Intrahepatic cholestasis of pregnancy (ICP), also called obstetric cholestasis, is a liver disorder that affects 0.5–0.7% of all pregnancies in the UK and is characterised by pruritus and raised serum bile acid levels. It can lead to prematurity, fetal distress, and intrauterine death. There is a familial component to the disease with parous sisters of affected patients having a 12-fold increased risk of developing ICP. The course and severity of the disease is variable and its genetic aetiology is likely to be complex.

Candidate genes for ICP include those that are mutated in different types of inherited cholestasis: progressive familial intrahepatic cholestasis types 1–3 (PFIC1–3) and benign recurrent intrahepatic cholestasis (BRIC). Homozygous inactivating mutations of the ATP8B1 (or FIC1) gene, which encodes a P-type ATPase, have been identified in PFIC1 and BRIC. PFCI2 and PFPC3 have been shown to be caused by homozygous inactivating mutations in the bile salt export pump (ABCB11/BSEP) and the multidrug resistance 3 (ABCB4/MDR3) genes, respectively. Both of these molecules are members of the ATP binding cassette (ABC) family of transmembrane proteins that play a role in a variety of transport functions in many organisms.

To date, mutations in ICP have been demonstrated in the ABCB4 gene. Children with PFIC3 have raised serum levels of gamma-glutamyl transpeptidase (γ-GT), and a small proportion of heterozygous mothers develop cholestasis of pregnancy. Homozygous alterations within ATP8B1 cause either PFIC1 or BRIC, depending on the localisation and functional effect of the variation. Clinical studies have shown that both diseases can coincide with ICP in a minority of cases. These data suggest that variations in the ATP8B1 gene may influence susceptibility to ICP. However, multipoint linkage analysis and haplotype segregation studies performed in two Finnish pedigrees suggest that the FIC1 locus is not implicated in women from these families. Also, no ATP8B1 mutations have been reported in ICP cases of any ethnic origin to date.

We hypothesised that alterations in the human ATP8B1 gene influence susceptibility to ICP. To assess the contribution of ATP8B1 gene variants to ICP, we sequenced the complete coding region from 16 women with ICP and normal γ-GT levels and subsequently examined a cohort of 182 ICP cases for the presence of variants leading to amino acid alterations.

Clinical (in vivo) phosphorus-31 magnetic resonance spectroscopy (31P MRS) is a non-invasive technique which can be used to provide direct localised biochemical information on hepatic metabolic processes. We hypothesised that the presence of ATP8B1 gene variants in women with ICP would be associated with metabolite abnormalities in the liver and we therefore performed in vivo hepatic 31P MRS in two women with the 208G>A mutation in ATP8B1 and in another six ICP cases that did not have a coding mutation.

**METHODS**

The study conformed to the guidelines outlined by the 1975 Declaration of Helsinki and permission was obtained from the ethics committees of the Hammersmith Hospitals NHS

**Abbreviations:** ICP, intrahepatic cholestasis of pregnancy; PFIC, progressive familial intrahepatic cholestasis; BRIC, benign recurrent intrahepatic cholestasis; MRS, magnetic resonance spectroscopy; γ-GT, gamma-glutamyl transpeptidase; NTP, nucleotide or nucleoside triphosphate; PME, phosphomonoester; PDE, phosphodiester; GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; ABC, ATP binding cassette; PCR, polymerase chain reaction; IQR, interquartile range
Trust, London (REC 93/4047 and 97/5197). Written informed consent was obtained from each patient.

ICP was diagnosed in pregnant women with pruritus who had no rash apart from dermatitis artefacta, and confirmation of the diagnosis was made with raised serum liver transaminases and/or bile acids. Women were excluded if another hepatic disorder was diagnosed following identification of abnormal hepatitis serology (hepatitis A, B, and C), raised antimitochondrial antibodies, or biliary obstruction following ultrasound examination. Women were also excluded from the analysis if hepatic impairment did not resolve postnatally, with the exception of cyclical and exogenous oestrogen induced cholestasis. As hospitals have different normal ranges for liver transaminase levels, the upper end of the normal range in pregnancy was assumed to be 80% of the level quoted outside pregnancy for each hospital, consistent with published studies, and any values above this were considered to be abnormal. The normal range for bile acids was \(14\) \(\mu\)mol/l. In addition to the 16 ICP cases that were sequenced, \(\gamma\)-GT was not raised in 26 further cases. \(\gamma\)-GT was raised in 34 cases and had not been measured in the remaining cases.

