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ORIGINAL ARTICLE

Association study of genetic polymorphism in *ABCC4* with cyclophosphamide-induced adverse drug reactions in breast cancer patients

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Cyclophosphamide (CPA)-based combination treatment has known to be effective for breast cancer, but often causes adverse drug reactions (ADRs). Hence, the identification of patients at risk for toxicity by CPA is clinically significant. In this study, a stepwise case–control association study was conducted using 403 patients with breast cancer who received the CPA combination therapy. A total of 143 genetic polymorphisms in 13 candidate genes (*CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5*, *ALDH1A1*, *ALDH3A1*, *GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*, *ABCC2* and *ABCC4)*, possibly involved in the activation, metabolism and transport of CPA, were genotyped using 184 cases who developed either ⩾grade 3 leukopenia/neutropenia or ⩾grade 2 gastrointestinal toxicity and 219 controls who did not show any ADRs throughout the treatment. The association study revealed that one SNP, rs9561778 in *ABCC4*, showed a significant association with CPA-induced ADRs (Cochran–Armitage trend's *P*-value=0.00031; odds ratio (OR)=2.06). Subgroup analysis also indicated that the SNP rs9561778 was significantly associated with two major ADR subgroups; gastrointestinal toxicity and leukopenia/neutropenia (Cochran–Armitage trend's *P*-value=0.00019 and 0.014; OR=2.31 and 1.83). Furthermore, the SNP rs9561778 showed an association with breast cancer patients who were treated with CA(F) drug regimen-induced ADR (Cochran–Armitage trend's *P*-value=0.00028; OR=3.13). The SNPs in *ABCC4* might be applicable in predicting the risk of ADRs in patients receiving CPA combination chemotherapy.
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INTRODUCTION

Cyclophosphamide (CPA) is one of the most widely used anticancer drugs in the treatment of hematological malignancies and a variety of solid tumors including breast cancer.[1] CPA is frequently used together with other chemotherapeutic agents; with anthracyclin (adriamycin, epirubicin) termed the CA regimen, with methotrexate and 5-fluorouracil (CMF), with adriamycin and 5-flurouracil (CAF), or with 5-fluorouracil (CF).[2] The CPA-based combination treatment has been known to be effective for breast cancer, but often causes adverse drug reactions (ADRs), such as leukopenia/neutropenia, and gastrointestinal symptoms such as vomiting, anorexia and nausea (http://www.cancercare.on.ca/pdfdrugs/cyclopho.pdf).

CPA is a prodrug that requires metabolic activation to exert its effect. After CPA administration, the drug is metabolized to 4-hydroxycyclophosphamide (4-OH-CPA) by CYP2B6 and CYP2C9 as well as to a lesser extent by CYP3A4 and CYP3A5 in the liver.[3–5] The 4-OH-CPA interconverts rapidly with its tautomer, aldophosphamide and then degrades spontaneously to form phosphoramide mustard, which is a therapeutically active component. Both 4-OH-CPA and aldophosphamide are detoxified by glutathione (GSH) conjugation catalyzed by multiple glutathione *S*-transferases (GSTA1, GSTM1, GSTP1 and GSTT1) and by aldehyde dehydrogenase (ALDH1A1 and ALDH3A1) to carboxycyclophosphamide.[6,7] Thus, hepatic metabolism is the primary route of CPA elimination. In addition, it has been reported that transporters such as ABCC2 (also known as MRP2)[8] and ABCC4 (also known as MRP4)[9] are known to be involved in transport of CPA and its metabolites.

Most of the drug-metabolizing enzymes and transporters contain a wide range of genetic polymorphisms, which might cause a large interindividual variability in the plasma concentration of drugs. Furthermore, anticancer therapies are notoriously known to have a narrow therapeutic range; a higher concentration in the patient's body causes toxicity and a lower concentration reduces the efficacy of the drugs. Hence, the role of pharmacogenomics, which is expected to provide a predictive way for severe drug toxicity, is greatly essential.

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To our knowledge, many of the current publications, which revealed association analysis with ADRs induced by CPA combination therapy, concentrated only on enzymes involved in the activation and detoxification of CPA, and many of them focused on ADRs by CPA combination therapy for other diseases such as systemic lupus erythematosus and lupus nephritis, but not for cancer.[10–12] Hence, the objective of this study is to discover SNPs associated with CPA-induced ADRs in patients with breast cancer using a case–control association study, focusing on not only the drug-metabolizing enzymes, but also the transporters, which might also have an important role in pharmacokinetics of CPA or its active forms.

MATERIALS AND METHODS

Subjects

All the samples were recruited at BioBank Japan (http://biobankjp.org), which has a collaboration network of 66 hospitals throughout Japan, with written informed consent. In this study, patients who revealed ADRs of ⩾grade 3 leukopenia or neutropenia, or those with ⩾grade 2 gastrointestinal toxicity induced by CPA combination therapy were defined as cases (ADR), whereas controls (non-ADR) were defined as patients who had shown no toxicity during CPA-based combination therapy.

