

ONLINE MUTATION REPORT

ABCB4 gene sequence variation in women with intrahepatic cholestasis of pregnancy

R Müllenbach, K J Linton, S Wiltshire, N Weerasekera, J Chambers, E Elias, C F Higgins, D G Johnston, M I McCarthy, C Williamson

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Intrahepatic cholestasis of pregnancy (ICP), also known as obstetric cholestasis, is a liver disease of pregnancy that complicates 0.7% of pregnancies in the UK.^{1,2} ICP causes maternal pruritus and hepatic impairment and can cause fetal death, spontaneous prematurity, and sudden intrauterine death.³⁻⁶ A diagnosis of ICP is made by the demonstration of abnormal liver function test results, and in particular the serum bile acids are raised.⁷⁻¹⁰ This is thought to be a consequence of abnormal bile transport across the hepatocyte canalicular membrane. Clinical features are heterogeneous and the aetiology is likely to be complex.

Insights to the genetic aetiology of ICP have come from studies of the childhood liver disease progressive familial intrahepatic cholestasis (PFIC), a condition which is divided into three subtypes. Children with PFIC1 and 2 have low concentrations of biliary bile acids and low to normal gamma-glutamyl transpeptidase (GGT) in the serum. PFIC3 patients have high serum levels of GGT and bile which lacks phospholipid but has a normal biliary bile acid concentration.¹¹ Homozygous mutations of the *ABCB4* (also called *MDR3* or *mdr2* in the mouse) gene have been described in pedigrees with PFIC3.¹¹⁻¹³ The *ABCB4* protein is a member of the ATP binding cassette (ABC) family of membrane transporters.¹⁴⁻¹⁶ One of the normal functions of *ABCB4* is to transport phosphatidylcholine across the hepatocyte canalicular membrane. The fact that expression is not only found in hepatocytes but also in B lymphocytes, heart, and muscle suggests that it may also transport other substrates. However, homozygous knockouts of the homologous (>90% identity at the amino acid level) murine *mdr2* only had hepatic effects.¹⁷

Several heterozygous mothers of children with PFIC3 have symptoms consistent with ICP.¹¹⁻¹³ In a large consanguineous pedigree with coexisting PFIC3 and ICP, three of the six mothers with ICP had pregnancies complicated by unexplained intrauterine death.¹² Four of the six women were investigated and shown to be heterozygotes for the *ABCB4* mutation for which the proband was a homozygote.¹² In a recent study of 31 patients with PFIC3, *ABCB4* mutations were found in 17 cases, and three heterozygous mothers were either diagnosed or suspected of having ICP.¹³

More frequently, ICP occurs in women with no family history of PFIC. Based on the observation that children with PFIC3 and *ABCB4* gene mutations have a raised serum GGT, we previously investigated a subgroup of eight women with ICP and a raised GGT and found a missense mutation in the *ABCB4* gene in one case.¹⁸ This mutation resulted in lack of functional protein at the cell surface. Both homozygous and heterozygous *ABCB4* mutations have also been described in three of six cases of women with cholesterol gallstones in association with ICP, and with no family history of PFIC3.¹⁹

In an attempt to elucidate the contribution of sequence variants in *ABCB4* in the pathogenesis of ICP, we sequenced the complete coding region from 14 women with ICP and raised GGT. The frequencies of variants found in these cases and in eight cases that were previously sequenced¹⁸ were com-

Key points

- Intrahepatic cholestasis of pregnancy (ICP) affects 0.7% of pregnancies in the UK.
- Our aim was to assess the role of *ABCB4* sequence variants in a large number of cases of ICP (n=184).
- To identify new and/or frequent variants, we sequenced all coding exons in 14 cases. We then established the frequency of these variants and 11 previously published mutations in *ABCB4* in our cohort.
- We have identified a new variant R150K that segregates with ICP in a UK pedigree and that is present in one additional case.
- A comparison of genotype frequencies between case and control groups showed that the allele frequency of one SNP (743A>T) is significantly different in all ICP cases (STATXACT p=0.045). This is more marked in the subgroup of cases with raised GGT (p=0.015).

pared in 184 ICP subjects and controls. In the majority of ICP cases in this cohort, the GGT level had not been measured. We also examined the cohort of 184 ICP patients for the presence or absence of known variations in the *ABCB4* gene and analysed three SNPs within the coding region for disease association.

Previous studies have investigated the role of *ABCB4* mutations in specific small subgroups of ICP cases, that is, a small number of cases with a raised GGT, three women with coexistent cholesterol gallstones with atypical clinical features, or the minority of cases with children affected by PFIC3. This is the first study of *ABCB4* variants in a larger group of ICP cases with and without a known raised GGT.

