The Pharmacogenomics Journal (2017) 17, 337–343

www.nature.com/tpj

ORIGINAL ARTICLE

Pharmacogenetics of *ABCB5*, *ABCC5* and *RLIP76* and doxorubicin pharmacokinetics in Asian breast cancer patients

S Lal[1], N Sutiman[2], LL Ooi[3], ZW Wong[4], NS Wong[4], PCS Ang[4] and B Chowbay[1,4,5]

This study investigated the impact of *ABCB5*, *ABCC5* and *RLIP76* polymorphisms on doxorubicin pharmacokinetics in Asian breast cancer patients ($N = 62$). Direct sequencing was performed to screen for previously identified *ABCC5* polymorphisms as well as polymorphisms in the exons and exon–intron boundaries of *ABCB5* and *RLIP76* genes. Genotype–phenotype correlations were analyzed using Mann–Whitney *U*-test. The homozygous variant allele at the *ABCC5* g.+*7161G>A* (rs1533682) locus was significantly associated with higher doxorubicin clearance (g.+*7161AA* vs g.+*7161GG*, CL/BSA ($Lh^{-1}m^{-2}$): 30.34 (25.41–33.60) vs 22.46 (15.04–49.4), $P = 0.04$). Homozygosity for the reference allele at the *ABCC5* g.-*1679T>A* locus was associated with significantly higher doxorubicinol exposure (g.-*1679TT* vs g.-*1679TA*, $AUC_{0-\infty}$/dose/BSA (hm^{-5}): 15.48 (6.18–67.17) vs 8.88 (3.68–21.71), $P = 0.0001$). No significant influence of the three newly identified *ABCB5* polymorphisms (c.*2T>C*, c.*343A>G* and c.*1573G>A*) on doxorubicin pharmacokinetics was observed. No polymorphisms were identified in the *RLIP76* gene. These findings suggest that *ABCC5* polymorphisms may explain partially the interpatient variability in doxorubicin disposition.

The Pharmacogenomics Journal (2017) **17,** 337–343; doi:10.1038/tpj.2016.17; published online 15 March 2016

INTRODUCTION

Doxorubicin is an anthracycline chemotherapy agent that is widely used for the curative, adjuvant and palliative treatment of various malignancies. In particular, the combination of doxorubicin and cyclophosphamide (AC regimen) is widely used in the adjuvant settings for the treatment of primary breast cancer. The concerted actions of various influx and efflux transporters that transfer doxorubicin across cellular membranes and the metabolizing enzymes responsible for its biotransformation have been postulated to have significant roles in determining intracellular doxorubicin levels and influencing its disposition (Figure 1). Its main metabolite, doxorubicinol, exhibits lower antitumor potency as compared with doxorubicin,[1,2] but has been shown to accumulate in cardiac tissues and is reportedly 10–30 times more potent than the parent drug at depressing cardiac contractility and causing doxorubicin-induced cardiotoxicity.[2,3] Variability in the pharmacokinetics of doxouribin and its metabolite doxorubicinol have therefore been postulated to contribute to the wide inter-individual variability in doxorubicin treatment outcomes and toxicity profiles.

Although several *in-vitro* studies have identified the prototypical ATP-binding cassette (ABC) transporter ABCB1 as the major candidate mediating doxorubicin resistance,[4–6] doxorubicin transport from multidrug-resistant cells that do not overexpress ABCB1 suggests that efflux mechanism(s) distinct from ABCB1 may be involved in altering its disposition and mediating doxorubicin resistance.[7–9] We have previously reported on the pharmacogenetic influence of transporters belonging to the ABC superfamily (ABCB1, ABCG2) and the solute carrier family (SLC22A16), drug-metabolizing enzymes such as carbonyl reductases 1 and 3 (CBR1 and CBR3, respectively), as well as regulatory nuclear receptors, such as the pregnane-X-receptor (PXR), in relation to doxorubicin pharmacokinetics in Asian breast cancer patients.[10–13] However, more recent studies have highlighted the potential importance of other transporters and proteins in modulating the disposition of doxorubicin, including ABCB5, ABCC5 and RLIP76.

ABCB5 is the third member of the human ABC transporter family.[14–16] Similar to ABCB1, ABCB5 has been shown to mediate drug resistance in human cancer cells.[17,18] ABCB5-mediated efflux of doxorubicin has previously been identified as the mechanism underlying resistance to doxorubicin in ABCB5-expressing G3361 human melanoma cells.[17] Specifically, melanoma cells expressing ABCB5 had significantly lower doxorubicin accumulation compared with those that did not express ABCB5.[17] More recently, Yang *et al.*[19] also found that ABCB5 was upregulated in doxorubicin-resistant breast MCF-7 clones. These findings suggest that ABCB5 may contribute to alterations in the disposition and intracellular levels of doxorubicin as well as doxorubicin resistance phenotype.

ABCC5 (Canalicular multispecific organic anion transporter C, MRP5/ABC33) belongs to the ABCC subfamily.[20] Unlike the long type of ABCC proteins, it belongs to the short type of ABCC proteins that lacks of an N-terminal transmembrane domain.[21] As an efflux transporter, ABCC5 has been shown to mediate the ATP-dependent transport of several anticancer drugs, including doxorubicin, and has been reported to confer resistance to a number of chemotherapy agents, including methotrexate, and the thymidilate synthase inhibitor raltitrexed. In a study by Yoshida *et al.*[22], analysis of *ABCC5* mRNA levels in doxorubicin-resistant human lung cancer cells SBC-3/ADM, AdR MCF-7 and K562/ADM showed markedly high expression of *ABCC5* transcripts relative to their respective parental cell lines. Pratt *et al.*[23] further demonstrated that ABCC5-transfected HEK cells had a twofold higher

[1]Laboratory of Clinical Pharmacology, Division of Medical Sciences, Humphrey Oei Institute of Cancer Research, National Cancer Centre, Singapore; [2]Clinical Pharmacology, SingHealth, Singapore; [3]Division of Surgical Oncology, National Cancer Center, Singapore; [4]Division of Medical Oncology, National Cancer Center, Singapore and [5]Clinical Sciences, Duke-NUS Graduate Medical School, Singapore. Correspondence: Professor B Chowbay, Laboratory of Clinical Pharmacology, Division of Medical Sciences, Humphrey Oei Institute of Cancer Research, National Cancer Centre, Singapore 169610, Singapore.
E-mail: ctebal@nccs.com.sg
Received 3 August 2015; revised 4 February 2016; accepted 9 February 2016; published online 15 March 2016

