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Genetic influence of *DPYD**9A polymorphism on plasma levels of 5-fluorouracil and subsequent toxicity after oral administration of capecitabine in colorectal cancer patients of South Indian origin

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Abstract

Objectives: High interindividual variability was reported with capecitabine toxicities among colorectal cancer (CRC) patients. *DPYD**9A polymorphism was reported responsible for grade 3 or 4 toxicities. Finding the phenotypic association between *DPYD**9A polymorphism and 5-fluorouracil (5-FU) plasma levels will give a better prediction for toxicity susceptibility.

Methods: A total of 145 CRC patients were included in the final analysis. Each patient received capecitabine of 1,000 mg/m² twice daily for the first 14 days of a 21 day cycle. 5-FU levels were measured at two-time points 2 and 3 h post capecitabine administration across the 1st and 4th cycles of chemotherapy. 5-FU levels were measured using liquid chromatography and tandem mass spectrometry (LC-MS/MS). Genotyping analysis was done by real-time PCR (RT-PCR).

Results: The mean 5-FU drug levels measured during the 1st cycle at time points 2 and 3 h were found to be 267 ng/mL ± (29) and 124 ng/mL ± (22) respectively. Whereas, the observed 5-FU levels in the 4th cycle were 275 ng/mL ± (28) and 130 ng/mL ± (26) respectively. Patients with 5-FU levels in the range of 281–320 and 141–160 ng/mL at 2 and 3 h respectively showed a higher risk for the hand-foot syndrome (HFS) and thrombocytopenia. No association was

found between *DPYD**9A polymorphism and 5-FU drug levels measured at time point 2 h across both the cycles. However, the drug levels measured at 3 h were found to be significantly different across the *DPYD**9A genotypes. Individuals with GG genotype showed significantly higher 5-FU levels when compared to AA genotype.

Conclusions: *DPYD**9A polymorphism had a significant influence on the plasma levels of 5-FU after capecitabine administration. The 5-FU levels measured at 3 h corresponding to elimination $t_{1/2}$ was significantly higher in patients with GG genotype compared AA genotype.

Keywords: C_{max} ; *DPYD**9A; elimination half-life ($t_{1/2}$); 5-FU drug levels; genotyping; liquid chromatography and tandem mass spectrometry (LC-MS/MS); RT-PCR; toxicity.

Introduction

Capecitabine is an oral prodrug and is converted to an active metabolite, 5-fluorouracil (5-FU) with the help of thymidine phosphorylase (TP) enzyme. The enzyme was found in higher levels in tumour cells when compared to normal healthy tissue. This explains the preferential activation of capecitabine and for 3–4 fold higher levels of 5-FU in the tumour cells when compared to adjacent healthy tissue [1]. Capecitabine exerts its chemotherapeutic activity in several ways. The main mechanism of action is by inhibition of thymidylate synthase (TYMS) enzyme, which plays an important role in the folate homocysteine cycle and pyrimidine synthesis pathways. Also, the active metabolite (5-FU) can be incorporated directly into the RNA and DNA ultimately leading to cell death [2].

Capecitabine in combination with oxaliplatin (CAPOX) was found equivalent or non-inferior to 5-FU infusion based regimens like FOLFOX or FOLFIRI in the management of advanced or metastatic colorectal cancer (CRC) [3, 4]. Oral capecitabine is preferred over 5-FU infusion as it

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simplifies care by precluding the need for frequent central venous access or infusion pumps [5]. Also, capecitabine treatment was found to be safe and better tolerable when compared to 5-FU infusion. The incidence of haematological toxicities such as anaemia, grade 3 or 4 neutropenia were found to be lower when compared to 5-FU infusion [6]. However, capecitabine treatment was associated with its own self-limiting toxicities such as hand-foot syndrome (HFS), diarrhoea, vomiting and thrombocytopenia [7]. These toxicities found to affect the treatment efficacy either by imposing dose modification or treatment delay or even drug discontinuation.

