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## Combinations of Polymorphisms in Genes Involved in the 5-Fluorouracil Metabolism Pathway Are Associated with Gastrointestinal Toxicity in Chemotherapy-Treated Colorectal Cancer Patients

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### Abstract

**Purpose:** The purpose of this study was to investigate whether specific combinations of polymorphisms in genes encoding proteins involved in 5-fluorouracil (5-FU) pharmacokinetics and pharmacodynamics are associated with increased risk of treatment-induced toxicity.

**Experimental Design:** We analyzed two cohorts of 161 and 340 patients, the exploration and validation cohort, respectively. All patients were treated similarly with 5-FU-based adjuvant chemotherapy. We analyzed 13 functional polymorphisms and applied a four-fold analysis strategy using individual polymorphisms, haplotypes, and phenotypic enzyme activity or expression classifications based on combinations of functional polymorphisms in specific genes. Furthermore, multifactor dimensionality reduction analysis was used to identify a genetic interaction profile indicating an increased risk of toxicity.

**Results:** Alleles associated with low activity of methylene tetrahydrofolate reductase (*MTHFR*) were associated with decreased risk of toxicity [OR<sub>Exploration</sub> 0.39 (95% CI: 0.21–0.71, *P* = 0.003), OR<sub>Validation</sub> 0.63 (95% CI: 0.41–0.95, *P* = 0.03)]. A specific combination of the *MTHFR* 1298A>C and thymidylate synthase (*TYMS*) 3'-UTR (untranslated region) ins/del polymorphisms was significantly associated with increased toxicity in both cohorts [OR<sub>Exploration</sub> 2.40 (95% CI: 1.33–4.29, *P* = 0.003), OR<sub>Validation</sub> 1.81 (95% CI: 1.18–2.79, *P* = 0.007)]. The specific combination was also associated with increased cumulative incidence and earlier occurrence of severe toxicity during treatment.

**Conclusions:** Our results indicate that *MTHFR* activity and a specific combination of the *MTHFR* 1298A>C and *TYMS* 3'-UTR ins/del polymorphisms are possible predictors of 5-FU treatment-related toxicity. *Clin Cancer Res*; 17(11); 1–8. ©2011 AACR.

### Introduction

5-Fluorouracil (5-FU)-based adjuvant therapies for colorectal cancer reduce the risk of relapse and prolongs survival (1). A major issue is toxicity from therapy, which

may lead to adverse symptoms, incur delay in treatment, dose reductions, or premature cessation of treatment.

Theoretically, systemic toxicity is increased by constitutive factors that decrease 5-FU elimination or increase 5-FU activity in nontumor tissues. This is evidenced by several studies showing that factors influencing 5-FU clearance, such as sex, age, and dihydropyrimidine dehydrogenase (*DPYD*) activity, also influence the risk of toxicity (2–4). The type and severity of toxicity also depend on dose and mode of application (5).

In addition to treatment and demographic factors, genetic variation may be an important contributor to the interindividual differences in experienced toxicity. Many of the genes that encode proteins involved in 5-FU metabolism contain polymorphisms that change their enzymatic activity or expression levels. Several studies have implicated genetic polymorphisms in thymidylate synthase (*TYMS*), methylene tetrahydrofolate reductase (*MTHFR*), and *DPYD* as risk factors for severe toxicity (3, 4, 6–19) and a few studies have also implicated orotate phosphoribosyl transferase (*OPRT*) and *VEGFA* polymorphisms (15, 17, 20). The results have been contradictory, probably due to