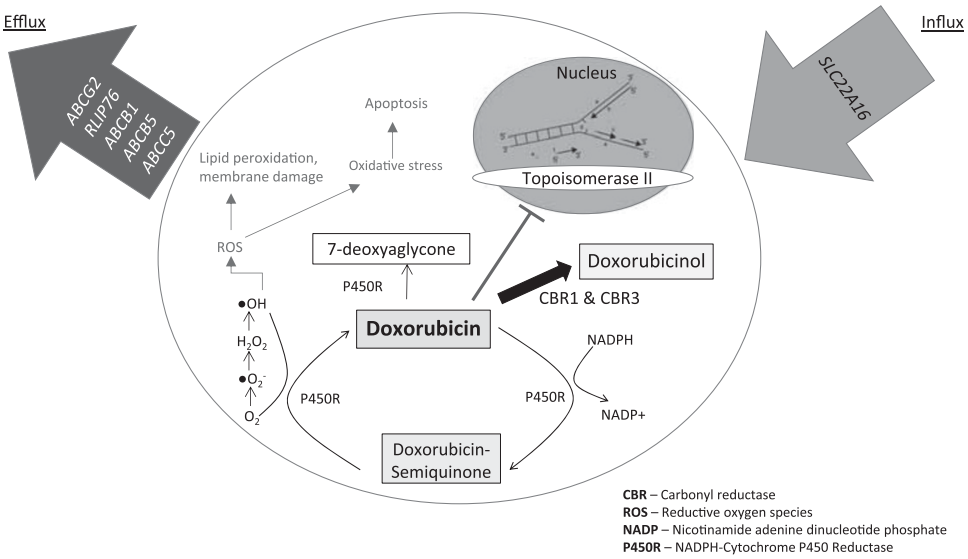

Figure 1. Pharmacology of doxorubicin.

resistance to doxorubicin than non-transfected cells. Taken together, these findings suggest that *ABCC5* expression and activity may contribute to variability in doxorubicin disposition observed in cancer patients.

RLIP76 (Ral-binding protein 1, 76 kD) is a multifunctional GTPase-activating transporter protein that is ubiquitously expressed.[24] RLIP76 share some similarities with the ABC transporters. The sequence of its ATP-binding sites in the N-terminal (69GKKKGK74) and *C*-terminal domains (418GGIKDLSK425), for instance, are similar with the P-loop in the ABC transporters.[25] However, transmembrane helices have not been identified in the *RLIP76* sequence. Taking into account the overexpression of RLIP76 in some cancer cells, the ability of these cells to develop resistance to anticancer agents has highlighted the potential importance of RLIP76 as a target for cancer therapy in recent years. Indeed, RLIP76 has been demonstrated to be involved in the energy-dependent efflux of doxorubicin.[26] RLIP76-overexpressing cells have also been shown to exhibit markedly increased efflux of doxorubicin and acquired resistance to doxorubicin-induced cytotoxicity.[27,28] Indeed, the inherently low intracellular accumulation of doxorubicin because of RLIP76-mediated efflux in non-small cell lung cancer has been attributed as the primary mechanism underlying doxorubicin resistance.[29,30] These findings suggest that transport by RLIP76 may prevent normal and malignant tissues from developing doxorubicin-induced toxicities. Although the *RLIP76* gene is polymorphic, the impact of *RLIP76* polymorphisms on doxorubicin disposition has not been evaluated in doxorubicin-treated cancer patients, including in the Asian population.

The present exploratory study examined the pharmacogenetics of additional efflux transporters, specifically *ABCB5*, *ABCC5* and *RLIP76* and their possible influence on the pharmacokinetics of doxorubicin in the same subset of Asian breast cancer patients. The pharmacogenetic profiles of the *ABCB5*, *ABCC5* and *RLIP76* genes were screened in three healthy Asian populations, namely the Chinese, Malays and Indians and the genotypic–phenotypic effects of identified polymorphisms in these genes were subsequently investigated in Asian breast cancer patients receiving the combination of doxorubicin and cyclophosphamide as adjuvant chemotherapy.

MATERIALS AND METHODS

Healthy subjects

The healthy population consisted of the three predominant ethnic groups in Singapore (Chinese, $n = 100$; Malays, $n = 100$; Indians, $n = 100$), for whom ethnicities were confirmed against their National Registry Identification Cards. Participants provided both written as well as verbal informed consent to participate in the study. The study was approved by the ethics review committee of the National Cancer Center, Singapore.

Breast cancer patients

Patients with histologically confirmed invasive breast cancer and receiving adjuvant chemotherapy with doxorubicin and cyclophosphamide were recruited ($n = 62$). Both verbal and written informed consent was obtained from all patients. The Patient Information and Consent forms, along with the study protocol were approved by the institutional ethics committee at the National Cancer Centre, Singapore. Patient selection criteria have been reported previously.[12]

Genotyping of *ABCB5*, *ABCC5* and *RLIP76* polymorphisms

Genomic DNA was extracted from the peripheral blood samples (5 ml) using the phenol–chloroform extraction method as described previously.[12] Direct DNA sequencing was performed to screen for polymorphisms in the exon and exon–intron boundaries of the *ABCB5* (Genbank accession no: AB353947) and *RLIP76* (Genbank accession no: NM_006788) genes, as well as *ABCC5* polymorphisms (5′-UTR: g.-*1990G>A*, g.-*1821T>C*, g.-*1679T>A*, g.-*1205C>T*, g.-*793C>A*, g.-*889T>C*; intron 5: i.*374C>T* (rs3749438); exon 8: c.*1145A>G*; exon 9: c.*1185T>C* (rs1132776); exon 12: c.*1782T>C* (rs939336); intron 1: i.*1834C>T* (rs4148557); intron 2: i.*7980C>T* (rs2292997); exon 25: c.*3624C>T* (rs3749442); exon 30: c.*4896G>A* (rs3749445), c.*5557A>G* (rs562) and 3′-UTR g.+*6272A>G* (rs1000002), g.+*7161G>A* (rs1533682)) as identified by Gwee *et al.*[31]

