Eur J Clin Pharmacol (2012) 68:389–395
DOI 10.1007/s00228-011-1134-0

PHARMACOGENETICS

Relationship of drug metabolizing enzyme genotype to plasma levels as well as myelotoxicity of cyclophosphamide in breast cancer patients

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Received: 17 June 2011 / Accepted: 28 September 2011 / Published online: 20 October 2011

Abstract
Purpose The cytotoxic drug cyclophosphamide (CP) is bioactivated into 4-hydroxy-cyclophosphamide (4-OH-CP) through cytochrome P450 enzymes and cleared through aldehyde dehydrogenase and glutathione S-transferase. This prospective study analyzes the influence of drug metabolizing enzyme genotype on (1) plasma 4-OH-CP:CP ratio and (2) myelotoxicity in breast cancer patients on 500 mg/m^2 cyclophosphamide.
Methods Sixty-eight female breast cancer patients on FAC (fluorouracil, adriamycin, cyclophosphamide) were included. Genotyping of cytochrome P450 enzymes CYP2B6, CYP2C9, CYP2C19, CYP3A5, aldehyde dehydrogenase (ALDH3A1), and glutathione S-transferase (GSTA1) was done either through RFLP or pyrosequencing. Plasma CP and 4-OH-CP were measured immediately and 1 and 2 h after the end of infusion through LC-MS. The leukocyte count was determined on day 10 and 20 after chemotherapy.
Results At CP dose of 500 mg/m^2, the 4-OH-CP:CP ratio was negatively affected by *CYP2C19*2* genotype (p=0.039) showing a gene-dose effect. Moreover *ALDH3A1*2* genotype increased 4-OH-CP:CP ratio (p=0.037). These effects did not remain significant in a univariate analysis of variance including all genotypes. *GSTA1*B* carriers were at increased risk of severe leucopenia (OR 6.94; 95% CI 1.75–27.6, p=0.006).
Conclusion The myelotoxicity in patients receiving FAC is related to the activity of the phase-II enzyme GSTA1 but is independent of the formation of 4-OH-CP.

Keywords Pharmacogenetics · Cyclophosphamide · 4-Hydroxycyclophosphamide · CYP2C19 · ALDH3A1 · GSTA1

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Introduction

Breast cancer is the most prevalent malignancy among females around the world, with a recently reported rise [1].

There has been a tremendous surge in attempts to elucidate diagnostic and prognostic markers of breast cancer and in particular to optimize the outcome individually as well as to prevent severe side effects caused by the cytostatic therapies.

Cyclophosphamide (CP) is commonly applied in the treatment of breast cancer among other indications. It is used at a dose of 500 mg/m^2 in conjunction with doxorubicin (Adriamycin®=A) and either fluorouracil (F) or a taxane (T) as FAC or TAC protocols, respectively, which are regarded as a cost effective first line chemotherapy regimen [2].

CP is an inactive prodrug that undergoes metabolic activation to 4-hydroxycyclophosphamide (4-OH-CP) mainly by hepatic cytochrome P450 (CYP) *2B6*, *2 C9*, *2 C19*, and *3A4* [3, 4]. 4-OH-CP is transformed non-enzymatically to its isomer aldophosphamide, which is either inactivated to carboxyphosphamide through aldehyde dehydrogenase (ALDH) or disintegrated into the ultimate bivalent alkylating nucleophile phosphoramide mustard. The reactive nucleophiles are inactivated especially by glutathione S-transferase (GSTA1) [5]. For details of the activation and detoxification pathway, see [6]. GSTs also play an important role in disposition of doxorubicinol, the active metabolite of doxorubicin [7].

Previous studies indicated an impact of *CYP2C19* genotype on the elimination constant of cyclophosphamide [4, 8], whereas others reported an impact of *CYP2B6* variants [9, 10]. However, only *CYP2C19* genotype has been found to have an impact on the elimination constant of cyclophosphamide at a dose <1,000 mg/m^2 [4].

