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Influence of dihydropyrimidine dehydrogenase gene (*DPYD*) coding sequence variants on the development of fluoropyrimidine-related toxicity in patients with high-grade toxicity and patients with excellent tolerance of fluoropyrimidine-based chemotherapy

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Alterations in dihydropyrimidine dehydrogenase gene (DPYD) coding for the key enzyme (DPD) of fluoropyrimidines (FPs) catabolism contribute to the development of serious FPs-related toxicity. We performed mutation analysis of DPYD based on cDNA sequencing in 76 predominantly colorectal cancer patients treated by FPs with early development of high (grade 3-4) hematological and/or gastrointestinal toxicity. Six previously described [85T>C (C29R), 496A>G (M166V), 775A>G (K259E), 1601G>A (S534N), 1627A>G (I543V), IVS14+1G>A, 2194G>A (V732I)] and two novel [187A>G (K63E) and 1050 G>A (R357H)] non-synonymous DPYD variants were found in 56/76 (73.7%) high-toxicity patients. Subsequently, these alterations were analyzed in 48 patients with excellent long-term tolerance of FPs and in 243 controls and were detected in 37/48 (77.1%) and 166/243 (68.3%) cases, respectively. Analysis of these alterations as risk factors for development of toxicity in pooled FPs-treated population demonstrated that C29R negatively correlated with overall gastrointestinal toxicity (OR = 0.48; 95%CI 0.23–1.0) and M166V in women protected against overall hematological toxicity and neutropenia (both OR = 0.26; 95%CI 0.07-0.89), whereas IVS14+1G>A (found in five high-toxicity patients only) increased risk of mucositis in overall population (OR = 7.0; 95%CI 1.1-44.53), and thrombocytopenia in women (OR = 10.8; 95%CI 1.24-93.98). Moreover, we identified a strong association of V732I with leucopenia (OR = 8.17; 95%CI 2.44 – 27.31) and neutropenia (OR=2.78; 95% CI 1.03-7.51). Our data enabled characterization of "high risk" haplotypes (carriers of IVS14+1G>A or V732 lacking M166V) representing small (22% female and 11% male patients), population in high risk of serious hematological toxicity development, and in patients with "lower risk" that unlikely develop serious hematological toxicity [carriers of M166V without IVS14+1G>A and V732I in females (32% women), and non-carriers of C29R, M166V, IVS14+1G>A, and V732I in males (46% men)]. Our results indicate that genotyping of several DPYD variants may lead to stratification of patients with respect to the risk of serious hematological toxicity development during FPs treatment.

Key words: dihydropyrimidine dehydrogenase gene [DPYD], fluoropyrimidines, 5-fluorouracil toxicity, mutation analysis, haplotypes, association study

Fluoropyrimidines – 5-fluorouracil (5-FU) and its derivates (e.g. capecitabine) – belong to the most frequently used anticancer drugs in treatment of solid cancers. Mechanism of action of 5-FU involves its anabolic conversions to 5-fluoropyrimidine nucleotides that exert profound inhibitory effect on thymidylate synthetase activity and interfere with RNA and DNA metabolism [1]. The development of severe toxicity is the critical complication of 5-FU-based therapy. It occurs in nearly one third of cases with progression to life-threatening complications in approximately 0.5% patients [2].

About 80% of 5-FU is quickly inactivated in catabolic pathway initiated by the rate-limiting enzyme dihydropyrimidine dehydrogenase (DPD; OMIM 274270; EC 1.3.1.2) catalyzing reduction of 5-FU to inactive 5-fluoro-5,6-dihydrouracil. Subsequent metabolic degradation involves dihydropyrimidinase

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(DPYS; OMIM 222748; EC 3.5.2.2) and finally β-ureidopropionase (UPB1; OMIM 606673; EC 3.5.1.6) converting fluoro-β-ureidopropionate to fluoro-β-alanine excreted in urine [3, 4]. Dihydropyrimidine dehydrogenase is ubiquitously expressed cytosolic enzyme with a high activity in the liver and mononuclear lymphocytes [5]. The dihydropyrimidine dehydrogenase gene (*DPYD*; OMIM 274270) consists of 23 exons distributed approximately over 900 kbp region of 1p22 [6, 7]. The DPD protein sequence is highly conserved among different species. The DPD molecule consists of 1025 amino acids (111 kDa) and it is organized in five functional domains [8]. The prosthetic groups involving one FAD, one FMN and four [4Fe-4S] promote electron transport during the redox reactions [9]. Physiologically, DPD homodimer catalyzes the first step in pyrimidine degradation: the NADPH-dependent reduction of uracil and thymine to the corresponding 5,6-dihydropyrimidines.

Serious 5-FU toxicity responsible for treatment discontinuation and death in some patients typically occurs in form of high-grade hematological and non-hematological complications (neutropenia, thrombocytopenia, diarrhea, stomatitis, and hand-foot syndrome). These adverse events results from increased bioavailability of 5-FU caused by decreased 5-FU catabolism in the liver tissue due to inherited decreased capacity of 5-FU catabolism or of liver function impairment. Both homozygotes and heterozygotes carrying mutations in *DPYD* altering structure of the enzyme were shown to have decreased 5-FU catabolism [10]. Over 50 different sequence variants of *DPYD* were described in patients with severe toxicity following 5-FU treatment so far, and several of them are referred to as disease-causing gene alterations [10–12]. Despite the widely accepted disease-causing mutations (e.g. IVS14+1G>A), the biological effect of many, mainly missense variants (e.g. M166V or C29R) remained controversial [13–17].

To address the contribution of *DPYD* alterations to the development of severe toxicity in 5-FU-treated patients we performed the mutation analysis of *DPYD* involving two extreme populations: patients with high-grade or life-threatening toxicity developed at the beginning of drug application vs. patients with very good tolerance of 5-FU treatment without any signs of major toxicity within long-lasting treatment period.

Patients and methods

Study design. Our retrospective pharmacogenomic study involved the two cancer patients' populations treated by fluoropyrimidine-containing regimens. The first one (termed "high-toxicity group") consisted of cancer patients developing high-grade (Grade 3-4; National Cancer Institute of Canada Common Toxicity Criteria scale; NCIC CTC) hematological (leucopenia, neutropenia or thrombocytopenia) toxicity and/or gastrointestinal (mucositis, emesis, diarrhea) toxicity during the first or the second cycle of 5-FU treatment. Dermatological adverse events were not considered to be indicative for selection of the high-toxicity patients [18, 19]. The second population (termed "low-toxicity group") involved patients with a high tolerance to 5-FU treatment. All individuals in this group received at least ten consecutive cycles of 5-FU-based chemotherapeutic regimens without development of hematological or gastrointestinal toxicity (i.e. the toxicities were evaluated as Grade 0-1 according to NCIC CTC). Patients with inadequate liver function tests were not enrolled to prevent biased 5-FU clearance. The population frequency of characterized *DPYD* alterations was analyzed in representative group of control population. The *DPYD* mutation analysis was performed in the high-toxicity group by bi-directional sequencing of *DPYD* mRNA (cDNA) isolated from peripheral blood lymphocytes. Only *DPYD* exons harboring alterations of coding sequence characterized in the high-toxicity group were subsequently analyzed in the low-toxicity group and the control population by DHPLC (Denaturing High-Performance Liquid Chromatography).

The primary endpoints of our study involved characterization of *DPYD* gene coding sequence alterations in 5-FU treated patients and analysis of haplotype(s) predisposing to the development of serious site-specific toxicity in 5-FU-treated patients.

Patients and genetic material. The high-toxicity group consisted of 76 individuals who were reported to develop serious toxicity (NCIC CTC Grade 3-4 toxicity) attributable to 5-FU during the first or second cycle of fluoropyrimidines-based therapy (Tab. 1). Five out of 76 high-toxicity patients (9.2%) died for the fatal toxic reactions (four with grade 4 hematological toxicities and one with gastrointestinal grade 4 toxicity). A total of 104 patients were recruited from oncology centers in the Czech Republic between January 2002 and May 2008, following signature of informed consent approved by ethical committee. Clinical characteristics were obtained via Internet-based form organized by Comprehensive Oncology Center (*www.koc.cz*) [20,21]. Each case reported by on-line formulary was reviewed by co-operating experienced oncologists' team to fulfill criteria of the high toxicity clearly attributable to 5-FU therapy. The oncologists' review board excluded 28 patients including 14 not fulfilling inclusion NCIC CTC criteria of high-toxicity, 5 patients treated by 5-FU containing regimens previously, and 9 patients with incomplete documentation.