Screening for novel variants in ATP8B1 in 182 cases

Using the diagnostic criteria described above, 182 women with ICP were identified. All coding exons of \(FIC1\) were sequenced in 15 Caucasian cases and one Caucasian/Asian mixed race case without raised \(\gamma\)-GT (table 1). The frequency of \(FIC1\) variants was then established in the additional 166 women with ICP. The majority (158) of these cases were Caucasian. The frequency of variants in a control population was investigated using DNA extracted from the blood of 120 parous women of Caucasian ethnicity who were recruited prospectively from the postnatal wards at Queen Charlotte’s hospital. They had no history of ICP or any other known hepatic disorder.

DNA was extracted from peripheral blood leucocytes as previously described and the 27 coding exons of \(ATP8B1\) were amplified using specific primers (sequence available on request). Polymerase chain reaction (PCR) products were purified using polyethylene glycol precipitation and sequenced using fluorescent dideoxy chain terminator chemistry following the manufacturer’s instructions (Big Dye version 3; Perkin-Elmer, Foster City, California, USA). The forward and reverse strands for each exon in each patient

<table>
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<th>Family history of ICP</th>
<th>Cyclical or OCP pruritus</th>
<th>Preg No</th>
<th>ICP in this preg</th>
<th>Max ALT* (&gt;20 U/l)</th>
<th>Max BA (&lt;14 (\mu)mol/l)</th>
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Table 1 Clinical features of 16 intrahepatic cholestasis of pregnancy (ICP) cases in whom all coding exons of \(ATP8B1\) were sequenced, and of two additional cases with the D70N variant

C, cyclical itch; OCP, oral contraceptive pill; ALT, alanine aminotransferase; BA, bile acid; M, meconium stained liquor; CTG, cardiotocograph abnormalities; IUD, intrauterine death; Y, yes; N, no; Preg, pregnancy; –, test was not performed or the result is not available.

*For some cases ALT was not available, and the aspartate aminotransferase level is given instead. The maximum value for ALT, AST, and BA is given for each affected pregnancy in each case.

†ICP cases with the D70N mutation
†‡ICP case with the R867C mutation
were analysed for heterozygous variations by visual inspection by two separate individuals (RM and NT), potential variants were confirmed by inspection of the reverse strand and, where possible, restriction enzyme digest from independent PCR amplifications using the same patient’s template DNA.

Confirmed exonic alterations were screened in the complete patient cohort using either restriction enzyme digestion where restriction sites were created or destroyed by the variant, or PCR fragment analysis using a denaturing high pressure liquid chromatography based assay (WAVE instrument; Transgenic, Crewe, UK).

For restriction analysis, 10 μl PCR reaction from the relevant exon was digested in a final volume of 15 μl containing 1 unit restriction endonuclease according to the manufacturer’s instructions (New England Biolabs, Hitchin, UK) and the products were separated on a 3% agarose gel.

For heteroduplex analysis, 10 μl PCR reactions were made up to a final volume of 20 μl by adding water, denatured for two minutes at 95°C, then cooled to room temperature at a rate of 1°C per minute, and stored on ice until further use. Optimum conditions for distinguishing hetero- from homozygous fragments were calculated using the “WAVE maker” software (Transgenic) and verified by running a known heterozygous product.

**Screening of 182 ICP cases for ATP8B1 variants previously reported in PFIC and BRIC**

To analyse the cohort for the presence of variations previously published in patients with PFIC1 and BRIC, exons 9, 15, 17, 20, 21, and 22 were sequenced in 182 ICP cases.

