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SHORT COMMUNICATION

Severe fluoropyrimidine-related toxicity: clinical implications of *DPYD* analysis and UH2/U ratio evaluation

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Abstract The fluoropyrimidines are commonly used in chemotherapeutic cancer medicine, but many patients still experience severe adverse side effects from these drugs. We observed a severe toxicity in a 50-year-old woman treated with capecitabine and docetaxel for a metastatic breast cancer. Since dihydropyrimidine dehydrogenase (DPD) is the main candidate for pharmacogenetic studies on 5-FU toxicity, the entire coding sequence and exon-flanking intronic regions of the *DPYD* gene were sequenced in the patient. None of the previously described deleterious variants were detected. Also, the haplotype-based analysis failed to reveal *DPYD* variations associated with 5-FU toxicity. We also evaluated the UH2/U ratio in plasma as an index of 5-FU pharmacokinetics. The UH2/U value did not demonstrate low DPD activity in the patient. We discuss the advantages and limitations of this approach, particularly concerning the clinical applications of 5-FU pharmacogenetics in the family setting.

Keywords 5-FU · Capecitabine · *DPYD* · UH2/U ratio

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Introduction

The fluoropyrimidines, such as 5-fluorouracil (5-FU) and its pro-drug capecitabine, are widely used antineoplastic drugs. Capecitabine was introduced in breast and colorectal cancer chemotherapy to reduce the fluoropyrimidine-induced toxicity, as its activation through a multistep process generates 5-FU preferentially in tumour cells. Nevertheless, 31–34% of patients treated suffer from dose-limiting toxicities with 0.5% up to 4% of the patients experiencing lethal toxicity [1, 2]. The challenge in fluoropyrimidine-based therapy remains to identify cancer patient at risk of toxicity.

The rate-controlling enzyme of endogenous pyrimidine and fluoropyrimidine catabolism is dihydropyrimidine dehydrogenase (DPD). DPD deficiency accounts for a substantial part of the 5-FU-induced toxicity [1] and was demonstrated in patients with lethal toxicity after administration of the pro-drug capecitabine [3, 4]. Several methods for the evaluation of DPD activity were developed [5, 6]. DPD activity measured in peripheral mononuclear cells (PBMC) has been considered as the reference technique for the functional evaluation of the enzymatic activity. However, it is difficult to implement in routine clinical protocols, and its precision largely depends on the isolated PBMC sub-fraction [7]. To predict adverse events caused by 5-FU, some authors suggested approaches based on pharmacokinetics parameters, such as the 5-FU test dose and the dihydrouracil/uracil ratio (UH2/U) in plasma [5]. The values of

plasma UH2/U were found to be highly correlated with those of 5-FU half-life and treatment toxicity. Therefore, the UH2/U values might be considered as an indirect evaluation of the DPD enzyme activity [8–10].

The genetic approach to DPD-associated toxicity was considered promising, as deleterious variants were found in the DPD coding gene, named dihydropyrimidine dehydrogenase (*DPYD*, chromosome 1p22). Three *DPYD* variants (IVS14 + 1G>A, c.2846A>T and c.1679T>G) were associated with fluoropyrimidine-induced toxicity. Subsequent analysis on large cohorts of patients suffering from 5-FU toxicity and control individuals demonstrated that *DPYD* is highly polymorphic [1, 11]. To date, the impact of common polymorphisms was not definitely assessed. The *DYPD* gene was also investigated at the haplotype level. According to the linkage disequilibrium (LD) structure estimated in the *DPYD* locus, the gene was partitioned into six haplotype blocks (A–F) identified through 36 tagging single-nucleotide polymorphisms (SNPs). Two haplotypes of block B were found significantly over-represented in patients with severe side effects [12].

A two-step approach based both on a pharmacokinetic-based test and on genetic analysis was recently suggested. It was shown that the best results can be obtained with, firstly, SNP detection combined to uracil plasma measurement then, secondly, UH2/U determination to confirm a DPD deficiency and/or to more precisely determine the residual level of activity [9]. However, there is as yet no consensus on the pharmacokinetics test and on the genetic analysis that should be performed prior to 5-FU treatment [5, 9].

We report on a patient with severe toxicities after capecitabine administration, who was investigated in order to elucidate the possible cause of the clinical phenotype by the means of a deep genetic analysis. After mutation analysis, we used the UH2/U ratio in plasma as an index of 5-FU pharmacokinetics. The predictive role of these investigations in the clinical setting is discussed.