Data on the relationship between *CYP2C19* genotype and efficacy or side effects are rare, e.g., the *CYP2C19*2* variant allele was associated with premature ovarian failure in lupus erythematosus–related nephritis [11]. A case report suggested that a fully functional *CYP2C19* and a compromised GST render the recipient excessively exposed to active metabolites of cyclophosphamide leading to severe leucopenia even at small doses of cyclophosphamide [12]. On the other hand, functional *ALDH3A1* has been associated with conferring resistance to cyclophosphamide [13].

This study aims to analyze the influence of genotypes of *CYP2B6, CYP2C9, CYP2C19, CYP3A5, ALDH31*, and *GSTA1* on plasma 4-OH-CP as a ratio to CP and iatrogenic leucopenia in a sample of 68 Pakistani breast cancer patients receiving the FAC regimen.

Materials and methods

Patients, treatment regimen, blood sampling, and toxicity assessment

The patients were recruited from two public-sector facilities, covering the majority of the population in Karachi. Ethics committee and institutional review board at Ziauddin University, Karachi, approved the study. After written informed consent, 68 chemotherapy naïve females suffering from infiltrating ductal carcinoma of the breast under care of a consultant oncologist were consecutively included in this study from April to September 2008. All patients received six cycles of FAC chemotherapy as fluorouracil 500 mg/m^2, doxorubicin 50 mg/m^2, and cyclophosphamide 500 mg/m^2 administered on a single day after every 21 days. The chemotherapy was administered only if their baseline (20th postchemotherapy day for cycles 2–6) vital signs, blood glucose, blood counts, liver function, renal function, and cardiac output were in the normal range (data not shown).

Chemotherapy was administered over a total time of approximately 3 h. Doxorubicin and cyclophosphamide were given as "infusion-1" over 30 min followed by fluorouracil as "infusion-2" over 2 h. Peripheral venous blood was collected serially through venipuncture at a suitable site (a) before infusion, (b) immediately after infusion-1, (c) 60 min after infusion-1, and (d) 120 min after infusion-1. Fourteen patients refused to give a sample at 120 min, hence that particular data point is not available for analysis. For each patient, sample (a) served as the control as well as the source for genotyping. An aliquot of 500 μL plasma from samples b, c, and d was immediately added to 50 μL of 2 M solution of semicarbazide hydrochloride to derivatize 4-OH-CP to the stable 4-OH-CP-semicarbazide as described by Huitema et al. [14].

Patient characteristics included age, ethnicity, clinical staging, and histological grading. Patients were followed up on the 10th day and 20th day after receiving chemotherapy. A total leukocyte count (measured on the 10th day postchemotherapy) below 2,500/mm^3 was considered severe leucopenia and warranted start of G-CSF or blood transfusion to prevent infections.

Chemicals and reagents All the chemicals and reagents were of MS grade. Acetonitrile and water were from Merck, Darmstadt, Germany; cyclophosphamide and semicarbazide hydrochloride were from Sigma-Aldrich, Steinheim, Germany; ifosfamide was from Baxter Oncology, Halle, Germany. Since 4-hydroxycyclophosphamide is very unstable, 4-hydroperoxycyclophosphamide (4-OOH-CP), which spontaneously converts into 4-hydroxycyclophosphamide upon dissolving in water, was purchased from IIT GmbH/NIO-MECH, Bielefeld, Germany. 4-Hydroxycyclophosphamide semicarbazide derivatization was accomplished by dissolving 4-hydroperoxycyclophosphamide directly in 2 M solution of semicarbazide hydrochloride to yield 1 mg/ml stock solution [14]. This work-up procedure was applied in an identical way to each patient sample.

