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Increased Risk of Severe Fluoropyrimidine-Associated Toxicity in Patients Carrying a G to C Substitution in the First 28-bp Tandem Repeat of the Thymidylate Synthase 2R Allele

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The fluoropyrimidines act by inhibiting thymidylate synthase (TS). Recent studies have shown that patients' risk of severe fluoropyrimidine-associated toxicity is affected by polymorphisms in the 5'-untranslated region of *TYMS*, the gene encoding TS. A G>C substitution in the promoter enhancer region of *TYMS*, rs183205964 (known as the 2RC allele), markedly reduces TS activity *in vitro*, but its clinical relevance is unknown. We determined rs183205964 in 1605 patients previously enrolled in a prospective multicenter study. Associations between putative low TS expression genotypes (3RC/2RC, 2RG/2RC, and 2RC/2RC) and severe toxicity were investigated using univariable and multivariable logistic regression. Activity of TS and *TYMS* gene expression were determined in peripheral blood mononuclear cells (PBMCs) of a patient carrying genotype 2RC/2RC and of a control group of healthy individuals. Among 1,605 patients, 28 patients (1.7%) carried the 2RC allele. Twenty patients (1.2%) carried a risk-associated genotype (2RG/2RC, $n=13$; 3RC/2RC, $n=6$; and 2RC/2RC, $n=1$), the eight remaining patients had genotype 3RG/2RC. Early severe toxicity and toxicity-related hospitalization were significantly more frequent in risk-associated genotype carriers (OR 3.0, 95%CI 1.04–8.93, $p=0.043$ and OR 3.8, 95%CI 1.19–11.9, $p=0.024$, respectively, in multivariable analysis). The patient with genotype 2RC/2RC was hospitalized twice and had severe febrile neutropenia, diarrhea, and hand-foot syndrome. Baseline TS activity and gene expression in PBMCs of this patient, and a healthy individual with the 2RC allele, were found to be within the normal range. Our study suggests that patients carrying rs183205964 are at strongly increased risk of severe, potentially life-threatening, toxicity when treated with fluoropyrimidines.

Introduction

The fluoropyrimidines 5-fluorouracil (5-FU), capecitabine, and tegafur are the backbone of chemotherapeutic treatment of gastrointestinal, breast, and head & neck cancers. Fluoropyrimidines act by inhibiting thymidylate synthase (TS), an enzyme which is crucial for DNA replication and repair, by providing the only *de novo* source of deoxythymidine monophosphate. While most patients tolerate treatment well, around 20% experiences severe, potentially lethal, treatment-related toxicity. The best recognized cause of intolerance to fluoropyrimidines is deficiency of the main 5-FU metabolizing enzyme, dihydropyrimidine dehydrogenase (DPD). Single nucleotide polymorphisms (SNPs) in *DPYD*, the gene encoding DPD, are now established predictors of fluoropyrimidine-associated toxicity, and upfront screening for these variants can improve patient safety.[1,2] The expression of TS is influenced by polymorphisms in *TYMS*, the gene encoding TS.[3–5] However, in contrast to *DPYD* variants, the clinical relevance of these polymorphisms as predictors of treatment-related toxicity is far from established.

TYMS is located on chromosome 18p11.32, consists of seven exons, and is ~16,000 bp long. The 5′-untranslated region (5'-UTR) of *TYMS* contains a variable number of 28-

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What's new?

Fluoropyrimidines are among the most commonly used anticancer drugs. Fluoropyrimidines act by inhibiting thymidylate synthase, encoded by the gene *TYMS*. A G>C substitution in the promoter enhancer region of *TYMS*, rs183205964, has been shown to reduce TS activity *in vitro*, but its effect in patients is unknown. We determined the clinical relevance of this variant as a predictor of severe fluoropyrimidine-induced toxicity in a cohort of 1605 patients treated with fluoropyrimidine-based chemotherapy, and demonstrate for the first time that rs183205964 is associated with risk of early severe toxicity.

bp tandem repeats (VNTR) which acts as an enhancer to the promoter and stimulates transcriptional activity.[3,4,6] The vast majority of individuals carry *TYMS* alleles that contain 2 or 3 repeats in this promoter enhancer region, designated 2R and 3R, with allele frequencies of ~0.47 and 0.53, respectively.[7] The VNTR affects expression of TS, and *in vitro* studies have shown that a 2R enhancer region produces 3.6 times less mRNA compared to 3R.[3] Similarly, tumor tissue with a 2R/3R genotype produced significantly less cellular TS protein compared to 3R/3R.[4] Kawakami showed that in addition to stimulating gene transcription, a higher number of repeats also confers a greater translational efficiency.[5] In line with these non-clinical observations, a recent meta-analysis has shown that, on treatment with capecitabine, patients carrying the 2R allele are at increased risk of severe fluoropyrimidine-associated toxicity.[7]