Clinical summary

A 49-year-old woman of Italian ancestry, with family history of breast and colorectal cancers and without relevant comorbidities, underwent neoadjuvant chemotherapy for locally advanced breast cancer (infiltrating ductal carcinoma) in May 2006. Treatment consisting of epirubicin and docetaxel (both drugs 60 mg/m^2, one administration every 21 days) was administered for four cycles of 21 days without toxicity, with complete clinical and radiological response. In September 2006, the patient underwent lumpectomy and axillary dissection. Due to cancer dissemination in dissected lymph nodes, it was not possible to ascertain any immunohistochemistry feature, so she received two further courses of chemotherapy followed by adjuvant locoregional radiotherapy and 20 mg/day tamoxifen. In February 2008, augmented serum tumour markers level (CEA and CA15.3) was found. Computed tomography (CT) showed liver and bone metastases. Fine-needle hepatic biopsy confirmed metastatic breast ductal carcinoma, ER and PGR negative, Her2 3+.

First-line combination chemotherapy was 21 days cycles with docetaxel 70 mg/m^2 (one intravenous infusion every 21 days, on day 1 of the cycle) and oral capecitabine 2,000 mg/m^2 (daily on day 1–14 followed by a 1 week break). On day 9 of the first cycle, the patient developed neutropenic fever (absolute neutrophil count below 500), nausea and vomiting, grade 2 diarrhoea and mucositis. She was immediately hospitalised and begun on supportive therapy with subcutaneous daily filgrastim, parenteral antibiotics and nutritional support. The duration of grade 4 neutropenia was 4 days. The complete resolution of symptoms occurred after 20 days.

In May 2008, combination therapy with low doses weekly paclitaxel (40 mg/m^2 day 1,8,15 q28) and trastuzumab (4 mg/kg the first time, then 2 mg/kg weekly) was started; paclitaxel was interrupted after the first cycle because of persistent grade 2 asymptomatic neutropenia. Treatment went on with only trastuzumab 6 mg/kg d1 q21 without toxicity for 14 months. CT showed complete liver response and bone stable disease. In March 2010, the patient developed multiple brain metastases, underwent palliative whole brain radiotherapy without clinical response and benefit, and died on June 2010.

Methods

Sample collection

After informed consent, blood sample was collected from the patient on May 2010. She was not under chemotherapy containing 5-FU. The blood sample in heparinized tube was drawn at 9:30 a.m. The control group included blood donors, free from pharmacologic therapy. Blood samples were collected in heparinized tubes, between 8:30 and 10 a.m. Plasma specimens were isolated readily after the collection and stored at −80°C until HPLC assay.

DPYD gene analysis

Genomic DNA was extracted from EDTA blood sample using standard phenol–chloroform method. The entire coding region of *DPYD* gene was amplified with 23 primer pairs corresponding to the 23 gene exons, the exon–intron boundaries and the non-coding regions including variants already described in literature (Table 1). All the *DPYD*

