LIVER

Intrahepatic cholestasis of pregnancy: the severe form is associated with common variants of the hepatobiliary phospholipid transporter *ABCB4* gene

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Gut 2007;**56**:265–270. doi: 10.1136/gut.2006.092742

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Revised 14 May 2006
Accepted 21 June 2006
Published Online First 4 August 2006

Background: Intrahepatic cholestasis of pregnancy (ICP) is characterised by troublesome maternal pruritus, raised serum bile acid levels and increased fetal risk. Mutations of the *ABCB4* gene encoding the hepatobiliary phospholipid transporter have been identified in a small proportion of patients with cholestasis of pregnancy. In a recent prospective study on 693 patients with cholestasis of pregnancy, a cut-off level for serum bile acid (≥40 μmol/l) was determined for increased risk of fetal complications.
Objectives: To investigate whether common combinations of polymorphic alleles (haplotypes) of the genes encoding the hepatobiliary ATP-binding cassette (ABC) transporters for phospholipids (ABCB4) and bile acids (ABCB11) were associated with this severe form of cholestasis of pregnancy.
Methods: For genetic analysis, 52 women with bile acid levels ≥40 μmol/l (called cases) and 52 unaffected women (called controls) matched for age, parity and geographical residence were studied. Gene variants tagging common *ABCB4* and *ABCB11* haplotypes were genotyped and haplotype distributions were compared between cases and controls by permutation testing.
Results: In contrast with *ABCB11* haplotypes, *ABCB4* haplotypes differed between the two groups (p = 0.019), showing that the severe form of cholestasis of pregnancy is associated with the *ABCB4* gene variants. Specifically, haplotype *ABCB4_5* occurred more often in cases, whereas haplotypes *ABCB4_3* and *ABCB4_7* were more common in controls. These associations were reflected by different frequencies of at-risk alleles of the two tagging polymorphisms (c.711A: odds ratio (OR) 2.27, p = 0.04; deletion intron 5: OR 14.68, p = 0.012).
Conclusion: Variants of *ABCB4* represent genetic risk factors for the severe form of ICP in Sweden.

Intrahepatic cholestasis of pregnancy (ICP) is a reversible cholestatic liver disease of undefined aetiology and pathogenesis. It is characterised by pruritus and raised serum bile acid levels (≥10 μmol/l), with onset in the second half of pregnancy and persisting until delivery.[1–3] Although essentially benign from a maternal viewpoint, ICP is associated with increased fetal risks, such as preterm delivery in 19–60%[4–6] and fetal distress in 22–41% of affected pregnancies.[4 7 8] Intrauterine fetal death has been reported in 0.8–1.6% of ICP cases,[4 5 7 8] but was detected in only 0.4% of women in a recent prospective study on ICP.[9] Fetal complications were correlated with maternal serum bile acid levels, increasing markedly when levels exceeded 40 μmol/l.[9]

The aetiology and pathogenesis of ICP is still unknown. Family clustering and varying incidence in different geographical regions suggest that genetic and environmental factors and their interactions probably play a part.[1] The hypothesis that genetically determined dysfunction of hepatocanalicular lipid transporters contributes to ICP was first supported by Jacquemin *et al*,[10] who reported the coexistence of ICP and a frameshift mutation in the *ABCB4* gene encoding the hepatobiliary transporter for phosphatidylcholine in a large consanguineous family. Since then, additional variants of the *ABCB4* gene have been identified in a small number of patients with ICP.[11–14] Also, single British and Finnish patients with ICP carried mutations in the *ATP8B1* gene encoding a potential membrane transporter for phosphatidylserine in the liver and intestine.[15 16]

The biliary secretion of bile acids and other biliary lipid constituents across the canalicular membrane of hepatocytes is mediated by a set of distinct ATP-binding cassette (ABC) transporters. The bile acid export pump ABCB11 and the phospholipid transporter ABCB4 are key players in this process. ABCB4 translocates the major biliary phospholipid, phosphatidylcholine (lecithin), from the inner to the outer leaflet of the canalicular membrane, and ABCB11 is the predominant bile acid efflux system of hepatocytes. Further, inherited deficiencies of ABCB11 and ABCB4 have been established as causes of progressive familial intrahepatic cholestasis type 2 and type 3, respectively.[17 18] These are chronic liver diseases that may rapidly progress to cirrhosis and often necessitate liver transplantation in early childhood. ABCB4 dysfunction is assumed to decrease biliary phospholipid levels, thereby causing toxic membrane damage via an excess of biliary bile acids, whereas ABCB11 deficiency causes intrahepatic retention of bile acids.

