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ORIGINAL ARTICLE

Contribution of dihydropyrimidinase gene alterations to the development of serious toxicity in fluoropyrimidine-treated cancer patients

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Abstract
Purpose Decreased 5-fluorouracil catabolism has been considered a major factor contributing to fluoropyrimidine (FP)-related toxicity. Alterations in the dihydropyrimidine dehydrogenase gene coding for the first and rate-limiting enzyme of FP catabolic pathway could explain toxicity in only a limited proportion of FP-treated patients. The importance of gene variants in dihydropyrimidinase (*DPYS*) coding for subsequent catabolic enzyme of FP degradation is not fully understood.
Methods We performed genotyping of *DPYS* based on denaturing high-performance liquid chromatography in 113 cancer patients including 67 with severe FP-related toxicity and 46 without toxicity excellently tolerating FPs treatment.
Results We detected nine *DPYS* variants including four located in non-coding sequence (c.-1T>C, IVS1+34C>G, IVS1-58T>C, and novel IVS4+11G>T), four silent (c.15G>A, c.216C>T, and novel c.105C>T and c.324C>A), and one novel missense variant c.1441C>T (p.R481W). All novel alterations were detected once only in patients without toxicity. The c.-1T>C and IVS1-58T>C variants were found to modify the risk of toxicity. The CC carriers of the c.-1C alleles were at higher risk of mucositis (OR = 4.13; 95% CI = 1.51–11.31; $P = 0.006$) and gastrointestinal toxicity (OR = 3.54; 95% CI = 1.59–7.88; $P = 0.002$), whereas the presence of the IVS1-58C allele decreased the risk of gastrointestinal toxicity (OR = 0.4; 95% CI = 0.17–0.93; $P = 0.03$) and leucopenia (OR = 0.29; 95% CI = 0.08–1.01; $P = 0.05$).
Conclusions Our results indicate that missense and nonsense variants in *DPYS* are infrequent, however, the development of serious primarily gastrointestinal toxicity could be influenced by non-coding *DPYS* sequence variants c.-1T>C and IVS1-58T>C.

Keywords Dihydropyrimidinase gene (DPYS) · 5-Fluorouracil · Toxicity · Denaturing high-performance liquid chromatography

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Introduction

Fluoropyrimidines (FPs) have been introduced into the clinical practice in the early sixties. Fifty years later, they still stand for the base pharmacotherapeutic option in the treatment of head and neck cancers, gastrointestinal cancer, breast cancer and other malignancies. The mechanism of action of 5-fluorouracil (5-FU) and its prodrugs (e.g. capecitabine) involves the inhibition of thymidylate synthase and interference with DNA and RNA metabolism in proliferating cells [1]. Despite its therapeutic value, the FP-based therapy is complicated by high frequency of adverse events. The development of toxicity influences numerous factors; except pharmacogenetic profile also treatment regimens and characteristics of treated populations. Overall, approximately 40% of patients experience grades 3–4 toxicity and

about 1% of them die due to therapy [2–4]. The most common toxicities observed with FP-based treatment include mucositis, diarrhea, neutropenia and thrombocytopenia.

Although the exact mechanism of serious FP-related toxicity is not currently fully understood, it has been proposed that the catabolic pathway responsible for initial degradation of over 80% of 5-FU plays the critical role. This pathway involves the three consecutive metabolic steps catalyzed by dihydropyrimidine dehydrogenase (DPD; OMIM 274270), dihydropyrimidinase (DPYS; OMIM 222748) and β-ureidopropionase (UPB1; OMIM 606673) enzymes [1, 5]. It has been shown that inactivating mutations of the dihydropyrimidine dehydrogenase (*DPYD*) gene coding for the first and rate-limiting enzyme in the (fluoro)pyrimidine degradation decrease its enzymatic activity [6, 7]. The carriers of *DPYD* alterations have increased FPs bioavailability in normal tissues through the inhibition of FPs catabolism, which subsequently leads to the serious toxicity development.

Many *DPYD* alterations have been characterized in patients with high-grade toxicity following 5-FU treatment, but only the c.1905+1G>A (IVS14+1G>A) resulting in exon 14 skipping has been shown to have clear impact on the development of FP-related toxicity in clinically meaningful, however, size limited subgroup of high-toxicity patients [8, 9]. The majority of *DPYD* alterations are probably polymorphisms with disputative role in toxicity development [10]. Except the *DPYD* mutation analyses and genotyping studies, numerous other methods analyzing *DPYD* promoter hypermethylation, genomic rearrangements, gene expression analyses (performed at the level of the gene transcript or the protein expression), and also many functional studies of DPD enzyme activity were introduced, however, even despite the combinatorial approaches, the establishment of robust and reliable test enabling the identification of patients at a risk of FP toxicity development is still an ongoing task [11–13].