Table 1 Patient genotype for the *DPYD* variants included in the study

Exon/ Intron	Nucleotide change	Aminoacid change	Nomenclature alias	RefSNP name	Patient genotype	References
2	c.61C>T	R21X		rs72549310	C/C	[1]
2	c.62C>A	R21Q	DPYD*12	rs72549310	C/C	[1]
2	c.74A>G	H25R			A/A	[18]
2	c.85T>C	C29R	DPYD*9A	rs1801265	T/T	[1, 7, 13]
3	c.187A>G	K63E			A/A	[19]
4	c.295-298delTCAT		DPYD*7		ins/ins	[7]
5	c.483+14A>G				A/A	[13]
5	c.483+18G>A			rs56276561	G/G	[20]
5	c.484-8C>T				C/C	[13]
6	c.496A>G	M166V		rs2297595	A/A	[1, 13]
6	c.545T>A	M182K			T/T	[1]
7	c.703C>T	R235W	DPYD*8	rs1801266	C/C	[7]
8	c.775A>G	K259E		rs45589337	A/A	[21]
8	c.818delT			rs34656878	T/T	[18]
8	c.850+113C>T				C/C	[13]
9	c.958+36A>G				A/A	[13]
8	c.775A>G	K259E		rs45589337	A/A	[21]
9	**c.958+118G>A**				**G/A**	
9	c.959-51T>G				T/T	[13, 20]
10	c.1003G>T	V335L	DPYD*11	rs72549306	G/G	[1]
10	c.1070G>A	R357H			G/G	[19]
10	c.1109-1110delTA				ins/ins	[20]
10	c.1129-15T>C			rs56293913	T/T	[13, 20, 21]
10	c.1129-28G>A				C/C	[13]
11	c.1156G>T	E386X	DPYD*12a		G/G	[1]
11	c.1218G>A	M406I		rs61622928	G/G	[13]
11	c.1236G>A	E412E		rs56038477	G/G	[13]
11	c.1340-119A>G				A/A	[13]
11	c.1340-106T>A			rs61789183	T/T	[13, 20, 21]
13	c.1601G>A	S534N	DPYD*4	rs1801158	G/G	[1, 13]
13	**c.1627A>G**	**I543V**	DPYD*5/DPYD*2Bc	rs1801159	**A/G**	[1, 13]
13	c.1679T>G	I560S	DPYD*13	rs55886062	T/T	[1]
13	c.1714C>G	L572V			C/C	[18]
13	**c.1740+39C>T**			rs2786783	**C/T**	[20, 21]
13	**c.1740+40A>G**			rs2811178	**A/G**	[20, 21]
14	c.1896T>C	F632F		rs17376848	T/T	[13]
14	c.1897delC		DPYD*3	rs72549303	ins/ins	[7]
14	c.1905+1G>A		DPYD*2a	rs3918290	G/G	[1, 7, 21]
14	**c.1906+123C>A**			rs56279429	**C/A**	[12]
15	c.1974+16G>A				G/G	[13]
15	**c.1974+75A>G**			rs72728438	**A/G**	[13, 20, 21]
16	**c.2058+101T>C**			rs1890138	**T/C**	[12]
16	**c.2058+145T>C**			rs1879374	**T/C**	[12]
18	c.2194G>A	V732I	DPYD*6	rs1801160	G/G	[1, 13]
18	c.2300-39G>A			rs12137711	G/G	[13, 20, 21]
19	c.2329G>T	A777S			G/G	[1]
21	c.2657G>A	R886H	DPYD*9Ba	rs1801267	G/G	[7]

Table 1 continued

Exon/ Intron	Nucleotide change	Aminoacid change	Nomenclature alias	RefSNP name	Patient genotype	References
22	c.2846A>T	D949V		rs67376798	A/A	[1, 13]
22	c.2907+55T>C			rs290854	T/T	
22	c.2908-69G>A			rs290855	G/G	[12]
23	c.2921A>T	D974V		rs72547602	A/A	[13]
23	c.2983G>T	V995F	DPYD*10	rs1801268	G/G	[7]
23	c.3067C>T	P1023S			C/C	[18]

All the variants spanning the coding region and previously described were summarised. Heterozygous variants identified in the patient are in bold

exons, except exon 1, were amplified with a standard PCR protocol using Platinum Taq DNA Polymerase (Invitrogen, CA, USA) at an annealing temperature of 58°C. *DPYD* exon 1 was amplified with GC-RICH PCR System (Roche Molecular Diagnostics, Meylan, France) at an annealing temperature of 60°C. Primer sequences and detailed procedures are available on request. Direct sequencing was performed on an ABI-3730 platform (Applied Biosystems, Foster City, CA, USA) according to standard procedures. Mutation analysis of the 23 amplicons was performed using the SeqScape software.

The tagging SNPs described by Amstutz et al. [12] were examined using the sequencing data obtained from mutation analysis of the *DPYD* gene.

The amplicons designed for the analysis contained 31 out of 36 tagging SNPs reported by Almstulz et al. [12] (see Fig. 1). All *DPYD* polymorphisms reported in relevant literature were also examined (Table 1). To assess the patient haplotype for block C (according to Almstuz et al. [12]), the amplicon 13 (see Table 1) was cloned by using the TOPO TA Cloning KIT (Invitrogen, Carlsbad, CA, USA) and examined by direct sequencing.

Plasma UH2/U ratio measurement

The UH2/U plasmatic ratio in patient and controls was obtained by adapting the HPLC method already described [10]. The calibration curves of peak areas versus concentrations of U and UH2 were linear giving a correlation coefficient (r^2) of 0.9997 and 0.9977, respectively. The patient UH2/U ratio was compared with the value distribution in 20 normal controls. The values in controls showed a normal distribution at the Kolmogorov–Smirnov test with the Lilliefors correction of the significance.

