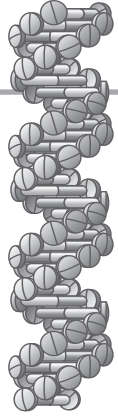

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Dihydropyrimidine dehydrogenase gene variation and severe 5-fluorouracil toxicity: a haplotype assessment

Aims: The importance of polymorphisms in the dihydropyrimidine dehydrogenase gene (*DPYD*) for the prediction of severe toxicity in 5-fluorouracil (5-FU)-based chemotherapy is still unclear. This study aims to assess the predictive value of *DPYD* variation with respect to previously described *DPYD* variants for 5-FU toxicity. It represents the first analysis of the gene at the haplotype level, also capturing potentially important genetic variation located outside the coding regions of *DPYD*. **Materials & methods:** The entire coding sequence and exon-flanking intronic regions of *DPYD* were sequenced in 111 cancer patients receiving fluoropyrimidine-based chemotherapy. *DPYD* haplotypes were inferred and their associations with severe 5-FU toxicity were assessed. **Results:** None of the previously described deleterious variants (IVS14+1G>A, c.2846A>T and c.1679T>G) were detected in 24 patients who experienced severe 5-FU toxicity. A potential association was observed between a haplotype containing three novel intronic polymorphisms (IVS5+18G>A, IVS6+139G>A and IVS9–51T>G) and a synonymous mutation (c.1236G>A), which was observed five- out of eight-times in patients with severe adverse effects. **Conclusion:** The association of a haplotype containing no nonsynonymous or splice-site polymorphisms indicates that additional important genetic variation may be located in noncoding gene regions. Furthermore, a comparison with other studies suggests that the relative importance of particular *DPYD* mutations (IVS14+1G>A and c.2846A>T) for predicting severe 5-FU toxicity differs geographically across Europe.

KEYWORDS: 5-fluorouracil ▪ capecitabine ▪ dihydropyrimidine dehydrogenase ▪ gene polymorphism ▪ haplotype

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The fluoropyrimidines, 5-fluorouracil (5-FU) and its prodrug capecitabine, have been the mainstay of chemotherapy in the treatment of various solid cancers for over 40 years, with approximately 2 million patients being treated worldwide each year [1]. Whereas genetic factors have been suggested to account for part of the estimated proportion of the 10–40% of patients who develop severe to life-threatening toxicity to 5-FU [2], no genetic predictor of severe 5-FU toxicity has proven to be reliable enough for routine clinical use [3,4]. As a key enzyme in the catabolism of 5-FU, dihydropyrimidine dehydrogenase (DPD) is the top candidate for pharmacogenetic studies on 5-FU toxicity, since a reduced DPD activity is thought to result in an increased half-life of the drug and thus, an increased risk of toxicity [1].

Numerous studies have investigated the DPD gene (*DPYD*) in the context of 5-FU sensitivity; however, most of them were based on small and biased patient samples, focusing exclusively on selected gene variants [5] and demonstrating conflicting results. Only recently, two large prospective screenings investigated *DPYD* in a more comprehensive manner [6,7]. Interestingly, the results of these two studies differ substantially; Morel *et al.* detected deleterious *DPYD* mutations in 29% of patients with severe 5-FU-related toxicity [6], whereas the fraction of toxicities explained by such *DPYD* variants in the second study [7] was much smaller (8%). Therefore, additional studies are needed to assess the predictive value of *DPYD* variation in the context of severe 5-FU toxicity.

Furthermore, previous studies focused exclusively on the coding region of *DPYD*. However, there is growing evidence that intronic mutations can have a significant functional impact, such as affecting the regulation of mRNA splicing [8]. For example, the deleterious effect of up to 50% of disease-causing mutations has been discovered to result from the disruption of the splicing code [9]. Moreover, such mutations that affect splicing need not be restricted to the well-defined splice sites, but they can also be located within exons or in introns that are close to, or far away from, the splice sites [8]. Consequently, intronic variation in *DPYD* may harbor important genetic information for the prediction of 5-FU toxicity.

Unfortunately, studying the complete intronic sequences of large genes, such as *DPYD*, is an extremely laborious task in large-scale

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screenings. However, owing to the extensive amount of linkage disequilibrium within a gene, haplotype-based analyses might lead to the detection of important haplotypes without the need for analyzing complete intronic sequences.

Therefore, the aims of the present study were:

- To assess the predictive value of previously described *DPYD* variants with respect to 5-FU toxicity;
- To investigate, for the first time, *DPYD* variation at the haplotype level in the context of 5-FU toxicity in a population of cancer patients of Caucasian origin.

To this end, we investigated not only the complete coding sequence of *DPYD*, but also the exon-flanking intronic regions, enabling the inference of recombination patterns within the gene and individual haplotypes, in order to gain information regarding the predictive value of genetic variation located between the investigated regions.

Materials & methods

Patients & sample collection

Between January 2006 and June 2007, a blood sample was collected from consenting patients who were scheduled for adjuvant or palliative 5-FU-based chemotherapy. Blood samples were collected before or during chemotherapy; patient and treatment characteristics, as well as adverse drug effects, during the first and second course of chemotherapy were assessed by detailed chart review according to Swiss law. Adverse effects were classified according to the common terminology criteria for adverse events (CTCAE) version 3.0 [101]; all toxicities corresponding to grade 3 and higher were considered severe.

The final study population consisted of 111 patients (40 women and 71 men). This included the first 98 patients and all additional nine patients who showed severe 5-FU-related toxicity in a prospective group of 183 patients, as well as four additional patients with known severe 5-FU-related toxicity. All patients from the prospective group were treated at the Bern University Hospital and the four additional patients with severe 5-FU toxicity were treated in hospitals from the same region. All patients suffered from solid malignant tumors, including 66 patients with colorectal cancer, 29 patients with other gastrointestinal tumors and 16 patients with nongastrointestinal tumors. The median age of the patients was 63 years, ranging from 32 to 89 years of age. A total of 26 patients received 5-FU as monotherapy or in combination with folinic acid; 38 patients received 5-FU plus folinic acid and oxaliplatin (FOLFOX); nine patients were treated with 5-FU, folinic acid and irinotecan (FOLFIRI); 13 patients received 5-FU plus a platinum-based compound (e.g., cisplatin or carboplatin); 13 patients were treated with capecitabine alone; 12 patients received other 5-FU-based chemotherapy regimens (e.g., 5-FU with epirubicin and cyclophosphamide). For some patients, these regimens were further combined with targeted therapies using monoclonal antibodies (e.g., bevacizumab and matuzumab). A total of 33 patients had previously received 5-FU as a first-line treatment, whereas the remaining 78 patients were 5-FU naive.