Transcription of *TYMS* is regulated by 2R and 3R due to 6 bp enhancer box (E-box) sequences, of CACTTG, that occur within the tandem repeats.[8] To these sequences, upstream stimulating factor 1 (USF-1) can bind, thereby stimulating transcription of *TYMS*. The 2R allele contains one functionally relevant E-box element, in the first repeat, while the 3R allele contains up to two binding sites, occurring in the first and the second repeat. Whether or not a second binding site is present in the second repeat of the 3R allele, depends on a G>C SNP that occurs at the 12th nucleotide of the second repeat in the CACTTG sequence. If present, the USF-1 binding site in the second repeat is abolished, and the transcriptional activity of the 3R allele containing the G>C SNP is reduced to approximately that of the 2R allele in *in vitro* studies.[8] The 3R allele containing the G>C SNP is commonly referred to as 3RC to distinguish it from the wild type 3RG allele.

In 2006, a novel G>C SNP was described for the first time in patients, occurring in the first repeat of the 2R allele. This variant, rs183205964, disrupts the last functional E-box sequence present in the 2R allele (Supporting Information Fig. 1). It was described to have an estimated allele frequency of 0.015–0.042, and is referred to as 2RC, to distinguish it from the wild type allele 2RG.[9,10] Independent studies have shown that this mutation markedly reduces TS expression *in vitro*, to a level lower than that of the 2RG allele.[8,11] Whether rs183205964 is associated with reduced TS activity in patients, and whether this results in greater sensitivity to fluoropyrimidines, has not been investigated. We hypothesized that patients carrying the putative low TS expression 2RC risk allele are at higher risk of fluoropyrimidine-associated toxicity on treatment with fluoropyrimidines, and conducted a pharmacogenetic study to determine the clinical relevance of this variant.

Material and Methods

Patients and study design

Sixteen-hundred thirty-one patients who were previously enrolled in a prospective multicenter study of *DPYD**2A genotype-guided dosing of fluoropyrimidines (NCT00838370) were considered for genotyping of *TYMS* in the context of this pharmacogenetic study (Fig. 1). The study population consisted of patients with cancer intended to undergo treatment with fluoropyrimidine-based anticancer therapy, either as single agent or in combination with other chemotherapy or radiotherapy, according to existing standard of care. Prior chemotherapy and radiotherapy were allowed. The primary endpoint of NCT00838370 was toxicity, which was recorded during each treatment cycle according to CTC-AE v3.0. Hematology (including neutrophils, leukocytes, and platelets) was monitored according to local protocol (prior to each cycle in most cases). Information on hospitalizations for toxicity and reasons for ending treatment were also collected for the purpose of the study. In study NCT00838370, patients were genotyped for *DPYD**2A prior to treatment using germline DNA. Heterozygous and homozygous *DPYD**2A variant allele carriers were treated with an initially reduced dose of the fluoropyrimidine during the first two cycles of treatment, followed by further dose individualization based on tolerability. Eighteen patients carrying the *DPYD**2A variant were excluded from the current analysis. No intervention was applied in the remaining 1,613 patients who proved to be wild type for *DPYD**2A; they were treated according to standard of care treatment regimens (Supporting Information Table 1). These 1,613 patients were considered eligible for inclusion in this analysis. All patients provided written informed consent prior to study procedures.

The association between rs183205964 and severe fluoropyrimidine-induced toxicity was investigated by comparing the risk of severe treatment-related toxicity between patients carrying 2RC risk-associated genotypes and patients not carrying risk-associated genotypes. Based on the available *in vitro* data on the relationship between *TYMS* alleles and putative low TS expression phenotypes, the following genotypes were considered risk-associated genotypes: 3RC/2RC, 2RG/2RC, and 2RC/2RC. Patients carrying the 3RG/2RC genotype were not included in the risk group, as they were expected to have higher TS activity in view of the 3RG allele.[12,13] The risk of severe treatment-related toxicity in the group of patients with risk-associated genotypes