High interindividual variability is noticed with capecitabine toxicities in CRC patients. The variability is partly due to the clinical (hepatic or renal function) and demographic factors (age, sex) related to the patients. However, much of the variability is linked to the genetic makeup of an individual [8]. With respect to capecitabine, the variability was mainly linked to *DPYD* gene polymorphisms [9, 10]. The *DPYD* gene codes for the enzyme dihydropyrimidine dehydrogenase (DPYD) which metabolizes 80% of the active metabolite of capecitabine (5-FU). The US Food and Drug Administration (FDA) and the European Medical Agency (EMA) approved drug label for capecitabine clearly warns for severe and life-threatening toxicities in the absence of DPYD enzyme [11, 12]. *DPYD**2A (c.1905+1G>A) polymorphism is the most commonly tested genetic variant and is responsible for severe and life-threatening toxicities with either capecitabine monotherapy or combinational treatment [13]. The clinical pharmacogenetic implementation consortium guidelines (CPIC) states the *DPYD**2A polymorphism results in the skipping of entire exon 14 and codes for a nonfunctional protein. The CPIC guidelines recommend about 30–50% of dose reduction in heterozygous (GA) patients and switching to an alternate drug in homozygous (AA) *DPYD**2A mutants [14]. In support to the above facts, a clinical pharmacokinetic study reported that heterozygous and homozygous *DPYD**2A carriers had 2.1 and 10 fold longer plasma half-life for 5-FU when compared to controls at a given single dose of 300 mg/m² capecitabine [8]. However, the major limitation of using *DPYD**2A polymorphism as toxicity predictor is its lower minor allele frequency (MAF) varying from 0.1 to 1% across different ethnicities [10]. In the Indians, the reported MAF of *DPYD**2A polymorphism was found to be 0.4% [15]. Interestingly, only about 50% of *DPYD**2A carriers actually develop severe toxicities, which limits its predictive power [16]. The observed sensitivity of *DPYD**2A polymorphism over toxicities was found to be only 5.5% with a positive predictive value of

0.46% [17]. A study by Gentile et al. found that besides *DPYD**2A, two additional polymorphisms such as *DPYD**9A (rs1801265, A>G) and *DPYD**6 (rs1128503, A > G) found to affect the metabolism of 5-FU and the haplotype combination of these single nucleotide polymorphisms (SNPs) (hap 7) have two-fold higher sensitivity in predicting 5-FU toxicities when compared to *DPYD**2A alone [18]. Unlike *DPYD**2A, the frequency of *DPYD**9A polymorphism was found higher among the Asian population and the reported MAF was about 27% in the South Asian population (Ensembl genome browser, 1,000 genome project). A recent study by Kushman et al. reported, *DPYD**9A was the common variant in their study cohort and found to have a significant influence on fluoropyrimidine associated grade 3–4 diarrhoea. [19] Also, the study emphasized for regular screening for *DPYD**9A polymorphism along with classic *DPYD**2A variant in patients who are scheduled to receive fluoropyrimidine-based chemotherapy. However, no studies have reported the phenotypic influence of *DPYD**9A polymorphism on 5-FU plasma levels after oral administration of capecitabine. Finding a phenotypic association will give a better prediction for toxicity susceptibility. With this background, we aimed to find the phenotypic association between *DPYD**9A polymorphism and 5-FU drug levels after oral administration of capecitabine in CRC patients of South Indian origin.

Materials and methods

Study design and subjects

In this prospective cohort study, we have included a total of 145 newly diagnosed and treatment-naïve CRC patient of South Indian origin in the final analysis. Patients of South Indian origin consists of subjects who are living in South Indian states (Tamil Nadu, Kerala, Andhra Pradesh) for at least three consecutive generations and speaking the native language.

