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Association between *ABCB4* variants and intrahepatic cholestasis of pregnancy

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The *ABCB4* gene encodes multidrug resistance protein 3(MDR3), which is a phosphatidylcholine(PC) transfer enzyme that transfers lecithin from the inner part of the phospholipid bilayer to the extracellular bile. The occurrence of intrahepatic cholestasis of pregnancy(ICP) is closely related to *ABCB4* variants, but there is limited research on this topic in southern Anhui, China. We sequenced *ABCB4* in pregnant women with ICP and healthy pregnant women to explore the relationship. A total of 30 patients diagnosed with ICP were selected as the study objects and 90 healthy pregnant women were selected as the control group. DNA was extracted from peripheral blood of ICP patients and healthy pregnant women, 27 exons were sequencing by Sanger sequencing. Polymerase chain reaction (PCR) was used to amplify those exons. PolyPhen2, Mutation Taster, Provean, SIFT and Mutpred2 were used to predict protein structure, and Pymol software was used to predict the impact of missense variant c.1954 A > G(p.Arg652Gly) on proteins. Four exonic variants of ABCB4 gene were detected in ICP patients and healthy pregnant women, including synonymous variants c.175 C > T, c.504 C > T,c.711 A > T and missense variant c.1954 A > G(p.Arg652Gly). The incidence of the missense variant c.1954 A > G(p.Arg652Gly) was 6/90 in healthy pregnant womenand 8/30 in ICP patients.In healthy pregnant women with the missense variant c.1954 A > G(p.Arg652Gly), no other exonic variants were found. In ICP patients with missense variant c.1954 A > G(p.Arg652Gly), other exonic variants were found. PolyPhen2, Mutation Taster, Provean, SIFT and Mutpred2 were used to predict that the four exonic variants were benign, while Pymol was used to showed that the missense variant was located in the linker region of MDR3 and had a slight impact on protein function. Among ICP patients with missense variant c.1954 A > G(p.Arg652Gly), patients with three exonic variants(c.504 C > T, c.711 A > T, c.1954 A > G) had higher γ-GT, TBA, ALT and AST than those with two exonic variants. *ABCB4* missense variant c.1954 A > G(p.Arg652Gly) requires the combination of other variants(c.175 C > T, c.504 C > T,c.711 A > T) to cause ICP symptoms, and when combined with other variants, it has a superimposed effect.

Keywords Intrahepatic cholestasis of pregnancy, Variant, *ABCB4*

Intrahepatic cholestasis of pregnancy (ICP) is a common gestational liver disease in the world, which is a disease with unclear etiology. It is more common in the middle and late stages of pregnancy and rapidly subsides after delivery. In most women, ICP will recur in subsequent pregnancies. It is reported that the incidence rate of ICP varies from 0.2 to 15.6% according to race and geographical location, with the highest incidence in Chile[1–4]. The typical symptoms of ICP are skin itching, mainly occurring in the palms and soles of the feet, accompanied by elevated serum total bile acids (TBA) and abnormal liver function indicators. Its greatest harm is spontaneous premature birth, amniotic fluid fecal staining, neonatal depression, fetal respiratory distress syndrome, and high risk of death[2,5–7].

The *ABCB4* gene is located on chromosome 7q21.1 and consists of 27 exons, with a total length of approximately 74kb[8,9]. The multidrug resistant protein3 (MDR3) encoded by *ABCB4* is responsible for transporting phosphatidylcholine (PC) on the hepatic tubular membrane from the inner lobule to the outer lobule. When mixed with bile acids, it can protect the bile duct epithelium from bile salt damage. *ABCB4* mutations can cause protein abnormalities, leading to bile duct damage and bile stasis[10–13]. Progressive familial intrahepatic cholestasis type 3 (PFIC3) that occurs during infancy or late adolescence, as well as adult onset

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syndromes such as low phospholipid associated gallstones (LPAC) or intrahepatic cholestasis of pregnancy (ICP), are all associated with *ABCB4* mutations[14].

The pathogenesis of ICP is multifactorial, with the interaction of genetic, hormonal, immune, and environmental factors[5]. Among genetic factors, mutations in *ABCB4* are believed to play a major role in the pathogenesis of ICP[15], with approximately 16% of ICP patients having *ABCB4* mutations[1]. Most studies on ICP are focused on European and American populations, with little research on the Chinese population. This article aims to clarify the relationship between *ABCB4* and genetic susceptibility to ICP by analyzing some unrelated populations in southern Anhui, China.

