, with pathological complete response pCR in two cases (20%).

Sohn and al[8] demonstrated on human cancer cells, a very high sensitivity to 5FU in the genotype 677T relative to 677C. In clinical trials, the impact of MTHFR gene polymorphism in tolerance and therapeutic efficiency is still controversial. Cohen[27] were the first to describe a link between C677T MTHFR gene polymorphism and tumor response to 5FU-based chemotherapy. In this study conducted on 43 patients with metastatic colorectal cancer, therapeutic response was achieved in all patients with 677TT (5 patients), it was 50% in patients with 677CC. In a retrospective study from Etienne and al

including 98 colorectal cancer patients with liver metastases receiving FUFOL, responsiveness was significantly linked to 677C→T genotype, with an increased response rate in 677TT tumors relative to 677CC (odds ratio = 1.88).[28] In contrast, a study by Marcuello and al failed to show a link between MTHFR polymorphisms and clinical response in 94 metastatic colorectal cancer patients receiving FU associated with irinotecan or oxaliplatin.[29] In Terrazzino Salvatore study, the aim was to evaluate the impact of MTHFR gene polymorphism on tumor regression, in 125 patients treated with concurrent preoperative radiation therapy associated at 5FU. Tumor regression was frequent in patients with genotype 677CC (57%) compared to genotype 677CT and 677TT, which were respectively (27% and 37%).[25]

Clinical studies on genetic variation exhibited the impact of MTHFR C677T polymorphism in colorectal cancer treated with 5FU on survival rate have mainly focused on patients undergoing treatment for metastatic disease and 5FU- based adjuvant chemotherapy. Most of the reported studies were conducted in a small number of patients (less than 150) and have shown varying results with almost equal numbers showing no effect, a positive effect, or a negative effect on survival, response.

CONCLUSION

In spite of the limited patient number, our study shows that the MTHFR 677 TT genotype can have a protective role of fluoropyrimidine toxicity, and it can be a predictive factor in therapeutic efficiency. This study will be continued, in order to include more patients and to analyze the second polymorphism in MTHFR gene (1298 A>C). Due to the encouraging results of this time limited study, further patient follow-up and study analysis is warranted.

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