Identification of two novel pathogenic variants of the NR1H4 gene in intrahepatic cholestasis of pregnancy patients

Hua Lai1,2†, Xianxian Liu1,3†, Siming Xin1,2, Jiusheng Zheng1,2, Huai Liu1,2, Yu Ouyang1,2, Huoxiu Yang1,2, Yang Zeng1,3, Yang Zou1,3* and Xiaoming Zeng1,2*

Abstract

Background: Intrahepatic cholestasis of pregnancy (ICP) can cause adverse pregnancy outcomes, such as spontaneous preterm delivery and stillbirth. It is a complex disease influenced by multiple factors, including genetics and the environment. Previous studies have reported that functioning nuclear receptor subfamily 1 group H member 4 (NR1H4) plays an essential role in bile acid (BA) homeostasis. However, some novel variants and their pathogenesis have not been fully elucidated. Therefore, this research aimed to investigate the genetic characteristics of the NR1H4 gene in ICP.

Methods: In this study, we sequenced the entire coding region of NR1H4 in 197 pregnant women with ICP disease. SIFT and PolyPhen2 were used to predict protein changes. Protein structure modelling and comparisons between NR1H4 reference and modified protein structures were performed by SWISS-MODEL and Chimera 1.14rc, respectively. T-tests were used to analyse the potential significant differences between NR1H4 mutations and wild types for 29 clinical features. Fisher’s test was conducted to test the significance of differences in mutation frequencies between ICP and the three databases.

Results: We identified four mutations: two novel missense mutations, p.S145F and p.M185L; rs180957965 (A230S); and rs147030757 (N275N). The two novel missense mutations were absent in 1029 controls and three databases, including the 1000 Genomes Project (1000G_ALL), Exome Aggregation Consortium (ExAC) and ChinaMAP. Two web-available tools, SIFT and PolyPhen2, predicted that these mutations are harmful to the function of the protein. Moreover, compared to the wild-type protein structure, the NR1H4 p.S145F and p.M185L protein structure showed a slight change in the chemical bond in two zinc finger structures. Combined clinical data indicate that the mutation group had higher levels of total bile acid (TBA) than the wild-type group. Therefore, we hypothesized that these two mutations altered the protein structure of NR1H4, which impaired the function of NR1H4 itself and its target gene and caused an increase in TBA.

Conclusions: To our knowledge, this is the first study to identify the novel p.S145F and p.M185L mutations in 197 ICP patients. Our present study provides new insights into the genetic architecture of ICP involving the two novel NR1H4 mutations.

*Correspondence: zouyang81@163.com; 18070038675@163.com
1 Hua Lai and Xianxian Liu have contributed equally to this work
2 Key Laboratory of Women's Reproductive Health of Jiangxi Province, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang 330006, Jiangxi, China

Full list of author information is available at the end of the article

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Background

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific liver disease characterized by skin pruritus and abnormal liver function, such as elevated liver enzymes and increased serum TBA (≥ 10 μmol/L), that appears in the second and third trimesters of pregnancy [1]. The symptoms and biochemical abnormalities usually rapidly disappear in the early postpartum period [2]. The incidence of ICP disease ranges from 1% to 15.6% depending on geographical location [3–5]. The recurrence rate of ICP in the next pregnancy reaches as high as 40–60% [1]. ICP has been associated with adverse perinatal outcomes, including premature birth and intrapartum death [1, 6, 7]. An elevated level of serum TBA will increase the risk of premature delivery and stillbirth [8, 9]. Therefore, untangling the genetic basis of ICP disease is very important.

Obviously, ICP is a complex disease that depends on multiple factors, including genetic background, metabolites of progesterone, oestrogens, seasons and environmental background [4, 10, 11]. Among them, familial clustering analysis in pedigree studies indicated a genetic predisposition for ICP disease [12–14]. To date, several bile acid homeostasis-related genes, including NR1H4, ATP Binding Cassette Subfamily B Member 4 (ABCB4), ATP Binding Cassette Subfamily B Member 11 (ABCB11) and ATP Binding Cassette Subfamily C Member 2 (ABCC2), have been reported. Moreover, multiple previous studies have identified genetic variants of the NR1H4, ABCB4, ABCB11 and ABCC2 genes that contribute to the development of ICP [15–20]. Among them, NR1H4 plays a central role in regulating bile acid metabolism.