Sequencing analysis

Genomic DNA was extracted from ethylenediaminetetraacetic acid (EDTA) blood samples using the BioRobot EZ1 (Qiagen [Hilden, Germany]) and the EZ1 DNA Blood 350 µl Kit (Qiagen). The 23 exons and flanking intronic regions of *DPYD* were amplified either separately or in multiplex reactions [10] using the primers and mixes given online in Supplementary Table 1. PCR reactions were performed following standard protocols (given in the Supplementary material) using either the Multiplex PCR Kit (Qiagen; exons 2–23) or the GC-rich PCR system (Roche Applied Science [Basel, Switzerland]; exon 1). All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), and were sequenced using one of the same primers as used for PCR amplification, the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems [ABI; CA, USA]) and an ABI Prism 3130xl Genetic Analyzer (ABI). Sequences were initially obtained in only one direction; ambiguous sequences or sequences of inferior quality were subsequently confirmed by sequencing both strands (Supplementary Table 1). On average, 47% of the analyzed DNA fragments were sequenced in both directions. Comparisons of the obtained sequences with the reference sequence (NM_000110) were performed using the software SeqScape v2.6 (ABI).

Statistical analyses

Deviations of genotype frequencies from the Hardy–Weinberg equilibrium were assessed for all polymorphic loci using the exact test implemented in the program Arlequin v3.11 [11]. The standardized linkage disequilibrium coefficients (D′ [12]) between pairs of loci were estimated using Hill's maximum likelihood method [13];

significance of genotypic disequilibrium was assessed using the log-likelihood ratio (G) test, which is implemented in the software Genepop version 4.0 [14] with the default settings. Individual haplotypes were estimated using the Excoffier–Laval–Balding (ELB) algorithm, implemented in Arlequin with the recommended settings [15]. Associations of single gene variants and haplotypes with severe 5-FU toxicity were assessed by performing Fisher's exact tests using unadjusted p-values and a threshold of $p < 0.05$ for statistical significance. In order to get an estimate of the chance of a false-positive discovery, the false discovery rate (FDR) was calculated for the frequency comparisons between patients with and without 5-FU toxicity in the different haplotype blocks according to Benjamini *et al.* [16]. Odds ratios with 95% CIs were calculated for significant associations of haplotypes with 5-FU toxicity, where applicable.

Results

5-FU-related toxicities

In the prospective group of 98 cancer patients treated with fluoropyrimidine-based chemotherapy, 11 (11%) experienced severe toxic side effects. Of the 24 patients with severe side effects, two experienced lethal toxicity (grade 5), four patients had grade 4 toxicity and 18 patients demonstrated grade 3 toxicity (Table 1). The most frequently observed toxicities were hematologic (leukopenia including neutropenic fever and anemia, thrombocytopenia) and gastrointestinal (oral and intestinal mucositis, diarrhea, nausea and vomiting). Dermatologic toxicities (hand–foot syndrome, hair loss and dry skin) were observed only rarely (Table 1). For seven of 21 patients with nonlethal severe 5-FU toxicity, treatment was discontinued because of the toxicity; treatment was continued with a reduced dose for five patients; treatment was continued using the same dose for five patients and four patients only experienced severe 5-FU toxicity during their last scheduled cycle of chemotherapy. For one patient with severe 5-FU toxicty, no information concerning any further continuation of treatment was available.

DPYD variation in 111 cancer patients

A total of 36 sequence variants were detected, all of which were SNPs, except for a T-insertion in intron 13 (IVS13+75insT; Supplementary Table 2). The variable sites included nine coding SNPs (seven nonsynonymous and two synonymous mutations). With the exception of the c.3025A>C (Thr1009Pro) mutation, all observed coding mutations have previously been described in the literature [6,17]. Of the 27 noncoding variations, only ten have been described previously. Rare allele frequencies of the observed sequence variants ranged from 0.45 to 42.30% (Supplementary Table 2) with 17 polymorphisms, including three coding variants, demonstrate frequencies of 5% or more. Only one polymorphism demonstrated a significant deviation from Hardy–Weinberg equilibrium (IVS18–39G>A; $p = 0.01$) and this was owing to a slight excess of homozygotes.

Severe 5-FU toxicity & individual *DPYD* variants

Our analysis revealed no association between the occurrence of severe toxicity following fluoropyrimidine treatment and any of the previously described deleterious *DPYD* variants (Table 2). In the 24 investigated patients with severe 5-FU-related toxicity, only four nonsynonymous *DPYD* mutations were observed, all of which were also observed at similar frequencies in patients without severe toxic side effects (Table 2). Furthermore, four functional *DPYD* variants were observed exclusively in patients without severe adverse reactions, including IVS14+1G>A and c.1679T>G – two mutations that were previously associated with 5-FU toxicity [6]. On the other hand, four intronic variants (IVS3–123G>C, IVS5+18G>A,

Table 1. Number of investigated cancer patients who experienced toxic side effects.

Toxicity type	Toxicity grade				
	5	*4*	*3*	*2*	*0–1*
Hematologic	2	1	12	18	78
Mucositis, stomatitis, diarrhea	-	2	8	6	95
Nausea, vomiting	-	1	5	4	101
Dermatologic	-	-	2	6	103
Infection	2	4	-	-	105
All (%)	2 (2)	4 (4)	18 (16)	19 (17)	68 (61)

Table 2. Observed nonsynonymous and splice-site mutations in *DPYD* in patients with and without 5-FU toxicity and p-value of association test.

Exon	Mutation	Effect	Rare allele frequencies			Genotype frequencies				p-value
			Total (N = 222)	*Grade ≥ 3 (N = 48)*	*Grade 0–2 (N = 174)*	*Grade ≥ 3*		*Grade 0–2*		
						N_{HET}	N_{HOM}	N_{HET}	N_{HOM}	
2	c.85T>C	Cys29Arg	0.221	0.25	0.213	10	1	27	5	0.56
6	c.496A>G	Met166Val	0.09	0.125	0.08	6	–	12	1	0.39
13	c.1601G>A	Ser534Asn	0.023	0.021	0.023	1	–	4	–	1.00
13	c.1627A>G	Ile543Val	0.203	0.146	0.218	7	–	32	3	0.32
13	c.1679T>G	Ile560Ser	0.005	–	0.006	–	–	1	–	1.00
14	IVS14+1G>A	del exon 14	0.005	–	0.006	–	–	1	–	1.00
18	c.2194G>A	Val732Ile	0.041	–	0.052	–	–	9	–	0.21
23	c.3025A>C	Thr1009Pro	0.005	–	0.006	–	–	1	–	1.00

N_{HET}: Number of heterozygous carriers; N_{HOM}: Number of homozygous carriers.

IVS6+139G>A and IVS9–51T>G) and one synonymous mutation in exon 11 (c.1236G>A) were observed at significantly higher frequencies in patients experiencing severe 5-FU toxicity (Supplementary Table 2).

Haplotype analysis

Linkage disequilibrium (LD) in *DPYD* was estimated using the 17 most frequent polymorphisms (rare allele frequency ≥ 0.05). A clear pattern of linkage blocks was revealed (Figure 1), which is in good agreement with data from the HapMap project (HapMap release 21a, January 2007 [102]). Therefore, for the inference of individual haplotypes, the gene was partitioned into six haplotype blocks (A–F) according to the observed LD pattern and the HapMap data (Figure 1 & Supplementary Table 3).