The low correlation of *DPYD* alterations with occurrence of serious and life-threatening 5-FU toxicity led to the analysis of other genes, including those in (fluoro) pyrimidine degradation pathway. Only limited data referring about *DPYS* alterations in patients with FP-related toxicity have been available so far. Mutation in *DPYS* c.833G>A (p.G278D) was found in one breast cancer patient with severe leucopenia and skin ulcers following the administration of continuous infusion of 5-FU with normal DPD activity [14]. Eighteen *DPYS* alterations were described recently by Thomas et al. [15] in healthy volunteers alongside to another nine known *DPYS* variants.

We investigated the presence of genetic alterations in *DPYS* in patients with severe (grades 3–4) FP-related toxicity and in patients with excellent tolerance of FP-based therapy without significant toxicity (grades 0–1). All these cancer patients were previously analyzed for the presence of mutations in *DPYD* [16].

Materials and methods

Patients and samples

The study population consisted of the two patients subgroups (Table 1). The first group consisted of 67 FP-treated

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

	High-toxicity group (toxicity grades 3–4) $N = 67$	Low-toxicity group (toxicity grades 0–1) $N = 46$
Demographic parameters		
Females, *N* (%)	34 (50.7)	22 (47.8)
Mean age, years ± SD	60.4 ± 10.7	56.4 ± 11.4
Age range, years	30–75	31–74
Males, *N* (%)	33 (49.3)	24 (52.2)
Mean age, years ± SD	62.6 ± 8.0	59.7 ± 9.3
Age range, years	42–73	36–77
Cancer diagnose, *N* (%)		
Orofacial cancer	1 (1.5)	0
Esophageal	2 (3.0)	0
Gastric	5 (7.5)	0
Colorectal	44 (65.7)	39 (84.8)
Biliary	2 (3.0)	1 (2.2)
Pancreatic	1 (1.5)	1 (2.2)
Pharyngeal	1 (1.5)	0
Breast	11 (16.4)	3 (6.5)
Unknown primary site	0	2 (4.3)
Chemotherapy regimens		
Bolus 5-FU	17 (25.4)	17 (37.0)
Continuous 5-FU/capecitabine	35 (52.2)	11 (23.9)
FOLFIRI	1 (1.5)	6 (13.0)
FOLFOX	13 (19.4)	8 (17.4)
Other	1 (1.5)	4 (8.7)
Toxicity grades III–IV according to NCI CTC; *N* (%)		
Gastrointestinal only	33 (49.3)	0
Hematological only	11 (16.4)	0
Gastrointestinal and hematological	23 (34.3)	0
Mucositis	21 (31.3)	0
Emesis	13 (19.4)	0
Diarrhea	35 (52.2)	0
Leucopenia	11 (16.4)	0
Neutropenia	33 (49.3)	0
Thrombocytopenia	11 (16.4)	0

patients that experienced severe grades 3–4 hematological (leucopenia, neutropenia or thrombocytopenia) and/or gastrointestinal (mucositis, emesis, diarrhea) toxicity according to the National Cancer Institute (NCI) common toxicity criteria (CTC) scale 3.0 during the first or the second cycle of FP-based therapy. These patients were referred to our center from Czech regional oncology departments using electronic online database system organized by Comprehensive Oncology Center (http://www.koc.cz) [17]. The second group included 46 patients treated at the Department of Oncology, General Teaching Hospital in Prague and the Department of Oncology Faculty Hospital in Pilsen between April 2003 and February 2007 who underwent at least ten cycles of FP-based chemotherapy without any dose reductions or delays, and experienced no or at most grade 1 toxicity according to NCI CTC criteria. Previously performed genotyping of *DPYD* coding sequence alterations did not find any *DPYD* variant in 18 out of 67 patients (29.8%) with serious toxicity and in 11 out of 46 patients (23.9%) with excellent tolerance of FP-based treatment [16]. All patients signed written informed consent, approved by the ethics committee of the General Teaching Hospital in Prague, Czech Republic. The population frequencies of frequent *DPYS* alterations characterized in high-toxicity patients were determined in anonymized control population consisted of 69 non-cancer individuals including 30 women [61.2 ± 12.2 years (mean age ± SD)] and 39 men [62.6 ± 13.6 years (mean age ± SD)].

