FULL PAPER

BJC

British Journal of Cancer (2013) 108, 2505–2515 | doi: 10.1038/bjc.2013.262

Keywords: DPYS; TYMS; MTHFR; CDA; 5-fluorouracil; capecitabine

Pharmacogenetic variants in the *DPYD*, *TYMS*, *CDA* and *MTHFR* genes are clinically significant predictors of fluoropyrimidine toxicity

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Background: Fluoropyrimidine drugs are extensively used for the treatment of solid cancers. However, adverse drug reactions are a major clinical problem, often necessitating treatment discontinuation. The aim of this study was to identify pharmacogenetic markers predicting fluoropyrimidine toxicity.

Methods: Toxicity in the first four cycles of 5-fluorouracil or capecitabine-based chemotherapy were recorded for a series of 430 patients. The association between demographic variables, *DPYD, DPYS, TYMS, MTHFR, CDA* genotypes, and toxicity were analysed using logistic regression models.

Results: Four *DPYD* sequence variants (c.1905 + 1G > A, c.2846A > T, c.1601G > A and c.1679T > G) were found in 6% of the cohort and were significantly associated with grade 3–4 toxicity ($P < 0.0001$). The *TYMS* 3′-untranslated region del/del genotype substantially increased the risk of severe toxicity ($P = 0.0123$, odds ratio (OR) = 3.08, 95% confidence interval (CI): 1.38–6.87). For patients treated with capecitabine, a *MTHFR* c.1298CC homozygous variant genotype predicted hand–foot syndrome ($P = 4.1 \times 10^{-6}$, OR = 9.99, 95% CI: 3.84–27.8). The linked *CDA* c. − 92A > G and *CDA* c. − 451C > T variants predicted grade 2–4 diarrhoea ($P = 0.0055$, OR = 2.3, 95% CI: 1.3–4.2 and $P = 0.0082$, OR = 2.3, 95% CI: 1.3–4.2, respectively).

Conclusion: We have identified a panel of clinically useful pharmacogenetic markers predicting toxicity to fluoropyrimidine therapy. Dose reduction should be considered in patients carrying these sequence variants.

The fluoropyrimidine drug 5-fluorouracil (5-FU) and the prodrug capecitabine have been extensively used for almost 5 decades (Ezzeldin and Diasio, 2004) either as monotherapy or in combination therapy for a variety of solid cancers including gastrointestinal tract and breast. However, adverse drug reactions are a major clinical problem, often necessitating dose reduction and treatment discontinuation. Diarrhoea, mucositis, myelosuppression and hand–foot syndrome are the most frequent and troublesome side effects. A meta-analysis of 1219 colorectal cancer (CRC) patients receiving 5-FU reported that grade 3–4 toxicity was encountered in 31–34% of patients, with 0.5% mortality (Cancer M-AGI, 1998). A significant proportion of these adverse drug reactions are likely to be the result of inter-individual genetic variation. Identification of the genetic factors underlying such variation would provide a basis for individualised patient dosing strategies and a significant advance on current empirical

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Received 16 November 2012; revised 26 March 2013; accepted 8 May 2013; published online 4 June 2013

dosing based solely on body surface area. To date, no robust pharmacogenetic markers of fluoropyrimidine toxicity have been validated for use as a standard of care for the routine management of patients with cancer (Ezzeldin and Diasio, 2008).

The metabolic pathways by which 5-FU and the prodrug capecitabine are converted to active nucleotide analogues are well described (Thorn *et al*, 2011) and have led to a number of candidate gene-based pharmacogenetic studies (Figure 1). As most of the administered 5-FU dose (80–90%) is degraded via dihydropyrimidine dehydrogenase (DPD, encoded by the *DPYD* gene), several studies have highlighted the role of DPD deficiency in the development of severe 5-FU related toxicity. These have been extensively reviewed by Amstutz *et al* (2011). Significant associations between variation in the *DPYS* gene encoding dihydropyrimidinase, the next step in fluoropyrimidine degradation and toxicity to 5-FU, have also been reported (Hamajima *et al*, 1998; van Kuilenburg *et al*, 2003).

Genetic polymorphisms in the promoter and 3′-untranslated regions (3′-UTR) of the *TYMS* gene are known to influence TS expression and have been associated with both toxicity and an improved clinical response (Horie *et al*, 1995; Kawakami and Watanabe, 2003; Lecomte *et al*, 2004; Mandola *et al*, 2004). Inhibition of TS requires binding of both 5,10-methylenetetrahydrofolate (5,10-MTHF) and 5-FdUMP. Methylene tetrahydrofolate reductase (MTHFR) catalyses the conversion of 5,10-MTHF to 5-methyltetrahydrofolate (5-MTHF). Studies correlating the *MTHFR* 677TT and *MTHFR* 1298CC variant homozygous genotypes with toxicity and response have been contradictory (De Mattia and Toffoli, 2009).

Capecitabine, a prodrug of 5-FU, is first hydrolysed by carboxyl esterases in the liver to form 5-fluorodeoxycytidine, which is then deaminated by cytidine deaminase (CDA) in liver and neoplastic tissue to form 5-fluorodeoxyuridine, which in turn is converted to 5-FU by thymidine phosphorylase (TP) (Hameed and Cassidy, 2011). Decreased CDA activity is predicted to lead to the accumulation of potentially toxic fluoro-cytidine metabolites. Variation in *CDA* expression has been linked to polymorphism in the *CDA* promoter region (Fitzgerald *et al*, 2006) and has been suggested to impact on both gemcitabine (Gilbert *et al*, 2006) and capecitabine metabolism (Mercier *et al*, 2009; Caronia *et al*, 2011).

In this series of 430 patients treated with fluoropyrimidine-based chemotherapy for predominantly colorectal malignancy, we identify a panel of pharmacogenetic markers predicting toxicity.