A total of 43 different haplotypes were identified across the six haplotype blocks, 14 of which were observed at frequencies of 5% or more (Supplementary Table 3). No significant differences in haplotype frequencies were observed between patients with and without severe 5-FU-related toxicity in haplotype blocks A, C, D, E and F ($p > 0.27$; Supplementary Table 3). In haplotype block B, haplotype frequencies differed significantly between the two groups ($p = 0.0199$; FDR: 0.12) with two haplotypes (B3 and B6; indicated in bold in Table 3) being significantly over-represented in patients with severe toxic side effects ($p = 0.013$, odds ratio: 6.63, 95% CI: 1.52–28.82; and $p = 0.046$, odds ratio: not defined, respectively). All polymorphisms that were found to be associated with severe 5-FU toxicity in the locus-by-locus analysis (Supplementary Table 2) were located within this haplotype block, and four of them were combined in haplotype B3 (IVS5+18G>A, IVS6+139G>A, IVS9–51T>G and c.1236G>A). Of the seven patients carrying this haplotype, four developed severe 5-FU toxicity (57%) and the only homozygous carrier experienced lethal 5-FU toxicity. In addition, the frequency of haplotype B3 appeared to be positively correlated with increasing toxicity grade (Supplementary Figure 1). Surprisingly, the haplotype is comprised of a synonymous SNP in exon 11 (c.1236G>A) and three intronic polymorphisms (IVS5+18G>A, IVS6+139G>A and IVS9–51T>G), and it does not contain any nonsynonymous or splice-site mutations. The other haplotype associated with severe 5-FU toxicity, haplotype B6, was observed twice, exclusively in patients experiencing severe toxicity (Table 3) and contains only one intronic variant position (IVS3–123G>C). Interestingly, one of the two carriers of this haplotype experienced lethal 5-FU toxicity. In addition, three other haplotypes were observed only once, and exclusively in patients with severe 5-FU toxicity, in other haplotype blocks (haplotypes B14, C7 and E9; Supplementary Table 3).

Discussion

This study presents the first comprehensive analysis of *DPYD* at the haplotype level in the context of severe toxic side effects following fluoropyrimidine administration in a patient sample of Caucasian origin. Sequencing of, on average, 160 base pairs of additional intronic sequence per exon more than doubled the number of detected polymorphic positions with allele frequencies 5% or more compared with earlier studies [17,18], enabling the estimation of LD and haplotype blocks within the gene. Using this approach, a novel haplotype was found to be over-represented in patients

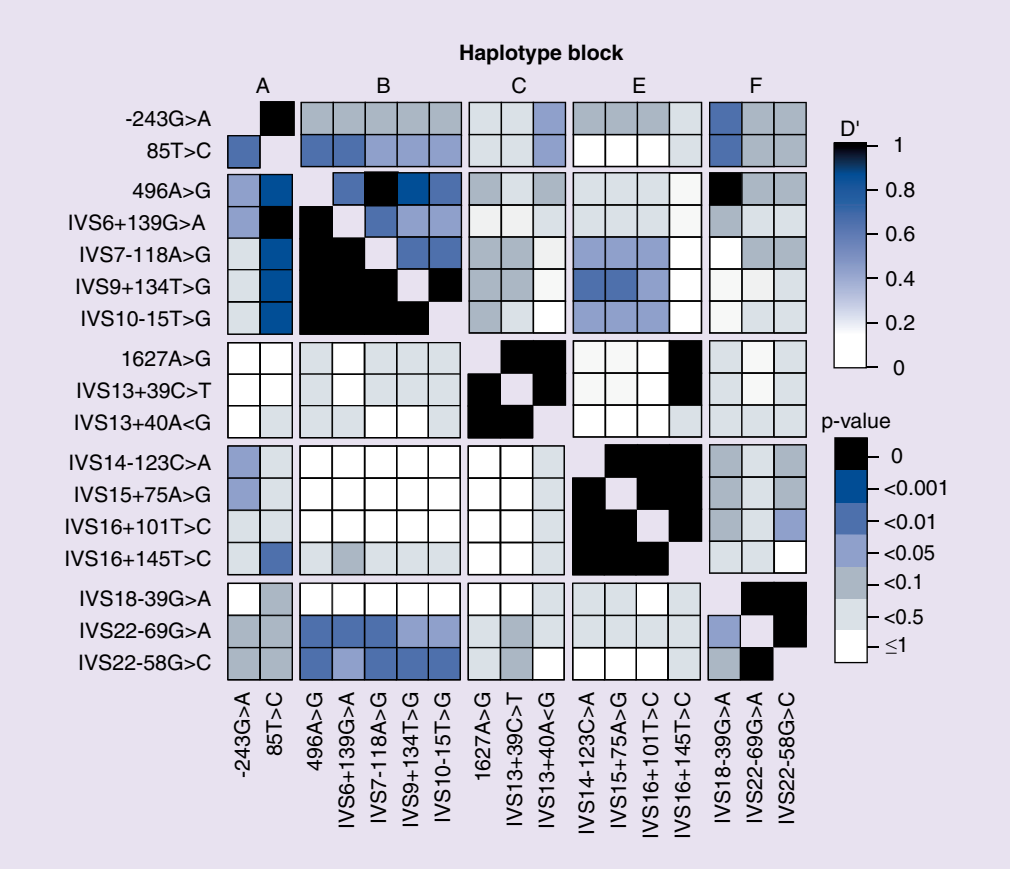

Figure 1. Linkage disequilibrium between 17 frequent *DPYD* polymorphisms. Genotypic disequilibrium p-values (below diagonal) and standardized LD coefficients (D'; above diagonal) are given. Haplotype block partitioning for individual diplotype inference is shown. Haplotype block D (comprised of exon 14) is not shown because this block did not contain any frequent polymorphisms.

with severe 5-FU toxicity, although previously reported associations of 5-FU toxicity with known deleterious mutations could not be confirmed.

Interestingly, this haplotype, B3 (Table 3), was comprised of four polymorphisms that have no direct effect on the amino acid sequence of DPD. Of the variant positions contained in this haplotype, only the synonymous c.1236G>A substitution in exon 11 has been observed in other studies [7,17], whereas the intronic regions containing the other three polymorphisms were not investigated in these studies. Consequently, it is not known if these other patients were, in fact, carriers of the same haplotype. Therefore, functional consequences of this haplotype are currently unknown and are being investigated. In this context, it is also important to note that such functional consequences need not necessarily be caused by one of the four observed SNPs in this haplotype. As haplotype B3 encompasses a large genomic region ranging from the end of intron 3 to the beginning of intron 11, a causative variant may be located anywhere within this region. In addition, the association of haplotype B3 was detected on the background of various chemotherapy regimens; therefore, the data should be interpreted with caution and requires replication in additional larger patient samples. On the other hand, such background variation will most likely reduce the power to detect genetic effects, and thus, our results may indicate a robust association of a particular haplotype with 5-FU toxicity.

In addition, haplotype B6 (two observations) and three other haplotypes (single observations) were detected exclusively in patients with severe toxicity. Of course, these rare observations require further investigation in larger patient samples in order to distinguish true associations from spurious ones. However, among these haplotypes, C7 may be of particular interest since it contains a T-insertion polymorphism in a homopolymer run of eight T residues in proximity to the exon 13 splice site. Interestingly, several studies have demonstrated that changes in the length of such intronic poly(T) sequences can have a profound effect on mRNA splicing [19,20].

