Original Article

DIHYDROPYRIMIDINE DEHYDROGENASE DEFICIENCY (DPD) IN GI MALIGNANCIES: EXPERIENCE OF 4-YEARS

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ABSTRACT

Objectives: 5-Fluorouracil (5-FU) is an integral part of treatment of GI malignancies. While normal DPD enzyme activity is rate limiting in 5-FU catabolism, its deficiency could increase concentrations of bioavailable 5-FU anabolic products leading to 5-FU related toxicity syndrome.

Methodology: Twenty-three patients were tested for DPD deficiency after excessive toxicities from 5-FU and/or capecitabine. DPD activity was evaluated by Peripheral Blood Mononuclear Cell (PBMC) radioassay, genotyping of *DPYD* gene by Denaturing High Performance Liquid Chromatography (DHPLC), or 2-^{13}C uracil breath test (UraBT).

Results: Of 23 patients with excessive toxicities from 5-FU and/or capecitabine, 7 (30%) were DPD deficient with a median age of 66 years, M:F ratio = 1.3:1 and ethnicities included Caucasian (71%), African-American (14%) and South-Asian (14%). DPD activity ranged from 0.064 – 0.18nmol/min/mg. Three patients were treated with bolus 5-FU/LV, two with capecitabine, and two with high dose bolus 5-FU with 2', 3', 5'-tri-*O*-acetyluridine. Toxicities included mucositis (71%), diarrhea (43%), skin rash (43%), memory loss/altered mental status (43%), cytopenias (43%), nausea (29%), hypotension (14%), respiratory distress (14%) and acute renal failure (14%) Re-challenge with capecitabine in one patient after the Mayo regimen caused grade 3 hand-foot syndrome. Genotypic analysis of the *DPYD* gene in one patient with severe leucopenia demonstrated a heterozygous mutation (IVS14+1 G>A, DPYD). The UraBT in two patients revealed 1 to be DPD-deficient (DOB_{50} of 112.8; PDR of 49.4%) and borderline normal values (DOB_{50} of 130.9; PDR of 52.5%) in a second patient. There were 2 toxicity-related deaths among DPD-deficient patients (28%).

Conclusions: DPD deficiency was observed in several ethnicities. Akin to 5-FU, capecitabine can also lead to severe toxicities in DPD-deficient patients. Screening patients for DPD deficiency prior to administration of 5-FU or capecitabine using UraBT could potentially lower risk of toxicity. Future studies should validate this technique.

KEY WORDS: 5-Fluorouracil, Fluoropyrimidines, Capecitabine, Uridine, DPD, DPYD gene, Neutropenia, HFS, Mucositis.

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INTRODUCTION

5-Flurouracil (5-FU) and its derivatives (e.g. capecitabine) are widely prescribed in the management of gastrointestinal cancers. Despite widespread use, approximately 31-34% of cancer patients develop severe 5-FU related toxicities.[1] DPD is the rate-limiting enzyme responsible for 80-85% of 5-fluorouracil (5-FU) catabolism while 5-20% of the 5-FU is being excreted virtually intact in the urine.[2-4] 1-3% of 5-FU enters the DNA and RNA anabolic

pathways (Fig-1) and is responsible for its antitumor activity and systemic toxic effects. Therefore, even at a minute 5-FU dose, patients with severe DPD enzyme deficiency could experience a marked surge in plasma 5-FU concentrations (C_{max}), which will in turn lead to increased 5-FU anabolism in susceptible tissues e.g. GI tract, hair, and bone marrow.[1-3,5] The DPD deficiency consist of exaggerated 5-FU toxicities including mucositis, hair loss, diarrhea, neutropenia, skin rash and neurologic toxicities.[3,5-7] The mortality rate is almost 100% in those patients with complete DPD deficiency when exposed to 5-FU as best exemplified by this fatal case to topical 5-FU. Fortunately, complete DPD deficiency is extremely rare in the general population whereas 3-5% of cancer patients are considered partially DPD deficient as defined by an enzyme activity that is less than the lower limit of the 95% distribution range.[6,7] However, up to 43-59% of cancer patients who experienced severe to life-threatening toxicities to 5FU were considered partially or borderline DPD deficient.[8,9] DPD deficiency is a familial syndrome as a result of the allelic mutations within the DPYD gene.[10] Although DPD enzyme activity can be assayed from peripheral blood mononuclear cells in a specialized laboratory, routine phenotypic and genotypic screenings for DPD deficiency prior 5-FU therapy are not yet available.[4] In contrast to the incidence of approximately 3-5% in the Caucasian population, we recently reported that the incidence of DPD deficiency is slightly higher in the African American population (8%).[12] Data regarding toxicity of capecitabine, an oral fluoropyrimidine in DPD deficient patients are scarce. We recently reported severe toxicities from capecitabine in two patients with DPD deficiency.[13]

