Associations Between *ABCG2* Gene Polymorphisms and Isolated Septal Defects in a Han Chinese Population

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Breast cancer resistance protein (BCRP) in the placenta, encoded by the *ABCG2* gene in humans, plays an essential role in regulating fetal exposure to toxicants and the maintenance of cellular folic acid homeostasis. This study aimed at exploring the associations between 421C>A and 34G>A polymorphisms within the *ABCG2* gene of the children and isolated septal defects in a Han Chinese population. An age- and gender-matched case-control study involving 210 pairs was conducted. Genotyping of the *ABCG2* gene polymorphisms was performed by sequencing. Forty-six placental tissues and umbilical cords from healthy Han Chinese mothers with uncomplicated pregnancy were collected to investigate the impact of these two polymorphisms on the transcription and translation activities of the *ABCG2* gene. The results showed that there were no differences in the genotype distributions and allele frequencies of 421C>A polymorphism. For the 34G>A polymorphism, more cases were carriers of the GA/AA genotypes (adjusted odds ratio [OR]: 1.6, 95% confidence interval [CI]: 1.0–2.3). The *ABCG2* mRNA and protein expression did not differ among the three genotypes of 421C>A polymorphism. For the 34G>A polymorphism, the *ABCG2* mRNA and protein expression of the GG genotype was significantly higher than that of the AA genotype. In conclusion, 34G>A polymorphism in the *ABCG2* gene of the children is associated with isolated septal defects in a Han Chinese population, presumably through regulation of BCRP expression in the placenta.

Introduction

WORLDWIDE, CONGENITAL HEART DEFECT (CHD) is one of the most common birth defects, occurring in 7–8 per 1000 live births in China (Liu *et al.*, 2013). After years of study, a certain proportion of CHDs still has no identifiable cause, but is commonly believed to involve the complex interaction of multiple environmental and genetic factors (Jenkins *et al.*, 2007; Pierpont *et al.*, 2007). Substantial epidemiological data have demonstrated that several toxicants exposure and/or a lack of folic acid supplementation periconceptionally is associated with an increased risk of CHD (Hanke and Jurewicz, 2004; Bailey and Berry, 2005; Thulstrup and Bonde, 2006; Jenkins *et al.*, 2007; Kishi *et al.*, 2008; Gilboa *et al.*, 2012; Lupo *et al.*, 2012). However, not all women exposed to toxicants periconceptionally give birth to a child with CHD, and not all population subgroups benefit equally from folic acid sup-

plementation. The reasons for such phenomenon remain to be experimentally elucidated.

The developing fetus is a highly sensitive, fragile tissue whose uncomplicated development mainly depends on optimal in-uterus conditions. The placenta is obviously considered the crucial organ responsible for successful ontogeny. Evidence strongly suggests that the placenta expresses a range of transporters which are capable of controlling the transplacental dispositions of many toxicant agents, thereby playing a crucial role in fetal protection against maternal toxins (Myllynen *et al.*, 2005, 2009; Ceckova *et al.*, 2006; Atkinson *et al.*, 2007; Behravan and Piquette-Miller, 2007; Myren *et al.*, 2007; Vahakangas and Myllynen, 2009; Prouillac and Lecoeur, 2010; Ni and Mao, 2011; Iqbal *et al.*, 2012). Of main interest are the ATP-binding cassette (ABC) transporters, particularly the P-glycoprotein (P-gp) and the breast cancer resistance protein (BCRP), which are most intensively studied

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currently. They are encoded by the *ABCB1* and *ABCG2* gene in humans, respectively (Mao, 2008; Hahnova-Cygalova *et al.*, 2011).

Our previous study has demonstrated that 3435C>T polymorphism in the *ABCB1* gene of the children increases the risk of CHD when the mothers are exposed to phthalates and alkylphenolic compounds periconceptionally, particularly for isolated septal defects (Wang *et al.*, 2013). As the P-gp, BCRP also has the capacity to actively extrude a wide range of toxicants back to the maternal compartment, thus preventing potentially harmful compounds from entering the fetal compartment. Moreover, toxicants that are already present in the fetal circulation can also be removed by this transporter (Mao, 2008; Hahnova-Cygalova *et al.*, 2011). BCRP expression and its efflux activity in placenta have been confirmed in a variety of *in vitro* and *in vivo* studies (Jonker *et al.*, 2000, 2002; Kolwankar *et al.*, 2005; Ceckova *et al.*, 2006; Evseenko *et al.*, 2006; Enokizono *et al.*, 2007; Zhang *et al.*, 2007; Gedeon *et al.*, 2008). Besides its protective role, the BCRP, which is currently the only recognized ABC transporter that exports mono- and polyglutamates of folic acid, has been shown to be down-regulated under conditions of both long- and short-term folic acid deprivation, suggesting that BCRP plays an essential role in the maintenance of cellular folic acid homeostasis (Ifergan and Assaraf, 2008). Therefore, not only folic acid deficiency alone, but also increased exposure to toxicants due to consequently lowered BCRP expression might jeopardize normal fetal development. In light of findings mentioned earlier, it is conceivable that the inter-individual variability in expression of BCRP in placenta is also likely to be associated with fetal susceptibility to CHD.