Study procedure

The study was approved by the Institute Scientific Advisory Committee (JSAC Reg.No.JSAC 34/6/2016) and Institute Ethics Committee (JEC Reg.No: 25-5-2016). Principles of the declaration of Helsinki and good clinical practice guidelines were strictly followed during the course of the study. Patients were enrolled in the study based on the eligibility criteria and after obtaining informed consent. The inclusion criteria were newly diagnosed and treatment-naïve CRC patients of either gender above 18 years of age and who were scheduled to receive CAPOX as the standard treatment care. Patients who were previously treated with 5-FU based regimens like FOLFOX or FOLFIRI, pregnant, lactating women and patients with

abnormal liver function (serum transaminases ≥ 3 times the normal value) or renal function (serum creatinine greater than 1.5 g/dL) were excluded from the study. Each patient received oxaliplatin 130 mg/m² infusion over 2 h on day 1 and oral capecitabine of 1,000 mg/m² in divided doses twice daily for the next 14 days of a 21-day cycle. For measuring drug levels of 5-FU, 3 mL of the blood sample was collected at two-time points i.e., at 2 and 3 h post capecitabine oral administration during the 1st and 4th cycle of chemotherapy. The blood samples are subjected to centrifugation at 2,500 rpm for 10 min for the separation of plasma from the blood components. The supernatant plasma was collected and stored at -80°C for the estimation of 5-FU plasma levels using liquid chromatography and tandem mass spectrometry (LC-MS/MS). The remaining whole blood sediment was utilized for the DNA extraction and for further downstream genotyping analysis of *DPYD*9A* polymorphism.

DNA extraction and genotyping analysis

Five millilitres of whole blood was used for DNA extraction. Genomic DNA was extracted from the White Blood Cells (WBC) by the manual phenol-chloroform method [20]. The quantity and quality of the extracted DNA were analysed using biophotometer (Eppendorf AG 22331, Hamburg, Germany). The quantity of the extracted DNA was analysed at the absorbance wavelength of 260 nm whereas, the quality of the DNA was checked at the absorbance ratio of A260/280 nm wavelength. The absorbance ratio reading ranging of 1.7–2 was considered as the minimum indicator for good quality of DNA. Each DNA sample was diluted to an optimal concentration of 50 ng/ μL for further downstream genotyping analysis. The PCR reaction was performed using a 10 μL of the final volume containing 2.5 μL of extracted DNA, 5 μL of TaqMan universal master mix (2x), 0.25 μL of TaqMan assay kit (40x) and 2.25 μL of Milli-Q water. The genotyping analysis of *DPYD*9A* polymorphism was carried out in duplicates using a validated TaqMan SNP genotyping kit (rs1801265) by real-time PCR (Applied Biosystems; Life Technologies Corporation, Carlsbad, CA, USA). Absolute quantification and allelic discrimination analysis were done by using sequence detection software (SDS) 7300, Version 1.4.

LC-MS/MS analysis

Plasma levels of 5-FU were measured by using Liquid Chromatography-Mass Spectrometry (LC-MS/MS). 5-Bromouracil (5-BU) was used as an internal standard for the estimation procedure whereas, pure 5-FU powder was used as a reference standard [21]. The mobile phase for liquid chromatography was prepared with acetonitrile and formic acid. Mobile phase A was prepared by adding 0.25 mL of formic acid (final concentration 0.1%) in 250 mL of Milli Q water and sonicated for 15 min for removing any air bubbles. Mobile phase B was prepared with 250 mL of acetonitrile and sonicated for 3 min. The final mobile phase used for the separation was a combination of A and B in a ratio of 30:70. The prepared mobile phase was then infused in the chromatography system in an isocratic method for analyte separation. The solid phase used was a C₁₈ column (Xterra MS, 5 μm pore size, 3.9 $\times$ 150 mm length). The needle wash solution was prepared by 80:20 ratio of methanol and Milli Q water respectively. The seal wash was prepared by 90:10 ratio of Milli Q water and methanol respectively. The column wash solution was prepared

by 50:50 ratio of methanol and Milli Q water. Other related chromatography and multiple reaction monitoring (MRM) conditions are listed in Table S3 of Supplementary Material.