Table 3. Haplotype frequencies in patients with and without 5-FU toxicity in haplotype block B (intron 3 to exon 10).

ID	IVS3–123G>C	IVS5+18G>A	c.496A>G	IVS6+139G>A	IVS7–118A>G	IVS7–78A>G/C	IVS9+134T>G	IVS9–85A>C	IVS9–51T>G	IVS10+77G>A	IVS10–15T>C	c.1236G>A	Frequency (%)			% Tox*
													Total	*Grade ≥ 3*	*Grade 0–2*	
B1	G	G	A	G	A	A	T	A	T	G	T	G	180 (81)	34 (71)	146 (84)	19
B2	G	G	G	A	G	A	G	A	T	G	C	G	17 (7.7)	5 (10)	12 (6.9)	29
B3	**G**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**G**	**G**	**T**	**A**	**8 (3.6)**	**5 (10)**	**3 (1.7)**	**63**
B4	G	G	A	G	A	A	G	A	T	G	C	G	5 (2.3)	0 (0)	5 (2.9)	0
B5	G	G	A	G	G	A	G	A	T	G	C	G	2 (0.9)	1 (2.1)	1 (0.6)	50
B6	**C**	**G**	**A**	**G**	**A**	**A**	**T**	**A**	**T**	**G**	**T**	**G**	**2 (0.9)**	**2 (4.2)**	**0 (0)**	**100**
Single observation													8 (3.6)	1 (2.1)	7 (4.0)	13

Bold indicates haplotypes with a significant ($p < 0.05$) association with 5-FU toxicity.
**Percentage of haplotype carriers with severe 5-FU toxicity.*
5-FU: 5-fluorouracil; Tox: Toxicity.

Haplotype B6 is also an interesting candidate for further studies, since one of the two carriers experienced lethal 5-FU toxicity.

On the other hand, previously reported associations of 5-FU toxicity with known deleterious mutations could not be confirmed in this study. In contrast to our findings, Morel *et al.* detected strong associations between two *DPYD* mutations (IVS14+1G>A and c.2846A>T) and severe 5-FU toxicity in a much larger patient sample of similar heterogeneity with respect to the chemotherapy regimen and tumor type as investigated in the present study [6]. To assess the probability of this lack of association occurring as a result of the smaller sample size, we calculated the statistical power to detect an association in the magnitude, as observed in [6], for the two mutations in our sample [103]. The estimated power was 83%, indicating that the differences between the two studies are unlikely to be attributable to random sampling error. In addition, this calculation is likely to represent an underestimation of the true power to detect an existing association, since in the present study the complete coding sequence was screened in contrast to only a few selected SNPs analyzed in the aforementioned screening. Therefore, our results add to the number of studies with strongly differing results with respect to known deleterious *DPYD* mutations (Table 4).

Interestingly, when considering the varying geographic origins of the participants in different studies (Table 4), the disagreement between different screenings may point towards regional differences in the frequencies of particular mutations, even within Caucasian subjects, as this is also the case for rare disease-causing mutations [21]. Therefore, the effectiveness of a pretherapeutic screening tests for these *DPYD* variants may differ depending on the geographic and ethnic origin of the target population. The discrepancies between different studies are most evident for the IVS14+1G>A splice-site mutation, where the observed frequencies in patients with severe toxicity range from 0 to 28% (Table 4). Interestingly, both studies that detected this mutation at very low frequencies were carried out in Southern France, whereas it was observed at higher frequencies in studies with patients originating from Northern Europe, suggesting that a frequency gradient of this mutation exists throughout Europe. Although the investigated 5-FU treatment regimens differed between some of these studies (Table 4), which may also partly account for the differing results, it is worth

Table 4. Predictive potential of known functional *DPYD* variants across different studies.

Study	Mutations investigated	IVS14+1 $F_{CARRIER}$ (%)	IVS14+1 F_{TOX} (%)	All variants F_{TOX} (%)	Associated variant positions (cDNA)	N_{TOX}	N_{NOT}	Patients' region of origin	Treatment regimens investigated	Ref.
van Kuilenberg *et al.*	IVS14+1	1.8	28	28	IVS14+1	60	–	Netherlands	Unknown	[22]
Morel *et al.*	22 selected SNPs	2.2	14	29.5	IVS14+1, c.2846, c.1679	44	443	Northern France	5-FU, 5-FU-P, FOLFIRI, FOLFOX, FEC	[6]
Schwab *et al.*	8 selected SNPs	1.9	5.5	8.2	IVS14+1, c.2846	54*	206*	Germany	5-FU	[7]
Collie-Duguid *et al.*	10 exons	–	0	7.1	c.1679	14	1	Unknown	Unknown	[24]
Magne *et al.*	IVS14+1	–	2.2	2.2	–	93	–	Southern France	5-FU, 5-FU-P, FOLFIRI, FOLFOX, FEC	[23]
Ciccolini *et al.*	IVS14+1	–	0	0	–	80	–	Southern France	5-FU, 5-FU-P, FOLFIRI, FOLFOX, CAPE	[28]
Amstutz *et al.*	Coding region	0.9	0	0	–	24	87	Switzerland	5-FU, 5-FU-P, FOLFIRI, FOLFOX, CAPE	This study

*Minimum number of patients genotyped, some SNPs were analyzed in more patients.
5-FU: 5-fluorouracil; CAPE: Capecitabine; $F_{CARRIER}$: Carrier frequency; FEC: 5-FU, epirubicin and cyclophosphamide; FOLFIRI: 5-FU, folinic acid and irinotecan; FOLFOX: 5-FU, folinic acid and oxaliplatin; F_{TOX}: Frequency in patients with toxicity; N: Number of patients; N_{TOX}: Number of patients with severe 5-FU toxicity; N_{NOT}: Number of patients without severe 5-FU toxicity; P: Platinum-based compound.

noting that findings from studies with similar heterogeneity with respect to chemotherapy regimen ([6,22,23], this study) also demonstrate remarkable frequency differences. The findings for the c.2846A>T substitution are similar. Morel *et al.* [6] observed this mutation at a relatively moderate carrier frequency (2%) whereas it was not observed in our screening and detected only five-times in 656 patients [7], again, indicating the frequency differences of this mutation between different study populations.

Furthermore, the c.1679T>G substitution, which has consistently been reported to be associated with low DPD activity and severe 5-FU toxicity [6,24,25], was observed here, for the first time, in a patient without severe 5-FU toxicity. Various studies, including our own, have thus now demonstrated that not all carriers of a specific deleterious gene variant develop severe 5-FU toxicity [6,7]. Moreover, a recent study [7] revealed a sex-dependent effect of IVS14+1G>A; male carriers of the mutation had a high risk of toxicity, whereas this risk was not significantly elevated for female carriers. In agreement with these findings, the only observed carrier of IVS14+1G>A in our study who did not experience severe side effects was, indeed, female. Therefore, to determine the value of genetic screening for these mutations prior to fluoropyrimidine treatment, not only the sensitivity, but also the specificity, of such tests needs to be assessed in more detail. However, for haplotype B3, which was observed at a higher frequency in patients with severe 5-FU toxicity in our study, there is no indication of such a sex-dependent effect, as two of the four carriers with severe adverse side effects were female and two were male. In the three carriers who did not experience 5-FU toxicity, two were male and one was female.