To date, evidence has suggested that several single nucleotide polymorphisms (SNPs) in the *ABCG2* gene are associated with altered BCRP expression and functionality, of which 421C>A (rs2231142) and 34G>A (rs2231137) SNPs occur at a relatively high frequency in most ethnicities and were widely proved to be associated with susceptibility of several diseases (Ieiri, 2012). From this background, we hypothesized that these two polymorphisms within the *ABCG2* gene may potentially increase the risk for CHD through altering BCRP expression in placenta. Therefore, for the first time, a case-control study was conducted to explore the associations between 421C>A and 34G>A polymorphisms in *ABCG2* gene and CHD in a Han Chinese population. In addition, maternal placentas and umbilical cords were collected to examine the effect of these two polymorphisms on the *ABCG2* gene transcription and translational expression.

Materials and Methods

Ethics statements

Informed written consent was obtained from the parents, who were on the behalf of their children as well, about their participation in the study after the nature of this study had been fully explained to them. Informed written consent was also obtained when we collected the placental tissue and umbilical cords. The study was performed according to the principles of Good Clinical Practice and the Declaration of Helsinki and approved by the University Committee on Human Subjects at Sichuan University.

Study participants

An age- and gender-matched case-control study was conducted from March 2012 to April 2013. In total, 210 children with isolated septal defects and 210 matched children without any congenital malformations were enrolled. All cases and controls were recruited from the Department of Pediatric Cardiology in West China Second University Hospital, Sichuan University. All of them were born in Sichuan province, and their birth mothers were local residents. Since our department is a pediatric cardiology interventional center, the diagnosis of all cases was confirmed by cardiac catheterization. Meanwhile, the diagnosis of all control children was checked by two experienced pediatric cardiologists according to medical records. To minimize recall bias of exposure by the mother to the greatest extent, all children recruited were younger than 3 years old. Children with a family history of CHD and 22q11 deletion or other chromosomal abnormalities were excluded. After the exclusion, 206 children with septal defects and 202 controls were used for analysis. All recruited children are unrelated. The included CHD phenotypes were perimembranous ventricular septal defect (Pm VSD, $n=158$), secundum atrial septal defect (s-ASD, $n=48$).

It is essential to correct for further risk factors of CHDs when we explore the associations between 421C>A and 34G>A polymorphisms of the *ABCG2* gene and CHDs. Therefore, a questionnaire survey was conducted when we collected the children's blood. It had been described in detail in our previous study (Wang *et al.*, 2013).

The placenta is formed by both fetal (chorionic plate and chorionic villi) and maternal (decidua basalis) tissues. The fetal placenta consists of syncytiotrophoblast and cytotrophoblast layers. The BCRP is located in the maternal-facing apical membrane of the syncytiotrophoblast and the fetal blood vessels of the villous core, both of which are of fetal origin (Weier *et al.*, 2008; Prouillac and Lecoœur, 2010; Mason *et al.*, 2011; Ni and Mao, 2011; Iqbal *et al.*, 2012; Staud *et al.*, 2012). Therefore, only the genotypes of 421C>A and 34G>A polymorphisms in the *ABCG2* gene of the children (but not mothers) might be associated with altered BCRP expression and transport activity in placenta, thereby modifying the inter-individual susceptibility to CHDs. Thus, in the present study, only children's blood was collected for analysis.

Placenta and umbilical cords collection

Forty-six human placentas and umbilical cords were consecutively obtained from mothers who delivered at the Department of Gynecology and Obstetrics of our hospital by a well-trained nurse. Immediately after delivery, large tissue cores through the full thickness of the placenta were obtained in each quadrant, avoiding chorionic plate tissue and areas with obvious evidence of thrombosis or other abnormalities. Meanwhile, the umbilical cords were also collected. The tissues were immediately frozen in liquid nitrogen and stored at -80°C until use. Moreover, the maternal and neonatal clinical data were also collected, including maternal age, maternal ethnicity, health conditions, pregnancy complications, any medication consumptions, and neonatal birth outcomes (e.g., sex, gestational duration, birth length, birth weight, and placental weight).