A total of 216 breast cancer patients comprising 76 cases (ADR) and 140 controls (non-ADR), were collected from June 2003 to March 2006; this served as the first exploratory samples set (1st set) in this study (Table 1). Another independent set of samples was collected from April 2006 to November 2007, which consist of 108 ADR cases and 79 non-ADR controls, and was subsequently added into the study as independent second set samples. Age difference between case and control groups in this study was not statistically significant (*P*-value=0.73). The grade of toxicity was classified in accordance with the National Cancer Institute—Common Toxicity Criteria version 2.0. This project was approved by the ethics committees at The Institute of Medical Science, The University of Tokyo, Tokyo, Japan.

Selection of SNPs and genotyping

A total of 141 SNPs (tagSNPs and functional SNPs) and two deletion polymorphisms in 13 candidate genes that are involved in activation, detoxification and transportation of CPA (*CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5*, *ALDH1A1*, *ALDH3A1*, *GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*, *ABCC2*, and *ABCC4*), were genotyped. The selection criteria of the tagSNPs were based on the measures of linkage disequilibrium (LD) with r^2 value ⩾0.8 and minor allele frequency (MAF) of >10% from the HapMap database (http://www.hapmap.org/). For *CYP2C19*, *CYP3A4* and *CYP3A5*, only functional SNPs were tested because of the poor information on tagSNPs on these gene loci. All the SNPs were genotyped using multiplex polymerase chain reaction (PCR)-invader assay[13] or direct sequencing. Lastly, for *GSTM1* and *GSTT1*, only gene deletion analysis was performed as described previously, as these two genes were frequently deleted in our population.[14]

Table 1 Patients' characteristics

	Case (ADR)	*Control (non-ADR)*
The number of samples		
First set	76	140
Second set	108	79
Total	184	219
Mean age at diagnosis	57.7	52.0
Types of adverse drug reaction		
≧Grade 3 leucopenia or neutropenia	91	219
≧Grade 2 gastrointestinal toxicity	118	219

Strategy of the study and statistical analysis

The strategy of this study was in a stepwise manner. Association analysis was performed by using the Cochran–Armitage trend test. The first genotyping was performed using 76 ADR cases and 140 non-ADR controls. SNPs that show *P*-value<0.05 in the Hardy–Weinberg equilibrium were excluded from further evaluation. SNPs that showed a *P*-value of <0.05 in the Cochran–Armitage trend test were further genotyped in an additional 108 ADR cases and 79 non-ADR controls. Lastly, the data of the first and the second sets were merged to evaluate its association with the ADR. In addition, during multiple testing, Bonferroni correction was applied, to further assess the significance level of the association. Subgroup analysis, such as types of ADR developed after the individual received CPA combination therapy and types of chemotherapy regimen for breast cancer, was also evaluated. All the statistical analyses and haplotype analyses were performed using the PLINK program[15] and haploview software,[16] respectively.

RESULTS

Association study with ADRs by CPA-based combination therapy

A total of 143 polymorphisms in 13 candidate genes were genotyped by using the first set of samples consisting of 76 ADR cases and 140 non-ADR controls. Among them, eight SNPs (rs4918766 in *CYP2C9*, rs1614102, rs9561778, rs4148532, rs1729775, rs1751070, rs4771912 and rs8001444 in *ABCC4*) showed possible association with ADRs induced by CPA combination therapy, yielded a *P*-value of <0.05 in the Cochran–Armitage trend test (Table 2). Considering the low statistical power because of the number of the exploratory sample set, three SNPs, rs1934968 in *CYP2C9*, rs7988595 and rs870004 in *ABCC4*, which showed some trends of association, were also examined in the further study. Hence, a total of 11 SNPs were genotyped using an independent second set of samples. Three SNPs located in *CYP2C9* (rs1934968) and *ABCC4* (rs9561778 and rs4148532), revealed *P*-values of less than 0.05 (Table 3). However, only one SNP rs9561778 in *ABCC4* was considered to be significantly associated with ADR by CPA combination therapy after applying strict Bonferroni's correction (Cochran–Armitage trend's *P*-value=0.00031; Bonferroni-adjusted *P*-value=0.044; OR=2.06; 95% CI=1.36–3.11; Table 3). Hence, we further genotyped all the tagSNPs in this gene, to facilitate haplotype analysis and subgroup analysis. Haplotype analysis (data not shown) revealed that the association of a single SNP (rs9561778; permutation *P*-value=0.0031, OR=2.06) with ADR by CPA was stronger than that of a risk haplotype (permutation *P*-value=0.011, OR=1.89).

Subgroup analysis

We also performed subgroup analyses by using five SNPs located within the LD block including the significantly associated SNP (rs9561778), according to the types of ADRs. For the first subgroup analysis, we divided cases into two major subgroups; one is gastrointestinal toxicity of ⩾grade 2 (GI) and leukopenia/neutropenia of ⩾grade 3 (LN). We found that rs9561778 showed significant association with both the gastrointestinal toxicity and leukopenia/neutropenia, yielding similar trends of odds ratio (Cochran–Armitage trend's *P*-value=0.00019 and 0.014; OR=2.31 and 1.83; 95% CI=1.45–3.68 and 1.10–3.05, respectively; Table 4).