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### Translational Relevance

This study examined the association of specific combinations of genetic polymorphisms in the *DPYD*, *MTHFR*, *OPRT*, *VEGFA*, and *TYMS* genes, which encode proteins involved in the pharmacokinetics and dynamics of 5-fluorouracil, with treatment-related toxicity in 2 patient populations: an exploration cohort and a validation cohort. Genetically determined low activity of *MTHFR* was associated with decreased risk of toxicity, whereas a genetic interaction profile consisting of the *MTHFR* 1298A>C and *TYMS* 3'-UTR (untranslated region) ins/del polymorphisms was significantly associated with increased toxicity. Treatment-related toxicity is a major cause of suboptimal treatment in cancer, and pretreatment risk stratification would improve treatment quality considerably. This study represents one of the few validated studies of combinatorial effects of functional polymorphisms. The findings may have important clinical implications because genotyping is highly reproducible, accurate, and easily implemented compared with other more complicated profiling methods, e.g., mRNA expression profiling.

treatment differences, different patient populations, inadequate statistical analyses, and chance findings.

Most of these studies investigate the association of individual polymorphisms with severe toxicity, whereas the associations of haplotypes (7) and genetic interactions with 5-FU toxicity have largely been overlooked, partly due to lack of reliable methods to study genetic interactions.

Theoretically, the 5-FU metabolic phenotype is better explained by multigene- and pathway-oriented analysis rather than single gene analysis.

The purpose of this study was to investigate whether individual polymorphisms, haplotypes, phenotypic classifications based on functional polymorphisms, and specific gene-gene interactions were associated with increased risk of toxicity in adjuvant 5-FU-based chemotherapy of colorectal cancer.

We used 2 independent cohorts treated with adjuvant 5-FU-based chemotherapy; 1 cohort for exploratory analyses and another cohort for validating the exploratory analyses. The primary endpoint was severity of gastrointestinal toxicity.

## Patients and Methods

### Populations

**Exploration cohort.** This cohort consisted of prospectively collected patients from Italy (19). The 161 patients were recruited from 1999 to 2005 and follow-up ended 2008. DNA was isolated from peripheral blood mononuclear cells. All patients were Dukes' stage B2 and C and all patients were treated with surgery and the Mayo regimen (Folinic Acid 20 mg/m<sup>2</sup>, 5-FU 425 mg/m<sup>2</sup>/day, days 1–5, every 28 days, 6 cycles). If toxicity of grade more

than 2 occurred, the next doses were reduced by 25% to 50% or stopped, depending on toxicity level. The protocol allowed for dose reduction by 25% in the oldest patients. The local ethics committees approved the study and each patient provided written informed consent.

**Validation cohort.** A total of 340 Caucasian patients with Dukes' stage B2, C, and D treated at Rigshospitalet (Copenhagen University Hospital, Denmark) with surgery and the Mayo regimen (Isovorin 10 mg/m<sup>2</sup>, 5-FU 425 mg/m<sup>2</sup>/day, days 1–5, every 28 days, 6 cycles) from 1996 to 2003 were retrospectively included (18). DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tumor specimens, containing at least 50% tumor tissue. Clinical data and tumor pathology was reviewed retrospectively as previously described (18). Last follow-up date was August 30, 2007. The local ethics committee approved this study (protocol H-KF-01-201/03, amendment 18370).

**Toxicity grading.** In the exploration, cohort toxicity was recorded and graded according to World Health Organization (WHO) criteria (21) and in the validation cohort toxicity was recorded according to the Common Toxicity Criteria (CTC; National Cancer Institute, Common Toxicity Criteria version 2.0, [http://ctep.cancer.gov/protocoldevelopment/electronic\\_applications/docs/ctcv20\\_4-30-992.pdf](http://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcv20_4-30-992.pdf)). Toxicity was dichotomously classified into none-to-moderate (grades 0–2) and severe (grades 3 and 4) toxicity for the multifactor dimensional reduction method.

The type of toxicities included were stomatitis/pharyngitis, nausea/vomiting, and diarrhea, these were transformed into a gastrointestinal toxicity score by taking the maximum toxicity experienced in any of the categories. This translated into a score going from 0 to 4 on both scales (Supplementary Table S1).