Pairwise correlation analysis between polymorphisms

Pairwise correlations between these polymorphisms and previously reported polymorphisms in *ABCB1*, *ABCG2*, *SLC22A16*, *CBR1*, *CBR3* and *PXR* genes were quantified by $|D'|$ and rho square (r^2) values (Haploview, v4.2, Daly Lab, Broad Institute, Cambridge, MA, USA). The following polymorphisms were included: *ABCB1* (*c.1236C>T* (rs1128503), *c.3435C>T* (rs1045642)), *ABCG2* (*c.421C>A* (rs2231142)), *SLC22A16* (*c.146A>G* (rs714368), *c.755T>C* (rs6907567), *c.1226T>C* (rs12210538), *c.312T>C* (rs6907567)), *CBR1* (g.-48G>A, c.219G>C (rs25678), +967G>A, c.693G>A, c.627C>T), *CBR3* (c.11G>A (rs8133052), c.255T>C, c.279C>T, c.606G>A, c.730G>A) and *PXR* (-31273A>G (rs9832958),

Table 1. Summary of doxorubicin pharmacokinetic parameters in Asian breast cancer patients

Pharmacokinetic parameters	*Median (range)*	*CV, %*
Doxorubicin		
C_{max}/dose/BSA (m^{-5})	34.1 (4.5–97.7)	57.9
$AUC_{0-\infty}$/dose/BSA (hm^{-5})	17.5 (6.2–67.2)	49.9
$t_{1/2}$ (h)	15.4 (4.7–24.6)	20.5
CL (Lh^{-1})	36.0 (11.3–89.4)	36.5
CL/BSA ($Lh^{-1}m^{-2}$)	22.9 (8.7–55.7)	34.1
V_{ss}/BSA (Lm^{-2})	237.5 (53.9–703.7)	47.5
Doxorubicinol		
C_{max}/dose/BSA ($10^{-2}m^{-5}$)	0.33 (0.15–2.0)	76.0
$AUC_{0-\infty}$/dose/BSA (hm^{-5})	9.6 (3.7–24.3)	41.9
$t_{1/2}$ (h)	27.5 (6.3–112.4)	52.7

Abbreviations: AUC, area under the plasma concentration-time curve; BSA, body surface area; CV, coefficient of variation.

-1570C>T (rs3814055), -566A>C (rs1523127), -298G>A (rs2276706), -23914T>G (rs3814056), -6994T>C (rs2472677), -601A>G (rs7643645), IVS2+55A>G (rs1464603), IVS2+78A>G (rs1464602), IVS2-1131C>T (rs2472680), IVS3+72T>G (rs3732356), IVS3+648T>C (rs2472681), IVS4 +285G>A (rs3732357), IVS5+845C>A (rs2472682), IVS5-93A>G (rs6785049), IVS6-17C>T (rs2276707), 1448G>A, 1437G>A (rs3732358), 1792A>G (rs3732359), 1944T>C (rs3732360), 2180A>G (rs6438550), 2617A>C (rs3814057), 2654T>C (rs3814058)).[10–13]

Doxorubicin administration and pharmacokinetic analysis

Intravenous doxorubicin was administered at 60 mg m^{-2} over 20 min with standard pre-medications, including intravenous dexamethasone 10 mg, diphenhydramine 50 mg, cimetidine 300 mg or ranitidine 50 mg. Intravenous cyclophosphamide was administered at a dose of 600 mg m^{-2} over 30 min. Each cycle lasted 3 weeks. Blood samples for pharmacokinetic analysis of doxorubicin were collected at 0, 5, 15 and 30 min, and at 1, 4, 8 and 24 h. Reverse-phase high-performance liquid chromatography with fluorescence detection was used for the quantification of plasma concentrations of doxorubicin and its major metabolite, doxorubicinol, as previously described.[12]

Pharmacokinetic parameters were determined using non-compartmental methods with a non-linear regression program on WinNonLin version 2.1 (Pharsight, Mountain View, CA, USA). Peak plasma concentrations (C_{max}) were directly identified from the concentration-time curves of each individual. The trapezoidal rule was used to calculate the area under the plasma concentration-time curve (AUC) from time zero to the time (t) of the last detectable concentration ($AUC_{0\rightarrow t}$). AUC was extrapolated to infinity ($AUC_{0-\infty}$) by adding $C_t/\lambda z$ to $AUC_{0\rightarrow t}$, where C_t was the last detectable plasma concentration and λz is the elimination rate constant.

Statistical analyses

The chi-square (χ^2) test was used to assess for the departure of genotype frequencies from Hardy–Weinberg equilibrium. The statistical differences between the pharmacokinetic parameters among the genotype groups were analyzed by the nonparametric Mann–Whitney *U*-test, with statistical significance set at $P<0.05$. The effects of demographics and clinical characteristics, such as ethnicity, age, body surface area (BSA), on the pharmacokinetic parameters of doxorubicin and doxorubicinol were evaluated using the univariate linear regression analysis. In addition, univariate linear regression analysis was performed to evaluate the associations between polymorphisms reported in this study as well as previously reported studies,[10–13] as listed above, with the pharmacokinetic parameters of doxorubicin. Covariates found to be significant ($P<0.05$) in the univariate analysis were included in the multivariate regression model and goodness-of-fit of the model was assessed using R^2. All statistical analyses were performed using STATA v7.0 (College Station, TX, USA) and SPSS (Chicago, IL, USA).

Table 2. Genotype and allele frequency of *ABCB5* polymorphisms in healthy subjects and Asian breast cancer patients

Populations	N	*Genotype frequencies,* N *(%)*			*Allele frequencies*	
*c.*2T>C *(rs34603556)*		TT	TC	CC	T	C
Healthy subjects						
Chinese	100	92 (92)	8 (8)	0 (0)	0.96	0.04
Malays	100	90 (90)	10 (10)	0 (0)	0.95	0.05
Indians	100	77 (77)	23 (23)	0 (0)	0.89	0.12
Asian cancer patients	62	58 (93.5)	4 (6.5)	0 (0)	0.97	0.03
*c.*343A>G *(rs2301641)*		AA	AG	GG	A	G
Healthy subjects						
Chinese	100	77 (77.8)	17 (17.1)	5 (5.1)	0.86	0.14
Malays	100	67 (70.5)	22 (23.2)	5 (5.3)	0.83	0.17
Indians	100	80 (80)	17 (17)	3 (3)	0.89	0.11
Asian cancer patients	62	57 (91.9)	4 (6.5)	1 (1.6)	0.95	0.05
*c.*1573G>A *(rs6461515)*		GG	GA	AA	G	A
Healthy subjects						
Chinese	100	20 (20)	2 (2)	78 (78)	0.21	0.79
Malays	100	17 (17.3)	16 (16.3)	65 (66.3)	0.26	0.74
Indians	100	22 (22)	12 (12)	66 (66)	0.28	0.72
Asian cancer patients	62	2 (3.2)	12 (19.4)	48 (77.4)	0.13	0.87

Abbreviation: *N*, number of subjects.