NR1H4 is both a key modulator of hepatocyte-protective pathways and a therapeutic target for cholestatic liver disease [21]. NR1H4 is a BA-activated transporter factor that is responsible for BA homeostasis and acts by binding to DNA response elements through the NR1H4 DNA binding domain (DBD) in the promoter of target genes (such as ABCB4, ABCB11 and ABCC2), thereby activating their transcription [22–24]. Moreover, the C-terminal region of NR1H4 has a highly conserved ligand binding domain (LBD), which determines the specificity of NR1H4 ligands. These ligands include farnesoid derivative, BA, unsaturated fat, hepatocyte factor-1 and steroid compound [25, 26]. NR1H4 has four different isoforms: a1, a2, a3 and a4. The first two isoforms, which are expressed in the human liver, have a different N-terminus than the other two isoforms [27, 28]. In liver tissue, when raising hepatocyte BA levels, NR1H4 regulates bile flow by directly inducing gene expression (ABCB4, ABCB11 and ABCC2) to stimulate hepatic bile export [29, 30]. Conversely, NR1H4 represses the expression of bile acid import (NTCP) [31] and key enzymes (CYP7A1 and CYP8B1) [32] in the bile acid synthesis pathway through the induction of short heterodimer partner (SHP) [31] in the liver and growth factor 19 (FGF19)/FGF15 [33] in the intestine. In addition, NR1H4 +/− transgenic mice exhibited BA pool sizes [34]. Therefore, NR1H4 maintained a stable TBA level in hepatocytes by regulating TBA synthesis, transport, secretion and metabolism.

Considering that women with ICP exhibited elevated serum BAs and NR1H4 mutations resulted in altered BA levels, we hypothesized that NR1H4 mutations might also exist in ICP samples. Here, we recruited a total of 197 Han Chinese women with ICP and analysed the entire coding region of the NR1H4 gene. A total of 4 mutations, including two novel missense mutations in NR1H4, were identified in our ICP samples for the first time.

Methods

Samples and features

We recruited 197 patients diagnosed with ICP disease based on clinical symptoms (skin pruritus) and laboratory investigations (fasting TBA ≥ 10 μmol/L, etc.) between 2018 and 2020. Peripheral blood samples from 197 patients with ICP disease were collected from the Department of Obstetrics, Jiangxi Provincial Maternal and Child Health Hospital in Nanchang, China. In addition, we recorded a total of twenty-nine available clinical characteristics, which included age, body mass index (BMI), gestational weeks at diagnosis, gravidity and parity; the level of ion concentration covering K, Na, Cl, Ca, Mg and P; the counts of white blood cells (WBCs), red blood cells (RBCs), platelets (PLTs), and red blood cell distribution width. SD (RDW-SD); the level of serum biochemical indices including TBA, aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), total cholesterol (CHOL), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), uric acid (UA); and the outcomes of pregnant women and newborn babies, including birth weight, bleeding count and Apgar score. The clinical features were determined as described previously [20, 35]. Briefly, the ion concentration and serum biochemical index were examined by an AU5800 automatic biochemical analyser (Beckman...
Coulter, Inc., USA). Routine blood tests were determined by a Sysmex-xn-2000 automatic blood cell analyser (Sysmex Corporation, Japan).

Summary statistics for all the above clinical features investigated in 197 ICP patients are shown in Table 1. Of these samples, 151 clinical data points were described in our previous study [20, 35]. In addition, 1029 samples without ICP disease were also recruited. The present study followed the tenets of the Helsinki Declaration, and the ethics approval was approved by the Institutional Review Board of Jiangxi Provincial Maternal and Child Health Hospital in China. Each participating woman gave written informed consent (Additional file 1).