**Genotyping.** Extraction and genotyping methods for tissue and samples have been described elsewhere (18, 19).

We selected 13 polymorphisms for analysis (Table 1). DNA samples were analyzed for single nucleotide polymorphisms (SNP) using the fluorogenic 5'-nuclease assay (TaqMan\_SNP Genotyping Assay Made to Order on an ABI 7900 HT, Applied Biosystems). Genotypes were determined in a reaction mix as follows: 25  $\mu$ L containing 10 ng DNA, 14  $\mu$ L primer/probe mix with TaqMan Universal PCR Master mix (Applied Biosystems) according to the manufacturer's instructions. PCR amplification was carried out with an initial step of 95°C for 10 minutes followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute (Applied Biosystems 7900HT Sequence Detection System). The fluorescence profile of each well was measured in an Applied Biosystems 7900HT Sequence Detection System, and the results were analyzed with Sequence Detection Software (SDS 2.3, Applied Biosystems). Positive and negative controls with known genotypes were included on each plate. Reproducibility was checked by re-genotyping 10% of the samples, which showed a 100% agreement. *TYMS* was genotyped using RFLP methods as described previously (19). The greater number of failed genotyping

**Table 1.** Polymorphisms included in this work

Gene (location)	rs ID	Alleles	Protein/mRNA	Functional effects
<i>MTHFR</i> (1p36.3)	1801133	c.665C>T (c.677C>T)	Ala222Val	Decreased activity
	1801131	c.1286A>C (c.1298A>C)	Glu429Ala	Decreased activity
<i>DPYD</i> (1p22)	3918290 (DPYD2a)	c.1905+1G>A	Del (exon 14)	No activity
	1801265 (DPYD9a)	c.85T>C	Cys29Arg	Decreased activity?
	2297595	c.496A>G	Met166Val	Decreased activity
	1801159 (DPYD5)	c.1627A>G	Ile543Vval	Decreased activity?
<i>OPRT</i> (UMPS) (3q13)	1801019	c.638G>C	Gly213Ala	Increased activity
	3772809	c.1336A>G	Ile446Val	Decreased activity?
<i>VEGFA</i> (6p12)	3025039	g.19584C>T (936C>T)	3'-UTR	Decreased expression
	833061	g.4534C>T (-460 T>C)	Promoter region	Increased expression
<i>TYMS</i> (18p11.32)	45445694	(CCGCGCCAC TTGGCCTG CCTCCGTCCCG) 2/3/4/7/8/9 (vast majority 2-3 repeats)	5'-UTR VNTR 28 bp, (mRNA start pos 43)	Increased expression with increasing number of repeats
	34743033*	(CCGCGCCACTTCG CCTGCCTCCGT CCCG) 2/3/4	5'-UTR G > C 12th bp in VNTR repeat	C-allele: decreased transcription
	34489327	-/TTAAAG	3'-UTR (mRNA start pos 1530)	Deletion: decreased expression

attempts in the validation cohort was due to DNA being isolated from FFPE tissue in contrast to the exploration cohort where DNA was isolated from peripheral blood mononuclear cells.

**Phenotypic classification of enzyme activity based on functional polymorphisms.** Two common *MTHFR* SNPs cause reduced enzyme activity in homozygous individuals (22–24): the *MTHFR* 677C>T polymorphism induces an Ala-to-Val substitution in the catalytic domain (70% reduction in activity), whereas the *MTHFR* 1298A>C polymorphism induces a Glu-to-Ala substitution in a regulatory domain (30%–40% reduction in activity). Compound heterozygosity leads to a 40% to 50% reduction in enzyme activity (23, 24). Based on the presence or absence of both variant alleles *MTHFR* activity was defined as normal or low, where the presence of 2 or more variant alleles was defined as low activity (Table 2).

Based on the variable number of tandem repeats (VNTR), G>C SNP in the 5'-UTR (VNTR SNP) and the 3'-UTR 6 bp ins/del polymorphism *TYMS* expression was classified as high, intermediate, or low expression (Table 2). Studies have shown that the *TYMS* VNTR and an insertion/deletion polymorphism are associated with mRNA stability and *TYMS* expression, with the 3 repeat and insertion alleles, respectively, being associated with increased *TYMS* expression, whereas the G>C base change in the second VNTR repeat decreases the transcriptional activity of the *TYMS* gene (25–27).