Table 1 Baseline characteristics of patients

Characteristic		Number (%)
Source hospital	KIRAN[a]	48 (70.6)
	JPMC[b]	20 (29.4)
Age group (years)	25–29	2 (2.9)
	30–34	6 (8.8)
	35–39	15 (22.1)
	40–44	14 (20.6)
	45–49	10 (14.7)
	50–54	11 (16.2)
	55+	10 (14.7)
Ethnicity	Mohajir[c]	36 (52.9)
	Baloch	10 (14.7)
	Punjabi	9 (13.9)
	Sindhi+Katchi[d]	7 (10.3)
	Bengali	4 (5.9)
	Pashtoon	2 (2.9)
Body mass index	25.3±5.2	–
Body surface area (m^2)	1.54±0.16	–
Involved side	Right	34 (50.0)
	Left	33 (48.5)
	Bilateral	1 (1.5)
Family history for cancer	Positive	10 (14.7)
	Negative	58 (85.3)
Tumor size	Upto 2 cm	3 (4.4)
	2–5 cm	27 (39.7)
	>5 cm	21 (30.9)
	Any size with direct extension to chest wall	15 (22.1)
	Could not be determined	2 (2.9)
Node positive disease	Negative	19 (27.9)
	Positive	44 (64.7)
	Could not be determined	5 (7.4)
Metastasis	Absent	53 (77.9)
	Present	14 (20.6)
	Could not be determined	1 (1.5)
TNM stage	I	0
	II a	14 (20.6)
	II b	14 (20.6)
	III a	11 (16.2)
	III b	14 (20.6)
	IV	14 (20.6)
	Could not be determined	1 (1.5)
Grading	I	6 (8.8)
	II	32 (47.1)
	III	22 (32.4)
	Could not be determined	8 (11.8)
ER status	Positive	25 (36.8)
	Negative	22 (32.4)
	Not available	21 (30.9)
PR status	Positive	22 (32.4)
	Negative	25 (36.8)
	Not available	21 (30.9)
Her2/neu status	Positive	14 (20.6)
	Negative	5 (7.4)
	Not available	49 (72.1)
Chemotherapy intent	Adjuvant	39 (57.4)
	Neoadjuvant	14 (20.6)
	Palliative	15 (22)

[a] KIRAN (Karachi Institute of Radiotherapy and Nuclear Medicine) is a specialized oncology hospital situated at the northern end of the city

[b] JPMC (Jinnah Postgraduate Medical Center) is a tertiary care hospital in Karachi

[c] Literally, immigrants. The Urdu-speaking majority of Karachi, whose ancestors migrated from India, when Pakistan came into being

[d] Only two patients

Pharmacokinetic analysis

CP and 4-OH-CP (as semicarbazide) plasma concentrations were quantified by HPLC-ESI-MS at mass-to-charge ratios (m/z) of 283 and 356, respectively. Ifosfamide was used as internal standard (m/z 283, eluted earlier than CP). The LC-MS platform LCMS-2010EV (Shimadzu, Japan) was used equipped with a Pursuit XRs C18 column (150×2.0 mm, 3 micron) and pre-column (Varian, CA, USA). Gradient elution at a total flow rate of 0.2 ml/min was applied [acetonitrile/water: 15% (0–7 min), 30% (7–8 min), 75% (8–9 min), 15% (9–10 min), 15% (10–16 min)].

Plasma samples were purified by solid-phase extraction [15] using Bond Elut C18 cartridges, 1 ml (Varian). After conditioning with 1 ml acetonitrile and 1 ml water, plasma samples were loaded followed by washing with 2 ml water. Finally, samples were eluted with 100% acetonitrile, evaporated to dryness, and reconstituted in 1 ml water.

The minimum limit of detection was 10 ng/ml for cyclophosphamide and 100 ng/ml for 4OH cyclophosphamide applying a signal-to-noise ratio of 3:1. The validation study involved concentrations of 0.125, 0.25, 0.5, and 1.0 μg/ml. The precision (coefficient of variance) was 7% at the lowest and 5% at the highest validation level; the mean accuracy for CP was 90%, whereas for 4-OH-CP, it was 103%. All determinations were performed six times. The analyte stability was confirmed at −80°C over 4 weeks.