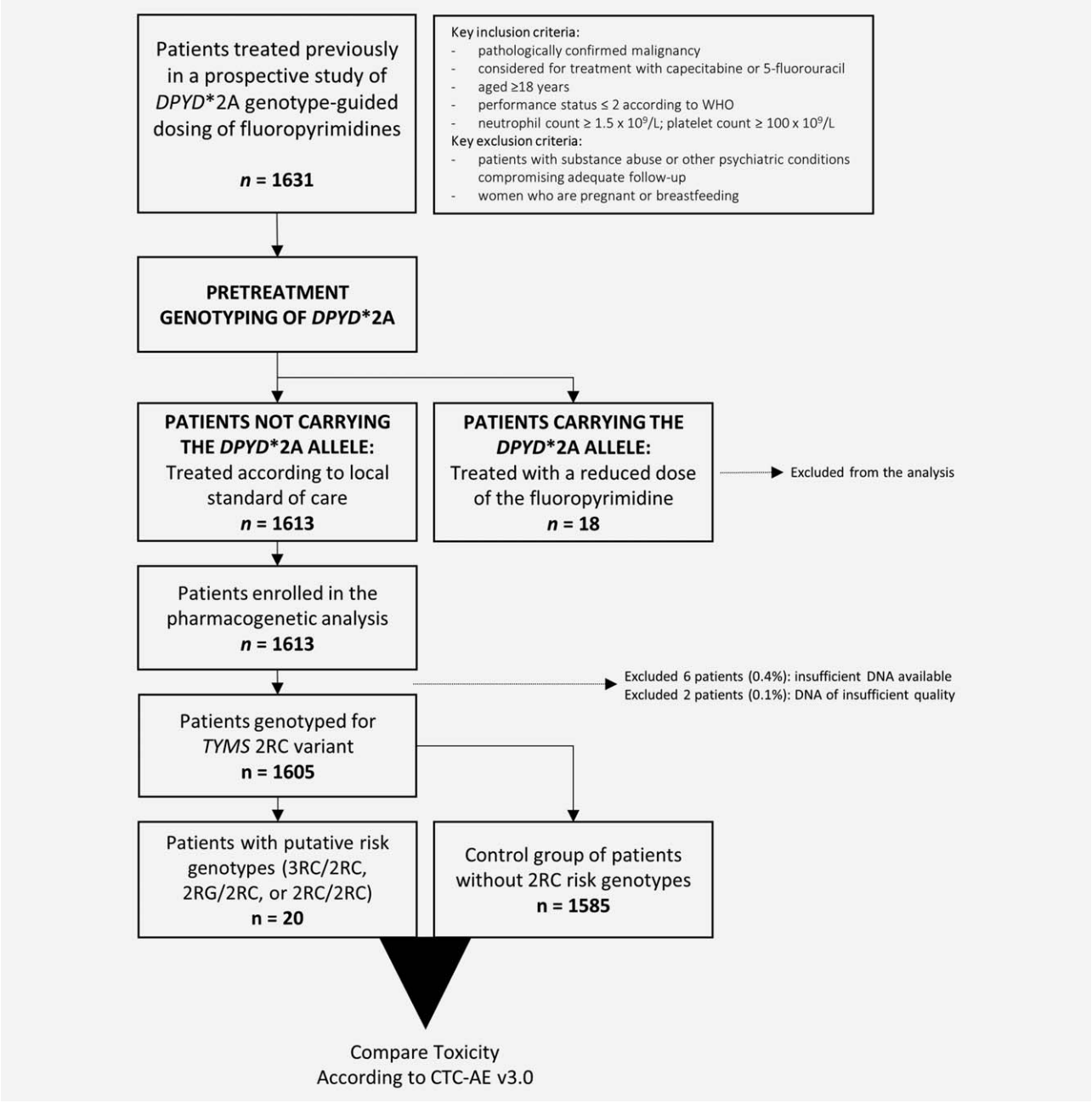

Figure 1. Flow chart of the study. *DPYD*: dihydropyrimidine dehydrogenase (gene); *TYMS*: thymidylate synthase (gene); CTC-AE: common toxicity criteria for adverse events (version 3.0).

was compared to that of the rest of the population in univariable and multivariable analysis. In a separate, exploratory, analysis the association between the 3RG/2RC genotype and risk of severe toxicity was investigated.

Genotyping of *TYMS* and *DPYD*, thymidylate synthase activity assays, and *TYMS* gene expression

A synopsis is provided here (details in Appendix). All patients were screened for the VNTR in the 5'-UTR of *TYMS* (rs34743033), the G>C SNP in the second repeat of the 3R allele (rs2853542), and the G>C SNP in the first repeat of the 2R allele (rs183205964) using PCR. To investigate the effect of the 2RC risk-associated allele on TS enzyme activity, we determined TS activity in peripheral blood mononuclear cells (PBMCs) of a patient with a homozygous 2RC/2RC genotype, a healthy control with a heterozygous (3RC/2RC) genotype, and 18 healthy controls with non-2RC genotypes, using a fully validated radioassay.[14] In addition to TS activity, relative *TYMS* mRNA expression was determined in PBMCs of the patient with the homozygous 2RC/2RC genotype, a healthy control with a 3RC/2RC genotype, and 19 healthy controls (Jacobs *et al.*, submitted).

The prevalence of four *DPYD* variants known to be associated with fluoropyrimidine-induced toxicity, 2846A>T, 1679T>G, 1236G>A, and 1601G>A, was compared between groups of patients with risk-associated genotypes and controls to exclude DPD deficiency as a possible confounding factor.[28]

Determination of PBMC dihydropyrimidine dehydrogenase enzyme activity and the *CYP3A4*22* allele

To exclude other possible causes of intolerance to fluoropyrimidines in the single patient carrying the 2RC/2RC genotype, DPD activity in PBMCs of this patient was determined, as described previously.[15,27] In addition, as the patient was treated with the combination of capecitabine and docetaxel, we investigated potential CYP3A4 deficiency, which could lead to increased exposure to docetaxel, in the same patient, by determining the most clinically relevant dysfunctional *CYP3A4* allele, *CYP3A4*22*, using a commercial RT-PCR assay (Applied Biosystems, Foster City, CA, USA).[29]