**Statistics.** For univariable and multivariable analyses, several ordinal logistic regression models were considered with toxicity grade as an ordinal response variable increasing from grade 0 to 4. The ordinal logistic regression (OLR) models were computed under the proportional odds assumption and therefore this assumption was formally tested before applying the OLR model. This is equivalent to fit several binary logistic regression models simultaneously with common regression coefficients but possibly different intercepts that depend on whether the toxicity grade is  $i$  or more ( $i = 0-3$ ). In the exploration cohort, none of the clinical factors were significantly associated to outcome. Therefore, an OLR model adjusted for age and sex, which in a meta-analysis have been shown to be associated with toxicity (5), was used to confirm associations. Assuming a prevalence of 40% of risk genotypes, a 40% incidence of severe gastrointestinal toxicity, 80% power and a sample size of 160, then the detectable OR is 2.5.

The Kaplan–Meier and the log-rank tests were adopted to estimate and compare cumulative incidences of severe toxicity. Multiple regression analysis of the independent effects of the molecular markers on cumulative incidence of severe toxicity was carried out using the Cox Proportional Hazards model. Mann–Whitney  $U$  test,  $\chi^2$  test, or Fisher's exact test were applied to assess differences or proportions of clinical and genetic data.

The multifactor dimensionality reduction method, a nonparametric method, was used to study interaction

**Table 2.** Phenotypic classification of MTHFR activity and TYMS expression based on polymorphisms

	Activity level	MTHFR 677 <sup>a</sup> + MTHFR1298 <sup>b</sup>
MTHFR	Normal	CC + AA or AC CT+ AA
	Low	TT + AA or AC or CC CT + AC or CC CC + CC
	Expression level	TYMS 5'-UTR + TYMS 3'-UTR
TYMS	High	2G/3G or 3G/3G or 3C/3G + ins/ins 3G/3G + ins/del
	Intermediate	3G/3G + del/del 2G/3G or 3C/3G + ins/del 2G/2G or 3C/3C or 2G/3C + ins/ins
	Low	2/2 or 2/3 or 3C/3C or 3C/3G + del/del 2/2, 3C/3C, 2G/3C + ins/del

<sup>a</sup>The wildtype genotype for MTHFR677 is CC.

<sup>b</sup>The wildtype genotype for MTHFR1298 is AA.

between the polymorphisms in relation to toxicity (28). This method is implemented in the MDR software, which was used for interaction analyses (version 2.0 beta 8). Toxicity was dichotomized in to none-to-moderate (grades 0–2) and severe toxicity (grades 3 and 4). Patients with missing data for polymorphisms were excluded from the analysis. In the genetic interaction analysis, the ratio between patients with nonsevere toxicity to patients with severe toxicity for each genotype combination was evaluated; if patients with severe toxicity were overrepresented compared with patients with nonsevere toxicity, then these genotype combinations were considered to be associated with a high risk of toxicity development. This procedure was carried out across 10-fold cross-validation samples to avoid overfitting and was repeated for all possible combinations of up to 3 polymorphisms. The genotype combination with the highest test sample accuracy (fraction of correctly classified patients) was considered the combination that best predicted toxicity and was selected for further analysis. A *P*-value for the statistical significance of the accuracy was obtained using 10,000-fold permutation testing (<https://sourceforge.net/projects/mdr/files/mdrpt/>).

Haplotype associations were tested using FamHap software specifically designed to test haplotype and diplotype associations with outcome as well as haplotype interactions and haplotype inference (29, 30). Linkage disequilibrium between the relevant polymorphisms and Hardy-Weinberg (HW) equilibrium was calculated using Haploview v4.2 (31, 32).