From a prospective cohort study on 693 women with ICP, we selected a subgroup with severe ICP (n = 52). In these patients, we studied whether common sets of polymorphisms (haplotypes) covering most of the genetic variation in the hepatobiliary transporter genes *ABCB4* and *ABCB11* differed from haplotypes observed in healthy controls.

PATIENTS AND METHODS

The cohort with ICP and controls

Between 1 February 1999 and 31 January 2002, all pregnant women in the Västra Götaland region of Sweden were

Abbreviations: ABC, ATP-binding cassette; ICP, intrahepatic cholestasis of pregnancy; SNP, single-nucleotide polymorphism

prospectively screened for ICP, defined as otherwise unexplained pruritus of pregnancy combined with fasting bile acid levels ⩾10 μmol/l. The epidemiological results of this study have been published recently.[9] In Sweden, all pregnancies are monitored by midwives in local antenatal clinics. All 106 antenatal clinics and the six departments of obstetrics in the region participated in the study.

In all, 45 485 pregnancies leading to deliveries were recorded during the study period, and 937 women (2.1%) complained of generalised pruritus during pregnancy, 693 (1.5%) of whom had ICP. On the basis of prospectively recorded fetal complication rates, an increased fetal risk was observed exclusively in patients with bile acid levels ⩾40 μmol/l.[9] Therefore, we defined a mild and a severe form of ICP, the women with severe ICP had bile acid levels ⩾40 μmol/l.[9] Women with severe ICP (n = 130; 96 in the observational study[9] and 34 in the treatment study[19]) were invited to participate in the genetic study. Forty eight women who accepted and all additional patients (n = 4) who presented with severe ICP shortly after the end of these two studies were enrolled in the genetic study after they gave written informed consent; no more than these four additional patients were available for haplotype analysis. For control purposes, 52 healthy women without ICP were recruited. All controls reported uneventful pregnancies and were matched with respect to age, parity and geographical residence.

The study was approved by the ethics committee of the Faculty of Medicine at the University of Göteborg, Göteborg, Sweden.

Liver chemistry tests

Blood samples were obtained after a 12-h fast. Total serum bile acid concentrations were analysed with an enzymatic colorimetric method (Enzabile, Biostat Diagnostic Systems, Stockport, UK). Activities of serum transaminases and γ-glutamyl transpeptidase, and serum bilirubin levels were analysed with standard laboratory methods.[9]

Genotyping

Genomic DNA was isolated from EDTA-anticoagulated blood using the QIAamp (Qiagen, Hilden, Germany) protocol. DNA concentrations were determined fluorometrically (Bio-Rad Laboratories, Hercules, California, USA), using the dye PicoGreen (Molecular Probes, Leiden, The Netherlands).

In general, single-nucleotide polymorphisms (SNPs) were genotyped using solution-phase hybridisation reactions with 5′ nuclease and fluorescence detection (TaqMan assays) on the ABI PRISM 7000 Sequence Detection System (Applera, Norwalk, Connecticut, USA). Primer and probe sequences for allele-specific polymerase chain reactions are available at http://www.appliedbiosystems.com. Polymerase chain reactions contained 5–20 ng genomic DNA, 1× TaqMan Universal Master Mix, 900 nM of each primer and 200 nM of VIC-labelled and FAM-labelled probes in 25 μl-reactions (Applera). Amplification conditions were 95℃ for 10 min, 40 cycles at 92℃ for 15 s and 60℃ for 1 min.