**Table 1** Descriptive statistics of twenty-nine clinical characteristics of 197 ICP patients

| Characteristics        | N  | Mean  | SD   | Min  | Max  |
|------------------------|----|-------|------|------|------|
| Basic information      |    |       |      |      |      |
| Age (years)            | 197| 29.42 | 5.26 | 17.00| 43.00|
| Gestational age (days) | 192| 256.12| 23.28| 215.00| 290.00|
| BMI (kg/m²)            | 183| 25.82 | 3.92 | 17.08| 38.50|
| Gravidity (times)      | 189| 2.40  | 1.55 | 1.00 | 8.00 |
| Parity (times)         | 188| 0.63  | 0.78 | 0.00 | 4.00 |
| Serum biochemical index|    |       |      |      |      |
| K (mmol/L)             | 187| 4.01  | 0.36 | 3.20 | 6.40 |
| Na (mmol/L)            | 186| 137.34| 2.21 | 132.00| 143.00|
| Cl (mmol/L)            | 186| 104.08| 2.62 | 97.00 | 112.00|
| Ca (mmol/L)            | 186| 2.36  | 0.17 | 2.00 | 2.90 |
| Mg (mmol/L)            | 186| 0.81  | 0.14 | 0.60 | 1.89 |
| P (mmol/L)             | 186| 1.15  | 0.19 | 0.70 | 1.60 |
| WBC (x 10⁹)           | 196| 8.55  | 2.70 | 4.11 | 24.23|
| RBC (x 10⁹)           | 196| 3.84  | 0.41 | 2.96 | 5.52 |
| PLT (x 10⁹)           | 196| 197.76| 58.84| 75.00| 412.00|
| RDW-SD (fL)           | 196| 46.09 | 4.86 | 36.20| 67.30 |
| ALT (U/L)             | 197| 103.14| 127.27| 3.00| 595.00|
| AST (U/L)             | 197| 87.23 | 98.98| 12.00| 509.00|
| TBA (μmol/L)          | 197| 42.51 | 38.11| 4.20 | 286.80|
| TBL (μmol/L)          | 195| 14.95 | 7.48 | 5.70 | 64.80 |
| DBIL (μmol/L)         | 195| 6.96  | 6.12 | 0.90 | 49.50|
| IDBIL (μmol/L)        | 195| 8.01  | 3.48 | 2.70 | 26.90|
| CHOL (mmol/L)         | 189| 6.38  | 1.67 | 3.16 | 13.25|
| TG (mmol/L)           | 189| 3.61  | 1.58 | 1.20 | 11.10|
| HDL (mmol/L)          | 189| 1.95  | 0.50 | 0.92 | 4.06 |
| LDL (mmol/L)          | 189| 2.79  | 1.31 | 0.04 | 7.34 |
| UA (μmol/L)           | 187| 326.49| 91.76| 71.00| 701.00|
| Outcomes of pregnancy women and newborn baby |    |       |      |      |      |
| Birth weight (kg)     | 159| 3.07  | 0.74 | 1.23 | 5.30 |
| Apgar score (1–10)    | 158| 9.39  | 1.24 | 6.00 | 10.00|
| Bleeding count (mL)   | 156| 261.89| 104.15| 90.00| 810.00|

**Evolutionary conservation analysis**

The evolutionary conservative analysis of p.S145Fand p.M185L were performed in 26 representative species, including Chimpanzee, Gibbon, Macaque, Olive baboon, Gelada, Marmoset, Prairie vole, Mouse, Rat, Alpine marmot, Rabbit, Domestic yak, Cow, Goat, Sheep, Sperm whale, Arabian camel, Chacoan peccary, Pig, Dog, Dingo, Cat, Leopard, Horse and Elephant, through the genomic alignments of the Ensembl Genome Browser.

**Protein structural modelling**

The protein template of modelling between the reference and modified (p.S145F and p.M185L) mutations of the NR1H4 gene were detected using the SWISS-MODEL repository database (http://www.expasy.org/). Then, we compared the protein models simultaneously with the Chimera 1.14rc package.

**Statistical analysis**

The summary function was used to perform the descriptive statistics on the clinical data of 197 samples with ICP disease. The t.test function was conducted to analyse
the potential association of 29 clinical data between ICP samples with or without NR1H4 mutations. The \( P \) values were two sided, and the results were considered significantly different at \( P < 0.05 \). The frequency significant difference for NR1H4 mutations between 197 ICP samples and databases were analysed by Fisher’s test function. All the analyses were completed with R software. Logistic regression analysis was performed to assess the clinical parameters (age, gestational age, BMI, gravidity and parity) with the mutations.

**Results**

**NR1H4 mutations**

We sequenced 9 exon fragments of the NR1H4 gene and detected a total of four mutations, including three missense mutations in exons 2, 3 and 4 and one synonymous mutation in exon 5 with 3 samples in 197 ICP patients.

Two out of three missense mutations were novel (novel-1, novel-2) (Fig. 1, Additional file 1, Table 3) and were identified in a 40- and 21-year-old ICP individual, respectively. Using the web-available tools SIFT and PolyPhen2, the influence of the two novel mutations on protein function was predicted to be damaging. Furthermore, these two mutations were absent from 1029 controls without the ICP, 1000G_ALL (http://www.internationalgenome.org/), and ExAC (http://exac.broadinstitute.org/) databases. There was a significant difference (\( P = 0.018 \)) in the frequency for two novel mutations between 197 ICP samples and the ChinaMAP (http://www.mbiobank.com/) database.