Mutation analysis of *DPYS* gene

Genomic DNA was isolated from whole blood using Wizard genomic DNA purification kit (Promega). The entire *DPYS* coding sequence was amplified from DNA in amplicons covering coding exons (exons 1–9) and flanking intronic regions. Primers (Generi-Biotech) for PCR amplification were designed based on the *DPYS* sequence (NCBI NT_008046.15; GI:51467074) and are listed in Table 2. The PCR reaction contained 1.5–2.5 mM $MgCl_2$, 0.3 mM of each primer, 0.25 mM of each dNTP, 0.6 U of *Taq* Gold DNA polymerase (Applied Biosystems) and 60 ng of template DNA. Following the initial denaturation for 10 min at 95°C, amplification was carried out in 38 cycles (30 s at 95°C, 45 s at 57°C, 40 s at 72°C) followed by the final extension for 10 min at 72°C. The denaturation at 98°C for 5 min and slow renaturation of PCR amplicons was introduced for subsequent DHPLC (denaturing high-performance liquid chromatography) analysis. PCR products were separated on 1% agarose gel electrophoresis to confirm the presence of a single amplified PCR product.

The DHPLC analysis of the unpurified PCR fragments was performed on the WAVE3500 (Transgenomic) analyzer using DNASep cartridge and original buffers (Transgenomic) under conditions specified in Table 2. Samples with aberrant elution profiles on DHPLC chromatograms were bi-directionally sequenced from independently amplified PCR amplicons using amplification

Table 2 The design of the primers, composition of PCR mixes used for amplification of nine coding exons (exons 1–9) of *DPYS*, and temperatures for DHPLC analysis of amplicons predicted by Navigator ver. 1.64 (Transgenomic) software and determined experimentally

Exon	Primers (5′–3′) (F: forward/R: reverse primer)	Size (bp)	$MgCl_2$ (mM)	DMSO (%)	DHPLC Temp (°C)
1	F: CAGCCCGGGCCGAGTAGGACC	381	1.5	5	66.9
	R: GCTGAGGACCCCGGGACGACTG				67.4
2	F: TGCCCAAGTGCTTACAAATGCAC	311	1.5	0	56.3
	R: TTGTGCAGAGTGAGGACAAGAGG				
3	F: GAATGCAACGTTTTAGAGAAGGTTG	333	1.5	0	55.6
	R: GGCCCAATCATCTTCACCTTATG				57.6
4	F: AACAGATCAAAAGCCTGGCATTGC	367	1.5	0	57.6
	R: GCAGAGGCTACAGACGTGGAC				59.1
5	F: GTAGATATTACCCTCTGCTAGGGGAGA	350	1.5	0	55.1
	R: GAGGATCCAGATGGGAGGACG				58.1
6	F: GGAAAGGTTATGGCAGTTTCCTCC	268	1.5	0	58.2
	R: GATCCTGGCTGAAGAACTAGGCA				
7	F: GGCGAAGAAATTAGGTGCAGTTCTG	353	1.5	0	55.5
	R: GGAGAAGCGGCTGATTAATGCC				
8	F: CAAGTGAGCTGGTGATGATCCAGA	320	1.5	0	57.6
	R: CAGACATCCAGAAGCAGGAGACAG				59.3
9	F: GCTCTCGCAATTCATAAAGGGAGC	266	2.5	0	57.2
	R: TTTCATATGCTGTCATGCAGGAAGAC				60.2

primers and BigDye Terminator sequencing kit ver. 3.1 (Applied Biosystems) on ABI3130 analyzer (Applied Biosystems).

Statistical analysis

The differences in frequencies of found genetic alterations between analyzed groups were calculated by the χ^2 test. The odds ratios (OR) and 95% confidence intervals (CI) of OR were computed for the determination of differences between investigated groups of patients. As alternative of logistic regression, the χ^2 test was used. The relations between the investigated parameters were computed by the Spearman's correlation coefficients. The comparison of variables in given groups and subgroups, considering the distribution of these variables, was performed by a non-parametric Wilcoxon's 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 χ^2 test in linkage disequilibrium analyzer 1.0 software [18].

