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# Clinical validation study of genetic markers for capecitabine efficacy in metastatic colorectal cancer patients

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*Pharmacogenet Genomics. 2015 Jun;25(6):279-88*

## Abstract

**Background & aim:** Pharmacogenetic studies continue to search for pretreatment predictors of chemotherapeutic efficacy and toxicity in metastatic colorectal cancer (mCRC). Both genome wide association (GWA) studies and candidate gene studies have yielded potential genetic markers for chemosensitivity. We conducted a clinical association study, validating the effect of specific genetic markers cited in recently published papers on the efficacy of the oral 5-FU pro-drug capecitabine.

**Patients & methods:** Germline DNA was collected for 268 mCRC patients from the CAIRO trial, a multicenter phase III trial, randomizing between combined or sequential first-line treatment with capecitabine, irinotecan and oxaliplatin. Genotyping was performed for eight single nucleotide polymorphisms (SNPs), using high resolution melting curves. Four SNPs are located in the *MTRR* gene, and another four SNPs showed significant association with 5-FU cytotoxicity in a recent *in vitro* GWA study. Primary endpoint was progression free survival (PFS); secondary endpoints were objective response and overall survival (OS).

**Results:** In patients receiving capecitabine monotherapy, rs4702484, located in *ADCY2* and close to *MTRR*, was associated with slightly reduced PFS for homozygous wildtype patients (CC 6.2 vs. CT 8.0 months, P = *0.018*). For the other selected genetic markers, we found no association with PFS, OS or radiologic response upon treatment with capecitabine, either in the total study population, or the capecitabine monotherapy subgroup.

**Conclusion:** With the exception of rs4702484, we found no evidence of an effect on capecitabine chemosensitivity of any of the studied SNPs. More specifically, variants in MTRR are not likely associated with capecitabine efficacy.

## Background

Colorectal cancer is the third leading cause of cancer death worldwide.[1] Survival is strongly dependent on disease stage.[2] For patients presenting with distant irresectable metastases, systemic therapy is indicated with the objective of prolongation of survival and sometimes cure if downsizing permits secondary resection of metastases. Fluoropyrimidines, including the oral pro-drug capecitabine, remain the cornerstone of chemotherapeutic treatment, although treatment options have expanded and now include oxaliplatin and irinotecan, as well as the monoclonal vascular endothelial growth factor (VEGF) inhibitor bevacizumab and the endothelial growth factor receptor (EGFR) blockers panitimumab and cetuximab.[3] Despite the fact that systemic therapy significantly improves median survival, a substantial portion of patients do not benefit from this. Chemotherapy is sometimes accompanied by severe adverse events, which can delay or even abrogate further treatment. There is an urgent need to preemptively identify patients who will both tolerate and benefit from a specific chemotherapeutic schedule. Up until now, no germline molecular markers have been identified that may predict for the efficacy of chemotherapy.[4] Pharmacogenetics may provide such a tool, by identifying genetic predictors for both efficacy and toxicity.[5]

Up to now, most studies searching for pretreatment genetic markers in colorectal cancer have used a pathway-based approach. This has led to the identification of *UGT1A1**28 as a risk factor for increased toxicity (specifically neutropenia) after treatment with irinotecan[6], and *DPYD**2A as a risk factor for severe and sometimes lethal toxicity in response to fluoropyrimidine therapy.[7] However, this candidate gene approach is limited by our a priori knowledge of the genes involved in the pathway, and is therefore unable to identify novel markers in genes not previously associated with the drug under investigation. Recently, a genome wide association (GWA) study applying a hypothesis free approach was published, identifying single nucleotide polymorphisms (SNPs) with putative influence on cytotoxicity of capecitabine in human lymphoblastoid cell lines (LCL).[8] The most significant marker was located near the gene encoding for 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (methionine synthase reductase, MTRR). As part of the methionine-folate pathway, MTRR is involved in fluoropyrimidine cytotoxicity. (Figure 1) Furthermore, variation in this gene has been implicated in colorectal carcinogenesis.[9]

The number of genes and polymorphisms that are being implicated as pretreatment biomarkers has expanded rapidly, necessitating validation of reported results.

In this study, we tested eight single nucleotide polymorphisms (SNPs), selected for their location within the *MTRR*-gene or their significance in the recent GWA paper by O'Donnell and co-workers[8], for their association with progression free survival (PFS) in a clinical trial population of 268 metastatic colorectal cancer (mCRC) patients who were treated with first-line capecitabine-based chemotherapy.

