Sequence analysis of bile salt export pump (*ABCB11*) and multidrug resistance p-glycoprotein 3 (*ABCB4*, *MDR3*) in patients with intrahepatic cholestasis of pregnancy

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Intrahepatic cholestasis of pregnancy (ICP) is a liver disorder associated with increased risk of intrauterine fetal death and prematurity. There is increasing evidence that genetically determined dysfunction in the canalicular ABC transporters bile salt export pump (BSEP, *ABCB11*) and multidrug resistance protein 3 (MDR3, *ABCB4*) might be risk factors for ICP development. This study aimed to (i) describe the extent of genetic variability in *BSEP* and *MDR3* in ICP and (ii) identify new disease-causing mutations. Twenty-one women with ICP and 40 women with uneventful pregnancies were recruited between April 2001 and April 2003. Sequencing of *BSEP* and *MDR3* spanned 8–10 kb per gene and comprised the promoter region and 100–350 bp of the flanking intronic region around each exon. DNA sequencing of polymerase chain reaction fragments was performed on an ABI3700 capillary sequencer. *MDR3* promoter activity of promoter constructs carrying different ICP-specific mutations was studied using reporter assays. A total of 37 and 51 variant sites were detected in *BSEP* and *MDR3*, respectively. Three non-synonymous sites in codons for evolutionarily conserved amino acids were specific for the ICP collective (*BSEP*, N591S; *MDR3*, S320F and G762E). Furthermore, four ICP-specific splicing mutations were detected in *MDR3* [intron 21, G(+1)A; intron 25, G(+5)C and C(−3)G; and intron 26, T(+2)A]. Activity of the mutated *MDR3* promoter was similar to that observed for the wild-type promoter. Our data further support an involvement of *MDR3* genetic variation in the pathogenesis of ICP, whereas analysis of *BSEP* sequence variation indicates that this gene is probably less important for the development of pregnancy-associated cholestasis. *Pharmacogenetics* 14:91–102
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Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a liver disorder associated with increased maternal and fetal morbidity (e.g. risk of prematurity and intrauterine fetal death). Usually, ICP occurs in the third trimester of pregnancy and resolves spontaneously after delivery. The onset of ICP is typically heralded by the development of pruritus, which may precede laboratory abnormalities [1]. The main biochemical finding is an increase in total serum bile acid concentrations, which may be the first laboratory finding. Other facultative laboratory findings reflecting cholestasis are an elevation in serum alkaline phosphatase and total bilirubin, while gamma glutamyl transpeptidase levels are normal or only mildly elevated [2–4].

The cause of ICP is unknown, but oestrogens are likely to play a pathophysiological role because they have been shown to cause cholestasis in both experimental and clinical conditions [5]. Furthermore, ICP occurs during the third trimester, when serum concentrations of oestrogens reach their peak. ICP is also more common in twin pregnancies, which are associated with higher levels of circulating oestrogens compared to singleton pregnancies [6]. In accordance with these findings, cases of cholestasis have also been reported in non-pregnant women with a personal or a family history

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of ICP after being started on oral contraceptives [7]. ICP may also be associated with alterations in progesterone metabolism, and the administration of progesterone may constitute a risk factor for ICP [8].

On the other hand, there is increasing evidence that genetically determined dysfunction in the canalicular ATP-Binding Cassette (ABC)-transporters bile salt export pump (BSEP, *ABCB11*) and multidrug resistance protein 3 (MDR3, *ABCB4*) might be risk factors for the development of ICP. BSEP and MDR3 are two highly conserved members of the family of multidrug resistance P-glycoproteins, which are involved in the secretion of cholephilic compounds from the liver cell into the bile canaliculus. Under physiological conditions, BSEP appears to be the predominant bile salt efflux system of hepatocytes and mediates cellular excretion of numerous conjugated bile salts such as taurine- or glycine-conjugated cholate, chenodeoxycholate and deoxycholate. MDR3 was shown to function as an ATP-dependent phospholipid flippase, translocating phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane [9].