Results

Mutation analysis of the *DPYS* gene

We performed genotyping of *DPYS* variants in coding and flanking intronic sequences in the population of 113 cancer patients treated by FPs using the DHPLC-based analysis. We detected nine *DPYS* alterations including four variants located in non-coding sequence [known c.-1T>C, IVS1+34C>G, IVS1-58T>C, and novel IVS4+11G>T], four silent mutations [known c.15G>A (p.S5S) and c.216C>T (p.F72F), and novel c.105C>T (p.L35L) and c.324C>A (p.G108G)], and one novel missense variant c.1441C>T (p.R481 W) in exon 8 (Table 3; Fig. 1). Five *DPYS* variants occurred within single amplicon covering exon 1 and flanking intronic sequences including two highly frequent polymorphisms c.-1T>C and c.216C>T (p.F72F). Eleven different genotypes containing combinations of exon 1 variants were detected in our sample collection resulting in very complex patterns of DHPLC chromatograms. For this reason, the bidirectional sequencing of exon 1 in all patients' samples was implemented. Other fragments covering *DPYS* exons 2–9 were resolved by DHPLC analysis enabling also clear evaluation of exon 2 containing frequent polymorphism IVS1-58T>C (Fig. 2). The frequencies of common *DPYS* variants in general population (69 non-cancer controls) were scored using direct sequencing for exon 1 and by DHPLC analysis for exon 2 (Table 3).

The frequencies of common *DPYS* variants c.-1T>C and IVS1-58T>C slightly differed among all three analyzed groups (Table 3). The minor allele frequency (MAF) of c.-1T>C was 89/134 (0.66) in patients with high toxicity, 52/92 (0.57) in patients without toxicity and 75/138 (54%) in control population ($P = 0.06$). The lowest IVS1-58T>C MAF was found in patients with severe toxicity [63/134 (0.47)] comparing to that in patients without toxicity and controls [55/92 (0.59) and 79/138 (0.57), respectively; $P = 0.04$]. We found significant differences in c.-1T>C frequency of recessive homozygotes compared with heterozygotes and wild-type homozygotes (CC vs. CT + TT) between FP-treated patients with severe toxicity (32/67; 48%) and patients without toxicity (12/46; 26%; OR = 2.59; 95% CI = 1.15–5.85; $P = 0.02$). Frequencies of all other *DPYS* variants did not differ between studied groups. All novel *DPYS* alterations were detected only in patients without toxicity and with excellent long-term tolerance of FP-based chemotherapy. The new and the only missense variant—the transition of c.1441C>T leading to the change of arginine 481 to tryptophan (p.R481W)—was detected in one woman with rectal carcinoma treated by FPs with no signs of any clinically apparent toxicity. In this patient, the c.1441C>T mutation was presented alongside the three others *DPYS* variants—previously described c.-1T>C, the silent mutation c.15G>A (S5S), and the novel intronic variant IVS4+10G>T.

Contribution of individual *DPYS* coding sequence alterations to the development of serious toxicity

The risk of individual high-grade site-specific toxicity was analyzed in carriers of c.-1T>C, IVS1-58T>C and c.216C>T alleles (reaching MAF >0.05) in pooled population consisting of all cancer patients analyzed in the study ($N = 113$). Recessive homozygotes carrying c.-1 CC alleles were in significantly higher risk of serious (grades 3–4) mucositis (OR = 4.13) and overall gastrointestinal toxicity (OR = 3.54) compared with individuals carrying heterozygote c.-1TC or wild-type c.-1TT allelotype and showed also trend to higher frequency of serious diarrhea (OR = 2.12; Fig. 3). The presence of minor alleles of IVS1-58 (i.e. IVS1-58 TC and CC allelotypes) negatively correlated with the risk of serious gastrointestinal toxicity (OR = 0.40) and with borderline significance protected also against grades 3–4 leucopenia (OR = 0.29; Fig. 3).

Our analysis involved samples of 19 patients with high-grade toxicity (17 of them suffered from grades 3–4

Table 3 The frequency of genetic alterations in *DPYS* gene found in populations of patients with and without fluoropyrimidines-mediated toxicity, respectively and in control non-cancer population

