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# Sequence variation in the *ATP8B1* gene and intrahepatic cholestasis of pregnancy

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Intrahepatic cholestasis of pregnancy (ICP) is a cholestatic condition that may affect women during the third trimester of pregnancy. Symptoms experienced by these women generally resolve spontaneously following delivery, but prior to delivery the fetus is at increased risk of intrauterine distress and sudden intrauterine death. The genetic etiology of most cases of ICP is unknown, although heterozygous carriers of mutations causing progressive familial intrahepatic cholestasis (PFIC) diseases may experience ICP. When examining linkage to known cholestasis genes, affected members of four Finnish ICP families shared haplotypes around *ATP8B1*, the gene responsible for PFIC1. This gene was subsequently screened in 176 familial and sporadic ICP patients. A total of 17 sequence changes were detected, five exonic and 12 intronic. No intronic change was associated with ICP in sporadic cases. Four intronic changes segregated with ICP in three families, a different change in each of two families and three changes in another family, although the significance of this is currently unknown. Three exonic changes were nonsynonymous, one (in exon 23) is probably a polymorphism while two predict novel amino-acid replacements (N45T and K203R). These changes, in exons 2 and 7, were detected in one individual each, and may have predisposed these individuals to ICP. In conclusion, although the exon 2 and 7 changes may have functioned as risk alleles, *ATP8B1* is probably not a major gene contributing to the occurrence of ICP. *European Journal of Human Genetics* (2005) 13, 435–439. doi:10.1038/sj.ejhg.5201355  
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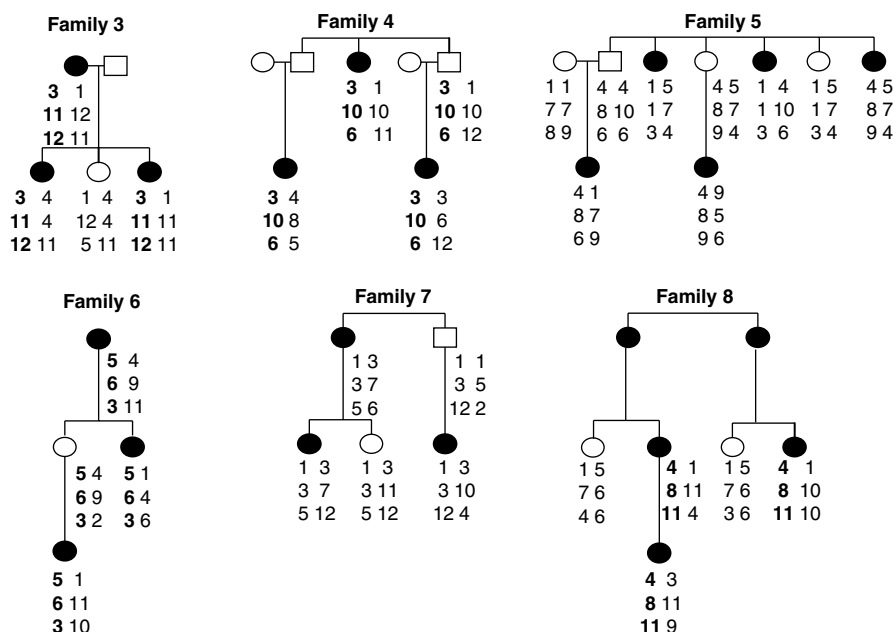
## Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a cholestatic condition complicating otherwise normal pregnancies. ICP usually arises in affected women during the third trimester and is characterized by generalized and possibly intense itching and increased levels of total bile acids and

transaminases in maternal serum. While this condition can be particularly uncomfortable for the mother, the prognosis is generally good, as symptoms resolve spontaneously soon after delivery and leave no permanent liver damage. The outcome for the fetus is less certain, as ICP is associated with increased rates of premature birth, intrauterine distress and also sudden intrauterine death.<sup>1,2</sup> In Scandinavian countries, ICP occurs in 0.5–1.5% of pregnancies.<sup>3,4</sup>

The genetic etiology of ICP is largely unknown, and likely to be heterogenous. Currently, investigation of the genetic background of ICP is focused mainly on candidate

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**Figure 1** Genotypes for microsatellites D18S977, D18S849 and D18S1144 flanking the *ATP8B1* gene for six Finnish ICP families. Shared haplotypes are in bold. Pedigrees and microsatellite genotypes for Families 1 and 2 are detailed in Savander *et al.*<sup>3</sup>

genes, particularly those causing cholestatic diseases. Mutations in three genes, *ATP8B1*, *ABCB11* and *ABCB4*, cause the recessively inherited progressive familial intrahepatic cholestasis (PFIC) types 1, 2 and 3. Heterozygous mothers of children with PFIC3 may experience ICP.<sup>5–7</sup> Subsequent screening has revealed, however, that mutations in *ABCB4* are responsible for ICP in only a minority of women,<sup>8,9</sup> and the importance of *ABCB11* is yet to be determined.<sup>9–11</sup>