MATERIALS AND METHODS

Women with ICP were identified either by the clinicians responsible for their care or by the UK Obstetric Cholestasis Patient Organisation (OCPO). The diagnostic criteria for ICP were met if women had persistent pruritus and biochemical confirmation of the diagnosis in the absence of other known liver disease (including pre-eclampsia with abnormal liver function, the HELLP syndrome, acute fatty liver of pregnancy, primary biliary cirrhosis, and ultrasound abnormality that could result in biliary obstruction). Women were excluded from the analysis if the hepatic impairment did not resolve postnatally, with the exception of cyclical and exogenous oestrogen induced cholestasis. Women with dermatoses of pregnancy that can result in pruritus were also excluded. Abnormal liver function was defined as one or more of raised serum bile acids and/or raised serum transaminases (alanine aminotransferase (ALT), aspartate aminotransferase (AST) or GGT) in at least one pregnancy. As hospitals have different

Table 1 Clinical and biochemical features of women with ICP and raised GGT

Case No	Fetal distress	Max ALT* (<28 U/l)	Max BA (<14 µmol/l)	Max GGT (<30 U/l)
1	N	89	103	68
2	N	204	189	43
3	N	27 (AST)	NT	72
4	N	71	17	51
5	N	164	195	117
6	N	89	383	47
7	Y	48	82	66
8	N	185	119	147
9	Y	369	66	106
10	Y	81	NT	107
11	Y	559	†	†
12	N	626	23	64
13	Y	73	16	121
14	Y*	203	143	57
All cases (n=34)	38% (13/34)	Mean=262.1 (SEM=40.8)	Mean=98.6 (SEM=17.9)	Mean=80.7 (SEM=6.8)

*Indicates pregnancy complicated by intrauterine death. †ICP diagnosed in an overseas unit; referred as a case with raised GGT, but the original biochemical data are currently not available. NT = not tested.

Table 2 Published mutations not identified in 184 women with ICP

Mutation	Exon	Diagnostic digest or methodology	Reference
Exon 6 insertion and/or deletion	6	Sequencing	11, 13
959C>T	9	<i>Bam</i> HI digest (cuts in WT only)	19
1302 A>G	12	<i>Cac</i> 8I digest (cuts in variant only)	13
1307 G>A	12	<i>Ha</i> ellI digest (cuts in WT only)	13
1327insA	12	PCR incl ³² P run on 6% PAA	19
Exon 14 insertion and/or deletion	14	PCR incl ³² P run on 6% PAA	11 13
1669 C>A	14	<i>Sex</i> AI digest (cuts in variant only)	18
1712delT	14	<i>Rsa</i> I digest (cuts in WT only)	12
2901 C>T	23	<i>Taq</i> I digest (cuts in WT only)	11

PAA = polyacrylamide

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 case, consistent with published studies,²⁰ and any values above this were considered to be abnormal. The normal range for bile acids was <14 µmol/l. Serum bile acid results were available in 53% of cases. There were only three cases with raised serum bile acids and liver transaminases within the normal range. Women known to have hepatitis C infection were excluded from the study.

A total of 184 women with ICP were identified; 34 had a raised GGT, 39 had a normal GGT, and the GGT was not measured in 111 cases.

All 184 women were screened for the presence of the *ABCB4* mutations previously reported in ICP. In addition, DNA from 14 patients with ICP and a raised GGT and from one normal control was sequenced using primers for the coding exons of *ABCB4*.

Table 1 summarises the fetal complications and biochemical abnormalities that were found in the 14 cases with raised GGT that were sequenced in this study. Table 1 also summarises the mean values for the ALT, serum bile acids, and GGT for all of the 34 cases with raised GGT. The biochemical results are from samples that were taken during the pregnancy or within 24 hours of delivery. Fetal distress was diagnosed if there were CTG abnormalities or meconium stained liquor. There were two pregnancies complicated by intrauterine death; one occurred in a female fetus at 36 weeks' gestation (case 14 in table 1) and the other in a 37 week male fetus (case not summarised in table 1).

To check for the presence of sequence variants in normal subjects, we used a panel of 65 parous women who had no history of cholestasis in pregnancy.

This study has ethical approval from the Hammersmith and Queen Charlotte's Hospital Trust Ethics Committee, and informed consent was obtained from all subjects.