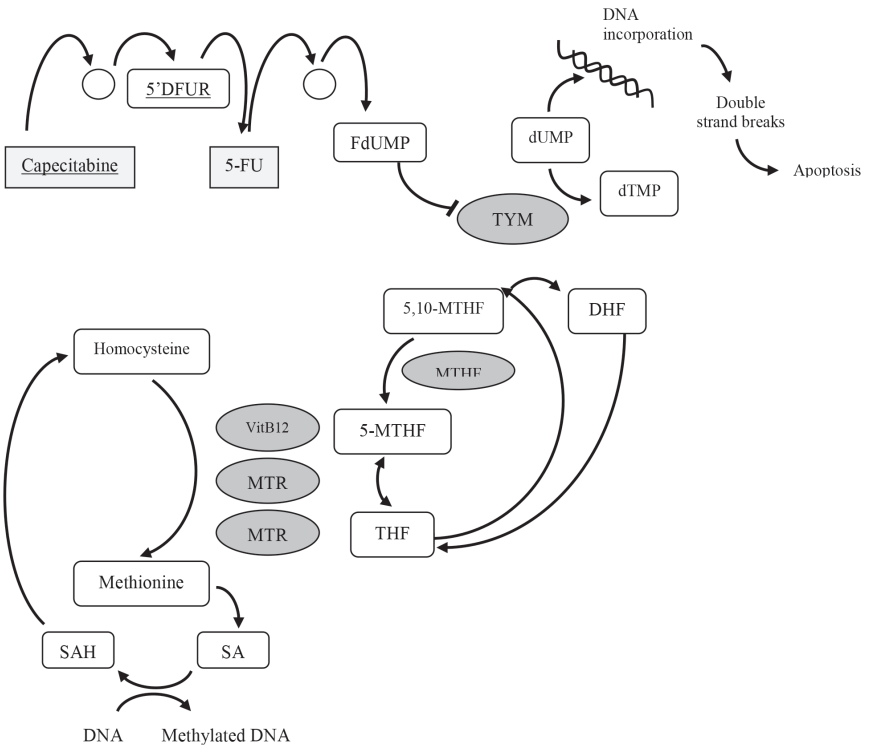


*Figure 1. Schematic overview of pathways involved in cellular response of fluoropyrimidines*

5-FU, 5-fluoro-uracil; DHF, dihydrofolate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FdUMP, fluoro-deoxyuridine monophosphate; MTHF, methylene tetrahydrofolate; MTHFR, methylene tetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; SAH, S-adenosyl homocysteine; SAM S-adenosyl methionine; THF, tetrahydrofolate. Figure based on: M. Whirl-Carrillo, et al.[31]

## Patients and methods

### Clinical association study

Patients were recruited from the CAIRO trial, a multicenter open label randomized phase III clinical trial, comparing sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in a total of 803 mCRC patients.[10] A total of 268 patients were included in this pharmacogenetic study, of whom 127 received first-line capecitabine monotherapy, and 141 patients received first-line capecitabine plus irinotecan combination therapy. Patients with mCRC were enrolled in the CAIRO study between January 2003 and December 2004, by the Dutch Colorectal Cancer Group (DCCG) in 74 hospitals in The Netherlands. Inclusion criteria were a WHO performance score of 0-2 and adequate renal, hepatic and bone marrow function. A history of previous adjuvant chemotherapy was allowed, only if the last administration was

given six months prior to randomization. Main exclusion criteria were: serious concomitant disease preventing the safe administration of chemotherapy and other malignancies in the past five years. Capecitabine (1250 mg/m2, bid) was administered on day 1-14 in the monotherapy group, every three weeks. In the combination therapy group, capecitabine (1000 mg/m2, bid) was given on day 1-14, and irinotecan (250 mg/m2) on day 1, in a three weekly cycle. Tumor response was assessed by computed tomography (CT)-scan, every nine weeks, using Response Evaluation Criteria for Solid Tumors (RECIST, version 1.0).

The CAIRO study was approved both by the Central Committee on Research involving Human Subjects (CCMO) and by the local ethics committees of all participating centers. As sample collection for this pharmacogenetic substudy was initiated later than the CAIRO clinical trial and not all study centers participated, the number of patients included in the pharmacogenetic analyses is limited to 268 patients. All included patients gave written informed consent before inclusion for the main study and the pharmacogenetic side study.

## SNP selection and genotyping

Four SNPs were selected from the results of a recently published *in vitro* GWA study[8] (rs4702484, rs8101143, rs576523 and rs11722476), based on their genome-wide significance levels in meta-analysis. A fifth SNP (rs361433) showed near genome-wide significance in this study. Unfortunately, no primers could be designed for this marker and it was therefore not included in our analyses. The GWA study suggested involvement of *MTRR* in capecitabine cytotoxicity. Although the *MTRR* gene has been suggested to be involved in colorectal carcinogenesis[11], current knowledge on the effect of *MTRR* polymorphisms on efficacy of fluoropyrimidine treatment in colorectal carcinoma is limited to one publication. In that study, no association of *MTRR* genotype with PFS was found.[12] To further investigate the predictive effect of *MTRR* polymorphisms in colorectal cancer treatment, four additional SNPs were selected based on their location in the this gene and citation in recent pharmacogenetic papers (rs1801394, rs10380, rs162036 and rs1532268). Variants in *MTHFR* (rs1801133 and rs1801131) and *TYMS* (rs34743033, rs11540151 and rs11280056) were also included as covariates, because an effect on capecitabine efficacy has been suggested for these polymorphisms.[11;13;14] *DPYD*2A* (IVS14+1G>A) was not included as a covariate in the model, because of the low estimated population frequency (minor allele frequency 0.003[16]). Furthermore, it was previously shown that individual SNPs in *DPYD,* including *DPYD*2A,* did not influence treatment efficacy in our patient group.[7]

Peripheral EDTA-blood samples were collected and stored at -20°C before DNA isolation. Germline DNA was extracted with the Magnapure LC (Roche Diagnostics, Almere, The Netherlands) according to manufacturer's instructions.