Materials and methods

Patients

30 ICP patients from Yijishan Hospital in Wuhu were selected as the experimental group, with an average age of (26.93 ± 3.25) and an average gestational age of (35.53 ± 2.42), and 90 healthy pregnant women were selected as the control group, with an average age of (27.19 ± 2.47) and an average gestational age of (36.62 ± 1.16). ICP patients met the ICP diagnostic criteria in the Guidelines for Diagnosis and Treatment of Intrahepatic Cholestasis of Pregnancy (2015)[16]. Inclusion criteria: All patients had no history of diabetes, hypertension, liver and kidney diseases and other complications. Blood was collected from all patients before delivery and stored in a -20 ℃ freezer.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Yijishan Hospital of Wannan Medical College. All patients provided written informed consent.

DNA extraction

DNA was extracted from peripheral blood of ICP patients and healthy pregnant women, and 27 exons of *ABCB4* were amplified by polymerase chain reaction (PCR) and sequenced[17].

Polymerase chain reaction (PCR)

We designed primers for 27 exons of *ABCB4*, the NCBI version number of *ABCB4* is NM_000443.4. The designed upstream and downstream primers were BLAST validated in NCBI, synthesized by Shanghai Jierui Biotechnology Co., Ltd., and used for PCR amplification (Additional Table 1).

PCR reaction conditions and reaction system: PCR reaction was performed according to 50 μl system. The concentration was 5 μg/μl template DNA 2 μl, Taq enzyme 2.5 U, 10×Buffer(including Mg^{2+}) 5 μl, including 25 mM Tris-HCl pH 8.5, 50 mM KCl and 2 mM $MgCl_2$. dNTP is 0.2mmol/L, and the upstream and downstream primers with a concentration of 10μM are 0.5 μl each. Add ddH2O to 50 μl. Pre denature at 98 ℃ for 5 min, denature at 94℃ for 30 s, and add Taq enzyme (Promega company product) at 20 s; Refolding at 65℃ for 45 s, extending at 72℃ for 1 min, a total of 33 cycles, and finally extending at 72℃ for 4 min. The agarose gel electrophoresis at a concentration of 2% was used to verify that the PCR product had a unique specific band with the correct amplicon size.

DNA sequencing

PCR products were purified and recovered using the DNA Gel Extraction Kit (AXYGEN). The purified PCR products and upstream and downstream primers were sent to Beijing Nosai Genomics Research Center Co., LTD for sequencing.

Predict protein structure

Polyphen2 (http://genetics.bwh.harvard.edu/pph/)[18], MutationTaster(https://www.mutationtaster.org/)[19], Provean, SIFT (http://sift.jcvi.org/)[20] and Mutpred2 (http://mutpred.mutdb.org/)[21] were used to predict protein damage. Pymol software was used to predict structural missense variation of p.Arg652Gly protein.

Statistical analysis

GraphPad Prism8.0 software was used to perform statistical analysis on the data. Measurement data in mean ± standard deviation($\bar{x} \pm s$) indicates that ANOVA analysis of variance is used for comparison between multiple groups, and $P < 0.05$ indicates a statistically significant difference.

Results

DNA sequencing results

We detected four exonic variants in *ABCB4* in ICP patients and healthy pregnant women, with a missense variant c.1954 A > G, and 3 synonymous variants c.175 C > T, c.504 C > T, c.711 A > T (Table 1) (Additional Fig. 1).Missense variant c.1954 A > G was found in 8 ICP patients and 6 healthy pregnant women. In 6 healthy

Position	DNA change	Protein change	MAF in gnomAD	Database ref
7:87092185	c.175 C > T	Leu59Leu	0.1595	rs2302387
7:87082292	c.504 C > T	Asn168Asn	0.5198	rs1202283
7:87079406	c.711 A > T	ILe237ILe	0.1934	rs2109505
7:87056176	c.1954 A > G	Arg652Gly	0.08949	rs2230028

Table 1. Four exonic variants of *ABCB4* in ICP identified in this study.

	c.175 C > T variant	c.504 C > T variant	c.711 A > T variant	c.1954 A > G variant
Control	31/90	43/90	41/90	6/90
ICP	7/30	18/30	9/30	8/30

Table 2. Frequency distribution of four exonic variants in healthy pregnant women and ICP patients.