TABLE 1. SOCIO-DEMOGRAPHIC AND LIFESTYLE CHARACTERISTICS OF MOTHERS AND CHILDREN

Variables ^a	Cases n=206	Controls n=202	p-Value ^b
Characteristics of children			
Age at intake (months)	26.30±7.40	26.20±7.60	
Gender (male)	90 (43.7)	88 (43.6)	
Gestational age			0.270
>42 weeks	3 (1.5)	4 (2.0)	
37–42 weeks	195 (94.7)	183 (90.6)	
<37 weeks	8 (3.9)	15 (7.4)	
Birth weight (kg)	3.12±0.49	3.15±0.58	0.568
Birth length (cm)	49.87±1.96	50.10±2.10	0.264
Twins (yes)	7 (3.4)	6 (3.0)	0.806
Artificial fertilization (yes)	8 (3.9)	5 (2.5)	0.418
Characteristics of mothers			
Age at births (years)			0.223
≥35	17 (8.3)	12 (5.9)	
20–35	163 (79.1)	173 (85.6)	
≤20	26 (12.6)	17 (8.4)	
Education level			<0.001
Illiteracy	1 (0.5)	8 (4.0)	
Primary school	32 (15.5)	26 (12.9)	
Middle school	88 (42.7)	47 (23.3)	
High school	44 (21.4)	47 (23.3)	
College or higher	41 (19.9)	74 (36.6)	
BMI (kg/m ²) ^c			0.967
Underweight	40 (19.4)	37 (18.3)	
Normal	153 (74.3)	150 (74.3)	
Overweight	13 (6.3)	15 (7.4)	
Gravity			0.608
1	73 (35.4)	79 (39.1)	
2	60 (29.1)	61 (30.2)	
3	37 (18.0)	36 (17.8)	
≥4	36 (17.5)	26 (12.9)	
History of previous abortions			
Spontaneous abortion	12 (5.8)	11 (5.5)	0.868
Artificial abortion			0.398
0	104 (50.5)	116 (57.4)	
1	57 (27.7)	48 (23.8)	
2	33 (16.0)	24 (11.9)	
≥3	12 (5.8)	14 (6.9)	
Previous stillbirth (yes)	6 (2.9)	3 (1.5)	0.326
Ectopic pregnancy (yes)	2 (1.0)	2 (1.0)	0.984
Preconceptional ^d			
Folic acid (yes)	24 (11.7)	30 (14.9)	0.340
Smoking (yes)	8 (3.9)	8 (4.0)	0.864
Alcohol consumption (yes)	13 (6.3)	17 (8.4)	0.415
Tea drinking (yes)	25 (12.1)	21 (10.4)	0.579
Coffee drinking (yes)	4 (1.9)	4 (2.0)	0.978
Toxicants exposure (yes)	86 (41.7)	60 (29.7)	0.011
Medication use (yes)	71 (34.5)	48 (23.8)	0.017
Settlement			0.125
Urban	90 (43.7)	108 (53.5)	
Rural	84 (40.8)	71 (35.1)	
Suburban	32 (15.5)	23 (11.4)	

^aQuantitative data and categorical data were expressed as mean±SD and *n* (%), respectively.

^bMann–Whitney *U*-test was used to compare means; chi-square test was used to compare proportions, and Fisher's Exact test was also used if not matched. A two-tailed *p*-value was chosen as the level of significance.

^cBMI was calculated as weight divided by height (m) squared and it was divided into four categories: underweight (<18.5 kg/m²), normal (18.5–23.9 kg/m²), overweight (24.0–27.9 kg/m²), and obese (≥28 kg/m²), in accordance with the criteria specially formulated for Chinese (Wang *et al.*, 2013).

^dThe periconceptional period was defined as 4 weeks before conception until the end of first trimester. The maternal use of folic acid periconceptionally was defined as the daily use of at least 400 µg folic acid during the complete periconceptional period. Inconsistent users were classified as nonusers. We defined smoking, alcohol consumption, tea drinking, coffee drinking, and medication use as any use periconceptionally. We assessed occupational exposure to chemicals by applying a JEM, with a focus on endocrine-disrupting chemicals. Environmental exposure only included living in a newly decorated house for at least 1 month periconceptionally. Given the relatively small number of exposed parents in any particular environmental and occupational category, statistical analysis for toxicant exposure was performed, including both environmental and occupational exposure (Wang *et al.*, 2013).

BMI, body mass index; JEM, job exposure matrix.

Only placentas and umbilical cords from healthy Han Chinese mothers with uncomplicated pregnancy and neonates with a gestational age between 38 and 41 weeks and a birth weight between 2500 and 4000 g were included. Those samples from mothers with chronic diseases (e.g., hepatitis, hypothyroidism, polycystic ovary syndrome, and sexually transmitted infections), pregnant complications (e.g., gestational diabetes mellitus, pregnancy-induced hypertension, intrahepatic cholestasis of pregnancy, and placenta previa), and any medication use were excluded.

