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Original Article

Prevalence of ABCB4 polymorphisms in gallstone disease in han-Chinese population

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Abstract: ATP Binding Cassette Transporter A4 (ABCB4) is a sterol export pump that regulates excretion of biliary cholesterol. We tested association between ABCB4 polymorphisms and gallstone disease using meta-analysis. In a cross-sectional study, 296 subjects were recruited from a hospital-based population. Total of 171 subjects were diagnosed as gallstone disease by abdominal ultrasonography from three cohort studies. We evaluated prevalence of ABCG8 *rs11887534* (D19H) as a positive control, and the ABCB4 *rs1202283* and *rs2230028* polymorphisms on Chinese population were screened by meta-analysis and genotyped using TaqMan® SNP assay. Stata/SE 11.0 software and random-effects model were used in meta-analyzing 3 cohort between study heterogeneity. Four studies including three cohorts were used for final meta-analysis. In allelic model, minor alleles of ABCB4 *rs1202283* (OR = 0.41, 95% CI: 0.25-0.67, *P*<0.001) and of ABCB4 *rs2230028* (OR = 0.12, 95% CI: 0.06-0.22, *P* = 0.001) were associated with an increased risk for gallstone disease in Europeans. Funnel plot and Egger's test suggested absence of publication bias. Concentration of total cholesterol, low-density lipoprotein cholesterol (LDLC) (*P* = 0.015) and high-density lipoprotein cholesterol (HDLC) (*P* = 0.028) were significantly higher in subjects with gallstones disease than controls. ABCB4 *rs1202283* (heterozygote AG) (*P*<0.0001), *rs2230028* (heterozygote CT) (*P* = 0.023) and ABCG8 *rs11887534* (heterozygote CG) (*P* = 0.006) were significantly associated with gallstone disease in Chinese population. Genetic risk associated with ABCB4 *rs2230028* (homozygote GG) polymorphism was dominated in asymptomatic gallstone disease (95% C.I.: 0.219-0.768; *P* = 0.005). In conclusion, carriers of ABCB4 *rs1202283, rs2230028* are at an increased risk for gallstone disease, while ABCB4 rs2230028 is associated with asymptomatic gallstone disease.

Keywords: Gallstone disease, single nucleotide polymorphism, ATP binding cassette transporter A4

Introduction

Gallstone disease is common in Western countries [1]. In China, the prevalence of gallstone disease has increased to 10.8% in the general population compared to 5% five years ago. While an additional 2.97% gallstone disease subjects have a previous history of cholecystectomy. The majority of the gallstone disease is associated with high cholesterol. Recently, studies have suggested that genetic factors, in interaction with environmental factors, are the predisposition to gallstone disease [2]. Katsika *et al*. [3], suggested that genetic heredity contributes 25% of factors to gallstone formation after an elegant analysis of data from Swedish twins.

Since late 1980s', studies have attempted to reveal susceptible genes associated with gallstone disease in different populations. The possible genes studies included apolipoprotein (Apo)-E [4-7], Apo-B, [5, 6] and cholesterol alpha-hydroxylase [5, 6]. In the last decade, the ABCB4 [adenosine triphosphate (ATP)-binding cassette, sub-family B (MDR/TAP), member 4] transporter located in the hepatocyte canalicular membrane was identified as involving in the transport of phosphatidylcholine into bile. Mutation in the *ABCB4* gene may lead to low levels of biliary phosphatidylcholine, resulting in enhanced cholesterol precipitation and formation of crystals and gallstones. The first evidence that human gallstone disease might be caused by a single gene defect came [8, 9].

Table 1. List of references

References	Year	Ethnicity	SNPs	Methods	Number	
					Case	Control
Oliver Rosmorduc *et al*.	2003	French	Exon 4, 5, 6, 8, 16	PCR-RFLP	108	66
Karl Esten Nakken *et al*.	2008	Norwegian	Exon 5, 6, 13, 14, 15, 16	AD	16	95
Bronsky *et al*.	2010	Czech	Exon 6, 16	PCR-RFLP	35	150
Milan Jirsa *et al*.	2014	Czech	Exon 6, 16	AD	370	150

AD: allelic discrimination; PCR: polymerase chain reaction; RFLP: restricted fragment length polymorphism.