The low-toxicity group included 48 individuals with excellent tolerance of 5-FU therapy (NCIC CTC Grade 0-1 toxicity) treated by more than ten cycles of fluoropyrimidines-based regimens (Tab. 1). Fifty-three patients were enrolled at the Departments of Oncology, Faculty Hospitals in Prague and Pilsen between April 2003 and July 2007. During re-evaluation of clinical data, five individuals were discharged from low-toxicity group due to presence of the toxicity higher than grade 0-1.

The non-cancer control population (103 males and 140 females), aged 55.7 ± 17.0 years (mean ± SD), consisted of randomly selected adult persons examined at the Department of Clinical Biochemistry and Laboratory Medicine, General Teaching Hospital in Prague between January 2003 and No-

vember 2005, excluding those with primary cancer diagnosis (Tab. 1). All patients and controls were Caucasians from the Czech Republic.

Total RNA was extracted from blood samples obtained only from high-toxicity patients. The blood samples were preserved in PAX Gene collection tubes (BD Diagnostics) and RNA was isolated using PAX Gene Blood RNA kit (Qiagen) according to manufacturers protocols. The blood samples for DNA isolation in EDTA-containing collection tubes (BD Diagnostics) were obtained from all individuals. Genomic DNA was isolated using Wizard DNA isolation kit (Promega) according to manufacturer.

Mutation analysis of the DPYD gene. Mutation analysis of entire *DPYD* coding region in high-toxicity patients' samples was performed by sequencing of cDNA. Two μg of total RNA was used for cDNA synthesis with 5U of SuperScriptIII Reverse Transcriptase (Invitrogene) and 150 pmol of random hexanucleotides (Roche) under the manufacturer instruction. Whole coding sequence of *DPYD* mRNA (including flanking 5' and 3' UTRs) was amplified in three overlapping fragments using 2.5 μl of cDNA, 0.5 U Gold Taq polymerase (Applied Biosystems), 0.5 μl 50xdNTP's (Invitek), 2.5 μl 10x Gold Taq PCR buffer (Applied Biosystems), 0.75 μl 50 mM $MgCl_2$, and 3 pmol of each PCR primer (Generi-Biotech; Tab. 2) in 25μl volume. The PCR protocol consisted of denaturation at 96°C for 10 min, followed by 34 cycles (30 s at 95°C, 45 s at 68°C and 2 min at 72°C), and final extension at 72°C for 10 min. Purified PCR products (JETQUICK PCR Product Purification Spin Kit; Genomed) were sequenced on ABI3130 analyzer (Applied Biosystems) using BigDye Terminator Mix ver. 3.1 (Applied Biosystems) and primers listed in Tab. 2. All *DPYD* alterations characterized by cDNA sequencing were confirmed by sequencing of genomic DNA (Tab. 3).

Only the exons (Tab. 3), in which alterations of coding sequence occurred in the high-toxicity patients' samples, were analyzed in samples from the low-toxicity and control population groups. Amplicons covering exons and flanking intron sequences were PCR amplified in 25 μl volume containing 100 ng gDNA, 0.25 U Gold Taq DNA polymerase, 0.5 μl 50xdNTP's 2,5 μl 10x Gold Taq PCR buffer and 30 pmol of each PCR primer under conditions specified in Tab. 3. Following denaturation/ renaturation step, 5 μl PCR aliquots were subjected to DHPLC analysis (WAVE 3500 System; Transgenomic) under conditions specified in Tab. 3. The amplicons covering *DPYD* exons 2 and 18 were reanalyzed by DHPLC

Table 1. Basic characteristics of the patients' study populations treated by 5-FU-based chemotherapeutic regimens.

	High-toxicity group (toxicity grade 3-4) N = 76	Low-toxicity group (toxicity grade 0-1) N = 48
Demographic parameters		
Females; N (%)	40 (52.6)	23 (47.9)
– mean age; years ± SD	61.5 ± 10.2	56.2 ± 11.2
– age range; years	30 – 75	31 – 74
Males; N (%)	36 (47.4)	25 (52.1)
– mean age; years ± SD	62.1 ± 7.5	59.4 ± 9.3
– age range; years	42 – 73	36 – 77
Cancer diagnose; N (%)		
– orofacial	1 (1.3)	0
– esophageal	3 (4.0)	0
– gastric	5 (6.6)	0
– colorectal	51 (67.1)	40 (83.4)
– biliary	2 (2.6)	1 (2.1)
– pancreatic	1 (1.3)	1 (2.1)
– pharyngeal	1 (1.3)	0
– breast	12 (15.8)	3 (6.2)
– unknown primary site	0	3 (6.2)
Chemotherapy regimens		
Bolus 5-FU	19 (25.0)	17 (35.4)
Continuous 5-FU / capecitabine	40 (52.6)	12 (25.0)
FOLFIRI	1 (1.3)	6 (12.5)
FOLFOX	14 (18.4)	9 (18.8)
Other	2 (2.7)	4 (8.3)
Toxicity grade 3-4 according to NCIC CTC; N (%)		
– gastrointestinal only	35 (46.1)	0
– hematological only	13 (17.1)	0
– gastrointestinal and hematological	28 (37.3)	0
– mucositis	24 (31.6)	0
– emesis	14 (18.4)	0
– diarrhea	41 (54.0)	0
– leucopenia	14 (18.4)	0
– neutropenia	40 (52.6)	0
– thrombocytopenia	14 (18.4)	0

Table 2. PCR primers used for initial amplification of three overlapping amplicons from *DPYD* cDNA covering the whole coding sequence [DPD1+DPD4 (1005 bp), DPD5+DPD8 (1096 bp), and DPD9+DPD14 (1504 bp); marked by asterisk], and for bi-directional sequencing (all listed primers). Position in DPD mRNA (NCBI reference number: NM_000110.3) indicated.

Primer	Position in mRNA	Sequence (5'→3')
DPD1*	48-69	TTGAGGACGCAAGGAGGGTTTG
DPD2	552-531	TAATGGGTCCCTCTTCAGTGGC
DPD3	460-481	ACCCACTTGGTCTGACTTGTGG
DPD4*	1052-1031	ACTGCCTTTGGCTACAAGTGGC
DPD5*	972-993	GATGCCATCTTCCAAGGCCTGA
DPD6	1542-1521	CACCACCTGCAAATACCCATGC
DPD7	1470-1491	AACAGATGGGGTCTCCCAGAAG
DPD8*	2067-2046	TCTTGGCAAGTTCCGTCCAGTC
DPD9*	1950-1972	ACGGCTGCATATTGGTGTCAAAG
DPD10	2501-2479	TTCAGCAGAGTCAATTCCACCAG
DPD11	2420-2441	TGCTTTGAGAGCTGTGACCTCC
DPD12	3048-3027	GAAACCCACCTGCCCACCATAA
DPD13	2917-2983	GCAACGTAGAGCAAGTTGTGGC
DPD14*	3453-3431	AAGACAACTGGCAGTGAACATCC

Table 3. Sequences of PCR primers for amplification of *DPYD* fragments covering exons with intron-exon boundaries from gDNA used for DHPLC analyses in the low-toxicity patients group and the control population group.