The impaired expression and function of these transporters has been shown to be the cause of different hereditary cholestatic syndromes, such as familial intrahepatic cholestasis type 2 (PFIC2) and 3 (PFIC3). It was observed that mothers of children with a PFIC3 phenotype often experience typical recurrent episodes of ICP. Interestingly, MDR3 deficiency in obstetric cholestasis has frequently been associated with a high γ-GT form of ICP, which is in parallel to PFIC3 patients, who exhibit a high γ-GT phenotype [10,11]. This led to the hypothesis that the heterozygous state for an *MDR3* gene defect represents a genetic predisposition for ICP [7].

Furthermore, recent data indicate an association between single nucleotide polymorphisms in *BSEP* with cholestasis of pregnancy [12]. Interestingly, data obtained both *in vitro* and *in vivo* indicate that BSEP can be inhibited by oestrogen metabolites [13,14]. Therefore, genetically determined decreased expression of this canalicular transporter might be a susceptibility factor in conditions with high circulating oestrogen levels, such as pregnancy.

To date, reports associating intrahepatic cholestasis of pregnancy with genetically determined dysfunction of MDR3 and BSEP are based upon case observations or genotyping for defined marker polymorphisms. The extent of genetic variation in these two genes in pregnant women with and without symptoms of cholestasis has not been investigated. Thus, the aim of the present study was two-fold: (i) to describe the extent of genetic variability in *BSEP* and *MDR3* in cholestatic and healthy pregnant control women and (ii) to search for new disease-causing mutations in a collective of unrelated Caucasian women with pregnancy-associated cholestasis.

Methods

After approval by the Ethics Committee of the University Hospitals of Zurich, Munich and Bonn and written informed consent from participating individuals, blood samples for DNA extraction were obtained from 21 women with ICP and 40 control women with normal pregnancies. All patients were unrelated individuals of Caucasian origin and self-specified their ethnic background. DNA samples from ICP and control women were obtained in collaboration with the Department of Gynaecology and Obstetrics at the University Hospital Zurich, the Department of Internal Medicine at the University Hospitals of Munich and Bonn and the Swiss Association for the Study of the Liver (SASL 16 Study).

Diagnosis of ICP was based upon the following criteria: (i) a clinical history of pruritus with or without jaundice, occurring in the second or third trimester of pregnancy; (ii) the presence of laboratory abnormalities suggestive of ICP: fasting serum bile acid values >20 μmol/l (normal range <10 μmol/l) and/or serum alkaline phosphatase levels >150 U/l (normal range <150 U/l) and/or alanine aminotransferase levels >50 U/l (normal range <50 U/l); and (iii) spontaneous resolution of clinical symptoms and laboratory findings after delivery. Furthermore, quantification of γ-GT levels in patients with obstetric cholestasis allowed the distinction of ICP forms with normal and elevated γ-GT levels. γ-GT levels were expressed as times the upper limit of normal (N) (Table 1).

Sequencing

Genomic and cDNA sequences were derived from known sequences (*BSEP*: GenBank accession number AC008177 for promoter and exon 1–21, AC069165 for exon 22–28 and NM_003742 for cDNA; *MDR*3: AC005068.2 for noncoding exons –3 to 1 and coding exon 2 and 3, AC006154 for exons 4–12, AC0005045 for exons 13–28 and NM_000443 for cDNA). Primers for genomic DNA were designed to span all exons and at least 100 bp of flanking intronic sequence at the 5′ and 3′ end of each exon. The DNA sequences of purified polymerase chain reaction (PCR) fragments were analysed on an ABI3700 capillary sequencer (ABI, Weiterstadt, Germany) and assembled using the phred-Phrap, consed and polyphred software University of Washington). Details regarding the primers, optimized PCR conditions and subsequent purification and sequencing of the fragments are available by e-mail from: info@epidauros.com

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Table 1 *BSEP* genetic variability