We ascertained the results of TaqMan assays by direct Big Dye Termination cycle sequencing on the ABI PRISM 310 Genetic Analyzer (Applera). The 5-bp deletion in intron 5 of the *ABCB4* gene[20] was visualised on silver-stained polyacrylamide gels.[21]

Genotype analysis

Consistency in genotype frequencies with Hardy–Weinberg equilibrium was confirmed using an exact test[22] and visualised in triangular de Finetti diagrams,[23] using software by T Wienker and T Strom (http://ihg.gsf.de/snps.html).

To test for association, we compared the distribution of alleles and genotypes in contingency tables by χ^2 test and Armitage's trend test, respectively.[24]

Haplotype analysis

Haplotypes are defined as particular combinations of SNP alleles present on single chromosomes. The haplotype structure of the human *ABCB4* and *ABCB11* genes has been defined by Pauli-Magnus *et al*,[20] on the basis of resequencing of the genes in 56 and 93 healthy Caucasians, respectively. We identified the minimum sets of SNPs tagging the common haplotypes (frequencies >3%) of both genes using the Best Enumeration of SNP Tags algorithm.[25]

Haplotypes were reconstructed from the genotype data of cases and controls using the PHASE algorithm v.2.0, which is based on bayesian inference.[26] To allow referral to specific haplotypes, we have followed the nomenclature used by Pauli-Magnus *et al*[20] (ie, *ABCB11_1*, *ABCB11_2*, ...), irrespective of the actual haplotype frequencies observed in our study.

To test for significant differences in haplotype distributions between patients with ICP (hereafter called cases) and controls, permutation tests were performed, as implemented in the PHASE algorithm.[26] The permutation test checks the null hypothesis that case and control haplotypes are a random sample from a single set of haplotype frequencies versus the alternative that cases are more similar to each other than to controls. Haplotypes of cases and controls were permuted 10 000 times, which generates empirical p values at the 0.05 level while controlling for multiple testing.

Additional statistical analysis

Means, standard deviations (SDs), standard errors (SE) medians and ranges were calculated as indicated. Student's t test was used to compare differences in continuous variables between groups. Dichotomous variables were compared using Fisher's exact test. All tests were conducted at a significance level of 0.05.

RESULTS

Clinical characteristics of cases

We included 52 cases with serum bile acid concentrations ⩾40 μmol/l in our study. The mean (SD) age of the patients was 30.1 (5.1) years. Heredity for pruritus of pregnancy was reported by 19 (37%) women. The parous women (n = 24) had experienced a total of 36 pregnancies, of which 27 (75%) had been associated with pruritus of pregnancy. Intrauterine fetal deaths had occurred in 2/36 pregnancies (5.6%), both associated with pruritus. Gallstone disease (cholecystectomy or symptomatic, ultrasound-verified gallstones) was reported by 5 (9.6%) women.

In our study, the 52 women developed symptomatic ICP after a mean of 29.1 (SD 6.4) weeks of gestation. The mean serum bile acid concentration was 111 (SD 64) μmol/l. Bile acid levels were correlated significantly with bilirubin levels ($r = 0.38$, $p = 0.005$) and alanine aminotransferase activities ($r = 0.51$, $p<0.001$). Serum bilirubin levels and γ-glutamyl transpeptidase activities were raised in 44% and 20% of the patients, respectively. Spontaneous premature birth in singleton pregnancies occurred in 27% of the pregnancies, whereas the total prematurity rate (including inductions, preterm caesarean sections or multiple pregnancies) was 33%. Asphyxial events (operative delivery due to asphyxia, umbilical arterial pH <7.05 or Apgar Score <7 at five minutes) occurred in 13% of the pregnancies. Meconium staining of amniotic fluid and green staining of placenta and membranes (indicating a longer period of meconium passage) were registered in 60% and 44% of the cases, respectively.

