

# Analysis of mutations of *MDR3* exons 9 and 23 in infants with parenteral nutrition-associated cholestasis

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Received May 13, 2014; Accepted January 8, 2015

DOI: 10.3892/etm.2015.2800

**Abstract.** The aim of this study was to investigate mutations of multidrug resistance 3 (*MDR3*) exons 9 and 23 in infants with parenteral nutrition-associated cholestasis (PNAC). A total of 41 infants with PNAC were enrolled in the study. Genomic DNA was extracted from the peripheral venous blood leukocytes of each patient and *MDR3* exons 9 and 23 were amplified by polymerase chain reaction. One patient was identified who carried a frameshift mutation in *MDR3* exon 23 (C.2793) that was caused by the insertion of a single adenine residue, while mutations were not found in *MDR3* exon 23 in the other 40 patients. The clinical features of the patient with the *MDR3* exon 23 frameshift mutation included high serum  $\gamma$ -glutamyl transferase levels, the absence of biliary dilatation and deformity in magnetic resonance cholangiopancreatography, and abnormal electrical capacitance tomography imaging of the liver. No mutations in *MDR3* exon 9 were identified in any of the patients. All 41 PNAC patients recovered following oral ursodeoxycholic acid treatment. The C.2793 frameshift mutation in *MDR3* exon 23 is potentially associated with the development of PNAC in infants.

## Introduction

Since Dudrick *et al* first reported the application of parenteral nutrition (PN) in newborns in 1968 (1), the prognosis of preterm infants has markedly improved. However, in 1971, Peden *et al* reported the case of a premature infant who developed severe liver function damage owing to the application of total parenteral nutrition (TPN) (2). The autopsy of this patient revealed the presence of intrahepatic cholestasis, bile duct dilatation

and cirrhosis. Since then, parenteral nutrition-associated cholestasis (PNAC) in preterm infants has garnered increasing attention.

The pathogenesis of PNAC has not been elucidated until recently. PNAC is considered to be caused by a variety of factors, including premature birth, low birth weight, long PN duration, a lack of fasting, gastrointestinal irritation, infection, intestinal bacterial overgrowth, bacterial translocation, TPN solution nutrient imbalances, lack of trace elements and toxic ingredients in PN (3,4). These factors cause liver damage, degeneration, and fat deposition in the liver (5,6). In recent years, a number of studies have shown that *MDR3* mutations or the decreased expression or dysfunction of *MDR3* causes bile phospholipid deficiency, bile stone formation and the obstruction of small bile ducts, and may affect bile metabolism, thereby causing cholestasis (7,8). The purpose of the present study was to investigate whether mutations of *MDR3* exons 9 and 23 were present in infants with PNAC.

## Materials and methods

**Subjects.** A total of 41 infants with PNAC were enrolled in the study between June 2011 and December 2013. PNAC diagnostic criteria included: PN for >14 days, jaundice, a direct bilirubin level of >1.5 mg/dl, discolored stools, elevated liver enzymes, and the exclusion of biliary atresia, choledochal cysts, bile duct dilatation, surgery-induced disease, viral infection (hepatitis A, B or C and cytomegalovirus) and metabolic diseases (9).

Informed consent for participation in the study was received from all the subjects' guardians, and the protocol was approved by the ethics committee of the Zhongshan People's Hospital Affiliated to Sun Yat-Sen University (Zhongshan, China).

**Study design.** Blood cultures were routinely checked for cytomegaloviruses, syphilis and *Toxoplasma gondii*, and the blood levels of glucose, C-reactive protein and indicators of liver function [alanine aminotransferase (ALT), aspartate aminotransferase (AST),  $\gamma$ -glutamyl transferase ( $\gamma$ -GGT), total bilirubin, direct bilirubin and total bile acid] were measured weekly. Genomic DNA was extracted from the peripheral venous blood leukocytes collected from