Genotyping

DNA was extracted from leucocytes of the whole blood and from the umbilical cord by a commercial SE blood DNA isolation kit (D3471-02; Omega Bio-Tek, Norcross, GA) and a commercial tissue DNA isolation kit (D3396-01; Omega Bio-Tek), respectively. Three hundred ninety-one- and 371-bp fragments were amplified using 0.25 μ L Taq DNA polymerase (Takara, Dalian, China), 2.5 μ L 10 $\times$ PCR buffer, 1 μ L dNTP (2.5 mM), 0.5 μ L forward primer (10 pM), 0.5 μ L reverse primer (10 pM), and 30 ng of genomic DNA in a total volume of 25 μ L for 421C>A and 34G>A polymorphism, respectively. PCR conditions were 34 cycles of 30 s at 95°C, 30 s at 64/55°C, and 1 min at 72°C, preceded by an initial denaturation of 5 min at 95°C, and followed by a final extension of 7 min at 72°C for 421C>A and 34G>A,

respectively. PCR products were purified using a Watson DNA Purification Kit (Watson Company, Shanghai, China) and were directly sequenced on an ABI 3730 (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The primers sequences used were as follows:

5'-TAGCAGGCTTTGCAGACATC-3' (*ABCG2* 421C>A-F), 5'-ATCCACACAGGGAAAGTCCT-3' (*ABCG2* 421C>A-R)

5'-TTGTGCCTGTCTTCCCATTTA-3' (*ABCG2* 34G>A-F), 5'-CGACAAGGTAGAAAGCCACTC-3' (*ABCG2* 34G>A-R).

RNA extraction and cDNA synthesis

Total RNA was extracted from 50 to 100 mg of frozen placental tissue by TRIzol (Invitrogen, Life technologies, Carlsbad, CA) and subjected to qualitative and quantitative measurements using a spectrophotometer (GeneQuant 100; GE Healthcare, Fairfield, CT). One microgram of total extracted RNA was reverse transcribed to cDNA using Prime Script RT reagent kit with gDNA eraser (DRR047A; Takara) according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (qRT-PCR) was performed with cDNA templates and SsoFast EvaGreen Supermixture (Bio-Rad Laboratories, Hercules,

TABLE 2. THE GENOTYPE DISTRIBUTIONS AND ALLELE FREQUENCIES OF 421C>A AND 34G>A POLYMORPHISMS OF THE *ABCG2* GENE

SNP	Genotyping distribution	Controls n (%)	Cases n (%)	p	OR (95% CI)	Adjusted p ^a	Adjusted OR (95% CI) ^a
421C>A	C/C	103 (51.0)	107 (51.9)	0.963		0.911	
	C/A	83 (41.1)	84 (40.8)				
	A/A	16 (7.9)	15 (7.3)				
	Hardy-Weinberg equilibrium p-value	0.05	0.05				
	Dominant model			0.808	1.1 (0.5-2.3)	0.967	1.0 (0.6-2.0)
	C/C or C/A	186 (92.1)	191 (92.7)				
	A/A	16 (7.9)	15 (7.3)	0.848	1.0 (0.7-1.5)	0.850	0.9 (0.7-1.5)
	Recessive model						
	C/C	103 (51.0)	107 (51.9)	0.800	1.0 (0.8-1.4)		
	C/A or A/A	99 (7.9)	99 (7.3)				
34G>A	Co-dominant model			0.078		0.095	
	C allele	289 (71.5)	298 (72.3)				
	A allele	115 (28.5)	114 (27.7)	0.029	1.6 (1.0-2.3)	0.033	1.6 (1.0-2.3)
	GG	94 (46.5)	74 (35.9)				
	GA	85 (42.1)	108 (52.4)				
	AA	23 (11.4)	24 (11.7)				
	Hardy-Weinberg equilibrium p-value	0.05	0.05	0.933	1.0 (0.5-1.8)	0.851	1.1 (0.6-2.0)
	Dominant model						
	AA/GA	108 (53.5)	132 (64.1)	0.104	0.8 (0.6-1.1)		
	GG	94 (46.5)	74 (35.9)				
	Recessive model						
	GG/GA	179 (88.6)	182 (88.3)				
	AA	23 (11.4)	24 (11.7)				
	Co-dominant model						
	G allele	273 (67.6)	256 (62.1)				
	A allele	131 (32.4)	156 (37.9)				

^aAdjusted by maternal educational level, maternal medication use, and toxicants exposure periconceptionally. CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.

CA). Briefly, sequences were amplified using 5 μ L reaction mixture, 0.3 μ L forward primer (10 pM), 0.3 μ L reverse primer (10 pM), 3.4 μ L nuclease-free H₂O, and 1 μ L cDNA in a total volume of 10 μ L. PCR conditions were 39 cycles of 30 s at 95°C, 30 s at 61°C, and 1 min at 72°C, preceded by an initial denaturation of 3 min at 95°C, and followed by a continuous melt curve from 65°C to 95°C. All the primer sets were tested to ensure the efficiency of amplification over a range of template concentrations. All samples were amplified in triplicate. Expression level of ABCB1 transcript was represented by the mean of triple tests. The relative expressions of ABCB1 mRNA were normalized to expression of GAPDH using $2^{-\Delta\Delta ct}$ method.