The other missense mutation rs180957965 (p.Ala230Ser) was identified in a 30-year-old sample (ICP12), and the synonymous mutation rs147030757 (p.Asn275Asn) were identified in three ICP patients (ICP1, ICP69 and ICP107). These mutations were all absent in the controls and had a low frequency of databases, ranging from 0.00018 to 0.0057. There was a significant difference in the frequency of the missense mutation rs180957965 (\( P = 0.036 \)) and the synonymous mutation rs147030757 (\( P = 1.63 \times 10^{-5} \)) between 197 ICP patients and the ExAC database. In addition, rs147030757 showed a significant frequency difference between the ICP population and 1000G_ALL (\( P = 0.001 \)).

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**Table 2** Primers used for sequencing the coding regions of the NR1H4 gene

| Exon | Amplicon (bp) | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|------|--------------|------------------------|-----------------------|
| 1    | 418          | TGAACAAGAACCCACCT      | ATCTTCACACAAAGTCC    |
| 2    | 523          | ACTCCTACACCATACGCAAAC  | GCAATTGTCAGGAAATTCA  |
| 3    | 609          | TAGGCTCTGCTGCGATAG     | GGTGTCATTACCCCTTT    |
| 4    | 553          | CTACAAACTTGCCCTTCC     | TTCTGTCGGAAACACT     |
| 5    | 415          | TCGTCTGCTATATGCCC      | ATCAAGAATAAGGGAGA    |
| 6    | 487          | TGAGTGCTCCACCATATC     | GAACAGACTGCTCTTCT    |
| 7    | 465          | AATGGAAGCTTGTTGAT      | GCCTTCTTTTGCTCTTC    |
| 8    | 580          | GATTCACTAAATCCCCATCT   | GCAGAATTATAGGCTACT   |
| 9    | 749          | GGCAGAAGCTAGTTGTA      | CTGAGTGAAACTGGTTA    |
Table 3  Screening for mutations in the NR1H4 gene in 197 pregnant women with ICP disease

| Exon | Patient | SNP     | Chr | Position | Alleles | Protein change | SIFT | PolyPhen2 | MAF in controls | 1000G_ALL | ExAC | ChinaMAP | P₁ | P² | P³ |
|------|---------|---------|-----|----------|---------|----------------|------|------------|----------------|-----------|------|----------|----|----|----|
| Exon2 | ICP127  | Novel-1 | 12  | 100904880| C/T     | Ser145Phe      | 0.999| 0.00022  | Not present     | 0         | –    | –        | 0.018 |    |    |
| Exon3 | ICP53   | Novel-2 | 12  | 100926313| A/T     | Met185Leu      | 0.981| 0.00080  | Not present     | 0         | –    | –        | 0.018 |    |    |
| Exon4 | ICP12   | rs180957965 | 12 | 100928727| G/T     | Ala230Ser      | 0.015| 0.815    | 0.000080        | 0.00018  | 0.00029 | 0.17  | 0.036 | 0.44|
| Exon5 | ICP1,69,107 | rs147030757 | 12 | 100930352| C/T     | Asn275Asn      | –   | –        | 0.001         | 0.00022  | 0.0057 | 0.0027 | 0.63e-05 | 0.11|

Significant differences were underlined

P₁ the significance of differences in frequencies between 197 ICP patients and 1000G_ALL, P² the significance of differences in frequencies between 197 ICP patients and ExAC, P³ the significance of differences in frequencies between 197 ICP patients and ChinaMAP
Clinical features of ICP patients with NR1H4 mutations
The clinical and biochemical features of the six ICPs with 4 mutations are presented in Table 4. Serum bile acids were increased in all six patients with NR1H4 mutations. The serum TBA levels of the patients identified with novel-1 and novel-2 were 46.4 and 113.2 μmol/L, respectively (Table 4). The patient with novel-1 had one child after experiencing six previous pregnancies. The TBA level of the patient ICP12 with the missense mutation rs180957965 was 12 μmol/L, and ICP1, ICP69 and ICP107 patients with a synonymous mutation rs147030757 were had TBA levels of 18.9, 27.5 and 46.4 μmol/L, respectively. Furthermore, the concentrations of CHOL and TG for the six patients with NR1H4 mutations were higher than the reference values (CHOL: 0–5.2 mmol/L; TG: 0.34–1.69 mmol/L).

Evolutionary conservative analysis and protein structural modelling
Evolutionary conservation analysis showed that these two novel mutations (p.S145F and p.M185L) were highly conserved among the 26 species, ranging from human to elephant (Fig. 2).

