A novel heterozygous *NR1H4* termination codon mutation in idiopathic infantile cholestasis

Xiu-Qi Chen, Lin-Lin Wang, Qing-Wen Shan, Qing Tang, Ya-Nan Deng, Shu-Jun Lian, Xiang Yun

Nanning, China

Background: **This study aimed to evaluate the genetic effect of the *NR1H4* gene in the pathogenesis of idiopathic infantile cholestasis of Chinese subjects in Guangxi, China.**

Methods: **Seventy-eight patients with idiopathic infantile cholestasis served as a study group and 95 infants without cholestasis as controls. Genomic DNA was extracted from peripheral venous blood leucocytes by phenol chloroform procedures. Polymerase chain reaction (PCR) was used to amplify all coded exons of *NR1H4*, and single-strand conformation polymorphism (SSCP) was used to analyze all amplification fragments. The PCR products with abnormal bands in SSCP were sequenced using an ABI 3100 sequencer.**

Results: **A novel heterozygous termination codon mutation in *NR1H4* exon 5 (*NR1H4* R176X, CGA-TGA) was found in one of the 78 patients. The patient with mutation R176X had high levels of bilirubin, alanine aminotransferase, γ-glutamyltransferase, cirrhosis and ascites despite biliary tract flushing procedures and drug therapy. In the other patients and controls, no mutation was detected.**

Conclusions: **Heterozygous termination codon mutation of *NR1H4* R176X was found in idiopathic infantile cholestasis. The novel mutation is useful to establish particular characteristics for differential diagnosis of idiopathic infantile cholestasis and to determine the influence of such gene defects in the prognosis.**

World J Pediatr 2012;8(1):67-71

Author Affiliations: Department of Pediatrics, First Affiliated Hospital, Guangxi Medical University, Nanning, 530021 Guangxi, China (Chen XQ, Wang LL, Shan QW, Tang Q, Deng YN, Lian SJ, Yun X)

Corresponding Author: Lin-Lin Wang, Department of Pediatrics, First Affiliated Hospital, Guangxi Medical University, Nanning, 530021 Guangxi, China (Tel: +86-771-5356505; Fax: +86-771-5356781; Email: WLL276@yahoo.com.cn)

doi: 10.1007/s12519-011-0299-z

Key words: **cholestasis;**
farnesoid X receptor;
infant;
mutation;
NR1H4

Original article

Introduction

Cholestasis can be divided into extrahepatic and intrahepatic cholestasis. Extrahepatic cholestasis is caused by extrahepatic diseases such as bile duct injury, obstruction and congenital biliary atresia. Intrahepatic cholestasis was due to intrahepatic bile duct injury and damaged liver cells. Intrahepatic bile duct injury is characterized by intrahepatic bile duct hypoplasia and paucity. About 40%-50% of infantile intrahepatic cholestasis cases are idiopathic due to unknown causes; 5%-10% of them have persistent inflammation or fibrosis, and a few develop severe liver diseases, such as cirrhosis. Despite the unknown cause of idiopathic infantile cholestasis, it is suggested that genetic factors had a relation with cholestasis, such as familial intrahepatic cholestasis-1 deficiency in progressive familial intrahepatic cholestasis type 1 (PFIC-1), bile salt export pump gene (BSEP) deficiency in PFIC-2,[1,2] and multidrug resistance protein 3 (MDR3) deficiency in PFIC-3.[3]

Farnesoid X receptor (FXR) encoded by the *NR1H4* gene is a member of the nuclear receptor superfamily of ligand-activated transcript factors. As a metabolic regulator, FXR plays a key role in hepatoprotection by maintaining the homeostasis of liver metabolism, involving bile acid, cholesterol and lipid metabolism.[4,5] Variation of *NR1H4* as a factor for development of gallstones indicates complex interactions of *NR1H4* alleles.[6] It was reported that four of *NR1H4* functional variants (-1g>t, W80R, M1V, M173T) may predispose to intrahepatic cholestasis of pregnancy.[7] The decreased FXR activity is also found to be associated with PFIC-1.[1] In idiopathic infantile cholestasis, however, *NR1H4* variants are rarely reported. This study aimed to determine whether *NR1H4* mutations take place in idiopathic infantile cholestasis.