RESULTS

Patient demographics

As previously reported, the Asian breast cancer patients consisted of Chinese ($N=46$, 74%), followed by Malays ($N=11$, 18%) and Indians ($N=5$, 8%). The median age, height and BSA of the patients were 51 years (range: 29–73 years), 154 cm (range: 144–168 cm) and 1.52 m^2 (range: 1.23–1.95 m^2), respectively.

Doxorubicin pharmacokinetics

Plasma samples for pharmacokinetic analysis were available in 52 of the 62 recruited Asian breast cancer patients. The pharmacokinetics parameters of doxorubicin and doxorubicinol in these patients are summarized in Table 1. Wide interpatient variability in the pharmacokinetic parameters of doxorubicin and doxorubicinol was observed and has been described previously.[12] Heights and weights were shown to be significant covariates affecting doxorubicin $AUC_{0-\infty}$ ($P<0.015$ and $P<0.0001$, respectively) and C_{max} ($P<0.028$ and $P<0.0001$, respectively). Age and ethnicity were shown to be significant covariates affecting doxorubicin half-life ($P<0.018$). Markers of hepatic (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, bilirubin) and renal (serum creatinine) functions were not shown to significantly influence doxorubicin pharmacokinetic parameters.

ABCB5, *ABCC5* and *RLIP76* genotype distributions

Three novel polymorphisms were identified by direct sequencing of the coding regions of the *ABCB5* gene. All polymorphisms were nonsynonymous transitions: c.*2T>C* (exon 1; rs34603556), c.*343A>G* (exon 2; rs2301641) and c.*1573G>A* (exon 12; rs6461515) polymorphisms resulting in the p.M1T, p.K115E and p.E525K amino-acid changes, respectively. Table 2 summarizes the

Table 3. Genotype and allele frequencies of *ABCC5* polymorphisms in Asian breast cancer patients ($N = 62$)

Polymorphisms	*NCBI dbSNP ID*	*Regions*	*Genotypes,* N *(%)*			*Allele frequencies*	
g.*-1990G>A*	—	5′-UTR	*GG*	*GA*	*AA*	*G*	*A*
			40 (64.5)	21 (33.9)	1 (1.6)	0.81	0.19
g.*-1821T>C*	—	5′-UTR	*TT*	*TC*	*CC*	*T*	*C*
			40 (64.5)	21 (33.9)	1 (1.6)	0.81	0.19
g.*-1679T>A*	—	5′-UTR	*TT*	*TA*	*AA*	*T*	*A*
			27 (43.5)	35 (56.5)	0	0.72	0.28
g.*-1205C>T*	—	5′-UTR	*CC*	*CT*	*TT*	*C*	*T*
			8 (12.9)	26 (41.9)	28 (45.2)	0.66	0.26
g.*-889T>C*	—	5′-UTR	*TT*	*TC*	*CC*	*T*	*C*
			60 (96.8)	2 (3.2)	0	0.98	0.02
g.*-793C>A*	—	5′-UTR	*CC*	*CA*	*AA*	*C*	*A*
			27 (43.5)	34 (54.8)	1 (1.6)	0.71	0.29
i.*374C>T*	rs3749438	Intron 5	*CC*	*CT*	*TT*	*C*	*T*
			26 (41.9)	26 (41.9)	10 (16.1)	0.63	0.37
c.*1145A>G*	—	Exon 8	*AA*	*AG*	*GG*	*A*	*G*
			27 (44.3)	27 (44.3)	7 (11.5)	0.66	0.37
c.*1185T>C*	rs1132776	Exon 9	*TT*	*TC*	*CC*	*T*	*C*
			12 (19.4)	5 (8.1)	45 (72.6)	0.23	0.77
c.*1782T>C*	rs939336	Exon 12	*TT*	*TC*	*CC*	*T*	*C*
			5 (8.5)	16 (27.1)	38 (45.8)	0.22	0.78
i.*1834C>T*	rs4148557	Intron 1	*CC*	*CT*	*TT*	*C*	*T*
			35 (56.5)	22 (35.5)	5 (8.1)	0.74	0.26
i.*7980C>T*	rs2292997	Intron 2	*CC*	*CT*	*TT*	*C*	*T*
			26 (41.9)	35 (56.5)	1 (1.6)	0.70	0.30
c.*3624C>T*	rs3749442	Exon 25	*CC*	*CT*	*TT*	*C*	*T*
			30 (50.8)	25 (42.4)	4 (6.8)	0.72	0.28
c.*4896G>A*	rs3749445	Exon 30	*GG*	*GA*	*AA*	*G*	*A*
			24 (38.7)	27 (43.5)	11 (17.7)	0.60	0.40
c.*5557A>G*	rs562	Exon 30	*AA*	*AG*	*GG*	*A*	*G*
			24 (38.7)	27 (43.5)	11 (17.7)	0.60	0.40
g.*+6272A>G*	rs1000002	3′-UTR	*AA*	*AG*	*GG*	*A*	*G*
			11 (17.7)	28 (45.2)	23 (37.0)	0.40	0.60
g.*+7161G>A*	rs1533682	3′-UTR	*GG*	*GA*	*AA*	*G*	*A*
			32 (51.6)	23 (37.1)	7 (11.3)	0.70	0.30

genotype and allele frequencies of the *ABCB5* polymorphisms identified in the healthy subjects of three Asian ethnic groups and the breast cancer patients. All genotype frequencies conformed to Hardy–Weinberg equilibrium. The genotypic and allelic frequencies of these *ABCB5* polymorphisms have been previously reported.[32] The c.*2T>C* (rs34603556) polymorphism was approximately twofold higher in the healthy Indians (23%) compared with the Chinese (8%) and Malay (10%) populations. The allelic frequency of the c.2*C* variant was also significantly higher among the healthy Indian population (0.12) compared with the Chinese (0.05, $P = 0.007$) and Malay (0.04, $P = 0.026$) populations. The *ABCB5* c.*343A>G* (rs2301641) and c.*1573G>A* (rs6461515) polymorphisms did not differ significantly in frequencies between the three healthy Asian ethnic groups. Among cancer patients, the c.*343A>G* (rs2301641) variant allele was significantly lower in frequency (0.05) compared with the healthy Chinese population (0.14), whereas the frequencies of *ABCB5* c.*2T>C* (rs34603556) and c.*1573G>A* (rs6461515) were similar among the breast cancer patients as well as healthy subjects.