A short amplicon high resolution melting (HRM) assay was designed for each SNP and genotype allocations were confirmed by conventional Sanger sequencing. Genotype calls were made using the Call-IT 2.0 software. Oligonucleotide sequences and annealing temperatures are available on request. As quality control, all HRM assays were validated on a panel of DNA

from 18 healthy individuals. In addition, negative controls (water) were included in each run. Samples failing initial genotyping were repeated and in this run samples with confirmed genotypes were included as positive controls. By repeating HRM and sequencing samples, more than 5% of samples were genotyped in duplicate. *MTHFR* rs1801133 (677C>T) and *MTHFR* rs1801131 (1298 A>C) genotypes were determined using commercially available Taqman genotyping assays and analyzed on 7500 realtime PCR system (Lifetechnologies, Bleiswijk, The Netherlands) according to manufacturer's protocol. The VNTR polymorphism in the *TYMS* 5'-untranslated region (TSER, rs34743033), including the additional G/C SNP in the second base pair for 3-repeat individuals (rs11540151), was determined by direct sequencing. The *TYMS* 1494del6bp polymorphism in the 3'-untranslated region (rs11280056) was also determined using a pre-designed Taqman genotyping assay.

## Data and statistical analysis

The primary endpoint of this study was progression free survival (PFS), which was calculated from the date of randomization until the first observation of disease progression or death from any cause. Secondary endpoints were overall survival (OS), objective response and clinical benefit. OS was calculated as the interval from randomization until death from any cause or until the date of last follow-up. Response to chemotherapy was assessed in all patients who completed at least 3 cycles of treatment. Objective response was determined as either complete or partial response. Clinical benefit was determined as stable disease, complete or partial response.

We chose not to include analyses for SNP effects on treatment toxicity. *In vitro* experiments, such as performed by O'Donnell and co-workers[8], are useful in examining cytotoxic effects of chemotherapeutic drugs, but do not take into account the multitude of patient-related factors that influence adverse events in clinical practice. We therefore believe that these *in vitro* results cannot be extrapolated to predict fluoropyrimidine-induced toxicity.

Differences in PFS and OS according to genotype were determined by Kaplan-Meier survival curves and log-rank testing. Multiple regression analysis was performed assessing the effect of genotype on PFS and OS by Cox regression analysis, treating gender, age, treatment arm and LDH at baseline as covariates. Variants in *MTHFR* (rs1801133 and rs1801131) and *TYMS* (rs34743033, rs11540151 and rs11280056) were also included as covariates, because these polymorphisms have been associated with efficacy of fluoropyrimidine therapy by others.[11;13;14] Although we previously showed that these SNPs did not affect treatment efficacy in our patient group[15], they were nonetheless included to minimize bias. Data are expressed as medians and 95 percent confidence intervals (95% CI). Additionally, the ten percent of all patients showing the longest PFS times and the ten percent of patients showing the shortest PFS times were selected and genotype distributions were compared between these groups by the Chi-squared test. The association of objective response and clinical benefit with genotypes was determined by cross tabulation and the Chi-squared test. All analyses were performed for the treatment population as a whole and for patients in the capecitabine monotherapy group separately. Conservative Bonferroni-correction for multiple testing would lead to the

adoption of a significance level of $\alpha = 0.05/8 = 0{\cdot}00625$, if all genetic markers are assumed to be unrelated. Earlier research has shown that there is a moderate amount of linkage disequilibrium between MTRR polymorphisms[11], and these SNPs are therefore not completely independent. We confirmed the presence of linkage disequilibrium between these polymorphisms in our population, using Haploview. (Figure 2A and B) A more lenient correction was therefore adopted, with a significance level of $\alpha = 0.05/5 = 0{\cdot}01$.

Genotype distributions were tested for agreement with those expected under Hardy-Weinberg equilibrium using the Chi-squared test, with a statistical cut-off value of $\chi^2 \geq 3.84$.

All statistical analyses were performed using SPSS software, version 20.0 (IBM Corp., Armonk, New York, USA).

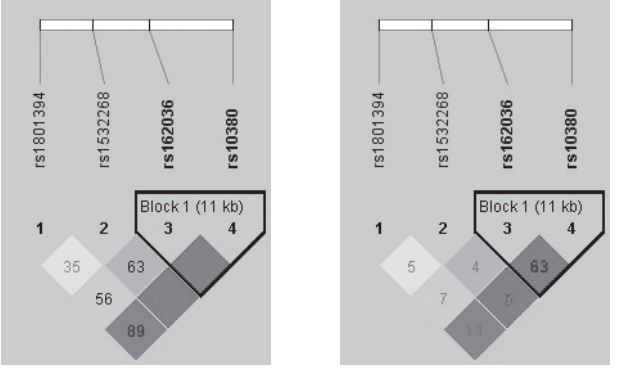


*Figure 2. Linkage disequilibrium analyses for polymorphisms located in the gene encoding for 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR)*