MATERIALS AND METHODS

Patients and clinical data. A series of 430 patients were recruited from oncology clinics forming part of a regional cancer network in South East London, UK. Ethical approval was obtained from St Thomas' Hospital Research Ethics Committee (07/H0802/143) and written consent was provided by all patients. For inclusion in the study, patients had to fulfil the following criteria: (1) World Health Organisation performance status <2; (2) life expectancy $\geqslant 3$ months; (3) any previous chemotherapy completed $\geqslant 6$ months ago; and (4) adequate haematological and cardiac status. Although the study was retrospective, clinical outcome data were obtained from standardised oncology outcome records completed at each clinic visit. Pre-treatment evaluation included a complete physical examination and recording of the following information: (1) baseline patient demographics (age, sex and ethnicity) and medical history; (2) diagnosis of tumour and staging (tumour, node, metastasis system); (3) current chemotherapy regimen (drug, dosing regimen) and (4) baseline blood analyses. Patients were assessed for treatment tolerance and had full blood count, renal function and liver function monitored before each chemotherapy cycle. All chemotherapy related toxicity in the first four cycles of treatment was recorded according to the National Cancer Institute

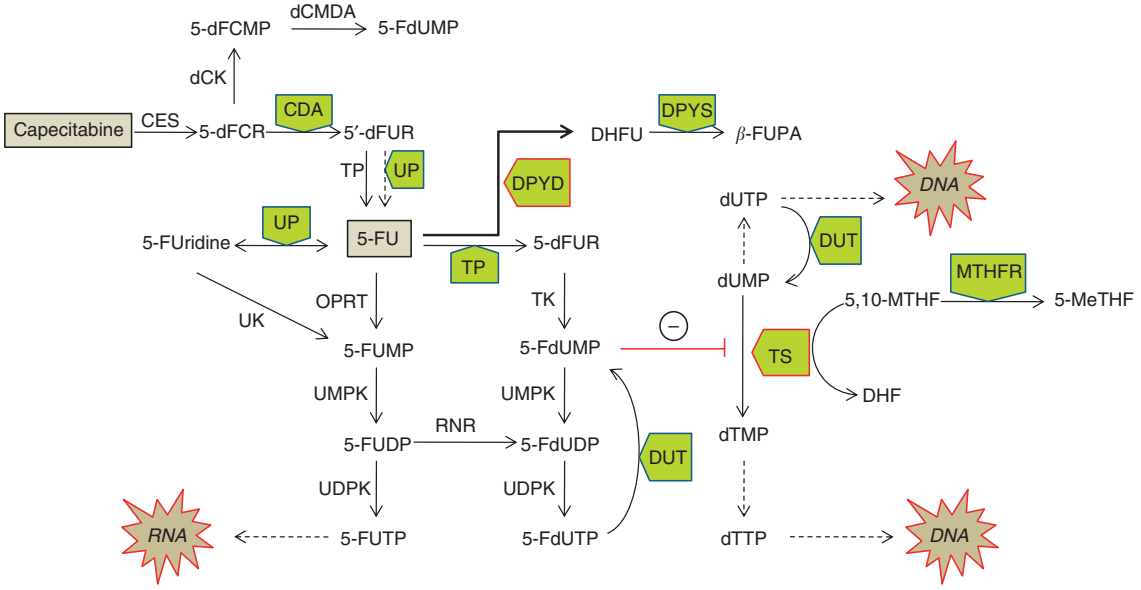

Figure 1. Capecitabine and 5-FU metabolism. Enzymes: Carboxyl esterase (CES), deoxycytidine kinase (dCK), deoxycytidine monophosphate deaminase (dCMDA), cytidine deaminase (CDA), thymidine phosphorylase (TP), uridine phosphorylase (UP), dihydropyrimidine dehydrogenase (DPYD), dihydropyrimidinase (DPYS), orotate phosphoribosyltransferase (OPRT), uridine kinase (UK), uridine monophosphate kinase (UMPK), uridine diphosphate kinase (UDPK), ribonucleotide reductase (RNR), thymidine kinase (TK), thymidine synthase (TS), deoxyuridine triphosphatase (DUT), methylene tetrahydrofolate reductase (MTHFR). Metabolites: deoxyfluorocytidine riboside (5′-dFCR), deoxyfluorocytidine monophosphate (5′-dFCMP), deoxyfluorouridine monophosphate (5-FdUMP), deoxyfluorouracil (5′-dFUR), fluorouracil (5-FU), fluorouridine (5-FUridine), fluorouracil monophosphate (5-FUMP), fluorouracil di, tri-phosphate (5-FUDP, 5-FUTP), deoxyfluorouracil di, tri-phosphate (5-FdUDP, 5-FdUTP), deoxyuridine mono, tri –phosphate (dUMP, dUTP), deoxycytidine mono, tri-phosphate (dTMP, dTTP), 5,10-methylenetetrahydrofolate (5,10-MTHF), 5-methyltetrahydrofolate (5-MeTHF), dihydrofolate (DHF), dihydrofluorouracil (DHFU), beta-fluoroureido propionic acid (β-FUPA).

Table 1. Polymorphisms genotyped

Gene	SNP name	Nucleotide change	Amino-acid substitution	Allele frequency in our cohort
DPYD	rs3918290	1905 + 1G > A	Exon 14 skipping	0.0047
DPYD	rs2297595	496 A > G	M166V	0.0988
DPYD	rs1801266	703 C > T	R235W	Not polymorphic
DPYD	rs1801158	1601 G > A	S534N	0.0186
DPYD	rs67376798	2846 A > T	D949V	0.0058
DPYD	rs55886062	1679 T > G	I560S	0.0012
DPYD	rs75017182		Intronic	0.0176
DPYD	—	1156 G > T	E386X	Not polymorphic
DPYD	—	295-298 del TCAT	Frameshift	Not polymorphic
DPYS	rs61758444	1423 C > T	R475X	Not polymorphic
DPYS	rs36027551	541 C > T	R181W	0.0023
DPYS	rs34895123	937 A > T	N313Y	Not polymorphic
DPYS	rs2669429		Intronic	0.4988
TYMS	rs59755869	298 G > C	E100Q	Not polymorphic
TYMS	rs596909	470 G > T	G157V	Not polymorphic
TYMS	rs11540152	349 T > C	F117L	Not polymorphic
TYMS	rs11540153	500 C > T	T167I	Not polymorphic
TYMS	rs59755869	298 G > C	E100Q	Not polymorphic
TYMS	rs34489327	1494del6b	3′-UTR 6 bp deletion	0.6779
TYMS	rs34743033	2R/3R (TSER*2/TSER*3)	TS enhancer region 28 bp repeat	0.5128
MTHFR	rs1801133	677 C > T	A222V	0.2849
MTHFR	rs1801131	1298A > C	E429A	0.2965
CDA	rs602950	c. − 92A > G	5′-UTR	0.2910
CDA	rs2072671	c.79A > C	K27Q	0.3115
CDA	rs532545	c. − 451C > T	Promoter region	0.3032
CDA	rs3215400	c. − 943insC	Promoter region	0.4221

Abbreviations: CDA = cytidine deaminase; DYPD = dihydropyrimidine dehydrogenase; DYPS = dihydropyrimidinase; MTHER = methylene tetrahydrofolate reductase; SNP = single-nucleotide polymorphism; TSER = TYMS promoter enhancer region; UTR = untranslated region

Common Toxicity Criteria version 3. Patient outcome data were not disclosed to investigators undertaking the genetic analysis.

Laboratory methods. DNA was extracted from EDTA whole blood using the QIAamp DNA Mini Kit (Qiagen Ltd, Crawley, UK).