Table 2. Baseline data for 296 recruited subjects

IndexIndex	Gallstones (n = 171)	No stones (n = 125)	P
Sex ratio (M:F)	72:99	51:74	0.905†
Age (years)	46.1±9.2	53.2±5.6	0.267
BMI (kg/m^2)	24.9±3.1	24.6±2.9	0.889
AST (units/l)	34.07±17.8	30.5±20.4	0.668
ALT (units/l)	33.2±19.3	29.2±21.6	0.867
LDLC (mg/dl)	130.7±35.1	113.3±28.9	0.015*
HDLC (mg/dl)	46.8±10.5	51.8±12.0	0.028*

Two-sample Student's *t* test, except †Pearson χ^2 test. Values are presented as mean ± s.d. BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDLC: low-density lipoprotein cholesterol; HDLC: high-density lipoprotein cholesterol. *P represents the significant differences between the Gallstones group and No stones group. *: *P* value <0.05.

These studies showed that *ABCB4* gene mutations represented a genetic risk factor in a symptomatic and recurring form of gallstone disease in young adults. Furthermore, gallstone formation is a consistent feature of *ABCB4* knockout mice [10]. Accordingly, *ABCB4* genotyping could be used to confirm the diagnosis of LPAC in young adults who present with symptomatic gallstone disease and allow familial screening [9]. With the understanding of ABCB4 as important in regulating biliary cholesterol content and cholesterol absorption, studies on association of *ABCB4* polymorphisms and gallstone disease have been published [8, 11-18]. The most studied loci are ABCB4 exon 6 (*Rs1202283*) and exon 16 (*Rs2230028*). Due to difference in allele frequency at each polymorphic locus between different ethnicities, the associations between these SNPs and gallstone disease have not been consistent. Furthermore, the functional roles of these polymorphic loci have not been fully clarified. Additionally, no study has ever determined whether any difference exists for these SNPs are associated with gallstone disease risk in Chinese people.

In this study, we evaluated the association between polymorphisms at ABCB4 Exon 6 (*Rs1202283*), Exon 16 (*Rs2230028*) and gallstone disease using meta-analysis. We evaluated two loci attaining a genome-wide significance level of a *P* value less than 0.01 in this meta-analysis of four studies including 529 gallstone patients and 461 controls. Then we screened a Chinese population with these two polymorphisms and examined the risk stratification and their interaction with age, sex, body mass index (BMI) status and symptoms in gallstone disease. According to the likely mode of inheritance extracted from the meta-analysis for each locus, we illustrated that these two loci are associated with the gallstone disease risk of Chinese people by TaqMan® SNP genotyping assay.

Materials and methods

Literature search

We searched public databases PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), Embase (http://www.embase.com), ISI Wed of Knowledge (http://isiknowledge.com), Wanfang (http://www.wanfangdata.com.cn) and China Biological Medicine (CBM) (http://cbm.imicams.ac.cn) with the last update as of May 2015. The keywords used for search were 'gallstone disease' and 'ATP Binding Cassette Transporter A4 or ABCB4' combined with 'gene or variants or polymorphism or alleles', all of which were MeSH terms (Medical Subject Headings in the US National Library of Medicine). Only studies published in English or Chinese were identified. Afterwards, the full texts of the retrieved articles were scrutinized to ensure that data of interest was included. If two or more studies shared the same studied populations, the study with the small size was excluded. If more than one geographical or ethnic population were