Exon	Primer	Sequence (5'→3')	bp	T_a [1] (°C)	$MgCl_2$ [2] (mM)	DHPLC temp[3] (°C)	Gradient of Buffer B[4] (%)
2	DPYD01	ACACATTGTTTATGCTGTCTTTAG	161	58	2.5	56.5	46.3 – 55.3
	DPYD02	TGAAATAGTGTATCAGTGGTACTT					
3	DPYD03	GGTATGCATATTTTTCATGAGTCC	293	61	1.5	57.0	52.5 – 61.5
	DPYD04	GTGGCAATGAACTCATTTGTTC					
6	DPYD05	TTTAACCATGACAATTGATTTCCC	279	61	1.5	57.0	54.1 – 63.1
	DPYD06	GTTTTGCTCCATCATTTCTGAC					
8	DPYD13	AATCTCATAGAATTTTTGGCTGAC	270	61	3.0	53.0	54.8 – 63.8
	DPYD14	AGTCATTCTTCTGGATATTGCTAG					
10	DPYD19	AGTCATTCTTCTGGATATTGCTAG	355	61	2.0	60.3	52.2 – 61.2
	DPYD20	TTGACAATTTCAACATTCTAGCG					
11	DPYD21	TGGTGAAAGAAAAAGCTGCAT	381	61	2.5	57.1	46.3 – 55.3
	DPYD22	AACAGACAATTGCATCACACA					
13	DPYD07	AGATGTAATATGAAACCAAGTATTGG	392	55-48	2.5	50.9 58.2	56.9 – 65.9
	DPYD08	TTAATGTGTAATGATAGGTCTTGTC					
14	DPYD09	CTTTGTCAAAAGGAGACTCAATATC	255	61	2.5	57.6	52.5 – 61.2
	DPYD10	TCACCAACTTATGCCAATTCTC					
18	DPYD11[5]	TGAATGGGTTTTAACTATCGTGTC	201	61	1.5	60.4	50.8 – 59.8
	DPYD12[5]	AAGTGGGCAACACCTACCAG					

1) Annealing temperatures and; 2) $MgCl_2$ concentrations used for PCR. 3) Oven temperatures and 4) gradient of acetonitrile-containing Buffer B (%) used for DHPLC analyses. 5) Primers designed by Fischer J *et al.* [17].

after adding of equimolar amount of wild-type PCR product and denaturation/ renaturation step to disclose presence of recessive homozygotes. Samples with aberrant chromatograms detected by Navigator software ver. 1.6.4 (Transgenomic) were sequenced.

Statistical analyses. The odds ratios (OR) and 95% confidence intervals (CI) of OR were computed for determination the risks factors between investigated groups. As alternative of logistic regression, the Chi-Square test was used. The relations between the investigated parameters were computed by the Spearman correlation coefficients. The comparison of variables in given groups and subgroups, considering to the distribution of this variables, was performed by a non-parametric Wilcoxon test. All statistical analyses were performed using CRAN 2.4.0. software (http://www.r-project.org/). For genotype-phenotype correlations, the individual toxicities in pooled high- and low-toxicity groups were analyzed as individual variables (mucositis, diarrhea, emesis, leucopenia; neutropenia; thrombocytopenia), and also as the overall gastrointestinal or the overall hematological toxicity numerically equal to the highest grade of particular toxicity in a given group.

Genotype frequencies in the analyzed populations were estimated using chi-square test in Linkage Disequilibrium Analyzer (LDA) 1.0 software [22].

Results

DPYD alterations in the group of high-toxicity patients. The *DPYD* cDNA sequencing enabled fast screening of variants within coding sequence and alterations affecting *DPYD* mRNA splicing (e.g. IVS14+1G>A) in the group of high-toxicity cancer patients. At least one gene alteration affecting DPD protein composition was found in 56 out of 76 (73.7%) samples in this group.

Two novel *DPYD* missense variants [c.187A>G (p.K63E) and c.1050 G>A (p.R357H)] were found, each occurring as the only *DPYD* alteration in two high-toxicity patients (Fig. 1). The c.187A>G transition leads to change of a highly conservative lysine 63 to glutamate (K63E; data not shown) localized within exclusively α-helical domain I containing two Fe-S clusters [8]. The homology modeling (Fig. 1A) based on crystallography data of porcine DPD enzyme (93% identity to human homologue) indicates the proximity of lysine 63 to FAD binding site in domain II [8, 14]. The change of lysine 63 to negatively charged glutamate might therefore influence the electron transfer pathway. The second newly described transition c.1050G>A causes replacement of less conservative arginine 357 to histidine (data not shown), however, the *in silico* modeling of this alteration (Fig. ″1B) showed that the missense mutation affects the outer loop protruding outside from the NADPH binding domain III and hence the possibility of significant alteration of DPD protein structure is probably low. This hypotheses support the results of Align GVGD missense mutation predictive tool (http://agvgd.iarc.fr/index.php; data not shown) classifying the K63E to high-risk Class C55 with risk 2.5-3.0, whereas the R357H was classified in low risk Class C0 with risk 0.9 – 1.1 [23].

The c.1905+1G>A (IVS14+1G>A) mutation resulting in exon 14 skipping was found in five out of 76 (6.6%) high-

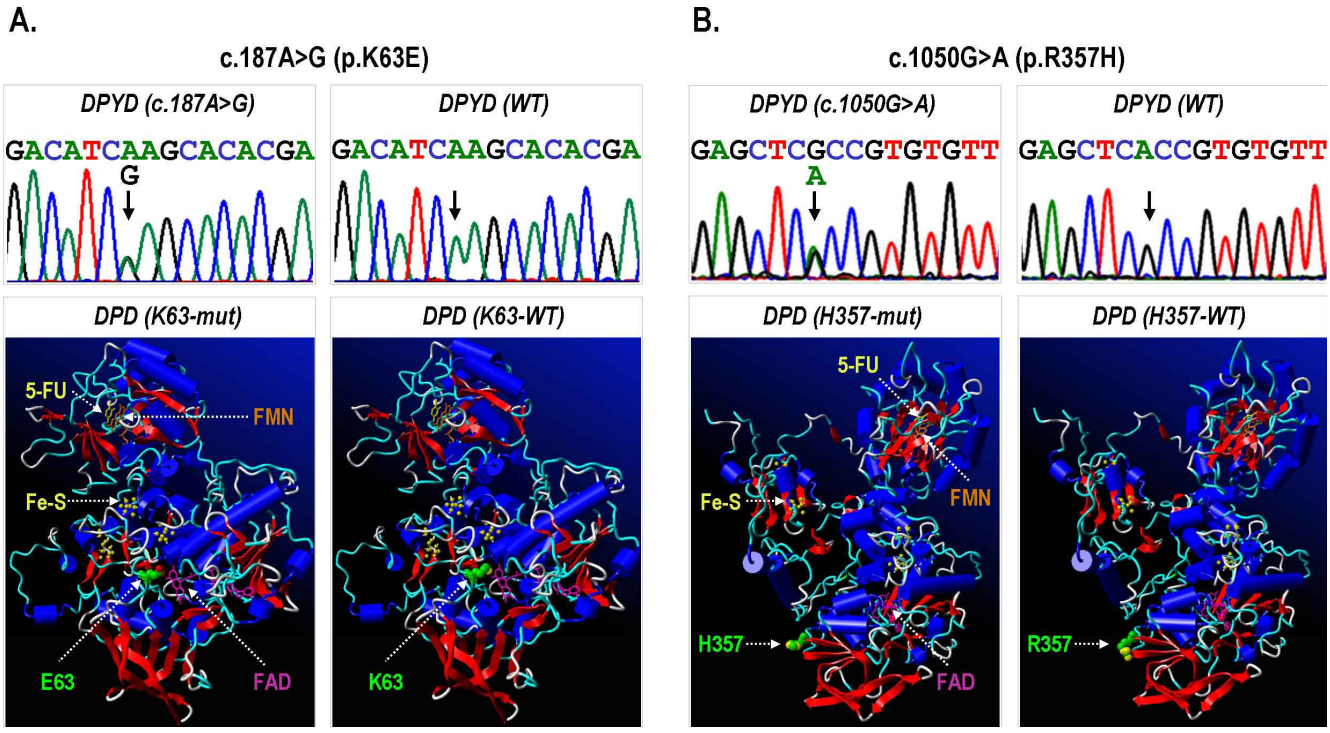

Figure 1. The sequencing chromatograms of the mutant and control wild-type sequences (upper panels) and structural localization of the mutated and corresponding wild-type amino acid residues within monomeric DPD protein (lower panels) depict the c.187A>G (A) and c.1050G>A (B) ***DPYD*** **mutations. The position of A to G (A) and G to A (B) transitions and corresponding sites in wt sequence in sequencing chromatograms (upper panels) are marked by the arrows. The 3D representative views of altered and corresponding wt DPD protein structures (lower panels) were drawn with YASARA Dynamics ver. 6.3.21 (www.yasara.org) using coordinates 1h7w and 1gth of porcine DPD available from Protein Data Bank (www.pdb.org) and published by Dobritzch** *et al.* **[8]. The binding cofactors (FAD, Fe-S clusters and FMN), contributing to the electron transfer to 5-FU are depicted as wire-frame structures and indicated by arrows. The altered residues (green letters) are shown as the green space-fill models with highlighted oxygen (red) and nitrogen (yellow) atoms within DPD protein colored according to its secondary structure.**