To further investigate the possible effects of the p.S145F and p.M185L variants on protein structure, the reference and the modified protein structure of NR1H4 gene were compared using UCSF Chimera 1.14rc. These two variants were located in the DNA Table 4

| Characteristics | Novel-1 (ICP127) | Novel-2 (ICP53) | rs180957965 (ICP12) | rs147030757 (ICP1) | rs147030757 (ICP69) | rs147030757 (ICP107) |
|-----------------|-----------------|-----------------|---------------------|-------------------|-------------------|---------------------|
| Basic information |                 |                 |                     |                   |                   |                     |
| Age (years)     | 40              | 21              | 30                   | 26                | 27                | 27                  |
| Gestational age (weeks) | 38 ± 5 | 30 ± 1 | 39 ± 6 | 40 ± 3 | 37 ± 6 | 28 |
| BMI (kg/m²)     | 28.2            | 22              | 20.4                 | 25.4              | 24.6              | 22.2                |
| Gravida (times) | 6               | 1               | 2                    | 1                 | 1                 | 5                   |
| Parity (times)  | 1               | 0               | 0                    | 0                 | 0                 | 4                   |
| Serum biochemical index |           |                 |                     |                   |                   |                     |
| K (3.5–5.1, mmol/L) | 4.1       | 4.3             | 4.2                  | 4                 | 4.2               | 3.7                 |
| Na (135–145, mmol/L) | 137       | 142             | 143                  | 135               | 140               | 140                 |
| Cl (96–108, mmol/L) | 105       | 109             | 104                  | 102               | 102               | 108                 |
| Ca (2.1–2.9, mmol/L) | 2.2        | 2.2             | 2.14                 | 2.49              | 2.34              | 2.4                 |
| Mg (0.6–1.1, mmol/L) | 0.8        | 0.91            | 0.82                 | 0.74              | 0.86              | 0.7                 |
| P (0.85–1.51, mmol/L) | 1         | 0.97            | 1.13                 | 1.37              | 1.24              | 0.9                 |
| WBC (3.69–9.16, × 10^9/L) | 6.45   | 7.6             | 17.59                | 7.61              | 5.81              | 5.49                |
| RBC (3.68–5.3, × 10^12/L) | 3.8       | 3.31            | 3.99                 | 3.86              | 3.8               | 3.28                |
| PLT (101–320, × 10^9/L) | 164       | 196             | 194                  | 144               | 188               | 238                 |
| RDW-SD (37–54, fl) | 51.6     | 50.4            | 44.8                 | 57                | 41.5              | 43.9                |
| ALT (0–35, U/L)  | 198             | 44              | 76                   | 12                | 40                | 7                   |
| AST (0–35, U/L)  | 196             | 53              | 51                   | 22                | 40                | 15                  |
| TBA (0–10, μmol/L) | 46.4        | 113.2           | 12                   | 18.9              | 27.5              | 46.4                |
| TBL (3.4–20.5, μmol/L) | 31.5    | 10.1            | 14                   | 13.6              | 11.9              | 9.1                 |
| DBIL (0–5, μmol/L) | 22.4       | 7.2             | 6                    | 5                 | 2.5               | 4.5                 |
| IDBIL (0–14, μmol/L) | 9.1       | 2.9             | 8                    | 8.6               | 9.4               | 4.6                 |
| CHOL (0–5.2, mmol/L) | 5.79      | 5.52            | 5.73                 | 6.21              | 7.44              | 6.07                |
| TG (0.34–1.69, mmol/L) | 3.97      | 2.47            | 3.13                 | 2.22              | 4.89              | 3.17                |
| HDL (0.9–2, mmol/L) | 1.59       | 1.83            | 1.6                  | 2.32              | 2.29              | 2.27                |
| LDL (0–3.74, mmol/L) | 2.4        | 2.57            | 2.71                 | 2.88              | 2.93              | 2.36                |
| UA (155–357, μmol/L) | 339       | 257             | 348                  | 282               | 411               | 131                |
| Outcomes of pregnancy women and newborn baby | | | | | | |
| Birth weight (kg) | 3.8           | –               | 3                    | 3.55              | 3.85              | –                   |
| Apgar score (1–10) | 9            | –               | 8                    | 10                | 9                 | –                   |
| Bleeding count (mL) | 400     | –               | 300                  | 250               | 350               | –                   |

1 Abbreviations refer to the footnotes in Table 1
binding region of the NR1H4 gene (Fig. 3A). For the variant p.S145F, compared with the reference 3D model of protein structure, the mutation has a slight change in the chemical bond in the two zinc finger structures rich in Cys amino acids at positions 137, 140, 154, 173 and 192 (Fig. 3B). Similarly, for another

| Human (Wild) | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Human (Mutation) | V C G D R A F G Y H Y N A | C V M D M Y L R R K C Q E |
| Chimpanzee | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Gibbon | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Marmoset | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Prairie vole | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Mouse | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Rat | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Alpine marmot | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Rabbit | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Domestic yak | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Cow | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Goat | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Sheep | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Sperm whale | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Arabian camel | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Chacoan peccary | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Pig | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Dog | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Dingo | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Cat | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Leopard | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Horse | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |
| Elephant | V C G D R A S G Y H Y N A | C V M D M Y M R R K C Q E |