Variant number	Amplicon	DNA position	cDNA position	Nucleotide reference	Nucleotide variant	AA change	ICP ($n=42$)	Control ($n=80$)
Pro 1		Intron −1	(−2080)	CTCT	delCTCT		0.132	0.162
Pro 2		Intron −1	(−1952)	T	C		0.286	0.556
Pro 3		Intron −1	(−1820)	G	A		0.143	0.125
Pro 4		Intron −1	(−1746)	G	A		0.150	0.122
Pro 5		Intron −1	(−1275)	G	A		0.000	0.014
Pro 6		Intron −1	(−1239)	G	A		0.143	0.365
Pro 7		Intron −1	(−1155)	T	C		0.381	0.681
Pro 8		Intron −1	(−1009)	T	C		0.000	0.014
Pro 9		Intron −1	(−906)	C	T		0.000	0.026
Pro 10		Intron −1	(−603)	T	C		0.000	0.014
4.1.	Amplicon 4	Intron 3	(−20)	T	C		0.000	0.075
5.1.	Amplicon 5	Intron 5	(+8)	G	A		0.000	0.032
6.1.	Amplicon 6	Exon 6	402	C	T	Syn	0.000	0.038
6.2.	Amplicon 6	Intron 6	(+16)	G	A		0.000	0.038
10.1.	Amplicon 10	Intron 9	(−69)	C	T		0.000	0.038
10.2.	Amplicon 10	Intron 9	(−31)	C	T		0.000	0.038
10.3.	Amplicon 10	Intron 9	(−17)	G	A		0.000	0.075
10.4.	Amplicon 10	Intron 9	(−15)	A	G		0.857	0.650
10.5.	Amplicon 10	Exon 10	957	A	G	Syn	0.000	0.075
10.6.	Amplicon 10	Intron 10	(+18)	A	T		0.000	0.038
12.1.	Amplicon 12	Intron 11	(−91)	C	T		0.000	0.013
12.2.	Amplicon 12	Exon 12	1244	G	A	R415Q_c	0.000	0.013
12.3.	Amplicon 12	Intron 12	(+73)	G	T		0.000	0.013
13.1.	Amplicon 13	Exon 13	1331	T	C	A444V_c	0.833	0.513
13.2.	Amplicon 13	Intron 13	(+70)	C	T		0.833	0.514
14.1.	Amplicon 14	Intron 13	(−45)	C	G		0.028	0.000
14.2.	Amplicon 14	Intron 14	(+32)	T	C		0.833	0.513
15.1.	Amplicon 15	Exon 15	1772	A	G	N591S_c	0.026	0.000
15.2.	Amplicon 15	Exon 15	1791	G	T	Syn	0.026	0.000
17.1.	Amplicon 17	Exon 17	2029	A	G	M677V	0.000	0.056
18.1.	Amplicon 18	Exon 18	2134	T	C	Syn	0.000	0.013
19.1.	Amplicon 19	Intron 18	(−17)	C	A		0.619	0.583
20.1.	Amplicon 20	Intron 19	(−17)	T	C		0.684	0.813
22.1.	Amplicon 22	Intron 22	(−36)	G	A		0.000	0.013
24.1.	Amplicon 24	Exon 24	3084	G	A	Syn	0.325	0.413
26.1.	Amplicon 26	Exon 26	3561	A	G	Syn	0.000	0.016
28.1.	Amplicon 28	Intron 27	(−34)	G	A		0.368	0.423

cDNA numbers are relative to the ATG site and based on the cDNA sequence from GenBank accession number NM_003742. The amino acid (AA) position is indicated for those variants in the coding exonic sequence. Numbering of intronic variants is shown with respect to the corresponding exon. Intronic variants are designated with (+) or (−) for mutations located upstream or downstream of an exon, respectively. _c designates evolutionary conserved amino acids. Nucleotide changes in the intronic region are from the accession numbers AC008177 (promoter and exon 1–21) and AC0691165 (exon 22–28). Allele frequencies were calculated for patients with intrahepatic cholestasis of pregnancy (ICP) and controls.