Patients	c.175 C > T variant	c.504 C > T variant	c.711 A > T variant	c.1954 A > G variant
ICP-9	–	y	y	y
ICP-14	–	y	–	y
ICP-15	–	y	–	y
ICP-18	–	–	y	y
ICP-19	–	–	y	y
ICP-25	–	y	y	y
ICP-27	–	–	y	y
ICP-30	y	–	–	y
Control-2	–	–	–	y
Control-4	–	–	–	y
Control-9	–	–	–	y
Control-15	–	–	–	y
Control-23	–	–	–	y
Control-28	–	–	–	y

Table 3. Distribution of other exonic variants in ICP patients with c.1954 A > G variant and in healthy pregnant women with c.1954 A > G variant. Y: carry this variant.

Variant location	Protein change	PolyPhen2[a]	Mutation Taster[b]	Provean[c]	SIFT[d]	Mutpred2[e]
c.175 C > T	Leu59Leu	-	0.00023(N)	-	-	-
c.504 C > T	Asn168Asn	-	0.94094(N)	-	-	-
c.711 A > T	ILe237ILe	-	4.23E-15(N)	-	-	-
c.1954 A > G	Arg652Gly	0.06	0.99999(N)	-0.1	0.355	0.03

Table 4. *ABCB4* variant information and damage prediction. [a]Polyphen2 can only detect missense variant (c.1954 A > G), with a score between 0 and 1. The closer it is to 0, the less impact it has on the protein; The closer to 1, the greater the impact on protein. [b]The Mutation Taster score ranges from 0 to 1, indicating the probability of the mutation occurring. [c]Provean score of less than − 2.5 indicates deleterious, more than − 2.5 indicates neutral. [d]SIFT score less than 0.05 indicates probable pathogenic, and a SIFT score greater than 0.05 indicates probable non-pathogenic. [e]Mutpred2 score less than 0.5 indicates probable non-pathogenic, and a SIFT score greater than 0.5 indicates probable pathogenic. N: Polymorphism.

pregnant women who carried the missense variant c.1954 A > G, no other exonic variants were found. But in 8 ICP patients with missense variant c.1954 A > G, other exonic variants were found (Table 2). Among the 8 ICP patients with c.1954 A > G variant, there were 6 ICP patients with 2 variants, including 1 patient with c.175 C > T variant, 2 patients with c.504 C > T variant, and 3 patients with c.711 A > T variant. And there were 2 ICP patients with 3 variants, all of whom carried the c.504 C > T and c.711 A > T variants (Table 3).

Protein structure prediction

Polyphen2, MutationTaster, Provean, SIFT and Mutpred2 suggest that exonic variants may be nonpathogenic (Table 4) and missense variant c.1954 A > G are highly unconserved in species(Additional Table 2), including *P.toglytes*, *M.mulatta*, *M.musculus*, *G.gallus*, *T.rubripes*, *D.rerio*, *D.melanogaster and C.elegans*. Pymol software was used to show that the missense variant c.1954 A > G(p.Arg652Gly) was located in the linker region of the protein (Fig. 1).

c.1954 A > G variant(p.Arg652Gly)

The missense variant c.1954 A > G may need to be combined with other exonic variants to cause the symptoms of ICP and has a superimposed effect (Table 5).