Fig. 2 Evolutionary conservation analysis of the NR1H4 p.S145F and p.M185L mutation among 26 vertebrates, ranging from chimpanzee to elephant. The amino acids serine (S) and methionine (M) in the red horizontal line were highly conserved

Fig. 3 The genetic features of NR1H4. A The distribution of the NR1H4 variants. NR1H4 is a 486-amino acid protein containing two DBD regions and one LBD region. Schematic representation of NR1H4 NM_001206993.1 cDNA and protein showing the locations of two novel possible pathogenic variants p.S145F and p.M185L detected in two out of 197 patients with ICP disease. Effects of NR1H4 p.S145F. B and p.M185L variants. C on the protein structure. The three-dimensional models of reference and modified (p.S145F and p.M185L) NR1H4 showed gold and blue rounded structures, respectively. The enlarged portion showed that the two DBD regions have small changes in the chemical bond lengths. DBD: DNA-binding domain; LBD: ligand-binding domain. D Comparison of the expression level of the NR1H4 gene between two healthy pregnant women and 4 patients with ICP. The expression level of NR1H4 was higher in the ICP group than in the healthy group. The difference did not reach the significance level (P = 0.22)
Fig. 3 (See legend on previous page.)
novel missense mutation, p.M185L, there is a change in the chemical bond at positions 137, 157, 189 and 192 (Fig. 3C).

To further explore the genetic basis of NR1H4, we analysed the mRNA expression level of the NR1H4 gene in placental tissue between two healthy pregnant women and four patients with ICP using NCBI GEO databases (GEO accession: GSE46157) from the Du Q et al. report [36]. The results showed that the expression of NR1H4 was upregulated in the ICP group (Fig. 3D), even though the difference was not significant ($P = 0.22$).

### Correlation analysis

The potential correlation of NR1H4 four mutations and 29 available clinical and laboratory data are presented in Table 5. The results showed that the mutation group had higher TBA levels, TBIL levels, and bleeding amounts and a lower Apgar score. In addition, it was found that only the level of Na ions was significantly ($P = 0.014$) higher in the mutation group (139.50 mmol/L) than in the wild-type group (137.27 mmol/L). The associations between the clinical parameters (age; odds ratio (OR) = 0.965); 95% confidence intervals (CI): 0.823–1.132; gestational age (OR = 1.001; 95% CI: 0.982–1.019); BMI