toxicity patients (Tab. 4). Six previously reported missense variants [c.85T>C (p.C29R), c.496A>G (p.M166V), c.775A>G (p.K259E), c.1601G>A (p.S534N), c.1627A>G (p.I543V), c.2194G>A (p.V732I)] were present solely or in various combinations in 53 out of 76 high-toxicity patients (69.7%). Two silent mutations [c.1236 G>A (E412) and c.1896T>C (F632)] were detected in two and four out of 76 high-toxicity patients, respectively (Tab. 4). We did not find any significant differences in frequencies of mutations between male and female high-toxicity patients.

DPYD alterations in the group of low-toxicity patients and control population. The exons carrying non-synonymous changes in the high-toxicity group were analyzed in 48 samples from the low-toxicity cancer patients group and 243 samples from non-cancer controls using DHPLC. This analysis revealed 37 carriers of at least one *DPYD* alteration in the group of 48 (77.1%) low-toxicity patients (Tab. 4). Exon 14 skipping mutation (IVS14+1G>A) and rare missense variants (K63E, K259E, and R357H) were not detected in any of the low-toxicity patient. The significant differences were found in frequencies of C29R carriers [26/76 (34.2%) of high-toxicity vs. 26/48 (54.2%) of low-toxicity patients; $p = 0.03$]. The significant difference was found in frequency of M166V within the low-toxicity group between males (12/23; 52.2%) and females (6/25; 24.0%; $p = 0.04$). Compared to high-toxicity patients, the significant difference was found in female subgroup for frequency of C29R carriers [12/40 (30.0%) of high-toxicity vs. 14/23 (60.9%) of low-toxicity female patients; $p = 0.02$] and M166V carriers [10/40 (25.0%) of high-toxicity vs. 12/23 (52.2%) of low-toxicity female patients; $p = 0.03$].

Analysis of control population identified 166 out of 243 individuals (68.3%) carrying at least one *DPYD* gene alteration (Tab. 4) with no difference in frequencies of particular alterations between males and females. Statistically significant difference between controls and the high-toxicity group was found in frequency of IVS14+1G>A carriers [5/235 (2.10%) of control vs. 5/76 (6.6%) of high-toxicity individuals; $p = 0.048$], being pronounced in the female carriers subgroups [3/140 (2.1%) vs. 4/40 (10%); $p = 0.02$]. The women subpopulations differed also in frequency of V732I carriers [11/140 (7.9%) in the control population vs. 8/40 (20%) in the high-toxicity patients; $p = 0.03$]. The frequency of S534N was significantly lower in control

Table 4. Frequencies of *DPYD* alterations.

Exon/intron	DPYD alteration (protein change)	Allelotype	High-toxicity patients N (%)	Low-toxicity patients N (%)	Control population N (%)
e2	**c.85T>C (C29R)**	Homo	2 (2.6)	1 (2.1)	13 (5.3)
		Het	24 (31.6)	25 (52.1)	95 (39.1)
		WT	50 (65.8)	22 (45.8)	135 (55.6)
e3	**c.187A>G * (K63E)**	Homo	0	0	0
		Het	1 (1.3)	0	0
		WT	75 (98.7)	48 (100)	243 (100)
e6	**c.496A>G (M166V)**	Homo	0	1 (2.1)	7 (2.9)
		Het	20 (26.3)	17 (35.4)	55 (22.6)
		WT	56 (73.7)	30 (62.5)	181 (74.5)
e8	**c.775 A>G (K259E)**	Homo	0	0	0
		Het	1 (1.3)	0	1 (0.4)
		WT	75 (98.7)	48 (100)	242 (99.6)
e10	**c.1050 G>A * (R357H)**	Homo	0	0	0
		Het	1 (1.3)	0	0
		WT	75 (98.7)	48 (100)	243 (100)
e11	**c.1236 G>A (E412)**	Homo	0	0	N.D.
		Het	2 (2.6)	2 (4.2)	N.D.
		WT	74 (97.4)	46 (95.8)	N.D.
e13	**c.1601G>A (S534N)**	Homo	0	0	0
		Het	5 (6.6)	6 (12.5)	8 (3.3)
		WT	71 (93.4)	42 (87.5)	235 (96.7)
e13	**c.1627A>G (I543V)**	Homo	0	0	3 (1.2)
		Het	22 (29.0)	11 (22.9)	69 (28.4)
		WT	54 (71.0)	37 (77.1)	174 (70.4)
e14	**c.1896T>C (F632)**	Homo	0	0	0
		Het	4 (5.3)	4 (8.3)	32 (13.2)
		WT	72 (94.7)	44 (91.7)	211 (86.8)
i14	**IVS14+1G>A (e14 del)**	Homo	0	0	0
		Het	5 (6.6)	0	5 (2.1)
		WT	71 (93.4)	48 (100)	238 (97.9)
e18	**c.2194G>A (V732I)**	Homo	0	0	1 (0.4)
		Het	12 (15.8)	7 (14.6)	19 (7.8)
		WT	64 (84.2)	41 (85.4)	223 (91.8)

* Novel *DPYD* alterations.

population compared to the low-toxicity group [8/243 (7.9%) vs. 6/48 (12.5%); $p = 0.006$]. The frequency of M166V female carriers was significantly lower in controls vs. low-toxicity group [35/140 (25%) vs. 12/23 (52.2%); $p = 0.008$].

The DHPLC analysis performed in the low-toxicity and control groups revealed presence of five intronic variants flanking to analyzed exons (IVS9-51T>G, IVS10-15T>C, IVS12-11G>A, IVS13+39C>T, and IVS13+40A>G). All these variants of unknown significance were described previously in various populations and were not considered for further analysis in our study [12, 24, 25]. The strong association of IVS13+39C>T with c.1627A>G (I543V) reported in previous studies was also observed in our samples (data not shown) [16, 26].

DPYD haplotypes in analyzed populations. All variants detected in *DPYD* coding sequence in three analyzed populations were in Hardy-Weinberg equilibrium ($p>0.05$), with the only exception for I543V ($p = 0.02$) in the low-toxicity group. Identified were 46 different haplotypes encompassing all alterations affecting *DPYD* coding sequence (Tab. 5). The strongest statistically significant linkage was apparent between C29R and M166V (R = 0.425; $p<0.0001$) in the all analyzed populations.