Methods

Patients

Seventy-eight infants with idiopathic infantile cholestasis were enrolled in the study, and 95 healthy infants without cholestasis served as a control group. The patients were selected according to the following criteria: (1) a history of chronic cholestasis onset of unknown origin in the neonatal/infantile period; (2) elevated concentrations of total bile acid and total bilirubin (TB), mainly marked conjugated bilirubin (CB); (3) increased concentration of serum alanine aminotransferase (ALT); (4) presence of hepatomegaly or hepatosplenomegaly; and (5) absence of viral infections (such as hepatitis A virus, hepatitis B virus, hepatitis C virus, cytomegalovirus, herpes simplex virus, rubella virus, toxoplasma gondii, tubercule bacillus and treponema). The infants of the control group did not suffer from cholestasis. In addition, serum TB, CB, ALT, aspartate aminotransferase (AST) and γ-glutamyltransferase (γ-GT) were recorded.

Informed consent was obtained from the guardians of the infants, and the study protocol was approved by the institutional ethical committee of the First Affiliated Hospital of Guangxi Medical University, China.

Polymerase chain reaction (PCR) amplified all coded exons of *NR1H4*

Genomic DNA was extracted from leucocytes in peripheral venous blood by the phenol chloroform method, and the DNA concentration was quantified by spectrophotometry. The oligonucleotide primers for PCR analysis were designed to cover all coded exons of *NR1H4* as well as the intron-exon boundaries (Table). Primary PCRs for generated *NR1H4* fragments were generally performed in a reaction volume of 50 μL with 100 ng genomic DNA, 1.5 unit Taq polymerase (Fermentas), 10×PCR buffer (Fermentas), 1.5 mmol/L $MgCl_2$ (Fermentas), 200 μmol/L deoxynucleoside-5-triphosphates (Takara), and 20 μmol each primer. PCR conditions included an initial denaturation step at 95ºC for 5 minutes, followed by 30 cycles of denaturation at 94ºC for 45 seconds, annealing at 52ºC-60ºC for 30-50 seconds in accordance with conditions between different exons and extension at 72ºC for 50 seconds or 1 minute. The PCR reaction was terminated after an extension step at 72°C for 5-10 minutes.

Single-strand conformation polymorphism (SSCP) and genomic DNA sequence analysis

NR1H4 mutations search was performed by SSCP. The PCR products were excised from 2% agarose gel and purified. SSCP analysis was performed on the PCR fragments that contained all coding exons. Samples (2 μL PCR product + 4 μL loading dye solution, with 10% sucrose and 0.05% bromchlorphenol blue[8]) were denatured at 98ºC for 5 minutes, and then chilled on ice to generate a single-strand DNA. Electrophoreses were run at two different temperatures. The first temperature was calculated in an empirical formula T = [80×C/(A+1)]/{2.71×[C/ (A+1)]}[8] given by professor LI Wei. The latter temperature was run at 4ºC. The electrophoretic temperature was controlled by an incubator (MIR-153, Sanyo). DNA bands were revealed by the silver-stain method. DNA bands of SSCP shift different from the others were considered for DNA sequence analysis with ABI 3100. The results of sequence were compared with the normal sequences of *NR1H4* in GenBank (NC_000012.11).

Results

In the patient group, the mean TB was 194.43±13.33 μmol/L (nomal ≤ 20 μmol/L). The mean level of CB was 105.75±6.44 μmol/L, and the mean serum ALT