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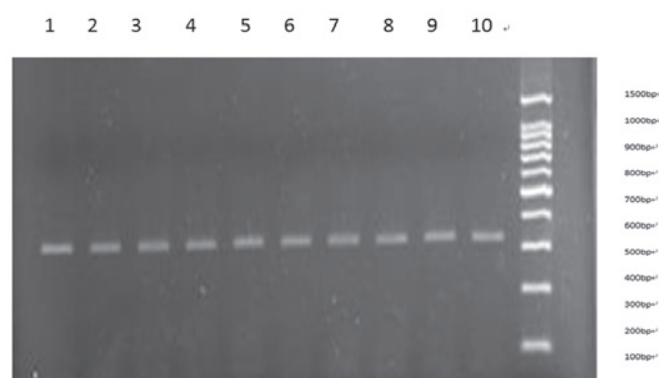
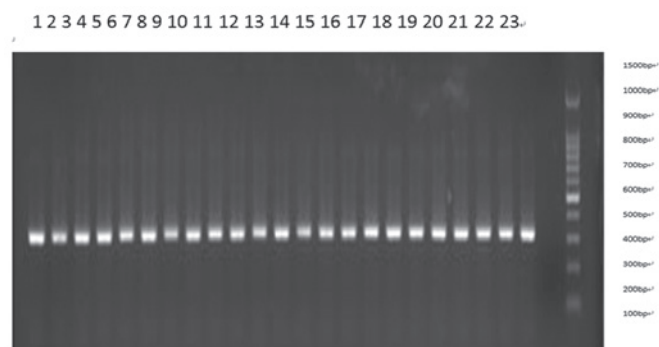
**Key words:** parenteral nutrition associated-cholestasis, multidrug resistance 3 gene, mutation, clinical features

Table I. Primers for *MDR3* gene exons 9 and 23, annealing temperature and amplified fragment length.

Exon	Primer direction	Primer sequence	Annealing temperature	Length of product
Exon 9	Forward	5'-GGGTTCATTACCTTGACTGAC-3'	58°C	433 bp
	Reverse	5'-CTGGACAGTGGAAAGATTAC-3'		
Exon 23	Forward	5'-AGCCGTGCTCTTTCCACT-3'	54°C	329 bp
	Reverse	5'-ATCCCTGACCTCATCTTTGG-3'		

each patient. Samples were stored at -80°C. Genomic DNA was extracted using the phenol-chloroform method (10). Polymerase chain reaction (PCR) was used to amplify *MDR3* exons 9 and 23. Mutations in *MDR3* exons 9 and 23 were detected using a restriction enzyme digestion method and by sequencing analysis using an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The DNA sequences of each patient were compared with the human genome sequence for *MDR3*. The patients were examined by electrical capacitance tomography (ECT) with the intravenous injection of Tc<sup>99m</sup>-ethyl hepatic iminodiacetic acid (EHIDA; 5 mCi), and also by magnetic resonance cholangiopancreatography (MRCP) and hepatobiliary ultrasound.

**Materials and genetic analysis.** DNAzol BD reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), isopropanol, 75% ethanol, QL-901 Vortex Shaker (Kylun-Bell Lab Instruments Co., Ltd, Haimen, China) and a high-speed centrifuge (Eppendorf, Hamburg, Germany) were used for the extraction of genomic DNA. The extracted DNA was examined and primers were synthesized by PCR-restriction fragment length polymorphism (PCR-RFLP), using a PCR machine (C1000; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers were designed using Primer 5 software (Premier Biosoft, Palo Alto, CA, USA). PCR was used to amplify exon 9 and 23 of the *MDR3* gene. Each PCR reaction mixture contained 45 µl Platinum PCR SuperMix (Invitrogen Life Technologies), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2 µl template DNA and ddH<sub>2</sub>O. The primers for *MDR3* exons 9 and 23 and their respective annealing temperatures are shown in Table I. PCR conditions included an initial denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 15 sec, and annealing at 72°C for 30 sec. The PCR reaction was terminated after an extension step at 72°C for 5 min. The PCR products were observed following 1% agarose electrophoresis (Bio-Rad Laboratories, Inc.) using an automatic Bio-Rad Gel Doc™ XR gel imaging system (Bio-Rad Laboratories, Inc.) and a DL2000 DNA marker (Takara Biotechnology Co., Ltd., Dalian, China). *MDR3* DNA was amplified by PCR (Bio-Rad Laboratories, Inc.), and the mutations of *MDR3* exons 9 and 23 were detected using the restriction enzyme (sex AI) (Bio-Rad Laboratories, Inc.) digestion method, using an ABI Prism 3100 sequencer (Applied Biosystems). For the DNA sequence analysis, the DNA sequence was compared with the human genome sequence of the *MDR3* gene in Genbank (<http://www.ncbi.nlm.nih.gov/Genbank>; accessed 01/06/2011). Gene muta-

Figure 1. Amplified fragments of *MDR3* gene exon 9. Lanes 1-9, amplified fragments of *MDR3* gene exon 9 of parenteral nutrition-associated cholestasis infants; M, DNA marker DL 2000.Figure 2. Amplified fragments of *MDR3* gene exon 23. Lanes 1-9, amplified fragments of *MDR3* gene exon 23 of parenteral nutrition-associated cholestasis infants; M, DNA marker DL 2000.

tions were analyzed using BioEdit protein contrast software obtained from [www.piaodown.com](http://www.piaodown.com) (accessed 30/08/2011).