Pharmacogenetic analysis Genotyping for *CYP2B6*4* (785A >G; rs2279343), **5* (1459 C >T; rs3211371), and **6* (516 G >T; rs3745274 and 785A >G); *CYP2C9*2* (430 C >T; rs1799853) and **3* (1075A >C; rs1057910); *CYP2C19*2*

Table 2 Plasma CP and 4-OH-CP levels (μg/ml)

	Number	Minimum	Maximum	Mean	Standard deviation
Cyclophosphamide					
Baseline[a]	68	2.35	5.27	4.03	0.53
After 60 min	67	2.88	4.56	3.83	0.37
After 120 min	54	2.84	5.76	3.74	0.46
4-OH-Cyclophosphamide					
Baseline[a]	68	0.33	4.35	1.84	0.82
After 60 min	67	0.73	3.11	1.68	0.59
After 120 min	53	0.59	3.15	1.55	0.55

[a] At the end of cyclophosphamide infusion

(681 G >A; rs4244285); *CYP3A5*3* (6986A >G; rs776746); *GSTA1* –69 C >T; rs3957357 and –52 G >A; rs3957356 (representing *GSTA1*A* and *GSTA1*B* haplotypes); and *ALDH3A1*2* (985 C >G; rs2228100) was performed by PCR-RFLP or pyrosequencing as described previously [2, 6, 16].

Statistical analysis The data were analyzed using SPSS® v.17.0. The sample size was calculated under the assumption that a change in metabolic ratios of 1.5 would be clinically significant. A *post hoc* analysis regarding genotype differences between the present study population and Caucasians revealed a statistical power of 89% using StatCalc® software. Student's *t*-test was applied for continuous variables and χ^2 (with Yates' correction for continuity) or Fisher's exact test for categorical variables. Mann-Whitney *U* test was applied followed by Jonckheere-Terpstra trend test where appropriate. Univariate/multivariate analysis was used to identify genotypes that significantly predict the outcome. Logistic regression analysis was applied to calculate the risk of severe leucopenia in relation to genotypes. All analyses were two-sided and only a *p*-value less than 0.05 was considered significant.

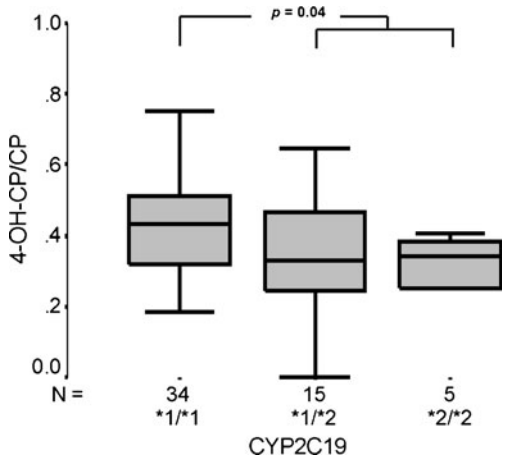

Fig. 1 Influence of *CYP2C19* genotype on metabolic ratio (4-OH-CP:CP). The data are arranged in boxplots representing the metabolic ratio after 120 min. The 4-hydroxylation of cyclophosphamide is significantly influenced by the *CYP2C19* genotype

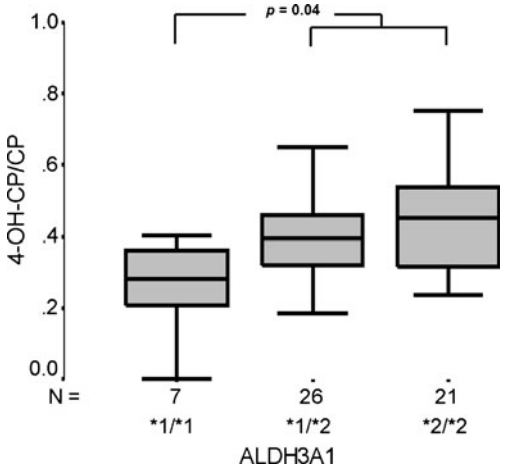

Fig. 2 Influence of *ALDH3A1* genotype on metabolic ratio (4-OH-CP:CP). The data are arranged in boxplots representing the metabolic ratio after 120 min. A lower level of 4-OH cyclophosphamide in the case of homozygous **1/*1 ALDH3A1* genotype reflects higher metabolic clearance