Table. PCR amplicon information for *NR1H4*

Fragment	Primer sequences (5'-3')	Product size (bp)
Exon 3	F: 5-TCT CCT AAC CAA CCC TTC C-3 R: 5-GCA TTT CCT CAT GTA TTT CC-3	259
Exon 4	F: 5-AAC CAT TAC GCC AAA C-3 R: 5-GAG GCT CTG TCT CCA CA-3	401
Exon 5	F: 5-TCA GTG TTT CTC CCA CA-3 R: 5-TGA CAT CTG CCT CCT T-3	227
Exon 6	F: 5-TCT GTG ATT GGT GAA GTC T-3 R: 5-CAG TTC CTG CCA GTC TT-3	214
Exon 7	F: 5-ACA TCT TCC CTA ACA CCT-3 R:5-GGC AAT AAA AAC TCC AG-3	200
Exon 8	F: 5-TAG TAA GAT GGG TTT TC-3 R: 5-CTA ATC TGT GGG CAC-3	279
Exon 9	F: 5-TAG GAT TTC AGA CTT TGG ACC-3 R: 5-ACA AAC TCT TTT ACC ATT AGC C-3	183
Exon 10	F: 5-ACT TTC CCC CAC AGG-3 R: 5-GCA GAA TTA TAG GCT ACT TT-3	253
Exon 11	F: 5-TAT GCT GAA TTA ATG CTT TTC CAC-3 R: 5-GAG AGA GAA AAA GGA GCT AGA CCC-3	342

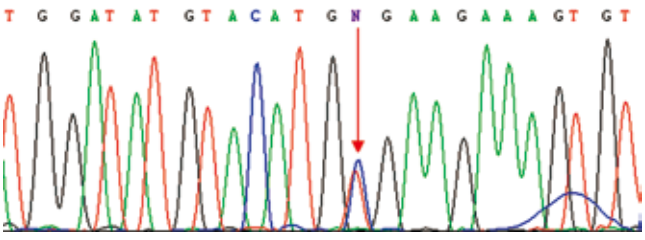

Fig. Sequence of the termination codon mutation, *NR1H4* R176X (CGA-TGA).

was 123.80±11.63 U/L (nomal ≤40 U/L). The mean AST was 273.86 ± 27.90 U/L (normal ≤40 U/L) and γ-GT was 308.60±46.30 U/L (nomal ≤50 U/L). All of the patients had hepatomegaly and 62 of them had splenomegaly. In the control group, all the biochemical markers were normal (P<0.01).

In our study one patient had a mutation in exon 5. The mutation sequencing showed a heterozygous substitution (CGA-TGA) in codon 176 (Fig.), which creates a stop codon R176X in exon 5 (GenBank NP_005114.1). This was a novel heterozygous termination codon mutation. But in the other patients and controls, there were no relevant mutations detected.

The peak levels of TB and CB of the patient with R176X mutation was 348.2 μmol/L and 193.6 μmol/L respectively. The levels of ALT, AST, and γ-GT were 72 U/L, 402 U/L, and 61 U/L respectively. The patient received a surgery for biliary tract flushing and T-tube drainage procedures due to the failure of drug treatment. Two days after surgery, the TB was 256.4 μmol/L, CB 133.0 μmol/L, ALT 98 U/L, and γ-GT 69 U/L. Three weeks after surgery, the TB was 170.3 μmol/L, CB 103.6 μmol/L, ALT 219 U/L, and γ-GT 195 U/L. But five weeks after surgery, the TB was 334.4 μmol/L, CB 185.6 μmol/L, ALT 143 U/L, and γ-GT 144 U/L. The patient was subjected to persisted drug administration. Ultrasonography showed a small amount of ascites and cirrhosis. Histopathologically, liver cells and bile capillaries were more extensive cholestasis and interstitial fibrosis. But most of the other patients without this mutation had good prognosis.

Discussion

We found a new heterozygous mutation (CGA-TGA) in an infant with idiopathic intrahepatic cholestasis. The mutation created a termination codon (R176X) in exon 5. Since exon 5 coded a DNA-binding domain in *NR1H4* encoding sequence,[9] the stop codon mutation may lead to a peptide missing and loss of FXR regulatory function, which may result in intrahepatic cholestasis. This finding is supported by clinical characteristics in our patient. Two days after surgery, the TB and CB declined due to the flushing of the biliary tract procedures. But three weeks after surgery, the TB and CB were increased and five weeks later, they were higher than those at three weeks although the patient had been given persistent drug therapy. At last, the infant developed cirrhosis and ascites. While the other children without this mutation had a good prognosis. The findings suggest that *NR1H4* R176X mutation may lead to the functional loss of FXR and failure to adjust bile synthesis and secretion. But this mutation was found only in one patient; in the other patients and controls no variants were observed. It was confirmed that *NR1H4* variants may predispose to intrahepatic cholestasis in pregnancy. And decreased FXR activity is also associated with PFIC.[1]

The *NR1H4* R176X mutation detected in children from Guangxi province of China was compared with the data (rs35177932 and rs61755050) from the International HapMap Project for the Chinese population and also the National Center for Biotechnology Information (NCBI) Variation Database (dbSNP). This mutation is located in the position C874T (NM_005123.2), and has not been reported. This mutation will provide some insights into *NR1H4* in Chinese people.