Preparation of standard solutions for the calibration curve

The stock solutions of 1 mg/mL of 5-FU and 5-BU were prepared by dissolving in methanol-water (50:50 ratio). Working concentrations of 5-FU (10 and 1 $\mu\text{g/mL}$) were prepared from the stock solution. Working standards for 5-FU of different concentration ranging from 1 ng to 1,000 ng/mL were prepared using drug-free plasma for the calibration curve. The quality controls (QC) of three different concentrations, 75, 300 and 500 ng/mL were prepared to verify the accuracy and precision of the analytic method during the period of sample analysis. The working concentration of 5-BU (100 ng/mL) was prepared with methanol-water (50:50 ratio). The calibration curve was created to determine the unknown concentration of an analyte based on the known standards. The developed method was validated on accounting for linearity of a calibration curve, the slope of the curve, recovery of the standards and matrix effect. A linear curve indicates a proportional increase in signal (response) to the concentration of standards, whereas the slope of the curve, recoveries and matrix effect were measures of sensitivity [22]. The formulae used for calculating the response of the standards and unknown concentration in normalization to the internal standard (5-BU) were given in the Supplementary Material.

Extraction procedure

The extraction of samples in the present study was done by a simple protein precipitation method using acetonitrile. To each of the 200 μL of the patient sample and calibration standards (1–1,000 ng/mL) an equal amount of 600 μL of acetonitrile containing 60 ng of internal standard i.e., 5-BU was added and vortexed for 30 s for uniformity. In the next step, all the analytes were subjected to centrifugation at 13,500 rpm for 5 min for protein precipitation. Following this 100 μL of clear supernatant was separated and 20 μL of the same was injected into LC-MS/MS for measuring the levels of 5-FU.

Statistics

The continuous data were expressed as mean $\pm$ standard deviation. The genotype frequencies of *DPYD*9A* variant were expressed in percentages by direct gene count method. The genotype frequencies were tested for Hardy Weinberg equilibrium (HWE) by using the chi-square test. The overall association between genotypes of *DPYD*9A* polymorphism and 5-FU plasma drug levels were analysed by one way ANOVA test. A p-value of <0.05 was taken as a significant statistical difference. A Dunnett's post hoc test was done for multiple comparisons. A simple logistic regression analysis was done to find the association between toxicities and drug levels and. A multiple linear regression analysis was done for accounting the influence of non-genetic predictors on 5-FU plasma levels. An adjusted p-value of ≤ 0.01 was taken as a significant association in multiple linear regression analysis. All the statistical analysis were performed using SPSS IBM software (Version 20.0)

Results

Genotype frequency and distribution

The study patients were from different South Indian states of India. Majority of the patients belong Tamilian ethnicity; n=111 (76%) followed by Andhra; n=20 (14%) and Kerala; n=14 (10%). On genotyping analysis, the observed major (A) and minor allele (G) frequencies were found to be 74 and 26% respectively. The genotype frequencies were AA (56%), AG (36%), and GG (8%). All the genotypes were found to be in HWE (p-value=0.3) on chi-square analysis (Table 1).

Calibration curve

The calibration curve was linear with a correlation coefficient (r^2)=0.999630. The slope value of the curve was equal to 0.7 (Figure S1). The observed retention time (RT), recoveries, peak area for the reference and internal standards are shown in the Supplementary File (Table S2). The elution time of the standards was about 5 min however, the total run time was increased to an extra minute to avoid carry-over effect. So the total run time for each sample was 6 min. The LC-MS/MS predicted mass chromatogram of 5-FU in control 7 (250 ng/mL) and patients samples (Sample 6 and 10) was depicted in Figure S2 of the Supplementary Material.

Measured 5-FU levels in patient's samples

The measured mean 5-FU drug levels at time points 2 and 3 h post capecitabine administration during the 1st cycle were found to be 267 ng/mL $\pm$ 29 and 124 ng/mL $\pm$ 22 respectively. Similarly, the drug levels measured during the 4th cycle at 2 and 3 h were found to be 275 ng/mL $\pm$ 28 and 130 ng/mL $\pm$ 26 respectively (Table 2). The 5-FU levels

measured at 2 h correspond to concentration maximum (C_{max}) whereas at 3 h correspond to first elimination half-life ($t_{1/2}$). The drug levels of 5-FU across 1st and 4th cycle were similar which indicate no drug accumulation over chemotherapy cycles (Table 2).

