# Bile acid transport correlative protein mRNA expression profile in human placenta with intrahepatic cholestasis of pregnancy

*Tao Cui, MD, Yufang Liu, MD, Xiaoliang Men, MD, Zhou Xu, MD, Lin Wu, MD, Shuyun Liu, MD, Aiyun Xing, MD.*


## ABSTRACT

**الأهداف:** تحليل ظهور البروتينات المترابطة لناقل الحمض الصفراوي في المشيمة مع ركود الصفراء داخل الكبد أثناء الحمل (ICP).

**الطريقة:** أجريت الدراسة في قسم أمراض النساء والولادة مستشفى جامعة غرب الصين الثاني بجامعة ستشوان – الصين، خلال الفترة ما بين مارس 2007م إلى أكتوبر 2008م. تم تطبيق الوقت الفعلي (RT–PCR) من أجل قياسات ظهور (mRNA) لعدد 8 بروتينات مترابطة لنواقل الحمض الصفراوي، و OATP1A2، وOATP1B1، وMRP1، و MRP2، و AE2، وBSEP، وFIC1، في المشيمة الطبيعية (n=20) وأولئك اللواتي يعانين من ICP n=20.

**النتائج:** تم اكتشاف جميع النواقل ماعدا OATP1B1 و (BSEP). كانت مستويات OATP1A2 وAE2 mRNA أعلى بينما كانت مستويات (FIC1) أقل لدى مجموعة (ICP).

**خاتمة:** قد يكون لدى التبديل في بروتينات الربط لنواقل الحمض الصفراوي AE2، (OATP1A2) و(FIC1) صله في ركود الصفراوي الجنيني لدى ICP.

**Objectives:** To analyze the expression of bile acid transport correlative proteins in the placenta of patients with intrahepatic cholestasis of pregnancy (ICP).

**Methods:** This case-control study was performed in the Department of Gynecology and Obstetrics, West China Second University Hospital, Sichuan University, Chengdu, Sichuan, China from March 2007 to October 2008. Real time reverse transcriptase polymerase chain reaction was applied for the mRNA expression measurement of 8 bile acid transport correlative proteins, organic anion transporting polypeptide (OATP)1A2, OATP1B1, multidrug resistance protein (MRP)1, MRP2, anion exchanger (AE)2, bile salt export pump (BSEP), multidrug resistance 3, and familial intrahepatic cholestasis (FIC)1, in normal human placentas (n=20) and those with ICP (n=20).

**Results:** All the transcripts except OATP1B1 and BSEP were detected. Both OATP1A2 and AE2 mRNA were higher while FIC1 was lower in ICP patients.

**Conclusions:** The alteration of bile acid transport correlative proteins OATP1A2, AE2, and FIC1 may be involved in the fetal cholestasis of ICP.


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*From the Department of Gynecology and Obstetrics (Cui, Wu, Liu S, Xing), West China Second University Hospital, Sichuan University, Department of Gynecology and Obstetrics (Xu), Sichuan Provincial Hospital of Women and Children, Chengdu, Sichuan, Department of Gynecology and Obstetrics (Liu Y), Affiliated Hospital of Binzhou Medical College, Binzhou, Shandong, Department of Gynecology and Obstetrics (Men), Daxing Maternal and Child Health Hospital of Beijing, Beijing, China.*

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*Address correspondence and reprint request to: Dr. Aiyun Xing, Department of Gynecology and Obstetrics), West China Second University Hospital, Sichuan University, No. 20, Section 3, Ren Min Nan Lu, Chengdu, Sichuan 610041, China. Tel. +86 (28) 85503857. Fax. +86 (28) 85559065. E-mail: aiyun-x@126.com*