FXR is acknowledged as a bile acid-activated nuclear receptor. *BSEP* and *MDR3* are the target genes of FXR. FXR through a DNA-binding domain is combined to a highly conservative inverted repeat sequence (IR-1) of *BSEP* or *MDR3*.[10,11] Bile acids, FXR agonist chenodeoxycholate and synthetic agonist GW4064[12,13] can activate the transcription of *BSEP* and *MDR3* gene expression[14,15] via a direct binding of FXR/retinoid X receptor α-heterodimers.[16] Chen et al[17] reported that *BSEP* and *MDR3* were down-regulated during early-stage obstructive cholestasis in children. The down-regulation of the major bile transport pathway was probably controlled mainly by down-regulation of FXR. This response could decrease the intracellular bile acid level as well as the biliary pressure. Therefore, if the function of FXR drops suddenly, it may reduce the expression of *BSEP* and *MDR3*, increasing the degree of intrahepatic cholestasis. *BSEP* and *MDR3* have been confirmed to be related to genetic pathogenesis of intrahepatic cholestasis.[18]

FXR also regulates the synthesis of bile in liver cells through two channels. Firstly, bile acids are synthesized in hepatocytes and secreted across the bile canalicular membrane by ABC transporters which are regulated by FXR. Secondly, *SHP*, another FXR target gene,[16] can inhibit the expression of biosynthetic enzyme cholesterol 7-alpha hydroxylase (Cyp7α1).[19,20] By inhibiting the activity of this enzyme, it can reduce the synthesis of bile. The FXR null rat model also convinced that FXR/BAR played a central role in bile acid homeostasis.[21] FXR will not be able to inhibit the synthesis of bile if its function disappears.

Furthermore, FXR selectively inhibits NF-kappa B-mediated hepatic inflammatory response but maintains or even enhances the cell survival response. FXR is a negative mediator of hepatic inflammation which makes it play a critical role in hepatoprotection.[4,22] Also, FXR

can increase the canalicular bile acid output and the excretion of unconjugated bile acids that protects against cholic acid-induced liver toxicity.[23] Hence FXR plays an important role in the metabolic process of bile acid. The loss of function of FXR due to the *NR1H4* mutation will result in bile acid metabolism disorder.

There are some limitations in this study. Firstly, only one case of the mutation in *NR1H4* was detected and our views were based on the stop codon mutation and poor prognosis of the patient. This study was conducted in infants from Guangxi, and a small sample size of this study may affect the positive rate. Hence multi-center studies with large samples are required to discover more mutations. Secondly, this was only a preliminary study on *NR1H4* in the pathogenesis of idiopathic infantile cholestasis. More studies should be considered on genetic familial pedigree analysis. Moreover, genetics or *in vitro* related experiments are needed to confirm FXR in the pathogenesis of idiopathic infantile cholestasis.

In conclusion, a termination codon mutation in *NR1H4* R176X is very important to establish particular characteristics in the differential diagnosis of idiopathic infantile cholestasis as well as the influence of such gene defect in the prognosis. This mutation may be a new genetic metabolic etiology of idiopathic infantile intrahepatic cholestasis.

Acknowledgements

We thank Prof. Li Wei for his empirical formula provided to us and suggestions on the Single-Strand Conformation Polymorphism.

Funding: This study was supported by a grant from the Foundation of Guangxi Scientific Research and Technological Development Projects (Bureau of Science & Technology of Guangxi: No.0816004-6).

Ethical approval: This study was approved by the local ethical committee of the First Affiliated Hospital of Guangxi Medical University, China.

Competing interest: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this study.

Contributors: Wang LL contributed the conception and design of the study and revised the paper. Chen XQ performed the experiments and wrote the paper. Shan QW and Tang Q analyzed the data and edited the manuscript. Deng YN, Lian SJ and Yun X collected the specimens and performed some of the experiments.