Table 1 *ABCB11* genotypes and haplotypes

Haplotypes	htSNP (codon position)						Haplotype frequencies*	
	Intron −1 (−1952)	Intron −1 (−1155)	Exon 13 (1331)	Intron 18 (−17)	Intron 19 (−17)	Exon 24 (3084)	Cases (%)	Controls (%)
ABCB11_1	T	T	C	A	C	G	16.6 (1.6)	16.9 (1.7)
ABCB11_2	C	C	T	A	C	G	17.2 (1.8)	17.2 (2.3)
ABCB11_3	C	C	T	C	T	A	6.0 (1.6)	7.4 (1.9)
ABCB11_4	T	T	C	C	T	A	11.6 (1.7)	10.9 (1.3)
ABCB11_5	T	T	C	C	C	A	3.7 (1.2)	5.8 (1.5)
ABCB11_6	C	C	C	A	C	G	7.7 (1.6)	4.1 (1.4)
ABCB11_7	T	C	C	A	C	G	5.0 (1.4)	6.5 (1.8)
ABCB11_9†‡	C	C	C	C	T	A	5.6 (1.3)	5.4 (2.0)
ABCB11_10‡	T	T	T	A	C	G	6.1 (0.9)	4.1 (0.9)
ABCB11_11‡	T	C	C	C	T	A	3.5 (1.2)	6.5 (2.0)
ABCB11_12‡	C	C	T	C	C	A	3.7 (1.1)	5.2 (1.4)

Case; woman with intrahepatic cholestasis of pregnancy; control, healthy woman without intrahepatic cholestasis of pregnancy; htSNP, haplotype-tagging single-nucleotide polymorphism.
Values are mean (SE).
*Haplotype frequencies do not differ significantly between cases and controls (p = 0.23), as indicated by permutation tests with 10 000 permutations.[26]
†The haplotype *ABCB11_8* is omitted, as it was observed at a frequency of <3% in both cases and controls.
‡As haplotypes *ABCB11_9–ABCB11_12* were observed but not designated in the study by Pauli-Magnus *et al*,[20] numbers are assigned according to their frequencies in this study.

Genetic analysis

The Best Enumeration of SNP Tags algorithm identified a minimum set of five polymorphisms tagging the common haplotypes (frequencies >3%) of the *ABCB4* gene and six haplotype-tagging SNPs covering the *ABCB11* gene (tables 1 and 2). These tagging variants define six common *ABCB4* and 11 common *ABCB11* haploytpes with population frequencies >3% in our study. The tagging polymorphisms were subsequently genotyped in the 52 patients with severe ICP and 52 matched controls. None of the genotype frequencies deviated from the frequencies expected for a large, randomly mating population, as shown by accordance with an exact test for Hardy–Weinberg equilibrium.

Table 1 shows that the distributions of *ABCB11* haplotypes do not differ between cases and controls. However, *ABCB4* haplotypes differ significantly between both groups (table 2), as confirmed by permutation testing (see Methods, p = 0.019, 10 000 permutations). The haplotype *ABCB4_5* occurs more frequently in cases than in controls (5.8% *v* 0.9%), whereas haplotypes *ABCB4_3* and *ABCB4_7* are more common in controls (8.7% *v* 5.4% and 10.2% *v* 1.7%, respectively).

Table 3 shows that these associations are reflected by significantly different allele and genotype frequencies of the polymorphisms tagging the *ABCB4* haplotypes 3, 5 and 7. For the haplotype-tagging SNP c.711A→T, which tags haplotypes *ABCB4_3* and *ABCB4_7*, allele frequencies differed significantly between cases and controls (c.711A: odds ratio 2.27, 95% confidence interval (CI) 1.04 to 4.96, p = 0.037). The common OR for c.711A→T genotypes was 2.07 (p = 0.047). Allele frequencies of the SNP c.1954A→G (tagging haplotype *ABCB4_7* only) also differed between cases and controls (OR 3.98, 95% CI 1.08 to 14.72, p = 0.027). Of note, six cases were heterozygous carriers of the deletion in intron 5; this was not observed in any control in our study (OR 14.68, p = 0.012; table 3).

The supplementary figs A and B (provided online at http://www.gutjnl.com/supplemental) show de Finetti diagrams illustrating genotype and allele frequencies of the tagging polymorphisms. In these triangular diagrams, the frequencies of homozygotic genotypes are plotted using the left and right diagonal axes for homozygotes, whereas the frequencies of heterozygotes are shown on the left vertical axis. Consistency with Hardy–Weinberg equilibrium is indicated by the parabola, and allele frequencies are depicted by the intersection with the bottom perpendicular. For both *ABCB4* polymorphisms, the positions within the triangular diagrams and the allele frequencies differ significantly between patients with severe ICP and controls.