Statistical considerations and data analysis

Demographic and clinical characteristics of patients with risk-associated genotypes and the control group were described and differences were tested using the Student's *t*-test, the Mann–Whitney U test, Fisher's exact test, or the Chi-square test, where appropriate. The 2RC risk allele was tested for deviation from Hardy–Weinberg equilibrium using the exact test.[16] To test associations with toxicity, we considered the maximum grade toxicity experienced during the first cycle. An analysis of the entire treatment duration was regarded not informative in view of the fact that there was a wide variety of treatment durations in this heterogeneous daily-care patient population. Global (any) toxicity and individual types of toxicity, gastrointestinal, hematological, and hand-foot syndrome (HFS), were dichotomized as absent to moderate (grade 0, 1, or 2) vs. severe (grade 3, 4, or 5). Associations between risk-associated genotypes and toxicity or toxicity-related outcomes, which included treatment-related hospitalization and treatment discontinuation due to adverse events, were tested in univariable and multivariable logistic regression models, adjusting for factors known to be associated with risk of toxicity in patients treated with fluoropyrimidines: age (continuous), gender (female or male), and treatment regimen (5-FU-based, capecitabine monotherapy, capecitabine plus platinum, capecitabine plus taxane, capecitabine-based triplet combination, capecitabine plus other drug, or capecitabine plus radiotherapy). The planned starting dose of capecitabine was highly collinear with type of regimen and was not predictive of toxicity after adjustment for treatment regimen; it was therefore not included in the models. Also tumor type, disease stage, or previous treatment were not predictive of toxicity and not included as covariates.

In view of the low frequency of the homozygous variant genotype, we tested for an association between the 2RC risk allele and severe toxicity using a dominant model (wild type *vs.* heterozygous or homozygous). A log-additive model was explored as well. Because the 2RC allele occurs within the VNTR, the effect of the VNTR polymorphism on risk of toxicity was also investigated, assuming a log-additive model for the VNTR, with correction for clinical covariates.

Associations between risk-associated genotypes and severe toxicity or toxicity-related outcomes were reported as an odds ratio (OR) and a 95% confidence interval (CI), with corresponding p values. For all statistical tests the threshold for significance was set at $p < 0.05$. All statistical analyses were performed in R v3.1.0.

Results

Patients and *TYMS* genotyping

As shown in Figure 1, 1,605 out of 1,613 patients (99.5%) were genotyped for rs183205964. Among 1,605 patients, 28 patients had the 2RC variant allele (1.7%). Genotypes with the 2RC allele containing in total ≤ 1 intact USF binding sites were considered risk-associated genotypes, as a reduced activity of *TYMS* is expected for these genotypes compared to genotypes with ≥ 2 USF binding sites.[8] There were 20 patients carrying a putative risk-associated genotype (2RG/2RC, $n = 13$; 3RC/2RC, $n = 6$; 2RC/2RC, $n = 1$). Eight patients carried the presumed non-risk 2RC genotype 3RG/2RC (containing 2 USF binding sites). The allele frequency of the 2RC allele in the studied population was 0.009, and there was no statistically significant deviation from Hardy–Weinberg equilibrium ($p = 0.120$). The characteristics of the patients with risk-associated genotypes as well as the rest of the population are summarized in Table 1, the treatment regimens that patients received are summarized in Supporting Information Table 1. There were no significant differences between groups with regard to patient characteristics.

Association of the 2RC allele with fluoropyrimidine-associated toxicity

The frequencies of toxicity and toxicity-related outcomes occurring during the first cycle are summarized in Table 2 (frequencies by treatment regimen are available in Supporting Information Table 2). The association between risk-associated genotypes and severe fluoropyrimidine-induced toxicity during the first cycle was investigated in univariable and in multivariable logistic regression analyses, assuming a dominant model, the results of which are shown in Table 3. In univariable analysis, risk of global severe toxicity was significantly higher in patients with risk-associated genotypes than in the control group (OR 3.0, 95%CI 1.06–8.22, $p = 0.039$). There was a trend toward increased incidence of early gastrointestinal toxicity (OR 3.4, 95%CI 0.98–11.9, $p = 0.054$). Also hematological toxicity appeared to be more frequent, but not significantly (OR 3.4, $p = 0.259$). Of the patients with 2RC risk-associated genotypes, none had early severe HFS, compared to 1% of the patients in the control group. Treatment-related hospitalization and treatment discontinuation due to adverse events were both significantly more frequent in patients with risk-associated genotypes in univariable