Recently, hypermethylation of the *DPYD* promoter region has been proposed as an alternative mechanism for DPD deficiency and thus, as a cause of severe 5-FU toxicity in patients where no inactivating *DPYD* mutation has been discovered [26]. However, no other study has so far been able to reproduce these promising findings [7], and in addition, in a subsample of patients from the study presented here, no evidence for *DPYD* promoter methlyation was found [27].

Conclusion

In summary, the above findings question the value of pretreatment screening for isolated *DPYD* variants for the prediction of 5-FU toxicity, since the frequencies of these variants appear to vary substantially among different patient populations. Thus, more screenings of large prospective patient samples are needed to determine the true predictive potential of specific *DPYD* gene variants. Furthermore, a combined investigation of *DPYD* with other genes involved in the metabolism or mechanism of action of 5-FU,

Executive summary

Dihydropyrimidine dehydrogenase & 5-fluorouracil toxicity

- Genetic variation in the dihydropyrimidine dehydrogenase gene (*DPYD*), encoding for the key enzyme of 5-fluorouracil (5-FU) catabolism, is thought to be important for drug response and toxicity; however, no genetic predictor has so far proven to be reliable enough for routine clinical use.
- This study investigates the complete coding region of *DPYD* and presents the first analysis at the haplotype level in the context of severe 5-FU toxicity.

Severe 5-FU toxicity & individual* DPYD *variants

- Previously associated *DPYD* variants (IVS14+1G>A, c.2846A>T and c.1679T>G) did not predict severe 5-FU toxicity in any of the investigated patients.
- An increased frequency of the IVS14+1G>A mutation in patients with 5-FU toxicity in Northern Europe compared with study populations from Southern Europe suggests that geographic differences exist in the frequencies of particular deleterious *DPYD* mutations.

DPYD *haplotype analysis*

- Sequencing of exon-flanking intronic regions led to the discovery of novel frequent intronic polymorphisms, enabling the inference of linkage disequilibrium across the entire gene, haplotype blocks and individual *DPYD* haplotypes.
- A haplotype comprised of three intronic and one synonymous mutation was found to be associated with severe 5-FU toxicity and several rare haplotypes were observed exclusively in patients with severe side effects.
- Haplotype analysis may lead to the discovery of new potentially important genetic variation located outside the coding region that may affect gene regulation or splicing and thus may be of functional importance.

and other drugs administered in combination therapies, may result in an improved prediction of toxicity from these chemotherapies. Finally, the haplotype-based approach used in this study identified new candidates located outside the coding sequence and well-defined splice sites of *DPYD*, which may be of value for the prediciton of 5-FU toxicty. Thus, our results indicate that further studies of noncoding polymorphisms with potential regulatory effects could lead to the discovery of other, as yet uncharacterized, *DPYD* variants associated with 5-FU toxicity, and thus, to a more comprehensive insight into fluoropyrimidine pharmacogenetics.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Bibliography

Papers of special note have been highlighted as:
- ▪ of interest
- ▪▪ of considerable interest

1 Ezzeldin H, Diasio RB: Dihydropyrimidine dehydrogenase deficiency, a pharmacogenetic syndrome associated with potentially life-threatening toxicity following 5-fluorouracil administration. *Clin. Colorectal Cancer* 4, 181–189 (2004).

2 Levy E, Piedbois P, Buyse M *et al.*: Toxicity of fluorouracil in patients with advanced colorectal cancer: Effect of administration schedule and prognostic factors. *J. Clin. Oncol.* 16, 3537–3541 (1998).

3 Ezzeldin HH, Diasio RB: Predicting fluorouracil toxicity: can we finally do it? *J. Clin. Oncol.* 26, 2080–2082 (2008).

4 Soong R, Diasio RB: Advances and challenges in fluoropyrimidine pharmacogenomics and pharmacogenetics. *Pharmacogenomics* 6, 835–847 (2005).

▪▪ **Recent and comprehensive review on 5-fluorouracil (5-FU) pharmacogenetics.**

5 Lim W-T, McLeod HL: Should screening for DPD deficiency be mandatory before 5-FU exposure? *Onkologie* 27, 531–533 (2004).

6 Morel A, Boisdron-Celle M, Fey L *et al.*: Clinical relevance of different dihydropyrimidine dehydrogenase gene single nucleotide polymorphisms on 5-fluorouracil tolerance. *Mol. Cancer Ther.* 5, 2895–2904 (2006).

▪▪ **First large prospective screening investigating *DPYD* variants in the context of 5-FU toxicity.**

7 Schwab M, Zanger UM, Marx C *et al.*: Role of genetic and nongenetic factors for fluorouracil treatment-related severe toxicity: a prospective clinical trial by the German 5-FU toxicity study group. *J. Clin. Oncol.* 26, 2131–2138 (2008).

▪▪ **Largest prospective study investigating 5-FU toxicity.**

8 Pagani F, Baralle FE: Genomic variants in exons and introns: identifying the splicing spoilers. *Nat. Rev. Genet.* 5, 389–396 (2004).

9 Wang G-S, Cooper TA: Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat. Rev. Genet.* 8, 749–761 (2007).

▪ **Review on the importance of genetic variation affecting gene splicing in the context of genetic diseases.**

10 Horn MP, Mäder-Heinemann G, Andrey-Zürcher G, Largiadèr CR: Mutation screening of the medium-chain acyl-CoA dehydrogenase (*MCAD*) and the ornithine transcarbamylase (*OTC*) genes by multiplex PCR. *Clin. Chem. Lab. Med.* 47, 56–59 (2009).

11 Excoffier L, Laval G, Schneider S: Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evol. Bioinform. Online* 1, 47–50 (2005).

12 Lewontin RC: The interaction of selection and linkage. I. General considerations; heterotic models. *Genetics* 49, 49–67 (1964).

13 Hill WG: Estimation of linkage disequilibrium in randomly mating populations. *Heredity* 33, 229–239 (1974).

14 Rousset F: GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Resour.* 8, 103–106 (2008).

15 Excoffier L, Estoup A, Cornuet J-M: Gametic phase estimation over large genomic regions using an adaptive window approach. *Hum. Genomics* 1, 7–19 (2003).

16 Benjamini Y, Hochberg Y: Controlling the false discovery rate – a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B.* 57, 289–300 (1995).

17 Seck K, Riemer S, Kates R *et al.*: Analysis of the *DPYD* gene implicated in 5-fluorouracil catabolism in a cohort of Caucasian individuals. *Clin. Cancer Res.* 11, 5886–5892 (2005).

18 Ezzeldin H, Okamoto Y, Johnson MR, Diasio RB: A high-throughput denaturing high-performance liquid chromatography method for the identification of variant alleles associated with dihydropyrimidine dehydrogenase deficiency. *Anal. Biochem.* 306, 63–73 (2002).

19 Zuccato E, Buratti E, Stuani C, Baralle FE, Pagani F: An intronic polypyrimidine-rich element downstream of the donor site modulates cystic fibrosis transmembrane conductance regulator exon 9 alternative splicing. *J. Biol. Chem.* 279, 16980–16988 (2004).