Twenty-seven pairs of exon specific primers¹⁸ were used to amplify the 27 coding exons of the *ABCB4* gene together with the respective exon/intron boundaries and sequenced using amplification primers and the FS+ Dye Terminator sequencing kit (PE Applied Biosystems) according to the manufacturer's instructions. Products were run on an ABI 3100 genetic analyser (PE Applied Biosystems), and analysed by comparison to the Genbank sequence file M23234.

To screen for previously published mutations,^{11-13 18 19} exons of interest were amplified from genomic DNA using standard polymerase chain reaction (PCR) methodology (<http://info.med.yale.edu/genetics/ward/tavi/PCR.html>). Screening for known mutations was performed using the appropriate restriction endonuclease (table 2).

Screening for microdeletions of 1-7 bp^{11 12 19} was performed by adding 1 µCi of ³²P-dCTP per PCR reaction before PCR amplification. Post-amplification, products were denatured for two minutes at 96°C and separated on a 6% denaturing polyacrylamide gel (Sequagel, National Diagnostics).

Plasmid pMDRI-wt encodes a hexahistidine tagged P-gp1.²¹ R148 of P-gp1 (the equivalent of *ABCB4* R150) was mutated to lysine by site directed mutagenesis using the mutagenic oligonucleotide 5'-GGAAGACAAATTCACAAAA TTAAAAACAGTTT-3', as described previously¹⁸; codon 148 is underlined. The same oligonucleotide introduced an *Apo*I restriction site (italicised) without change to the codon sense. This allowed the success of the mutagenesis reaction (and subsequent cloning of the mutated DNA fragment) to be monitored by restriction digest. The mutated DNA fragment

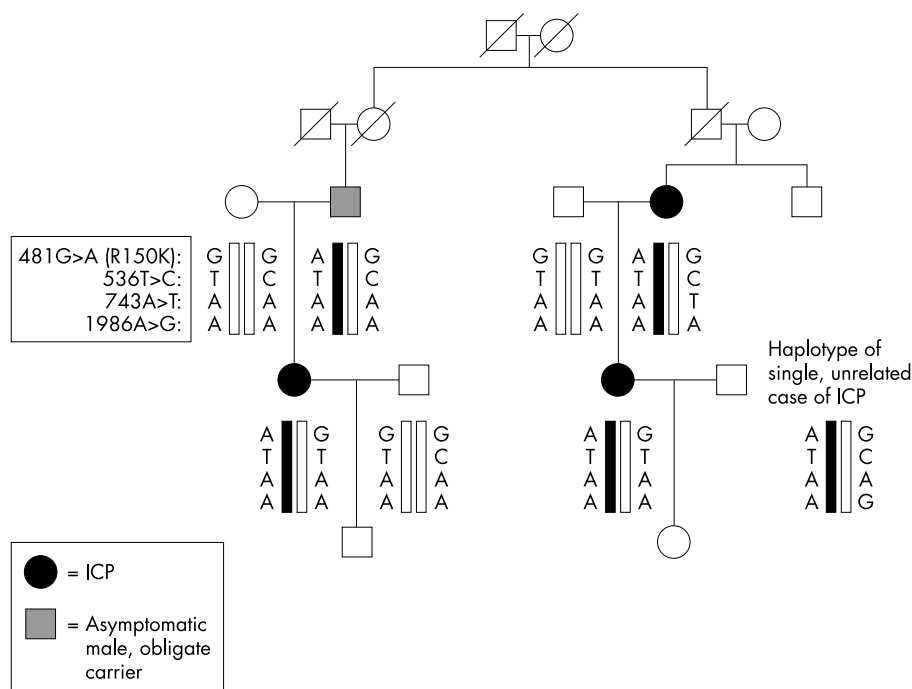


Figure 1 Pedigree showing transmission of R150K and associated haplotypes for three markers in a case of familial ICP. The haplotype of the single unrelated case of ICP in whom the mutation was identified is also shown.

was used to replace the wild type sequence of pMDR1-wt (to generate pMDR1-R148K). The mutated sequence of pMDR1-R148K was verified by DNA sequencing. Plasmids pMDR1-wt and pMDR1-R148K were transfected into human embryonic kidney (HEK) 293T cells, as described previously.¹⁸ The function of R148K-P-gp was compared with the wild type P-gp by flow cytometry using a dual labelling assay to measure both the level of P-gp on the cell membrane and its ability to extrude the fluorescent dye rhodamine 123 (R123, Sigma Aldrich) as described previously.¹⁸