Results

The baseline characteristics of 68 consecutive breast cancer patients are presented in Table 1. The mean age (±SD; range) was 44 (±9.7; 25–70) years. All patients received six cycles of FAC chemotherapy protocol. Their hospital stay and pretreatment were similar. All major ethnic groups in Pakistan were represented in this study. There was a tendency to present with advanced disease at an early age. None of the patients presented at stage I, whereas 28% had a node negative disease. All investigated genotypes were in Hardy-Weinberg equilibrium. The genotype frequency data are presented elsewhere [6].

Plasma values of CP and 4-OH-CP are given in Table 2. On stratifying CP pharmacokinetics to single genotypes, neither the elimination of the parent compound CP nor of the metabolite 4-OH-CP was significantly influenced by any genotype of the investigated metabolic enzymes. Also application of the univariate analysis of variance revealed no further statistical significance. However, analysis of the ratio between 4-OH-CP and CP, which reflects the metabolic capacity of the involved enzymes, indicated that the 4-OH-CP:CP ratio after 120 min was significantly affected by *CYP2C19*2* (p=0.039) and *ALDH3A1*2* (p=0.037), and there was also a significant gene-dose effect for *CYP2C19* (Jonkheere-Terpstra test, p=0.045) but not for *ALDH3A1* (p=0.13). The carriers of the wild-type *CYP2C19*1/*1* genotype

Table 3 4-OH-Cyclophosphamide-to-cyclophosphamide ratio in relation to drug metabolizing enzyme genotype

Gene		w/w			w/v			v/v			p^a		
	Ratio 4-OH-CP:CP	Number	Mean	S.D.	Number	Mean	S.D.	Number	Mean	S.D.	(w/w vs. w/v)	(w/v vs. v/v)	(w/w vs. w/v + v/v)
CYP2B6[b]	At 60 min	11	0.40	0.20	39	0.46	0.12	12	0.37	0.12	0.12	0.03	0.09
	At 120 min	6	0.37	0.14	32	0.41	0.14	12	0.38	0.12	0.60	0.46	0.56
CYP2C9[c]	At 60 min	46	0.42	0.13	15	0.47	0.14	6	0.44	0.21	0.39	0.53	0.55
	At 120 min	42	0.41	0.14	9	0.41	0.15	3	0.34	0.14	0.86	0.23	0.73
CYP2C19[d]	At 60 min	39	0.46	0.14	20	0.40	0.14	8	0.38	0.11	0.17	0.78	0.09
	At 120 min	34	0.43	0.13	15	0.36	0.17	5	0.33	0.07	0.13	0.76	0.04
CYP3A5[e]	At 60 min	1	0.51	-	26	0.47	0.14	37	0.42	0.15	0.52	0.32	0.29[f]
	At 120 min	1	0.46	-	20	0.41	0.15	30	0.41	0.14	0.74	0.79	0.89[f]
ALDH3A1[d]	At 60 min	9	0.42	0.17	31	0.42	0.12	27	0.46	0.15	0.97	0.44	0.75
	At 120 min	7	0.29	0.19	26	0.40	0.10	21	0.44	0.15	0.06	0.34	0.04

[a] Mann-Whitney U-test

[b] w/w = *1/*1, w/v = *1/*4, *1/*5 or *1/*6, v/v = *4/*5, *5/*5, *6/*6 or *6/*7

[c] w/w = *1/*1, w/v = *1/*2 or *1/*3; v/v = *3/*3

[d] w/w = *1/*1, w/v = *1/*2; v/v = *2/*2

[e] w/w = *1/*1, w/v = *1/*3, v/v = *3/*3

[f] (*1/*1 + *1/*3) vs. *3/*3

exhibited a 1.19-fold higher ratio than heterozygote and a 1.3-fold higher ratio than homozygote *CYP2C19*2* allele carriers (Fig. 1). In contrast, the carriers of the wild-type *ALDH3A1*1/*1* genotype had lower 4-OH-CP:CP ratios after 2 h than heterozygotes (factor 0.72) or homozygotes (factor 0.66, Fig. 2). Although *CYP2C9* low-activity genotypes also demonstrated lower formation of 4-OH-CP, this finding did not reach statistical significance (Table 3). These effects did not remain significant in a univariate analysis of variance including all genotypes.