20 Hegde S, Lenox LE, Lariviere A *et al.*: An intronic sequence mutated in flexed-tail mice regulates splicing of Smad5. *Mamm. Genome* 18, 852–860 (2007).

21 Estivill X, Bancells C, Ramos C; Consortium BCMA: Geographic distribution and regional origin of 272 cystic fibrosis mutations in European populations. *Hum. Mutat.* 10, 135–154 (1997).

22 van Kuilenburg ABP, Meinsma R, Zoetekouw L, Gennip AHV: High prevalence of the IVS14+ 1G>A mutation in the dihydropyrimidine dehydrogenase gene of patients with severe 5-fluorouracil-associated toxicity. *Pharmacogenetics* 12, 555–558 (2002).

23 Magné N, Etienne-Grimaldi M-C, Cals L *et al.*: Dihydropyrimidine dehydrogenase activity and the IVS14+1G>A mutation in patients developing 5FU-related toxicity. *Br. J. Clin. Pharmacol.* 64, 237–240 (2007).

▪ **Largest series of 5-FU toxicity case reports investigated for dihydropyrimidine dehydrogenase activity and the IVS14+1G>A mutation.**

24 Collie-Duguid ES, Johnston SJ, Powrie RH *et al.*: Cloning and initial characterization of the human *DPYD* gene promoter. *Biochem. Biophys. Res. Commun.* 271, 28–35 (2000).

25 Johnson MR, Wang K, Diasio RB: Profound dihydropyrimidine dehydrogenase deficiency resulting from a novel compound heterozygote genotype. *Clin. Cancer Res.* 8, 768–774 (2002).

26 Ezzeldin HH, Lee AM, Mattison LK, Diasio RB: Methylation of the *DPYD* promoter: an alternative mechanism for dihydropyrimidine dehydrogenase deficiency in cancer patients. *Clin. Cancer Res.* 11, 8699–8705 (2005).

27 Amstutz U, Farese S, Aebi S, Largiadèr CR: Hypermethlyation of the *DPYD* promoter region is not a major pedictor of severe toxicity in 5-fluorouracil based chemotherapy. *J. Exp. Clin. Cancer Res.* 27, 54 (2008).

28 Ciccolini J, Mercier C, Evrard A *et al.*: A rapid and inexpensive method for anticipating severe toxicity to fluorouracil and fluorouracil-based chemotherapy. *Ther. Drug Monit.* 28, 678–685 (2006).

▪ **Study of 80 toxicity cases where the IVS14+1G>A mutation was not detected in any of the investigated patients.**

Websites

101 Common terminology criteria for adverse events v3.0 (CTCAE) http://ctep.cancer.gov/reporting/ctc_v30.html

102 The International HapMap Project www.hapmap.org/

103 Power for association with errors (PAWE) web tool http://linkage.rockefeller.edu/pawe

Supplementary Material: PCR amplification protocols

PCR reactions were performed using GeneAmp 9700 Thermal Cyclers (Applied Biosystems [ABI, CA, USA]). Exons 2–23 were amplified using the Multiplex PCR kit (Qiagen [Hilden, Germany]) following the Q-Solution protocol given by the manufacturer (Qiagen, Multiplex PCR Handbook 07/2004) with 35 amplification cycles, an annealing temperature of 58°C and a total reaction volume of 25 µl containing approximately 200 ng of genomic DNA. For the amplification of exon 1, the GC-rich PCR system (Roche Applied Science [Basel, Switzerland]) was used with an initial denaturation step of 3 min at 96°C followed by 45 cycles of 30 s at 96°C, 30 s at 60°C and 45 s at 72°C, and a final extension step of 10 min at 72°C. A reaction volume of 25 µl contained 1 µl of genomic DNA, 10 pmol of each primer, 0.5 µl of PCR Nucleotide Mix (Promega [WI, USA]), 5 µl of GC-rich resolution solution, 12.5 pmol of additional $MgCl_2$, 5 µl of GC-rich reaction buffer and 0.5 µl of GC-rich enzyme mix.

Supplementary Table 1. PCR primer mixes, amplified fragment sizes and percentage of sequences obtained in both directions (second strand) per exon.

Exon	PCR mix	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)	Second strand (%)
1	Single	AATGCAGTTGCCCCTCAAACA	TCTTCAGTCACTGACATTCAGAGGA	825	70
2	Single	TGGGAGACTAAGGTGGGAGGAT	CATTGTGTCATTAGGCAAAACAATT	691	48
3	A	ATGAATGCTACCCAATTAAAGTGGT	TGAGGCTTAACATTTATGCAGCTTC	411	73
4	A	CCCAAGAGGAGTGCCAAAGAT	AAACAAAAAACAATGAACCTGGATT	482	95
5	A	ATGTTTGTCGTAATTTGGCTGTTTAA	TCCTTTTTGTAGCTAAGCTGCTGA	410	93
6	B	TTAGCCATAACTCCTCATCTACTTGACA	ATAAATATTGCTTCAAGCCAACTGC	581	51
7	B	TCAATAAGAATGTAGATGTCCTCATGC	TTCTGCTTCTGCCTGATGTAGC	391	7
8	Single	TGCATTTAGCCCTTAATAGAACATGT	TTTCTTCCTAGAGATTCTCACTGGTG	575	41
9	Single	GATGTTTTCCTCTAAGAATGACATTATTTC	CTTGAAGCAATTTTTCATGATGTAGTT	456	22
10	B	GGAATAAAACTGTCTTTCAATGAAGCA	TGTCTGAATTAGAAAAGAAACAATTATGTG	499	58
11	C	GGTGTAAAGAAAAAGCTGCATATTGAC	TTAATGTTCTTTTCAATACTTGCCACTT	524	41
12	Single	ACAGTTGTTTGAATCCCTGGAAC	CCTGGCCCAATTTTTAATCAACTA	513	100
13	Single	TCATACTGCCTTTGAAATTAAAAGGC	GACAGAAAGGAAGGAAAGAAACTAAAGAT	609	62
14	A	CAAAAATGTGAGAAGGGACCTCA	TCTATGCATCAGCAAAGCAACTG	410	59
15	D	GCCCCAAATGTCATCCAGTG	CAAGGGACCGCTTTTATAGAATAAA	476	30
16	D	CCTCACAAGATAGCTGTGATGCA	TTCAGCTTCCCTCATTTTCGA	411	6
17	D	AGCTCATTGTCAAGTTGGATTTGTC	GCATGAGTCCAGGTGTAAATCTCCT	423	16
18	C	TGAAGAACTTTGAGGAGAAGACATGTT	AATAGAATTTGTGCAAGACCTTATCTTG	484	18
19	C	TTTGTCCAGTGACGCTGTCATC	GGTTCGTAAGCCCTCAACAGGA	464	28
20	D	CCCCATCTCCAGACGGCTAC	GAAATCACATCCAGGAGGCAC	448	22
21	C	CGGAACCTGATACCGAGAAGAC	TTTTCACCATGGACAGATGTTTTTA	477	14
22	C	TTGTATAAAAACAGGAAAATGCTGAGTG	CCATATTATAAGGGTGACAGGACAGAA	400	55
23	B	TCCTCTGTCAGCTCAACTGTTGC	GAACATCCAATTAACTGCCACAC	591	40

Supplementary Table 2. *DPYD* variants, allele and genotype frequencies in patients with and without severe 5-FU toxicity, and p-value of association test.