Table 1. Patient characteristics according to *TYMS* 2RC genotype

Characteristics	Control ($n = 1,585$)	Risk-associated genotypes combined ($n = 20$)	Individual risk-associated genotypes[1]			p-values[2]
			3RC/2RC ($n = 6$)	2RG/2RC ($n = 13$)	2RC/2RC ($n = 1$)	
Age, median (range)	61 (21–89)	63 (49–78)	68 (51–78)	63 (49–78)	50	0.398
Sex						
Male	712 (55%)	6 (30%)	0 (0%)	6 (46%)	0 (0%)	0.258
Female	873 (45%)	14 (70%)	6 (100%)	7 (54%)	1 (100%)	
Tumor type						
Colorectal cancer (locally adv.)	524 (33%)	11 (55%)	4 (67%)	7 (54%)	0 (0%)	0.115
Colorectal cancer (metastatic)	319 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Gastric cancer (locally adv.)	112 (7%)	1 (5%)	0 (0%)	1 (8%)	0 (0%)	
Gastric cancer (metastatic)	114 (7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Breast cancer (locally adv.)	117 (7%)	2 (10%)	0 (0%)	1 (8%)	1 (100%)	
Breast cancer (metastatic)	245 (15%)	5 (25%)	2 (33%)	3 (23%)	0 (0%)	
Other (*e.g.*, HNSCC, SCCAC)	154 (10%)	1 (5%)	0 (0%)	1 (8%)	0 (0%)	
Treatment						
Capecitabine monotherapy	417 (26%)	7 (35%)	2 (33%)	5 (38%)	0 (0%)	0.125
Capecitabine plus radiotherapy	426 (27%)	10 (50%)	3 (50%)	7 (54%)	0 (0%)	
Capecitabine plus taxane	63 (4%)	1 (5%)	0 (0%)	0 (0%)	1 (100%)	
Capecitabine plus platinum	377 (24%)	1 (5%)	1 (17%)	0 (0%)	0 (0%)	
Capecitabine triplet	112 (7%)	1 (5%)	0 (0%)	1 (8%)	0 (0%)	
Capecitabine plus other	22 (1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
5-FU-based	168 (11%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Daily dose of capecitabine in mg/m^2, median (range)	2000 (500–2500)	1650 (1650–2500)	2000 (1500–2500)	1650 (1600–2500)	1650	0.102
Origin						
Caucasian	1519 (96%)	20 (100%)	6 (100%)	13 (100%)	1 (100%)	0.972
Other	86 (4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Previous chemotherapy						
No	1230 (78%)	16 (80%)	6 (100%)	9 (69%)	1 (100%)	0.936
Yes	355 (22%)	4 (20%)	0 (0%)	4 (31%)	0 (0%)	
DPYD **SNPs**[3]						
No	1418 (90%)	19 (90%)	5 (83%)	12 (92%)	1 (100%)	1.000
Yes	163 (10%)	2 (10%)	1 (17%)	1 (8%)	0 (0%)	

[1]The number of intact upstream stimulating factor (USF) binding sites for the different risk-associated genotypes was 1 for 3RC/2RC, 1 for 2RG/2RC, and 0 for 2RC/2RC.
[2]Student's *t*-test (age), Mann–Whitney U test (dose of capecitabine), Fisher's exact test (sex, origin, previous chemotherapy, *DPYD* SNPs), or Chi-square test (tumor type, treatment).
[3]Two patients in the risk group had a mutation in *DPYD*: one had 1601G>A (2RG/2RC) and one had 1236G>A (3RC/2RC). Of these patients, the former had no toxicity (grade 0) and the latter had grade 3 toxicity as maximum toxicity.
Abbreviations: *TYMS*: thymidylate synthase (gene); HNSCC: head & neck squamous cell carcinoma; SCCAC: squamous cell carcinoma of the anal canal; 5-FU: 5-fluorouracil; *DPYD*: dihydropyrimidine dehydrogenase (gene); SNPs: single nucleotide polymorphisms.

analysis. Also after adjustment for age, gender, and treatment regimen, risk of global severe toxicity and treatment-related hospitalization remained significantly increased in patients with risk-associated genotypes.

When the association between the 2RC allele and treatment-related toxicity was evaluated in multivariable analysis using a log-additive model, similar results were obtained for the association with global toxicity (OR 3.0, 95%CI 1.07–8.42, $p = 0.037$), gastrointestinal toxicity (OR 2.2, 95%CI 0.65–7.16, $p = 0.208$), hematological toxicity (OR 4.0, 95%CI 0.90–18.0, $p = 0.068$), treatment-related hospitalization (OR 3.7, 95%CI 1.29–10.8, $p = 0.015$), and treatment

Table 2. Maximum toxicity grade during the first cycle of treatment according to *TYMS* 2RC genotype