**A.** Numbers in squares represent D' values between the respective SNPs. D' for rs10380 and rs162036, as well as for rs10380 and rs1532268 are 1. **B.** Numbers in squares represent hundredfold R-square values.

| | Total N= 265 | Capecitabine monotherapy N= 126 | Combination therapy N= 139 | P-value* |
|---|---|---|---|---|
| **Age** | | | | *0.60* |
| Age at randomisation, median (range) | 62 (27-81) | 61 (27-78) | 62 (37-81) | |
| **Sex** | | | | *0.90* |
| Male | 161 (61%) | 76 (60%) | 85 (61%) | |
| Female | 104 (39%) | 50 (40%) | 54 (39%) | |
| **Performance status** | | | | *0.57* |
| PS 0 | 153 (58%) | 77 (61%) | 76 (55%) | |
| PS 1 | 96 (36%) | 42 (33%) | 54 (39%) | |
| PS 2 | 16 (6%) | 7 (6%) | 9 (6%) | |
| **LDH at randomisation** | | | | *0.89* |
| Normal | 174 (66%) | 82 (65%) | 92 (66%) | |
| >Upper limit of normal | 91 (34%) | 44 (35%) | 47 (34%) | |
| **Previous adjuvant therapy** | | | | *0.70* |
| Yes | 235 (89%) | 113 (90%) | 122 (88%) | |
| No | 30 (11%) | 13 (10%) | 17 (12%) | |

*Table 1. Baseline characteristics*

LDH, Lactate dehydrogenase
* Significance level for the difference in distribution between "Capecitabine monotherapy" and "Combination therapy".

# Results

## Clinical data

Baseline characteristics are shown in Table 1. Baseline characteristics were not significantly different between both treatment groups. The majority of patients (61%) were male and median age at randomization was 62 years (range 27-81 years). Baseline characteristics were evaluated for their relationship with SNP-genotypes, and no associations were found (data not shown).

## Genotyping data

Genotyping was successful for all SNPs in 248 of 268 patients (93%). Three samples failed genotyping for three or more SNPs, and these were excluded from the statistical analysis. For individual SNPs, genotyping results ranged from 96% for rs1081394 to 100% for rs1532268.

Genotype frequencies are shown in Table 2. Genotype distributions were consistent with Hardy Weinberg equilibrium (HWE), except for rs11722476 ($\chi^2 = 4.38$) and rs4702484 ($\chi^2 = 4.86$). However, allele frequencies are consistent with those reported by others[16] and HWE would have been met in both cases with the addition of even one homozygous variant-type sample.

| SNP | Chrom | Position | Gene | Mutation | Observed / expected MAF* | Genotype frequencies# |
|---|---|---|---|---|---|---|
| rs576523 | 1q23.3 | 159012700 | *intergenic* | A>G | 0.01 / 0.07 | 98.9 – 1.1 – 0 |
| rs11722476 | 4q22.3 | 95389862 | *SMARCAD1* | G>A | 0.40 / 0.45 | 33.0 – 54.2 – 12.9 |
| rs4702484 | 5p15.31 | 7702860 | *ADCY2* | C>T | 0.12 / 0.15 | 76.1 – 23.9 – 0 |
| rs1801394 | 5p15.31 | 7923973 | *MTRR* | G>A | 0.42 / 0.38 | 34.8 – 46.8 – 18.4 |
| rs1532268 | 5p15.31 | 7931179 | *MTRR* | G>A | 0.38 / 0.26 | 37.7 – 48.0 – 14.3 |
| rs162036 | 5p15.31 | 7938959 | *MTRR* | A>G | 0.14 / 0.23 | 74.8 – 21.8 – 3.4 |
| rs10380 | 5p15.31 | 7950191 | *MTRR* | C>T | 0.09 / 0.19 | 81.7 – 18.3 – 0 |
| rs8101143 | 19p12 | 21747976 | *intergenic* | A>G | 0.29 / 0.18 | 51.5 – 38.6 – 9.9 |

*Table 2. Genotype frequencies*

ADCY2, adenylate cyclase type 2; MAF, minor allele frequency; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; SMARCAD1, SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily A, containing DEAD/H box 1; SNP, single nucleotide polymorphism.

* Observed minor allele frequencies in our population. Expected minor allele frequencies, based on those reported in: Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine.(dbSNP Build ID: 36.3). http://www.ncbi.nlm.nih.gov/SNP/.

# Genotype frequencies are displayed as percentages homozygous wildtype – heterozygous – homozygous variant type.

## Association with capecitabine efficacy

*Association with PFS*

Updated PFS data were available for all but three patients. Updated OS data were available for 243 patients. In the remaining cases censored data were used for the analyses. Results for the association analyses are shown in Table 3.

Considering the total study population, we found no difference in PFS according to genotype for any of the SNPs. Also, when comparing genotypes between patients with the longest and those with the shortest PFS times, no significant differences in genotype distributions were found for any of the SNPs (P = *0.183* for rs1801394, to P = *1.000* for rs576523, data not shown).