Single-nucleotide polymorphisms were genotyped by TaqMan assay (Applied Biosystems, Warrington, UK) following the manufacturer's instructions using an Agilent Mx3005P RT–PCR instrument (Agilent Technologies, Edinburgh, UK) and are shown in Table 1. The nine candidate *DPYD* sequence variants genotyped were identified from the literature and from previous research in our laboratory (Loganayagam *et al*, 2010). Two common MTHFR deficiency associated sequence variants c.677C > T and *c.1298A > C* were genotyped. Coding region non-synonymous and non-coding region variants in *CDA* and *DPYS* were identified from the online single-nucleotide polymorphism (SNP) registry (http://www.ncbi.nlm.nih.gov/SNP) and included variants reported in the literature to be associated with response to fluoropyrimidine therapy or in the case of CDA, to modulate mRNA expression.

The *TYMS* 5′-UTR region containing the 28 bp 2R/3R tandem repeat polymorphism was amplified using primers forward 5′-CTC CGT TCT GTG CCA CAC C-3′ and reverse 5′-GTC TGT AAG GCG AGG AGG AC-3′, designed using the web-based tool primer3 (http://frodo.wi.mit.edu/) and synthesised by MWG Biotech, (Ebersberg Germany). PCR products were amplified using Hot Start DNA Polymerase (Rovalab, Teltow, Germany). PCR conditions were 1 min denaturation at 94 °C then 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 48 °C and 30 s extension at 72 °C and a last cycle consisting of 5 min extension at 72 °C. PCR products were purified using QIAquick PCR purification kit (Qiagen Ltd). Dye-terminator cycle sequencing was performed using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). Excess dye terminators were removed using Agencourt CleanSeq (Beckman Coulter Limited, High Wycombe, UK). Samples were run on an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems). Sequences were analysed by Mutation Surveyor Local v3.20 (Softgenetics LLC, State College, PA, USA). The TYMS 5′-UTR polymorphisms were classified by both the number of repeats and the number of functional upstream stimulatory factor (USF)-binding sites (depending on G > C or C > G SNPs within the tandem repeat). The homozygous double-repeat variant genotype was designated 2R/2R, the heterozygous genotype was designated as 2R/3R, and the homozygous triple repeat as 3R/3R. Variants with higher number of repeats were grouped into 3R for statistical analysis.

TYMS c.1494del6b 3′-UTR polymorphism was genotyped by amplifying the sample twice. Both amplifications shared the same forward primer 5′-ATTACAACAGGTCGTACAATTATGGC-3′, and differed in the reverse primer. The positive 5′-CTTTATTAT AGCAACATATAAAACAACTATAACT-3′ primer was designed to anneal when the 6 bp sequence was present, and the negative 5′-TTTATTATAGCAACATATAAAACAACTATA AAGT-3′ reverse

primer annealed specifically to the DNA sequence when the 6 bp fragment was absent. The fragments amplified with each set of primers were ~673 bp long. Each sample was run in consecutive lanes on a 2% standard agarose gel and scored for the presence or absence of the 673 bp band. A PCR blank and genotype controls were included in each series of PCR reactions.

Statistical analysis. Differences in the demographic and clinical characteristics of patients with and without toxicity were tested using *t*-tests (continuous variables) or Fisher's exact test (discrete variables). A baseline clinical model for toxicity was determined using logistic regression and backward stepwise selection used to identify relevant clinical characteristics to be included in the model. For the genetic association testing, sex, age and ethnicity (coded as European *vs* non-European ancestry) were included in all models as standard covariates, together with GFR.

The frequency of each SNP was compared with the published frequencies for Caucasian individuals in dbSNP and all SNPs were tested for departure from Hardy–Weinberg equilibrium. Association was tested for two toxicity outcome measures: diarrhoea, neutropenia and mucositis in the first four cycles of treatment were dichotomised as either mild to moderate (grade 0–2) or severe (grade 3–4). In capecitabine patients, hand–foot syndrome was dichotomised as grades 0–1 and 2–3. For statistical analysis of rare variants in a gene, carriers of at least one rare variant were identified, and Fisher's exact test was used to test for association across pooled variants with toxicity. For common variants, two analysis strategies were performed. First, a gene-based logistic regression analysis was performed across all common variants in the gene to assess cumulative evidence for association with toxicity. Second, analysis of each variant was performed using an allele-based Fisher's exact test, and with a logistic regression model, assuming an additive genetic model within and between variants, and including baseline clinical covariates. A gene-based logistic regression model was used to predict toxicity effects (0–2 *vs* 3–4) with all variants genotyped within the gene, together with explanatory variables of sex, age at initiation, GFR and ethnicity (Caucasian, non-Caucasian) included in all models. Separate models were fitted for each gene. In the initial analysis, genotypes were included as an additive model (0, 1, 2). Dominant and recessive models were tested to follow-up our significant results and when testing prior hypotheses from other studies. The number of repeats (in the *TYMS* promoter enhancer region (TSER) was analysed as a continuous variable.

Models were tested against the baseline model with only clinical factors included (sex, age, European ancestry and GFR). Akaike's information criterion was used to determine the SNPs to be included in the backward stepwise regression, and a likelihood ratio test was used to compare the final model with the baseline clinical model. A global model of all genetic variants was also analysed, to test for risk effects across genes for all patients and for capecitabine patients and the predictive ability of models with DPYD rare variants was compared with a model including all variants and clinical predictors. Predictive ability was assessed by the area under the curve (AUC) of the receiver-operating characteristic curve. Statistical analysis was performed using R v2.7.0.

A Bonferroni correction for multiple testing of 5 genes (*DPYD, DYPS, TYMS, MTHFR, CDA*) was applied, giving a significance threshold 0.01 ($=0.05/5$) for association *P*-values. Applying this across the common variants tested ($n=12$) would give a Bonferroni-corrected *P*-value of 0.0042.

RESULTS

Of the 430 patients recruited to the study, 186 (43%) were treated with 5-FU-based chemotherapy and 244 (57%) were on capecitabine-based therapy. Demographics and clinical characteristics are shown in Table 2. The majority of patients (85%) were treated for CRC. Of the 430 patients, only 19 patients (4%) had less than a full initial fluoropyrimidine dose, receiving a 75% dose owing to poor renal function.