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Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific disorder, which occurs mainly in the third trimester of pregnancy and is characterized by pruritus and biochemical cholestasis. While it usually implies a benign outcome for the mother, ICP may 5-fold increase the incidence of fetal distress, stillbirth, and premature delivery. When the condition is untreated, the perinatal mortality has been found to range between 11-20%.[1] The incidence of ICP varies throughout the world. In Chengdu, China, for example, a retrospective analysis disclosed a high incidence of 5% of ICP in 23917 pregnant women over a period of 10 years.[2] Elevated bile acid may be crucial for the pathophysiology of fetal adverse effects.[3] Evidence suggests the presence of some bile acid transporters in human placenta,[4] but the placental expression profile of bile acid transporters in ICP remains to be established. We hypothesized that an unusual bile acid transport across placenta between the maternal and fetal circulation is the potential reason for fetal cholestasis in ICP, and bile acid transport correlative proteins may play an important role. We chose typical bile acids transporters, which stand for different sites and functions in hepatocytes to explore their functions in placenta and determine whether they are involved in the pathogenesis of ICP. Using real time reverse transcriptase polymerase chain reaction (RT-PCR), we compared the placental expression levels of 8 transporters, namely, organic anion transporting polypeptide-A (OATP-A, OATP1A2), OATP-C (OATP1B1, OATP-C), multidrug resistance protein (MRP)1, MRP2, anion exchanger 2 (AE2), bile salt export pump (BSEP), multidrug resistance 3 (MDR3), and familial intrahepatic cholestasis-1 (FIC1), in women of the normal third trimester and in those with ICP.

**Methods.** The study was performed from March 2007 to October 2008 in the Department of Gynecology and Obstetrics, West China Second University Hospital, Sichuan University, Chengdu, Sichuan, China and was approved by the Ethics Committee of West China Second University Hospital of Sichuan University. Informed consent was obtained from all participants. Normal third trimester placental samples (control group, n=20) and placentas of ICP (n=20) were obtained following cesarean section between 34 and 40 weeks' gestation.

***Clinical samples collection.*** The following criteria were required for the diagnosis of ICP: 1) presence of pruritus in the second or third trimester; 2) elevated liver function parameter (fasting serum total bile acid >10 μmol/L; aminotransferases >40 IU/L for alanine aminotransferase [ALT] and >37 IU/L for aspartate aminotransferase [AST]); 3) absence of other causes of itching and of liver dysfunction, such as obstructive gallstone disease, viral hepatitis, infectious, metabolic, or drug-related liver diseases; and 4) confirmed postnatal resolution of pruritus and liver function tests. Exclusion criteria for our study were the presence of pre-existing liver disease (such as hepatitis B and C, primary biliary cirrhosis, familial cholestatic liver disease), skin diseases, allergic disorders, and symptomatic cholelithiasis. Women were also excluded if hepatic impairment did not resolve postnatally, with the exception of cyclical and exogenous oestrogen induced cholestasis. All placentas were obtained within 15 minutes after separation from the uterus. Small sections of placenta, free of maternal decidua and fetal membranes, were wiped free of blood using phosphate buffer solution prior to snap freezing in liquid nitrogen and stored at -80°C. Total RNA was isolated from these samples within 3 months.

***Total RNA isolation and reverse transcription.*** Fifty to one hundred milligrams of placenta specimen fragments were homogenized, and total RNA extracted after completing the protocol for TRIzol (Invitrogen Ltd, California, USA). Four μg of total RNA was reverse transcribed into cDNA after DNase treatment by using the Revert Aid™ Frist Strand cDNA Synthesis Kit (MBI, Vilnius, Lithuania), mixing 4μl reaction buffer, 20nM dNTP, 20 unit of RNAse inhibitor, 200 units of RT enzyme and 0.2μg random hexamer primers in a 20μl volume according to the manufacturers' instruction.

***Primers design and TaqMan probes.*** Sequences for all genes were derived from GenBank. All primers and probes were designed using the Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, USA) and were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co, Ltd, Shanghai, China (**Table 2**). Beta-actin was used as the housekeeping gene. The RT-PCR products were sequenced using the TaqMan primers to confirm that they were from the target gene.

***Quantitative TaqMan real-time RT-PCR.*** The OATP1A2, OATP1B1, MRP1, MRP2, AE2, BSEP, MDR3, and FIC1 mRNA were quantified with TaqMan probes. One μl of cDNA was amplified by PCR, using 75nM magnesium chloride, 9nM deoxyribonucleotide triphosphate (dNTP), 10pM gene specific forward and reverse primers, 1.5U Taq enzyme, 15.34 μl double distilled $H_2O$, and 10nM gene specific dual labeled TaqMan probe in a 30μl volume. The PCR was performed on the FTC-2000C-II Real-time FQ-PCR system (Funglyn Biotech Co. Ltd, Toronto, Canada) in 96-well plates with initial steps of 2 minutes at 94°C, and then 45 cycles of 20 seconds at 94°C, different seconds in annealing phase and 40 second extension phase at 60°C. The annealing temperature of OATP1A2 was 54°C, OATP1B1 -52°C, MRP1 - 60°C, MRP2 - 56°C, AE2 - 56°C, BSEP -54°C, MDR3 -54°C, FIC1 -58°C, and β-actin gene -52°C. The PCR amplification of each sample was performed in triplicate. Liver cDNA

was used as a positive control. A non-template control was also included for each run as a negative control.