The genotype and allelic frequency of the *ABCC5* polymorphisms among Asian breast cancer patients were in agreement with previously published reports in healthy Asian populations (Table 3).[31] No polymorphisms were identified in the exon and exon–intron boundaries of the *RLIP76* gene in all the three Asian ethnic groups.

Pairwise correlations between polymorphisms

The *ABCC5* and *PXR* genes are both located on chromosomes 3. The *ABCB1* and *ABCB5* genes are both located on chromosomes 7. The *SLC22A16* and *ABCG2* genes are located on chromosomes 6 and 4, respectively, whereas *CBR1* and CBR3 genes are both located on chromosome 21. Although a classic linkage disequilibrium assessment could not be performed between the single-nucleotide polymorphisms in these genes as linkage disequilibrium analysis would necessitate the assessed single-nucleotide polymorphisms to be on the same chromosome, we performed pairwise correlation analysis between all the single-nucleotide polymorphisms that have been studied in these genes and the results are presented in Supplementary Table 1. Minimal correlations were observed between these single-nucleotide polymorphisms (pairwise $r^2 < 0.161$), suggesting that most of them are independent from one another.

Genotypic–phenotypic correlates

Pairwise comparisons failed to show any significant associations between the *ABCB5* c.*2T>C* (rs34603556), c.*343A>G* (rs2301641) and c.*1573G>A* (rs6461515) polymorphisms and the pharmacokinetic parameters of doxorubicin and doxorubicinol.

With regards to *ABCC5* polymorphic variants, significant genotypic–phenotypic correlations were observed with *ABCC5* g.*+7161G>A* (rs1533682) and g.*-1679T>A* polymorphisms (Table 4). Specifically, breast cancer patients homozygous for the variant *g.+7161A* (rs1533682) allele had significantly higher clearance of doxorubicin (CL/BSA ($Lh^{-1}m^{-2}$), median: 30.34; range: 25.41–33.60) when compared with patients who were homozygous for the g.*+7161G* allele (CL/BSA ($Lh^{-1}m^{-2}$), median: 22.46; range: 15.04–49.4, $P = 0.04$). In addition, breast cancer patients homozygous for the g.*-1679T* allele had significantly higher exposure levels of doxorubicinol

Table 4. The influence of *ABCC5* g.*+7161G>A* (rs1533682) and g.*-1679T>A* polymorphisms on doxorubicin pharmacokinetics in Asian breast cancer patients

Pharmacokinetic parameters	*ABCC5 genotypes median (range)*			*Pairwise* P-*values*[a]		
g.+7161G>A (rs1533682)	***GG (N=28)***	***GA (N=19)***	***AA (N=5)***	***GG vs AA***	***GG vs GA***	***GA vs AA***
Doxorubicin						
$AUC_{0-\infty}$/dose/BSA (hm^{-5})	18.3 (6.18–38.03)	18.46 (7.12–67.17)	15.89 (9.82–18.76)	0.24	0.91	0.26
C_{max}/dose/BSA (m^{-5})	34.01 (4.55–97.70)	38.67 (7.00–80.72)	20.91 (10.42–41.59)	0.67	0.99	0.65
CL/BSA ($Lh^{-1}m^{-2}$)	22.46 (15.04–49.4)	21.57 (8.68–55.66)	30.34 (25.41–33.60)	0.04	0.53	0.06
Doxorubicinol						
$AUC_{0-\infty}$/dose/BSA (hm^{-5})	9.61 (5.79–21.71)	9.84 (5.41–24.34)	8.43 (3.68–20.63)	0.33	0.39	0.66
C_{max}/dose/BSA (m^{-5})	0.39 (0.15–1.22)	0.33 (0.16–2.00)	0.31 (0.19–0.76)	0.32	0.91	0.53
g.-1679T>A	***TT (N=26)***	***TA (N=26)***	***AA (N=0)***	***TT vs TA***		
Doxorubicin						
$AUC_{0-\infty}$/dose/BSA (hm^{-5})	19.78 (9.04–31.63)	15.48 (6.18–67.17)		0.17		
C_{max}/dose/BSA (m^{-5})	41.28 (10.90–97.70)	29.06 (4.55–77.67)		0.23		
CL/BSA ($Lh^{-1}m^{-2}$)	22.34 (13.80–55.66)	24.52 (8.68–49.40)		0.47		
Doxorubicinol						
$AUC_{0-\infty}$/dose/BSA (hm^{-5})	15.48 (6.18–67.17)	8.88 (3.68–21.71)		0.0001		
C_{max}/dose/BSA (m^{-5})	0.33 (0.15–2.00)	0.37 (0.18–0.79)		0.38		

Abbreviations: AUC, area under the plasma concentration-time curve; BSA, body surface area; *N*, number of subjects. [a]$P < 0.05$ was considered statistically significant.

($AUC_{0-\infty}$/dose/BSA (hm^{-5}), median: 15.48; range: 6.18–67.17) as compared with patients who were heterozygous carriers of the polymorphism ($AUC_{0-\infty}$/dose/BSA (hm^{-5}), median: 8.88; range: 3.68–21.71, $P = 0.0001$).

Multivariate analysis conducted with all the polymorphisms studied in the *ABCB5* and *ABCC5* genes, as well as *ABCB1*, *ABCG2*, *SLC22A16*, *CBR1*, *CBR3* and *PXR* genes, revealed that only *CBR1* +967G>A genotype status contributed significantly to the overall variability in doxorubicin clearance, CL/BSA ($P = 0.037$) after adjustments for multiple covariates. The effects of other polymorphisms in the *ABCB5*, *ABCC5*, *ABCB1*, *ABCG2*, *SLC22A16*, *CBR1*, *CBR3* and *PXR* genes were not significantly associated with the pharmacokinetics of doxorubicin.