Table 2. Demographics and clinical characteristics of 430 patients treated with fluoropyrimidine therapy

Demographic details	Number of patients (%)
Males	247 (57)
Females	183 (43)
Mean age (years)	61.9 (range 20–83)
Ethnicity *N* (%)	
Caucasian	364 (84.7)
Afro-Caribbean	50 (11.6)
South Asian	12 (2.8)
South East Asian	4 (0.9)
Cancer type *N* (%)	
Colorectal	364 (84.7)
Other Gastrointestinal[a]	62 (14.4)
Cancer of unknown primary	4 (0.9)
Treatment type *N* (%)	
Adjuvant	206 (47.9)
Neo-adjuvant	18 (4.2)
Palliative	206 (47.9)
Chemotherapy regimens	
5-FU + folinic acid or leucovorin	35 (8.1)
5-FU + oxaliplatin	96 (22.3)
5-FU + irinotecan	16 (3.7)
5-FU + mitomycin C	9 (2.1)
5-FU + epirubicin + cisplatin	30 (7.0)
Capecitabine monotherapy	79 (18.4)
Capecitabine + oxaliplatin	155 (36.1)
Capecitabine + mitomycin C	3 (0.7)
Capecitabine + epirubicin + cisplatin	7 (1.6)
Fluoropyrimidine type	
5-FU	186 (43)
Capecitabine	244 (57)

Abbreviation: 5-FU = 5-fluorouracil.
[a]Includes gastric, oesophageal, anal and hepatobilliary cancers.

The fluoropyrimidine dose was reduced to 75% of the initial dose in 126/430 patients, 104 of whom were dose reduced due to grade 3–4 toxicity (Table 3). Grade 3–4 diarrhoea, mucositis or neutropenia tended to occur in a polyvisceral syndrome in the first four cycles of treatment. There were no reported cases of mortality secondary to toxicity. Treatment was stopped in 31 out of 430 patients due to grade 3–4 toxicity and in 7 patients due to either disease progression ($n=2$), persistent line sepsis ($n=1$), frailty ($n=2$) or patient choice ($n=2$). The characteristics of patients with and without grade 3–4 toxicity (diarrhoea, mucositis or neutropenia) are shown in Table 4. In agreement with previous studies, patients with grade 3–4 toxicity were older than those with grade 1–2 toxicity ($P=0.025$, Student's *t*-test) and the average GFR values were significantly lower in the patients with grade 3–4 toxicity ($P=0.0014$, Student's *t*-test). There was no difference in toxicity according to gender ($P=0.426$), by Caucasian/non-Caucasian ethnicity ($P=0.351$), tumour stage ($P=0.8618$) or by the type of fluoropyrimidine drug ($P=1.000$). In capecitabine-treated

Table 3. Major types of toxicity in patients receiving fluoropyrimidine-based chemotherapy in the first four cycles of treatment

Toxicity type	Grade 0–2, *n* (%)	Grade 3–4, *n* (%)
Diarrhoea	362 (84)	68 (16)
Mucositis	415 (97)	15 (4)
Neutropenia	387 (90)	43 (10)
All toxicity[a]	326 (76)	104 (24)

[a]Diarrhoea, mucositis or neutropenia

patients, hand–foot syndrome was assessed on a severity scale 0–3, with 55 of 244 patients (23%) having severity score of 2–3.

Pharmacogenetic markers for fluoropyrimidine toxicity and clinical response. The allele frequencies in the study cohort are shown in Table 1, and were similar to those reported in online databases. All genotypes were in Hardy–Weinberg equilibrium. The genotyping success rate for all polymorphisms studied was >99%.

Four rare *DPYD* variants predict grade 3–4 toxicity. The study cohort was genotyped for nine *DPYD* sequence variants of which six variants were polymorphic in the cohort. Four rare *DPYD* sequence variants (c.1905+1G>A, c.2846A>T, c.1601G>A and c.1679T>G) known to be associated with DPD deficiency were present in 24 patients in heterozygous or compound heterozygous genotypes. All 24 patients experienced grade 3–4 diarrhoea, mucositis and/or neutropaenia in the first four cycles of chemotherapy (Table 5), $P<10^{-16}$, logistic regression. Two patients were compound heterozygous for the variants c.1601G>A/c.1905+1G>A and c.1601G>A/c.2846A>T, and both suffered grade 4 toxicity within the first two cycles, requiring hospital admission for 16 and 19 days, respectively. Of the three patients with heterozygous c.1905+1G>A genotypes, two patients experienced toxicity in the first two cycles of therapy, and continued therapy after 25 and 50% dose reductions. The third patient experienced grade 3–4 toxicities in cycles 3–4 and discontinued therapy. Ten of fourteen patients heterozygous for the c.1601G>A variant experienced 3–4 toxicity during the first two cycles of therapy. Eight patients tolerated a 25% dose reduction, two discontinued therapy, the remaining patient was not dose adjusted and suffered grade 4 toxicity in the subsequent cycle. Four patients experienced toxicity in cycle 3 and three tolerated a 25% dose reduction with one patient withdrawing from therapy. Three of four patients with heterozygous c.2846A>T genotypes experienced early grade 3–4 toxicity, one of whom discontinued and two continued therapy after 25% dose reductions. Toxicity was delayed to cycle 3 in the fourth patient and this patient tolerated a 25% dose reduction. The single patient with a heterozygous c.1679T>G variant genotype tolerated a 25% dose reduction after experiencing toxicity in cycles 3–4. The sensitivity, specificity, positive and negative-predictive values for the DPYD variants and toxicity are shown in Table 6. When all four variants were considered, the positive-predictive value for toxicity was 98% with a specificity of 100%.

Overall, the 104 patients experiencing grade 3–4 toxicity were admitted to hospital for a total of 423 days compared with 65 days for the 326 patients with grade 0–2 toxicity. Although the 24 patients carrying a c.1601G>A, c.1679T>G, c.1905+1G>A or c.2846A>T sequence variant comprised just 6% of the cohort, admissions for these patients accounted for 171/488 admission days or 35% of the total.

The *DPYD* intronic variant rs75017182 (c.1129-5923C>G) has been reported to be associated with mis-splicing of an intronic sequence into *DPYD* mRNA and 5-FU toxicity (van Kuilenburg *et al*, 2010). In our study, only heterozygous patients were found ($n=15$), none of which carried one of the rare variants analysed above. This variant, rs75017182, was not associated with grade 3–4 diarrhoea, mucositis or neutropaenia overall ($P=0.2210$). When the 24 patients with a deleterious DPYD genotype were excluded, the association between a heterozygous rs75017182 genotype and toxicity strengthened, but did not reach significance ($P=0.088$). The positive-predictive value for this variant was just 40% (Table 6). The common variant *DPYD* c.496A>G (p.Met166Val) was not associated with toxicity ($P=0.3957$).