For rs4702484, PFS for homozygous wildtype patients was 7.5 months (95% CI: 6.4-8.5 months), versus 7.8 months (95% CI: 5.9-9.6 months) for heterozygous patients (P = *0.351*), with no patients carrying the rs4702484 homozygous variant genotype. However, when patients in the capecitabine monotherapy group were considered separately, a borderline significant effect of rs4702484 genotype was seen. (Table 4 and Figure 3A) PFS for patients with the homozygous wildtype genotype was 6.2 months (95% CI: 5.6-6.7), versus 8.0 months (95% CI: 6.2-9.8) for heterozygous patients (P univariate = *0.018*). This result did not remain statistically significant in multivariate analysis (P multivariate = *0.029*).

| Variant | | Objective respons | P-value | Clinical benefit | P-value | PFS, median (95% CI) | HR (95% CI) | P-value univariate/ multivariate | OS median (95% CI) | HR (95% CI) | P-value univariate/ multivariate |
|---|---|---|---|---|---|---|---|---|---|---|---|
| rs576523 | AA | 34% | | 89% | | 7.6 (6.8-8.4) | *ref* | | 19.6 (17.5-21.8) | *ref* | |
| *(intergenic)* | AG | 67% | | 67% | | 8.9 (0-19.6) | 2.04 (0.66-3.46) | | 14.8 (0.0-34.1) | 1.19 (0.00-2.63) | |
| | GG | NA | *0.275* | NA | *0.292* | NA | - | *0.206/0.327* | NA | | *0.604/0.812* |
| rs11722476 | GG | 37% | | 89% | | 6.8 (5.4-8.3) | ref | | 17.7 (14.3-21.1) | *ref* | |
| *(SMARCAD1)* | GA | 31% | | 90% | | 7.8 (7.0-8.6) | 1.26 (0.82-1.70) | | 21.4 (18.3-24.5) | 1.29 (0.83-1.74) | |
| | AA | 44% | *0.317* | 88% | *0.949* | 6.7 (4.2-9.3) | 1.16 (0.74-1.58) | *0.953/0.578* | 20.0 (17.0-23.1) | 1.29 (0.79-1.77) | *0.870/0.494* |
| rs4702484 | CC | 36% | | 89% | | 7.5 (6.4-8.5) | *ref* | | 19.5 (17.1-21.8) | *ref* | |
| *(ADCY2)* | CT | 31% | | 90% | | 7.8 (5.9-9.6) | 1.2 (0.89-1.55) | | 19.8 (14.0-25.5) | 1.22 (0.87-1.57) | |
| | TT | NA | *0.539* | NA | *1.000* | NA | NA | *0.351/0.229* | NA | | *0.759/0.271* |
| rs1801394 | GG | 36% | | 90% | | 8.1 (6.8-9.4) | ref | | 21.6 (17.6-25.5) | *ref* | |
| *(MTRR)* | GA | 34% | | 91% | | 6.8 (5.9-7.8) | 1.12 (0.71-1.53) | | 19.4 (17.2-21.7) | 0.86 (0.42-1.30) | |
| | AA | 35% | *0.953* | 81% | *0.223* | 8.3 (6.7-9.9) | 0.96 (0.57-1.34) | *0.617/0.614* | 21.0 (16.7-25.3) | 0.90 (0.50-1.30) | *0.799/0.794* |
| rs1532268 | GG | 37% | | 84% | | 6.8 (5.5-8.1) | *ref* | | 19.8 (16.6-22.9) | *ref* | |
| *(MTRR)* | GA | 31% | | 91% | | 8.0 (6.8-9.2) | 0.85 (0.44-1.27) | | 18.9 (17.4-20.4) | 1.17 (0.72-1.62) | |
| | AA | 39% | *0.537* | 97% | *0.054* | 7.0 (4.7-9.2) | 0.78 (0.38-1.19) | *0.134/0.493* | 21.6 (16.4-26.8) | 1.35 (0.92-1.78) | *0.961/0.352* |
| rs162036 | AA | 35% | | 91% | | 7.9 (7.1-8.7) | ref | | 19.5 (17.0-22.0) | ref | |
| *(MTRR)* | AG | 32% | | 85% | | 6.5 (5.5-7.5) | 1.21 (0.45-1.97) | | 18.7 (14.9-22.4) | 1.20 (0.41-1.99) | |
| | GG | 33% | *0.934* | 67% | *0.039* | 6.6 (6.3-7.0) | 1.51 (0.71-2.32) | *0.498/0.380* | 26.0 (0.0-66.5) | 1.39 (0.55-2.23) | *0.653/0.619* |
| rs10380 | CC | 36% | | 90% | | 7.8 (7.0-8.5) | *ref* | | 19.5 (17.3-21.6) | *ref* | |
| *(MTRR)* | CT | 28% | | 85% | | 6.2 (5.6-6.9) | 0.85 (0.48-1.22) | | 21.0 (15.9-26.2) | 0.88 (0.51-1,26) | |
| | TT | NA | *0.391* | NA | *0.300* | NA | NA | *0.339/0.402* | NA | NA | *0.837/0.532* |
| rs8101143 | AA | 33% | | 89% | | 7.0 (5.9-8.1) | *ref* | | 21.9 (18.2-25.6) | *ref* | |
| *(intergenic)* | AG | 32% | | 88% | | 8.0 (6.7-9.3) | 1.35 (0.87-1.84) | | 19.5 (18.2-20.7) | 1.23 (0.69-1.77) | |
| | GG | 50% | *0.242* | 92% | *0.886* | 8.3 (5.7-11.0) | 1.18 (0.68-1.67) | *0.597/0.379* | 21.2 (2.7-39.8) | 1.46 (0.91-2.01) | *0.412/0.308* |