For the second subgroup analysis, we evaluated the association of *ABCC4* genotypes with the ADR induced by the CA(F) (cyclophosphamide and anthracyclin with or without 5-fluorouracil) drug regimen because the CA(F) regimen is one of the most major combination therapies for breast cancer. The numbers of cases treated with these regimens were the most in our CPA combination therapy cases (ADR: 146 cases and non-ADR: 80 controls). Thus, we consider that this combination possesses some statistical power to be analyzed. This subgroup analysis revealed that the SNP rs9561778 in *ABCC4*

Table 2 Relationship between single nucleotide polymorphisms (SNPs) in candidate genes and ADR risk induced by CPA combination therapy

						MAF			*HWE*	
CHR	*Gene*	*SNP*	*Allele1*	*Allele2*	*Position/effect*	*ADR*	*Non-ADR*	*Cochran–Armitage trend P-value*	*ADR*	*Non-ADR*
6	*GSTA*	rs9367495	C	T	3′ UTR GSTA1	0.16	0.09	0.059	0.38	1.00
6		rs9395826	G	A	3′ UTR GSTA5	0.34	0.29	0.26	0.61	0.53
6		rs7739421	A	G	Intron 6 of GSTA5	0.18	0.20	0.68	0.71	0.79
6		rs9370155	G	C	Intron 5 of GSTA5	0.16	0.19	0.44	0.68	0.41
7	*CYP3A5*	rs776746	T	C	Intron_3	0.24	0.22	0.64	1.00	0.62
7	*CYP3A4*	rs28371759	C	T	Pro293Leu	0.03	0.01	0.22	1.00	1.00
7		rs12721627	G	C	Ser185Thr	0.02	0.00	0.092	1.00	1.00
9	*ALDH1A1*	rs4646548	G	A	3′ UTR	0.46	0.40	0.20	0.47	0.72
9		rs348471	G	A	Intron 12	0.48	0.42	0.26	0.36	0.86
9		rs1330291	T	C	Intron 11	0.08	0.10	0.66	1.00	0.62
9		rs4646544	C	A	Intron 7	0.09	0.10	0.65	0.08	1.00
9		rs8187929	A	T	Phe177Ile	0.03	0.04	0.72	1.00	1.00
9		rs13959	T	C	Exon 3 syn.	0.45	0.43	0.61	0.50	0.22
9		rs647880	A	G	Intron 1	0.46	0.49	0.60	0.16	0.50
9		rs10156653	T	C	Intergenic	0.46	0.50	0.47	0.64	0.24
9		rs3003989	C	T	Intergenic	0.05	0.03	0.47	0.14	1.00
9		rs7853400	G	A	Intergenic	0.38	0.42	0.41	0.81	0.49
10	*CYP2C9*	rs1074145	A	G	Intergenic	0.29	0.25	0.35	0.26	0.021
10		rs10509679	A	G	Intron 4	0.32	0.28	0.42	0.19	0.10
10		rs4918766	A	G	Intron 5	0.54	0.44	0.045	0.25	0.50
10		rs1057910	C	A	Tyr358Leu	0.05	0.05	0.99	1.00	1.00
10		rs1934968	T	C	Intron 7	0.22	0.30	0.057	0.74	1.00
10		rs11188133	G	A	5′ UTR	0.44	0.51	0.15	0.82	0.31
10	*CYP2C19*	rs4986893	A	G	Ter212Trp	0.18	0.13	0.20	1.00	1.00
10		rs4244285	A	G	Exon 5 syn.	0.31	0.26	0.26	0.29	0.045
10	*ABCC2*	rs2804398	T	A	Intron 7	0.14	0.12	0.59	0.62	0.41
10		rs2756109	T	G	Intron 7	0.36	0.34	0.80	0.13	0.19
10		rs11190291	T	C	Intron 11	0.14	0.14	0.99	0.34	0.14
10		rs2002042	T	C	Intron 19	0.31	0.32	0.92	0.03	0.17
10		rs3740065	G	A	Intron 29	0.35	0.36	0.91	0.80	0.14
10		rs12762549	C	G	Intergenic	0.42	0.44	0.76	0.35	0.010
10		rs2862691	T	C	Intergenic	0.23	0.21	0.65	1.00	0.44
11	*GSTP1*	rs612020	T	C	Intergenic	0.20	0.20	0.86	0.47	1.00
11		rs614080	G	A	5′ UTR	0.38	0.35	0.55	0.47	0.57
11		rs1695	G	A	Val105Ile	0.17	0.14	0.35	0.44	0.72
13	*ABCC4*	rs4148542	G	A	Intron 30	0.50	0.48	0.67	0.06	0.50
13		rs9561765	A	G	Intron 30	0.27	0.27	0.99	0.39	0.28
13		rs6492763	T	C	Intron 30	0.46	0.51	0.35	0.37	0.31
13		rs2182262	T	C	Intron 29	0.26	0.23	0.43	0.37	1.00
13		rs4148540	T	C	Intron 29	0.24	0.28	0.41	0.76	0.30
13		rs1614102	A	G	Intron 26	0.30	0.21	0.037	0.42	0.44
13		rs1751031	G	A	Intron 26	0.28	0.33	0.28	0.39	0.08
13		rs9561778	T	G	Intron 26	0.24	0.13	0.0086	0.75	0.71
13		rs931110	G	A	Intron 26	0.53	0.47	0.29	0.65	0.12
13		rs4148532	T	A	Intron 21	0.22	0.14	0.032	1.00	0.73
13		rs17234998	T	C	Intron 20	0.16	0.21	0.22	0.19	0.015
13		rs1751055	T	C	Intron 20	0.28	0.34	0.23	0.78	0.19
13		rs2698243	C	T	Intron 20	0.47	0.51	0.39	0.36	0.17
13		rs1729775	A	G	Intron 20	0.28	0.18	0.027	1.00	1.00