Table 4. Characteristics of patients with grade 0–2 *vs* grade 3–4 neutropaenia, mucositis and diarrhoea

Feature	Grade 0–2 (*n*, %)	Grade 3–4 (*n*, %)	*P*-value
No. of patients	326 (76%)	104 (24%)	
Sex			
Male Female	191 (77%) 135 (74%)	56 (23%) 48 (26%)	0.4260
Age, years			
Mean Range	61 20–80	64 27–83	0.0250
European ancestry	279 (86%)	85 (82%)	0.3507
Glomerular filtration rate (ml min^{-1} 1.73 m^{-2})			
Mean Range	88 22–169	81 35–125	0.0014
Tumour stage			
T1 N0 to T4 N4 Metastatic	164/326 (50%) 162/326 (50%)	54/104 (52%) 50/104 (48%)	0.8618
Fluoropyrimidine type			
5-FU Capecitabine	141 (76%) 185 (76%)	45 (24%) 59 (24%)	1.0000

Abbreviation: 5-FU = 5-fluorouracil.
P-value from *t*-test (continuous variables) or Fisher's exact test (discrete variables).

***DPYS*, *MTHFR* and *TYMS* polymorphisms.** The association between polymorphisms in *DPYS*, *TYMS* and *MTHFR* and toxicity is shown in Table 7. Owing to the highly significant association between the four rare *DPYD* sequence variants (c.1905+1G>A, c.2846A>T, c.1601G>A or c.1679T>G) and grade 3 and 4 toxicity, the 24 patients with variant *DPYD* genotypes were excluded from subsequent analysis. Of the four *DPYS* coding sequence variants tested, two were not polymorphic in the cohort (rs34895123 (c.937T>A, p.Asn313Tyr) and rs61758444 (c.1423C>T p.Arg475Ter)). The rare coding region variant rs36027551 (*DPYS* c.541C>T, p.Arg181Trp) was polymorphic but was not significantly associated with toxicity (5 carriers observed; 1 carrier experienced toxicity; $P=1.00$). The common intron 1 sequence variant rs2669429 (c.265-58T>C) previously reported to protect against side effects (Fidlerova *et al*, 2010) was not significantly associated with protection from diarrhoea, mucositis and/or neutropaenia (Table 7; $P=0.513$).

The five *TYMS*-coding region sequence variants reported in SNP databases and listed in Table 1 were not polymorphic in the cohort. Sequencing of the *TYMS* promoter region revealed considerable variation, with the number of repeats varying from 2 to 4. No association between repeat number and toxicity was seen in the either logistic regression model ($P=0.080$) or Fisher's exact

Table 5. Variant *DPYD* genotypes and fluoropyrimidine toxicity

Variant genotype	Cycle 1–2 toxicity (*n*)	Cycle 1–2 dose reduction	Cycle 3–4 toxicity (*n*)	Cycle 3–4 dose reduction
c.1905+1G>A heterozygous	2	One patient dose reduced by 50%, the other by 25%	1	Withdrew from therapy
c.1905+1G>A/c.1601G >A compound heterozygous	1	Grade 4 toxicities, withdrew from therapy	—	—
c.2846A>T heterozygous	3	1 Patient withdrew from therapy, 2 patients tolerated 25% dose reduction	1	25% Dose reduction
c.2846A>T/c.1601G>A compound heterozygous	1	Withdrew from therapy	—	—
c.1601G>A heterozygous	10	8 Patients were dose reduced by 25%, two patients withdrew from therapy	4	3 Patients 25% dose reduction, 1 patient discontinued therapy
c.1679T>G	—	—	1	25% Dose reduction

Abbreviation: DPYD = dihydropyrimidine dehydrogenase.

Table 6. Sensitivity, specificity, positive- and negative-predictive values of variant genotypes significantly associated with toxicity

Variant	Toxicity and grade	Genotype	Sensitivity	Specificity	Positive-predictive value	Negative-predictive value
All fluoropyrimidine-treated patients						
c.1601G>A	DMN 3–4	wt *vs* het	14	100	>99	78
c.1905+1G>A	DMN 3–4	wt *vs* het	3	100	>99	76
c.2846A>T	DMN 3–4	wt *vs* het	4	100	>99	77
c.1679T>G	DMN 3–4	wt *vs* het	1	100	>99	76
c.1601+1905+2846+1679 variant	DMN 3–4	wt *vs* het+compound het	23	100	>99	80
c.1129-5923C>G	DMN 3–4	wt *vs* het	6	97	40	76
TYMS 3′-UTR c.1494del6b	DMN 3–4	wt *vs* hom	47	54	25	76
TYMS 3′-UTR c.1494del6b	DMN 3–4	wt *vs* het and hom	81	9	22	59
Capecitabine-treated patients						
CDA c.−92 A>G c.−451C>T	Diarrhoea 2–4	wt *vs* het and hom	61	58	40	77
MTHFR 1298CC	HF 2–3	wt *vs* hom	26	96	64	82
MTHFR 1298A>C	HF 2–3	wt *vs* het and hom	55	49	24	79

Abbreviations: DMN = diarrhoea, mucositis and neutropaenia; HF = hand–foot syndrome; UTR = untranslated region.

test, classifying $\leqslant 2$ variants and >2 variants ($P=0.140$). The number of functional USF-binding sites present in the repeat region, which is a function of additional single-nucleotide substitutions in the repeat region, did not predict toxicity. The gene-based analysis of *TYMS* variants showed some evidence of association ($P=0.031$), which would not withstand correction for multiple testing of genes. Further analyses of these variants by genotype did not strengthen the association.

Given the reported influence of the *TYMS* 3′-UTR c.1494del6b variant on mRNA stability, further exploration suggested a recessive effect for the deletion with Fisher's exact test showing a significant association between the rare homozygous del/del genotype and diarrhoea, neutropaenia and mucositis ($P=0.0123$, OR = 3.08, 95% confidence interval (CI): 1.38–6.87).

The *MTHFR* 677C>T and 1298A>C variants were not significantly associated with grade 3–4 diarrhoea, mucositis and neutropenia in an analysis including both 5-FU and capecitabine-treated patients (Table 7).

Full modelling analysis of all genotyped variants showed the best fitting model with rare DPYD variants, the number of functional USF-binding sites in the 5′-UTR of TYMS, and the TYMS 3′-UTR deletion (full results shown in Supplementary Materials). However, adding the common variants provided little increase in predication compared with a model with the clinical variables only, with AUC increasing from 0.72–0.74 (Figure 2).