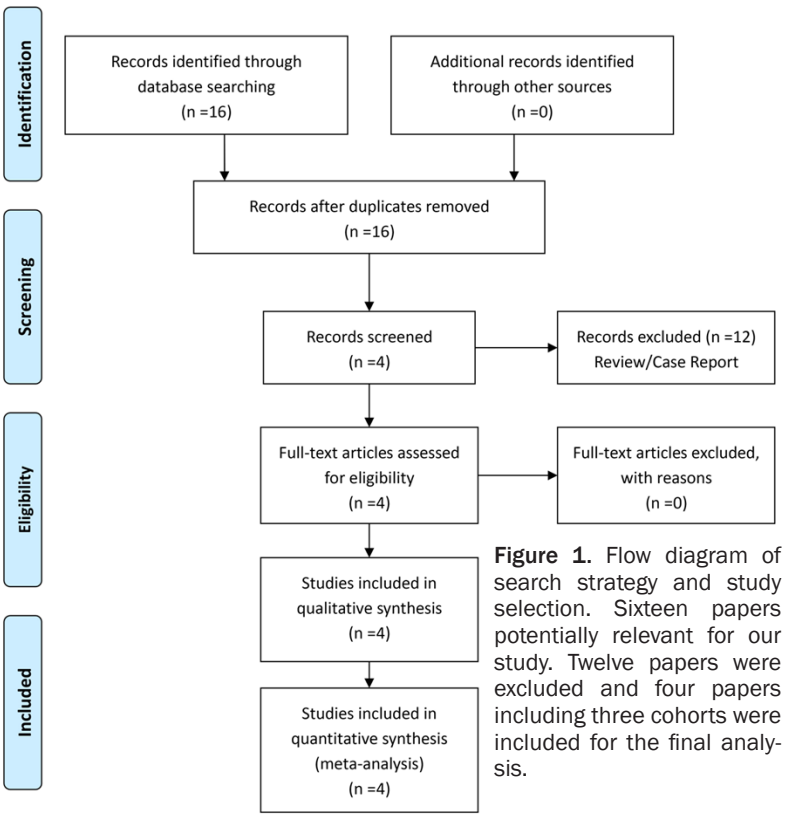

Figure 1. Flow diagram of search strategy and study selection. Sixteen papers potentially relevant for our study. Twelve papers were excluded and four papers including three cohorts were included for the final analysis.

included in one paper, each ethnical population was considered separately.

Study criteria

Inclusion criteria were listed as the followings: 1. evaluation of the association between ABCB4 polymorphism and gallstone disease; 2. case-control study using either hospital-based or population-based designs; 3. genotype/allele counts of *ABCB4* polymorphisms between cases and controls for estimating binary variable. Gallstone disease was diagnosed by ultrasonography or operation. Studies were excluded if they were published in a non-English and non-Chinese language or published in conference abstract form only.

Extracted information

We abstracted the following information from qualified studies (**Table 1**), including first author's last name, publication date, population ethnicity, methods of diagnosis of gallstone, study design, methods of genotyping and the genotype in cases and controls. Information such as cases and controls' age, gender and BMI were also collected.

Subjects

For the validation study, all the cases and controls were recruited in the Department of Biliary-Hepatic Surgery at The Affiliated Hospital of Guizhou Medical University, the Gallstone Disease Biobank project. The study protocol was approved by Ethical Committee at The Affiliated Hospital of Guizhou Medical University and written informed consent was obtained from all participants. We examined a sub-cohort that consisted of 296 individuals who were randomly selected from the total cohort using a random selection algorithm. The whole blood samples were obtained from these individuals. Demographic characteristic were described in **Table 2**. Among these patients, whole blood sample was collected from 171 patients with gallstone disease and 125 gallstone-free patients. All samples were stored in -80°C until analysis. Patients with hepatic, renal or endocrine disorders were not included in the study.

SNP genotyping assay

We used MagCore® Genomic DNA Whole Blood Kit (RBC Bioscience, Taipei, Taiwan) for purification of total DNA (including genomic and mitochondrial DNA) from whole blood by MagCore® auto-extraction instrument. Analysis was performed with the TaqMan® SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). The assay was designed for human SNP genotyping studies with the precision of TaqMan® reagent-based chemistry, *ABCG8* gene D19H (C_26135643_10 for *rs11887534*) as positive control in Chinese [19], and the *ABCB4* gene Exon 6 (C_8317490_30 for *rs1202283*) and Exon 16 (C_25472183_30 for *rs2230028*) were evaluated. Detection of the above gene variants was performed according to the SNP genotyping reaction protocol, under the following conditions, 95°C for 10