***Statistical analysis.*** The Ct value for each sample was obtained and the relative gene expression for each sample was calculated using the 2(-Delta Delta C(T) method.[5] Data analysis was performed using SPSS version 11.0.1. Comparisons between the control group and ICP group were made using the 2-independent samples test. A value of $p<0.05$ was considered statistically significant.

**Results.** The clinical details of the subjects studied are described in **Table 1**. There were no significant differences in maternal age, gravidity, and parity between ICP and control group. The gestational age of the ICP group is a little shorter than control group ($p=0.014$). The OATP1A2, MRP2, AE2, MDR3, and FIC1 transcripts were detected in both normal third trimester and ICP placentas. The MRP1 transcript was detected in 18 of 20 normal third trimester, and 19 of 20 ICP placentas. The OATP1B1 and BSEP transcripts were not detected in either the normal or the ICP placentas (**Figures 1a & 1b**). Placental OATP1A2 expression in the ICP group was up regulated 1.38 times that of the control group (2.25±1.74 versus 1.63±2.16, $p=0.036$, 95% CI: 0.07-1.80), the AE2 expression was up regulated 1.72

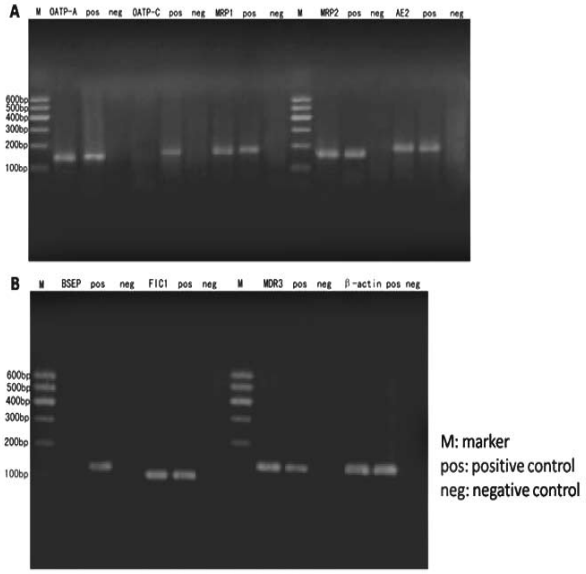


**Figure 1 -** The electropherogram of OATP1A2, OATP1B1, MRP1, MRP2, AE2, BSEP, MDR3, and FIC1 gene fragments after amplification showing: a) OATP1A2, OATP1B1, MRP1, MRP2 and AE2 gene fragments; b) BSEP, MDR3, and FIC1 gene fragments. OATP-A: OATP1A2; OATP-C: OATP1B1. The human liver expression of all the proteins is 100%.

**Table 1 -** Clinical parameters in control and ICP groups.

| Parameters | Normal pregnancy n=20 mean ±SD | ICP n=20 mean ±SD | *P*-value |
|---|---|---|---|
| Gestational age (weeks) at delivery | 38.33±1.69 | 37.16±1.47 | 0.014 (<0.05)* |
| Maternal age (year) | 29.50±4.02 | 29.35±3.57 | 0.678 (>0.05) |
| Gravidity | 2.45±1.43 | 2.10±1.07 | 0.664 (>0.05) |
| Parity | 1.30±0.57 | 1.25±0.44 | 0.565 (>0.05) |

*$p<0.05$. There were no significant difference in maternal age, gravidity, parity between intrahepatic cholestasis of pregnancy (ICP) and control group ($p>0.05$) The gestational age of ICP group is a little shorter than control group ($p<0.05$).