Exon/intron	*DPYS* alteration (protein change)	Allelotype	High-toxicity patients *N* (%)	Low-toxicity patients *N* (%)	Control population *N* (%)
e1	c.-1T>C[a] (as 129T>C)[b] (unknown)	Homo	32 (47.8)	12 (26.1)	21 (30.4)
		Het	25 (37.3)	28 (60.9)	33 (47.8)
		WT	10 (14.9)	6 (13.0)	15 (21.7)
e1	c.15G>A[b] (none, S5S)	Homo	1 (1.5)	0	0
		Het	7 (10.4)	3 (6.5)	4 (5.8)
		WT	59 (88.1)	43 (93.5)	65 (94.2)
e1	c.105C>T[c] (none; L35L)	Homo	0	0	0
		Het	0	1 (2.2)	0
		WT	67 (100.0)	45 (97.8)	69 (100.0)
e1	c.216C>T[a,b] (none; F72F)	Homo	0	1 (2.2)	1 (1.4)
		Het	19 (28.4)	13 (28.3)	21 (30.4)
		WT	48 (71.6)	32 (69.6)	47 (68.1)
i1	IVS1 + 34C>G[b] (unknown)	Homo	0	0	n.d.
		Het	0	1 (2.2)	n.d.
		WT	67 (100.0)	45 (97.8)	n.d.
i1	IVS1-58T>C[b] (unknown)	Homo	19 (28.4)	18 (39.1)	20 (29.0)
		Het	25 (37.3)	19 (41.3)	39 (56.5)
		WT	23 (34.3)	9 (19.6)	10 (14.5)
e2	c.324C>A[c] (none; G108G)	Homo	0	0	0
		Het	0	1 (2.2)	0
		WT	67 (100.0)	45 (97.8)	69 (100.0)
i4	IVS4+11G>T[c] (unknown)	Homo	0	0	n.d.
		Het	0	1 (2.2)	n.d.
		WT	67 (100.0)	45 (97.8)	n.d.
e8	c.1441C>T[c] (R481W)	Homo	0	0	n.d.
		Het	0	1 (2.2)	n.d.
		WT	67 (100.0)	45 (97.8)	n.d.

Variants described by [a]van Kuilenburg et al. [14], [b]Thomas et al. [15], [c]novel variants described in this study

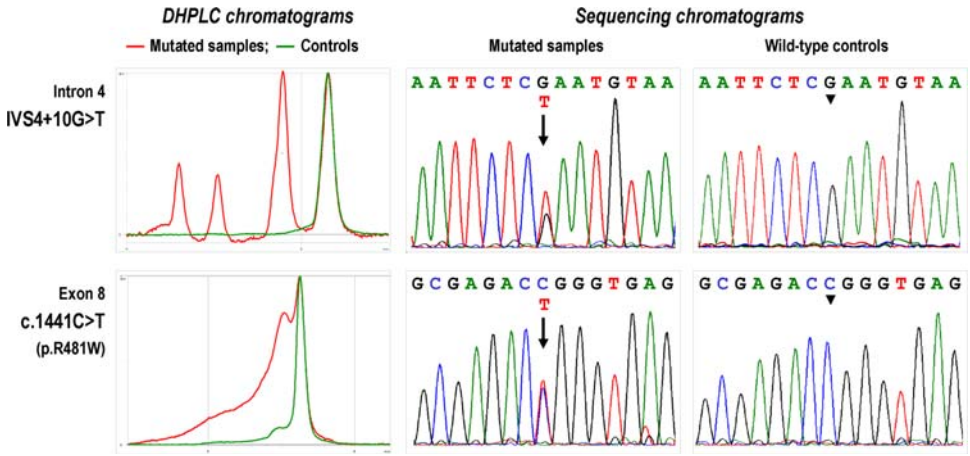

Fig. 1 Novel non-synonymous and intronic *DPYS* sequence alterations described in our study using DHPLC analysis and confirmed by direct sequencing. The elution profiles of DHPLC chromatograms of the altered samples (*red curves*) compared with wild-type samples (*green curves*). Position of *DPYS* sequence variants in sequencing chromatograms is indicated by an *arrow*, the same position in wild-type controls by an *arrowhead*

gastrointestinal toxicity) and 11 patients without toxicity in which we found no alteration affecting *DPYD* coding sequence in our previous study [16]. All these patients carried c.-1T>C and/or IVS1-58T>C alterations in *DPYS* gene; carriers of c.-1 CC alleles were found in 9/19 (47%) of high-grade toxicity patients and in 3/11 (27%) of patients

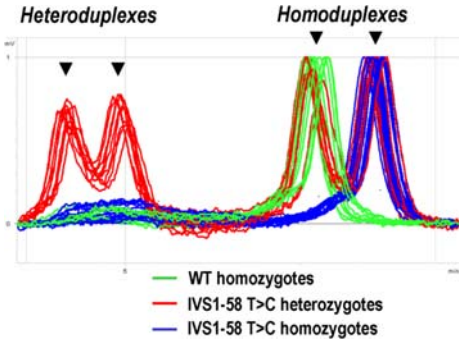

Fig. 2 The result of typical DHPLC experiment shows a good resolution of different genotypes containing IVS1-58T>C in amplicon covering exon 2. The *peaks* representing heteroduplexes in heterozygotes (*red*) and homoduplexes in wild type (*green*) and recessive (*blue*) homozygotes are indicated by the *arrowheads*

without toxicity, whereas the frequency of IVS1-58TT carriers was similar in both the groups [5/19 (26%) and 3/11 (27%), respectively].