Any positive associations, be it individual polymorphisms or haplotypes, functional classifications based on individual polymorphisms or genetic interactions, were tested in the exploration and validation cohorts with OLR models to get interpretable effect measures alongside *P*-values.

OLR modeling and all other analyses, except MDR and Haplotype analyses, were carried out using the SAS Statistical Package Version 9.2 (SAS Institute Inc.).

## Results

### Population characteristics and genotyping

Comparing the 2 cohorts showed that the patients in the validation cohort were younger, with more advanced disease and there were a higher proportion of rectal cancer patients (Table 3).

*DPYD 1905 + 1G>A* was excluded from the analysis due to too low allele frequency with zero variant alleles in the exploration cohort. The genotype distribution of *MTHFR677*, *DPYD 85T>C*, and the *TYMS* polymorphisms were different between the 2 cohorts (Supplementary Table S1). All the polymorphisms were in HW equilibrium in the exploration cohort.

There was linkage disequilibrium between *MTHFR677* and *MTHFR1298* (Exp:  $D' = 1.00$ ,  $r^2 = 0.38$ , Val:  $D' = 0.97$ ,  $r^2 = 0.19$ ) and to a lesser degree between *DPYD 85T>C* and *DPYD 496A>G* (Exp:  $D' = 0.67$ ,  $r^2 = 0.25$ , Val:  $D' = 0.69$ ,  $r^2 = 0.17$ ) and between the *TYMS* VNTR and ins/del polymorphisms (Exp:  $D' = 0.66$ ,  $r^2 = 0.22$ , Val:  $D' = 0.19$ ,  $r^2 = 0.02$ ).

### Genotype and haplotype associations

None of the genotypes were individually associated with increasing toxicity in both cohorts (Supplementary Table S2). Phenotypic classification of MTHFR enzyme activity was associated with gastrointestinal toxicity events (Table 4). Low activity of MTHFR seems to be protective against gastrointestinal toxicity in both cohorts when adjusted for age and sex [OR<sub>Exploration</sub> 0.39 (95% CI: 0.21–0.71), OR<sub>Validation</sub> 0.63 (95% CI: 0.41–0.95)]. In relation to specific toxicities, the only significant association was with decreased severe stomatitis/pharyngitis OR<sub>Exploration</sub> 0.40 (95% CI: 0.20–0.80,  $P = 0.01$ ) in the exploration cohort but low activity is also protective against severe diarrhea [OR<sub>Exploration</sub> 0.48 (95% CI: 0.18–1.26), OR<sub>Validation</sub> 0.62 (95% CI: 0.33–1.15)] in both cohorts (Supplementary Table S4).

The phenotypic classification of *TYMS* expression was not associated with toxicity (Table 4).

Haplotype analysis did not reveal any associations with severe toxicity (data not shown), whether using the haplotypes based on tightly linked loci or any other haplotypes.

### Gene–gene interactions

We found a gene–gene interaction between the *MTHFR* 1298 SNP and the *TYMS* 3'-UTR polymorphism with a maximum accuracy of 0.70 (10,000-fold permutation

**Table 3.** Population characteristics, genotypes, and associations with toxicity

	Cohort characteristics			Univariate association with toxicity			
	Exploration	Validation	<i>P</i>	Exploration cohort		Validation cohort	
				OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
Age (median, range)	66.7 (34–87)	61.4 (20–85)	<0.001	1.00 (0.98–1.03)	0.71	1.00 (0.98–1.02)	0.88
Sex			<0.001		0.15		0.04
Female	54	169		1.55 (0.86–2.82)		1.51 (1.03–2.24)	
Male	107	171		Reference		Reference	
Stage			<0.001		0.67		0.57
II	60	37		Reference		Reference	
III	101	265		1.13 (0.64–2.02)		0.80 (0.43–1.50)	
IV	0	38		–		0.64 (0.28–1.46)	
Tumor site			<0.001		0.74		0.92
Colon	154	272		Reference		Reference	
Rectum	7	68		0.80 (0.20–3.10)		0.97 (0.60–1.58)	
Maximum toxicity <sup>a</sup>			<0.001				
0	18	18					
1	33	95					
2	47	134					
3	43	88					
4	18	5					