To investigate the role of pharmacogenetics in detoxification, the side effect of acquired leucopenia was assessed in relationship to drug metabolizing enzyme genotypes (Table 4). Loss of at least one functional allele of GSTA1 predisposed to severe leucopenia (p=0.004; OR 4.48; 95% CI 1.58–12.7). This observation remained valid even after binary logistic regression analysis correcting for age and all other dichotomous genotypes (OR 6.94; 95% CI 1.75–27.6, p=0.006). Additional analyses did not reveal any significant correlation between any 4-OH-CP:CP ratios and presence of leucopenia (data not shown).

Table 4 Association of total leukocyte count at 10th post chemotherapy day to the genotype of drug metabolizing enzymes

Gene	Genotype	Number (%)		p^a
		≤2,500 mm^{-3}	>2,500 mm^{-3}	
CYP2B6	*1/*1 *1/variant variant/variant	19 (73.1) 7 (26.9)	30 (83.3) 6 (16.7)	0.33
CYP2C9	*1/*1 *1/*2, *1/*3, **2/*3, *3/*3	20 (71.4) 8 (28.6)	25 (64.1) 14 (35.9)	0.53
CYP2C19	*1/*1 *1/*2 + *2/*2	13 (46.4) 15 (53.6)	26 (66.7) 13 (33.3)	0.10
CYP3A5	*1/*1 + *1/*3 *3/*3	14 (50) 14 (50)	14 (38.9) 23 (61.1)	0.37
ALDH3A1	*1/*1 *1/*2 + *2/*2	3 (10.7) 25 (89.3)	7 (17.9) 32 (82.1)	0.42
	*1/*1 + *1/*2 *2/*2	15 (53.6) 13 (46.4)	25 (64.1) 14 (35.9)	0.39
GSTA1 -69/-52	*A/*A *A/*B + *B/*B	17 (60.7) 11 (39.3)	10 (25.6) 29 (74.4)	0.004

[a] Chi-squared test

Discussion

In vitro studies have shown, though inconsistently, that several cytochrome P450 enzymes including *CYP2B6* [3, 17], *CYP2C9* [18, 19], *CYP2C19* [18, 20], and *CYP3A4/5* [19] are capable of 4-hydroxylation of cyclophosphamide.

In a study conducted on lupus nephritis patients who received pulsed low dose cyclophosphamide treatment [11], *CYP2C19*2* carriers had a significantly lower risk of developing premature ovarian failure, indicating less bioactivation of cyclophosphamide. Timm et al. [4] demonstrated that metabolic clearance of cyclophosphamide is significantly dependent on *CYP2C19* genotype at CP doses <1,000 mg/m^2.

In contrast, Nakajima et al. [9] could not demonstrate any effect of *CYP2C19* on CP pharmacokinetics in a larger but heterogeneous cohort. Some earlier studies had shown that *CYP2C19* has low affinity (high *Km*) [18, 20], whereas *CYP2C9* has high affinity (low *Km*) hydroxylase activity for cyclophosphamide [18, 19].

In the absence of the limitations encountered by Timm et al. [4] (no 4-OH-CP levels could be determined and no outcome data were available), our study confirms that *CYP2C19* influences the bioactivation of cyclophosphamide and shows a gene-dose effect. All patients in our present study were female breast cancer patients who received low dose cyclophosphamide as a part of the FAC protocol. Their baseline hepatic and renal functions were within normal limits, the pretreatment was similar, and they were treated in a similar fashion. The in vivo involvement of *CYP2C9* genotypes in cyclophosphamide metabolism seems to have a minor impact. In our study, *CYP2C9* genotypes showed a trend similar to *CYP2C19* but did not reach statistical significance, confirming observations by Timm et al. [4], Ekhart et al. [8], and Xie et al. [10].