## Results

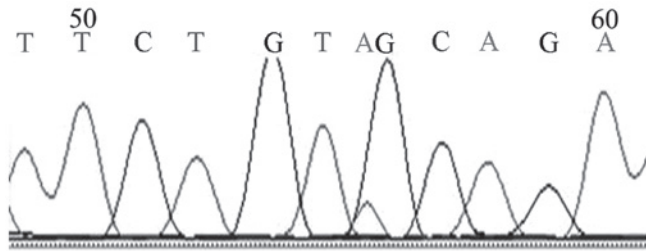
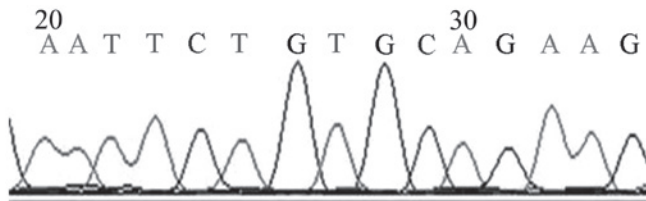
**Electrophoresis of PCR-amplified *MDR3* fragments.** The PCR-amplified fragments of *MDR3* exons 9 and 23 were clearly visible when electrophoresis was performed. The product lengths were as expected (Figs. 1 and 2).

**DNA sequencing.** DNA sequencing analysis revealed that there was only one patient with a frameshift mutation in *MDR3* exon 23. This mutation involved the insertion of an adenine residue at position 2,793 (C.2793 mutation; Fig. 3).

Table II. Comparison between the patient with a C.2793 frameshift mutation and the group without mutation.

Group	No. of cases	ALT (U/l)	T-BIL ( $\mu\text{mol/l}$ )	D-BIL ( $\mu\text{mol/l}$ )	TBA ( $\mu\text{mol/l}$ )	r-GGT (U/l)
With mutation	1	140	150.3	80.2	90.6	116
Without mutation	40	119 $\pm$ 19.8	126.2 $\pm$ 22.3	69.7 $\pm$ 10.3	73.9 $\pm$ 11.2	59 $\pm$ 5

The values of indicators of liver function for the without mutation group are presented as mean  $\pm$  standard deviation. ALT, alanine aminotransferase; T-BIL, total bilirubin; D-BIL, direct bilirubin; TBA, total bile acid; r-GTT, r- $\gamma$ -glutamyl transferase.

Figure 3. DNA sequencing of the *MDR3* gene exon 23 frameshift mutation (one case).Figure 4. DNA sequencing of the *MDR3* gene exon 23 with no mutation (40 cases).Figure 5. DNA sequencing of the *MDR3* gene exon 9 with no mutation (41 cases).

*MDR3* exon 23 mutations were not identified in the other 40 infants with PNAC (Fig. 4). No *MDR3* exon 9 mutations were identified in any of the 41 infants with PNAC that were included in this study (Fig. 5).

**Clinical features.** The levels of ALT, total bilirubin, direct bilirubin, total bile acid and r-GGT in the patient with the C.2793 frameshift mutation in *MDR3* exon 23 were higher than those in the other patients who did not carry the mutation (Table II).

When the patient with the mutation was examined by ECT, the position of the liver was normal and the distribution of radioactivity in the liver was sparse. The results showed that there was no development in the gallbladder, biliary tract and

intestines, whereas there was obvious development in the kidney and bladder. Hepatobiliary dynamic ECT imaging indicated that the excretion function of the liver was abnormal (Fig. 6). MRCP revealed that the morphology, size and lobes of the liver were normal. No abnormal signals were observed in the liver. Dilatation of the intrahepatic and extrahepatic bile ducts and the pancreatic duct was not observed. The gall bladder was normal and no signs of biliary calculi were present. The size and morphology of the pancreas and spleen were normal (Fig. 7). Hepatobiliary ultrasound showed that the size of the liver was normal and no biliary dilatation and deformity were observed in the patient with the *MDR3* exon 23 frameshift mutation (Fig. 8). All 41 PNAC patients recovered following oral treatment with ursodeoxycholic acid (10 mg/kg/day). The course of treatment was  $\sim$ 125 $\pm$ 12 days.