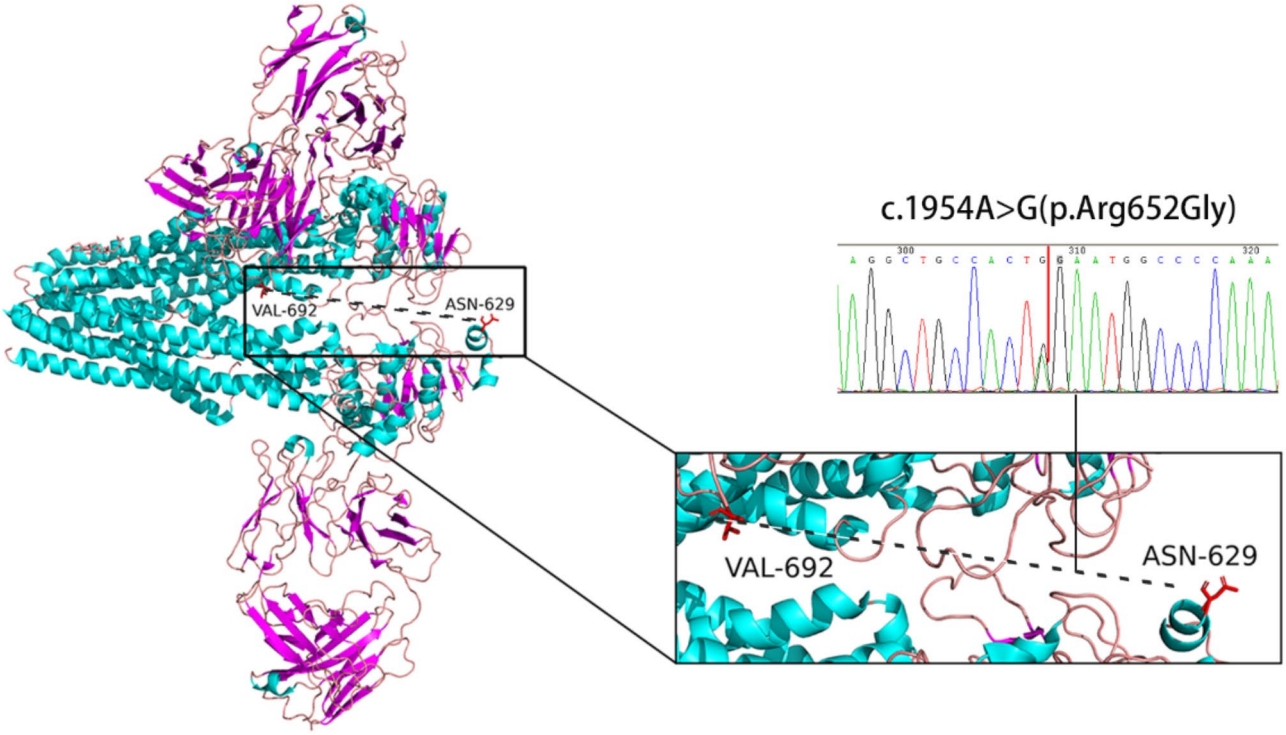

Fig. 1. The location of missense variant c.1954 A > G(p.Arg652Gly) in the linker region of MDR3 protein was analyzed by Pymol software.

	ICP-b ($\bar{x}\pm$ s)	ICP-t ($\bar{x}\pm$ s)	*P*
γ-GT (U/L)	33.3 ± 27.8	108 ± 15.6	0.013
TBA (μmol/L)	14.4 ± 9.7	40.4 ± 22.2	0.045
ALT (U/L)	102.8 ± 98.4	424.5 ± 242.5	0.026
AST (U/L)	88.7 ± 59.9	292.0 ± 94.9	0.007

Table 5. Biochemical findings in 8 ICP patients carrying the c.1954 A > G variant. ICP-b: 6 patients with ICP who carry the c.1954 A > G variant and one other exonic variant. ICP-t:2 patients with ICP who carry the c.1954 A > G variant and two other exonic variants. γ-GT: gamma glutamyl transferase. TBA: total bile acids. ALT: alanine transaminase. AST: aspatate aminotransferase.

Discussion

The incidence rate of ICP varies according to different geographical locations. It is most common in South America. In Europe, North America and Australia, about 1–2% of pregnant women suffer from ICP[1,22]. In southwest China, the incidence rate of ICP reached 2.8%[23]. Racial and regional differences are important factors in studying ICP and *ABCB4* mutations. Since Jacquemin first discovered a T deletion at the 1712nd base of the 571st codon of *ABCB4* in a proband of a cholestasis family, a mutation was subsequently discovered in four patients of this family[15]. Subsequent studies detected a wide range of *ABCB4* mutations in ICP patients, but *ABCB4* mutations have different outcomes in different racial populations. Wasmuth found that c.175 C > T and c.1954 A > G are genetic influencing factors for the onset of severe ICP in Swedes, while c.504T > C did not show any association with the disease[24]. Bacq believes that it is meaningful for Caucasian ICP patients with c.504T > C, while c.175 C > T and c.1954 A > G did not show any association with the disease[25].