The primer sequences used were as follows:

5'-TATAGCTCAGATCATTGTACAGTC-3' (ABCG2-F), 5'-GTTGGTCGTCAGGAAGAAGAG-3' (ABCG2-R)

5'-GAAGGTGAAGGTCGGAGTC-3' (GAPDH-F), 5'-GAAGATGGTGATGGGATTTC-3' (GAPDH-R).

Western blot analysis of BCRP

One hundred milligrams placenta tissues were homogenized in buffer containing 1 mL RIPA (P0013B; Beyotime, Shanghai, China) and 10 μ L complete protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO). The homogenate was then centrifuged at 12,000 *g* for 5 min at 4°C. Protein concentration was determined using enhanced BCA

protein assay kit (P0010S; Beyotime) according to the manufacturer's protocol. Total protein (50 μ g/lane) was separated on 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked in 5% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween-20 (PBST). Thereafter, blocked membranes were incubated overnight at 4°C with monoclonal primary antibody for BCRP (ab130244, 1:300; Abcam, Cambridge, United Kingdom) and GAPDH (ab9484, 1:7500; Abcam). After extensive washing with PBST, membranes were incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibodies (No. 107724; ZSGB-BIO, Beijing, China). After extensive washing with PBST, protein-antibody complexes were visualized by the enhanced chemiluminescence's detection system. The optical density (OD) of each band was measured using Gelpro32 software. The relative OD of BCRP for each blot was normalized internally to GAPDH.

Statistical analysis

All materials were coded, data were doubly input using EPIDATA 3.1 version, and all analyses were conducted with SPSS 17.0 version. Socio-demographic and life-style characteristics were compared between the groups using Mann–Whitney *U*-test for quantitative variables and

TABLE 3. THE GENOTYPE DISTRIBUTIONS AND ALLELE FREQUENCIES OF 421C>A AND 34G>A POLYMORPHISMS OF THE ABCG2 GENE STRATIFIED BY PERIMEMBRANOUS VENTRICULAR SEPTAL DEFECT

SNP	Genotyping distribution	Controls n (%)	Pm VSD n (%)	p	OR (95% CI)	Adjusted p ^a	Adjusted OR (95% CI) ^a
421C>A	C/C	103 (51.0)	83 (52.5)	0.925		0.790	
	C/A	83 (41.1)	64 (40.5)				
	A/A	16 (7.9)	11 (7.0)				
	Hardy–Weinberg equilibrium <i>p</i> -value	0.05	0.05				
	Dominant model			0.732	1.2 (0.5–2.6)	0.848	1.0 (0.5–2.0)
	C/C or C/A	186 (92.1)	147 (93.0)				
	A/A	16 (7.9)	11 (7.0)	0.771	1.1 (0.7–1.6)	0.785	0.9 (0.6–1.5)
	Recessive model						
	C/C	103 (51.0)	83 (52.5)	0.711	1.1 (0.8–1.5)		
	C/A or A/A	99 (7.9)	75 (47.5)				
34G>A	Co-dominant model			0.711	1.1 (0.8–1.5)		
	C allele	289 (71.5)	230 (72.8)				
	A allele	115 (28.5)	86 (27.2)	0.046		0.082	
	GG	94 (46.5)	54 (34.2)				
	GA	85 (42.1)	86 (54.4)				
	AA	23 (11.4)	18 (11.4)				
	Hardy–Weinberg equilibrium <i>p</i> -value	0.05	0.05	0.018	1.7 (1.1–2.6)	0.022	1.7 (1.1–2.6)
	Dominant model						
	AA/GA	108 (53.5)	104 (65.8)	0.999	1.0 (0.5–1.9)	0.948	1.0 (0.5–2.0)
	GG	94 (46.5)	54 (34.2)				
	Recessive model			0.085	0.8 (0.6–1.0)		
	GG/GA	179 (88.6)	140 (88.6)				
	AA	23 (11.4)	18 (11.4)				
	Co-dominant model						
	G allele	273 (67.6)	194 (61.4)				
	A allele	131 (32.4)	122 (38.6)				

^aAdjusted by maternal educational level, maternal medication use, and toxicants exposure periconceptionally.

Pm VSD, perimembranous ventricular septal defect.

chi-square test for categorical variables. Fisher's Exact test was also used if not matched.

The chi-square test was used to test for deviation from Hardy–Weinberg equilibrium and to compare allelic and genotypic frequencies among the groups. For SNP alleles, we calculated three categories of *p*-values for the different probable function of the mutant allele as follows: dominant, recessive, and co-dominant. Then, multivariate logistic regression analysis was performed to correct for the risk factors of isolated septal defects. The odds ratios (ORs) and 95% confidence intervals (CIs) were adjusted for the following confounding factors: maternal education level, maternal medication use, and toxicant exposure periconceptionally (Table 1).