## Discussion

With the development of neonatology, the survival rate of premature infants has increased significantly. TPN has played an important role in this improvement (11); however, with the wide application of PN, the prevalence of PNAC in infants has increased. Severely affected patients may develop malnutrition, biliary cirrhosis and liver failure, which may even result in mortality (12-14). With the development of genomics, mounting evidence indicates that genetic factors such as *MDR3* mutations are important in the pathogenesis of intrahepatic cholestasis, including low phospholipid-associated cholelithiasis (14-16), progressive familial intrahepatic cholestasis type 3 (17-19), intrahepatic cholestasis of pregnancy (20-22), fibrosing cholestatic liver disease (23) and sclerosing cholangitis (24).

The formation and excretion of bile involves bile transport to basolateral hepatocytes and across biliary duct membranes. The most important aspect of bile dynamics is bile acid secretion from the blood into the bile duct. The active transport of bile acid within the liver and of soluble substances across canalicular hepatocyte membranes are the rate-limiting steps in bile formation. Active transport requires several ATP export pump proteins [ATP-binding-cassette (ABC)-transport proteins]. Bile salt transport is mediated by the bile salt export pump. The multidrug resistance phospholipid glycoprotein *MDR3* is a specialized ABC-transporter that serves as the primary phospholipid transporter through the tubular membranes (25-27).

ATP-binding cassette subfamily B member 4 (ABCB4) transporters have phosphatidylcholine floppase activity. They translocate phosphatidylcholine from the inner to the



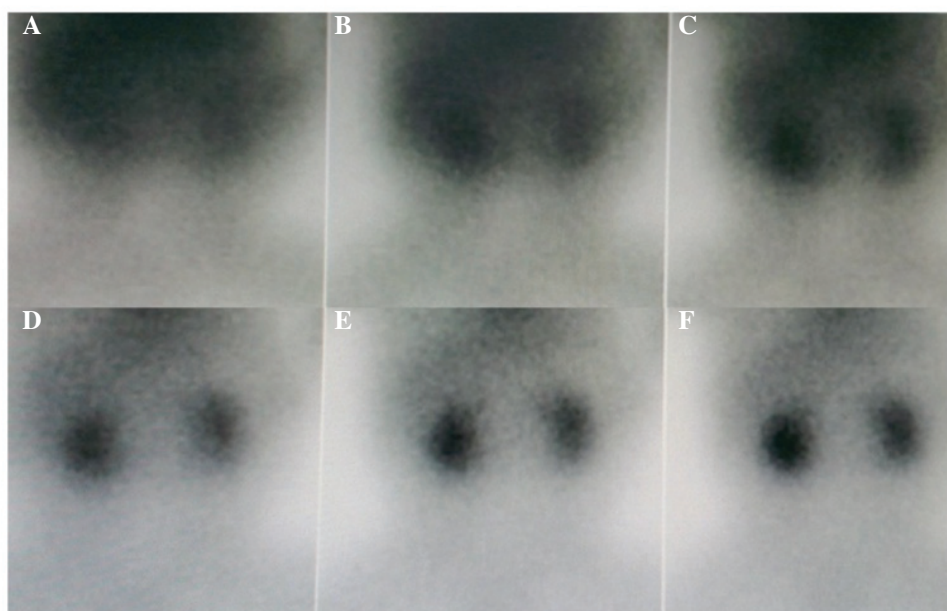


Figure 6. Electrical capacitance tomography imaging in the case with a C.2793 frameshift mutation in exon 23 of *MDR3* at (A) 30 min, (B) 1 h, (C) 2 h, (D) 4 h, (E) 6 h and (F) 24 h after intravenous Tc99m-EHIDA 5mCi.