Aldehyde dehydrogenases catalyze the formation of the inactive carboxyphosphamide from aldophosphamide thus contributing to detoxification. Therefore, it could be hypothesized that increased expression of *ALDH3A1* may play a role in the development of tumor resistance. Indeed, transfection of *ALDH3A1* expression vectors has been shown to decrease the sensitivity to cyclophosphamide [21], but Ekhart et al. [8] could not find an effect of *ALDH3* genotypes on the pharmacokinetics of 4-OH-CP in a cohort of patients treated with high dose cyclophosphamide.

However, we could demonstrate that 4-OH-CP:CP ratio after 120 min was significantly influenced by *ALDH3A1* in a gene-dose-related manner. After a univariate analysis of variance however, no genotype remained significant, possibly due to the limited sample size. The toxicity of the chemotherapy was analyzed with respect to the leukocyte count. Glutathione conjugation catalyzed by polymorphic *GSTA1* contributes to the detoxification of reactive nucleophiles of cyclophosphamide [22]. This is also applicable to doxorubicin. However, there are not enough data that suggest a substantial preventive role of *GSTA1* on the doxorubicin-induced cytotoxicity in vivo, especially in the lower doses commonly used in chemotherapy. There is some evidence in vitro for high doses of doxorubicin [23]. Therefore, *GSTA1*, the most abundant hepatic isoenzyme, was included to assess its effect on cyclophosphamide clearance in this study. In accordance with Ekhart et al. [8], Nakajima et al. [9], and Timm et al. [4], no effect of *GSTA1* polymorphism could be demonstrated on plasma drug or metabolite levels. However, we could demonstrate that it strongly influences the myelotoxicity. Carriers of *GSTA1*A/*A* showed 5.7-fold less likelihood of experiencing a leukocyte drop below 2.5×10^9/L. As a consequence, they also required minimal correction of blood count and needed less vigorous antimicrobial therapy. This significant finding is in contrast to a recent paper by Yao et al. [24] showing no impact of *GSTA1* but of *GSTP1*. In that study however, only one tagging SNP of *GSTA1* was investigated. Since GSTs conjugate reactive nucleophiles, loss of function is reflected in terms of the susceptibility to toxicity rather than altering plasma levels of the parent drug. At present, these SNPs may be suitable to estimate or explain adverse effects. Further studies are needed to assign any role in a priori dose adapting.

In conclusion, we could confirm the importance of *CYP2C19* as a modulator of 4-OH-CP formation in a single approach. In addition, *ALDH3A1* remained the only further significant factor contributing to 4-OH-CP clearance. The myelotoxicity of CP treatment, however, was associated with genotypes of the detoxifying phase-II enzyme *GSTA1*. Carriers of *GSTA1*B* were at significant increased risk of leucopenia compared to *GSTA1*A/A* subjects.

Acknowledgments The research grant was provided by Higher Education Commission Pakistan and Ziauddin University, Karachi, Pakistan. The authors are thankful to all the administration and staff of Karachi Institute of Radiotherapy and Nuclear Medicine (KIRAN) and Jinnah Postgraduate Medical Center (JPMC), Karachi, Pakistan, as well as Dr. Rubina Ghani, Molecular and Pathological Laboratories, Karachi; Prof. N.A. Jafarey, Ziauddin University, Karachi; Dr. Sandra Lächelt, Dr. Denisa May, Jutta Finger, and Marion Pauer of the Institute of Experimental and Clinical Pharmacology, CAU, Kiel, for their support.

The study is dedicated to Dr. Muhammad Noorul Afsar [1931–1991; MBBS (Dhaka, Bangladesh), DTM&H (Liverpool, UK)] for his philanthropic general practice and life-long support of rational drug use.

Conflict of interest It is declared that there is no conflict of interest regarding this study.

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