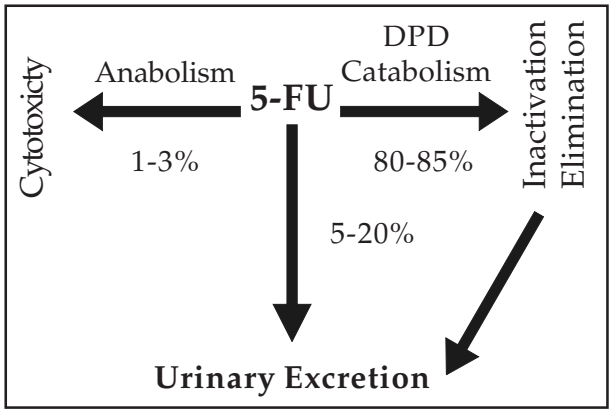

Figure-1: Metabolism of 5-FU and role of DPD as the rate-limiting enzyme.

80-85% of 5-FU is catabolized to inactive metabolites by DPD and only 1-3% of the 5-FU mediated cytotoxic effects on tumor cells and normal tissue through anabolic actions. Increased anabolism of 5-FU in patients with DPD deficiency is responsible for wide spectrum of life-threatening 5-FU toxic effects.

Therefore, we collected data on the patients who were evaluated for DPD deficiency upon development of severe toxicity secondary to 5-FU or capecitabine and present in this paper. Their clinical manifestations, results of DPD expression by different methods and important aspects of these cases are discussed.

METHODOLOGY

Subjects: Patients were tested for DPD deficiency after excessive toxicities from 5-FU and/or capecitabine at University of Alabama at Birmingham (UAB) between 2001 and 2005. DPD activity was evaluated by PBMC radioassay, genotyping of *DPYD* gene by Denaturing High Performance Liquid Chromatography (DHPLC), or 2-^{13}C uracil breath test (UraBT) as described below. Informed consent was obtained from all subjects prior to initiation of this Institutional Review Board (IRB) approved protocol.

1:- *Peripheral Blood Mononuclear Cell (PBMC) DPD radioassay:* To minimize variation resulting from the circadian rhythm in DPD activity, sixty milliliters of whole blood was drawn from a peripheral vein into heparinized vacutainers at approximately 12 p.m. Peripheral blood mononuclear cells were isolated by separation on a ficoll gradient, washed three times with Phosphate Buffered Saline (PBS), placed in an ice-bath, and lysed by sonication. The lysed cells were then centrifuged to remove cellular debris and the cytosol was collected. The protein concentration of the cytosol was quantitated by a Braford assay.[14]

2:- *DPD Radioassay:* The DPD radioassay is described in greater detailed elsewhere.[15]

Two-hundred-fifty µg of cytosolic protein was added to a reaction mixture containing NADPH and [6-^{14}C]-5-FU. The reaction mixture was incubated at 37 °C for 30 minutes. One-hundred-thirty µl aliquots of the reaction mixture were removed every 5 minutes during the incubation period and added to an equal volume of ice-cold ethanol to terminate the reaction. This mixture was incubated overnight at -80°C, thawed, and then filtered prior to HPLC analysis. Reversed-phase HPLC was used to separate [6-^{14}C]-5-FU from its catabolite, [6-^{14}C]-5-FUH_2. The amount of [6-^{14}C]-5-FUH_2 formed at each time point was quantitated and then graphed against time as described elsewhere.[15] From these data, the formation rate of [6-^{14}C]-5-FUH_2 was calculated. DPD enzyme activity was determined by standardizing the formation rate of [6-^{14}C]-5-FUH_2 to the amount protein used in the reaction mixture (i.e. nmol/min/mg protein). Subjects were considered to be DPD deficient by radioassay when their PBMC DPD activity was ≤0.18 nmol/min/mg protein.[15]