### Table 5 The potential correlation of NR1H4 mutations with clinical and laboratory data in 197 ICP patients

| Characteristics | Wild type | Mutation | $P$ value |
|-----------------|-----------|----------|-----------|
| Basic information | | | |
| Age (years) | 29.45 ± 5.24 (n = 191) | 28.50 ± 6.35 (n = 6) | 0.66 |
| Gestational age (days) | 256.29 ± 22.81 (n = 186) | 250.83 ± 37.49 (n = 6) | 0.57 |
| BMI (kg/m²) | 25.89 ± 3.94 (n = 177) | 23.80 ± 2.83 (n = 6) | 0.14 |
| Gravida (times) | 2.39 ± 1.53 (n = 183) | 2.67 ± 2.25 (n = 6) | 0.67 |
| Parity (times) | 0.63 ± 0.75 (n = 182) | 0.83 ± 1.60 (n = 6) | 0.52 |
| Serum biochemical index | | | |
| K (mmol/L) | 4.01 ± 0.36 (n = 181) | 4.08 ± 0.21 (n = 6) | 0.63 |
| Na (mmol/L) | 137.27 ± 2.15 (n = 180) | 139.50 ± 3.02 (n = 6) | 0.014* |
| CL (mmol/L) | 104.04 ± 2.61 (n = 180) | 105.00 ± 2.97 (n = 6) | 0.38 |
| Ca (mmol/L) | 2.36 ± 0.17 (n = 180) | 2.30 ± 0.14 (n = 6) | 0.37 |
| Mg (mmol/L) | 0.82 ± 0.14 (n = 180) | 0.81 ± 0.08 (n = 6) | 0.38 |
| P (mmol/L) | 1.15 ± 0.19 (n = 180) | 1.10 ± 0.18 (n = 6) | 0.55 |
| WBC ($\times 10^3$) | 8.56 ± 2.64 (n = 190) | 8.43 ± 4.58 (n = 6) | 0.91 |
| RBC ($\times 10^{12}$) | 3.84 ± 0.42 (n = 190) | 3.67 ± 0.30 (n = 6) | 0.32 |
| PLT ($\times 10^3$) | 197.56 ± 59.10 (n = 190) | 204.00 ± 54.36 (n = 6) | 0.79 |
| RDW-SD (fL) | 46.03 ± 4.83 (n = 190) | 48.20 ± 5.81 (n = 6) | 0.28 |
| ALT (U/L) | 104.40 ± 128.54 (n = 191) | 62.83 ± 70.74 (n = 6) | 0.43 |
| AST (U/L) | 88.00 ± 99.84 (n = 191) | 62.83 ± 67.00 (n = 6) | 0.54 |
| TBA (μmol/L) | 42.46 ± 38.25 (n = 191) | 44.07 ± 36.68 (n = 6) | 0.92 |
| TBIL (μmol/L) | 14.95 ± 7.48 (n = 189) | 15.03 ± 8.29 (n = 6) | 0.98 |
| DBIL (μmol/L) | 6.93 ± 6.10 (n = 189) | 7.93 ± 7.26 (n = 6) | 0.69 |
| IDBIL (μmol/L) | 8.04 ± 3.51 (n = 189) | 7.10 ± 2.69 (n = 6) | 0.52 |
| CHOL (mmol/L) | 6.39 ± 1.69 (n = 183) | 6.13 ± 0.69 (n = 6) | 0.69 |
| TG (mmol/L) | 3.62 ± 1.59 (n = 183) | 3.31 ± 0.99 (n = 6) | 0.63 |
| HDL (mmol/L) | 1.94 ± 0.50 (n = 183) | 1.98 ± 0.35 (n = 6) | 0.84 |
| LDL (mmol/L) | 2.80 ± 1.33 (n = 183) | 2.64 ± 0.24 (n = 6) | 0.77 |
| UA (μmol/L) | 327.54 ± 91.69 (n = 181) | 294.67 ± 96.65 (n = 6) | 0.39 |

| Outcomes of pregnancy and newborn baby | | | |
| Birth weight (kg) | 3.06 ± 0.75 (n = 155) | 3.55 ± 0.39 (n = 4) | 0.09 |
| Apgar score (1–10) | 9.40 ± 1.25 (n = 154) | 9.00 ± 0.82 (n = 4) | 0.24 |
| Bleeding count (mL) | 260.82 ± 102.76 (n = 153) | 316.67 ± 170.17 (n = 3) | 0.32 |

1 Abbreviations refer to the footnotes in Table 1
2 The total number of patients for wild type group
3 The total number of patients for mutation group
4 *P < 0.05, the level of Na ion was significantly difference between wild-type group and mutation group
The mutant has a slight change in the chemical bond of NR1H4 in the first and second zinc finger of the DBD of NR1H4. Two novel mutations S145F and M185L were also located covering much larger cohorts suggests that these variants are rare. The allele frequencies of the three missense mutations (MAF = 0.0025) and one synonymous mutation (MAF = 0.007) were lower in this study. According to previous studies, low-frequency and rare variants with large effect sizes contribute to complex traits and diseases [40–42]. Therefore, we hypothesized that the allele frequency and the size effect of mutations have a larger effect on TBA levels. In this study, combining the prediction results with the website available tools SIFT and PolyPhen2 and protein structural modelling, we suspected that the novel mutations contributed more to the development of ICP than the other two. Therefore, it is also likely reasonable that there is no significant difference in TBA levels between wild-type and NR1H4 mutations even though the mutation group tended to be associated with higher TBA levels when considering the allele frequency and size effect. Except for the ICP caused by the NR1H4 mutations, we speculated that other gene mutations (such as ANO8, ATP-binding cassette transporter family, bile acid receptors) [20, 35, 43], epigenetic regulators (microRNAs, DNA methylation and histone modification) [44–46], oestrogen and progesterone sulfate metabolites [10, 47], hypoxia [48] and the immune system [49], among other factors [50], may be responsible for the remaining ICP patients in this study.