Table 2 Continued

CHR	Gene	SNP	Allele1	Allele2	Position/effect	MAF		Cochran–Armitage trend P-value	HWE	
						ADR	Non-ADR		ADR	Non-ADR
13		rs9561784	G	A	Intron 20	0.19	0.21	0.64	0.45	0.19
13		rs1729741	G	A	Intron 19	0.13	0.18	0.23	1.00	0.57
13		rs1751070	G	C	Intron 19	0.23	0.15	0.029	0.33	0.31
13		rs2619313	T	C	Intron 19	0.24	0.23	0.78	0.03	0.46
13		rs997777	T	A	Intron 19	0.38	0.39	0.85	0.15	0.48
13		rs4148508	T	A	Intron 19	0.16	0.15	0.64	0.68	0.17
13		rs1479390	A	C	Intron 19	0.27	0.31	0.34	1.00	0.84
13		rs7988595	C	A	Intron 19	0.19	0.27	0.057	1.00	0.83
13		rs7988271	T	C	Intron 19	0.10	0.16	0.10	0.54	0.75
13		rs3765534	T	C	Lys757Glu	0.11	0.17	0.14	1.00	0.54
13		rs1729764	G	A	Intron 16	0.33	0.32	0.93	1.00	0.85
13		rs4148500	T	C	Intron 15	0.21	0.19	0.57	0.30	0.41
13		rs9561797	G	A	Intron 14	0.28	0.36	0.080	0.57	0.26
13		rs12429339	A	T	Intron 14	0.16	0.17	0.70	0.38	1.00
13		rs1729786	A	G	Intron 13	0.30	0.23	0.12	0.42	0.34
13		rs1189458	C	T	Intron 13	0.31	0.37	0.24	0.79	0.46
13		rs6492768	G	A	Intron 13	0.48	0.46	0.69	0.50	1.00
13		rs1751003	A	G	Intron 13	0.15	0.16	0.88	0.67	0.74
13		rs1887162	T	G	Intron 13	0.22	0.27	0.31	0.51	0.67
13		rs10161985	T	C	Intron 11	0.45	0.43	0.66	0.16	0.39
13		rs1564352	T	G	Intron 11	0.33	0.35	0.80	1.00	0.71
13		rs10162199	T	C	Intron 11	0.33	0.30	0.61	1.00	0.69
13		rs3843689	G	A	Intron 11	0.29	0.30	0.81	0.58	0.84
13		rs2766474	A	G	Intron 11	0.15	0.16	0.76	0.65	0.75
13		rs1557069	G	A	Intron 10	0.24	0.22	0.76	1.00	0.47
13		rs4773843	T	C	Intron 10	0.13	0.15	0.59	1.00	1.00
13		rs9561802	A	G	Intron 10	0.17	0.15	0.61	1.00	0.74
13		rs1678374	T	C	Intron 9	0.41	0.40	0.96	1.00	0.28
13		rs4148486	T	C	Intron 9	0.33	0.33	0.95	0.43	0.70
13		rs7319330	C	T	Intron 9	0.47	0.50	0.65	1.00	0.73
13		rs4148481	C	T	Intron 9	0.40	0.41	0.88	1.00	0.59
13		rs1678384	A	G	Intron 8	0.05	0.07	0.63	1.00	0.012
13		rs4148478	C	T	Intron 8	0.29	0.31	0.67	0.58	0.55
13		rs1751022	T	C	Intron 8	0.24	0.21	0.52	0.11	0.61
13		rs1751025	G	C	Intron 8	0.30	0.29	0.78	1.00	0.83
13		rs4773844	T	C	Intron 8	0.27	0.29	0.79	0.24	0.53
13		rs17268170	T	C	Intron 8	0.07	0.07	0.81	1.00	1.00
13		rs9556465	T	G	Intron 8	0.07	0.09	0.41	1.00	0.60
13		rs1751029	A	G	Intron 8	0.19	0.18	0.78	0.06	0.25
13		rs2274408	T	C	Intron 7	0.45	0.42	0.54	0.65	0.22
13		rs9524827	C	T	Intron 6	0.38	0.37	0.87	0.32	0.71
13		rs873706	T	C	Intron 5	0.37	0.37	0.95	0.63	0.35
13		rs11568658	T	G	Trp187Gly	0.07	0.10	0.43	0.33	0.61
13		rs9524831	C	A	Intron 4	0.26	0.28	0.75	0.37	0.52
13		rs7330519	T	C	Intron 4	0.49	0.50	0.84	0.35	0.49
13		rs4773856	A	G	Intron 4	0.22	0.22	0.98	0.75	1.00
13		rs9561814	C	T	Intron 4	0.17	0.18	0.82	0.03	0.77
13		rs7333234	A	G	Intron 4	0.32	0.34	0.79	0.43	0.70
13		rs4148456	G	A	Intron 3	0.09	0.09	0.94	1.00	1.00
13		rs9561817	T	C	Intron 3	0.37	0.38	0.89	0.32	0.58
13		rs4258481	C	G	Intron 3	0.51	0.49	0.69	0.10	0.86
13		rs12427972	A	G	Intron 3	0.28	0.32	0.42	0.24	0.69
13		rs2892715	A	G	Intron 3	0.16	0.11	0.14	0.0022	1.00
13		rs4148450	G	A	Intron 3	0.28	0.32	0.39	0.26	0.84
13		rs10508019	T	C	Intron 3	0.22	0.28	0.23	0.33	0.67
13		rs4148440	A	G	Intron 3	0.45	0.47	0.71	0.35	0.12
13		rs4148436	C	T	Intron 2	0.27	0.22	0.30	0.37	0.80
13		rs4148434	A	G	Intron 1	0.20	0.16	0.33	0.06	0.34