Results

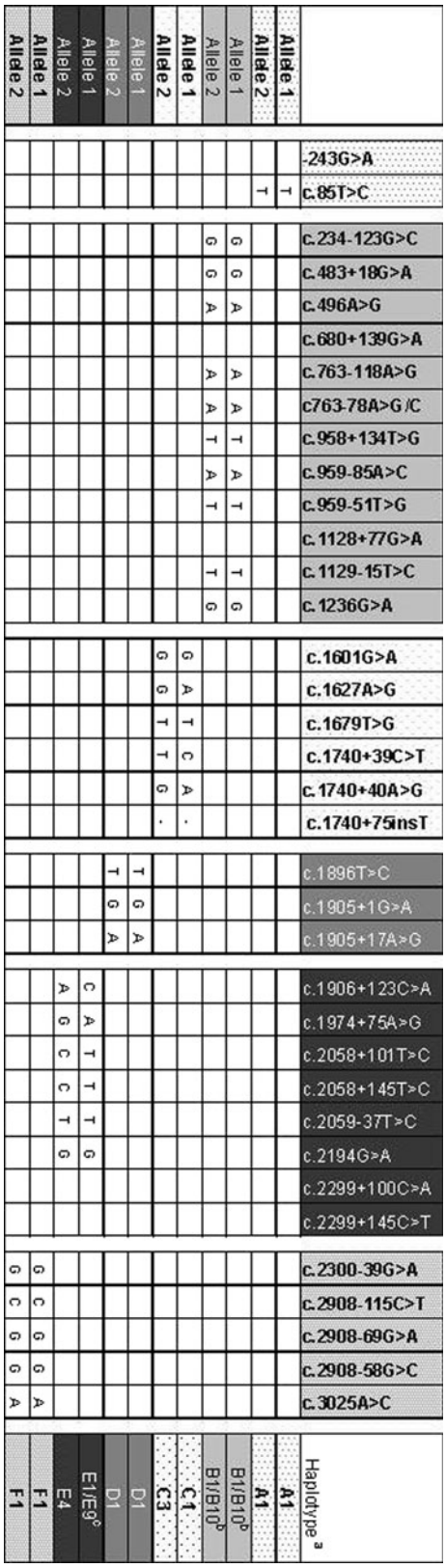
Fifty-two genomic variants spanning the *DPYD* gene and known as either mutations or polymorphisms were examined. One additional single-nucleotide change (c.958+118G>A) not reported in literature and in relevant databases was detected. Eight heterozygous and no homozygous variants were found. Direct sequencing did not identify deleterious variants (i.e. c.1905+1G>A, c.2846A>T and c.1679T>G). One heterozygous non-synonymous nucleotide change was found in exon 13 (c.1627A>G, p.I543 V, also named DPYD*5). Table 1 reports the patient genotype for each polymorphic site.

The patient haplotypes were inferred comparing the genotype of tagging SNPs with the haplotype definitions given in Amstutz et al. [12]. The haplotypes could be unambiguously inferred for blocks A, C, D and F (see Fig. 1). For block B and E, as two tagging SNPs were not genotyped, the possible haplotypes were reported. The latter was inferred based on the expected frequency. To determine unambiguously the allelic phase previously inferred for block C, the amplicon 13, corresponding to *DPYD* exon 13, was cloned and sequenced in both chromosomes. The patient carried the C1 and C3 haplotypes, thus confirming the previously inferred ones (Fig. 1).

The UH2/U ratio assessed in the patient was 4.31. Though the observed value was lower than the mean recorded in normal controls, it did not clearly deviate from the reference distribution (mean 5.26, SD 2.08; median 4.58; range: 2.48–5.26).

Discussion

We described the clinical course of a patient with breast cancer who suffered from severe toxicity after administration of capecitabine. Given the patient's positive family history, and the higher risk of occurrence of cancer in relatives, our investigation was initially aimed at assessing whether a specific *DPYD* genotype was responsible for the severe adverse reaction. We assumed that genetic analysis could be of value in the family context, as the identification of a clear pathogenic anomaly in the proband can lead to offer predictive testing to relatives at high risk of toxicity in case of



◀ **Fig. 1** Patient haplotypes pertaining to the *DPYD* gene observed in the present case (the haplotype profile was drawn according to Amstutz et al. [12]). [a]Haplotype classification according to Amstutz et al. [12]. [b]For block B, the patient could carry both the B1 and the B10 haplotypes. [c]For block E, it could be inferred that the patient carries not only the E1 and E4 haplotypes, but also the rare E9 could be inferred

fluoropyrimidine-based therapy. In addition, we estimated the UH2/U ratio, as it was suggested that the combination of *DPYD* mutation search and a pharmacokinetic-based test give better results, in terms of accuracy and speed, as compared to either single analysis [9].