Influence of selected haplotypes in the *DPYS* gene on the serious toxicity development

Based on correlations between individual *DPYS* sequence variants and serious site-specific toxicity, we analyzed the impact of compound haplotypes (considering both c.-1 and IVS1-58 alleles) on the development of all analyzed toxicity types and overall gastrointestinal and hematological toxicities (grade 0–2 vs. 3–4) using logistic regression. The analysis included all patients (consisted of the pooled high- and low-toxicity groups). This pooled population was categorized according to the selected haplotypes as follows:

Category 1 "High risk" patients ($N = 26$; 23%)—carrying allelotypes independently increasing the risk of FPs toxicity—c.-1 CC together with IVS1-58 TT alleles.

Category 2 "Lower risk" patients ($N = 63$; 56%)—carrying allelotypes independently reducing the risk of FPs toxicity—c.-1 (TT or TC) together with IVS1-58 (CT or CC) alleles.

Category 3 "Unsorted" patients ($N = 24$; 21%)—patients not included in category 1 or 2.

We identified a strong correlation between analyzed haplotypes and risk of serious overall gastrointestinal toxicity and mucositis. The overall gastrointestinal toxicity grades 3–4 occurred in 19/26 (73%) individuals of "high risk" group compared with 37/87 (43%) others ("lower risk" + "unsorted" patients; OR = 3.67; 95% CI = 1.40–9.63; $P = 0.008$) and in 24/63 (38%) "lower risk" patients compared with 32/50 (64%) others ("high risk" + "unsorted" patients; OR = 0.35; 95% CI = 0.16–0.75; $P = 0.007$). Severe mucositis (grades 3–4) was present in 8/26 (31%) individuals of "high risk" group compared to 13/87 (15%) others ("lower risk" + "unsorted" patients; OR = 2.53; 95% CI = 0.91–7.02; $P = 0.08$) and in 7/63 (11%) "lower risk" patients compared with 14/50 (28%) others ("high risk" + "unsorted" patients; OR = 0.32; 95% CI = 0.12–0.87; $P = 0.03$). A statistically insignificant trend was shown for leucopenia affecting 5/26 (19%) individuals from "high risk" patients' category compared with 6/87 (7%) others ("lower risk" + "unsorted" patients; OR = 3.22; 95% CI = 0.89–11.56; $P = 0.07$), whereas no correlation was apparent for any other types of analyzed toxicities.