Table 2 Continued

CHR	Gene	SNP	Allele1	Allele2	Position/effect	MAF		Cochran–Armitage trend P-value	HWE	
						ADR	Non-ADR		ADR	Non-ADR
13		rs9590216	T	C	Intron 1	0.45	0.46	0.93	0.24	0.11
13		rs9561820	T	C	Intron 1	0.31	0.29	0.63	0.17	0.010
13		rs4148431	A	G	Intron 1	0.44	0.45	0.84	0.48	0.023
13		rs7328332	T	C	Intron 1	0.14	0.10	0.23	1.00	1.00
13		rs870004	A	G	Intron 1	0.18	0.26	0.063	0.69	0.65
13		rs8001475	C	T	Intron 1	0.29	0.35	0.24	0.78	0.45
13		rs4148426	G	C	Intron 1	0.18	0.25	0.11	0.68	0.34
13		rs9524873	A	G	Intron 1	0.26	0.22	0.40	0.25	0.010
13		rs4771910	C	T	Intron 1	0.30	0.35	0.40	0.58	0.70
13		rs10508017	T	C	Intron 1	0.24	0.23	0.83	0.34	0.025
13		rs4148424	T	C	Intron 1	0.34	0.36	0.56	0.61	1.00
13		rs4148422	C	T	Intron 1	0.37	0.36	0.92	0.80	0.26
13		rs34665760	A	G	Intron 1	0.24	0.30	0.21	0.75	0.84
13		rs4773872	T	C	Intron 1	0.28	0.36	0.084	0.40	0.57
13		rs4771912	G	A	Intron 1	0.24	0.33	0.045	1.00	0.33
13		rs4773875	T	G	Intron 1	0.43	0.35	0.10	0.34	0.70
13		rs8001444	T	C	Intron 1	0.37	0.47	0.041	0.80	1.00
17	*ALDH3A1*	rs758427	T	C	5′ near gene	0.49	0.49	0.88	0.82	0.73
17		rs11657205	T	C	5′ near gene	0.49	0.48	0.82	1.00	0.49
17		rs11204411	C	T	5′ near gene	0.20	0.22	0.67	1.00	0.45
17		rs57555435	A	G	Exon 10 syn.	0.14	0.14	0.85	0.34	0.040
17		rs2228100	G	C	Ala329Pro	0.32	0.33	0.82	1.00	0.85
17		rs3744692	A	G	Glu309Gly	0.03	0.05	0.36	1.00	1.00
17		rs2072330	A	T	Exon 6 syn.	0.45	0.44	0.83	0.64	0.06
17		rs887241	T	G	Ala134Ser	0.03	0.04	0.73	1.00	1.00
19	*CYP2B6*	rs7254579	C	T	5′ near gene	0.46	0.49	0.57	0.24	0.24
19		rs4802101	T	C	5′ near gene	0.39	0.32	0.17	0.81	0.56
19		rs4803415	T	C	Intron 1	0.23	0.19	0.35	0.52	0.28
19		rs4803419	T	C	Intron 3	0.45	0.49	0.49	0.64	0.61
19		rs3745274	T	G	His172Gln	0.13	0.17	0.32	1.00	1.00
19		rs2279343	G	A	Arg262Lys	0.16	0.22	0.13	1.00	0.81
19		rs2279345	T	C	Intron 5	0.36	0.31	0.28	1.00	0.32
19		rs7255374	T	C	Intron 8	0.19	0.22	0.51	1.00	0.80
19		rs1042389	C	T	3′ UTR	0.30	0.26	0.46	0.09	0.83