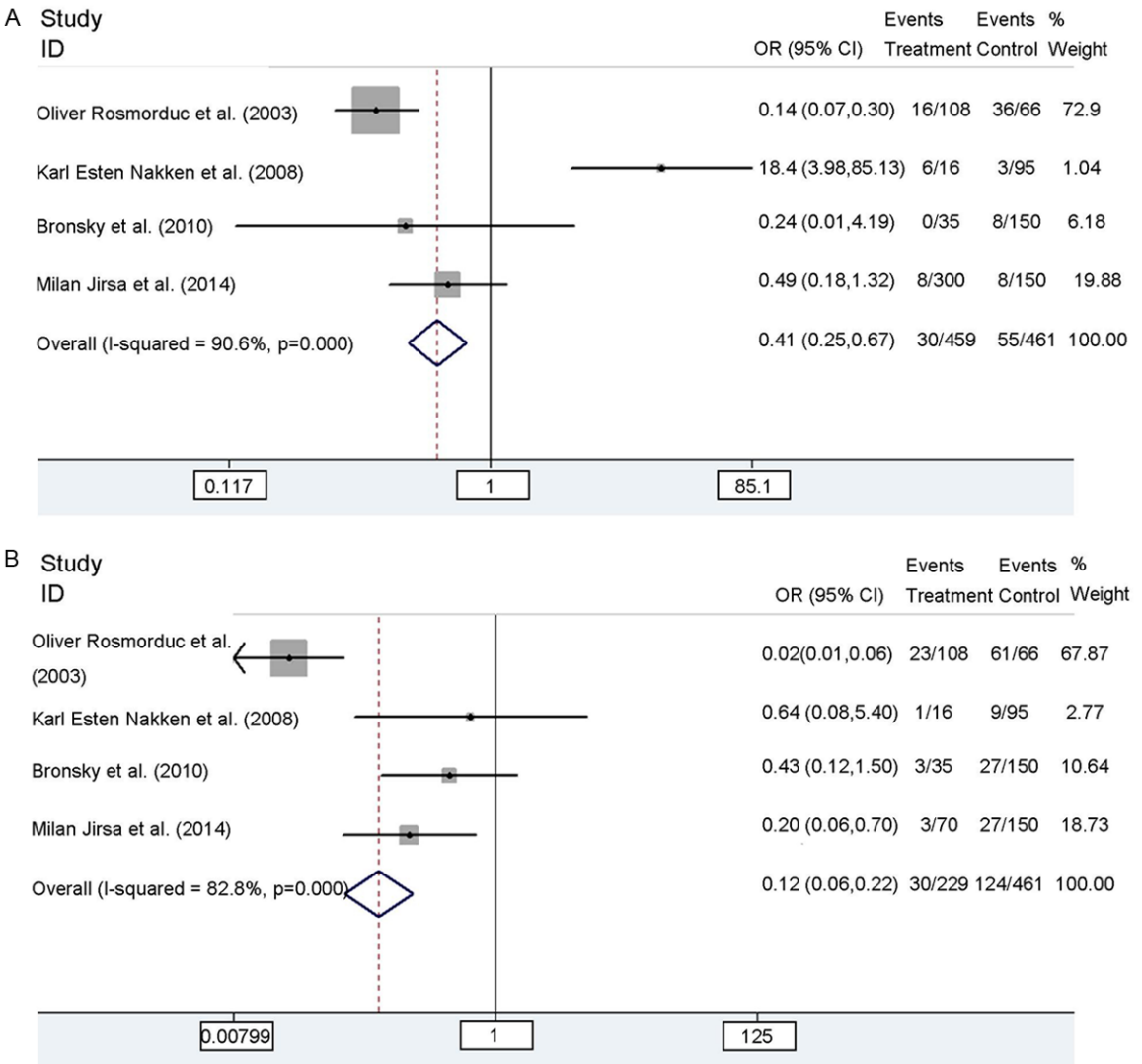

Figure 2. Pooled random-effect-based odds ratio of gallstone disease for ABCB4 polymorphism. A. Minor alleles of Exon 6 polymorphism (R = 0.41, 95% CI: 0.25-0.67, *P*<0.001) was related with an increased risk of gallstone disease in European. B. Exon 16 polymorphism (OR = 0.12, 95% CI: 0.06-0.22, *P* = 0.001) was also related with an increased risk of gallstone disease in Europeans.

min, then 40 cycles of 95°C for 15 s alternating with 60°C for 90 s. The Agilent Mx3000p™ sequence detection system (Agilent Technologies, Santa Clara, CA, USA) was used to detect gene variants using VIC® and FAM™ dyes.

Statistical analysis

The meta-analysis was performed in Stata/SE 11.0 software (StataCorp LP, College Station, USA). We used the random-effects model with the method of DerSimonian & Laird to pool effect-size estimates from the individual studies. Heterogeneity was estimated qualitatively using the Mantel-Haenszel method [20], and quantified using the I^2 statistic, with the ranges of 0 to 100% (I^2 = 0-25%, no heterogeneity; I^2 = 25-50%, moderate heterogeneity; I^2 = 50-75%, large heterogeneity; I^2 = 75-100%, extreme heterogeneity) [21]. Publication bias was evaluated by using funnel plots and the Egger test [22].