3:- *Rapid Oral 2-^{13}C uracil breath test (UraBT):* The UraBT principle and methodology is described in greater detail elsewhere.[16] To minimize variation resulting from a circadian rhythm in DPD activity,[2] the UraBT protocol started at approximately 8 a.m. Fasting subjects were weighed and an aqueous solution containing 6 mg/kg 2-^{13}C-uracil (Cambridge Isotope Laboratories Inc., Andover, MA) was formulated. Subjects donated 3 baseline breath samples into 1.2 L bags (Otsuka Pharmaceuticals, Tokushima, Japan) prior to ingestion of the oral solution. Twenty-one post-dose breath samples were collected into 100ml breath bags (Otsuka Pharmaceuticals, Tokushima, Japan) during the 180 minute period immediately following ingestion. Post-dose breath samples were collected every 5 minutes for the first 30 minutes and every 10 minutes thereafter. The concentration of $^{13}CO_2$ in breath, reported in delta over baseline (DOB) notation, was determined by infrared spectrophotometry (Meretek, Lafayette, CO).[17] These data were graphed [DOB (y axis) vs. time (x axis)] and C_{max}, T_{max}, and DOB_{50} ($^{13}CO_2$ concentration in breath 50 minutes following 2-^{13}C-uracil ingestion) were determined by inspection. The percentage dose of 2-^{13}C-uracil, recovered in the breath as $^{13}CO_2$ (PDR), was calculated as described elsewhere.[18] Subjects were considered to be DPD deficient by UraBT when their DOB 50 < 128.9 DOB.[16]

4:- *DPYD Genotyping:* The genotype of the *DPYD* gene was analyzed using DHPLC to assess the coding region and promoter as described previously.[19] All *DPYD* sequence variants identified by DHPLC were confirmed by DNA sequencing using a dideoxynucleotide chain termination method (Big Dye Kit; Applied Biosystems, Foster City, CA) and capillary electrophoresis on an ABI 310 Automated DNA Sequencer (Applied Biosystems, Foster City, CA).

RESULTS

Twenty-three patients with GI malignancies that included small intestine, gastric, pancre-

Table-I: Patient Demographics who developed severe untoward toxicities following administration of 5-FU and capecitabine (n = 23)

Characteristic	*No. of Patients*
Patients Enrolled	23
Men	11
Women	12
Median Age, y (range)	66 (48-75)
Race (Caucasian : Black: South Asian: Hispanic)	19:2:1:1
Diagnosis	
Colorectal	8
Pancreatic	9
Hepatocellular	1
Gastric	4
Small Intestine	1
Regimen	*Dose*
5-FU	
Bolus	2
Mayo	1
Roswell	1
ECF	1
High dose 5-FU + PN401	2
Capecitabine	
Single agent	9
+ XRT	5
+ oxaliplatin	1

atic, hepatocellular cancer, and colorectal cancer were evaluated after they experienced severe toxicities to either 5-FU or capecitabine (Table-I). Seven out of these 23 (30%) patients with excessive toxicities following 5-FU or capecitabine were found to DPD deficient. The patients ages ranged from 51-75 years, the male:female ratio was 1.3:1 and ethnicities included Caucasian (71%), African-American (14%) and South-Asian (14%). Three were treated with 5-FU/LV (2 Roswell; 2 Mayo); 2 with capecitabine (1800mg/m^2); & 2 with high dose bolus 5-FU (1400mg/m^2) combined a prodrug of uridine (2', 3', 5'-tri-*O* acetyluridine)

Among these 7 DPD-deficient patients, DPD activity ranged from 0.064 – 0.18nmol/min/mg (normal range 0.182-0.688nmol/min/mg protein). Genotypic analysis of *DPYD* gene in one patient (no peripheral blood mononuclear cells due to neutropenia) on high dose 5-FU and 2', 3', 5'-tri-*O*-acetyluridine demonstrated a heterozygous mutation (IVS14+1 G>A, DPYD*2A). The UraBT was performed in two of the seven patients. The UraBT demostrated one to be DPD-deficient (DOB_{50} of 112.8; PDR of 49.4%), and the second to have borderline normal values (DOB_{50} of 130.9; PDR of 52.5%) in the second patient.