Multiple regression analysis did not show any significant association between liver chemistry tests and *ABCB4* genotypes; however, mean (SD) serum bile acids concentrations tended to be higher in patients carrying at least one at-risk *ABCB4* allele

Table 2 *ABCB4* genotypes and haplotypes

Haplotypes	htSNPs and deletions (codon position)*					Haplotype frequencies†	
	Intron 5 del AGAAA (−66 to −62)	Exon 6 (504)	Exon 8 (711)	Exon 16 (1954)	Intron 26 (−16)	Cases (%)	Controls (%)
ABCB4_1	0	T	A	A	C	45.5 (2.2)	50.5 (1.2)
ABCB4_2	0	C	A	A	C	21.6 (2.0)	17.5 (1.0)
ABCB4_3	0	C	T*	A	C	5.4 (0.8)	8.7 (0.8)
ABCB4_4	0	T	A	A	T*	11.3 (2.1)	8.0 (1.1)
ABCB4_5	1*	T	A	A	C	5.8 (0.6)	0.9 (0)
ABCB4_7‡	0	C	T*	G*	C	1.7 (0.2)	10.2 (0.4)

Case; woman with intrahepatic cholestasis of pregnancy; control, healthy woman without intrahepatic cholestasis of pregnancy; del, deletion; htSNP, haplotype-tagging single-nucleotide polymorphism.
Values are mean (SE).
*Asterisks indicate haplotype-tagging alleles.
†Haplotype frequencies differ significantly between cases and controls (p = 0.019), as indicated by permutation tests with 10 000 permutations.[26]
‡The haplotype *ABCB4_6* is omitted, as it was observed at a frequency of <3% in both cases and controls.

Table 3 Distributions of alleles and genotypes for selected *ABCB4* tagging polymorphisms in 52 patients with intrahepatic cholestasis of pregnancy and 52 matched controls

	Number (frequency) of alleles or genotypes		Tests for association*		
ABCB4 tagging polymorphism	Cases (2n = 104)	Controls (2n = 104)	OR	95% CI	p value
c.711A	93 (0.89)	82 (0.79)	2.27	1.04 to 4.96	0.037
c.711T	11 (0.11)	22 (0.21)			
c.711A/A	42 (0.81)	33 (0.63)	2.07		0.047
c.711A/T	9 (0.17)	16 (0.31)			
c.711T/T	1 (0.02)	3 (0.06)			
c.1954A	101 (0.97)	93 (0.89)	3.98	1.08 to 14.72	0.027
c.1954G	3 (0.03)	11 (0.11)			
c.1954A/A	49 (0.94)	42 (0.81)	5.43		0.033
c.1954A/G	3 (0.06)	9 (0.17)			
c.1954G/G	0 (0)	1 (0.02)			
wt intron 5	46 (0.88)	52 (1.00)	14.68	0.81 to 267.66	0.012
del intron 5	6 (0.12)	0 (0)			

Case; woman with intrahepatic cholestasis of pregnancy; control, healthy woman without intrahepatic cholestasis of pregnancy; del, deletion; wt, wild type.
*OR, 95% CI and p values were determined using contingency table statistics (see Methods). ORs were calculated relative to low-risk alleles (c.711T, c.1954G, no deletion in intron 5).

(146 (30) *v* 107 (9) μmol/l, p = 0.160). γ-Glutamyl transpeptidase activities, on the other hand, did not differ. In our cohort, only 3 of 13 (23%) patients for whom serum γ-glutamyl transpeptidase activities were available had raised levels and only one patient carried an at-risk *ABCB4* allele.