Contribution of individual DPYD coding sequence alterations to the development of serious 5-FU toxicity. The overall gastrointestinal and hematological toxicity, as well as individual toxicities were analyzed as phenotypes potentially affected by individual *DPYD* coding sequence alterations in the pooled group (N = 124) including all cancer patients from the high- and low-toxicity groups. The logistic regression analysis revealed that several *DPYD* missense variants may negatively or positively influence risk of toxicity development in the

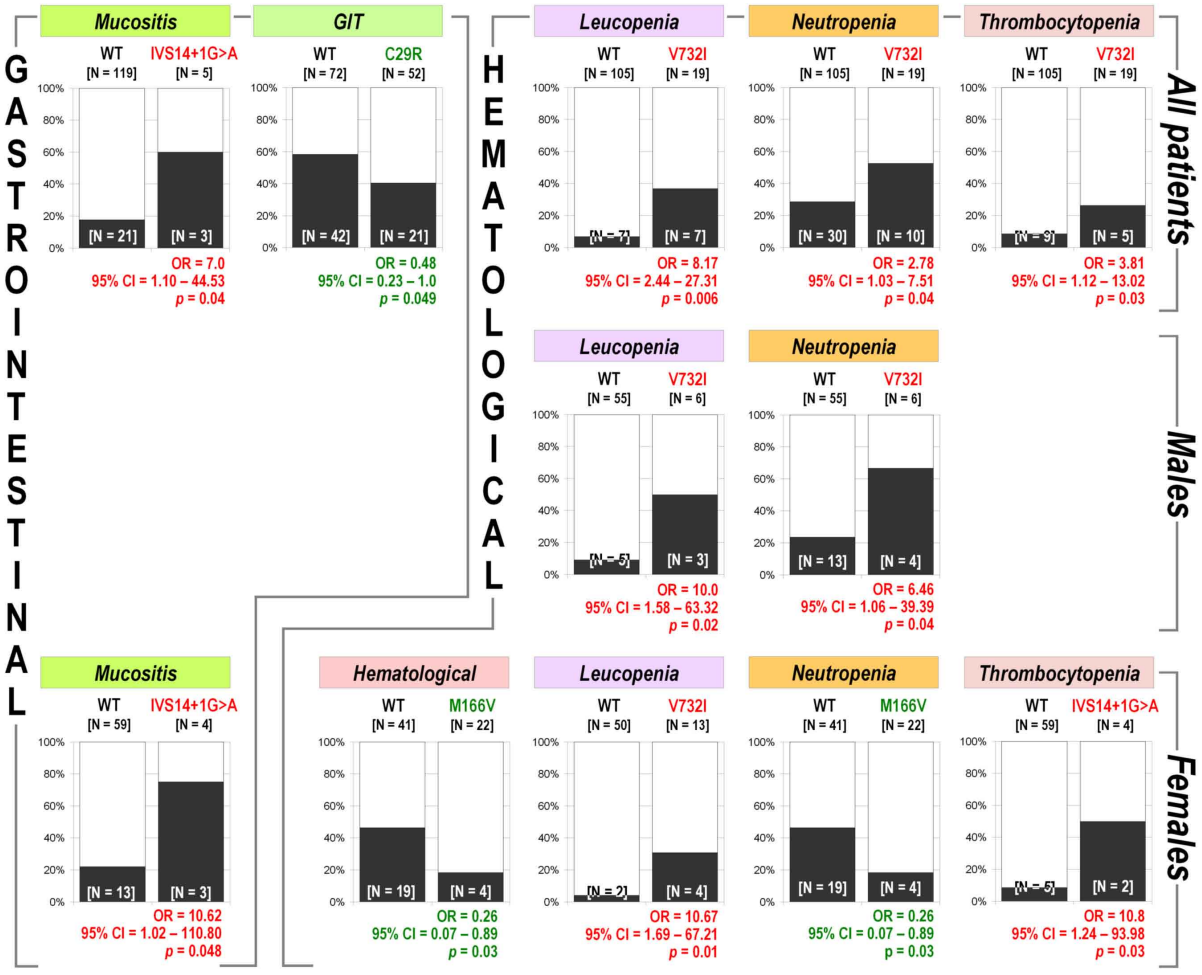

Figure 2. The percentages of all 5-FU treated patients with grade 0-2 toxicity (white bar) and grade 3-4 toxicity (black bar with a number indicating the count of individuals suffering from grade 3-4 toxicity) separated according to presence of given alteration (wild-type in left column and carriers of at least one mutated allele in right column). The *DPYD* alterations decreasing risk of toxicity in their carriers are depicted in green letters; alterations increasing risk of toxicity in their carriers are depicted in red letters. Only the statistically significant results from logistic regression analysis of the patients' groups with toxicity grade 0-2 vs. 3-4 are shown ($p < 0.05$). Male and female population (upper raw; N = 124); male population (middle raw; N = 61); female population (lower raw; N = 63). GIT = overall gastrointestinal toxicity; OR = odds ratio; CI = confidence interval.

overall populations or in the males and females subgroups, respectively. A protective effect was shown for two alterations – c.85T>C (C29R) and c.496A>G (M166V), whereas carriers of c.1905+1G>A (IVS14+1G>A) and c.2194G>A (V732I) were at significantly higher risk of serious site-specific toxicity development (Fig. 2). No association was found for c.1601G>A (S534N), c.1627A>G (I543V), and rare *DPYD* alterations. The novel *DPYD* variants c.187A>G (K63E) and c.1050 G>A (R357H) were found only in one woman (with neutropenia grade 4 and diarrhea grade 3) and one man (with diarrhea grade 4), respectively. Rare alteration c.775A>G (K259E), reported previously twice in high- and low-toxicity German patients, respectively, was found in combination with C29R and V732I in one man (with leucopenia grade 3 and neutropenia grade 4) [14, 27].

Carriers of TC or CC mutant allele (C29R) showed 2.1-times lower risk (OR = 0.48; Fig. 2) for development of overall serious gastrointestinal toxicity (grade 0-2 vs. 3-4) comparing to the patients carrying wild type (TT) allele. This protective effect of mutated allele C29R was even stronger considering patients developing gastrointestinal toxicity grade 0-2 vs. 4 (OR = 0.19; 95% CI = 0.04 – 0.96; $p = 0.04$). A borderline statistical significance was shown for the female carriers of C29R comparing to that carrying wild-type allele in a protection against overall hematological toxicity development (grade 0-2 vs. 3-4; OR = 0.35; 95% CI = 0.12 – 1.08; $p = 0.07$), however, we found significant difference in this group analyzing high-grade hematological toxicity grade 0-2 vs. 4 (OR = 0.21; 95% CI = 0.05 – 0.86; $p = 0.03$). Similarly, carrying of at least C29R allele protected with borderline statistical significance neutropenia