MDR3 promoter constructs

DNA from three different ICP patients was used for the generation of three different *MDR3* promoter constructs covering 11 distinct promoter mutations found in ICP patients. *MDR3*-P1 contained the reference sequence, *MDR3*-P2 contained a combination of five promoter sites, designated as Pro 1, Pro 4, Pro 11, Pro 14 and Pro 15 (Table 2), and *MDR3*-P3 contains a combination of eight promoter sites, denominated as Pro 3, Pro 5–7, Pro 9–11 and Pro 15 (Table 2). PCR fragments were generated from patients DNA using the following primers: forward: 5′-GTACATGACA AACGCGTTGTAAAGTTAGGGGTG-3′ and reverse: 5′-GCCTCAAGATGGATCCCAGCCTGAGGAGAAA CCACAGC-3′. PCR fragments were cut with *Bam*H/*Mlu*I and cloned into a *Bgl*II/*Mlu*I site of the pGL3 vector (Promega Catalys AG, Wallisellen, Switzerland). The sequence was confirmed by full resequencing of the three constructs.

MDR3 promoter assay

HepG2 and human hepatoma (Huh7) cell lines were purchased from ATCC and maintained in RPMI640 (Sigma, St Louis, Missouri, USA) supplemented with 10% fetal calf serum (Gibco BRL, Cheshire, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL). For transactivation assays, cells were grown for 3 days in medium containing 10% charcoal-stripped bovine calf serum and then selected at 90–95% density in 24-well plates. For transient transfection, 1.5 µl of Lipofectamine 2000 reagent (Gibco BRL) and 500 ng of plasmid DNA were used per well. Plasmid DNA comprised 450 ng of *MDR3* promoter construct and 50 ng pSV-β-galactosidase plasmid.

Cells were lysed with passive lysis buffer (PBL; Promega Catalys AG) 24 h after transfection. Luciferase activity was quantified by using the luciferase assay system (Promega Catalys AG) in a Lumat LB 9507-2

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Table 2 ***MDR3*** **genetic variability**

Variant number	Amplicon	DNA position	cDNA position	Nucleotide reference	Nucleotide variant	AA change	ICP ($n=42$)	Control ($n=80$)
Pro 1	amplicon −3	Intron −4	(−394)	T	G		0.050	0.066
Pro 2	Amplicon −3	Intron −4	(−394)		insA		0.025	0.000
Pro 3	Amplicon −3	Intron −4	(−10)	C	T		0.028	0.000
Pro 4	Amplicon −3	Exon −3	(−410)	T	C	Non-coding	0.053	0.066
Pro 5	Amplicon −3	Exon −3	(−358)	A	G	Non-coding	0.026	0.000
Pro 6	Amplicon −2	Intron −3	(−31)	C	T		0.024	0.000
Pro 7	Amplicon −2	Exon −2	(−229)	C	T	Non-coding	0.095	0.154
Pro 8	Amplicon −2	Intron −2	(+234)	C	T		0.024	0.000
Pro 9	Amplicon −1	Intron −2	(−221)	C	T		0.095	0.150
Pro 10	Amplicon −1	Intron −2	(−204)	A	G		0.095	0.150
Pro 11	Amplicon 1	Intron −1	(−301)	C	G		0.167	0.225
Pro 12	Amplicon 1	Intron −1	(−292)	G	A		0.000	0.013
Pro 13	Amplicon 1	Intron −1	(−285)	T	C		0.000	0.013
Pro 14	Amplicon 1	Intron −1	(−220)	C	T		0.071	0.063
Pro 15	Amplicon 1	Intron −1	(−201)	C	G		0.167	0.138
Pro 16	Amplicon 1	Intron −1	(−184)	T	C		0.333	0.275
Pro 17	Amplicon 1	Exon 1	(−10)	C	A	Non-coding	0.024	0.000
4.1.	Amplicon 4	Exon 4	175	C	T	Syn	0.095	0.154
5.1.	Amplicon 5	Intron 4	(−61)	C	T		0.000	0.025
5.2.	Amplicon 5	Intron 5	(+113)	A	G		0.167	0.213
6.1.	Amplicon 6	Intron 5	(−62)	AGAAA	delAGAAA		0.026	0.027
6.2.	Amplicon 6	Exon 6	459	T	C	Syn	0.026	0.000
6.3.	Amplicon 6	Exon 6	504	C	T	Syn	0.525	0.500
6.4.	Amplicon 6	Exon 6	523	A	G	T175A_c	0.025	0.000
8.1.	Amplicon 8	Exon 8	711	A	T	Syn	0.167	0.213
9.1.	Amplicon 9	Intron 8	(−36)	T	G		0.000	0.016
9.2.	Amplicon 9	Exon 9	959	C	T	S320F_c	0.050	0.000
12.1.	Amplicon 12	Intron 11	(−88)	T	delT		0.125	0.103
12.2.	Amplicon 12	Intron 11	(−70)	A	G		0.000	0.015
12.3.	Amplicon 12	Intron 12	(+86)	G	A		0.024	0.000
12.4.	Amplicon 12	Intron 12	(+130)	G	T		0.067	0.227
13.1.	Amplicon 13	Intron 12	(−40)	G	A		0.950	0.919
14.1.	Amplicon 14	Intron 13	(−198)	A	C		0.000	0.013
14.2.	Amplicon 14	Exon 14	1584	G	C	E528D	0.000	0.013
16.1.	Amplicon 16	Exon 16	1954	A	G	R652G	0.100	0.163
16.2.	Amplicon 16	Intron 16	(+55)	A	G		0.100	0.145
17.1.	Amplicon 17	Intron 17	(+16)	T	C		0.975	0.879
18.1.	Amplicon 18	Intron17	(−139)	A	G		0.024	0.000
18.2.	Amplicon 18	Exon 18	2285	G	A	G762E_c	0.024	0.000
19.1	Amplicon 19	Exon 19	2324	C	T	T775M_c	0.000	0.013
20.1.	Amplicon 20	Intron 20	(+40)	A	G		0.100	0.176
21.1.	Amplicon 21	Intron 21	(+1)	G	A	Splicing	0.025	0.000
21.2.	Amplicon 21	Intron 21	(+98)		insTGT		0.000	0.013
21.3.	Amplicon 21	Intron 21	(+158)	T	C		0.150	0.064
23.1.	Amplicon 23	Intron 23	(+65)	T	A		0.000	0.013
25.1.	Amplicon 25	Intron 25	(+5)	G	C	Splicing	0.029	0.000
26.1.	Amplicon 26	Intron 25	(−3)	C	G	Splicing	0.028	0.000
26.2.	Amplicon 26	Intron 26	(+2)	T	A	Splicing	0.028	0.000
26.3.	Amplicon 26	Intron 26	(+53)	A	G		0.000	0.013
27.1.	Amplicon 27	Intron 26	(−16)	T	C		0.921	0.871
28.1.	Amplicon 28	Intron 27	(−72)	T	C		0.125	0.186