*Table 3. Association between genetic variants and efficacy of capecitabine in the total population*

CI, confidence interval; HR, Hazard ratio; NA, no patients carried this genotype; PFS, progression free survival; OS, overall survival.

| Variant | | Objective response | P-value | Clinical benefit | P-value | PFS, median (95% CI) | HR (95% CI) | P-value univariate/ multivariate | OS. median (95% CI) | HR (95% CI) | P-value univariate/ multivariate |
|---|---|---|---|---|---|---|---|---|---|---|---|
| rs576523 | AA | 20% | | 85% | | 6.3 (5.8-6.8) | *ref* | | 19.5 (16.7-22.2) | *ref* | |
| *(intergenic)* | AG | 100% | | 100% | | 8.9 ( - ) | 2.98 (0.96-5.00) | | 14.8 ( - ) | 2.19 (0.14-4.24) | |
| | GG | NA | 0.044 | NA | *1.000* | NA | NA | *0.083/0.290* | NA | NA | *0.201/0.454* |
| rs11722476 | GG | 23% | | 86% | | 6.1 (5.5-6.8) | *ref* | | 14.7 (7.8-21.5) | *ref* | |
| *(SMARCAD1)* | GA | 18% | | 85% | | 6.6 (3.7-9.5) | 1.13 (0.51-1.76) | | 19.4 (15.0-23.8) | 0.91 (0.22-1.59) | |
| | AA | 29% | 0.578 | 86% | *0.991* | 6.3 (5.7-6.9) | 0.98 (0.39-1.56) | *0.889/0.812* | 21.6 (17.6-25.6) | 1.02 (0.40-1.65) | *0.881/0.893* |
| rs4702484 | CC | 20% | | 84% | | 6.2 (5.6-6.7) | *ref* | | 19.2 (15.1-23.2) | *ref* | |
| *(ADCY2)* | CT | 24% | | 90% | | 8.0 (6.2-9.8) | 1.75 (1.25-2.25) | | 22.1 (15.6-28.6) | 1.56 (1.04-2.08) | |
| | TT | NA | 0.795 | NA | *0.559* | NA | NA | *0.018/0.029* | NA | NA | *0.457/0.096* |
| rs1801394 | GG | 22% | | 88% | | 6.6 (4.5-8.7) | *ref* | | 19.5 (11.9-27.0) | *ref* | |
| *(MTRR)* | GA | 17% | | 85% | | 6.1 (5.2-7.1) | 1.20 (0.58-1.82) | | 18.9 (15.6-22.1) | 1.42 (0.78-2.07) | |
| | AA | 33% | 0.259 | 80% | *0.642* | 8.3 (7.9-8.7) | 1.00 (0.44-1.56) | *0.616/0.729* | 22.1 (14.3-29.9) | 1.14 (0.56-1.71) | *0.816/0.516* |
| rs1532268 | GG | 27% | | 82% | | 6.6 (4.7-8.5) | *ref* | | 21.4 (17.2-25.5) | *ref* | |
| *(MTRR)* | GA | 18% | | 86% | | 6.1 (5.4-6.8) | 0.91 (0.21-1.61) | | 18.1 (13.7-22.5) | 1.26 (0.51-2.02) | |
| | AA | 21% | 0.515 | 93% | *0.612* | 6.1 (4.8-7.4) | 0.97 (0.32-1.62) | *0.915/0.948* | 19.8 (4.9-35.7) | 1.56 (0.86-2.27) | *0.808/0.396* |
| rs162036 | AA | 17% | | 86% | | 6.3 (5.4-7.1) | *ref* | | 18.1 (13.9-22.3) | ref | |
| *(MTRR)* | AG | 27% | | 85% | | 6.2 (5.4-6.9) | 1.97 (0.87-3.08) | | 24.1 (18.1-30.1) | 1.46 (0.39-2.53) | |
| | GG | 40% | 0.253 | 80% | *0.943* | 6.6 (6.4-6.9) | 2.59 (1.44-3.73) | *0.440/0.227* | 26.0 (0-55.8) | 1.56 (0.43-2.68) | *0.543/0.744* |
| rs10380 | CC | 21% | | 85% | | 6.3 (5.7-7.0) | *ref* | | 18.9 (15.0-22.7) | *ref* | |
| *(MTRR)* | CT | 23% | | 87% | | 6.2 (5.5-6.9) | 0.77 (0.25-1.29) | | 24.1 (18.3-30.0) | 0.90 (0.36-1.43) | |
| | TT | NA | 0.805 | NA | *1.000* | NA | NA | *0.876/0.330* | NA | NA | *0.720/0.681* |
| rs8101143 | AA | 20% | | 86% | | 6.1 (5.0-7.1) | *ref* | | 18.1 (10.5-25.6) | *ref* | |
| *(intergenic)* | AG | 21% | | 81% | | 6.6 (4.5-8.7) | 1.38 (0.61-2.15) | | 19.5 (16.7-22.2) | 1.00 (0.15-1.86) | |
| | GG | 33% | 0.582 | 92% | *0.630S* | 6.8 (3.5-10.2) | 1.23 (0.47-2.00) | *0.560/0.690* | 29.0 (15.2-42.8) | 1.17 (0.33-2.02) | *0.649/0.799* |