Toxicities included mucositis (71%), diarrhea (43%), memory loss/altered mental status (43%), cytopenias (43%), severe skin rashes (43%), nausea (29%), hypotension (14%), respiratory distress (14%), and acute renal failure (14%) (Table-II). Re-challenge with capecitabine in one patient after the Mayo regimen caused grade 3 HFS only on the dorsal surfaces of the hands. One patient on high dose 5-FU and 2', 3', 5'-tri-*O*-acetyluridine developed grade 3 facial skin rash as the worst toxicity, and grade 3 thrombocytopenia. There were 2 toxicity-related deaths among these DPD-deficient patients (28%): one on capecitabine and one on high dose 5-FU and 2', 3', 5'-tri-*O*-acetyluridine.

DISCUSSION

Although 5-FU is generally well tolerated at standard doses, approximately 40-60% of cancer patients that develop severe, life-threatening 5-FU toxicities are DPD-deficient.[8,9] Complete DPD deficiency is extremely rare, but 3-5% of all cancer patients are considered partially DPD-deficient, defined by enzyme activity that is less than the 95th percentile of the lower limit of the population.[10] However, up to 43-59% of cancer patients who experienced severe to life-threatening toxicities to 5-FU are considered partially or borderline DPD-deficient.[15] Gender differences in DPD expression and activity have been noted. A retrospective

Table-II: Severe Toxicity in patients with DPD deficiency
M – mucositis, D – diarrhea, N – nausea/vomiting, R – rash, C – cardiovascular toxicity

Patient (Age/sex)	*Diagnosis*	*Regimen*	*Dose (mg/m^2)*	*Toxicities*				
				M	*D*	*N*	*R*	*Other*
59/F	SMALL INTESTINE	5FU/LV-Roswell Park	FU 500 LV 200weekly	X		X		Hand-foot rash (HFS), memory loss
59/M	GASTRIC	5FU/LV – Mayo	FU 425LV 2005/28 days	X	X			Bruising
66/F	PANCREATIC	High dose 5FU + PN401	FU- 1400	X			X	Cytopenia, periorbital edema
60/F	HCC	CAP	1800-2000		X	X		Colon inflammation, hypotension, respiratory distress
52/M	RECTAL	5FU/LV – MayoCAP	FU 425LV 2005/ 28 days CAP 1800-2000	X			X	Dorsal hand rash
71/M	COLON	5FU/LV-Roswell Park	FU 500LV 200 weekly	X	X	X		confusion
75/M	PANCREATIC	High dose 5FU + PN401	FU- 1400					Weakness, confusion, pancytopenia

review of past clinical studies in which 5-FU was administered showed that women had a higher average grade of toxicity, more grade ≥ II hematological toxicities, and increase of moderate to severe mucositis compared to men.[20] In one series, tumors and normal tissue from 118 men and women were analyzed. In the tumor tissue, the DPD expression was lower among women, but in normal tissue there was not a significant difference between the genders.[21] Thus, this may provide an explanation as to why women have more sensitivity to 5-FU treatment. Experience from our group also has indicated that women have lower DPD enzyme activity compared to men as measured by the radioassay described above, with African-American women being the most affected.[12]

DPD deficiency is a familial syndrome as a result of the allelic mutations within the DPYD gene.[22] The DPYD*2A variant is the single most common mutation associated with DPD deficiency. It is associated with a glycine to arginine mutation in the GT 5' splicing recognition sequence of intron 14 which results in a 165-bp deletion in the DPD mRNA.[23] We have earlier demonstrated by genotype analysis of the DPYD gene that a homozygote genotype results in complete deficiency[15] while a heterozygote genotype results in partial deficiency of DPD.[24]