<sup>a</sup>The toxicity was measured on the WHO toxicity scale for the exploration cohort, whereas the CTC criteria were used for the validation cohort. Only gradings regarding gastrointestinal toxicity are included as defined in the Patients and Methods section.

testing,  $P < 0.001$ ). This genotype combination was also associated with toxicity in the validation cohort (Fig. 1, Table 4). This association was confirmed in OLR models and the association remained both in univariate and

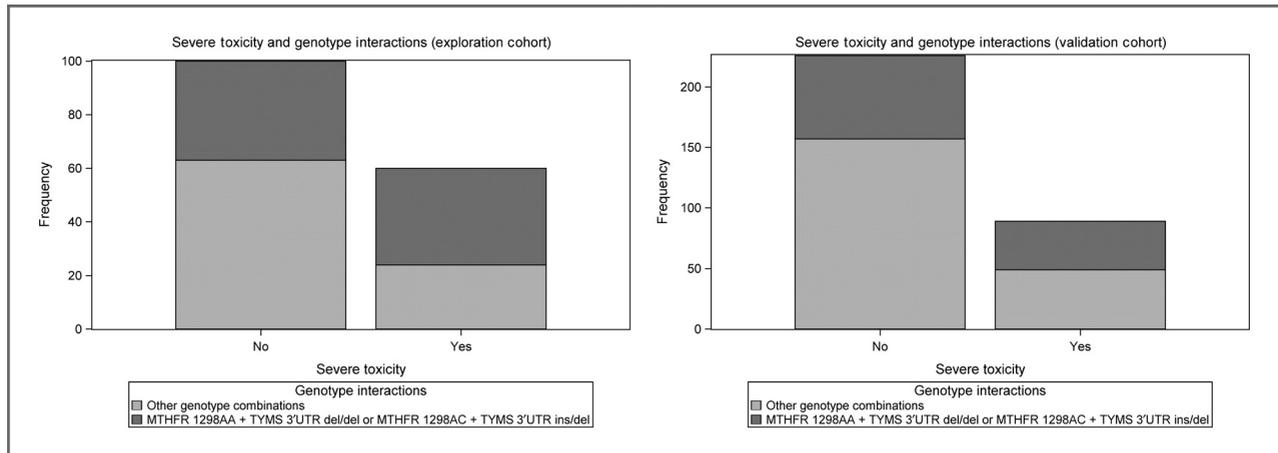
multiple variable analyses adjusted for age and sex [ $OR_{\text{Exploration}} 2.40$  (95% CI: 1.33–4.29),  $OR_{\text{Validation}} 1.81$  (95% CI: 1.18–2.79)]. In relation to specific toxicities, this MDR-derived genotype interaction was

**Table 4.** Association with gastrointestinal toxicity of phenotypic classifications and gene–gene interactions using ordinal logistic regression

	Exploration cohort <sup>a</sup>			Validation cohort <sup>a</sup>		
	<i>n</i> <sup>b</sup>	OR (95% CI)	<i>P</i>	<i>n</i> <sup>b</sup>	OR (95% CI)	<i>P</i>
MTHFR activity			0.002			0.03
Normal	62 (30)	1		190 (59)	1	
Low	97 (30)	0.39 (0.21–0.71)		124 (29)	0.63 (0.41–0.95)	
TYMS expression			0.87			0.35
High	20 (9)	1.01 (0.38–2.72)		30 (11)	1.73 (0.82–3.65)	
Intermediate	100 (38)	0.86 (0.43–1.69)		161 (42)	1.09 (0.70–1.68)	
Low	38 (13)	1		117 (31)	1	
Gene–gene interaction			0.003			0.007
Normal risk	87 (24)	1		206 (49)	1	
High risk (MTHFR 1298AC + TYMS 3'-UTR del/del or MTHFR 1298AA + TYMS 3'-UTR ins/del)	73 (36)	2.40 (1.33–4.29)		109 (40)	1.81 (1.18–2.79)	

<sup>a</sup>Models adjusted for the effects of age and sex.