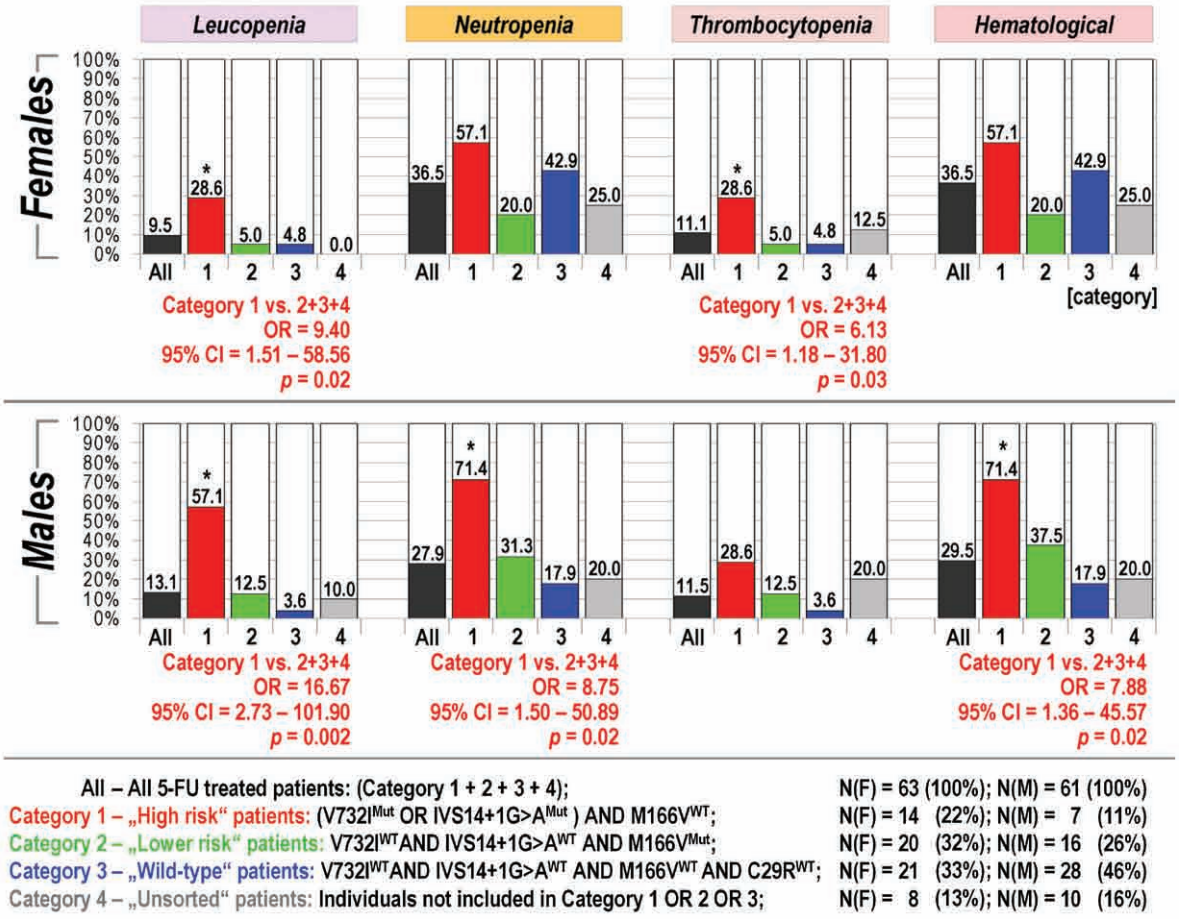

Figure 3. The percentages of patients with serious hematological toxicities grade 3-4 (depicted by numbers; color bars) and low toxicities grade 0-2 (white bars) in the females and males subgroups categorized according to analyzed haplotypes (category 1-4). The frequencies in overall male and female populations (All), not involved in the analysis are depicted. Only the statistically significant results ($p < 0.05$; marked by asterisk) of logistic regression analysis (OR = odds ratio; CI = confidence interval; p value) for given toxicities are listed bellow graphs.

development in women (grade 0-2 vs. 3-4; OR = 0.35; 95% CI = 0.12 – 1.08; $p = 0.07$), with significant influence in the female patients with neutropenia grade 0-2 vs. 4 (OR = 0.23; 95% CI = 0.06 – 0.94; $p = 0.04$).

Protective effects of M166V were shown in the female subpopulation only where carriers of this mutation conferred lover risk of overall serious hematological toxicity (grade 0-2 vs. 3-4; OR = 0.26; Fig. 2). The same result was found for neutropenia because in each of female patients with high-grade hematological toxicity (grade 3-4) occurred the high-grade neutropenia (grade 3-4). For both toxicities, there was found protective effect of M166V considering the most serious toxicity female patients grade 0-2 vs. 4 [(OR = 0.16; 95% CI = 0.03 – 0.81; $p = 0.03$) and (OR = 0.18; 95% CI = 0.04 – 0.87; $p = 0.03$), respectively].

The IVS14+1G>A, found only in one man and four women in the high-toxicity group, was associated with significantly higher risk of mucositis development in the overall patients population and female subgroup (OR = 7.0 and OR = 10.62, respectively; Fig. 2). The carriers of IVS14+1G>A in overall population showed statistically insignificant trend toward development of overall hematological toxicity (OR = 8.9), neutropenia (OR = 9.2), and thrombocytopenia (OR = 5.9; for all grade 0-2 vs. 3-4 $p < 0.07$). However, this mutation increased the risk of high-grade hematological toxicity (grade 0-2 vs. 4) in the whole population (OR = 11.3; 95% CI = 1.21 – 105.36; $p = 0.03$). In the female subpopulation, carriers of IVS14+1G>A were in high risk of thrombocytopenia (grade 0-2 vs. 3-4 OR = 10.8; Fig. 2), and considering the most serious toxicity grade 0-2 vs. 4, the risk of thrombocytopenia development was even higher (OR = 27.0; 95% CI = 1.20 – 605.59; $p = 0.04$).

The missense alteration V732I conferred a risk factor mainly of hematological toxicities development. It significantly increased the risk of leucopenia (grade 0-2 vs. 3-4) in the whole cancer patients' population (OR = 8.2) and in men

Table 5. The frequencies of haplotypes involving *DPYD* missense variants and IVS14+1 G>A detected in all three analyzed populations.

	IVS14+1G>A and missense DPYD alterations									Analyzed populations*		
#	C29R	K63E	M166V	K259E	R357H	S534N	I543V	IVS14+1	V732I	TOX N (%)	NETOX N (%)	POPUL N (%)
1	2	-	2	-	-	-	-	-	-	0	0	1 (0.4)
2	2	-	1	-	-	-	1	-	-	0	0	7 (2.9)
3	2	-	-	-	-	1	-	-	-	0	1 (2.1)	0
4	2	-	-	-	-	-	1	-	-	0	0	1 (0.4)
5	2	-	-	-	-	-	-	-	1	0	0	1 (0.4)
6	2	-	-	-	-	-	-	-	-	2 (2.6)	0	3 (1.2)
7	1	-	2	-	-	-	-	-	-	0	0	3 (1.2)
8	1	-	1	-	-	1	-	-	-	0	1 (2.1)	2 (0.8)
9	1	-	1	-	-	-	1	-	1	0	0	1 (0.4)
10	1	-	1	-	-	-	1	-	-	4 (5.3)	2 (4.2)	9 (3.7)
11	1	-	1	-	-	-	-	-	1	1 (1.3)	1 (2.1)	3 (1.2)
12	1	-	1	-	-	-	-	-	-	10 (13.2)	9 (18.8)	24 (9.9)
13	1	-	-	1	-	-	-	-	1	1 (1.3)	0	0
14	1	-	-	1	-	-	-	-	-	0	0	1 (0.4)
15	1	-	-	-	-	1	1	-	-	0	1 (2.1)	0
16	1	-	-	-	-	1	-	-	-	0	1 (2.1)	1 (0.4)
17	1	-	-	-	-	-	2	-	-	0	0	1 (0.4)
18	1	-	-	-	-	-	1	-	1	0	1 (2.1)	1 (0.4)
19	1	-	-	-	-	-	1	-	-	2 (2.6)	2 (4.2)	16 (6.6)
20	1	-	-	-	-	-	-	1	1	1 (1.3)	0	0
21	1	-	-	-	-	-	-	1	-	1 (1.3)	0	0
22	1	-	-	-	-	-	-	1	-	0	0	1 (0.4)
23	1	-	-	-	-	-	-	-	1	1 (1.3)	3 (6.3) [1)]	2 (0.8)
24	1	-	-	-	-	-	-	-	-	3 (3.9) [2)]	4 (8.3)	30 (12.3)
25	-	1	-	-	-	-	-	-	-	1 (1.3)	0	0
26	-	-	2	-	-	1	-	-	-	0	1 (2.1)	1 (0.4)
27	-	-	2	-	-	-	1	-	-	0	0	2 (0.8)
28	-	-	1	-	-	-	2	-	-	0	0	1 (0.4)
29	-	-	1	-	-	-	1	-	-	1 (1.3)	1 (2.1)	1 (0.4)
30	-	-	1	-	-	-	-	1	-	0	0	2 (0.8)
31	-	-	1	-	-	-	-	-	1	0	0	2 (0.8)
32	-	-	1	-	-	-	-	-	-	4 (5.3) [2)]	3 (6.3) [3)]	3 (1.2)
33	-	-	-	-	1	-	-	-	-	1 (1.3)	0	0
34	-	-	-	-	-	1	1	-	-	1 (1.3)	0	2 (0.8)
35	-	-	-	-	-	1	-	1	-	1 (1.3)	0	0
36	-	-	-	-	-	1	-	-	1	1 (1.3)	0	1 (0.4)
37	-	-	-	-	-	1	-	-	-	2 (2.6)	1 (2.1)	1 (0.4)
38	-	-	-	-	-	-	2	-	-	0	0	1 (0.4)
39	-	-	-	-	-	-	1	1	-	1 (1.3)	0	0
40	-	-	-	-	-	-	1	-	1	4 (5.3)	1 (2.1)	4 (1.6)
41	-	-	-	-	-	-	1	-	-	9 (11.8)	3 (6.3)	31 (12.8)
42	-	-	-	-	-	-	-	1	1	0	0	1 (0.4)
43	-	-	-	-	-	-	-	1	-	1 (1.3)	0	1 (0.4)
44	-	-	-	-	-	-	-	-	2	0	0	1 (0.4)
45	-	-	-	-	-	-	-	-	1	3 (3.9)	1 (2.1)	3 (1.2)
46	-	-	-	-	-	-	-	-	-	20 (26.3)	11 (22.9)	77 (31.7)
										76 (100)	48 (100)	243 (100)

- wild type alleles

*The statistically different frequencies comparing to control populations are depicted as [1)] p=0.008; [2)] p=0.04; [3)] p=0.03; (ANOVA test).