For the *ABCG2* mRNA and protein expression in the placenta, data were expressed as mean $\pm$ SEM. Haplotypes were constructed from genotype data using the PHASE software.

Shapiro–Wilk test and Q-Q plot were used to confirm that data about the *ABCG2* mRNA and protein levels among different genotype and haplotype groups come from a normal distribution. Then, differences among different genotypes and haplotypes were determined by one-way ANOVA followed by a Student–Newman–Keuls multiple-comparison *post hoc* test and *t*-test, respectively.

Power of this study was calculated by Quanto v1.2.4 (Garcia-Closas and Lubin, 1999), suggesting that the sample size of this study could detect an OR of 1.60 with a power of 0.66 for a genetic study under the assumptions of dominant model, 7‰ disease prevalence, 5% minor allele frequency, 1:1 case-to-control ratio, and 5% type I error rate (α).

A two-tailed *p*-value < 0.05 was chosen as the level of significance. Given that only 48 patients with s-ASD were recruited in the present study, statistical analysis of this subgroup might not be feasible due to the limited sample size. Therefore, stratified analysis of the different CHD phenotypes was only conducted for Pm VSD.

Results

The results were shown in detail as follows:

Socio-demographic and lifestyle characteristics of mothers and children are illustrated in Table 1 in detail. There are no significant differences in patient age, gender distribution, gestational age, birth weight, and birth length. Maternal education level, maternal medication use, or toxicants exposure periconceptionally are significantly associated with isolated septal defects.

The genotype distributions and allele frequencies of 421C $>$ A and 34G $>$ A polymorphisms of the *ABCG2* gene are listed in Tables 2 and 3. The genotype distributions of both polymorphisms are in accordance with Hardy–Weinberg equilibrium. There are no differences in the genotype distributions and allele frequencies of 421C $>$ A polymorphism. However, for the 34G $>$ A polymorphism, more cases are carriers of the *ABCG2* GA/AA genotypes, which are significantly associated with an increased risk of isolated septal defects (adjusted OR: 1.6, 95% CI: 1.0–2.3, *p*-value: 0.033). This significant association is slightly increased for Pm VSD (OR: 1.7, 95% CI: 1.1–2.6, *p*-value: 0.022).

The genotype distributions of 421C $>$ A polymorphism and 34G $>$ A polymorphism for the 46 umbilical cords are listed as follows: CC: 24 (52.2%), CA: 18 (39.1%), and AA:

TABLE 4. CLINICAL DATA OF MOTHERS AND NEONATES STRATIFIED BY GENOTYPES OF 421C $>$ A AND 34G $>$ A POLYMORPHISMS

SNP	Genotype	Maternal age (years)	Prepregnancy BMI ^a	Gestational duration (weeks)	Gravity	Parity	Gender (F)	Neonatal weight (g)	Neonatal length (cm)	Placental weight (g)
421C $>$ A	CC (<i>n</i> = 24)	30.95 $\pm$ 3.63	20.38 $\pm$ 2.98	39.92 $\pm$ 0.98	2.00 $\pm$ 1.12	1.15 $\pm$ 0.37	13 (54.2)	3363.25 $\pm$ 360.96	50.40 $\pm$ 1.39	566.10 $\pm$ 68.78
	CA (<i>n</i> = 18)	29.00 $\pm$ 3.73	21.02 $\pm$ 3.23	39.26 $\pm$ 0.92	2.50 $\pm$ 1.34	1.44 $\pm$ 0.62	9 (50.0)	3227.22 $\pm$ 213.83	49.78 $\pm$ 1.22	552.72 $\pm$ 76.00
	AA (<i>n</i> = 4)	29.33 $\pm$ 3.06	20.31 $\pm$ 3.41	39.10 $\pm$ 0.97	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	2 (50.0)	3208.33 $\pm$ 211.68	49.67 $\pm$ 0.58	563.33 $\pm$ 70.89
34G $>$ A	GG (<i>n</i> = 19)	30.21 $\pm$ 4.18	20.61 $\pm$ 3.70	39.47 $\pm$ 0.93	2.32 $\pm$ 1.34	1.21 $\pm$ 0.54	12 (63.2)	3264.21 $\pm$ 306.67	49.63 $\pm$ 1.30	540.89 $\pm$ 77.31
	GA (<i>n</i> = 20)	30.10 $\pm$ 2.85	20.81 $\pm$ 2.65	39.55 $\pm$ 1.08	2.25 $\pm$ 1.45	1.30 $\pm$ 0.47	9 (45.0)	3369.47 $\pm$ 299.82	50.26 $\pm$ 1.15	555.10 $\pm$ 83.94
	AA (<i>n</i> = 7)	30.14 $\pm$ 4.74	20.72 $\pm$ 2.97	39.71 $\pm$ 1.18	2.00 $\pm$ 1.27	1.33 $\pm$ 0.52	3 (42.9)	3456.43 $\pm$ 476.43	50.57 $\pm$ 1.72	578.29 $\pm$ 66.14
	<i>p</i> ^b	0.996	0.977	0.870	0.887	0.809	0.435	0.380	0.181	0.559

^aBMI was calculated as weight divided by height (m) squared.