The biochemical and gene expression data analysis were performed using SPSS® 13.0 for Windows® (SPSS, Chicago, Illinois, USA). The comparison among groups for categorical vari-

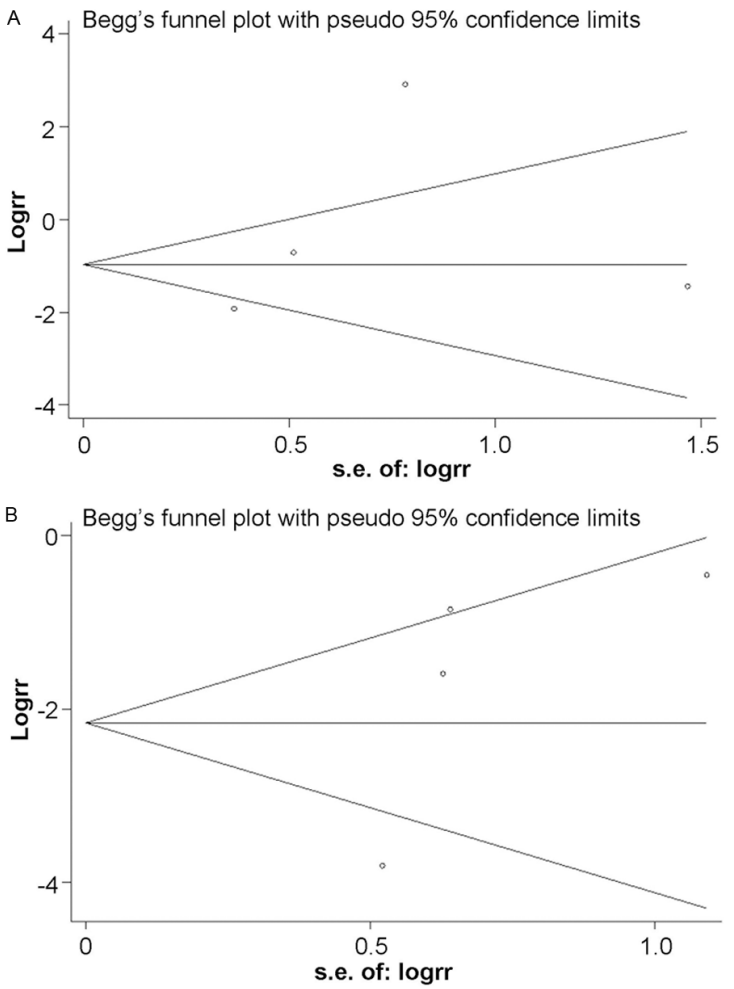

Figure 3. Begg's funnel plot of the Egger's test for publication bias of ABCB4 polymorphism and gallstone disease. The horizontal line in the funnel plot indicates the fixed-effects summary estimates, and the sloping lines indicate the expected 95% CI for a given standard error. A. *rs1202283*. B. *rs2230028*.

ables was performed with Pearson χ^2 tests or Fisher's exact tests. Continuous variables were compared by two-sample Student's *t* tests. Allelic frequencies were estimated by direct gene counting. A χ^2 test or a Fisher's exact test was used for testing Hardy-Weinberg equilibrium and power calculations for the sample size of each genotype. Haploview was used for estimating haploblocks, and haplotype analysis used a Hap-clustering program [23]. Binary logistic regression gave odds ratios of having gallstones under the influence of genotypes *ABCB4 rs1202283* and *rs2230028*. For the crude odds ratio, the presence or absence of gallstones was the dependent variable, and *rs1202283* or *rs2230028* was the independent variable. $P<0.05$ was considered significant with a two-tailed test.

Results

Association of rs1202283 and rs2230028 at ABCB4 with gallstone disease in European: meta-analysis

We identified 16 studies potentially relevant for our analysis. 12 papers were excluded, leaving four in the final analysis (**Figure 1**). The studies comprised of French, Norwegian and Czech cohorts. All studies had genotypic information for *ABCB4 rs1202283*, *rs2230028*. A summary of study population characteristics is presented in **Table 1**. In allelic model, minor alleles of *rs1202283* (pooled OR = 0.41, 95% CI: 0.25-0.67, $P<0.001$, **Figure 2A**) and *rs2230028* (pooled OR = 0.12, 95% CI: 0.06-0.22, $P = 0.001$, **Figure 2B**) were significantly associated with an increased risk of gallstone disease in Europeans.