Subgroup analysis: Capecitabine Cohort. Three sequence variants in the *CDA* promoter region, c.−92A>G (rs602950), c.−451C>T (rs532545) and c.−943insC (rs3215400) have been associated with increased *CDA* expression *in vitro*. After excluding 12 capecitabine patients with known *DPYD* mutations, the logistic regression model showed evidence of an association between the presence of the *CDA* c.−92A>G variant and diarrhoea, mucositis and neutropaenia ($P=0.052$), although this was not significant using Fisher's exact test ($P=0.38$). Further analysis of five toxicity phenotypes in a stepwise logistic regression including only the c.−92A>G polymorphism, revealed an association between a heterozygous or homozygous c.−92A>G genotype and the development of grade 2–4 diarrhoea ($P=0.002$) and dehydration ($P=0.042$) in the first four cycles of chemotherapy (Table 8). The c.−92G variant allele showed an additive effect with each c−92G allele present increasing the risk of diarrhoea two-fold

Table 7. Association of common genetic polymorphisms with grade 3–4 diarrhoea, mucositis and neutropenia, excluding patients with rare variant DPYD alleles

	Patients with toxicity levels (*n*)			*P*-value from			
Polymorphism	Grade 0–2	Grade 3–4	Grade 3–4, excluding rare DPYD variants	Fisher's exact test	Regression model	OR (95% CI)	Gene-based regression model
***DPYS* rs2669429**							
AA	90	21	17	0.7901	0.546	1.23 (0.86–1.78)	—
AG	148	56	44				
GG	85	25	18				
***TYMS* 5′-UTR repeats**							
2R/2R	76	35	27	0.2166	0.08	0.73 (0.52–1.04)	0.03101
2R/3R	157	40	31				
3R/3R	93	29	22				
***TYMS* 5′-UTR**							
No. of functional USF-binding sites							
1	7	1	1	0.5938	0.0835	0.62 (0.36–1.06)	
2	174	64	47				
3	119	30	24				
4	22	9	8				
5	4	0	0				
***TYMS* 3′-UTR**							
del/del	27	20	15	0.1309	0.2668	0.80 (0.54–1.19)	
in/del	148	35	30				
in/in	151	49	35				
***MTHFR* 677C>T**							
wt/wt	170	57	45	0.5575	0.8115	0.95 (0.64–1.41)	0.9686
wt/mut	123	38	28				
mut/mut	33	9	7				
***MTHFR* 1298A>C**							
wt/wt	163	50	39	1	0.8568	1.04 (0.70–1.52)	
wt/mut	133	46	35				
mut/mut	30	8	6				

Abbreviations: CI = confidence interval; del = deletion; DPYD = dihydropyrimidine dehydrogenase; DPYS = dihydropyrimidinase; mut = mutation; MTHFR = methylene tetrahydrofolate reductase; OR = odds ratio; USF = upstream stimulatory factor; UTR = untranslated region; wt = wild-type.
ORs are shown from the linear regression model.

(Fisher's exact test, $P = 0.0055$, OR = 2.3, 95% CI: 1.3–4.2). Similarly, the *CDA* c.−451C>T variant was also associated with the development of grade 2–4 diarrhoea in the first four cycles of chemotherapy ($P = 0.0082$, OR = 2.3, 95% CI: 1.3–4.2). There is a strong linkage disequilibrium between these two variants ($r^2 = 0.95$) and only 2.2% of haplotypes showed discordant alleles. The promoter variant c.−943insC was not associated with toxicity nor were any of the three *CDA* variants associated with hand–foot syndrome. A global model of all variants for toxicity showed the best fitting model with rare DPYD variants and the CDA c.−92A>G genotype. However, as with the full cohort, adding the common variants provided little increase in prediction, with AUC increasing only from 0.74 to 0.75 with the addition of common SNPs to a model with DYPD rare variants and clinical variables (Figure 2).

Unexpectedly, a subgroup analysis restricted to patients treated with capecitabine revealed a significant association between *MTHFR*677T and *MTHFR*1298C variant genotypes and hand–foot syndrome ($P = 0.0046$; logistic regression model). The best fitting model was a recessive model for *MTHFR* 1298CC genotypes, which significantly increased the risk of hand–foot syndrome (logistic regression, $P = 4.1 \times 10^{-6}$, OR = 9.99, 95% CI: 3.84–27.8).

DISCUSSION

We have previously reported that three DPYD variants (c.1905+1G>A, c.1679T>G (p.I560S) and c.2846A>T (p.D949V)) are prevalent in the UK population and are significant associated with grade 3–4 toxicity (Loganayagam *et al*, 2010). In the well-powered study reported here, we have confirmed these findings, and we have identified a fourth DPYD variant c.1601G>A (p.S534N) significantly associated with severe fluoropyrimidine toxicity. A total of 24 patients were heterozygous or compound heterozygous for these variants and all 24 patients experienced severe diarrhoea, mucositis and/or neutropenia in the first four cycles of therapy. In line with previous reports, DPD deficiency accounted for 23% of cases with grade 3–4 toxicity (Morel *et al*, 2006; Loganayagam *et al*, 2010). Patients with a variant DPYD allele comprised 6% of the cohort but accounted for a disproportionate 35% of all hospital admission days.

Dihydropyrimidine dehydrogenase deficiency is recognised as a significant cause of grade 3–4 toxicity (Amstutz *et al*, 2011) and the four *DPYD* variants we found to be associated with severe toxicity