<sup>b</sup>The number in parentheses indicates the number of patients with severe toxicities.



**Figure 1.** Gene-gene interaction analysis showed that patients with the high-risk genotype combination, derived from the multifactor dimensional reduction method, more frequently experienced severe toxicity from 5-FU than the other genotype combinations in both cohorts. Proportionally the gene-gene interaction group was overrepresented in the severe toxicity groups in both cohorts.

significantly associated with increased severe stomatitis/pharyngitis in both cohorts [ $OR_{\text{Exploration}}$  2.05 (95% CI: 1.03–4.09,  $P = 0.04$ ),  $OR_{\text{Validation}}$  2.15 (95% CI: 1.08–4.29,  $P = 0.03$ )]. Furthermore, it was associated with increased severe diarrhea [ $OR_{\text{Exploration}}$  1.90 (95% CI: 0.72–4.99,  $P = 0.19$ ),  $OR_{\text{Validation}}$  1.92 (95% CI: 1.06–3.47,  $P = 0.03$ )].

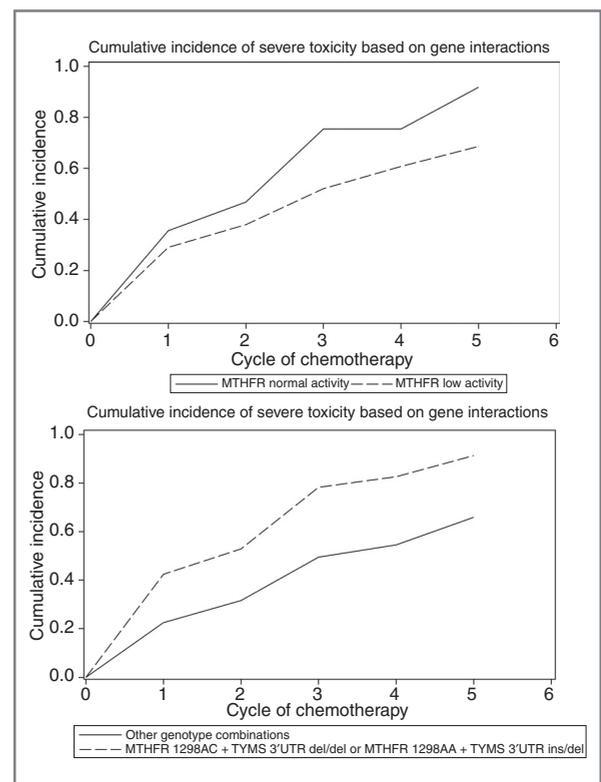
### Additional analyses

Information about at which treatment cycle maximum toxicity occurred was available only from the exploration cohort. We carried out a survival analysis to determine if the identified markers for increased severity of toxicity were associated with an increased cumulative incidence of severe toxicity (Supplementary Fig. S1). The cumulative incidence curves indicated an increased probability of severe toxicity in the gene-gene interaction group with duration of treatment and with early onset of severe toxicity. The median cycle for severe toxicity was cycle 2 in the interaction group compared with cycle 4 in the normal group (log-rank  $P = 0.001$ ; Table 4, Fig. 2). MTHFR activity showed a similar tendency with higher cumulative incidence of severe toxicity in the normal activity group but the analysis did not reach statistical significance (log-rank  $P = 0.09$ ; Fig. 2). The Cox Proportional Hazards model showed that the increased risk of severe toxicity in the gene-gene interaction group persisted after adjustment for sex and age (HR 1.85, 95% CI: 1.16–2.99,  $P = 0.01$ ).