Our series of patients noted DPD deficiency among several racial groups. A greater understanding of pharmacogenomic differences in DPD activity among different races is developing. Retrospective data from a phase III trial of 5-FU in the adjuvant setting shows a significant decrease in severe toxicity such as diarrhea, nausea and stomatitis in African-Americans compared to Caucasians. However, among African-Americans there was an increase in overall leucopenia and anemia, but not with respect to severe myelosuppression.[25] Differences in DPD activity between these two racial groups have been noted, with African-Americans having a lower level of mean DPD activity and a 3-fold higher incidence of DPD deficiency.[12] Among other racial groups, there are variable results. In a Japanese study of 150 healthy subjects, only one patient was found to be DPD deficient with an incidence of 0.7%.[26] Interestingly, there was a non statistically significant lower level of DPD activity among the women in this study. Another analysis of germline mutations among 107 Japanese cancer patients and healthy volunteers only detected an incidence of homozygous mutations in the DPYD gene of 0.2%.[27] One of the patients in this review is of East Indian origin. While the incidence of DPD deficiency in this population is not known, it is clearly present. A prior analysis of 13 healthy subjects found partial DPD deficiency in one patient.[28] In another series of cancer patients and healthy subjects of Indian, Malay and Chinese origin in Singapore, there was a statistically significant decrease in lymphocyte. Mean DPD activity among the Chinese population (0.06 nmol/min/mg/protien) compared to the Indian cohort (0.66 nmol/min/mg), but not when compared to the Malay group (0.31nmol/min/mg).[29] Overall, larger population studies in these ethnic groups are required to characterize the incidence of DPD deficiency and DPYD mutations further.

It is recommended that patients suspected of having DPD deficiency not receive further 5-FU chemotherapy regardless of the clinical indications because of the life-threatening 5-FU toxicities. This recommendation is further confounded by the therapeutic dilemma that tumors with low DPD levels are associated with improved 5-FU sensitivity[30,31] and better prognosis than high level DPD tumors.[32,33] Capecitabine offers an improved safety profile over bolus 5-FU and leucovorin[34] and is converted to active 5-FU preferentially in the tumor by a three-step enzymatic conversion, the last by thymidine phosphorylase.[35] In previous studies, the 5-FU concentration ratio in the tumor was almost 3-fold of that in normal tissue and 21-fold more in plasma, as compared to parenteral 5-FU levels in tumor, plasma or normal tissue which remained at 1.0.[36] Therefore, these pharmacological effects of capecitabine over parenteral 5-FU theory could be more evident in the setting of DPD deficient host. However, our analysis further highlights

that the safety of capecitabine in DPD-deficient patients needs to be evaluated, as mortality is a potential threat in these patients as evidenced by death of one of our patients. This patient was a 60 year-old Caucasian woman with hepatocellular cancer, who was treated with capecitabine[37] & subsequently developed vomiting, diarrhea, colonic mucosal wall thickening, respiratory distress and hypotension. Her DPD level was low at 0.064 nmol/min/mg.

Since the antitumor effects and the systemic toxicities associated with 5-FU are related to its metabolite FUTP, uridine has previously been examined for potential reduction of host toxicity, particularly when administered after the 5-FU administration allowing for an antitumor effect prior to "rescue" of the normal host cells. Uridine is a naturally occurring pyrimidine nucleoside that augments cellular UTP pools and competes with FUTP for incorporation into the host RNA of hematopoietic progenitor and gastrointestinal mucosal cells, thereby attenuating 5-FU/FUTP toxicity in normal tissues.[38,39] Preclinical and clinical studies have revealed that sustained uridine concentrations of at least 50μmol/L are required to confer protection from the toxic effects of 5-FU/FUTP to normal tissues.[40] Both hematopoietic and gastrointestinal mucosal progenitors efficiently incorporate exogenous uridine (via the "salvage pathway"), whereas most other tissues, including malignant tumors, favor the *de novo* pathway of uridine nucleotide biosynthesis, in which free uridine is not an intermediate.[41] Thus, exogenous uridine is more effective at competing with FUTP for incorporation into host RNA in normal tissues versus all solid tumors tested to date in murine systems. Although uridine has also been demonstrated to protect against 5-FU toxicity in humans, its low oral bioavailability, risk of fever and phlebitis, and the requirement for central venous access for parenteral administration limits its clinical utility.[38-41] PN401 (2', 3', 5'-tri-*O*-acetyluridine; Wellstat Therapeutics Corporation, Gaithersburg, MD) is an orally active prodrug of uridine, and is efficiently absorbed from the gastrointestinal tract and deacetylated, yielding uridine and acetate. In contrast to oral uridine, PN401 is not a substrate for the catabolic enzyme uridine phosphorylase and does not require the pyrimidine transporter for absorption. Consequently, administration of PN401 has a higher oral bioavailablility than uridine itself. Using PN401 rescue, it has been possible to increase the therapeutic index of 5-FU in BALB/c mice bearing advanced transplants of Colon 26 adenocarcinoma.[42] Further, in clinical trials, it has been possible to increase the dose of 5-FU resulting in a significant increase in antitumor activity without increased toxicity. The clinical feasibility of 5-FU dose escalation with PN401 has been demonstrated in phase I studies.[43] The demonstration in the Ashour study that toxicity reduction is still observed when PN401 is given 48 hours after a lethal dose of 5-FU[44] suggests that PN401 can be used as an antidote after accidental overdose of 5-FU (e.g. due to pump malfunction).