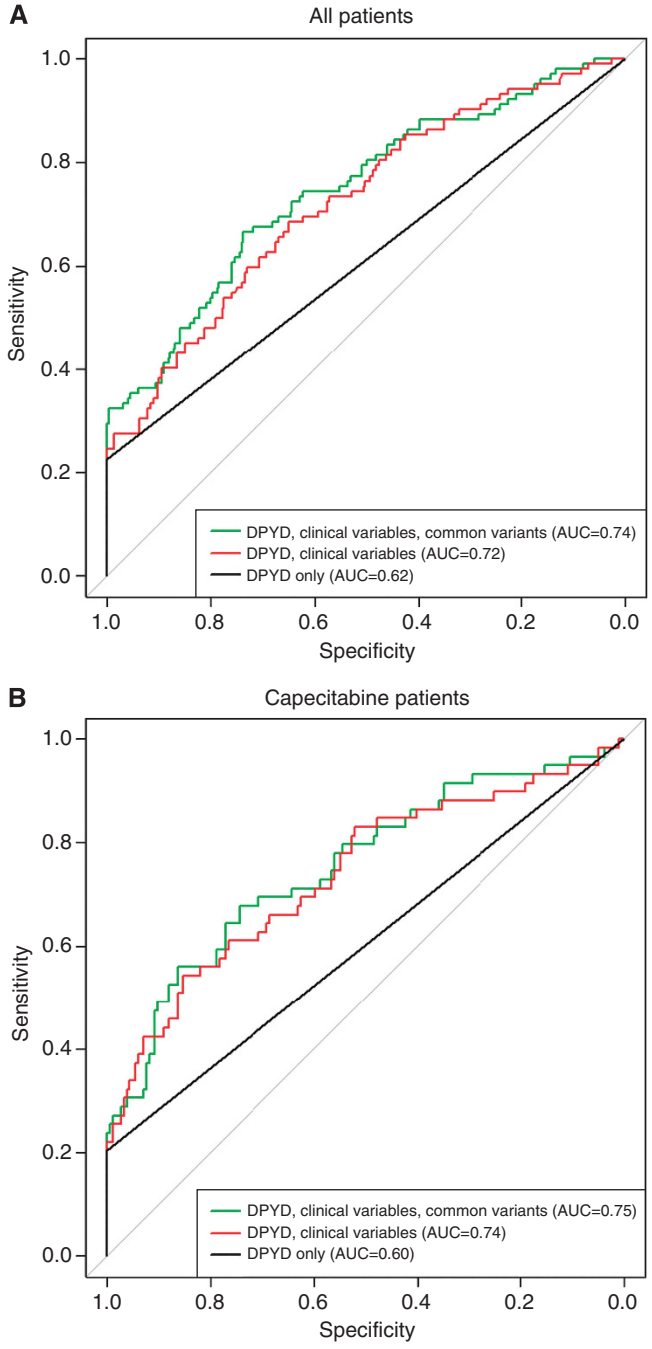

Figure 2. Receiver-operating characteristic curves for the prediction models with all variables (rare DPYD variants, common variants, clinical variables) for all patients (**A**) and the capecitabine cohort (**B**).

have been previously described in the literature as variants associated with decreased DPD enzyme activity and a high risk of severe 5-FU toxicity (van Kuilenburg *et al*, 2002; Seck *et al*, 2005; Morel *et al*, 2006; Loganayagam *et al*, 2010). The majority of patients 18/24 (75%) carrying a DPYD variant experienced severe toxicity in the first two cycles of therapy, with the remaining 6 patients experiencing severe toxicity in cycle 3 or 4. Although different in design, our study is broadly in line with the findings of Deenen *et al* (2011) who reported that not all patients carrying a DPYD variant experienced severe toxicity in the first two cycles of therapy, specifically, we and Deenen *et al* (2011), found that all patients heterozygous for the 1905 + 1G > A intron 14 splice site variant experienced severe toxicity, contrasting markedly with the findings of those of Schwab *et al* (2008) who reported toxicity in just 6/13 patients with the *DPYD* intron 14 splice variant. For the c.2846A > T variant we found 3/4 patients with a heterozygous genotype experienced severe toxicity in the first two cycles of therapy, not dissimilar to the 62% of patients experiencing toxicity within the first two cycles reported in the Deenen *et al* (2011) study and the 3/5 patients reported by Schwab *et al* (2008). The c.1601G > A variant is the most frequent toxicity-associated variant identified in our study, with 14 patients carrying the variant allele in a heterozygous genotype of whom 10 (71%) experienced early severe toxicity. These results differ markedly from those of Schwab *et al* (2008) where 2/7 cases carrying this variant experienced toxicity. Similarly, Amstutz *et al* (2009) reported that 1/5 patients heterozygous for this variant experienced toxicity. Interestingly, although Deenen *et al* (2011) did not find a significant association between the c.1601G > A variant and early toxicity, this study did report the specificity for this variant for any toxicity as 94% with a positive-predictive values of 83%, comparable to the specificity of 100% and positive-predictive value of > 99% found in our study (Table 6). There is additional evidence that the c.1601G > A (p.S534N) variant impacts on DPD activity (Seck *et al*, 2005) and the variant has been associated with severe toxicity in a series of smaller studies (Collie-Duguid *et al*, 2000; Gross *et al*, 2003). The single patient with a heterozygous c.1679T > G genotype experienced late toxicity after cycle 2.

We were unable to replicate the significant association between the deep intronic *DPYD* variant rs75017182 (c.1129-5923C > G), which is in disequilibrium with the synonymous exonic variant c.1236G > A, and toxicity. The intronic variant is reported to result in the insertion of 44 base pair sequence derived from intron 10 into *DPYD* mRNA and was found to be significantly ($P = 0.033$) overrepresented in a retrospective cohort of 203 cancer patients (van Kuilenburg *et al*, 2010) and in a c.1236G > A haplotype, with severe diarrhoea (Deenen *et al*, 2011). Similarly we found that the c.496A > G (p.Met166Val) variant was not significantly associated with toxicity, contrary to the report of Gross *et al* (2008).

Small case studies have suggested that deficiency of dihydropyrimidinase, the second enzyme in 5-FU catabolism, results in severe 5-FU toxicity (Hamajima *et al*, 1998; van Kuilenburg *et al*, 2003). However, in our study, only one out of the three *DPYS* coding SNPs genotyped was polymorphic in the cohort and no significant associations were found. The intron 1 sequence variant rs2669429 (c.265-58T > C), previously reported to protect against side effects (Fidlerova *et al*, 2010), was not significantly associated with protection from diarrhoea, mucositis and/or neutropaenia.

Thymidylate synthase is a key enzyme in thymidine nucleotide biosynthesis, and is the main intracellular target of the active 5-FU metabolite, FdUMP, which forms a ternary complex with TS and 5,10-MTHF (Pinedo and Peters, 1988). Variable numbers (2–9) of a 28-bp tandem repeat sequence (VNTR) are present in the 5′-UTR in the TSER with the most frequent being 2R and 3R repeats. The 2R repeat contains one USF-binding site and two are found in the 3R. Functional studies have shown a stepwise increase in TS transcription with an increasing number of tandem repeats (Horie *et al*, 1995). Additional polymorphic variation within repeats also alters USF-binding sequences and influences *TYMS* transcription (Kawakami and Watanabe, 2003; Mandola *et al*, 2003; Lincz *et al*, 2007). We found no significant associations between the number of *TYMS* 5′-UTR region repeats, or the number of functional USF-binding sites and toxicity. The findings from our study differ from a large prospective study where the high expression genotypes 2R/3R or 3R/3R were significantly associated with a lower risk of toxicity, (Schwab *et al*, 2008). Other studies have produced contradictory results (Largillier *et al*, 2006; Schwab *et al*, 2008; Sharma *et al*, 2008; Gusella *et al*, 2009). We would agree with a recent meta-analysis concluding that although variation in the *TYMS* 5′-UTR region may be associated with adverse reactions,

Table 8. Association of common genetic polymorphisms in MTHFR and CDA with grade 3–4 diarrhoea, mucositis and neutropenia in capecitabine patients