GSTM1 and GSTT1 deletion

Gene	ADR		Non-ADR		Fisher's exact P-value	Odds ratio
	Deletion	Wildtype	Deletion	Wildtype		
GSTM deletion	46	51	102	87	0.32	0.769
GSTT deletion	41	56	83	108	0.90	0.953

Abbreviations: ADR, adverse drug reaction; HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency.

showed a significant association with a higher odds ratio (Cochran–Armitage trend's *P*-value=0.00028; OR=3.13; 95% CI=1.68–5.83) with patients treated with the CA(F) regimen (Table 5).

DISCUSSION

Most of the genes encoding the enzymes involved in the activation and detoxification pathways are known to be highly polymorphic. There are several reports indicating that the polymorphisms in such genes were associated with the risk of the toxicity caused by CPA combination therapy, but there are significant inconsistencies in the results mostly because of the small sample size,[10–12,17,18] suggesting the urgent need to further confirm those reports. In this study, we examined a total of 141 SNPs and two gene deletions in 13 candidate genes that were considered to be involved in the activation (*CYP2B6*, *CYP2C9*,

Table 3 Replication study for single nucleotide polymorphisms (SNPs) showing *P*-values of <0.05 or trends of association in 1st set

					ADR			*Non-ADR*			*MAF*		*Cochran-Armitage*		*95% CI*		*Adjusted* P-*value*		
Chr	*SNP*	*Stage*	*Allele1*	*Allele2*	*11*	*12*	*22*	*11*	*12*	*22*	*ADR*	*Non-ADR*	*trend* P-*value*	*OR*[a]	*Lower*	*Upper*	*(BONF)*[b]	*Designation*	*Gene*
10	rs4918766	1st	A	G	19	44	13	29	65	46	0.54	0.44	0.045	2.37	1.19	4.70		INTRONIC	*CYP2C9*
		2nd	A	G	25	52	31	14	44	20	0.47	0.46	0.84	1.38	0.67	2.83			
		Total	A	G	44	96	44	43	109	66	0.50	0.45	0.13	1.38	0.89	2.15	1.00		
10	rs1934968	1st	T	C	4	25	47	12	59	66	0.22	0.30	0.06	1.74	0.99	3.08		INTRONIC	*CYP2C9*
		2nd	T	C	5	43	60	7	40	30	0.25	0.35	0.021	1.96	1.08	3.54			
		Total	T	C	9	68	107	19	99	96	0.23	0.32	0.0057	1.71	1.15	2.54	0.81		
13	rs1614102	1st	A	G	8	29	39	4	49	85	0.30	0.21	0.037	3.94	1.21	12.74		INTRONIC	*ABCC4*
		2nd	A	G	5	53	50	6	29	44	0.29	0.26	0.47	1.46	0.82	2.61			
		Total	A	G	13	82	89	10	78	129	0.29	0.23	0.025	1.56	1.05	2.32	1.00		
13	rs9561778	1st	T	G	5	26	45	3	31	104	0.24	0.13	0.0086	2.11	1.16	3.83		INTRONIC	*ABCC4*
		2nd	T	G	5	46	57	1	26	52	0.26	0.18	0.047	1.72	0.95	3.13			
		Total	T	G	10	72	102	4	57	156	0.25	0.15	0.00031	2.06	1.36	3.11	0.044		
13	rs4148532	1st	T	A	4	26	46	3	33	102	0.22	0.14	0.032	1.85	1.02	3.35		INTRONIC	*ABCC4*
		2nd	T	A	7	43	58	1	24	54	0.26	0.16	0.020	1.86	1.02	3.40			
		Total	T	A	11	69	104	4	57	156	0.25	0.15	0.00050	1.97	1.30	2.98	0.071		
13	rs1729775	1st	A	G	6	30	40	4	42	90	0.28	0.18	0.027	1.76	1.00	3.12		INTRONIC	*ABCC4*
		2nd	A	G	6	44	58	3	27	49	0.26	0.21	0.25	1.41	0.78	2.54			
		Total	A	G	12	74	98	7	69	139	0.27	0.19	0.013	1.60	1.07	2.40	1.00		
13	rs1751070	1st	G	C	2	31	43	1	39	96	0.23	0.15	0.029	1.84	1.03	3.30		INTRONIC	*ABCC4*
		2nd	G	C	3	38	67	2	32	45	0.20	0.23	0.55	1.23	0.69	2.22			
		Total	G	C	5	69	110	3	71	141	0.21	0.18	0.18	1.28	0.85	1.93	1.00		
13	rs7988595	1st	C	A	2	25	49	11	53	73	0.19	0.27	0.057	3.23	0.78	13.30		INTRONIC	*ABCC4*
		2nd	C	A	6	32	70	3	30	45	0.20	0.23	0.53	1.35	0.75	2.45			
		Total	C	A	8	57	119	14	83	118	0.20	0.26	0.047	1.50	1.01	2.25	1.00		
13	rs870004	1st	A	G	3	20	50	10	48	72	0.18	0.26	0.063	1.75	0.96	3.19		INTRONIC	*ABCC4*
		2nd	A	G	4	39	65	0	32	47	0.22	0.20	0.70	NA	0.74	NA			
		Total	A	G	7	59	115	10	80	119	0.20	0.24	0.20	1.32	0.88	1.98	1.00		
13	rs4771912	1st	G	A	4	28	42	12	65	56	0.24	0.33	0.045	1.80	1.02	3.20		INTRONIC	*ABCC4*
		2nd	G	A	5	57	46	2	41	36	0.31	0.28	0.54	1.87	0.41	8.55			
		Total	G	A	9	85	88	14	106	92	0.28	0.32	0.27	1.22	0.82	1.82	1.00		
13	rs8001444	1st	T	C	9	36	28	29	67	36	0.37	0.47	0.041	2.00	0.90	4.43		INTRONIC	*ABCC4*
		2nd	T	C	19	57	32	13	40	26	0.44	0.42	0.66	1.17	0.63	2.17			
		Total	T	C	28	93	60	42	107	62	0.41	0.45	0.24	1.36	0.81	2.29	1.00		