(OR = 10.0) and women (OR = 10.67), and thrombocytopenia (grade 0-2 vs. 3-4) in the whole population (OR = 3.18; Fig. 2). The V732I carriers in the overall population and the male subgroup were also at significant higher risk of neutropenia development (grade 0-2 vs. 3-4; Fig. 2), increased for overall and male patients developing high-grade neutropenia [grade 0-2 vs. 4: (OR = 3.97; 95% CI = 1.43 – 11.03; $p = 0.008$) and (OR = 7.63; 95% CI = 1.23 – 47.24; $p = 0.03$), respectively]. The overall hematological toxicity in V732I carriers was nearly significant in whole patients and in men comparing grade 0-2 vs. 3-4 toxicities [(OR = 2.65; 95% CI = 0.98 – 7.16; $p = 0.054$) and (OR = 5.86; 95% CI = 0.97 – 35.52; $p = 0.055$), respectively], and it was statistically significant in both groups comparing grade 0-2 vs. 4 toxicities [(OR = 3.58; 95% CI = 1.30 – 9.86; $p = 0.01$) and (OR = 6.83; 95% CI = 1.11 – 41.97; $p = 0.04$), respectively].

Analysis of selected haplotypes to the development of serious 5-FU toxicity. Considering the impact of individual alleles significantly influencing the risk of high-grade toxicity (C29R, M166V, IVS14+1G>A, and V732I), we analyzed four allelotypes (categories) with potential predictive value for prediction of serious hematological toxicities (grade 0-2 vs. 3-4):

1. The "high risk" patients category, consisted of carriers of the IVS14+1G>A or V732I mutations that simultaneously lacked the M166V (haplotypes #13, 18, 20, 21, 23, 35, 36, 39, 40, 43, 45 in Tab. 5) comprised 14 out of 63 (22%) females and 7 out of 61 (11%) males.
2. The "lower risk" patients category included the individuals without IVS14+1G>A and V732I mutations and carrying at least one mutated M166V allele (haplotypes #8, 10, 12, 26, 29, 32 in Tab. 5) consisted of 20 out of 63 (32%) females and 16 out of 61 (26%) males.
3. The category of "wild-type" patients not carrying any of considered mutations (haplotypes #25, 33, 34, 37, 41, 46 in Tab. 5) was represented by 21 out of 63 females (33%) and 28 out of 61 males (46%).
4. The eight out of 63 females (13%) and 10 out of 61 males (16%) remained unclassified according to these descriptors ("unsorted" patients category).

The impact of analyzed categories on serious toxicity development was performed by logistic regression analysis involving all patients treated by 5-FU-containing regimens with grade 0-2 vs. 3-4 (consisted of the pooled high- and low-toxicity groups) separated according to selected allelotypes (Fig. 3). Because the risk of toxicity development differed between men and women in our study as well as in previously published trials with 5-FU treated patients, we performed this analysis with respect to the gender groups [27, 19].

Female patients included in "high risk" category (category 1) were at significantly increased risk of leucopenia (OR = 9.4) and thrombocytopenia (OR = 6.13) compared to other women (categories 2+3+4; Fig. 3). This category showed a trend toward increased risk of neutropenia and overall hematological toxicity (for both OR = 3.02; 95% CI = 0.89 – 10.24; $p = 0.08$) compared to other women (categories 2+3+4; Fig. 3). Male "high-risk" patients (category 1) had increased risk of leucopenia (OR = 16.67), neutropenia (OR = 8.75) and overall hematological toxicity (OR = 7.88) compared to other men (categories 2+3+4; Fig. 3).

Only "lower risk" female patients (category 2) tended to decrease risk of neutropenia and overall hematological toxicity (for both OR = 0.32; 95% CI = 0.09 – 1.10; $p = 0.07$) compared to other women (categories 1+3+4; Fig. 3). This category was of no significant importance in the male subgroup.

Wild-type female patients (category 3) did not show any decreased risk for development of hematological toxicities. Moreover, the significant association was found for increased risk of serious emesis (grade 0-2 vs. 3-4) in female carriers of wt haplotype compared to other women (categories 1+2+4; OR = 8.0; 95% CI = 1.45 – 44.09; $p = 0.02$). Wild type male patients (category 3) tended to have decreased risk of leucopenia and overall hematological toxicity [(OR = 0.14; 95% CI = 0.02 – 1.20; $p = 0.07$) and (OR = 0.33; 95% CI = 0.10 – 1.10; $p = 0.07$), respectively] compared to other males (categories 1+2+4). Male patients in this category showed significantly lower frequency of leucopenia ($p = 0.005$), neutropenia ($p = 0.01$), and overall hematological toxicity ($p = 0.01$) compared to men from "high risk" category 1.

Predisposition of DPYD alterations to gastrointestinal cancer. Most of 5-FU treated patients were individuals with colorectal cancer (90 out of 124 patients; 72.6%). We analyzed frequencies of detected *DPYD* alterations in cancer patients vs. non-cancer controls as potential risk factors for colorectal cancer (i.e. cancer of colon, rectum, or anus). The carriers of c.1601G>A (S534N) were more frequent in the cancer patients group comparing to controls [8/90 (8.9%) vs. 8/243 (3.3%); OR = 2.87; 95% CI = 1.04 – 7.88; $p = 0.04$]. The female cancer patients subgroup compare to female controls showed higher frequencies of IVS14+1G>A [4/42 (9.5%) vs. 3/140 (2.1%); OR = 4.81; 95% CI = 1.03 – 22.41; $p = 0.046$] and V732I [9/42 (21.4%) vs. 11/140 (7.7%); OR = 3.20; 95% CI = 1.22 – 8.36; $p = 0.02$].

Discussion

The influence of DPD on tolerance of 5-FU-based therapy is widely accepted [10,28]. However, the involvement of other genetic factors [e.g. downstream enzymes of 5-FU degradation pathway – DPYS and UBP1, or other enzymes – thimidylate synthetase (TYMS; OMIM 188350) or methylenetetrahydrofolate reductase (MTHFR; OMIM 607093)] to the development of fluoropyrimidines-related toxicity has been also considered [27,29-31]. Since eighties, the numerous methods based on determination of DPD status were advised to predict serious 5-FU-related toxicity. The function tests have been used for measurement of DPD enzyme activity by 2-[^{13}C]-uracil breath test, or determining uracil/dihydrouracil or [^{14}C]-thymine/dihydrothymine plasma ratio [32–34]. Substantial drawbacks of function tests are a need for specialized technology approaches or the use of radiolabeled chemicals not available

worldwide [34]. The other strategy, used also for prediction of treatment efficacy, involves the expression analyzes of DPD by immunohistochemistry or at the level of mRNA [35–37]. Despite its easiness, many contradictory results regarding the predictive power of expression analyses were obtained [38, 39]. The influence of *DPYD* promoter methylation on *DPYD* gene expression was also considered, however contrary to initial reports latest studies demonstrated limited or no methylation of *DPYD* promoter in high-toxicity patients [27, 40–42]. The most common concept for prediction of serious toxicity in 5-FU-treated patients represents pharmacogenomic analyses. Numerous *DPYD* alterations were reported in published studies and case reports, however the vast majority represents missense gene variants with unknown impact on DPD enzyme activity. Moreover, the strong population differences in frequency of common *DPYD* gene alterations and haplotypes were described [12]. Recently, the common fragile site FRA1E was localized in *DPYD* gene, however the large deletions in *DPYD* were not studied so far [43]. Combinatory approaches implementing functional and expression analyses alongside to genotyping introduced by Morel *et al.* (2006) could enhance specificity and sensitivity of 5-FU toxicity detection, however it seems to be less suitable for routine clinical settings [44]. Therefore, despite substantial effort, there is currently unavailable a simple and reliable test predicting serious toxicity following 5-FU treatment [45, 46].