The pathogenesis of ICP is still unclear, and clinical and epidemiological studies suggest that ICP is related to genetics, hormones, and environment[26–29]. A recent study suggests that the phenotype of ICP is influenced by multiple environmental factors such as $PM_{2.5}$, O_3 and potential causes of various pregnancy complications. The ICP with elevated serum total bile acid level (TBA ≥ 10μmol/L) but no elevated serum total bile acid aminotransferase level or pruritus was defined as "monosymptomatic ICP" (ICPs). An ICP with elevated serum total bile acid levels and at least one symptom is defined as "multisymptomatic ICP" (ICPm).The occurrence of single symptom ICP is more attributed to endogenous risk factors, such as the expression of certain genes such as SLC10A1, while the occurrence of multi symptom ICP is more susceptible to external factors, such as environmental exposure[30]. In familial cluster analysis of pedigree studies, the higher incidence of ICP in mothers and sisters of ICP patients may suggest a genetic predisposition to the disease[31,32].

The results of this study show that there is little association between *ABCB4* synonymous variants c.175 C > T, c.504 C > T, and c.711 A > T with the incidence of ICP in ICP patients in southern Anhui. The missense variant c.1954 A > G is highly non conserved in other species. Through three-dimensional structural analysis, the missense variant is located in the linker region of MDR3 protein, and bioinformatics analysis shows that it has little effect on protein function. Previous studies abroad have shown that *ABCB4* missense variant c.1954 A > G may not lead to the occurrence of idiopathic gallstones in children[33]. The missense variant c.1954 A > G found in patients with progressive familial intrahepatic cholestasis had the same variant frequency as the control group, but skin itching and cholestasis occurred during pregnancy in a mother carrying this mutation. This indicates that the missense variant c.1954 A > G itself may not cause clinical symptoms and may require specific circumstances, such as pregnancy or combination with another variant, to cause clinical symptoms[34].

ABCB4 associated cholestasis is characterized by elevated levels of γ-GT, which may be due to a reduced concentration of phospholipids in bile, leading to impaired formation of mixed micelles, an increased relative concentration of bile salts, and subsequent toxic effects on the bile ducts[9,34–36]. According to Piatek K, genotype coexistence analysis suggests that mutant variants of genetic polymorphisms may have a superimposed effect on the development of ICP[37]. In our study, we found that the missense variant c.1954 A > G was found in both ICP patients and healthy pregnant women. The incidence of missense variant c.1954 A > G in ICP patients was slightly higher than that in healthy pregnant women. But in ICP patients with the missense variant c.1954 A > G, we found variants in other exons(c.175 C > T, c.504 C > T,c.711 A > T) that are not found in healthy pregnant women. Among ICP patients carrying the missense variant c.1954 A > G, ICP patients carrying three variants had higher γ-GT, TBA, ALT and AST than ICP patients carrying two variants, indicating an aggravation of ICP. This may suggest that the c.1954 A > G missense variant needs to combine with other variants(c.175 C > T, c.504 C > T,c.711 A > T) to cause clinical symptoms of ICP, and c.1954 A > G missense variant have superimposed effects when combined with other variants.

Data availability

The datasets generated during the current study are available in the [Clinvar] repositoryc.175 C> Thttps://www.ncbi.nlm.nih.gov/clinvar/variation/256158/？oq=rs2302387&m=NM_000443.4(ABCB4):c.175 C%3ET%20(p.Leu59=)c.504 C>Thttps://www.ncbi.nlm.nih.gov/clinvar/variation/198082/?oq=rs1202283&m=NM_000443.4(ABCB4):c.504 C%3ET%20(p.Asn168=)c.711 A>Thttps://www.ncbi.nlm.nih.gov/clinvar/variation/256166/？oq=rs2109505&m=NM_000443.4(ABCB4):c.711 A%3ET%20(p.Ile237=)c.1954 A>Ghttps://www.ncbi.nlm.nih.gov/clinvar/variation/256160/?oq=rs2230028&m=NM_000443.4(ABCB4):c.1954 A%3EG%20(p.Arg652Gly).

Received: 13 April 2024; Accepted: 22 January 2025
Published online: 26 January 2025

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Author contributions

Dekun Zhang, Tiechen Li and Qing Sun contributed to the conception or design of the study and drafted the manuscript. Yuhong Li, Shufeng He, Jing Shu and Qing Sun collected clinical data. Dekun Zhang analyzed data. Tiechen Li and Qing Sun reviewed and revised the manuscript. All authors reviewed and approved the final manuscript.

Disclaimer

Due to the long period of sample acquisition, we only received two samples of ICP in the past few months, and the conclusion of this study should be interpreted with caution. Further research is needed with a larger, more representative population sample and a more comprehensive statistical analysis to validate the results of this study and clarify any uncertainties.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-87909-5.

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