^bDifferences among three genotypes were determined by one-way ANOVA followed by Student–Newman–Keuls multiple-comparison *post hoc* tests.

FIG. 1. The *ABCG2* gene mRNA expression stratified by 421C>A and 34G>A polymorphisms. The *ABCG2* gene mRNA expression was determined by real-time quantitative polymerase chain reaction in human placenta. All data are presented as mean $\pm$ SEM. Differences among the three genotypes were determined by one-way ANOVA followed by Student–Newman–Keuls multiple-comparison *post hoc* tests. **(A)** The sample size for CC, CA, and AA genotypes of 421C>A polymorphism is 24, 18, and 4, respectively. The *ABCG2* gene mRNA expression does not differ among the three genotypes of 421C>A polymorphism (1.23 ± 0.19 , 1.52 ± 0.27 , and 2.12 ± 1.16 for CC, CA, and AA genotypes, respectively). **(B)** The sample size for GG, GA, and AA genotypes of 34G>A polymorphism is 19, 20, and 7, respectively. The *ABCG2* gene mRNA expression of the GG genotype was significantly higher than that of the AA genotype (1.85 ± 0.29 vs. 0.77 ± 0.20 , $p=0.023$), and heterozygous samples display an intermediate value (1.34 ± 0.20). * $p<0.05$, compared with the GG genotype of 34G>A polymorphism.

4 (8.6%) for 421C>A polymorphism; GG: 19 (41.3%), GA: 20 (43.4%), and AA: 7 (15.2%) for 34G>A polymorphism. Clinical data of mothers and neonates stratified by 421C>A and 34G>A genotypes are illustrated in Table 4. There are no significant differences in maternal age, body mass index, gestational duration, gravity, placental weight, neonatal weight, and length among three genotype groups of both polymorphisms. As shown in Figure 1, the *ABCG2* gene mRNA expression does not differ among the three genotypes of 421C>A polymorphism (1.23 ± 0.19 , 1.52 ± 0.27 , 2.12 ± 1.16 for CC, CA, and AA genotypes, respectively); in contrast, for the 34G>A polymorphism, the *ABCG2* gene mRNA expression of the GG genotype is significantly

higher than that of the AA genotype (1.85 ± 0.29 vs. 0.77 ± 0.20 , $p=0.023$), and the heterozygous samples display an intermediate value (1.34 ± 0.20). Meanwhile, consistent with the mRNA results, the BCRP relatively expression does not differ among the three genotypes of 421C>A polymorphism (Fig. 2A). On the contrary, for the 34G>A polymorphism, compared with the AA genotype, relatively higher BCRP expression is observed for the GG genotype ($p=0.034$), and the GA genotype displays an intermediate value (Fig. 2B).

On the basis of the haplotype analysis, four haplotypes are identified: G-A, G-C, A-A, and A-C (in order of 34G>A and 421C>A polymorphism). Their corresponding allelic

FIG. 2. Breast cancer resistance protein (BCRP) relatively expression stratified by 421C>A and 34G>A polymorphisms. Western blot of BCRP relatively expression in human placenta. All data are presented as mean $\pm$ SEM. Differences among the three genotypes were determined by one-way ANOVA followed by Student–Newman–Keuls multiple-comparison *post hoc* tests. **(A)** The BCRP relatively expression does not differ among the three genotypes of 421C>A polymorphism. **(B)** For the 34G>A polymorphism, compared with the AA genotype, relatively higher BCRP expression is observed for the GG genotype ($p=0.034$), and the GA genotype displays an intermediate value. * $p<0.05$, compared with the GG genotype of 34G>A polymorphism.

FIG. 3. Haplotype-phenotype (mRNA and protein expression) correlation analyses. All the haplotypes are not associated with significant mRNA and protein alteration. However, among umbilical cords carrying homozygotes for the C421 allele, both mRNA and protein of the *ABCG2* gene is significantly lower in homozygotes for the A34 allele than those for the G34 allele. * $p < 0.05$.

frequencies are 45.7%, 39.1%, 2.2%, and 13.0%, respectively. As shown in Figure 3, the results of the haplotype-phenotype (mRNA and protein expression) correlation analyses show that all the haplotypes are not associated with significant mRNA and protein alteration. However, among umbilical cords carrying homozygotes for the C421 allele, both mRNA and protein of the *ABCG2* gene is significantly lower in homozygotes for the A34 allele than those for the G34 allele ($p < 0.05$).