Considering that BAs are toxic to the body, the excessive increase in BA levels has been depicted in different pathological contents. Moreover, several previous studies demonstrated that BAs have the ability to promote lipid absorption and biliary cholesterol secretion [16, 51, 52], indicating that BAs are associated with abnormalities in lipids. Saskia et al. reported that six out of 11 pregnant women with ICP having NR1H4 variants had symptomatic gallstones [16], and the remaining five did not have gallstone symptoms but had a family history of gallstones. The formation of gallstones is likely determined by the relative concentrations of TBA, CHOL and phospholipids in bile. In the present study, according to the clinical characteristics of 6 ICP cases with NR1H4 mutations, we found that the TBA levels, CHOL levels and TG levels were higher than the reference values. Therefore, we speculated that these ICP cases with NR1H4 variants have a high risk for gallstones. Bergheim et al. demonstrated that the possible mechanism of gallstones is the decrease in the expression of the NR1H4 gene [53]. Furthermore, Moschetta et al. prevented cholesterol gallstone disease by NR1H4 agonists in a mouse model, indicating that NR1H4 could be associated with cholesterol [54]. In addition, NR1H4 dysfunctions may occur during the progression associated with inflammatory bowel disease, colorectal cancer in the gut [55, 56], fibrosis and hepatocellular carcinoma in the liver [57, 58]. These results suggest that the variants affecting the structure and functions of NR1H4 lead to gut-liver axis diseases, and in the future, NR1H4 will be proposed as an emerging therapeutic target for both cholestatic and multiple metabolic diseases.

Our present study had several advantages. First, to our knowledge, only a few pathogenic mutations of the
NH1R4 gene, such as M173T, R176* and Tyr139_Asn140InsLys, have been identified thus far [16, 21]. Our findings broaden our understanding of the mechanism of NR1H4’s action on ICP disease. Second, NR1H4 mutations have been detected in ICP families [16, 21]. To date, no studies have uncovered genetic mutations in NR1H4 genes of hepatic disease among pregnant patients from a relatively large nationally representative sample (n = 197) in China and 1029 local healthy pregnant women. Third, the 29 clinical data of 197 ICP patients are relatively complete, which provides data supporting correlation analysis between mutations and clinical data. However, even though our results provided possible pathogenic variants, the causality between the two potential interesting candidate loci and ICP disease needs to be verified by validation functional experiments.

Conclusions
In summary, we reported two potential damaging mutations (p.S145F and p.M185L) in the NR1H4 gene in two out of 197 Chinese patients with ICP for the first time. Our findings provide new insights into the genetic architecture of ICP disease and suggest potential candidate variant targets for ICP clinical treatment.

Abbreviations
ICP: Intrahepatic cholestasis of pregnancy; NR1H4: Nuclear Receptor Subfamily 1 Group H Member 4; BA: Bile acid; 1000X_ALL: 1000 Genomes Project; ExAC: Exome Aggregation Consortium; TBA: Total bile acid; ABCB4: ATP Binding Cassette Subfamily B Member 4; ABCB11: ATP Binding Cassette Subfamily B Member 11; ABCC2: ATP Binding Cassette Subfamily C Member 2; DBD: DNA binding domain; LBD: Ligand binding domain; BMI: Body mass index; WBC: White blood cell; RBC: Red blood cell; PLT: Platelet; RDW-SD: Red blood cell distribution width; SD: AST: Aspartate transaminase; ALT: Alanine transaminase; TBIL: Total bilirubin; DBIL: Direct bilirubin; IDBIL: Indirect bilirubin; CHOL: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; OR: Odds ratio; CI: Confidence intervals.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12920-022-01240-w.

Acknowledgements
We want to express our great gratitude to the patients who participated in this study.

Author contributions
HLai and XL performed the experiments, analyzed the data, prepared the figures and drafted the manuscript. SX, JZ, HLiu, YOuyang and HY collected samples. YZeng performed the experiments. YZou and XZ performed the experiments, analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

Funding
The authors gratefully acknowledge the financial support of the National Science Foundation of Jiangxi Province (No. 20202BABL216010 and No. 20192BG70003) and the Science and Technology Plan of Jiangxi Provincial Health Commission (No. 20213076). The funders played no role in the design of the study, data collection, analysis, writing the manuscript or the decision to submit it for publication.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The present study followed the tenets of the Helsinki Declaration, ethics approval was approved by the Institutional Review Board of Jiangxi Provincial Maternal and Child Health Hospital in China, and each participating woman gave informed consent.

Consent for publication
All authors agree and have given consent for publication.

Competing interests
The authors have declared that no potential competing interests exist.

Author details
1 Key Laboratory of Women’s Reproductive Health of Jiangxi Province, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang 330006, Jiangxi, China. 2 Department of Obstetrics, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang 330006, Jiangxi, China. 3 Central Lab, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang 330006, Jiangxi, China.

Received: 4 November 2021   Accepted: 12 April 2022
Published online: 18 April 2022

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