In the patient described herein, *DPYD* mutation analysis did not identify deleterious variants. One variant allele in rs1801159, a non-synonymous polymorphism (I543V), was found (Table 1). The I543V variant had been described as a common polymorphism (minor allele frequency 28%) not associated with low DPD activity [1]. Seck and coworkers [13] subsequently examined DPD activity and *DPYD* mRNA expression in a large cohort of Caucasian healthy subjects, concluding that I543V does not affect DPD function. Nine heterozygous allelic variants were detected in intronic regions (Table 1). One (c.958+118G>A) was not reported in public databases (last access March 21, 2011) and in relevant literature. This variant is not located in proximity of the exon–intron junction and is not likely to affect splicing. However, a possible regulatory effect cannot be formally excluded, as functional studies were beyond the purpose of this study.

The *DPYD* gene spans over 840 Kb and six major haplotype blocks. As direct sequencing of the entire genomic region is currently unavailable at affordable costs, haplotype analysis could be an effective strategy to capture a substantial part of the genetic variations in the *DPYD* locus [12]. By using a rather laborious approach, we obtained the *DPYD* haplotype structure in the patient, but the comprehensive genetic analysis failed to demonstrate any variation unambiguously related to DPD deficiency. The patient, namely, did not present the two haplotypes (B3 and B6) found significantly over-represented in patients with severe toxic side effects [12]. B3 haplotype is tagged by the variants IVS5+18G>A, IVS9-51T>G and c.1236G>A; B6 haplotype is tagged by variant IVS3-123G>C; for all these variants, the patient was found to carry the homozygous wild-type allele (Table 1). We are aware, however, that rare haplotypes possibly associated with DPD deficiency cannot be detected using the tagging SNPs approach.

The genetic analysis performed in the present case, though comprehensive, is far from being exhaustive. Other *DPYD* anomalies possibly associated with gene expression (for example promoter and enhancers) might be located in non-coding regions that were not examined. Moreover, the unexplained 5FU-induced toxicity may be associated with deleterious variants located in other enzymes, such as thymidylate synthase, 5,10-methylenetetrahydrofolate

reductase and dihydropyrimidinase, involved in fluoropyrimidine anabolism and catabolism [4, 14]. In addition, the capecitabine to 5-FU metabolic pathway could play a role in capecitabine-induced toxicities. Mercier and coworkers demonstrated increased activity of cytidine deaminase (CDA) in a patient with severe capecitabine-associated toxicity, thus suggesting that CDA extensive metaboliser phenotype could exert the adverse effects through an increased activation to 5-FU [15]. Other enzymes involved in the same pathway, such as carboxyl esterase and thymidine phosphorylase, might be taken into account to partly explain the capecitabine-induced toxicities.

Recent literature regarding prediction of 5-FU toxicity addressed the validity of a two-step approach, based both on a pharmacokinetic-based test and on genetic analysis, which could be implemented to identify patients at risk, in whom an alternative therapy should be recommended [5, 9]. In the present work, the plasma UH2/U ratio was assessed through a rather simple method affordable in a clinical setting, provided that a pharmacology unit performing ready plasma isolation is available. The UH2/U ratio found in the patient did not clearly deviate from the reference distribution, though it was below the median value. However, this measurement should be considered a proxy of the actual 5-FU plasma clearance and has several limitations. Single measurements could reflect transitory fluctuations of uracil and dihydrouracil concentrations in plasma. Moreover, the procedure requires an accurate standardisation, as median values and distribution shapes reported in literature considerably vary [6, 10, 16]. Two ranges of UH2/U ratio, associated with toxicity and borderline toxicity, respectively, were proposed [10, 17], but the threshold values were not definitely assessed. As an additional limitation, in our study the control curve was obtained using plasma collected from blood donors, with unknown phenotype as regards to 5-FU toxicity.

Due to the limitations outlined above, the results from this experimental workflow should be taken cautiously. However, our findings underscored that a patient not considered at risk after this two-step approach could develop, in fact, a severe adverse reaction to 5-FU. Though this is not astonishing at a population level, has important implications from a clinical standpoint. The assumption that multiple genetic variants in different loci influence the phenotype has important implications in the family setting. The likelihood of carrying the same alleles at multiple loci exponentially decreases as the number of loci increases. As a result, even in first-degree relatives the genetic risk is in fact unpredictable for clinical purposes, unless the allelic variants at all involved loci are taken into account. Therefore, to interpret a complex phenotype such as the fluoropyrimidine-induced toxicity, a holistic approach entailing the evaluation of multiple risk alleles should be implemented and validated in clinical studies.

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Conflict of interest None.

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