Association between drug levels of 5-FU and dose-limiting toxicities of capecitabine

The levels of 5-FU observed during the time points 2 and 3 h post capecitabine administration during the 1st and 4th cycle of CAPOX chemotherapy were categorized into different ranges to find the association with the dose-limiting toxicities of capecitabine such as HFS, diarrhoea and thrombocytopenia. With respect to of 5-FU levels measured at 2 h, the individuals with the categorical range of 281–320 ng/mL showed 2 times higher risk for thrombocytopenia when compared to the reference range 210–240 ng/mL. No association was found with respect to other dose-limiting toxicities. In relation to the levels of the drug measured at 3 h, the patients in the range of 141–160 ng/mL showed 4 and 5 times higher risk for HFS and thrombocytopenia respectively when compared to the reference range of 100–120 ng/mL (Table 3).

Association between *DPYD**9A polymorphism and drug levels

A one-way analysis of variance (ANOVA) was used to determine the overall association between *DPYD**9A genotypes (AA vs AG vs GG) and 5-FU levels. No significant difference was found between *DPYD**9A genotypes and 5-FU drug levels measured at 2 h time point across both the CAPOX cycles. However, the drug levels measured at 3 h post capecitabine administration were significantly different across the genotypes in both the CAPOX cycles (Table 4). On performing a Dunnett's post hoc multiple comparison analysis we found the patients with GG

Table 1: Observed genotype frequencies and test for Hardy Weinberg Equilibrium (HWE).

Gene	Genotypes			Major allele	Minor allele	HWE-p-value
<i>DPYD</i> *9A	AA	AG	GG	A	G	0.3
Frequency, n (%)	81 (56)	52 (36)	12 (8)	107 (74)	38 (26)	

A p-value of >0.05 indicates the genotype frequencies are consistent with HWE.

Table 2: Drug levels of 5-FU at 2 and 3 h post capecitabine administration during the 1st and 4th cycles of chemotherapy.

S. no	Time points	Mean drug levels (ng/mL $\pm$ SD)		p-Value
		Cycle 1	Cycle 4	
1	2 h (C_{max})	267 $\pm$ 29	275 $\pm$ 28	0.2
2	3 h ($t_{1/2}$)	124 $\pm$ 22	130 $\pm$ 26	0.3

C_{max} , concentration maximum; $t_{1/2}$, elimination half-life.

Table 3: A simple logistic regression analysis for the association between different ranges of 5-FU measured at 2 and 3 h and major dose-limiting toxicities of CAPOX regimen.

S.No	Time point and drug levels	n (%)	p-Value-OR (CI)		
			HFS	Diarrhoea	Thrombocytopenia
2 h					
1	210–250 ng/ml	30 (21)	Ref	Ref	Ref
2	251–290 ng/ml	89 (61)	0.4–1.2 (0.2–1)	0.3–1 (0.6–2)	0.5–2 (0.8–3)
3	291–330 ng/ml	26 (18)	0.07–3.2 (0.8–12)	0.3–1.8 (0.7–11)	0.03 ^a –2 (1.4–10)
3 h					
1	100–120 ng/ml	35 (24)	Ref	Ref	Ref
2	121–140 ng/ml	86 (59)	0.8–1 (0.5–1)	0.1–1 (0.7–4)	0.06–2 (0.9–5)
3	141–160 ng/ml	24 (17)	0.03*–4 (1–17)	0.1–2 (0.7–11)	0.01 ^a –5 (1.4–18)

HFS, hand foot syndrome; ref, reference. ^a Indicate significant difference (p < 0.05).

genotype found to have significantly elevated drug levels (p-value=0.01) when compared to AA genotype. The Mean Difference (MD) was 15 and 24 in relation to 5-FU levels between AA vs GG genotype across 1st and 4th cycle respectively. Whereas with AG genotype we observed a trend but the difference was not statistically significant (Table 5).

Influence of non-genetic predictors on 5-FU drug levels

A multiple linear regression analysis was done for accounting the influence of non-genetic predictors such as liver function, renal function, performance status, age and sex on 5-FU drug levels at time point 3 h (1st and 4th cycle). We found no significant influence between the non-genetic factors and 5-FU drug levels. Even in the multiple linear regressions at an adjusted p-value ≤ 0.01 to be significant, we found the patients with GG genotype had significantly elevated 5-FU drug levels when compared to AA genotype. In patients with AG genotype, we observed a trend but the difference was not statically significant (Table 6).