In a previous study, Savander *et al.*<sup>3</sup> found no evidence for linkage to *ATP8B1*, *ABCB4* or *ABCB11* in two Finnish ICP families. Mutations in *ATP8B1* cause two distinct diseases, PFIC1 and benign recurrent intrahepatic cholestasis (BRIC).<sup>12</sup> Early reports suggested that PFIC1 and BRIC carriers may experience ICP.<sup>13,14</sup> PFIC1 manifests as cholestasis in infancy and progresses to end-stage liver disease before early adulthood. In contrast, BRIC may manifest at any age, occurs episodically, and resolves spontaneously leaving no permanent liver damage, thus resembling ICP. The aim of this study was to investigate the possible association between *ATP8B1* and ICP, by testing six additional families for linkage to *ATP8B1* and screening a large cohort of familial and sporadic ICP patients for *ATP8B1* sequence variation.

## Materials and methods

We screened 176 Finnish ICP patients, 34 affected women from eight ICP families and 142 sporadic patients who

experienced ICP but had no significant family history. All women fulfilled the study criteria for ICP (serum aspartate aminotransferase >35 U/l, serum alanine aminotransferase >35 U/l and/or serum bile acid values >8  $\mu$ mol/l with or without itching and negative for hepatitis B and C). Control groups (50 females, 50 males) were comprised of samples from 100 healthy Finnish blood donors, and of 100 CEPH samples chosen randomly from among those listed as grandparents. The study was approved by the institutional ethics committee and informed consent was obtained from all individuals.

Members of the six additional ICP families were genotyped at three microsatellite loci (D18S977, D18S849 and D18S1144) flanking *ATP8B1* (Figure 1). LOD scores were calculated with Genehunter version 2.1. Members of all eight ICP families and all sporadic cases were screened for variation in *ATP8B1* by denaturing high-performance liquid chromatography (dHPLC) (either an Agilent 1100 Series, Agilent Technologies, or a 3500HT WAVE, Transgenomic). Primer sequences and dHPLC conditions are in Table 1. Each exon was sequenced for all affected members of the eight ICP families, all samples exhibiting variant dHPLC traces and eight randomly chosen homozygote samples.

The location and description of the genomic changes are in reference to NM\_005603. Coding exons are numbered 2–28, as an additional 5' untranslated exon was recently discovered.<sup>15</sup> Differences in the proportion of heterozygotes between the sporadic ICP samples and both Finnish and CEPH controls were analyzed with two-tailed *t* tests,

**Table 1** Primer sequences, PCR annealing temperatures and dHPLC analysis temperatures for the 27 coding exons of *ATP8B1*

Exon	Primer sequences	Size (bp)	PCR annealing temp	dHPLC conditions
2	F TGCAGGCAGTATTCACCAA R CACGCAAAATAGACCGATCA	367	TD 60–50°C	T 58.3°C
3	F CTGCAATGAGCAGTTTCCAA R GTTACGTGGAAGGCAGGTGT	341	TD 60–50°C	T 56.7°C
4	F TGTAAGCTGTGGGACTTGTGA R TAGGCTGGTTCTGTGTATGAGG	275	TD 55–45°C	T 55.6°C
5 and 6	F TGACGGTGATGAGACTTGG R GGCGACAGAGCGAGACTCTA	527	TD 60–50°C	T 55.4°C
7 and 8	F TCCCTTGCCTGTAACCTAAATG R TTTAAATCAGGCCCATCGAG	261	TD 55–45°C	A 57°C
9 and 10	F AGGTTTCATGTCCAGGTATGG R TGGTTTTGATGGACAAAGGA	553	TD 55–45°C	A 56°C
11	F ATGCCTGGCCAAGAAGAGTA R CCCTTCTTCCTGCATTTGAA	326	TD 55–45°C	A 55°C
12	F GAAATGCAAGAGGTTGGAA R GGCACTATGTTGGGAGAAGG	319	TD 60–50°C	T 57.9°C
13	F TCCGAGCTCTCTACGGAAAA R AAATGAGTGACGGCTTCCAC	272	50°C	A 59°C
14	F AAGCAAAGCCAGGTAAGGAG R CAGCATCCCAAACGATTCTT	155	TD 60–50°C	T 58°C
15	F TGAAACCTTGCCTTTGAAGAA R GCCTGAGATGCCAGAGAAAC	274	TD 60–50°C	A 54°C
16	F GGGATTTCTCTCGCTTCCT R TGGGCACAAGCAACATCTAA	271	54°C	A 61°C
17	F CCGATACTGAAGTCTGCAC R TCAGAATCCCTTGACAGAAAGA	264	TD 55–45°C	T 56.3°C
18	F TTCTTTGCATTGGTGGATT R CCTTCTTCCATTGTGCCAGT	343	50°C	A 54°C
19	F GAGAGCAGCAACCAGGATG R TCATCTTGGGCAAAGGAAAC	308	52°C	A 55°C
20	F TGAGATGGGCAGATCACTTG R TTGCATTTGCAAAGATGAGC	299	51°C	A 55°C
21	F TCTCAGAGTCAAGGGCCTATTT R GCATCTAAAAGTGGCTCCAAA	267	TD 55–45°C	T 58.2°C
22	F TCTTGGGAATGGTACTCCTG R CCCTACACATTCCAGCCATT	425	55°C	A 56°C
23	F GGATGGTGAGCAAGAGCTTC R TAAGGAGACACAGCCCCAAA	497	54°C	A 57°C (I) <sup>a</sup> A 59°C (E)
24	F CATAGCAAAGACCCCCATCTC R CCTTGATGCCTGACAACAGA	361	51°C	T 58°C
25	F CAGGCTGCAACTTTTTGTGA R CACTGAATACGGCCAAATGAA	370	TD 55–45°C	A 59°C
26	F TCAAGCCACATCATGCCTAA R CCAGCCATTCCACCTTGAT	353	TD 55–45°C	T 54.5°C
27	F GGACTACAGGTGCACAC R AATTTTGCAGGAAACGTGCT	441	53°C	T 58.5°C
28	F GACAGAACTGCCTGCATCAA r TCCAACCCAAGGAGTTTGT	540	TD 50–45°C	T 60.2°C

