ONLINE MUTATION REPORT

A second heterozygous *MDR3* nonsense mutation associated with intrahepatic cholestasis of pregnancy

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Intrahepatic cholestasis of pregnancy (ICP, 147480) is a liver disorder unique to pregnancy, which usually manifests during late pregnancy and disappears spontaneously after delivery. Maternal symptoms are characterised by generalised pruritus with or without jaundice. The main biological features are increased serum alanine transaminase (ALT) activities and serum bile acid concentrations.[1–3] Serum gamma-glutamyl transpeptidase (GGT) activity remains within normal limits or is increased.[4 5] The occurrence of ICP carries a risk for the baby because of premature spontaneous delivery or sudden fetal death and pruritus may cause considerable discomfort for the mother.[1–3 6] Cholestasis frequently recurs in subsequent pregnancies and rarely during administration of oral contraceptives.[5] The cause of ICP is unknown. However, clinical and epidemiological studies suggest mainly hormonal and genetic factors.[1–3 7] Genetic factors have been suggested by the existence of familial cases and by the high incidence of ICP in some ethnic groups, such as the Araucanos Indians of Chile.[8] The multidrug resistance 3 (*MDR3*, *ABCB4*, 171060) gene, localised on 7q21.1, was first reported to be involved in ICP by de Vree *et al*.[9] In a large consanguineous family, subjects affected by a subtype of progressive familial intrahepatic cholestasis called PFIC3 were homozygous for a nonsense mutation in exon 23 (R957X) and women affected by ICP were heterozygous; in another family, the same authors reported a homozygous deletion of exon 6 (426-432del) in a PFIC3 patient with consanguineous, healthy parents.[9] A mutation in exon 14 of *MDR3* (1744delT) was identified in another large pedigree in which all PFIC patients were homozygous for the mutation and women with ICP were heterozygous.[10 11] Dixon *et al*[12] investigated eight women affected by ICP with increased serum GGT activity and no familial history of PFIC.[12] A missense mutation of exon 14 (A546D) was found in one patient. Recently, Rosmorduc *et al*[13] reported six adult patients with a peculiar form of cholelithiasis associated with *MDR3* gene mutations.[13] These patients suffered from mild chronic cholestasis with symptoms like biliary colic, pancreatitis, or cholangitis which recurred after cholecystectomy but which improved after ursodeoxycholic acid therapy. Among these six patients, three women showed symptoms during pregnancy. Two of them, from independent and non-consanguineous families, had a homozygous missense mutation in exon 9 (S320F) and the third woman had a heterozygous 1 bp insertion in exon 12 (1327insA), with an early stop codon.

The aim of this study was to search for *MDR3* mutations in these five exons among a group of women suffering from ICP.

Key points

- Intrahepatic cholestasis of pregnancy (ICP) is a liver disease unique to pregnancy characterised by maternal pruritus and abnormal liver function tests. The main risks for pregnancy are prematurity or sudden intrauterine death.
- Although the cause is unknown, genetic and hormonal factors are clearly associated with ICP. Recently, heterozygous mutations of the *MDR3* gene have been reported in affected women. The aim of this study was to search for mutations in five exons of *MDR3* in 20 women suffering from ICP.
- A heterozygous mutation in codon 144 (C→T) which introduces a stop codon (TGA) in exon 6 of *MDR3* was found in one patient. This new mutation confirms the role of *MDR3* in the pathogenesis of ICP.

MATERIALS AND METHODS

Patients

Twenty patients with ICP referred for hepatological consultation were studied. The criteria for the diagnosis of ICP were: (1) pruritus, (2) increased serum total bile acid concentrations and/or serum ALT activities, (3) absence of current viral infection (viral hepatitis, cytomegalovirus, or Epstein-Barr virus), (4) absence of biliary tract dilatation on ultrasound examination, (5) disappearance of pruritus and normalisation of liver function tests after delivery. Blood samples for this study and for the routine liver function tests were taken at the same time after obtaining informed consent.

The highest values for serum GGT activity before delivery (mean (SD)) were 33.8 (27.8) IU/l with a range of 11-134 IU/l (normal value in non-pregnant women ⩽15). Serum GGT values were normal in four patients out of 20. Controls were 100 DNA samples from unrelated women from the general population with a mean age of 30 years (range 22 to 40 years).

Mutation detection

Genomic DNA was extracted from peripheral venous blood leucocytes by standard procedures. We searched for mutations in exons 6, 9, 12, 14, and 23 of *MDR3*. Amplification reactions (PCR) were carried out in a final volume of 25 µl, using 100 ng genomic DNA in 50 mmol/l KCl, 10 mmol/l Tris-HCl, 1.5 mmol/l $MgCl_2$, 200 µmol/l of each dNTP, 0.01% gelatin, 1.5 IU *Taq* polymerase (Promega), and 20 to 50 pmol of each primer with specific sequences determined from the exon-intron boundaries of *MDR3* (contigs AC006154 and AC005045). Primer sequences are available from the authors (CG and CA). For SSCP analysis, 4 µl of the PCR products were diluted 1:1 with 95% formamide, 0.025% bromophenol blue, and 0.025% xylene cyanol, then heated at 95°C for five minutes and placed on ice. Denatured samples were loaded on a non-denaturing 15% acrylamide precast minigel (GeneGel Clean 15/24 from Amersham Pharmacia Biotech) and electrophoresis was performed at three different temperatures (10, 15, and 20°C) on GenePhor Electrophoresis Unit (Amersham Pharmacia Biotech) with a prerun for 10 minutes (200 V/12 mA) and a run for 1.5 hours (600 V/15 mA). The gels were silver stained with DNA Silver Staining Kit Plus One (Amersham Pharmacia