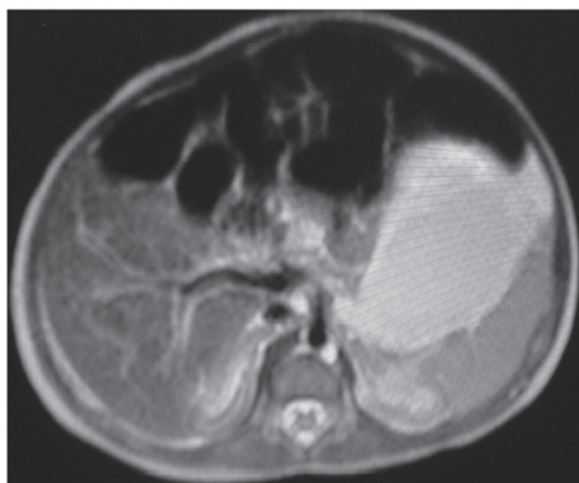


Figure 7. Magnetic resonance cholangiopancreatography (MRCP) imaging in the case with a C.2793 frameshift mutation in exon 23 of *MDR3*. MRCP revealed normal morphology, size and lobes of the liver, as well as no abnormal signals in the liver and no dilatation of the bile ducts.

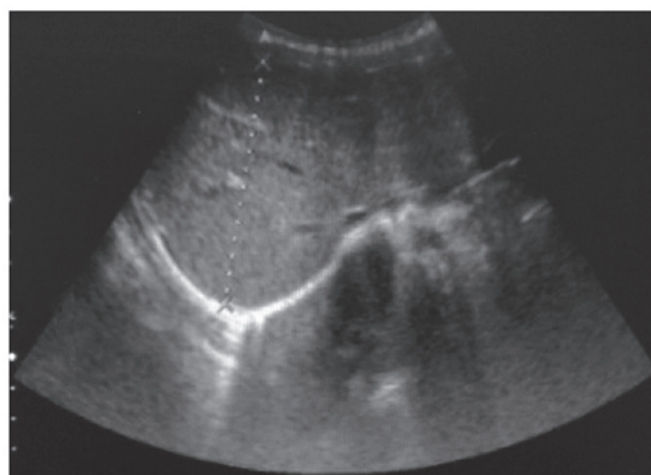


Figure 8. Hepatobiliary ultrasound in the case with a C. 2793 frameshift mutation in exon 23 of *MDR3*. Ultrasound showed normal size of the liver, no biliary dilatation or deformity.

outer leaflet of the canalicular membrane of hepatocytes, which renders phosphatidylcholine available for extraction into the canalicular lumen by bile salts. The role of *ABCB4* (MIM 171060) in this process is crucial, as demonstrated by the observation that its deficiency causes cholestatic liver diseases (28). The *ABCB4* gene, also known as *MDR3*, encodes a member of the MDR/TAP subfamily that is associated with multidrug resistance and antigen presentation. In humans, *ABCB4* is located on chromosome 7q21.1, contains 27 coding exons and spans ~74 kb (29). The pathophysiology associated with *ABCB4* alterations relates to the lack of phospholipid protection from the detergent effect of bile salts, which results in damage to the biliary epithelium, bile ductular proliferation, and potential progressive portal fibrosis. Since

the solubilization of biliary cholesterol depends on not only the concentration of the sterol, but also on the concentration of bile salt and phospholipid, a reduction in the rate of phospholipid excretion can also be a cause of gallstone formation. The reduced expression of *MDR3* reduces lecithin secretion and elevates vesicle cholesterol. It can also lead to bile duct damage, gallstone deposition, inflammation and biliary liver lesions (30-32).

The wide clinical spectrum of *ABCB4*-deficiency syndromes in humans encompasses cholestatic disorders, presenting from the neonatal period of life to late adulthood (8). In the present study, DNA sequencing analysis of *MDR3* exons 9 and 23 in 41 infants with PNAC identified only one patient who possessed a frameshift mutation in *MDR3* exon 23. This mutation was characterized by the insertion of a single adenine at position

2,793. *MDR3* exon 23 mutations were not identified in the other 40 infants with PNAC. All 41 patients recovered following oral ursodeoxycholic acid treatment. The clinical features of the patient with the *MDR3* exon 23 frameshift included high serum r-GT levels, the absence of biliary dilatation and deformity in MRCP, and abnormal ECT imaging of the liver. In the 41 PNAC patients, no *MDR3* exon 9 mutations were found. The frameshift mutation caused by the insertion of an adenine residue at position 2,793 may be associated with the pathogenesis of PNAC in infants. However, the majority of infants with PNAC in this study did not carry a mutation in *MDR3* exon 23. Therefore, future studies are required to confirm the correlation between this frameshift mutation in *MDR3* exon 23 and the incidence of PNAC in infants.

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