DISCUSSION

Screening the coding regions of the *ABCB5* gene in 300 healthy subjects of distinct Asian ethnic groups revealed three novel nonsynonymous polymorphisms (c.*2T>C* (exon 1; rs34603556), c.*343A>G* (exon 2; rs2301641) and c.*1573G>A* (exon 12; rs6461515)). Although differences were noted in the allele and genotype frequencies of *ABCB5* polymorphisms among the three Asian ethnic groups, genotype–phenotype analyses revealed a lack of significant associations between the identified *ABCB5* polymorphisms and doxorubicin pharmacokinetics in the Asian breast cancer patients. The potential contribution of ABCB5 in mediating doxorubicin transport and chemoresistance was first demonstrated in human malignant melanoma by Frank *et al.*[17] More recently, Kawanobe *et al.*[18] also reported that HEK293 cells transfected with *ABCB5* complementary DNA showed approximately 1.5-fold higher resistance to doxorubicin than the parental cells. However, the influence of *ABCB5* polymorphisms on the disposition of doxorubicin and its major metabolite doxorubicinol has not been investigated to date. Our results indicate for the first time that polymorphisms in the coding regions of the *ABCB5* gene do not significantly influence the disposition of doxorubicin and doxorubicinol.

It is important to note, however, that polymorphisms in both the intronic and promoter regions, which were not analyzed in this study, have been shown to influence *ABCB5* gene expression and activity.[33,34] Therefore, identifying the presence of *ABCB5* intronic and promoter variants in different populations may be important in elucidating their influence, if any, on the disposition of doxorubicin and doxorubicinol.

The sequencing of the *ABCC5* gene and its 3′-flanking region in Japanese by Saito *et al.*[35] identified 85 polymorphisms. Strong linkage disequilibrium between the 5′-UTR g.*-1205C>T* and 3′-UTR g.*+7161G>A* (rs1533682) polymorphisms has also been reported.[31] These two polymorphisms are separated by >100 kb, suggesting that the *ABCC5* gene is found within a region of strong linkage disequilibrium.[31] The *ABCC5* haplotype profiles were also observed to be highly similar between the Chinese and Malays, whereas those of Indian ethnicity had similar haplotype profiles with the Caucasians.[31] Similar to ABCB5, ABCC5 has also been shown to mediate resistance to doxorubicin, particularly in non-small cell lung cancer.[36,37] However, no studies to date have investigated the influence of the identified *ABCC5* polymorphisms on the pharmacokinetics of its substrates, including doxorubicin.

In this study, the *ABCC5* 3′-UTR g.*+7161G>A* (rs1533682) polymorphism was found to significantly influence the clearance of doxorubicin in our Asian breast cancer patients. Patients homozygous for the variant allele (*AA*) had approximately 35% significantly higher clearance of doxorubicin and 38% lower C_{max} values compared with the reference group (Table 4). It is not known, however, if the higher clearance and lower exposure levels of doxorubicin observed in patients carrying the 3′-UTR g.*+7161G>A* (rs1533682) polymorphism are associated with altered pharmacodynamic outcomes. Given that its antitumor potency is mainly attributed to the parent drug doxorubicin, the significantly higher clearance of doxorubicin observed in patients with the homovariant genotype status (AA) at this locus may potentially translate to poorer outcomes in these patients as compared with patients carrying wild-type alleles. However, further investigations with a larger sample size are necessary to confirm this.

Recently, Zhu *et al.*[38] demonstrated that miR-128 induced overexpression of ABCC5 in breast cancer cells and contributed to doxorubicin resistance. It is also interesting to note that the *ABCC5* gene is highly expressed in myocardial tissues, including endothelial and smooth muscle cells where it has a crucial role in the efflux transport of the second messenger 3′,5′-cyclic GMP.[39] The latter functions as a second messenger of nitric oxide, which regulates smooth muscle tone,[40,41] cardiac contractility[42] and cardiomyocyte hypertrophy.[43,44] It is thus reasonable to hypothesize that these regulatory efflux functions of ABCC5 in cardiomyocytes coupled with the higher clearance of doxorubicin associated with the *ABCC5* g.+*7161G>A* (rs1533682) polymorphism may serve as a protective mechanism against doxorubicin-induced cardiotoxicity, which merits further exploration.

Breast cancer patients homozygous for the *ABCC5 g.-1679T* allele were also found to have significantly higher exposure levels to doxorubicinol compared with patients who were heterozygous for the polymorphism. However, no significant influence on the exposure levels to doxorubicin was observed, and could be attributed to possible differences in the transport affinities of doxorubicin and doxorubicinol by the ABCC5 transporter protein. This finding is of potential clinical significance as doxorubicinol has been suspected to modulate doxorubicin-induced cardiotoxicity.[2] Doxorubicin-treated patients homozygous for the *ABCC5 g.-1679T* reference allele may thus be at potentially higher risks of developing cardiotoxicity. However, this needs to be further investigated in a larger study that is adequately powered to detect differences in incidence of toxicities among the different genotype groups. The functional impacts of the g.+*7161G>A* (rs1533682) and g.*-1679T>A* polymorphisms in altering doxorubicin and doxorubicinol substrate specificity of ABCC5 have not been elucidated and probably need further characterization.

Mechanisms of doxorubicin transport and resistance mediated by RLIP76 have been investigated in several studies as Awasthi *et al.*[26] demonstrated that an ATP-dependent uptake of doxorubicin into vesicles prepared from erythrocyte membrane, which do not express ABCB1 was completely inhibited by antibodies to RLIP76. The exceptionally broad substrate specificity (structurally unrelated anionic compounds, weakly cationic compounds (doxorubicin, dihydrodoxorubicin, daunomycin and vinblastine) and uncharged compounds (doxorubicinone, deoxydoxorubicinone and dihydrodoxorubicinone)) and its wide tissue distribution suggest that RLIP76 may function as an important efflux transporter for both xenobiotics and endobiotics.[45,46] In this study, no polymorphisms were identified, suggesting a lack of genetic variability in the *RLIP76* gene in the Asian ethnic groups. However, it is conceivable that contributions from *RLIP76* to variations in its activity or expression may arise from rare variants including those in the untranslated regions that were not detected in this study.