Discussion

The genotype frequencies of *DPYD*9A* polymorphism in the study was found to be in HWE. Therefore we can

Table 4: Association between 5-FU levels and *DPYD*9A* genotypes during the CAPOX chemotherapy (n=145).

Cycle number	Time points	Genotype	n	Mean drug levels, ng/mL ± SD	95% CI	p-Value
1st cycle						
	2 h	AA	81	265 ± 28	(260–276)	0.9
		AG	52	266 ± 29	(259–274)	
		GG	12	269 ± 26	(254–282)	
	3 h	AA	81	120 ± 15	(119–122)	0.01 ^a
		AG	52	124 ± 16	(121–125)	
		GG	12	136 ± 18	(132–140)	
4th cycle						
	2 h	AA	81	281 ± 29	(274–288)	0.5
		AG	52	279 ± 28	(277–293)	
		GG	12	276 ± 25	(265–286)	
	3 h	AA	81	118 ± 18	(116–120)	0.001 ^a
		AG	52	123 ± 19	(120–125)	
		GG	12	144 ± 22	(139–147)	

^a Significant difference.

consider the study population as not affected by evolutionary changes and a true representation of the actual population.

It was reported that after oral administration, capecitabine is rapidly and extensively absorbed from the gastrointestinal tract with almost 100% bioavailability. The time to reach peak concentration (*t*-max) was reported to be about 1_{1/2} h with a *C*_{max} of 3–4 mg/L and have a relatively shorter elimination half-life of 0.55–0.89 h. Whereas the pharmacokinetic parameters of cytotoxic moiety i.e., 5-FU were reported to be different from its parent compound. The *t*-max for 5-FU is achieved at 2 h with a *C*_{max} ranging of 0.22–0.33 mg/L. Also, the elimination half-life 5-FU was reported to be slightly longer (0.69–1.15 h) when compared to the parent compound [1, 5]. These pharmacokinetic parameters were found

Table 5: Dunnett's multiple comparison analysis between the genotypes of *DPYD**9A polymorphism and 5-FU drug levels at 3 h across the 1st and 4th cycle of CAPOX chemotherapy.

Cycle	Multiple comparisons	Mean difference	SE	Sig	95% (CI)
1st cycle					
1	AA vs. AG	2	1.1	0.06	(-0.09–5.2)
2	AA vs. GG	15	2.06	0.01 ^a	(10–20)
4th cycle					
1	AA vs. AG	4	1.7	0.05	(-0.06–7.5)
2	AA vs. GG	24	2.9	0.001 ^a	(18–31)

^a Significant difference. SE, standard error.**Table 6:** Multiple linear regression to find the association between *DPYD**9A genotypes and 5-FU drug levels by accounting the influence of non-genetic predictors.

S. no	Predictors	Estimate (SE)	Sig	95% CI
1	Genotype			
	AA (ref)	–	–	–
	AG	2.7 (1.2)	0.03	(0.3–5)
	GG	15 (2)	0.001 ^a	(11–19)
2	Age	–0.6 (0.1)	0.7	(–0.4–0.8)
3	Sex			
	Female (ref)	–	–	–
	Male	0.2 (1.2)	0.8	(–2.8–2.6)
4	Performance			
	0–1 (ref)	–	–	–
	2–3	–1.4 (1.2)	0.2	(–3.1–0.9)
5	Liver function			
	Normal (ref)	–	–	–
	Abnormal	1.5 (1.4)	0.2	(1.2–4.3)
6	Renal function			
	Normal (ref)	–	–	–
	Abnormal	–0.4 (1.7)	0.7	(–3–2.9)

Dependent variable: 5-FU drug levels. Predictors: Genotype, Age, Sex, performance, liver function, renal function. ^aSignificant difference. Adjusted p-value ≤ 0.01 was taken as statistically significant. Ref, reference.

unaltered overtime across the dose range of 500–3,500 mg/m²/day [23]. In the present study, the 5-FU levels measured at 2 h corresponding to $C_{\max}$ during 1st (267 ng/mL) and 4th cycle (275 ng/mL) of CAPOX chemotherapy were found within the reported range from the literature [1].