DNA sequence analyses showed no known mutations in exon 14 of the *ABCB4* gene for any patient tested (not shown). In particular, the C→A transversion in codon 546[11] and the single-nucleotide deletion starting in codon 571 (1712delT) previously described in single cases[10] were not detected, consistent with a previous report on Finnish patients with ICP.[27]

DISCUSSION

ICP is associated with maternal pruritus and increased fetal risk.[4–8 28] As the observational part of our ICP study showed that fetal complication rates did not increase until bile acid levels exceeded 40 μmol/l,[9] we focused our genetic analysis on these high-risk patients with severe ICP. This approach differs from previous studies on ICP genetics.[10–14 29–31] Our strategy, studying well-defined patients with more extreme phenotypes, is presumably more likely to identify clinically relevant susceptibility genes. Further, we recently showed that the subgroup of patients with severe ICP was more likely to benefit from treatment with ursodeoxycholic acid.[19] Ursodeoxycholic acid improved pruritus and all biochemical markers of cholestasis in patients with severe ICP. Mean bile acid levels decreased by 79%, bilirubin concentrations by 50% and alanine aminotransferase activities by 80% (all $p \leqslant 0.01$).[19]

It is still unclear whether environmental or genetic impairment of the bile-secretory apparatus is a prerequisite for liver injury in the case of ICP. Bile secretion normally depends on the integrity of an ensemble of membrane transport systems in hepatocytes and cholangiocytes.[1 32] The transport of all three major biliary lipids (ie, bile acids, phosphatidylcholine and cholesterol) across the canalicular membrane of hepatocytes into bile is mediated by ATP-dependent export pumps, known as ABC transporters.[33] Two major transporters are ABCB4 (formerly known as multidrug resistance gene 3, *MDR3*), which translocates phosphatidylcholine across the hepatocanalicular membrane,[34] and the bile acid export pump ABCB11, which is the predominant canalicular efflux system for conjugated bile acids.[35] Mutations in the genes encoding these hepatobiliary transporters have been identified as causative in progressive familial intrahepatic cholestasis in children.[12 18] In the light of these findings, *ABCB4* and *ABCB11* represent promising candidate genes, which could also determine the degree of cholestatic injury in patients with ICP. Thus, we investigated common variants of both genes in patients with severe ICP and matched controls. The study design was based on the expectation that there would be higher frequencies of the contributing genetic variants in patients than in a group of matched controls without the disease.

Pauli-Magnus *et al*[20] have defined the complete haplotype structure of the *ABCB4* and *ABCB11* genes by sequencing the promoters, the coding regions and the flanking intronic parts of the corresponding genes in a large number of individuals of European descent. Genetic variants have arisen from single historical mutational events and are therefore non-randomly associated with neighbouring alleles that were present on the particular chromosomal segments on which the mutation occurred (linkage disequilibrium). Haplotypes are defined as particular combinations of alleles that are inherited together on single chromosomes.[36] It is noteworthy that only few (<10) common haplotypes are observed in whole chromosomal regions, and these account for most of the genetic variation among individuals. In complex diseases, haplotype analysis is generally expected to yield more information than analysis of individual polymorphisms,[37] the rationale behind the design of the International HapMap Project that has released its first dataset recently.[36]

Owing to the block-like structure of linkage disequilibrium and the substantial correlations of SNPs with many of their neighbours,[36] the number of distinct combinations of SNP alleles encountered in human DNA samples is only a small fraction of the theoretical number of haplotypes if all alleles were distributed randomly. This haplotype structure is the basis for selection of a parsimonious set of SNPs, called haplotype-tagging SNPs, which represent most of the haplotype variation in a population. In our study, we identified the tagging polymorphisms using the Best Enumeration of SNP Tags algorithm,[25] which uses a powerful bayesian approach (PHASE) to reconstruct haplotypes,[26] and compared haplotype frequencies between cases and controls by permutation tests, thereby accounting for multiple testing.[26] Whereas *ABCB11* haplotypes did not differ between cases and controls (table 1), the distribution of *ABCB4* haplotypes differed significantly

between cases and controls, as determined by permutation testing ($p = 0.019$; table 2). The at-risk *ABCB4* haplotypes were characterised by tagging gene variants (deletion in intron 5, c.711A→T; table 3). The deletion in intron 5 tagging haplotype *ABCB4_5* was detected in 12% of the cases but not in controls, indicating a potentially much larger role for this *ABCB4* mutation in the case of severe ICP than previously acknowledged.