Exon 9 was screened for 923G→T (G308V) and 863T→C (L288S) found in PFIC1 patients of Amish or Polish origin.7 Exon 15 was screened for 1660G→A (D554N), described in Greenland cholestasis patients.26 Exon 17 was screened for 2097+2T→C (which leads to skipping of an exon and del645–699 at the protein level) described in a PFIC1 patient and 1982T→C (H661T) described in BRIC patients from 13 families of Western European origin.7 This mutation was shown to be present in 79% of BRIC cases with detectable ATP8B1 alterations.7 Exon 20 was screened for 2287-4CT→AA and 2304delGAAAACCGTG (795delGNR), described in a Dutch BRIC patient.7

Exon 21 was sequenced to screen for 2674G→A (G892R) described in a PFIC1 patient of European descent.7 Screening was not performed to identify additional mutations reported in Taiwanese and Japanese patients with low γ-GT cholestasis because none of the ICP patients in this study were from these ethnic groups.

**31P-MRS**

Two women with the D70N mutation and six additional ICP cases who did not have D70N or R867C were selected for in vivo hepatic 31P MRS. None was pregnant or was using hormonal contraception and none was overweight (body mass index <24 kg/m²). All eight provided written informed consent and were studied as close as possible to day 18 of the menstrual cycle (that is, days 16–20) to control for theoretical cyclical effects on hepatic metabolism and cholestasis, although no cyclical effects on 31P MR spectra have ever been noted in controls.22,23 In vivo hepatic 31P MR spectra were obtained on a 1.5 T Eclipse MR scanner (Philips, Cleveland, Ohio, USA), using a surface receive coil, dual tuned for 31P and proton (1H) operation. The 1H signal was used for shimming and to obtain a T1 weighted axial image (TR 800 ms, TE 16 ms, and 4 signal averages) for spectral localisation purposes. The 31P MR spectra were localised to the liver using an ISIS sequence voxel size 70×70×70 mm, TR = 10 000 ms, TE = 2 ms, and 64 signal averages.

**RESULTS**

**ATP8B1 gene variants identified in ICP cases**

Three exonic ATP8B1 variants were identified following automated DNA sequencing of 16 ICP cases. A heterozygous (208G→A) transition that results in the replacement of an aspartic acid by an asparagine at residue 70 of the protein (D70N) was found in exon 2 in one patient. This transition destroys a PvuI restriction site. This variant was found in two more Caucasian cases when the additional 166 ICP cases were assayed and it was not found in 120 healthy pregnant controls.
A heterozygous transition (2599G>T) that results in an arginine being replaced by a cysteine at residue 867 (R867C) was found in exon 21 in one patient. It destroys an Aci I site. This variant was not found in any additional cases when the remaining 166 were assayed, or in 120 healthy pregnant controls.

A heterozygous (2855G>A) transition that results in the replacement of an arginine by a glutamine at residue 952 (R952Q) was found in exon 22 in two out of the 16 patients that were sequenced. Heterozygotes were detected at similar frequencies in patients (31/364 chromosomes) and healthy control pregnancies (19/240 chromosomes). As homozygous variants of either “A” or “G” at position 2855 are not detected by the monoplex WAVE analysis performed, exon 22 was sequenced in the 166 additional cases to establish the frequency of “A” homozygotes. None was detected.

Screening of 182 ICP cases for variants previously reported in PFIC and BRIC

None of the variations from exons 9, 15, 17, 20, 21, or 22 that cause PFIC1 or BRIC in homozygous or compound heterozygous form was detected among 182 individuals with ICP. An additional ATP8B1 variant was identified in one ICP case following sequencing of exon 9. This heterozygous T>A transition (913T>A) results in the substitution of a phenylalanine by an isoleucine (F305I) and was found in one patient when screening the whole cohort of 182 for the presence of two mutations described in PFIC1 in exon 9. This variant creates an EcoRV site. It was detected in one of 120 healthy control pregnancies.