**Table 2 -** Sequence details of TaqMan primers and probe used for each gene.

| Gene name | Forward primer | Reverse primer | TaqMan probe |
|---|---|---|---|
| OATP1A2 | 5'-CCATTGGAACGGGAATAAAC-3' | 5'-TGCTCATCGCTGACAAGATT-3' | 5'-CAGGTCCTTTGTCGCACAGCC-3' |
| OATP1B1 | 5'-TTGCACTGGGTTTCCACTCAAT-3' | 5'-GACAAGCCCAAGTAGACCCTT-3' | 5'-TCCACCAACAACTGTGGCACAC-3' |
| MRP1 | 5'-CACACTGAATGGCATCACCTTC-3' | 5'-TCATTCTGAATCCAGGCCTGCT-3' | 5'-CCCTCCACTTTGTCCATCTCAGCC-3' |
| MRP2 | 5'-CACCATAAAGGACAACATCCTT-3' | 5'-AGGCTGATCCGCTGCTTCTG-3' | 5'-CTCTCCTCCCAGACTTGGAAATG-3' |
| AE2 | 5'-GAGGCCTTCTTCTCGTTCTG-3' | 5'-GTCTCATAGATGAAGATGAGTG-3' | 5'-CGGACCAGGAAGCTCCCCTC-3' |
| BSEP | 5'-GCCAGTTCTGTTCTCTACCAC-3' | 5'- TGTCAAATTGCTGTGGCAG-3' | 5'- CCTTGGCAGCTTGGACTATGTC-3' |
| MDR3 | 5'- CATTGGACACAGAAAGTGAAG-3' | 5'- CTTCCTGATGTCTGCATGTT-3' | 5'- CCTCAAACCCAGCGATGACATC-3' |
| FIC1 | 5'- TGGCAGGTTCAAAGTTGC-3' | 5'- GGTTTCTCCGTCCAGTTCTG-3' | 5'- CCAGCTGACATTCTCCTGCTGTC-3' |
| β-actin | 5'-GCCAACACAGTGCTGTCT-3' | 5'-AGGAGCAATGATCTTGATCTT-3' | 5'-ATCTCCTTCTGCATCCTGTC-3' |

OATP1A2 - OATP-A = organic anion transporting polypeptide-A; OATP1B1 - OATP-C = organic anion transporting polypeptide-C, MRP1 - multidrug resistance protein 1, MRP2 - multidrug resistance protein 2, AE2 - anion exchanger 2, BSEP - bile salt export pump, MDR3 - multidrug resistance 3, FIC1 - familial intrahepatic cholestasis-1

times (2.23±1.70 versus 1.30±0.89, $p$=0.038, 95% CI: 0.06-1.81), and the FIC1 expression in the ICP group was down regulated 1.65 times that of the control group (0.79±0.62 versus 1.30±0.93, $p$=0.049, 95% CI: -1.02-0.01). There was no significant difference in MRP1 (1.41±1.03 versus 1.40±0.97, p=0.975, 95% CI:-0.66-0.68), MRP2 (0.87±0.51 versus 1.29±0.83, $p$=0.062, 95% CI: -0.86-0.02) and MDR3 (1.54±1.75 versus 1.44±1.28, $p$=0.792, 95% CI: -0.86-1.12) gene expressions between the ICP and the control groups (**Figure 2**).

**Discussion.** Fetal liver can synthesize bile acid, but because it is immature, the hepatobiliary transporters are not detectable until shortly before birth.[6] Therefore, bile acid is transported from the fetal to the maternal circulation via placenta and eliminated by the maternal liver and kidney. Clinical and laboratory evidence suggested that poor fetal outcome in ICP may be related to increased bile acid concentration in the fetal circulation. As a result, the characterization of bile acid carriers in the placenta of ICP is needed. In this study, we have showed different mRNA expressions of genes that may influence bile acid transport in the placenta. This is the first study to explore various placental bile acid transport correlative proteins expressions in ICP. The OATP1A2 is a transporter responsible for transporting bile acids from blood to bile.[7] We assumed that the up-regulated OATP1A2 in ICP may be caused by its compensatory mechanism to release cholestasis of the fetus via transporting more bile acid to mother. The AE2 is an ATP-independent transporter, and previous studies showed that ICP induced an enhancement in efficiency of ATP-independent bile acid transport across the placenta.[8] The increased AE2 may facilitate placenta transport of bile acid from mother to fetus through the concentration gradient, which may induce or aggravate fetal cholestasis. The FIC1 deficiency induces the loss of canalicular phospholipid membrane asymmetry, which in turn may render it less resistant toward hydrophobic bile salt and impairs the function of the liver canalicular bile acid transporters.[9] Although the physiological role of FIC1 in the placental bile acid transport processes is not clear, it may be able to maintain membrane asymmetry.[10] On one hand, decreased FIC1 may impair the stable asymmetric distribution of phospholipids in trophoblast plasma membranes, which is required for normal function of the other bile transporters embedded therein. On the other hand, as an ATP-dependent transporter, reduced FIC1 may lead to less transshipment of bile acid from fetus to mother through the placenta. Thus, we assume that the reduced FIC1 expression in the placenta may interrupt the function of other bile acid transporters and be responsible for the fetal cholestasis in ICP. Increasing evidence shows that mutations in MDR3 are associated with ICP,[11] and we therefore initially assumed that MDR3 is the most probable bile acid transport correlative protein in placenta involved in the pathogenesis of ICP. However, the differences between the control and ICP placenta was not significant. Therefore, we suggested that MDR3 involvement in the pathogenesis of ICP may occur mainly through gene mutation instead of changes in its expression. Some researchers found that ursodeoxycholic acid-,[12] and dexamethasone-,[13] may induce the MRP2 expression in placenta or hepatocytes. Considering that some of our ICP placentas were obtained from the patients who accepted ursodeoxycholic acid or/and