*Table 4. Association between genetic variants and efficacy of capecitabine in patients treated with capecitabine monotherapy*

CI, confidence interval; HR, Hazard Ratio; NA, no patients carried this genotype; PFS, progression free survival; OS, overall survival.

*Association with OS*

None of the genetic markers showed interaction with OS in our data set. OS for rs4702484 was 19.5 months for homozygote wildtype (95% CI: 17.1-21.8 months) and 19.8 months (95% CI: 14.0-25.5 months) for heterozygotes (P = *0.759*) in the total study population; and 19.2 (95% CI: 11.9-27.0) versus 22.1 months (95% CI: 15.6-28.6) for the capecitabine monotherapy group (P univariate = *0.457*, P multivariate = *0.096*; Figure 3B).

When the capecitabine monotherapy group was evaluated separately, results for PFS and OS remained similar.(Table 4)

*Association with radiologic response*

Regarding radiologic response to capecitabine, no association with genotype was found for any of the selected SNPs. No effect of genotype was present, whether objective response was used as the outcome measure, or clinical benefit.(Table 3)

A trend toward significant results was found for the association with clinical benefit of rs1533268 (GG vs. GA vs. AA: 84% vs. 91% vs. 97%; P = *0.054*) and of rs162036 (AA vs. AG vs. GG: 91% vs. 85% vs. 67%; P = *0.039*). When both treatment arms were evaluated separately, results remained statistically significant for patients receiving capecitabine-irinotecan combination treatment (rs1533268: GG vs. GA vs. AA: 85% vs. 97% vs. 100%; P = *0.023*; and rs162036: AA vs. AG vs. GG: 96% vs. 85% vs. 50%; P = *0.001,* data not shown), but not for patients receiving capecitabine monotherapy (rs1533268: GG vs. GA vs. AA: 82% vs. 86% vs. 93%; P = *0.612*; and rs162036: AA vs. AG vs. GG: 86% vs. 85% vs. 80%; P = *0.943*).

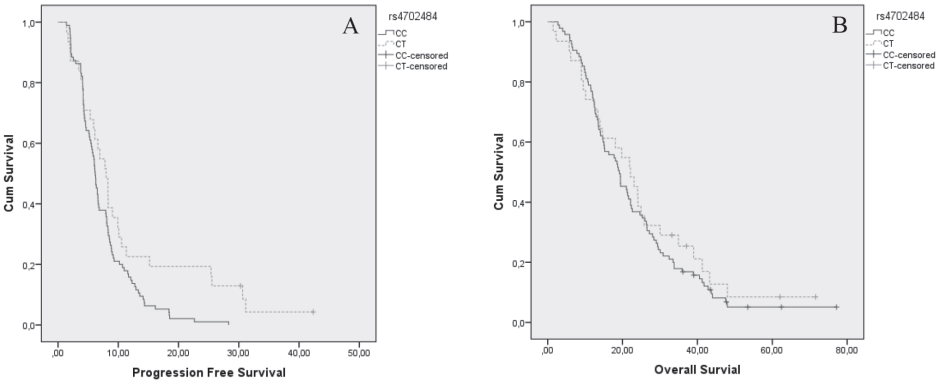


*Figure 3. Kaplan-Meijer survival curves for patients receiving capecitabine first-line monotherapy, according to genotype for rs4702484*

**A.** Progression free survival; **B.** Overall survival.

## Discussion

We designed this clinical pharmacogenetic association study to validate if a specific selection of SNPs implicated in recent pharmacogenetic papers was associated with efficacy of capecitabine in a large cohort of mCRC patients, treated with first-line capecitabine-based chemotherapy. The genetic markers were carefully chosen based on reports from previous studies.[8;11] In our evaluation of eight selected SNPs, we found a small, borderline significant effect of rs4702484 on PFS in a subgroup of patients treated with capecitabine monotherapy. However, none of the other genetic variants showed significant association with capecitabine efficacy, neither in the total study population, nor in patients receiving capecitabine monotherapy.

Rs4702484, located *intronic* in the gene encoding for adenylate cyclase type 2 (*ADCY2*) and 200kbp upstream of *MTRR*, was first implicated in capecitabine chemosensitivity in a recent GWA report by O'Donnell and co-workers.[8] Consistent with their results, we found PFS for patients carrying the rs4702484 heterozygous genotype was marginally better than for patients carrying the homozygous wildtype genotype, but only if they were treated with capecitabine monotherapy. As this did not translate, however, into a statistically significant overall survival benefit, the clinical implications of these findings remain uncertain.