cDNA numbers are relative to the ATG site and based on the cDNA sequence from GenBank accession number NM_000443. The amino acid (AA) position is indicated for those variants in the coding exonic sequence. Numbering of intronic variants is shown with respect to the corresponding exon. Intronic variants are designated with (+) or (−) for mutations located upstream or downstream of an exon, respectively. Positions in non-coding exons are relative to the ATG site and designated with (−). _c designates evolutionary conserved amino acids. Nucleotide changes in the intronic region are from the accession numbers AC005068.2 (non-coding exons 1–3), AC006154 (exons 4–12) and AC0005045 (exon 13–28). Allele frequencies were calculated for patients with intrahepatic cholestasis of pregnancy (ICP) and controls.

luminometer (Berthold, Bad Wildbad, Germany). β-galactosidase activity was quantified with a high-sensitivity assay (Stratagene, La Jolla, CA, USA) in a UV max kinetic mircoplate reader (Molecular Devices, Sunnyvale, California, USA) at 595 nm.

Results

DNA samples from 21 women with ICP and from a control group of 40 women with normal pregnancies were screened for the presence of genetic variations in *BSEP* and *MDR3*. Approximately 10 000 bp of the *BSEP* gene were screened including (i) non-coding exon 1 and 2400 bp of the upstream promoter region, and (ii) coding exons 2–28 and 100–350 bp of the intronic sequence around each exon. NM_003742 is the *BSEP* reference sequence, which was derived from the *BSEP* sequence published by Strautnieks *et al.* [15]. For *MDR3*, approximately 8000 bp were screened in-

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Fig. 1

Secondary structure of the bile salt export pump with non-synonymous coding region genetic variants. The transmembrane topology scheme was generated using TOPO (S. J. Johns and R. C. Speth, transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO/topo.html). Common variants are shown in green, ICP-specific variants are shown in red, variants specific for the control group are shown in blue.