31P MRS

No structural hepatic or bile duct abnormality was seen on MRI in any of the ICP cases. Compared with the mean values for normal volunteers, ICP cases had a significantly increased %PDE signal (median 41.15 (interquartile range (IQR) 5.90)) compared with controls (median 37.06 (IQR 6.49)) (Mann Whitney U test, p = 0.03) but there was no difference in the %βNTP signal (fig 2). These proportions were calculated with respect to the total 31P MR signal. The PME/PDE value was reduced in ICP cases (median 0.12 (IQR 0.07)) compared with controls (median 0.17 (IQR 0.07); Mann Whitney U test, p = 0.04) However, in both patients with the D70N mutation the %βNTP signal was lower than in the other ICP cases (that is, 12.55 and 12.56).

DISCUSSION

We identified two ATP8B1 mutations that resulted in an amino acid exchange (D70N and R867C) following DNA sequencing of 16 ICP cases. One variant was present in three of 182 ICP cases and the other was present in one case. Neither mutation was present in 120 parous controls. Thus it is possible that these variants play a role in the aetiology of ICP.

A recent report on ATP8B1 mutations in families with PFIC1 and BRIC revealed the presence of D70N as a compound heterozygote in combination with another missense mutation (1799G>A resulting in R600Q).19 It is possible that heterozygosity for D70N causes ICP without other features of BRIC. Alternatively, the women with D70N in the present study may have another abnormality in ATP8B1 that was not identified by sequencing the coding exons, or another ICP causing variant in a different gene. The most common ATP8B1 variant in BRIC, I661T, found in 79% of all BRIC cases with detectable ATP8B1 alterations,19 was not found in any of the ICP cases in this study, indicating that this BRIC associated variant did not cause ICP in the UK cases presented in this study. However, the data reported in our study cannot exclude I661T from having a role in the aetiology of ICP.

The function of FIC1, the ATP8B1 gene product, has not been established, nor has the mechanism by which variations in ATP8B1 result in cholestasis. It has been hypothesised that FIC1 is an aminophospholipid translocase which translocates phosphatidylserine from the outer to the inner leaflet of the canalicular plasma membrane.17 If this hypothesis is correct, this process could maintain the stable asymmetric distribution of phospholipids which is required for normal function of the transporters embedded therein. Thus a defect in FIC1 activity could influence the function of bile acid transporters without the molecule itself being involved in bile acid transport. Alternative functions for FIC1 have been proposed (for example, as a direct bile acid transporter or as a metal ion transporter).18 FIC1 is highly expressed in the small intestine and it has been proposed that mutations in PFIC1 patients may result in...
ATP8B1 mutations in intrahepatic cholestasis of pregnancy

It has been shown that complete absence of ATP8B1 mRNA in the ileum of patients with PFIC leads to substantial downregulation of FXR, a nuclear receptor involved in the regulation of bile acid metabolism, and this is another possible mechanism that results in cholestasis. However, abnormal intestinal bile acid absorption alone is unlikely to explain why ATP8B1 mutations cause cholestasis in pregnancy as the main bile acids that are raised in ICP are primary bile acids.

The subfamily of P-type ATPases to which FIC1 belongs includes at least 10 more members based on similarities in the protein sequence and positioning of the functional domains. Evidence for a discrete function for FIC1 within this subfamily of P-type ATPases comes from the observation that its expression pattern is different, as it is one of the few family members not expressed in the brain. Also, FIC1 is the only member of the family which has to date been identified as causing disease.

Residue R867 is localised within one of the "signature" motifs used to identify this subfamily of sequences. These diagnostic sequences were derived from alignments of 16 inferred protein sequences of subfamily members (from Schizosaccharomyces cerevisiae, S pombe, Caenorhabditis elegans, Plasmodium falciparum, and Drosophila melanogaster) and the motif containing R867 is found in all 16 of them. This implies that it may play a role that is central to this type of molecule. Furthermore, R867 is found in the same position in all human subfamilies of amphipath transporters. The motif is not conserved in Ca2+ transporting P-type ATPases, indicating that its function may relate to substrate specificity. D70N results in the substitution of aspartic acid, which is a polar charged amino acid with a pKA of 3.65, with an asparagine, a polar uncharged amino acid. This leads to a change in the charge at neutral pH from negative to neutral at the respective position.