Publication bias

We used Funnel plot and Egger's test to assess for publication bias. The resultant symmetrical funnel plot was symmetrical, consistent with an absence of publication bias. This was consistent with results of the Egger test for Exon 6 ($P = 0.461$) and Exon 16 ($P = 0.315$), **Figure 3A** and **3B**.

Association of rs1202283 and rs2230028 at ABCB4 with gallstone disease in Chinese

Allelic frequencies of *ABCB4 rs1202283* and *rs2230028* are shown in **Table 3**. The distributions of genotypes in the whole study group were in Hardy-Weinberg equilibrium. The allelic frequencies were different from those reported previously in the European-American population. The homozygote AA allele of *rs1202283* was quite rare (2.3%). Compared with Caucasians, the dominant allele distribution heterozy-

Table 3. Allelic frequencies of the polymorphisms in *ABCB4 rs1202283* and *rs2230028* in 296 subjects

Gene	Allele ratio	NCBI SNP reference	Frequency
ABCB4: Exon 6	AA, AG, GG	*Rs1202283*	23:194:79
ABCB4: Exon 16	CC, CT, TT	*Rs2230028*	0:288:8
ABCG8: D19H	CC, CG, GG	*Rs11887534*	0:286:10

NCBI: National Center for Biotechnology Information; SNP: single nucleotide polymorphism.

Table 4. Association of genotype frequency of *ABCB4 rs1202283* and *rs2230028* with gallstone development

	Gallstones (n = 171)	No stones (n = 125)	*P**
ABCB4: Exon 6			
Genotype			
AG	90	104	3.5E-8
AA + GG	81	21	
ABCB4: Exon 16			
Genotype			
CT	163	125	0.023
TT	8	0	
ABCG8: D19H			
Genotype			
CG	10	0	0.006
GG	161	125	

*Pearson χ^2 test.

gote CT of *rs2230028* was high (97.6%) and the homozygote CC genotype was absent in the study population. *ABCG8 rs11887534* (D19H) allele as a positive control exhibited the same results as previously reported in Chinese Taiwan population [19]. The association between the genotypes of *ABCB4 rs1202283* and *rs2230028*, and the presence of gallstones are shown in **Table 4**, with *ABCG8 rs11887534* (*P* = 0.006) as a positive control. There was a significant correlation of gallstone disease with the genotype distribution of *rs1202283* (*P* = 3.5E-8) and *rs2230028* (*P* = 0.023) polymorphisms. The minor alleles of *rs1202283* and *rs2230028* increased in patients with gallstones compared with controls. For example, in the *rs1202283* polymorphism, the frequency of the heterozygote AG genotype was 52.6% in the gallstone group compared with 83.2% in the control group. The frequency of the *rs2230028* heterozygote CT variant was 95.3% in the gallstone group compared with 100% in the control group.

ABCB4 rs1202283 GG genotype as risk with asymptomatic gallstone disease

For further analysis of the odds ratios of minor genotypes with gallstone disease, carriers of homozygote AA, heterozygote AG and homozygote GG genotypes of Exon 6 and those of homozygote CC, heterozygote CT and homozygote TT genotypes of Exon 16 were combined. The risk stratification of these minor genotypes for gallstone disease was determined and adjusted by age, sex, BMI and symptoms, which were well established as independent risk factors for gallstone disease. The *rs1202283* heterozygote AG and *rs2230028* homozygote TT variant contributed a significant and independent risk of gallstone formation. Epidemiological surveys demonstrated that the risk of asymptomatic gallstone disease increased with *rs1202283* homozygote GG genotype at *ABCB4*. The gallstone risk associated with *rs1202283* and *rs2230028* polymorphisms was further stratified into different symptoms groups. The result clearly demonstrated a significant risk of asymptomatic gallstone disease associated with *rs1202283* homozygote GG genotype than homozygote AA, heterozygote AG genotype (95% C.I.: 0.219-0.768; *P* = 0.005) and a greater risk than *rs2230028* (95% C.I.: 0.264-4.506; *P* = 0.906). On the basis of these two SNPs in *ABCB4*, a haplotype analysis study was conducted. There was no significant difference in the distribution of each haplotype between the gallstone disease and control groups (**Figure 4**).