Toxicity	Control ($n = 1,585$)	Risk-associated genotypes combined ($n = 20$)	Individual risk-associated genotypes		
			3RC/2RC ($n = 6$)	2RG/2RC ($n = 13$)	2RC/2RC ($n = 1$)
Global toxicity					
Grade 0–2	1424 (90%)	15 (75%)	5 (83%)	10 (77%)	0 (0%)
Grade 3–5	161 (10%)	5 (25%)	1 (17%)	3 (23%)	1 (100%)
Gastrointestinal toxicity					
Grade 0–2	1507 (95%)	17 (85%)	5 (83%)	11 (85%)	1 (100%)
Grade 3–5	78 (5%)	3 (15%)	1 (17%)	2 (15%)	0 (0%)
Hematological toxicity					
Grade 0–2	1521 (96%)	18 (90%)	6 (100%)	12 (92%)	0 (0%)
Grade 3–5	64 (4%)	2 (10%)	0 (0%)	1 (8%)	1 (100%)
Hand-foot syndrome					
Grade 0–2	1564 (99%)	20 (100%)	6 (100%)	13 (100%)	1 (100%)
Grade 3–5	21 (1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Hospitalization for toxicity					
No	1488 (94%)	16 (80%)	5 (83%)	11 (85%)	0 (0%)
Yes	97 (6%)	4 (20%)	1 (17%)	2 (15%)	1 (100%)
Treatment discontinuation due to toxicity					
No	1488 (94%)	16 (80%)	4 (67%)	11 (85%)	1 (100%)
Yes	97 (6%)	4 (20%)	2 (33%)	2 (15%)	0 (0%)

discontinuation due to adverse events (OR 2.2, 95%CI 0.73–6.52, $p = 0.165$).

We also investigated associations between 2RC and severe toxicity when considering the entire treatment duration. This analysis showed that risk of severe toxicity during the overall treatment duration was increased in patients with 2RC risk-associated genotypes, but not significantly (OR 1.5, 95%CI 0.56–4.09, $p = 0.408$), possibly as a result of the wide range of treatment durations.

As the 2RC allele occurs within the *TYMS* VNTR, we also investigated the effect of the VNTR on risk of toxicity. This analysis showed that there was no association between the VNTR and treatment-related toxicity (OR 1.1 for each additional 2R allele, 95%CI 0.85–1.36, $p = 0.545$).

The incidence of early severe toxicity in patients with the 3RG/2RC genotype was not significantly increased compared to the rest of the population, as the OR for global severe toxicity was 1.2 (95% CI 0.10–10.1, $p = 0.841$) in univariable analysis and 0.8 (95% CI 0.17–13.9, $p = 0.871$) in multivariable analysis. There were also no differences in risk of experiencing the individual types of toxicity (not shown).

Table 3. Univariable and multivariable analysis of risk for severe toxicity for patients carrying *TYMS* 2RC risk-associated genotypes vs. non-2RC genotypes

Outcome	OR	95% CI	*p* value
Univariable			
Global severe toxicity	3.0	1.06–8.22	0.039
Gastrointestinal severe toxicity	3.4	0.98–11.9	0.054
Hematological severe toxicity	3.4	0.41–27.8	0.259
Hospitalization for toxicity	3.8	1.26–11.7	0.018
Treatment discontinuation	3.6	1.17–10.8	0.025
Multivariable[1]			
Global severe toxicity	3.0	1.04–8.93	0.043
Gastrointestinal severe toxicity	2.7	0.73–10.0	0.136
Hematological severe toxicity	4.3	0.81–23.2	0.088
Hospitalization for toxicity	3.8	1.19–11.9	0.024
Treatment discontinuation	2.9	0.92–9.21	0.070

[1]The following covariates were included in multivariable analysis: age, gender, and treatment with concomitant chemotherapy or radiotherapy (effect estimates of the covariates are listed in Supporting Information Table 3).
Abbreviations: OR: odds ratio; CI: confidence interval.

Clinical course of treatment of the patient with the 2RC/2RC genotype

Since only one patient with a homozygous variant genotype was identified, this patient will be described in detail. She was a female patient, aged 54, with a triple negative $T_1N_0M_0$ ductal carcinoma, who received surgery, followed by radiotherapy up to 64.4 Gy, and was subsequently planned for adjuvant chemotherapy with capecitabine 825 mg/m^2 b.i.d. on days 1–14, combined with 75 mg/m^2 infusional docetaxel (day 1) for six cycles every 3 weeks (in the context of a clinical study[17]).

Nine days after starting treatment, she was hospitalized with high fever (40.5°C), severe neutropenia (0.4×10^9/L; grade IV) and leukocytopenia (1.9×10^9/L; grade III). Capecitabine was interrupted. The focus of the fever was thought to be an infection of the breast, and she was released on oral antibiotics 2 days later. Capecitabine was withheld for 5 weeks until wound healing had completed, and treatment was then restarted, at the same dose of capecitabine and docetaxel. On day 14 of cycle 2, she developed severe HFS (grade III); no neutropenia was noted. The dose of capecitabine was reduced to 75% in cycle 3, which was started 1 week later when HFS had subsided. The dose of docetaxel was left unchanged. On day 4 of cycle 3, she again developed HFS, grade III, and capecitabine was again interrupted. On day 8 of cycle 3, despite interruption of capecitabine, the patient was hospitalized with febrile neutropenia ($<0.1 \times 10^9$/L), HFS grade III, and diarrhea grade III. The patient was hospitalized for 3 days, and then continued to have HFS, which persisted as grade II until 14 days later, and then gradually faded away in 1 week. In view of the intolerable side-effects, it was decided to permanently discontinue treatment with capecitabine and docetaxel. An alternative treatment of six cycles of adriamycin (60 mg/m^2) and cyclophosphamide (600 mg/m^2) was initiated, which was well-tolerated. The patient is currently alive and disease-free, but continues to suffer from cognitive problems (related to information processing and working memory) ever since receiving chemotherapy with capecitabine and docetaxel.