In this manuscript we report toxicity in two patients who were treated with high dose 5-FU and 2', 3', 5'-tri-*O*-acetyluridine for pancreatic adenocarcinoma, who we subsequently demonstrated to be DPD deficient. Although the full details of the patients are not available for presentation at present, we believe that these two patients still developed severe toxicities from high dose 5-FU, despite being treated with 2', 3', 5'-tri-*O*-acetyluridine. This suggests that although 2', 3', 5'-tri-*O*-acetyluridine provides protection from toxicity related to 5-FU in most patients, patients with DPD deficiency may remain more susceptible to both hematologic and non-hematologic side effects of 5-FU or the DPD deficient patients may, in fact, require alternative dosing regimens of 2', 3', 5'-tri-*O*-acetyluridine and/or 5-FU to avoid toxicity or to treat accidental overdoses. Most importantly, however, it is crucial to recognize the potential for increased toxicity in this group of patients.

Although DPD enzyme activity can be assayed from peripheral blood mononuclear cells, routine phenotypic and genotypic screening for DPD deficiency prior the administrating 5-FU are not yet available.[44] Recently, we developed and validated an oral UraBT assay which may potentially be used as a screening

method to rapidly detect DPD deficiency in cancer patients prior to 5-FU administration.[16] This *in vivo* assay utilizes 2-^{13}C-uracil, which has a similar substrate affinity for the DPD enzyme as 5-FU. As the 2-^{13}C-uracil substrate is degraded by DPD and other enzymes of the pyrimidine catabolic pathway, the ^{13}C label is released as $^{13}CO_2$. The $^{13}CO_2$ present in the breath can then be quantitated by infrared spectrophotometry. Previously, we demonstrated DPD deficient individuals have an impaired ability to catabolize the 2-^{13}C-uracil, which results in altered $^{13}CO_2$ breath profiles (e.g. lower C_{max}, PDR, and DOB_{50}).

CONCLUSION

In this study, we describe the spectrum of toxicities of capecitabine in seven DPD-deficient patients, including death in two patients. Most assays that are currently available to detect this pharmacogenetic syndrome are too labor-and-time intensive to be routinely used to screen cancer patients prior to 5-FU administration. Recently, we developed and validated an oral UraBT which may potentially be used as a screening method to rapidly detect DPD deficiency in cancer patients prior to 5-FU administration. In addition to severe to life-threatening toxicities akin to 5-FU, capecitabine can lead to unusual variants of common toxicities in the DPD-deficient patients, including HFS. Further studies to evaluate the safety of capecitabine in these patients and the underlying mechanism of HFS are required. While some DPD-deficient patients may benefit, the 5-FU toxicity modulating role of PN401 in this setting will need to be further evaluated. Also, future trials should assess the difference in DPD activity between varying ethnic groups and genders. Currently, there is an FDA approved test to assay for the pharmacogenomic syndrome of the UGT1A1 mutation prior to irinotecan administration. Given the relatively high prevalence of DPD deficiency in the population (3-5%), encompassing several ethnic groups, and the frequent use of 5-FU in oncology practice, cancer patients would benefit from screening for DPD deficiency prior to initiating capecitabine or 5-FU therapy.

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