Discussion

In this study, we pooled data from 4 studies which comprised of French, Norwegian and Czech cohorts. The meta-analysis of samples were selected from 529 gallstone patients and 461 control samples, and the results showed that *ABCB4 rs1202283* and *rs2230028* mutations were associated with an increased risk of gallstone disease in Europeans. An obvious difference of gallstone prevalence between ethnicities was present.

Gallstone disease is highly prevalent in European, Pima, Hispanic populations, and is

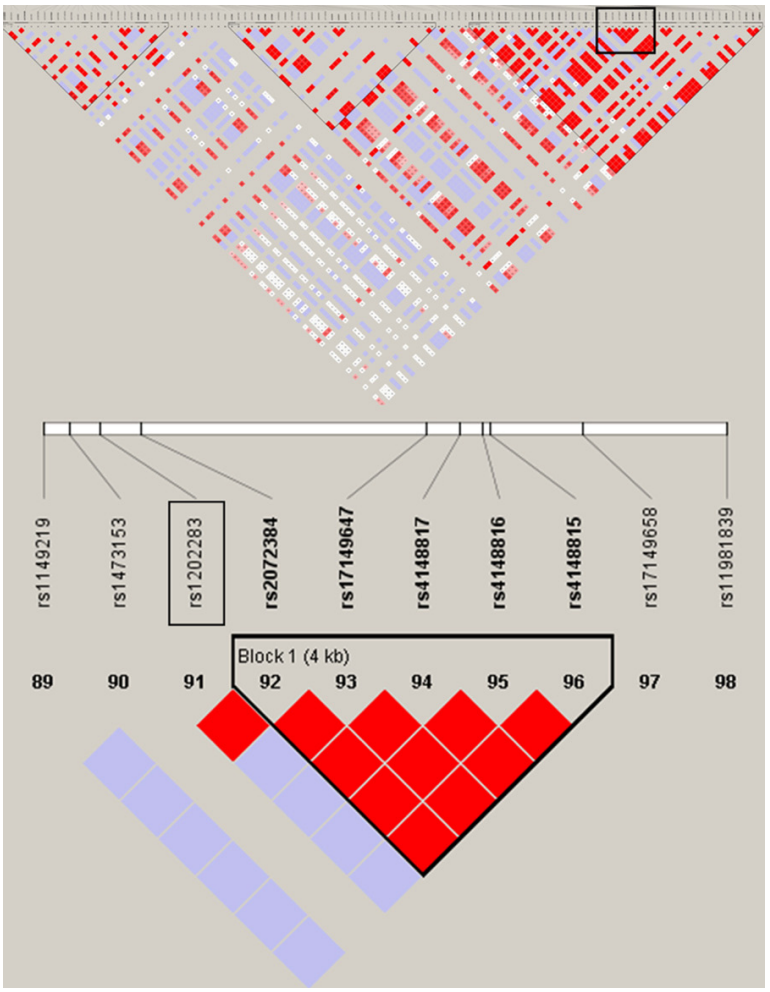

Figure 4. Overview of the physical and genetic structure of the ABCB4. The physical position of the investigated SNPs and a schematic representation of the gene structures are shown above. Haplotype blocks derived from the HapMap genotypes from individuals of Han Chinese Beijing (HCB) are outlined in black. No significant difference in the distribution of each haplotype between the gallstone disease and stone free groups.

We evaluated the two loci associated with gallstone disease attaining genome-wide significance in our meta-analysis as predictors of gallstone risk in 296 recruited subjects including 171 gallstone samples and 125 no gallstone controls. We found a significant correlation between the distribution of *rs1202283* (AG) and *rs2230028* (CT) polymorphisms and gallstone disease in the Chinese population. These similar results were present between *ABCB4* polymorphism in relation with gallstone disease in both populations. Furthermore, upon dividing the population into Chinese and non-Chinese, the association of *rs1202283* and *rs2230028* at *ABCB4* polymorphisms with gallstone disease did not change. The risk associated with Exon 6 (GG) was strong in asymptomatic gallstone disease. Additionally as a positive control, all the clinical conditions of *ABCG8* D19H were similar to the Chinese Taiwan population previously reported [19]. The study also found that serum levels of total cholesterol, LDLC were significantly lower and HDLC were higher in subjects with gallstones than in those without gallstones.

relatively lower in Asian, and lowest in African populations [1]. Gallstone disease is a common clinical entity, with a prevalence of 10-15% detected by ultrasonography in Western populations. The overall prevalence is lower in Asia, ranging from 3-15% [24-26]. This difference may relate to both dietary habits and genetic factors. The relationship between diet and gallstones is still controversial [27]. Cholesterol gallstones are responsible for 80-90% of all gallstones in Western countries [24, 25, 28]. As a result of westernization of dietary habits, the composition of gallstones has also altered: up to 77% in Chinese populations are now cholesterol gallstones [25]. Therefore, we divided the population into Asian and Western.