Since only one patient was homozygous for 2RC, we could not perform multivariable analysis to evaluate risk of severe toxicity. However, the patient experienced grade IV toxicity, which was very rare in the overall population (2.2%). The fact that the single patient with a homozygous 2RC genotype had grade IV toxicity, therefore, resulted in a statistically significant higher incidence of global grade $\geq$IV toxicity for the 2RC/2RC genotype compared to patients without this genotype ($p = 0.022$, Fisher's exact test).

DPD activity was found to be normal to high in this patient: 12.4 nmol/mg*hr (reference range: 6–14 nmol/mg/hr). Genotyping of *CYP3A4**22 showed that the mutation was not present.

Association of the 2RC allele with thymidylate synthase activity and TYMS gene expression in PBMCs

TS activity in the patient with the 2RC/2RC genotype was 0.066 nmol/mg/hr, which is close to the median activity of 0.072 nmol/mg/hr observed in controls (range: 0.031–0.134 nmol/mg/hr) and therefore not reduced (Supporting Information Fig. 2A). TS activity in PBMCs of the subject carrying the 3RC/2RC genotype was low but within the normal range (0.036 nmol/mg/hr). Likewise, *TYMS* mRNA expression levels in the 2RC variant allele carriers were very close to the median observed in controls with non-2RC genotypes (Supporting Information Fig. 2B).

Discussion

This is the first analysis investigating the clinical relevance of the G>C SNP at the 12th nucleotide of the first 28-bp tandem repeat of the 2R allele of in the 5'-UTR *TYMS*, which has been reported to result in significant reductions in *TYMS* gene expression *in vitro*, as a result of abolishing a USF-1 binding site.[8,11] In total 28/1,605 patients carried the risk allele, and therefore the observed allele frequency of 0.009 was lower than previously reported in Caucasians in smaller studies (0.015–0.042).[9,10] The higher frequency compared to other studies could be explained by sample-to-sample variation, and also publication bias may have contributed to the higher allele frequencies described in initial reports. True differences in the frequency of 2RC depending on ethnicity are also possible, as has been demonstrated for the *TYMS* VNTR polymorphism.[18] Our analysis showed that early severe fluoropyrimidine-associated toxicity and toxicity-related hospitalizations were more frequent among patients carrying 2RC risk-associated genotypes, 3RC/2RC, 2RG/2RC, or 2RC/2RC, indicating that these genotypes are likely to be associated with a higher risk of severe toxicity.

We identified one patient with a homozygous 2RC/2RC variant genotype. This patient experienced severe, recurrent, fluoropyrimidine-associated toxicity while being treated with a relatively low dose of capecitabine in combination with docetaxel. We were able to largely exclude other possible causes of intolerance to treatment in this patient. DPD enzyme activity was normal to high, and the disruptive *CYP3A4**22 allele was not found, making it less likely that either DPD deficiency or CYP3A4 deficiency (which could increase exposure to docetaxel) played a role in the observed toxicities. Considering the spectrum of toxicities observed (neutropenia, diarrhea, and HFS), it is not likely that they were related to docetaxel, and no other toxicities that raised suspicion toward docetaxel were present (*e.g.,* peripheral neuropathy, nail toxicity, or dysgeusia).

In contrast to what we expected based on *in vitro* studies, both PBMC TS activity and *TYMS* expression in the patient with the 2RC/2RC genotype and the control subject with the 3RC/2RC genotype were not found to be reduced. This was rather surprising in view of the observed course of treatment, and the statistical association between 2RC and severe toxicity. There are several possible explanations as to why PBMC TS activity was not reduced. Mandola *et al.*, who first studied the effect of USF-1 binding sites in *TYMS* on gene transcription, noticed that in the absence of USF-1 there was almost no difference in gene expression between 2RG and 2RC.[8] Only on stimulation with USF-1 differences in gene expression became apparent. It could be speculated that under normal conditions in mature PBMCs, the expression of TS is not so much dependent on stimulation by USF-1 (although PBMCs, as do almost all tissues, express USF-1[19]). It could be that only under conditions where increased TS expression is required, such as during cell growth or on challenge with a

dose of fluoropyrimidines, the contribution of USF-1 becomes important. There are no data to support this hypothesis, but it has been shown that the relative contribution of USF-1 in modulating gene transcription is cell type specific and is highly context-dependent.[20,21] For USF-2, a transcription factor similar to USF-1, it has been shown that its expression is repressed in quiescent mast cells, and only becomes active during cell growth.[22]