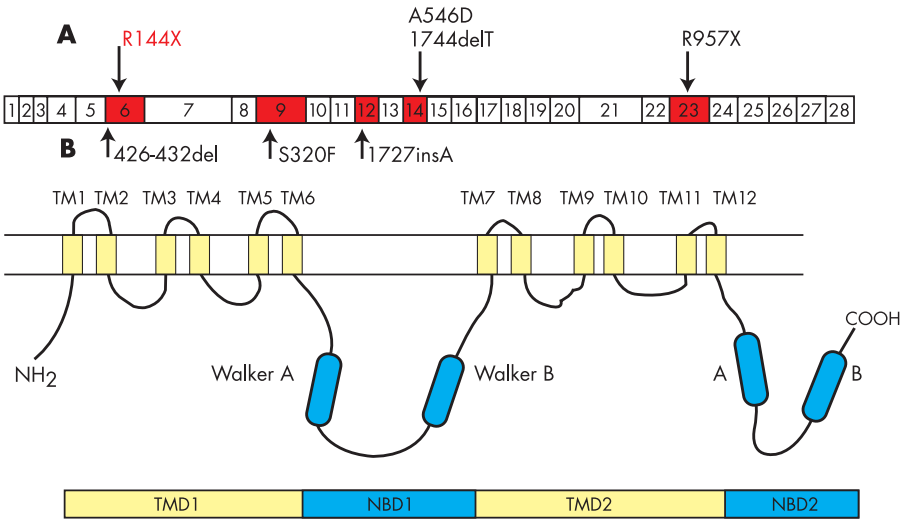

Figure 1 Intrahepatic cholestasis of pregnancy and *MDR3* mutations. In the red boxes, the five exons explored in the present study with (A) the localisation of the four mutations found in ICP, R144X (this report), 1744delT,[11] A546D,[12] R957X,[9] and (B) the localisation of 426-432del,[9] the first mutation found in a PFIC3 patient and the localisation of S320F and 1327insA found in women with cholestasis of pregnancy associated with chronic cholestatic liver disease.[13] Protein domains are shown in yellow boxes for the six segments of each transmembrane domain (TMD1 and TMD2) and in blue boxes for the two nucleotide binding domains (NBD1 and NBD2).

Biotech), according to the instructions supplied by the manufacturer, and air dried for 24 hours.

PCR products corresponding to SSCP positive samples were purified on agarose, then cycle sequenced with the ABI prism BigDye Terminator Cycle Sequencing Ready Reaction kit on a GeneAmp PCR System 9600 (PE Applied Biosystems). The purified products were run on an ABI Prism 377 sequencer (PE Applied Biosystems). The sequences obtained were compared to normal controls.

RESULTS

Among the 20 patients with ICP, two patients exhibited bands with altered mobility for exon 6. In the first patient, the sequence analysis showed a heterozygous substitution (CGA-TGA) in codon 144, which creates a stop codon (R144X) (fig 1). This patient was a 23 year old primiparous woman who suffered from pruritus from 26 weeks' gestation. The highest values for liver function tests during the course of ICP were: total bilirubin 36 µmol/l, serum ALT activity 1280 IU/l (normal ⩽35), serum total bile acid concentration 120.9 µmol/l (normal ⩽6), serum GGT activity 63 IU/l (normal ⩽15). Labour was induced at 36 weeks' gestation and a normal male infant was delivered weighing 2620 g. The pruritus disappeared one day after delivery. Four weeks after delivery serum ALT activity and total bile acid concentration were within normal limits (ALT 21 IU/l, serum total bile acid concentration 0.7 µmol/l) and serum GGT activity was 17 IU/l. The R144X mutation destroys a *Sal*I restriction site and we used a PCR restriction test to search for this mutation in the control group. No R144X mutation was detected in any of the 100 DNA samples.

In a second patient, a T/C substitution was observed at nucleotide 536, leading to a silent mutation (AAT>AAC, N168).

DISCUSSION

This study showed a new mutation in exon 6 of *MDR3* in a woman suffering from ICP. This R144X mutation is the most proximal truncating mutation reported in this disease, unique to pregnancy (fig 1). Codon 144 codes for an amino acid localised in the proximal part of the first hydrophobic transmembrane domain (TMD1). We can predict that this stop codon leads to a peptide missing the functional domains of MDR3. By contrast, we did not find the six mutations of *MDR3* already described in exons 6, 9, 12, 14, and 23 in patients suffering from ICP, PFIC, or cholestasis during pregnancy associated with chronic liver disease.[9–13]

The role of MDR3 is essential for the secretion of phosphatidylcholine into bile, as has been shown for its close murine homologue Mdr2.[15] The Mdr2 knockout mouse is completely unable to secrete phosphatidylcholine into bile and develops mild liver disease, since the augmentation of free biliary bile acids alters the canalicular membrane of the hepatocyte.[14 15] In patients with PFIC3, human bile salts are very hydrophobic and the absence of human phosphatidylcholine translocater coded by *MDR3* results in serious liver disease.[16]

The apparent heterozygous status of our patient is in agreement with previous studies in consanguineous families.[9 11] In these studies, women affected by ICP were heterozygous for the familial mutation, whereas patients affected by PFIC3 were homozygous.[9 10] Moreover, a homozygous state for the missense mutation S320F was observed in two women suffering from chronic liver disease with clinical and biological features outside pregnancy.[13]

The discovery of these mutations in *MDR3* opens the way for a better understanding of the pathogenesis of ICP.

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