A indicates exons analysed using the Agilent 1100 series, and T those run using the Transgenomic WAVE<sup>®</sup>.

<sup>a</sup>Different temperatures were needed to produce heteroduplex peaks for the exon 23 intronic (I) and exonic (E) SNPs.

where  $P \leq 0.01$  was taken to indicate a significant difference. The potential impact of amino-acid changes were assessed with the SIFT program,<sup>16</sup> which predicts tolerated and nontolerated amino-acid substitutions based on comparison to similar sequences.

## Results

When all eight families were included in the linkage analysis, single-point LOD scores were  $-1.99$  for D18S977,

$0.07$  for D18S849 and  $-0.48$  for D18S1144. Haplotypes were, however, shared by affected members in families 3, 4, 6 and 8 (Figure 1). When linkage was calculated for these families, the LOD scores were  $2.00$  for D18S977,  $2.18$  for D18S849 and  $2.79$  for D18S1144. Additional markers, haplotypes and linkage results are available from the authors.

Exonic base changes were detected in five exons (Table 2). One sporadic patient had a heterozygous  $c.134A > C$  change in exon 2, predicting the replacement of asparagine

**Table 2** ATP8B1 sequence variants in Finnish ICP patients

Exon	DNA variation (inferred amino-acid change)	Heterozygous ICP patients	% ICP (n = 142)	% Controls (n = 100)	% CEPH (n = 100)	SNP identifier <sup>a</sup>
<i>Exonic</i>						
2	c.134A>C (N45T)	1/142	0.7	0	0	—
3	c.246A>G	1/142	0.7	0	N/A	chr18:55158747
7	c.607A>G (K203R)	1/142	0.7	0	0	—
11	c.1014C>T	3/142	2.1	0	0	—
23	c.2855G>A (R952Q)	21/142	14.7	14	28	chr18:55107494
<i>Intronic</i>						
3	c.181-72G>A	22/142	15.5	23	8	—
6	c.554+122C>T	64/142	45.1	58	N/A	rs4940989
11	c.1029+35G>A	6/142	4.2	3	N/A	SNP 51293
17	c.1819-39_41delAA	58/142	40.8	36	28	—
20	c.2210-114T>C	71/142	50	40	N/A	rs17846
20	c.2210-45_50dupATAAAA	28/142	19.7	21	26	chr18:55119433
23	c.2932+59T>A	63/142	44.4	33	31	chr18:55107359
27	c.3401-175C>T	68/142	47.9	42	N/A	rs8097764
27	c.3401-167C>T	54/142	38:0	41	N/A	rs167603
27	c.3401-108C>T	64/142	45.1	48	36	—
27	c.3531+8G>T	37/142	26.1	32	30	chr18:55102583
28	c.3532-15C>T	57/142	40.1	41	37	chr18:55100951

Description of the genomic changes follows the current recommendations of the Human Genome Variation Society (<http://www.genomic.unimelb.edu.au/mdi/mutnomen/>). CEPH samples were not analyzed (N/A) for most SNPs described in the Entrez SNP database (NCBI).