### Discussion

In the present study, we investigated the association of 13 polymorphisms, in genes encoding enzymes involved in 5-FU pharmacodynamics and pharmacokinetics, with the risk and severity of toxicity in patients adjuvantly treated with 5-FU/LV. Our strategy was to explore haplotypes, phenotypic classifications, and gene-gene interactions in addition to single polymorphisms as relevant

biomarkers of toxicity. We found that MTHFR activity and a specific combination of MTHFR 1298A>C SNP and TYMS 3'-UTR ins/del polymorphisms were associated with risk and severity of toxicity (Table 4). Normal activity of MTHFR and the identified genotype combination were



**Figure 2.** Cumulative incidence of severe toxicity over the treatment cycles (data available from the exploration cohort only). Both normal activity of MTHFR and the gene-gene interaction group was associated with earlier onset and higher probability of severe toxicity the longer the treatment continued.

associated with almost a doubling of the risk (odds ratio) of experiencing increased severity of toxicity when adjusting for age and sex.

The ordinal toxicity scales are assumed to measure an underlying continuous scale of toxicity, so as long as the toxicity category measures the same entities, e.g., diarrhea, the differences in scale are immaterial in ordinal logistic regression, i.e., we are modeling shifts toward greater toxicity and not a specific toxicity grade per se. This allowed us to model the OR for increased severity of toxicity rather than just severe toxicity. The results indicate that the genetic classifiers are generally applicable irrespective of minor differences in patient populations and clinical evaluation of patients.

Classifiers based on gene–gene interactions, involving genes in the same drug metabolism pathway, should be better predictors of drug efficacy or side effects than individual polymorphisms or haplotypes of genes. Drug response is a complex trait that involves many proteins; from a biological point of view, it is to be expected that different metabolic routes compete and that the effect of 1 polymorphism on a drug metabolism pathway can be altered by other polymorphisms. The interaction identified by our analyses, *MTHFR* 1298AC + *TYMS* 3'-UTR del/del or *MTHFR* 1298AA + *TYMS* 3'-UTR ins/del, was a statistical interaction and further research is required to delineate the effects of different genotype combinations on drug pharmacokinetics and pharmacodynamics.

Previous studies have shown contradicting results with regard to the role of *MTHFR* in toxicity in relation to 5-FU chemotherapy (6, 13, 17, 33–35). The contradictory results may reflect that the *MTHFR* 677C>T and *MTHFR* 1298A>C SNPs were not combined according to phenotypic effects, differences in treatment regimens, and inadequate statistical analyses.

Our results seem robust for several reasons. Our results were validation in independent cohorts and with different statistical models. The cohorts were relatively large and both were treated with similar chemotherapy regimens. A combinatorial approach to genetic analyses was applied instead of the standard "one gene one phenotype" approach. Furthermore, the cohorts were included from

different geographical regions with different genotype distributions and still the associations remained.

The limitations of the study are that we cannot determine whether the associations apply to other types of 5-FU–based chemotherapies because we did not have a differently treated control group and we could not account for whether tumor genotypes are representative of germ line genotypes in the validation cohort; however, several studies have shown excellent concordance between germ line and tumor tissue genotypes (36). In our study, the only indication of loss of heterogeneity is for *TYMS* genotypes, but the consequently erroneous genotype assignment would only decrease the association and would only affect 2% to 3% of the genotypes (36). The differences in frequency for the 2 cohorts *MTHFR* 677C>T and *DPYD* 85T>C polymorphisms do not seem to be due to LOH as the other genotypes within these genes are similar. This discrepancy could be explained by patients being from different geographical regions or random differences. For *MTHFR*677, it has been shown that the genotype frequencies vary with geographical regions both within and outside Europe (37).

In conclusion, we have provided evidence for the following novel findings. Individual polymorphisms were not reproducibly associated with treatment-related toxicity. Normal activity of *MTHFR* and specific combinations of *MTHFR* and *TYMS* polymorphisms were associated with increased risk of 5-FU–related toxicity.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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