References

1 Chen F, Ananthanarayanan M, Emre S, Neimark E, Bull LN, Knisely AS, et al. Progressive familial intrahepatic cholestasis, type 1, is associated with decreased farnesoid X receptor activity. Gastroenterology 2004;126:756-764.
2 Jansen PL, Muller MM. Progressive familial intrahepatic cholestasis types 1, 2, and 3. Gut 1998;42:766-767.
3 Arnell H, Nemeth A, Anneren G, Dahl N. Progressive familial intrahepatic cholestasis (PFIC): evidence for genetic heterogeneity by exclusion of linkage to chromosome 18q21-q22. Hum Genet 1997;100:378-381.
4 Wang YD, Chen WD, Wang M, Yu D, Forman BM, Huang W. Farnesoid X receptor antagonizes nuclear factor kappaB in hepatic inflammatory response. Hepatology 2008;48:1632-1643.
5 Wang YD, Chen WD, Moore DD, Huang W. FXR: a metabolic regulator and cell protector. Cell Res 2008;18:1087-1095.
6 Kovacs P, Kress R, Rocha J, Kurtz U, Miquel JF, Nervi F, et al. Variation of the gene encoding the nuclear bile salt receptor FXR and gallstone susceptibility in mice and humans. J Hepatol 2008;48:116-124.
7 Van Mil SW, Milona A, Dixon PH, Mullenbach R, Geenes VL, Chambers J, et al. Functional variants of the central bile acid sensor FXR identified in intrahepatic cholestasis of pregnancy. Gastroenterology 2007;133:507-516.
8 Li W, Gao F, Liang J, Li C, Zhang H, Tang Z, et al. Estimation of the optimal electrophoretic temperature of DNA single-strand conformation polymorphism by DNA base composition. Electrophoresis 2003;24:2283-2289.
9 Kuipers F, Claudel T, Sturm E, Staels B. The farnesoid X receptor (FXR) as modulator of bile acid metabolism. Rev Endocr Metab Disord 2004;5:319-326.
10 Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem 2001;276:28857-28865.
11 Maloney PR, Parks DJ, Haffner CD, Fivush AM, Chandra G, Plunket KD, et al. Identification of a chemical tool for the orphan nuclear receptor FXR. J Med Chem 2000;43:2971-2974.
12 Wang H, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Mol Cell 1999;3:543-553.
13 Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999;284:1365-1368.
14 Plass JR, Mol O, Heegsma J, Geuken M, Faber KN, Jansen PL, et al. Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. Hepatology 2002;35:589-596.
15 Stedman C, Liddle C, Coulter S, Sonoda J, Alvarez JG, Evans RM, et al. Benefit of farnesoid X receptor inhibition in obstructive cholestasis. Proc Natl Acad Sci U S A 2006;103:11323-11328.
16 Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, et al. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol Cell 2000;6:517-526.
17 Chen HL, Liu YJ, Chen HL, Wu SH, Ni YH, Ho MC, et al. Expression of hepatocyte transporters and nuclear receptors in children with early and late-stage biliary atresia. Pediatr Res 2008;63:667-673.
18 Schuetz EG, Strom S, Yasuda K, Lecureur V, Assem M, Brimer C, et al. Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. J Biol Chem 2001;276:39411-39418.

19 Walters JR. Bile acids are physiological ligands for a nuclear receptor. Gut 2000;46:308-309.
20 Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, et al. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell 2000;6:507-515.
21 Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell 2000;102:731-744.
22 Krücken J, Delić D, Pauen H, Wojtalla A, El-Khadragy M, Dkhil MA, et al. Augmented particle trapping and attenuated inflammation in the liver by protective vaccination against Plasmodium chabaudi malaria. Malar J 2009;8:54.
23 Miyata M, Tozawa A, Otsuka H, Nakamura T, Nagata K, Gonzalez FJ, et al. Role of farnesoid X receptor in the enhancement of canalicular bile acid output and excretion of unconjugated bile acids: a mechanism for protection against cholic acid-induced liver toxicity. J Pharmacol Exp Ther 2005;312:759-766.

Received March 1, 2010
Accepted after revision May 4, 2010