	Patients with toxicity levels (*n*)			*P*-value from			
Polymorphism	Grade 0–2	Grade 3–4	Grade 3–4, excluding rare DPYD variants	Fisher's exact test	Regression model	OR (95% CI)	Gene-based regression model
***MTHFR* 677C>T**							
wt/wt	107	31	27	1	0.906	1.03 (0.61–1.71)	0.4328
wt/mut	60	23	16				
mut/mut	18	5	4				
***MTHFR* 1298A>C**							
wt/wt	84	34	25	0.2129	0.2358	0.73 (0.42–1.22)	
wt/mut	81	23	20				
mut/mut	20	2	2				
CDAc. −92A>G							
wt/wt	101	26	19	0.0764	0.05171	1.65 (1.00–2.75)	0.2216
wt/mut	66	26	21				
mut/mut	18	7	7				
CDA c.79A>C							
wt/wt	93	24	17	0.1366	0.09805	1.55 (0.92–2.61)	
wt/mut	73	29	24				
mut/mut	19	6	6				
CDA c. −451C>T							
wt/wt	97	26	19	0.1353	0.1041	1.52 (0.92–2.54)	
wt/mut	68	26	21				
mut/mut	20	7	7				
CDA c. −943insC							
wt/wt	63	16	16	0.9068	0.9551	0.99 (0.61–1.59)	
wt/mut	90	24	24				
mut/mut	32	7	7				

Abbreviations: CDA = cytidine deaminase; CI = confidence interval; del = deletion; mut = mutation; MTHRR = methylene tetrahydrofolate reductase; OR = odds ratio; wt = wild-type. ORs are shown from the linear regression model.

the effect is likely to be small and testing would not be clinically useful (Jennings *et al*, 2012).

The 6-bp deletion in the 3′-UTR region of the *TYMS* gene (*TYMS* c.1494del6b) has been reported to modulate gene regulation at a post-transcriptional level through decreased mRNA stability (Mandola *et al*, 2004). We found a significant association between the c.1494del6bp variant and toxicity. The presence of a homozygous del/del genotype approximately doubled the risk of grade 3–4 toxicity. However, other studies have failed to show an association between a homozygous del/del genotype and severe toxicity (Sharma *et al*, 2008; Braun *et al*, 2009; Lurje *et al*, 2009). With a specificity for the homozygous genotype and toxicity of 54% and a positive-predictive value of just 25%, we suggest that testing for this variant would not be clinically useful.

The *MTHFR* 677C>T and 1298A>C variant genotypes were not significantly associated with severe 5-FU toxicity overall. This is in agreement with previous published studies (Cohen *et al*, 2003; Ruzzo *et al*, 2007, 2008; Schwab *et al*, 2008) but contradicts the findings of others, which have reported significant associations between variant *MTHFR* genotypes and clinical outcomes (Sharma *et al*, 2008; Afzal *et al*, 2009; Gusella *et al*, 2009; Castillo-Fernandez *et al*, 2010).

In a subgroup analysis, we then analysed the 244 patients treated with the fluoropyrimidine prodrug capecitabine. The *MTHFR* c.1298A>C polymorphism was significantly associated with grade 2–3 hand–foot syndrome, which occurred in 22% of patients. Patients with the *MTHFR* 1298CC homozygous variant genotype were ten times more likely to develop hand–foot syndrome. Methylene tetrahydrofolate reductase catalyses the irreversible conversion of 5,10-MTHF to 5-MTHF. As 5,10-MTHF inhibits TS activity in conjunction with 5-FdUMP, reduced *MTHFR* activity, which is associated with increased levels of 5,10-MTHF, theoretically leads to more effective TS inhibition. The association between the *MTHFR* 1298CC genotype and hand–foot syndrome would be consistent with a localised increase in the conversion of capeticabine to active metabolites and increased inhibition of TS. Interestingly, TP, which catalyses the conversion of the intermediate 5-fluorodeoxyuridine to 5-FU, is highly expressed in the skin, and activity of this enzyme has been suggested to be a mechanism whereby a localised increase in the production of 5-FU leads to hand–foot syndrome (Milano *et al*, 2008). Variation in CDA has also been previously reported to be associated with hand–foot syndrome. Caronia *et al* (2011) found a significant association between the *CDA* c. −451C>T variant allele and grade 3 hand–foot syndrome, and a protective effect for the *CDA* rs315400insC variant (Caronia *et al*, 2011), although the latter variant was not significantly associated with toxicity in an earlier study (Ribelles *et al*, 2008). We were, however, unable to replicate these associations.

The *CDA* promoter region variants, c.−92 A>G (rs602950) and c.−451C>T (rs532545), have been associated with increased *CDA* expression *in vitro* (Fitzgerald *et al*, 2006) and we predicted that these variants would influence capecitabine metabolism and hence clinical outcome to therapy. In our study, the *CDA* promoter region variants, c.−92 A>G and c.−451C>T, were associated with grade 2–4 diarrhoea, and capecitabine-treated patients with variant genotypes twice as likely to develop diarrhoea in the first four cycles of chemotherapy. However, the positive-predictive value of 40% is relatively low and further studies are needed to determine whether testing for this variant is clinically useful.

In conclusion, our results, and those of others, provide convincing evidence that patients with the four variant *DPYD* genotypes c.1905+1G>A, c.1601G>A, c.1679T>G and c.2846A>T will experience grade 3–4 toxicity. The combined positive predicted value for the four variants is >99% with a negative-predictive value of 80%. We suggest there is sufficient evidence to justify testing for these variants before the start of therapy and dose reducing patients with variant genotypes. For the c.1905+1G>A variant, we agree with previous reports suggesting that the severity of side effects will be reduced by a dose reduction of 50–60% before the start of therapy (Deenen *et al*, 2011; Yang *et al*, 2011; van Kuilenburg *et al*, 2012), while patients carrying the other variant *DPYD* genotypes may benefit from a lesser dose reduction of 25%.

Diarrhoea and hand–foot syndrome have been reported to be amongst the most frequent toxicities requiring dose reduction patients on capecitabine therapy (Walko and Lindley, 2005). We have identified two markers in the *CDA* gene, which are significantly associated with grade 2–4 diarrhoea. There is, however, currently no evidence supporting a dose reduction strategy in patients with variant *CDA* genotypes. At the very least, these markers may be used by clinicians to anticipate severe diarrhoea and to prescribe appropriate doses of anti-diarrhoeal agents. We have also identified a homozygous MTHFR 1298CC variant genotype as a pharmacogenetic marker MTHFR for hand–foot syndrome in patients treated with capecitabine. Avoidance of hand–foot syndrome would add substantially to quality of life. Further studies are needed to determine whether capecitabine dose reduction would compromise clinical efficacy.

Finally, the majority of cases of severe toxicity remain unexplained. Pharmacogenetic variation in genes involved in the metabolism of co-therapies are likely to explain a subset of these adverse events.

ACKNOWLEDGEMENTS

We acknowledge funding from Purine Metabolic Patient Association (PUMPA), CANHELP and Guy's and St Thomas' Charity. We acknowledge support from the National Institutes of Health Research Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)