Discussion

This is the first study that investigates associations between the *ABCG2* polymorphisms and CHD in a Han Chinese population. Our data suggested that GA/AA genotypes of 34G>A polymorphism within the *ABCG2* gene of the children increase the risk of isolated septal defects, presumably through downregulation of BCRP in placenta.

Highly expressed in placenta throughout the whole gestational period, BCRP has the capacity to protect the fetus from the toxicants in the maternal circulation (Mao, 2008; Hahnova-Cygalova *et al.*, 2011). In *in vitro* studies, BCRP has been shown to mediate transports of mitoxantrone (Ceckova *et al.*, 2006) and glyburide (Gedeon *et al.*, 2008) in membrane vesicles prepared from human term placenta, as well as mitoxantrone and Hoechst 33342 in membrane vesicles isolated from the placental BeWo cells (Ceckova *et al.*, 2006) or primary human placental trophoblasts (Evseenko *et al.*, 2006). In *ex vivo* studies, human BCRP and rat Bcrp have been shown to significantly limit the maternal-to-fetal transport of PhIP (Myllynen *et al.*, 2008), glyburide (Pollex *et al.*, 2008), and cimetidine (Staud *et al.*, 2006) in the perfused human and rat placenta models, respectively. In pregnant mice deficient in P-gp, co-administration with the Bcrp inhibitor GF120918 caused a significant increase of

topotecan in the fetal compartment (Jonker *et al.*, 2000). Compared with those in wild-type pregnant mice, increased fetal/maternal plasma concentration ratios have also been reported for topotecan and phytoestrogens in the *abcg2*^{-/-} mice (Jonker *et al.*, 2002; Enokizono *et al.*, 2007). Recently, a study examined the role of Bcrp in determining fetal exposure of nitrofurantoin in pregnant mice, clearly suggesting that Bcrp significantly limited fetal distribution of nitrofurantoin (Zhang *et al.*, 2007). These *in vitro* and *in vivo* studies strongly suggested the role of BCRP/Bcrp in limiting fetal exposure of exogenous toxicants and in facilitating elimination of toxicants out of the fetal compartment. Besides the protective role of BCRP against potential toxicity of xenobiotics, several studies have suggested that BCRP played an essential role in the maintenance of cellular folic acid homeostasis (Ifergan and Assaraf, 2008). These findings collectively suggested that the *ABCG2* gene may modify the effect of maternal toxicants exposure and/or folic acid deficiency on CHD. This may explain why we observed an association between 34G>A polymorphism within the *ABCG2* gene of the children with isolated septal defects.

Before this study was conducted, only restricted knowledge about the effect of the *ABCG2* gene polymorphisms on the BCRP expression and activity in the placenta was available. In placentas of the Japanese population, Kobayashi *et al.* (2005) concluded that the 421C>A variant of the *ABCG2* gene led to decreased BCRP expression caused by post-transcriptional regulation and there were no difference in the mRNA and protein expression of the *ABCG2* gene among the three genotypes of 34G>A polymorphism. Evidence suggests that BCRP locates in the maternal-facing apical membrane of the syncytiotrophoblast and the fetal blood vessels of the villous core, both of which are of fetal origin. However, in the study described earlier, the genotype was determined by DNA isolated from whole

placental tissue, which comprises both maternal and fetal cell populations, thereby probably hampering a clear genotype–phenotype correlation. This shortcoming precludes any clear conclusion being made from these data. For the reasons mentioned earlier, umbilical cords were collected for fetal genotyping in the present study, which could reflect the real genotype of the syncytiotrophoblast. Our results demonstrated that there were no differences in the mRNA and protein expression among the three genotypes of the 421C>A polymorphism. On the contrary, for the 34G>A polymorphism, the *ABCG2* mRNA and protein expression level of the GG genotype was significantly higher than that of the AA genotype and the GA genotype displayed an intermediate value. In light of these findings, we hypothesized that lower expression of BCRP in placenta resulting from GA/AA genotypes of the 34G>A polymorphism might increase fetal exposure to toxicants, thereby increasing the risk of CHDs. This may explain why we observed an association between isolated septal defect with 34G>A polymorphism, but not 421C>A polymorphism. However, due to limited placental sample size and lack of measurement of BCRP transport activity, well-designed investigations with a large sample size on the relationship between these two polymorphisms and not only BCRP expression but, most importantly, BCRP transport activity in placenta are needed to address this issue.

In conclusion, we find an association between 34G>A polymorphism within the *ABCG2* gene of the children and isolated septal defect in a Han Chinese population, presumably through regulation of BCRP in placenta. These findings may open potential avenues for research in BCRP and its role in the etiology of CHDs. However, an undoubted limitation of our study was the modest size of the population. The results of the separate CHD phenotypes should be further investigated in much larger data sets.

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Disclosure Statement

None of the authors declared any conflicts of interest.

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