Abbreviations: ADR, adverse drug reaction; CI, confidence interval; MAF, minor allele frequency.
[a]OR: Odds Ratio of risk genotype vs non-risk genotype.
[b]BONF: Bonferroni correction; based on 143 independent effective tests.

Table 4 Association study of *ABCC4* genotypes with type of ADR occurrence induced by CPA combination therapy

				ADR			*Non-ADR*			*MAF*		*Cochran–Armitage*		*95% CI*	
SNP	*Allele1*	*Allele2*	*Type of ADR*	*11*	*12*	*22*	*11*	*12*	*22*	*ADR*	*Non-ADR*	*trend* P-*value*	*OR*[a]	*Lower*	*Upper*
rs9561778	T	G	GI[b]	7	49	62	4	57	156	0.27	0.15	0.00019	2.31	1.45	3.68
			LN[c]	4	34	53	4	57	156	0.23	0.15	0.014	1.83	1.10	3.05
rs931110	G	A	GI	34	55	29	44	110	63	0.52	0.46	0.11	1.59	0.95	2.67
			LN	32	41	18	44	110	63	0.58	0.46	0.0068	2.13	1.24	3.66
rs4148532	T	A	GI	8	47	63	4	57	156	0.27	0.15	0.00022	2.23	1.40	3.56
			LN	4	33	54	4	57	156	0.23	0.15	0.022	1.75	1.05	2.92
rs2698243	T	C	GI	29	64	25	43	116	58	0.52	0.47	0.18	1.36	0.80	2.31
			LN	31	42	18	43	116	58	0.57	0.47	0.015	2.09	1.21	3.60
rs1729775	A	G	GI	8	53	57	7	69	139	0.29	0.19	0.0029	1.96	1.24	3.09
			LN	5	33	53	7	69	139	0.24	0.19	0.22	1.31	0.80	2.16

Abbreviations: ADR, adverse drug reaction; CI, confidence interval; MAF, minor allele frequency.
[a]OR: Odds ratio of risk genotype vs non-risk genotype.
[b]GI: ⩾Grade 2 gastrointestinal toxicity,
[c]LN: ⩾Grade 3 leucopenia or neutropenia.

Table 5 Association study of *ABCC4* genotypes with CA(F)[a] regimens for breast cancer treatment

			ADR			*Non-ADR*			*MAF*		*Cochran-Armitage*		*95% CI*	
SNP	*Allele1*	*Allele2*	*11*	*12*	*22*	*11*	*12*	*22*	*ADR*	*Non-ADR*	*trend* P-*value*	*OR*[b]	*Lower*	*Upper*
rs9561778	T	G	8	60	78	1	16	61	0.26	0.12	0.00028	3.13	1.68	5.83
rs931110	A	G	29	64	53	26	38	14	0.42	0.58	0.0021	2.61	1.34	5.05
rs4148532	T	A	9	58	79	1	17	60	0.26	0.12	0.00063	2.83	1.53	5.22
rs2698243	C	T	27	68	51	24	40	14	0.42	0.56	0.0036	2.45	1.26	4.76
rs1729775	A	G	10	58	78	1	22	55	0.27	0.15	0.0060	2.08	1.17	3.73

Abbreviations: ADR, adverse drug reaction; CI, confidence interval; MAF, minor allele frequency.
[a]CAF: C; Cyclophosphamide, A; Anthracyclin (Epirubicin or Adriamycin), F; 5-fluorouracil.
[b]OR: Odds ratio of risk genotype vs non-risk genotype.