Despite the positive result for rs4702484, we could not confirm an effect on capecitabine chemosensitivity for the other SNPs from the GWA study in our patient population. Replication of GWA results is subject to statistical difficulty. Genome wide studies tend to overestimate the effect of the associated SNPs and these extreme results will be closer to the average when replicated in a second measurement.[17] To replicate these inflated results, large population sizes are necessary. Our patient sample is relatively small and lack of power may explain our inability to replicate the results found by O'Donnell et al.[8] For most SNPs, however, median values and confidence intervals are almost identical between genotype groups, without a trend towards an effect for the genetic markers. We therefore believe that increasing population size would not have led to substantially different results.

Furthermore, although the use of cell lines allows for analyses that would be unethical or infeasible in humans, it has certain disadvantages. Many pharmacokinetic influences, both genetic and non-genetic, are excluded. To partly circumvent this problem, the capecitabine metabolite 5'-deoxy-5-fluorouridine (5'DFUR) was used for the cited *in vitro* GWA study.[8] Although the impact of genetic variation in carboxylesterase (CES) and cytidine deaminase (CDD), both involved in the conversion of capecitabine to 5'DFUR *in vivo*, is still unclear[18;19], this may also partly explain the lack of replication in our patient group. In addition, tissue specific and tumor specific characteristics are eliminated when non-malignant cell lines, such as LCLs, are used.

We also studied four SNPs within *MTRR*. This gene has been implicated in the development of colorectal cancer[11] and is located in proximity to rs4720484. In our population of mCRC patients, the selection of four *MTRR* polymorphisms was not associated with capecitabine efficacy, which is consistent with results of another study showing no relation to efficacy of

adjuvant treatment with 5-FU in colorectal cancer patients.[20] Although a trend towards a significant effect was found for the association of rs1532268 and rs162036 with clinical benefit of capecitabine in combination therapy, this is probably due to statistical error associated with the small number of patients carrying the minor allele and with bias induced by multiple testing. Furthermore, since the effect is only present in patients treated with capecitabine-irinotecan combination therapy, this would imply an effect of this SNP on irinotecan, rather than capecitabine efficacy.

In designing this study, we hypothesized that *MTRR* could be important in capecitabine efficacy for two reasons. Firstly, MTRR as part of the folate pathway is involved in fluoropyrimidine pharmacodynamics (see also Figure 1). However, the relationship of fluoropyrimidine sensitivity to genetic variation in other components of this pathway, such as methylene tetrahydrofolate reductase (*MTHFR*), has not been confirmed[12;15;21], making an effect of *MTRR* genetic variation questionable. Secondly, polymorphisms in *MTRR* have been associated with colorectal carcinogenesis and as such may also influence disease prognosis. Whereas some authors described that cancer susceptibility genes show prognostic or predictive value in colorectal cancer patients[22;23], most studies found no correlation between these genes and survival of colorectal cancer patients, whether they were treated with chemotherapy[24] or not.[25;26] Furthermore, although two *MTRR* variants (*MTRR A66G*, rs1801394; and *MTRR C1793T*, rs10380) were associated with increased colorectal cancer risk in a case control study[11], these results were not replicated consistently in meta-analyses.[9;27] Based on these considerations, we believe our results should be seen as evidence that variation in *MTRR* is not essential in capecitabine chemosensitivity.

Genetic effects on drug metabolism have been recognized for decades.[28] Nevertheless, in today's practice, only few genetic markers have been integrated in algorithms for therapeutic control. The Pharmacogenetics Working Group of the Royal Dutch Pharmacists Association has provided pharmacogenetics-based therapeutic recommendations for 53 drugs, related to eleven genes.[29;30] Before implementation into clinical practice, extensive validation of genetic markers in varied patient groups is warranted. Validation studies like the present contribute to the conscientious transfer of basic research results into clinical practice. Although we believe the selected markers are not useful as pretreatment biomarkers of capecitabine efficacy in colorectal cancer, we cannot exclude an effect of some of these SNPs in other types of cancer and with other 5-FU derivatives. Further research therefore remains necessary.

## Acknowledgments

We thank the following CAIRO investigators for participating in the pharmacogenetic side-study:
J van der Hoeven-Amstelveen; D Richel, B de Valk- Amsterdam; J Douma-Arnhem; P Nieboer-Assen; F Valster-Bergen op Zoom; G Ras, O. Loosveld-Breda; D Kehrer-Capelle a/d Ijssel; M

Bos-Delft; H Sinnige, C Knibbeler-Den Bosch; W van Deijk, H Sleeboom-Den Haag; E Muller-Doetinchem; E Balk-Ede; G Creemers-Eindhoven; R de Jong-Groningen; P Zoon-Harderwijk; J Wals-Heerlen; M Polee-Leeuwarden; M Tesselaar-Leiden; R Brouwer-Leidschendam; P de Jong-Rotterdam; G Veldhuis-Sneek; D ten Bokkel Huinink-Utrecht; A van Bochove-Zaandam.

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