In our current survey, we studied the *DPYD* variants in patients suffering from high-grade 5-FU-related toxicity (high-toxicity patients) comparing to the patients with superior tolerance of fluoropyrimidines-based therapy (low-toxicity patients). We performed the genotyping of *DPYD* variants in the whole coding sequence in 76 high-toxicity patients and we detected the splicing site mutation IVS14+1G>A and eight different missense variants including two previously undescribed. The splicing mutation IVS14+1G>A was found in similar frequency in our high-toxicity patients (6.6%) compared to that in recent prospective German study (5.5%) published by Schwab *et al.* (2008), however, contrary to this study we did not find any low-toxicity patient carrying this alteration [27]. The frequency of IVS14+1G>A carriers in our control population of the Czech origin is relatively high (2.1%), comparing to that found in overall or cancer patients populations in the Central European region: Slovakia (0%), Poland (0.4%), Germany (1.1%) [47, 48] and exceeding the frequency in the Netherlands (1.8%) were the IVS14+1G>A testing has been advised for clinical utilization prior 5-FU therapy [49, 50].

For the two novel rare variants, each detected in patient with high toxicity, we hypothesize that c.187A>G (K63E) may potentially interfere with DPD enzymatic activity, but the c.1050G>A (R357H) could less likely influence the DPD enzyme function according to the prediction models of DPD protein structure. All non-synonymous *DPYD* changes that were found in 73.7% of high-toxicity patients were screened in low-toxicity patients group and non-cancer controls, where they were found in 77.1% and 68.3% of samples, respectively. Our results demonstrated that missense alterations C29R, M166V, S534N, I543V, and V732I representing the vast majority of detected variants are highly frequent *DPYD* polymorphisms in our population. Except the C29R and M166V, which we found in significantly higher frequency in the low-toxicity patients group, the frequency of others (S534N, I543V, and V732I) did not significantly differ between high- and low-toxicity patients and therefore, they can not be considered the causative factors of overall 5-FU toxicity. We found the strong segregation of C29R with M166V in our study population.

Further, we tested the frequency of analyzed mutations and polymorphisms in relationship to the frequencies of hematological and gastrointestinal toxicities in the pooled population of 124 patients treated by fluoropyrimidines. The C29R mutation represented mild (OR = 0.48) independent protective factor for overall gastrointestinal toxicity in the whole population, detected in 31/61 (51%) patients with gastrointestinal toxicity grade 0-2 and 21/63 (33%) of patients with gastrointestinal toxicity grade 3-4. We found that in women population the M166V mutation negatively correlated with overall hematological toxicity and neutropenia (OR = 0.26). For both toxicities, carriers of M166V accounted for 18/40 (45%) patients with toxicity grade 0-2 and 4/23 (17%) patients with toxicity grade 3-4. Contrary to this result, recent German study involving 89 patients with good and 39 patients with poor tolerance of fluoropyrimidines-based chemotherapy published by Gross *et al.* (2008) described the positive correlation of M166V with development of grade 3-4 fluoropyrimidine toxicity (OR = 4.42) in patients with gastroesophageal and breast cancer, but without significance for colorectal cancer patients [26]. Several differences in the design of studies could contribute to this contradictory results, including the higher proportion of the high-toxicity patients' population and more frequent colorectal cancer diagnoses in our study. Interestingly, the supplementary data from the other recent German multicenter prospective trial published by Schwab *et al.* (2008) involving patients treated by 5-FU monotherapy reported the allelic frequency of M166V as 8/60 (13%) and 10/108 (9%) for patients with toxicity grade 0-2 and 3-4, respectively, however, the effect of M166V itself was not evaluated in this study [27]. The two independent genetic factors positively correlating with toxicity in our study represented IVS14+1G>A and V732I. The IVS14+1G>A contributed to higher risk of mucositis development in overall population and mucositis and thrombocytopenia in women subgroup, however, these results need to be interpreted with caution, as only five carriers of this alterations in high-grade toxicity patients (four women) were detected in our study. The carriers of V732I alteration, that has been considered the polymorphism [16], were relatively frequent in our study and at significantly higher risk for neutropenia development. We found this alteration in 7/14 (50%) and in 12/110 (11%) patients with neutropenia grade 3-4 and 0-2, respectively. Moreover, all carriers of V732I in the high-grade neutropenia patients group suffered from neutropenia grade 4 and two of them (both women) were with lethal

outcome, following 5-FU or capecitabine in monotherapy. The presence of V732I significantly positively correlated also with development of thrombocytopenia in overall population, leucopenia and neutropenia in men and leucopenia in women. The aforementioned study of Schwab *et al.* (2008) found the frequency V732I as 38/1,146 (3.3%) alleles in patients with grade 0-2 fluorouracil toxicity and 14/220 (6.4%) alleles in patients with grade 3-4 toxicity [27]. The allelic frequency of V732I in study of Gross *et al.* (2008) did not differed between 89 no/mild toxicity and 39 grade 3-4 toxicity patients (0.10 and 0.09, respectively) [26].

Based on our results reflecting significant associations of several *DPYD* gene variants with particular site-specific toxicities differing in gender groups, we determined haplotypes considering possible protective effects of C29R and M166V and negative effects of IVS14+1G>A and V732I for development of fluoropyrimidines-related toxicity. The strongest significance we observed for the "high risk" patients category (carriers of IVS14+1G>A or V732 lacking M166V) represented by limited populations 14/63 (22%) women and 7/61 (11%) men, but identified in 4/6 (66%) female and 4/8 (50%) male patients with leucopenia grade 3-4, 8/23 (35%) female and 5/17 (29%) male patients with neutropenia grade 3-4, as well as in 4/7 (57%) female and 2/7 (29%) male patients with thrombocytopenia. The category described as the "lower-risk" (carriers of M166V allele without IVS14+1G>A and V732I mutations) displayed limited insignificant trend for protective role in female population only. Females carrying this haplotypes accounted for 20/63 (32%) from all female patients, but this haplotypes occurred only in 4/23 (17%) of women with neutropenia (or overall hematological toxicity) grade 3-4. On the other hand, the protective role of "wild-type" category (non carriers of C29R, M166V, IVS14+1G>A, and V732I) was observed in the male patients group consisting of 28/61 (46%) male patients. Male carriers of these haplotypes were found in only 1/8 (13%) of men with grade 3-4 leucopenia, in 5/17 (29%) men with grade 3-4 neutropenia, and in 5/18 (28%) men with overall hematological toxicity.

Despite these promising results we are aware that any clinical interpretation of our study should be performed with caution and confirmation of our results by further prospective trial involving the new unsorted fluoropyrimidine-treated patients, that may also resolved the sensitivity and specificity, and hence the usefulness this concept is essential. This ongoing independent study should verify if using this fast and easy analysis implementing genotyping of the only four *DPYD* gene variants (C29R, M166V, IVS14+1G>A, and V732I) may discriminate male and female patients recruiting from our "high risk" category in whom the fluoropyrimidines dose reduction or alternative treatment regimes application should be *a priori* considered, together with patients with "lower risk", that may benefit from regular fluoropyrimidines therapy with favorable safety profile. If these results will be proved, there would be also the time for reconsideration of biological rationale of alterations in *DPYD*. Despite the high frequency of genetic variants in *DPYD*, only little of them (representing relatively small portion of the high-toxicity patients' population) exert clear effect leading to abrogation of DPD enzyme activity and most of *DPYD* variants are polymorphisms with no easily apparent influence on development of serious toxicity. Further studies analyzing the large fluoropyrimidine-treated patients' populations as well as *in vitro* analyses with human tissue cultures and experiments on animal models are desired to clarify the role of these *DPYD* variants. Finally, our data indicate that the development of serious toxicity in fluoropyrimidine-treated patients may not only differ in men and women subpopulations, but the site-related toxicity should be also considered.

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