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cluding (i) 2000 bp of upstream promoter region including non-coding exons −3 to 1 and (ii) coding exons 2–28 and 100–350 bp of the intronic sequence around each exon. NM_000443 was used as reference sequence, which was derived from the *MDR3* sequence first published by van der Bliek *et al*. [16]. This variant, designated as variant A of the published *MDR3* sequences, represents the predominant form of the gene, which lacks both of the in-frame deletions that are found in variants B and C.

BSEP sequence variability

A total of 37 variant sites were found in *BSEP*, all of which were in Hardy–Weinberg equilibrium. The location and frequency of all variant sites in the different groups is shown in Table 1. Thirty-six variant sites were single nucleotide substitutions, which had only two alternative nucleotides and one was a 4-bp deletion in the promoter region. In the entire collective, 10 variants were in the promoter region, 17 were intronic variants and 10 were found in the coding region. Twelve of the variants were only found in a single chromosome; two were detected as doubletons. The 10 coding region variants included six synonymous and four non-synonymous changes. All four non-synonymous variants were located in the intracellular loop of BSEP (Fig. 1). Three coding region changes were polymorphisms that have already been described in a healthy Caucasian collective [17]: (synonymous: exon 24, G3084A; non-synonymous: exon 13, T1331C>V444A and exon 17, A2029G>M677V). Non-synonymous changes observed as singletons or doubletons in our sample set coded for the amino acid changes R415Q and N591S. Alignment of all mammalian *BSEP* sequences indicated that three of the four non-synonymous coding variants were in codons for evolutionarily conserved amino acids (R415Q, V444A and N591S) (Table 1).

The total number of variant sites was higher in the control group ($n = 34$) than in the ICP group ($n = 17$). This is in accordance with differences in sample size (40 controls, 21 ICP patients), which is expected to increase the number of rare variants detected in a population. Fourteen of the segregating sites were found in both groups and three (17.6%) were specific for the ICP patients. In the control group, 12 out of 20 specific sites were already observed in a bigger collective of Caucasian volunteers [17] (Pro 5, Pro 8–10, 4.1, 5.1, 10.1, 10.2, 10.3, 10.5, 12.3 and 18.1) (Table 1), resulting in eight (23.5%) specific variant sites in the control group. The three ICP-specific variants encoded for the following changes: intron 13, C-45G; exon 15, non-synonymous: A1772G>N591S and synonymous: G1791T.

MDR3 sequence variability

A total of 51 variant sites were found in *MDR3*, all of which were in Hardy–Weinberg equilibrium. The location and frequency of all variant sites in the different groups is shown in Table 2. Forty-seven variable sites were single nucleotide substitutions, which had only two alternative nucleotides. Two variable sites were deletions in intron 5 (5 bp) and intron 11 (1 bp) and two were insertions of 1 and 3 bp in the promoter and in intron 21, respectively. Seventeen variants were in the promoter region, 24 were intronic variants, including four splicing consensus variants, and 10 were found in the coding region. Twenty-four of the variants were only found in a single chromosome and two were detected as doubletons. The 10 coding region variants included four synonymous and six non-synonymous changes. Three non-synonymous variants were located in the intracellular loop of MDR3 and three in the transmembrane region (Fig. 2). Four coding region changes were polymorphisms (synonymous: exon 4, C175T and exon 6, C504T; non-synonymous: exon 6, A523G>T175A and exon 16, A1954G>R652G) also found in healthy Caucasians [17]. Non-synonymous changes newly observed as singletons or doubletons in our sample set coded for the following amino acid changes: S320F, E528D, G762E and T775M. Alignment of all mammalian *MDR3* sequences indicated that, with the exception of E528D and R652G, all non-synonymous coding variants were in codons for an evolutionarily conserved amino acid. The four splicing variants were located in intron 21, G(+1)A; intron 25, G(+5)C and C(−3)G; and intron 26, T(+2)A (Table 2).