Our findings confirm the association between ICP and the SNP c.711A→T detected previously in a large UK cohort of 184 patients with ICP with bile acid levels >14 μmol/l.[29] Both studies independently confirm the association between ICP and the common allele of this *ABCB4* SNP (designated 743A in the study by Müllenbach *et al*[29]). As noted previously,[29] it is not unusual that the common allele represents the risk-associated variant in complex diseases—for example, a common truncating splice-site mutation has been associated with sarcoidosis recently.[38] Similar frequency deviations for the SNP c.711A→T were observed in a recent smaller study comparing the *ABCB4* sequence in 21 patients with ICP (bile acid levels >20 μmol/l) and 40 controls,[13] but did not reach statistical significance. In the study by Pauli-Magnus *et al*,[13] the intron 5 deletion was detected in one patient with ICP and two controls, indicating that this polymorphism is not ICP specific, but study participants were not matched for age, parity or geographical residence.[13] Although we observed markedly different allele and genotype frequencies for the SNP c.1954A→G in our ICP cases and controls, it has been assumed that the resulting missense mutation (R652G) is a neutral polymorphism that occurs with similar frequencies in cases and controls.[12 17 29 39] However, in contrast with a French study,[12] in our Swedish patients and in patients from Switzerland,[13] ICP seemed to be more prevalent among carriers of arginine at codon 652, and sequence comparisons of *ABCB4* genes identifies that the glycine residue is conserved in other species such as rat and hamster.

The increased prevalence of *ABCB4* gene variants in ICP is consistent with the very strong association between ICP and gallstone disease in our observational study,[9] which detected a 15-fold increase in gallstone disease among patients with severe ICP. Notably, *ABCB4* mutations have been linked to a symptomatic and recurrent form of gallbladder and intrahepatic cholesterol cholelithiasis.[40 41]

Our study could not exclude that the non-severe form of ICP, which we defined by bile acid levels <40 μmol/l,[9] might also be associated with *ABCB4* haplotypes, but this group of patients was not available for genetic analysis. However, only exclusion of the genetic association in a large cohort of patients with mild ICP would unambiguously prove that ICP severity depends on genetic and not environmental factors.

The distributions of common *ABCB11* haplotypes did not differ between cases and controls. Our findings and those of Pauli-Magnus *et al*[13] showed more genetic variability in *ABCB4* than in *ABCB11* in patients with ICP. Thus, our genetic analysis does not support the idea that common *ABCB11* variants have a role in the pathogenesis of ICP. However, it is possible that as yet unidentified rare variants of the *ABCB11* gene might play a part in ICP pathogenesis, as a recent study on 57 Finnish patients with ICP[30] linked two intragenic *ABCB11* SNPs to ICP. Our findings are in line with a recent Finnish family study,[31] in which segregation of haplotypes and multipoint linkage analysis excluded *ABCB11* as being responsible for the pathogenesis of ICP in two families with dominant inheritance. *ABCB11* haplotype structure has also been investigated in patients with primary biliary cirrhosis, primary sclerosing cholangitis[20] or gallstone disease, without any specific findings.[42]

In conclusion, our study identifies a genetic association between common *ABCB4* gene variants and severe ICP in Swedish patients. Haplotype analysis was used to guide our search for relevant ICP-associated polymorphisms. Our findings confirm and extend the results of previous genetic association studies[13 29] for the clinically most important ICP subtype. We speculate that genetic risk profiling might help to stratify cases at the time of diagnosis, even before bile acid concentrations exceed levels that indicate an increased fetal risk.

ACKNOWLEDGEMENTS

We thank all colleagues and midwives from the Västra Götaland region, Sweden, for enrolling and taking care of study patients. Study nurses Ann Christiansson and Gunilla Kaplan (Göteborg, Sweden) and Maren Klöckner and Valerie Pucken (Bonn, Germany) were of invaluable help during clinical and experimental studies.

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Funding: This study was supported in part by grants from the Department of Research and Development (FoU), Västra Götaland, Sweden, the Ministry of Innovation, Science, Research and Technology of North-Rhine-Westphalia (MIWFT-NRW) and BONFOR, University of Bonn, Germany.

Competing interests: None declared.

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