Our results, combined with what is known from *in vitro* studies, support the hypothesis that rs183205964 induces a clinically relevant change in the cell's sensitivity to the effects of fluoropyrimidines. Risk of early severe toxicity was increased in the overall group of patients with risk-associated genotypes, which consisted of 19 patients who carried the 2RC allele in heterozygous form, and one patient with the 2RC/2RC genotype, suggesting that both patients with a heterozygous or a homozygous 2RC genotype are at increased risk of toxicity. The group consisted, however, of patients with different heterozygous genotypes, 3RC/2RC and 2RG/2RC. It is hypothetically possible that the extent by which expression of TS is affected by these genotypes is not equal, but we did not have sufficient statistical power to investigate these potential differences. Also based on the available *in vitro* data we cannot draw a firm conclusion as to whether there would be a difference in risk between these groups.

We defined, *a priori*, the patients with the 3RG/2RC genotype as not at risk, in view of the presence of the 3RG allele.[12,13] We identified eight such patients, and indeed found no indications for an increased risk of toxicity in these patients (OR 1.2 compared to the control group). We can, however, not exclude the possibility that we missed a potential association due to the small number of patients that could be studied.

If reduced compensatory TS expression is indeed a consequence of the 2RC variant, then patients carrying 2RC risk-associated genotypes should not be treated with full-dose fluoropyrimidines but should receive either an alternative treatment or treatment with a reduced dose of fluoropyrimidines. It remains to be established what degree of dose reduction would be safe in these patients. Dose-adaptation in patients with DPD deficiency is relatively straightforward, since reductions in DPD activity are associated with predictable changes in 5-FU clearance. For TS, however, predicting the safe and efficacious dose in case of reduced TS activity is less straightforward. First of all, it is unknown to what degree compensatory TS expression is reduced. Even if this could be measured accurately, then we do not know what the relationship is between TS expression and pharmacodynamic effect. The safe dose would have to be determined by careful dose-escalation and pharmacodynamic measurements during exposure to 5-FU. A second important issue is whether reduced-dose treatment in patients with reduced TS activity results in sufficient antitumor effect, as tumor TS activity might be higher than in normal tissue and less dependent on the germ line genotype, as shown for instance in colorectal cancer.[23] Considering these facts, and if our findings are confirmed in future studies, it would be logical to consider the presence of the 2RC allele an indication for treatment with a reduced dose or, if possible, with a non-fluoropyrimidine-based treatment regimen.

Several potential limitations of our study need to be highlighted. Although we analyzed a cohort of patients treated in a prospective study, our study was retrospective in nature, which potentially introduced bias. However, the fact that toxicity data were collected systematically for all patients and the fact that DNA was collected from nearly all participating patients (>99.5%), reduces the chances of information and selection bias being introduced. Secondly, patients were treated with a variety of treatment regimens and were of different ages and sex. It could be that these factors interact with the risk-associated genotypes, and it is possible that only subsets of patients are at increased risk (*e.g.,* patients treated with a certain regimen). This might have limited our ability to accurately measure the risk associated with the 2RC allele. However, the fact that this cohort represents a daily-care cancer population is also a potential strength of the study, as it shows that even in a heterogeneous population an effect of the variant allele could be detected. Risk of severe toxicity was not found to be increased when considering the entire treatment duration, which was expected in view of strong heterogeneity in duration of treatment. Future studies investigating more homogeneous populations should focus on longer treatment durations to further establish the clinical relevance of the 2RC allele as a predictor of fluoropyrimidine-associated toxicity. Lastly, a crucial aspect in determining the clinical validity of pharmacogenetic variants is that findings be replicated. Therefore, our findings need to be replicated in a second, independent population of patients treated with fluoropyrimidines, to confirm the clinical validity of rs183205964.

The role of *TYMS* genotypes as predictors of treatment-related toxicity in patients treated with fluoropyrimidines has only recently started to be recognized. Recently, Rosmarin *et al.* showed that rs2612091 in *ENOSF1* (enolase superfamily member 1), a gene adjacent to *TYMS* and known to regulate TS activity,[24,25] might explain previously observed associations between the *TYMS* VNTR polymorphism and fluoropyrimidine-associated toxicity.[26] *ENOSF1* rs2612091 genotyping should therefore be considered in future studies, as it might be an independent predictor in addition to the 2RC allele and *TYMS* VNTR, and may further improve the predictive value to identify patients at risk of toxicity.

We showed that patients carrying 2RC risk-associated genotypes were at increased risk of early fluoropyrimidine-associated toxicity. A patient with the not previously described 2RC/2RC genotype suffered recurrent severe fluoropyrimidine-associated toxicity while being treated with capecitabine 825 mg/m^2 twice daily. In contrast to what has been shown in *in vitro* studies, TS activity and *TYMS* expression were not found to be reduced in this patient. More research on the functional consequences of rs183205964 is now needed. While the associations found in our study require confirmation in an independent cohort, caution

should be taken in administering fluoropyrimidines to patients who are known to carry the rs183205964 variant allele, particularly if present in homozygous form.

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