In conclusion, the present pharmacogenetic analysis of *ABCB5*, *ABCC5* and *RLIP76* transporter proteins in Asian breast cancer patients suggest that the *ABCC5* g.+*7161G>A* (rs1533682) and *ABCC5* g.*-1679T>A* polymorphisms may significantly influence the pharmacokinetics of doxorubicin and doxorubicinol, respectively. No significant influence of the newly identified *ABCB5* polymorphisms on doxorubicin pharmacokinetic parameters was observed in this study. Taken together, the findings of this study as well as our previous findings on pharmacogenetic profiling across the doxorubicin pathway[10–13] further explain the importance of considering the polygenic impact of various functionally important candidate polymorphisms on the pharmacokinetics of doxorubicin and doxorubicinol.

Notably, the results of the multivariate analysis evaluating the relative contributions of all the polymorphisms studied to date in our Asian breast cancer patients revealed that only *CBR1* +967G>A remained significantly associated with doxorubicin clearance. However, the study may be limited by sample size and may not be adequately powered for such multivariate approach. Further studies should be explored in a larger cohort of breast cancer patients belonging to other ethnic groups.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by the National Medical Research Council (NMRC/0814/2003), Singapore.

REFERENCES

1 Chang BK, Brenner DE, Gutman R. Cellular pharmacology of doxorubicinol alone and combined with verapamil in pancreatic cancer cell lines. *Anticancer Res* 1989; **9**: 341–345.
2 Olson RD, Mushlin PS, Brenner DE, Fleischer S, Cusack BJ, Chang BK *et al.* Doxorubicin cardiotoxicity may be caused by its metabolite, doxorubicinol. *Proc Natl Acad Sci USA* 1988; **85**: 3585–3589.
3 Boucek RJ, Olson RD, Brenner DE, Ogunbunmi EM, Inui M, Fleischer S. The major metabolite of doxorubicin is a potent inhibitor of membrane-associated ion pumps. A correlative study of cardiac muscle with isolated membrane fractions. *J Biol Chem* 1987; **262**: 15851–15856.
4 Sharom FJ. ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics* 2008; **9**: 105–127.
5 Bao L, Hazari S, Mehra S, Kaushal D, Moroz K, Dash S. Increased expression of P-glycoprotein and doxorubicin chemoresistance of metastatic breast cancer is regulated by miR-298. *Am J Pathol* 2012; **180**: 2490–2503.
6 Pajic M, Iyer JK, Kersbergen A, van der Burg E, Nygren AOH, Jonkers J *et al.* Moderate increase in Mdr1a/1b expression causes *in vivo* resistance to doxorubicin in a mouse model for hereditary breast cancer. *Cancer Res* 2009; **69**: 6396–6404.
7 Versantvoort CH, Broxterman HJ, Pinedo HM, de Vries EG, Feller N, Kuiper CM *et al.* Energy-dependent processes involved in reduced drug accumulation in multidrug-resistant human lung cancer cell lines without P-glycoprotein expression. *Cancer Res* 1992; **52**: 17–23.
8 Allen JD, Jackson SC, Schinkel AH. A mutation hot spot in the Bcrp1 (Abcg2) multidrug transporter in mouse cell lines selected for doxorubicin resistance. *Cancer Res* 2002; **62**: 2294–2299.
9 Riganti C, Miraglia E, Viarisio D, Costamagna C, Pescarmona G, Ghigo D *et al.* Nitric oxide reverts the resistance to doxorubicin in human colon cancer cells by inhibiting the drug efflux. *Cancer Res* 2005; **65**: 516–525.
10 Lal S, Sandanaraj E, Wong ZW, Ang PCS, Wong NS, Lee EJD *et al.* CBR1 and CBR3 pharmacogenetics and their influence on doxorubicin disposition in Asian breast cancer patients. *Cancer Sci* 2008; **99**: 2045–2054.
11 Lal S, Wong ZW, Jada SR, Xiang X, Chen Shu X, Ang PCS *et al.* Novel SLC22A16 polymorphisms and influence on doxorubicin pharmacokinetics in Asian breast cancer patients. *Pharmacogenomics* 2007; **8**: 567–575.
12 Lal S, Wong ZW, Sandanaraj E, Xiang X, Ang PCS, Lee EJD *et al.* Influence of ABCB1 and ABCG2 polymorphisms on doxorubicin disposition in Asian breast cancer patients. *Cancer Sci* 2008; **99**: 816–823.
13 Sandanaraj E, Lal S, Selvarajan V, Ooi LL, Wong ZW, Wong NS *et al.* PXR pharmacogenetics: association of haplotypes with hepatic CYP3A4 and ABCB1 messenger RNA expression and doxorubicin clearance in Asian breast cancer patients. *Clin Cancer Res Off J Am Assoc Cancer Res* 2008; **14**: 7116–7126.
14 Dean M, Allikmets R. Complete characterization of the human ABC gene family. *J Bioenerg Biomembr* 2001; **33**: 475–479.
15 Frank NY, Pendse SS, Lapchak PH, Margaryan A, Shlain D, Doeing C *et al.* Regulation of progenitor cell fusion by ABCB5 P-glycoprotein, a novel human ATP-binding cassette transporter. *J Biol Chem* 2003; **278**: 47156–47165.
16 Moitra K, Scally M, McGee K, Lancaster G, Gold B, Dean M. Molecular evolutionary analysis of ABCB5: the ancestral gene is a full transporter with potentially deleterious single nucleotide polymorphisms. *PloS One* 2011; **6**: e16318.
17 Frank NY, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M *et al.* ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. *Cancer Res* 2005; **65**: 4320–4333.
18 Kawanobe T, Kogure S, Nakamura S, Sato M, Katayama K, Mitsuhashi J *et al.* Expression of human ABCB5 confers resistance to taxanes and anthracyclines. *Biochem Biophys Res Commun* 2012; **418**: 736–741.
19 Yang JY, Ha S-A, Yang Y-S, Kim JW. p-Glycoprotein ABCB5 and YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance. *BMC Cancer* 2010; **10**: 388.