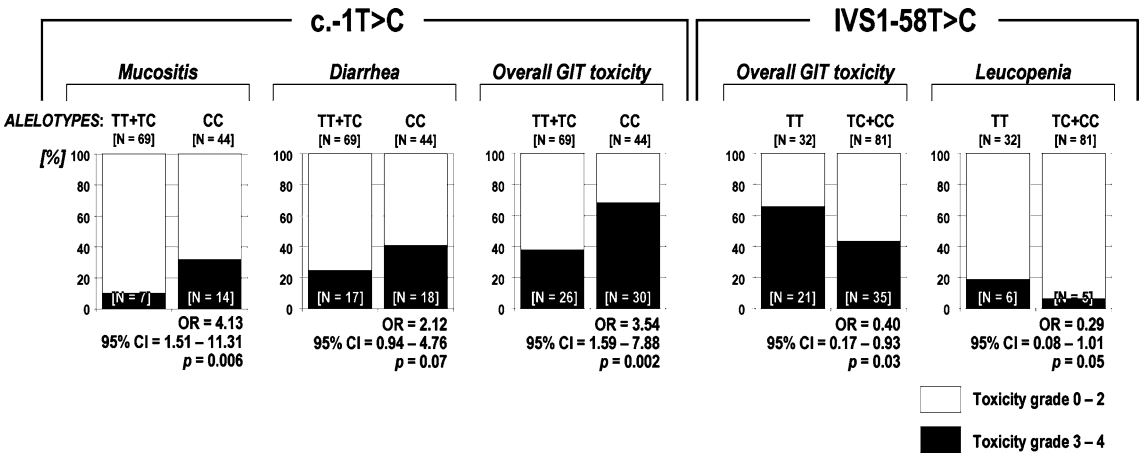

Fig. 3 Influence of c.-1T>C and IVS1-58T>C alterations on serious toxicity development in fluoropyrimidine-treated patients. The percentages of all fluoropyrimidine-treated patients with grades 0–2 toxicity (*white bar*) and grades 3–4 toxicity (*black bar* with a number indicating the count of individuals suffering from grades 3 to 4 toxicity) separated according to the presence of each alteration. *GIT* overall gastrointestinal toxicity, *OR* odds ratio, *CI* confidence interval

Discussion

Dihydropyrimidinase (DPYS, EC 3.5.2.2), the second enzyme in the (5-fluoro)uracil and thymine degradation pathway, naturally catalyzes the degradation of both dihydrouracil and dihydrothymine to *N*-carbamoyl-*β*-alanine and *N*-carbamoyl-*β*-aminoisobutyric acid, respectively. The fluorinated analogue of natural substrate of DPYS enzyme is the initial catabolite of 5-FU–fluoro-5,6-dihydrouracil (FUH_2). It has been proposed that similar to 5-FU also FUH_2 exerts cytotoxic effects suggesting that FUH_2 may contribute to 5-FU toxic profile [19].

The *DPYS* gene consisting of 10 exons comprising the region of over 80 kbp at 8q22 was characterized more than 10 years ago [20, 21]. The first nine exons code for 1,560 bp open reading frame translated to a protein composed of 519 amino acid residues [21]. The crystal structure of eukaryotic enzyme has been described recently [22]. The DPYS protein naturally occurs in the form of homotetrameric Zn^{2+}-containing metalloenzyme highly expressed in liver and kidney, but enzyme activity was found also in other tissues [23, 24].

DPYS deficiency (OMIM 222748) is a rare inborn metabolic syndrome characterized by dihydropyrimidinuria and variable clinical phenotype including epileptic attacks, febrile status, dysmorphic features, congenital microvillous atrophy and developmental retardation [21]. Sumi et al. [25] performed analysis of urine samples from 21,200 healthy Japanese infants and revealed two asymptomatic children with dihydropyrimidinuria estimating the incidence of DPYS deficiency to 1/10,000. Analysis of the affected family indicated that dihydropyrimidinuria is an autosomal recessive disorder. In the study of Hayashi et al. [26], only one man demonstrated significant dihydropyrimidinuria was found between 1,133 adults examined. The study of Hamajima et al. [21] described one frame shift and five missense *DPYS* alterations in six patients with dihydropyrimidinuria. The alterations c.1001A>G (p.Q334R) found in homozygous or heterozygous form in four asymptomatic Japanese patients and c.1078T>C (p.W360R) found in homozygous form in one female from Lebanon with clinical symptoms of DPYS deficiency were not found in 100 Japanese and 200 Caucasian control samples, respectively.

With respect to the 5-FU toxicity, the mutation analysis of *DPYS* has been studied only marginally. The case report published by van Kuilenburg et al. [14] described single female breast cancer patient, who experienced life-threatening toxicity after 5-FU administration. This female showed normal DPD, but strongly reduced DPYS enzymatic activity measured in peripheral blood mononuclear cells and the subsequent mutation analysis revealed the heterozygous missense variant c.833G>A (p.G278D) in exon five not present in any of 96 Dutch Caucasians control individuals. In recent study, Thomas et al. [15] performed genotyping of *DPYS* in 219 healthy asymptomatic volunteers of Caucasian and African American origin with phenotypical assessment of DPD enzyme activity and overall uracil catabolic pathway capacity using HPLC-based DPD enzyme radioassay and [2-^{13}C] uracil breath test, respectively. They found 18 different *DPYS* variants; all frequently occurring (with MAF >0.05) [c.-1T>C (denoted as 129T>C), c.15G>A (denoted as 144G>A), c.216C>T (denoted as 45C>T), IVS1+34C>G, and IVS1-58T>C] were identified also in our study, whereas the others probably represented rare *DPYS* variants (with MAF <0.012) that were not detected in our study populations.[1] Four novel and rare *DPYS* alterations described in our study probably do not alter DPYS enzymatic activity as all of them were found only once in patients with excellent tolerance of FP-based therapy. The novel missense variant c.1441 C>T (p.R481W) is localized within non-conservative helix α16 of the C-terminal tail of DPYS protein [22]. The results of our study involving patients with low and high tolerance of FP-based chemotherapy (together with the results of above-mentioned study of Thomas et al.) indicate that the majority of *DPYS* gene alterations is represented by frequent polymorphisms (e.g. c.-1T > C; IVS1-58T > C), whereas others, including missense variants, are rare *DPYS* abnormalities that can explain alteration of FP catabolism and hence the development of toxicity in very limited population of cancer patients.