<sup>a</sup>rs SNPs are detailed at <http://www.ncbi.nlm.nih.gov/SNP/>, SNP 51293 at <http://www.mutationdiscovery.com> and chr18: variants at <http://www.pharmgkb.org>.

with threonine. Another sporadic patient was heterozygous for a c.607A>G change in exon 7, predicting the replacement of lysine by glutamic acid. No control sample ( $n=200$ ) carried either of these changes. Of the 142 sporadic patients, 21 were heterozygous for a known c.2855G>A change in exon 23, predicting the replacement of arginine by glutamine. Heterozygotes for this change were equally frequent among ICP patients and Finnish controls (14.7 and 14% of samples), and twice as frequent among CEPH controls (28%). SIFT analysis predicted that the amino-acid replacements would be tolerated for the exon 7 change but not for the exon 2 and 23 changes. Synonymous changes were found in one patient in exon 3 and in three patients in exon 11, neither of which were detected in controls. All Finnish samples were homozygous for the alternate allele of a known SNP in exon 27 (rs222581, homozygous A).

Heterozygote frequencies did not differ between the sporadic ICP samples and controls for any intronic change (Table 2). Four changes segregated with ICP in three families: in Family 4 c.554+122C>T adjacent to exon 6, in Family 8 c.1819-39\_41delAA adjacent to exon 17 and in Family 6 c.181-72G>A adjacent to exon 3, c.554+122C>T and c.2210-114T>C adjacent to exon 20.

## Discussion

We detected five exonic base changes, three of which were novel, among 176 sporadic and familial ICP patients. Three

changes would result in the replacement of an amino acid. In addition, there were 12 intronic changes, three novel, none of which was associated with sporadic ICP in our sample group. As dHPLC can detect heterozygotes with high specificity and sensitivity, it is unlikely that we have missed any common, major ATP8B1 mutations contributing to ICP.

Heterozygotes for the c.2855G>A change in exon 23, in an area coding for part of the second cytoplasmic loop, were equally frequent among ICP patients and Finnish controls, and twice as frequent in CEPH controls. Although SIFT analysis predicted that replacement by glutamine would not be tolerated, it is probably a common sequence variant and therefore unlikely to contribute to ICP.

Base changes in exons 2 and 7 were each detected in one ICP patient. The c.134A>C exon 2 change occurs in a region coding for the N terminus of the protein, extending into the cytoplasm. No similar sequences were returned in a BLAST search with this exon; hence, the SIFT analysis predicted that this replacement would not be tolerated. Replacement of an acidic residue (arginine) with a polar residue (threonine), while both are small amino acids, may have an affect in this position. The c.607A>G exon 7 change, in an area coding for the first cytoplasmic loop, would result in the replacement of lysine by glutamic acid, the residue present in this approximate position in two of the closest human homologues of ATP8B1, ATP8B2 and ATP8B4. This replacement was, therefore, predicted to be tolerated. However, whether this change is a mutation or a polymorphism is unknown.

Intronic changes occurred with approximately equal frequencies in sporadic patients and controls. However, four intronic changes segregated with ICP in three families, with three changes in one family (Family 6). The significance of this is hard to determine. One plausible explanation could be that these alleles are linked to mutations in the *ATP8B1* promotor region, which remains to be identified. Mutations in this area may turn out to be important causes of cholestatic disease, particularly as the majority of both PFIC1 and BRIC patients do not have exonic mutations in *ATP8B1*.<sup>15</sup> Alternately, these changes could be linked to another, as yet unidentified, cholestasis gene.

PFIC and BRIC are recessively inherited diseases, while familial ICP appears to be dominantly inherited.<sup>5,17</sup> That ICP can occur in women heterozygous for mutations in cholestasis genes supports the idea that mutations that cause a serious disease in homozygous form may function as risk alleles in heterozygous form, although it is presently unknown whether the changes detected in this study predispose to ICP.

Recently, a number of studies have suggested associations between genetic polymorphisms and ICP,<sup>8,18–20</sup> which suggests that ICP may have a complex genetic background. Affected women experience cholestasis only when pregnant, and hence carry risk alleles that cause the disease only during this time of particular stress. Aside from mutations found in *ABCB4* in isolated individuals, the functional significance of most associations is unknown. Although our results indicate that *ATP8B1*, a putative ICP candidate gene, is not a major gene contributing to the occurrence of ICP in a large cohort of familial and sporadic Finnish ICP patients, the exon 2 and exon 7 changes may be risk alleles predisposing to ICP. Clearly, much further research is needed to determine the genetic etiology of this intriguing disease.

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