Gallstone formation results from a complex interaction between multiple predisposing genes and environmental factors. Genetic susceptibility for gallstone formation is influenced by well-known risk factors including age, sex, obesity, parity, and medication [24, 25, 28, 29]. In this study population, polymorphisms of *ABCB4* and age, sex, obesity, parity, and medication were not associated with gallstone disease in univariable analysis. However, we found GG allele of *rs2230028* polymorphism at *ABCB4* gene was a common allele predicting susceptibility to asymptomatic gallstone disease independent of ethnicities. Previous studies also showed that *Mdr2* gene knockout mice, the murine homolog of *ABCB4*, may develop

bile cholesterol crystals [30, 31]. The study by Oliver Rosmorduc *et al*. strongly suggested that *ABCB4* mutations may lead to cholesterol gallstone when residual ABCB4 activity and subsequent biliary phospholipid secretion decreases below a critical threshold, which may depend on the type of mutation and other host or environmental factors [9]. Consistent with previous studies [13], this finding supports the hypothesis that the Exon 6 at *ABCB4* mutation may affect the transporter function resulting in gallstone formation in earlier life. These polymorphisms may alter the conformation of ABCB4 transporters to modulate the intestinal absorption efficiency or the biliary secretion of cholesterol.

Epidemiological studies have indicated an association between low plasma HDLC levels and gallstone prevalence [26]. Super-saturation of bile with excess cholesterol is an essential step in gallstone formation. Bile is the major route for the elimination of excess cholesterol from the liver. In human studies, most of the cholesterol secreted into bile comes from circulating plasma lipoproteins, the free cholesterol carried by HDL particles binding to the scavenger receptor class B type 1 (SRB1), the uptake of cholesterol by LDL receptors or LDL receptor related proteins, and the dietary cholesterol in the chylomicron remnants. Newly synthesized cholesterol contributes very little (5-20%) to biliary cholesterol secretion [24, 26, 28, 32]. In cholesterol homeostasis, plasma HDLC plays a key role in reverse cholesterol transport to the liver. The biliary secretion of free cholesterol markedly increases in *Srb1* transgenic mice and transport of cholesterol into bile is mediated mainly by SRB1 [33]. More interestingly, the present study demonstrated that serum levels of total cholesterol, LDLC, and HDLC were significantly associated with abdominal pain in pancreatitis and other digestive system diseases [34, 35]. As previously reported, polymorphism in *ABCB4* was strongly associated (P = 9E-14) with the plasma LDLC concentrations on the high-cholesterol, high-fat diet. They indicated that genetic variation in *ABCB4*, or closely linked genes, was responsible for the dramatic differences among opossums in their LDLC response to an atherogenic diet [36, 37]. Polymorphisms of *ABCB4* and *ABCB4*-related genes may alter the transporter function, modify the intrahepatic cholesterol content and regulate the reverse cholesterol transport in cholesterol homeostasis. It is possible that gallstone formation may be a by-product of excess cholesterol excretion into bile to keep serum levels of cholesterol-containing lipoproteins lower and significantly associated with the risk of gallstone disease without symptoms, such as abdominal pain.

In summary, our results strongly support the role of *rs1202283* (AG) and *rs2230028* (CT) at *ABCB4*, and *rs11887534* (CG) at *ABCG8* were associated with the risk of gallstone disease. More notable is that the risk associated with *rs1202283* (GG) was strong in asymptomatic gallstone disease. In the near future, ABCB4 genotyping should be used to confirm the diagnosis of asymptomatic gallstone disease and allow familial screening. Depending on the results, long-term curative or prophylactic ursodeoxycholic acid therapy could be initiated early to prevent the occurrence or recurrence of this disease and its severe complications.

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Disclosure of conflict of interest

None.

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