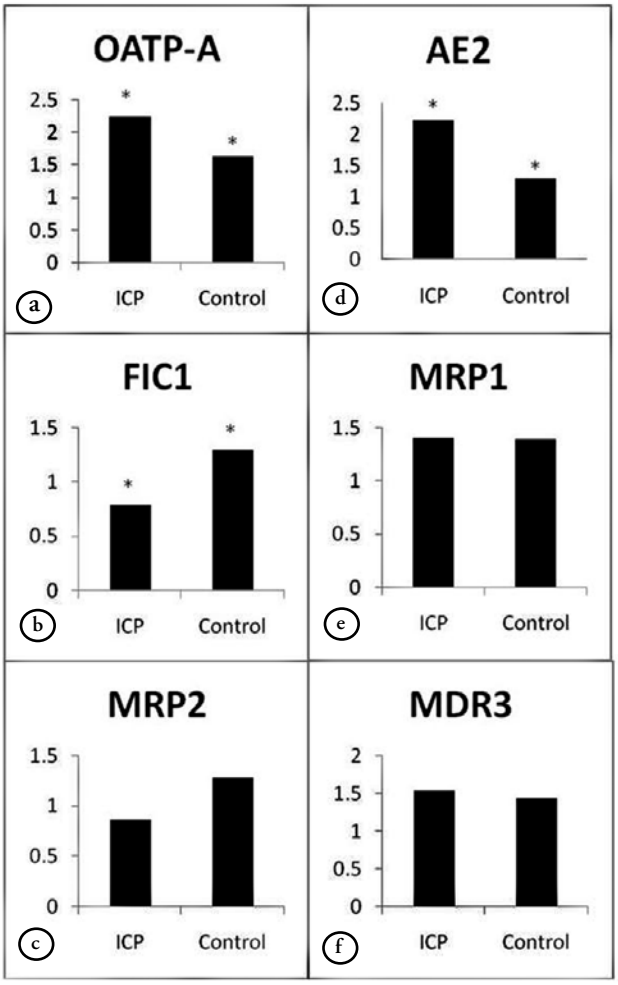


**Figure 2 -** The relative mRNA quantitation of **a)** OATP1A2, **b)** FIC1 **c)** MRP2, **d)** AE2, **e)** MRP1, and **f)** MDR3, in the ICP and control group. Placental OATP1A2 and AE2 expression in the ICP group was higher than that of the control group, while the FIC1 expression was lower than that of it. There was no significant difference in MRP1, MRP2, and MDR3 gene expressions between the ICP and the control groups. OATP-A: OATP1A2. Values represent the mean, *$p$<0.05.

dexamethasone administration before delivery, it is reasonable to assume that the drug may influence the results of our study. In addition, the small sample size of the study made it inappropriate to analyze data in the subgroup. Therefore, the exact expressions of MRP1 and MRP2 in ICP need further study. That OATP1B1 and BSEP mRNA may be too low to be detected suggested that they may not play a major role in the placental transport of bile acid or the pathophysiology of ICP.

In conclusion, we consider that the up-regulation of AE2, OATP1A2 and down-regulation of FIC1 in ICP placenta may participate in the pathogenic mechanism of fetal cholestasis or the compensatory mechanism of anti-cholestasis. The fetal cholestasis in ICP may be caused by the quantity or function alteration of bile acid transport correlative proteins. Nevertheless, the small sample size and some drug treatment may have influenced the results of our study. In addition, because bile acid transport correlative proteins are believed to exist on both the maternal and fetal sides of the placental trophoblast, it is necessary to conduct studies at the protein level to further investigate the role played by placental bile acid transport correlative proteins in the pathophysiology of ICP and their different functions on both sides of the maternal- fetal interface.

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