Although the D70N variant was not present in 120 parous controls in this study, it has been reported in two individuals of Caucasian origin. However, one of these was male and it is not stated whether the female carrier was parous. It is possible that D70N on its own is not of sufficiently high penetrance but leads to ICP only in combination with a second disadvantageous sequence variation or, alternatively, that the functional effect of the ATP8B1 variants reported in this study become clinically apparent as a consequence of the endocrine changes of pregnancy. Oestrogen treatment results in increased cholesterol content of membranes and reduced membrane fluidity, and this in turn reduces bile flow.

Oestrogen treatment also causes reduced protein mass and RNA expression of Ntcp, the principal sinusoidal bile acid transporter, and other sinusoidal organic anion transporters, Oatpl, and Oatp2 in the rat. Reduced protein mass of Oatp4 and Bsep, and reduced mRNA levels of murine mBsep. Either altered membrane fluidity, or reduced expression or function of the principal sinusoidal or canalicular bile acid transporters in pregnancy, in combination with loss of function of FIC1, could result in the development of cholestasis in a woman who is asymptomatic when not pregnant. It is also possible that the endocrine changes of pregnancy may influence the function of the mutant protein in a similar way to the K183R substitution in the thyrotrophin receptor that results in increased sensitivity to chorionic gonadotrophin in familial gestational hyperthyroidism.

It remains to be seen whether the MRS findings are truly of significance as subject numbers were small. However, there have been many studies looking at the utilisation of 31P MRS to assess liver disease, mainly in cirrhosis. These have reported good correlation with either an elevated PME resonance or decreased PDE resonance and functional capacity in cirrhotic livers. The ratio of PME to PDE has traditionally been viewed as an index of cell membrane turnover, the PME resonance including contributions from the precursors of phospholipid membrane synthesis, phosphocholine and phosphoethanolamine, as well as contributions from adenosine monophosphate and glycolytic intermediates. The PDE resonance contains information from membrane breakdown products, GPC and GPE, biliary phospholipid, and a contribution from endoplasmic reticulum. In women with a history of ICP, we observed an increase in %PDE, rather than the decrease seen in cirrhosis, a finding that has previously been reported in cholestatic liver transplant patients with chronic graft rejection. This has been interpreted as an increased or altered signal from biliary phospholipid, which probably underlies the findings in our patient group. It is possible that the abnormal %PDE signal is associated with an increase in the proportion of biliary phospholipid as a consequence of defective canalicular bile acid transport, and therefore a proportional reduction in biliary bile acid concentrations. The ICP cases also had a reduced PME/PDE ratio which may reflect increased cell membrane turnover.

Although there were no differences in hepatic ßNTP levels between cases and controls, it is intriguing that the two cases with D70N had lower levels than the other ICP cases.

It should be borne in mind that subjects in this study with 31P MRS abnormalities were not pregnant and were asymptomatic when the scans were performed. Therefore, the abnormal spectra may be indicative of underlying abnormalities of liver metabolism and biliary composition that only manifest as symptoms during pregnancy, but are not sufficiently severe to cause cholestasis in the non-pregnant state.

In summary, sequencing of the coding exons has revealed two ATP8B1 mutations in 16 ICP cases. The D70N mutation was present in another two affected women when 166 additional cases were screened for these variants. In vivo MRS studies of the liver in eight women with previous ICP, performed when they were not pregnant, demonstrated increased hepatic PDE and reduced PME/PDE values compared with normal controls. Two ICP cases with D70N had the lowest ßNTP levels of the ICP cases measured.

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Conflict of interest: None declared.

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Klomp LW


Mollenbach, Bennett, Tietlow, et al.

104


13


19


34


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ATP8B1 mutations in British cases with intrahepatic cholestasis of pregnancy

R Müllenbach, A Bennett, N Tetlow, N Patel, G Hamilton, F Cheng, J Chambers, R Howard, S D Taylor-Robinson and C Williamson

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