CYP2C19, *CYP3A4* and *CYP3A5*), detoxification (*GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*, *ALDH1A1* and *ALDH3A1*) and transportation of CPA (*ABCC2*, and *ABCC4*), and clarified that one SNP, rs9561778, in *ABCC4* was significantly associated with ADRs caused by CPA combination therapy.

ABCC4 is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABCC4 protein is expressed relatively ubiquitously in many organs including the kidney,[19] lung,[20] liver,[21] prostate,[22] brain,[23] pancreas,[24] lymphocytes[25] and platelets.[26] ABCC4 transports some of its substrates in GSH-dependent manner and depletion of intracellular GSH by GSH synthesis inhibitor, DL-buthionine-(S,R)-sulfoximine, blocks ABCC4-mediated export of the substrates, such as bile acid and cAMP.[27] A recent study indicated that CPA and/or its active metabolites are the substrates to ABCC4 because the *in vitro* CPA cytotoxicity was significantly enhanced by the addition of DL-buthionine-(S,R)-sulfoximine.[9]

The expression of ABCC4 in the kidney might have an important role in the elimination of CPA, and its metabolites from the body and genetic variations within this gene might affect the amount or nature of this transporter, resulting in the impairment of excretion and subsequent overdose manifestation. This idea was supported by several previous studies that showed specific localization of ABCC4 in the kidney at the apical membrane of proximal tubules and indicated its possible role as one of the efflux pumps for urinary excretion. The substrates for ABCC4 so far found are purine metabolites urate, cAMP, cGMP and methotrexate.[19,28,29] A recent report has suggested that not only CPA, but also its active metabolites are substrates to ABCC4,[9] and a significant proportion of them is likely to be excreted through the urine.[30] Hence, ABCC4 might act as one of the important efflux pumps for urinary excretion for both CPA and its metabolites. However, to prove the hypothesis that ABCC4 functions in the renal excretion of CPA and its metabolites, further studies are required. In addition, the expression of ABCC4 in the sinusoidal membrane of hepatocytes might facilitate the secretion of active metabolites of CPA produced from the liver into the systemic circulation. Variants on this gene might cause an excess efflux of CPA and its metabolites, which consequently increase systemic drug concentration in the body.

In this association study, one SNP (rs9561778) that showed a significant association with CPA-induced ADRs, was located in intron 26 of the *ABCC4* gene. Although two functional SNPs were also examined, we found no association of them with ADRs. Hence, we assume that rs9561778, some other variants in LD with it, or their combined haplotype possibly influence the expression levels of the gene product. The SNP function prediction software (FastSNP, http://fastsnp.ibms.sinica.edu.tw/pages/input_SNPListAnalysis.jsp) indicated that the SNP, rs9561778, might be located within a transcription factor binding site possibly within an intronic enhancer sequence and serve as a causative variant affecting the expression level of the gene. However, further functional analyses are required to clarify how this SNP influences the drug activity.

We found that rs9561778, which showed significant association with CPA-induced ADRs, possessed similar trends of odds ratio in both the gastrointestinal toxicity and leukopenia/neutropenia (OR=2.31 and 1.83, respectively), indicating that the two toxicities might be caused by an overdose manifestation of CPA, which leads to ADR development. We suspect that the impairment of ABCC4 might cause an insufficient CPA clearance and subsequent increase of the CPA concentration in the body, although further investigation is required. Furthermore, we observed associations of rs931110, rs2698243 and rs1729775 with either gastrointestinal toxicity or leukopenia/neutropenia (Table 4). These associations might be observed simply because of the LD with rs9561778, but the stronger association with one phenotype might be explained by the effect of these SNPs on the tissue-specific expression of ABCC4 and the tissue-specific clearance of the drug. However, this hypothesis should be validated by association analysis using larger samples as well as by a functional analysis of these SNPs.

We identified novel SNPs that might be significantly associated with ADRs in breast cancer patients treated with the CA(F) regimen. Although the number of samples used for this subgroup analysis was small, the SNP rs9561778 in *ABCC4*, which was significantly associated with the ADR induced by CPA combination therapy (Cochran–Armitage trend's *P*-value=0.00031; OR=2.06; 95% CI=1.36–3.11), revealed an even stronger association and higher OR with ADR induced by the CA(F) regimen for breast cancer (Cochran–Armitage trend's *P*-value=0.00028; OR=3.13; 95% CI=1.68–5.83). Although the other four SNPs located within the same LD block showed a similar trend of association, rs9561778 remained the strongest significantly associated SNP, further suggesting that this SNP might act as an important marker for risk of ADR induced by the CA(F) regimen.

In conclusion, through the candidate gene approach, associations between *ABCC4* genotypes and CPA-induced ADRs were identified. Although the association as well as the mechanism to induce ADRs should be further validated by using a larger number of samples or by molecular analysis, this study has contributed another piece of the puzzle into the mist of the prediction system, which may help in identifying patients at risk of CPA-induced ADRs and lead to a better prognosis and quality of life for patients with cancer.

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