Exon/ intron	Mutation	Effect	Allele frequencies			Grade ≥ 3		Grade 0–2		p-value
			Grade ≥ 3 (24)	*Grade 0–2 (87)*	*Total (111)*	N_{HET}	N_{HOM}	N_{HET}	N_{HOM}	
5′-UTR	-243G>A	–	0.063	0.046	0.05	3	–	8	–	0.707
2	c.85T>C	Cys29Arg	0.25	0.213	0.221	10	1	27	5	0.562
3	IVS3–123G>C*	–	0.042	–	0.009	2	–	–	–	0.046
5	IVS5+18G>A*	–	0.104	0.017	0.036	3	1	3	–	0.013
6	c.496A>G	Met166Val	0.125	0.08	0.09	6	–	12	1	0.392
6	IVS6+139G>A*	–	0.229	0.092	0.122	9	1	10	3	0.022
7	IVS7–118A>G*	–	0.146	0.092	0.104	7	–	14	1	0.289
7	IVS7–78A>G*	–	–	0.006	0.005	–	–	1	–	1.000
7	IVS7–78A>C*	–	–	0.006	0.005	–	–	1	–	1.000
9	IVS9+134T>G*	–	0.125	0.115	0.117	6	–	18	1	0.804
9	IVS9–85A>C*	–	–	0.006	0.005	–	–	1	–	1.000
9	IVS9–51T>G*	–	0.104	0.017	0.036	3	1	3	–	0.013
10	IVS10+77G>A*	–	–	0.006	0.005	–	–	1	–	1.000
10	IVS10–15T>C	–	0.125	0.109	0.113	6	–	17	1	0.797
11	c.1236G>A	Glu412Glu	0.104	0.017	0.036	3	1	3	–	0.013
13	c.1601G>A	Ser534Asn	0.021	0.023	0.023	1	–	4	–	1.000
13	c.1627A>G	Ile543Val	0.146	0.218	0.203	7	–	32	3	0.316
13	c.1679T>G	Ile560Ser	–	0.006	0.005	–	–	1	–	1.000
13	IVS13+39C>T	–	0.146	0.213	0.198	7	–	33	2	0.413
13	IVS13+40G>A	–	0.375	0.437	0.423	8	5	38	19	0.511
13	IVS13+75insT*	–	0.021	–	0.005	1	–	–	–	0.216
14	c.1896T>C	Phe632Phe	0.021	0.023	0.023	1	–	4	–	1.000
14	IVS14+1G>A	del exon 14	–	0.006	0.005	–	–	1	–	1.000
14	IVS14+17A>G*	–	–	0.006	0.005	–	–	1	–	1.000
14	IVS14–123C>A	–	0.125	0.247	0.221	6	–	35	4	0.079
15	IVS15+75A>G	–	0.125	0.247	0.221	6	–	35	4	0.079
16	IVS16+101T>C*	–	0.125	0.259	0.23	6	–	35	5	0.054
16	IVS16+145T>C*	–	–	0.063	0.05	–	–	9	1	0.127
16	IVS16–37T>C*	–	–	0.006	0.005	–	–	1	–	1.000
18	c.2194G>A	Val732Ile	–	0.052	0.041	–	–	9	–	0.211
18	IVS18+100C>A*	–	0.042	0.029	0.032	2	–	5	–	0.646
18	IVS18+145C>T*	–	0.021	–	0.005	1	–	–	–	0.216
18	IVS18–39G>A	–	0.125	0.121	0.122	2	2	15	3	1.000
22	IVS22–115C>T*	–	–	0.011	0.009	–	–	2	–	1.000
22	IVS22–69G>A	–	0.188	0.138	0.149	9	–	22	1	0.370
22	IVS22–58G>C	–	0.188	0.115	0.131	9	–	18	1	0.225
23	c.3025A>C*	Thr1009Pro	–	0.006	0.005	–	–	1	–	1.000

**Novel mutations.*
5-FU: 5-fluorouracil; N_{HET}: Number of heterozygous carriers; N_{HOM}: Number of homozygous carriers.

Supplementary Table 3. *DPYD* haplotype frequencies in patients grouped according to 5-FU toxicity and frequency of toxicity within haplotypes.