It has been recently shown that not only pathogenic mutations but also the occurrence of polymorphisms in *DPYD* (the most studied gene introducing FP catabolic pathway) could modify the risk of FP-related toxicity. The study of Gross et al. [27] described that occurrence of c.496A>G (p.M166V, that is considered to be a frequent non-synonymous polymorphism in *DPYD* gene) strongly correlates with FP-related overall high-grade toxicity. We reported recently the association of site-related toxicity with several haplotypes involving *DPYD* sequence; however, in contrast to the Gross et al. study [27], we found the negative correlation between carriage of M166V and gastrointestinal high-grade toxicity [16]. This discrepancy emerge several limitations of these studies including small sample sizes, different cancer diagnoses or possibility of distinct pathogenetic mechanisms leading to the development of different organ-specific toxicities. The clinical interpretation of these results must be made with caution,

[1] The IVS2-62G > T variant (with MAF = 0.42 and 0.25 for overall and Caucasian subpopulation, respectively in Thomas et al. study) was not detected in our population being localized outside DNA sequence amplified in amplicon covering exon 3 and flanking intronic sequences in our analysis.

until confirmatory data from large-scale population analyses become available.

In this study, using logistic regression analysis, we found the correlation between frequent *DPYS* variants and development of serious mainly gastrointestinal toxicity when c.-1CC-recessive homozygotes and IVS1-58TT-dominant homozygotes were at a increased risk of this toxicity. These results were confirmed by haplotype analysis involving both loci showing an increased risk of gastrointestinal toxicity for "high risk" patients carrying both c.-1CC and IVS1-58TT allelotypes. Possible negative effect of c-1C>T alteration on FP catabolism indirectly may also support the results of the study of Thomas et al. [15] in which carriers of c.-1T>C alteration represent 11/11 (100%) of individuals with discordant result of DPD activity and uracil breath test (normal DPD activity and deficient uracil breath test that were considered to defective in catabolic pathway downstream to DPD) but account for 150/194 (77%) individuals from group with physiological results of both phenotypical tests.

We can only speculate about the molecular effects of statistically significant *DPYS* polymorphisms. The c.-1C>T variant is localized within the Kozak consensus sequence (GCCRCCaugG) representing the critical regulatory element of translation initiation [28]. Alteration of nucleotide in −1 position has been reported to influence the expression of several other protein (e.g. annexin 5 [29, 30], CD40 [31], farnesoid X receptor [32]), however, in case of particular Kozak sequence (CTCGCTatgG) in *DPYS* gene, only low effect could be expected for T–C transition in position −1 considering the Kozak context rules [33]. The IVS1-58T>C alteration shown to have no effect on binding of analyzed splicing factors and splice/branch sites using ESEfinder 3.0 prediction tool [34]. Finally, it should be also considered that both significant *DPYS* polymorphisms are common in population and, hence, they unlikely could produce a strong phenotypical effect of (fluoro)pyrimidine catabolism. We suppose that, together with alteration(s) incident in other genes, they may contribute to the development of FP toxicity as the multivariate polygenic pharmacogenomic syndrome in which they may influence particularly the development of gastrointestinal-specific toxicity.

Conclusions

Data presented in our study demonstrate that non-synonymous alterations of *DPYS* gene in patients with serious FP-related toxicity are rare event, however, the allelotypes of untranslated sequence variants c.-1T>C and IVS1-58T>C could modify the risk of serious (grades 3–4) gastrointestinal toxicity and, to lesser extent, the risk of leucopenia development in patients treated by FPs. Despite that these results need to be confirmed in the future studies involving unselected population of FP-treated patients, we assume that haplotype analysis of genes coding for enzymes of (fluoro)pyrimidine catabolic pathway, including the *DPYS*, may represent an easy approach for the estimation of site-related toxicity risk in cancer patients treated by FPs.

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