Evidence for associations between *ABCB4* variants and ICP was sought for three common SNPs (536T>C, 743A>T, 1986A>G). For these analyses, three “nested” definitions of ICP cases were used. The first included only those women with ICP known to have raised GGT ($n=34$), the second comprised women with raised ($n=34$) or unknown GGT levels ($n=111$) (total $n=145$), and the third included all ICP women irrespective of GGT ($n=184$). Genotype frequencies in case and control ($n=65$) groups were compared using the Kruskal-Wallis test, implemented in STATXACT (Cytel Corporation, Cambridge, MA). Given the modest numbers of subjects in some of the groups, exact significance values are presented. Linkage disequilibrium between the loci was calculated using EH²² and PM.²³ Estimates of haplotype frequencies were obtained using TRANSMIT²⁴ and SNPHAP (<http://www-gene.cimr.cam.ac.uk/clayton/software/>), and differences in haplotype frequencies between controls and cases were determined by likelihood ratio testing. The significance of any difference in haplotype frequencies was determined by permutation (10 000 replicates).

RESULTS AND DISCUSSION

DNA sequence analysis of the 27 coding exons of the *ABCB4* gene, together with the respective exon-intron boundaries, was performed in 14 ICP patients with raised GGT levels, together with a normal control. In one patient (case 12 in table 1), a heterozygous DNA base change was identified in exon 6 (481G>A); this results in the substitution of the wild type arginine (R) with a mutant lysine (K) (R150K). This transition resulted in the introduction of an *MseI* restriction

site that allowed confirmation of the mutation, and for it to be excluded in 65 controls. Restriction digests showed this mutation in one of the additional 162 women in whom the coding exons of *ABCB4* had not been sequenced. This woman has a family history of ICP, with two affected relatives, one of whom has biochemical confirmation of the diagnosis with a raised GGT. R150K segregated with ICP in the latter pedigree (fig 1).

None of the published *ABCB4* mutations that cause ICP in the heterozygote mothers of children with PFIC3 was present in the 184 ICP cases recruited in this study (table 2). These results indicate that there is no clustering of the known *ABCB4* mutations in ICP, that is, the majority of mutations described to date^{11-13, 18} are unique. The only exceptions are 959C>T, which has been described in two patients with gallstones and ICP from two independent, non-consanguineous families,¹⁹ and the 481G>A (R150K) mutation reported in this study.

Three variants occurred in ICP cases and controls, 536T>C and 743A>T that were identified in this study (table 3) and 1986A>G that was previously reported.¹³

Genotype frequencies at the 536T>C or 1986A>G variants showed no association with ICP (in any of the three “case” definitions used). However, at 743A>T, genotype frequencies were markedly different, most notably in the comparison between ICP women with raised GGT and controls (exact midpoint $p=0.005$, corrected for the number of SNPs tested, assuming independence $p=0.015$). The association was still evident when the case sample was expanded to include those with unknown GGT as well (exact midpoint $p=0.014$, corrected to $p=0.042$) or to all women with ICP (for both, exact midpoint $p=0.015$, corrected to $p=0.045$). As the markers were in linkage disequilibrium (all pairs $p<0.002$ in controls, $p<0.0004$ in all cases), haplotype frequencies were estimated (by maximum likelihood methods) and compared between case and control groups. A significant difference in haplotype frequencies was evident in the comparison of raised GGT ICP women and control subjects ($p=0.013$), and was attributable to an excess of haplotypes carrying the common allele at 743A>T in affected cases. The haplotype frequencies were not significantly different in the other case/control comparisons.

Table 3 Allele frequencies of *ABCB4* variants identified following sequence analysis of the coding exons of *ABCB4* in 14 cases of ICP with raised GGT and of the previously reported 1986A>G variant¹³

DNA variant	Exon	Diagnostic method	Raised GGT (n=34)	Normal GGT (n=39)	Unknown GGT (n=111)	Controls (n=65)
481 G>A (R150K)	6	MseI digest	GG = 33 AG = 1 AA = 0	GG = 38 AG = 1 AA = 0	GG = 111 AG = 0 AA = 0	GG = 65 AG = 0 AA = 0
536 T>C	6	Sequencing	TT = 10 TC = 15 CC = 9	TT = 10 TC = 23 CC = 6	TT = 46 TC = 44 CC = 21	TT = 15 TC = 36 CC = 14
743A>T	8	HinfI digest	AA = 31 AT = 3 TT = 0	AA = 30 AT = 9 TT = 0	AA = 86 AT = 21 TT = 4	AA = 42 AT = 21 TT = 2
1986A>G (R652G)	16	TspRI digest	AA = 28 AG = 6 GG = 0	AA = 33 AG = 6 GG = 0	AA = 96 AG = 15 GG = 0	AA = 58 AG = 6 GG = 1
2372C>T	19	Cac8I digest	CC = 33 C/T = 1 TT = 0	CC = 39 C/T = 0 TT = 0	CC = 110 C/T = 1 TT = 0	CC = 65 CT = 0 TT = 0