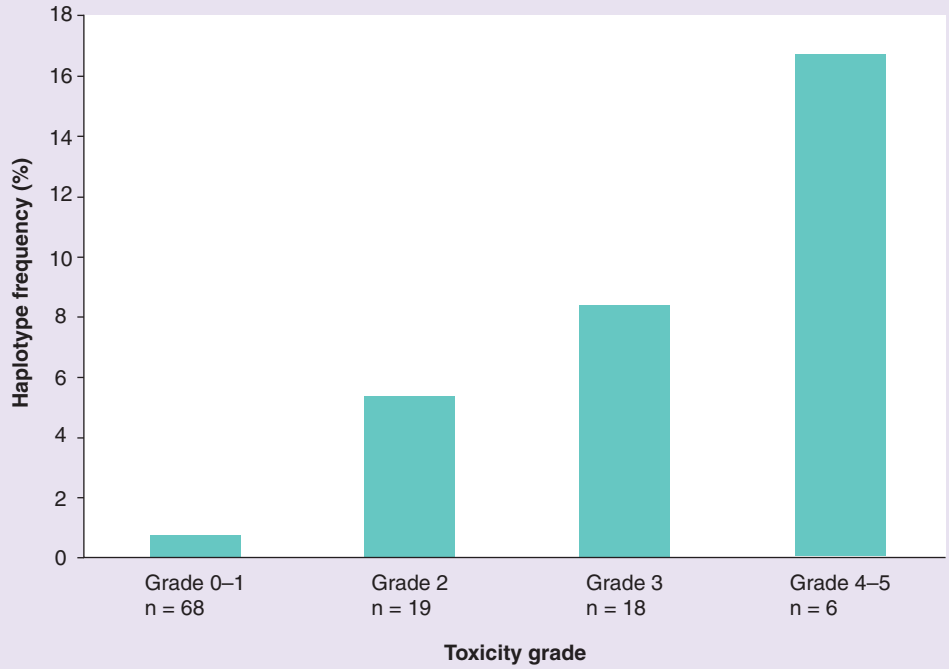
ID	Variant position																																	Frequency (%)			% Tox*	p-value‡
	-243G>A	c.85T>C	IVS3-123G>C	IVS5+18G>A	c.496A>G	IVS6+139G>A	IVS7-118A>G	IVS7-78A>G/C	IVS9+134T>G	IVS9-85A>C	IVS9-51T>G	IVS10+77G>A	IVS10-15T>C	c.1236G>A	c.1601G>A	c.1627A>G	c.1679T>G	IVS13+39C>T	IVS13+40G>A	IVS13+75insT	c.1896T>C	IVS14+1G>A	IVS14+17A>G	IVS14-123C>A	IVS15+75A>G	IVS16+101T>C	IVS16+145T>C	IVS16-37T>C	c.2194G>A	IVS18+100C>A	IVS18+145C>T	IVS18-39G>A	IVS22-115C>T	IVS22-69G>A	IVS22-58G>C	c.3025A>C	Total	Grade ≥ 3	Grade 0–2		
A1	G	T																																		173 (78)	36 (75)	137 (79)	21	0.777
A2	G	**C**																																		38 (17)	9 (19)	29 (17)	24	
A3	A	C																																		11 (5)	3 (6.3)	8 (4.6)	27	
B1			G	G	A	G	A	A	T	A	T	G	T	G																						180 (81)	34 (71)	146 (84)	19	0.0199
B2			G	G	**G**	**A**	**G**	A	**G**	A	T	G	**C**	G																						17 (7.7)	5 (10)	12 (6.9)	29	
B3			G	**A**	A	**A**	A	A	T	A	**G**	G	T	**A**																						8 (3.6)	5 (10)	3 (1.7)	63	
B4			G	G	A	G	A	A	**G**	A	T	G	**C**	G																						5 (2.3)	0 (0)	5 (2.9)	0	
B5			G	G	A	G	**G**	A	**G**	A	T	G	**C**	G																						2 (0.9)	1 (2.1)	1 (0.6)	50	
B6			**C**	G	A	G	A	A	T	A	T	G	T	G																						2 (0.9)	2 (4.2)	0 (0)	100	
B7			G	G	A	G	A	**G**	T	A	T	G	T	G																						1 (0.5)	0 (0)	1 (0.6)	0	
B8			G	G	A	G	A	**C**	T	A	T	G	T	G																						1 (0.5)	0 (0)	1 (0.6)	0	
B9			G	G	**G**	G	**G**	A	**G**	A	T	G	**C**	G																						1 (0.5)	0 (0)	1 (0.6)	0	
B10			G	G	A	G	A	A	T	A	T	**A**	T	G																						1 (0.5)	0 (0)	1 (0.6)	0	
B11			G	G	A	G	A	A	T	**C**	T	G	T	G																						1 (0.5)	0 (0)	1 (0.6)	0	
B12			G	G	A	G	**G**	A	T	A	T	G	T	G																						1 (0.5)	0 (0)	1 (0.6)	0	
B13			G	G	**G**	**A**	**G**	A	**G**	A	T	G	T	G																						1 (0.5)	0 (0)	1 (0.6)	0	
B14			G	G	**G**	**A**	**G**	A	T	A	T	G	T	G																						1 (0.5)	1 (2.1)	0 (0)	100	
C1															G	A	T	C	**A**	–															88 (40)	16 (33)	72 (41)	18	0.276
C2															G	A	T	C	G	–															82 (37)	23 (48)	59 (37)	28	
C3															**G**	**G**	T	**T**	G	–															44 (20)	7 (15)	37 (21)	16	
C4															**A**	A	T	C	**A**	–															5 (2.3)	1 (2.1)	4 (2.3)	20	
C5§															G	A	**G**	C	G	–															1 (0.5)	0 (0)	1 (0.6)	0	
C6															G	**G**	T	C	G	–															1 (0.5)	0 (0)	1 (0.6)	0	
C7§															G	A	T	C	**A**	**T**															1 (0.5)	1 (2.1)	0 (0)	100	
D1																					T	G	A												215 (97)	47 (98)	168 (97)	22	1.00
D2																					**C**	G	A												5 (2.3)	1 (2.1)	4 (2.3)	20	
D3																					T	**A**	A												1 (0.5)	0 (0)	1 (0.6)	0	
D4																					T	G	**G**												1 (0.5)	0 (0)	1 (0.6)	0	

Bolded letters indicate the variant (less frequent) alleles for each variant position.
**Percentage of haplotype carriers with severe 5-FU toxicity.*
‡Fisher's exact test p-value of haplotype frequency comparisons between patients with and without severe 5-FU toxicity.
§Haplotype that was inferred with a probability of ≤0.75.
5-FU: 5-fluorouracil; Tox: Toxicity.

Supplementary Table 3. *DPYD* haplotype frequencies in patients grouped according to 5-FU toxicity and frequency of toxicity within haplotypes.

ID	Variant position																																	Frequency (%)			% Tox*	p-value‡
	-243G>A	c.85T>C	IVS3-123G>C	IVS5+18G>A	c.496A>G	IVS6+139G>A	IVS7-118A>G	IVS7-78A>G/C	IVS9+134T>G	IVS9-85A>C	IVS9-51T>G	IVS10+77G>A	IVS10-15T>C	c.1236G>A	c.1601G>A	c.1627A>G	c.1679T>G	IVS13+39C>T	IVS13+40G>A	IVS13+75insT	c.1896T>C	IVS14+1G>A	IVS14+17A>G	IVS14-123C>A	iVS15+75A>G	IVS16+101T>C	IVS16+145T>C	IVS16-37T>C	c.2194G>A	IVS18+100C>A	IVS18+145C>T	IVS18-39G>A	IVS22-115C>T	IVS22-69G>A	IVS22-58G>C	c.3025A>C	Total	Grade ≥ 3	Grade 0–2		
E1																								C	A	T	T	T	G	C	C						165 (74)	41 (85)	124 (71)	25	0.284
E2																								**A**	**G**	**C**	T	T	G	C	C						30 (14)	4 (8.3)	26 (15)	13	
E3																								**A**	**G**	**C**	T	T	G	**A**	C						7 (3.2)	2 (4.2)	5 (2.9)	29	
E4																								**A**	**G**	**C**	**C**	T	G	C	C						7 (3.2)	0 (0)	7 (4)	0	
E5																								C	A	T	T	T	**A**	C	C						5 (2.3)	0 (0)	5 (2.3)	0	
E6																								**A**	**G**	C	C	T	**A**	C	C						4 (1.8)	0 (0)	4 (2.3)	0	
E7																								C	A	C	T	T	G	C	C						2 (0.9)	0 (0)	2 (1.1)	0	
E8																								**A**	**G**	C	T	C	G	C	C						1 (0.5)	0 (0)	1 (0.6)	0	
E9																								C	A	T	T	T	G	C	**T**						1 (0.5)	1 (2.1)	0 (0)	100	
F1																																G	C	G	G	A	161 (73)	33 (69)	128 (74)	20	0.678
F2																																G	C	**A**	**C**	A	29 (13)	9 (19)	20 (12)	31	
F3																																**A**	C	G	G	A	25 (11)	6 (13)	19 (11)	24	
F4																																G	C	**A**	G	A	4 (1.8)	0 (0)	4 (2.3)	0	
F5§																																**A**	**T**	G	G	A	2 (0.9)	0 (0)	2 (1.1)	0	
F6																																G	C	G	G	**C**	1 (0.5)	0 (0)	1 (0.6)	0	

Bolded letters indicate the variant (less frequent) alleles for each variant position.
**Percentage of haplotype carriers with severe 5-FU toxicity.*
‡Fisher's exact test p-value of haplotype frequency comparisons between patients with and without severe 5-FU toxicity.
§Haplotype that was inferred with a probability of ≤0.75.
5-FU: 5-fluorouracil; Tox: Toxicity.



Supplementary Figure 1. Distribution of haplotype B3 frequency according to toxicity grade. Numbers of patients in each toxicity group are given.