20 Dean M, Annilo T. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. *Annu Rev Genomics Hum Genet* 2005; **6**: 123–142.
21 Borst P, Elferink RO. Mammalian ABC transporters in health and disease. *Annu Rev Biochem* 2002; **71**: 537–592.
22 Yoshida M, Suzuki T, Komiya T, Hatashita E, Nishio K, Kazuhiko N *et al.* Induction of MRP5 and SMRP mRNA by adriamycin exposure and its overexpression in human lung cancer cells resistant to adriamycin. *Int J Cancer J Int Cancer* 2001; **94**: 432–437.
23 Pratt S, Shepard RL, Kandasamy RA, Johnston PA, Perry W, Dantzig AH. The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites. *Mol Cancer Ther* 2005; **4**: 855–863.
24 Sharma R, Singhal SS, Wickramarachchi D, Awasthi YC, Awasthi S. RLIP76 (RALBP1)-mediated transport of leukotriene C4 (LTC4) in cancer cells: implications in drug resistance. *Int J Cancer J Int Cancer* 2004; **112**: 934–942.
25 Awasthi S, Sharma R, Singhal SS, Zimniak P, Awasthi YC. RLIP76, a novel transporter catalyzing ATP-dependent efflux of xenobiotics. *Drug Metab Dispos Biol Fate Chem* 2002; **30**: 1300–1310.
26 Awasthi S, Singhal SS, Srivastava SK, Zimniak P, Bajpai KK, Saxena M *et al.* Adenosine triphosphate-dependent transport of doxorubicin, daunomycin, and vinblastine in human tissues by a mechanism distinct from the P-glycoprotein. *J Clin Invest* 1994; **93**: 958–965.
27 Awasthi S, Sharma R, Yang Y, Singhal SS, Pikula S, Bandorowicz-Pikula J *et al.* Transport functions and physiological significance of 76 kDa Ral-binding GTPase activating protein (RLIP76). *Acta Biochim Pol* 2002; **49**: 855–867.
28 Awasthi S, Singhal SS, Sharma R, Zimniak P, Awasthi YC. Transport of glutathione conjugates and chemotherapeutic drugs by RLIP76 (RALBP1): a novel link between G-protein and tyrosine kinase signaling and drug resistance. *Int J Cancer J Int Cancer* 2003; **106**: 635–646.
29 Singhal SS, Wickramarachchi D, Singhal J, Yadav S, Awasthi YC, Awasthi S. Determinants of differential doxorubicin sensitivity between SCLC and NSCLC. *FEBS Lett* 2006; **580**: 2258–2264.
30 Vatsyayan R, Chaudhary P, Lelsani PCR, Singhal P, Awasthi YC, Awasthi S *et al.* Role of RLIP76 in doxorubicin resistance in lung cancer. *Int J Oncol* 2009; **34**: 1505–1511.
31 Gwee PC, Tang K, Sew PH, Lee EJD, Chong SS, Lee CGL. Strong linkage disequilibrium at the nucleotide analogue transporter ABCC5 gene locus. *Pharmacogenet Genomics* 2005; **15**: 91–104.
32 Lal S, Mahajan A, Chen WN, Chowbay B. Pharmacogenetics of target genes across doxorubicin disposition pathway: a review. *Curr Drug Metab* 2010; **11**: 115–128.
33 Sauna ZE, Kimchi-Sarfaty C, Ambudkar SV, Gottesman MM. Silent polymorphisms speak: how they affect pharmacogenomics and the treatment of cancer. *Cancer Res* 2007; **67**: 9609–9612.
34 Conne B, Stutz A, Vassalli JD. The 3′ untranslated region of messenger RNA: a molecular "hotspot" for pathology? *Nat Med* 2000; **6**: 637–641.
35 Saito S, Iida A, Sekine A, Miura Y, Ogawa C, Kawauchi S *et al.* Identification of 779 genetic variations in eight genes encoding members of the ATP-binding cassette, subfamily C (ABCC/MRP/CFTR. *J Hum Genet* 2002; **47**: 147–171.
36 Oguri T, Achiwa H, Sato S, Bessho Y, Takano Y, Miyazaki M *et al.* The determinants of sensitivity and acquired resistance to gemcitabine differ in non-small cell lung cancer: a role of ABCC5 in gemcitabine sensitivity. *Mol Cancer Ther* 2006; **5**: 1800–1806.
37 Mourskaia AA, Amir E, Dong Z, Tiedemann K, Cory S, Omeroglu A *et al.* ABCC5 supports osteoclast formation and promotes breast cancer metastasis to bone. *Breast Cancer Res BCR* 2012; **14**: R149.
38 Zhu Y, Yu F, Jiao Y, Feng J, Tang W, Yao H *et al.* Reduced miR-128 in breast tumor-initiating cells induces chemotherapeutic resistance via Bmi-1 and ABCC5. *Clin Cancer Res Off J Am Assoc Cancer Res* 2011; **17**: 7105–7115.
39 Dazert P, Meissner K, Vogelgesang S, Heydrich B, Eckel L, Böhm M *et al.* Expression and localization of the multidrug resistance protein 5 (MRP5/ABCC5), a cellular export pump for cyclic nucleotides, in human heart. *Am J Pathol* 2003; **163**: 1567–1577.
40 Warren JB, Pons F, Brady AJ. Nitric oxide biology: implications for cardiovascular therapeutics. *Cardiovasc Res* 1994; **28**: 25–30.
41 Carvajal JA, Germain AM, Huidobro-Toro JP, Weiner CP. Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol* 2000; **184**: 409–420.
42 Flesch M, Kilter H, Cremers B, Laufs U, Südkamp M, Ortmann M *et al.* Effects of endotoxin on human myocardial contractility involvement of nitric oxide and peroxynitrite. *J Am Coll Cardiol* 1999; **33**: 1062–1070.
43 Maeda Y, Ikeda U, Oya K, Shimpo M, Ueno S, Urabe M *et al.* Adeno-associated virus-mediated transfer of endothelial nitric oxide synthase gene inhibits protein synthesis of rat ventricular cardiomyocytes. *Cardiovasc Drugs Ther Spons Int Soc Cardiovasc Pharmacother* 2001; **15**: 19–24.
44 Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA *et al.* Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* 1997; **57**: 3537–3547.
45 Sharma R, Gupta S, Singh SV, Medh RD, Ahmad H, LaBelle EF *et al.* Purification and characterization of dinitrophenylglutathione ATPase of human erythrocytes and its expression in other tissues. *Biochem Biophys Res Commun* 1990; **171**: 155–161.
46 Singhal SS, Sharma R, Gupta S, Ahmad H, Zimniak P, Radominska A *et al.* The anionic conjugates of bilirubin and bile acids stimulate ATP hydrolysis by S-(dinitrophenyl)glutathione ATPase of human erythrocyte. *FEBS Lett* 1991; **281**: 255–257.

Supplementary Information accompanies the paper on the The Pharmacogenomics Journal website (http://www.nature.com/tpj)