The approaches like measuring the area under the curve (AUC) or $C_{\max}$ or elimination $t_{1/2}$ and clearance (CL) of a specific drug in relation to genetic polymorphisms can be helpful in predicting the efficacy and toxicity susceptibility. A population pharmacokinetic study of capecitabine by Joerger et al. reported patients with *DPYD**9A polymorphism had decreased clearance for

5-FU and associated with a higher incidence of dose-limiting adverse effects such as diarrhoea and HFS [24]. The present study is first of its kind to study the phenotypic influence of *DPYD**9A polymorphism on 5-FU levels post capecitabine oral administration in South Indian CRC patients. The 5-FU levels measured at 2 h in the present study correspond to $C_{\max}$ whereas, at 3 h correspond to the elimination $t_{1/2}$ [1]. In the present study patients with drugs levels of 5-FU in the range of 281–320 ng/mL and 141–160 ng/mL measured at 2 and 3 h respectively found to be at a higher risk for developing HFS and thrombocytopenia with capecitabine treatment (Table 3). We found no difference in the 5-FU levels measured at 2 h across the *DPYD**9A genotypes in both the CAPOX cycles. This agrees with the fact that the *DPYD* gene plays no role in the activation of capecitabine to its active metabolite (5-FU). The genetic variations in the genes which are involved in the activation of capecitabine such as cytidine deaminase (CDD) or TP might better explain for the variation in the $C_{\max}$. We found a significant difference between *DPYD**9A genotypes and 5-FU levels measured at time point 3 h in both the CAPOX chemotherapy cycles. The GG genotype could have resulted in reduced *DPYD* enzyme activity and in turn, resulted for higher 5-FU levels compared to AA and AG genotype. The patients with GG genotype are the potential indicators for higher toxicities with capecitabine treatment when compared to AA and AG genotype.

In the present study, we did not study the genetic variations related to *CDD* gene, which is involved in the activation of capecitabine. The *CDD* gene polymorphism rs532545 (C>T) is one of the commonly tested genetic variants and found to be associated with capecitabine related toxicities [25]. A phenotypic association study addressing rs532545 polymorphism would have given a better prediction for the $C_{\max}$ variation in the patients. Not studying this polymorphism is one of the considerable limitations of the present study. Also, only dose-limiting toxicities of capecitabine like HFS, thrombocytopenia and diarrhoea were accounted and correlated with drug levels of 5-FU post capecitabine administration. Not accounting for overall haematological and non-haematological toxicities is one of the substantial limitations of the present study. Similarly, comparing the trough levels of 5-FU in relation to *DPYD**9A polymorphism would have given a better idea for variability. However, all the patients in the study cohort were outpatients and it was not feasible for us to collect the trough level samples. These were the few limitations of the present study and can be explored in future.

Conclusions

*DPYD**9A polymorphism had a significant influence on 5-FU levels post capecitabine oral administration. Patients with 5-FU drugs levels in the range of 141–160 ng/mL measured at 3 h showed a significantly higher risk for HFS and thrombocytopenia. The patients with GG genotype of *DPYD**9A polymorphism were found to higher plasma levels of 5-FU measured at 3 h when compared to AA and AG genotypes. Similar results were observed in multiple linear regression after adjusting for confounding factors like age, gender, liver and renal function.

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Author contributions: Ashok Varma: Sample collection, lab work, analysed data and wrote the manuscript. Jayanthi Mathaiyan, Biswajit Dubashi and DG Shewade: Contributed to the development of the study and interpretation of results. Rajan Sundaram: Contributed to the standardization of the LC-MS/MS method for measuring 5-FU levels. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The study was approved by the Institute Scientific Advisory Committee (JSAC Reg.No.JSAC 34/6/2016/) and Institute Ethics Committee (JEC Reg.No: IEC /25/5/2016). The study was carried out in accordance with the standards of the Institute ethics committee and with the principles of the declaration of Helsinki and good clinical practice guidelines.

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