The finding of an association between ICP and the 743A>T genotype was intriguing. The number of cases studied (especially in the high GGT subset) is, however, small. We are not aware of any equivalently sized (or better still, larger) data sets which would allow these findings to be corroborated by replication. Since the 743A>T variant does not result in any alteration of amino acid sequence, it is unlikely to affect the function of the protein. One possibility therefore is that it is in linkage disequilibrium with a functional variant elsewhere in the *ABCB4* gene. However, no variants meeting this description were found in this study. To pursue this further, it will be necessary to undertake extensive screening of intronic, 5' and 3' untranslated regions of *ABCB4*. Alternatively, 743A>T could be in linkage disequilibrium with another gene in close proximity to *ABCB4*. If the association between ICP and the 743A>T genotype is confirmed to be genuine, we will have shown an association between the common allele and ICP. This phenomenon is not remarkable in complex traits, for example, the PPAR γ Pro12Ala variant is associated with decreased risk of type 2 diabetes.²⁵

The use of assessing the functional consequences of *ABCB4* mutations by analysis of the equivalent P-gp1 mutant has been reported previously.¹⁸ *ABCB4* R150 is highly conserved within the ABC transporter family and is the equivalent of P-gp1 R148.

Flow cytometry of live cells was used to measure the relative abundance of P-gp on the cell surface and test the ability of transiently transfected cells to extrude the fluorescent P-gp1 substrate rhodamine 123 (R123). This indicated that the mutant protein was trafficked to the cell surface with similar efficiency to the wild type protein and signal transduction between the substrate binding and ATPase domains remained intact. Indeed, the mutant protein appeared to be equal in function to the wild type protein, at least for transport of R123 (data not shown).

Although the P-gp1 K148 mutant function did not differ from wild type (at least for the transport of R123), it is not possible to conclude that the equivalent mutant in *ABCB4* would be benign. *ABCB4* R150K may have a direct effect specific to the binding of phosphatidylcholine. Alternatively it may influence protein function in response to the hormonal changes of pregnancy.

Approximately 25% of women with ICP have a raised GGT, and we have previously suggested that *ABCB4* mutations may explain the aetiology of the condition in the majority of these

cases. However, the results of this study suggest that mutations in the coding exons of *ABCB4* do not cause ICP in such a high proportion of cases as we had previously proposed. We have not investigated hepatic RNA or protein expression of *ABCB4* as it is not clinically justified to obtain liver biopsies from women with ICP. We have also not investigated the *ABCB4* promoter for SNPs that may predispose women to cholestasis in pregnancy.

Familial and epidemiological studies,^{26, 27} as well as the heterogeneity of clinical course of ICP, indicate that it has a complex aetiology. Consequently, all molecules involved in bile acid metabolism may contribute to the aetiology of the condition, for example CYP7A1, the rate limiting enzyme in bile acid synthesis, transporter molecules BSEP, NTCP, MRP3, and MRP4 or nuclear receptors that influence bile acid metabolism.^{28–31} Sequence polymorphisms within the coding regions of all these molecules or their specific binding sites could be the reason for the shift from normal bile acid metabolism and transport to cholestasis under the influence of hormonal changes induced by pregnancy.

In summary, the current study has identified a new mutation (R150K) that cosegregates with ICP in a UK pedigree and an unrelated case. However, no functional effect was shown when R150K was expressed in P-gp1. We have shown that mutations in the coding region of *ABCB4* are not a common cause of ICP with raised GGT in our population. However, the finding of an association between 743A>T and ICP with raised GGT, and the identification of R150K indicate that this gene is implicated in the aetiology of ICP at least in a subgroup of women with raised GGT.

Authors' affiliations

R Müllenbach, J Chambers, D G Johnston, C Williamson, Institute of Reproductive and Developmental Biology, Faculty of Medicine, Imperial College, Hammersmith Campus, Du Cane Road, London W12 0NN, UK

R Müllenbach, K J Linton, N Weerasekera, C F Higgins, M I McCarthy, C Williamson, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Campus, Du Cane Road, London W12 0NN, UK

S Wiltshire, M I McCarthy, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, UK

S Wiltshire, M I McCarthy, Imperial College Genetics and Genomics Research Institute, Faculty of Medicine, Imperial College, Hammersmith Campus, Du Cane Road, London W12 0NN, UK

E Elias, Department of Gastroenterology, Queen Elizabeth Hospital, Birmingham B15 2TH, UK

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