The total number of variant sites was similar in the two groups (40 in ICP patients and 35 in the control goup). Given the lower number of individuals in the ICP group, this means a relative excess of variants in cholestatic women. Only 24 variants were found in both groups. Because T175A has already been detected in a larger cohort of healthy Caucasian volunteers [17], it cannot be counted as ICP-specific mutation, resulting in 15 (37.5%) and 11 (31.4%) variants specific for the ICP and the control group, respectively. The ICP-specific variants included two non-synonymous sites (heterozygous : exon 18, G762E; homozygous : exon 19, S320F) and one synonymous site (exon 6, T459C). The two ICP-specific non-synonymous sites were located in the transmembrane domain of the protein (Fig. 2). Furthermore, six intronic sites including the four splicing variants and six promoter sites, were ICP-specific (Table 2).

γ-GT values were available from 19 out of 21 patients with intrahepatic cholestasis of pregnancy (Table 3). Of these 19 patients, nine carried ICP-specific *MDR3* mutations and 10 did not. Seven out of these nine patients with ICP-specific MDR3 mutations (77%) had

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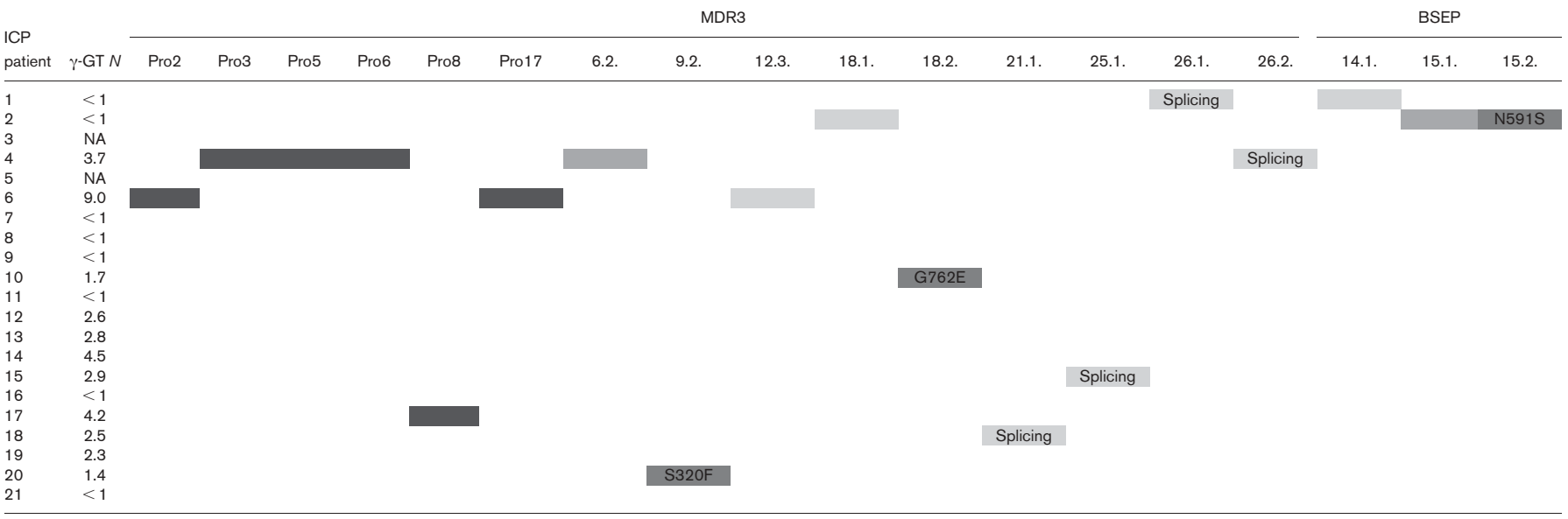
Table 3 Distribution of intrahepatic cholestasis of pregnancy-specific variants



ICP-specific variants are indicated with their respective variant number (Tables 1 and 2). Patients γ-glutamyl transferase levels (γ-GT) are expressed as *N*, where *N* is times the upper normal limit. NA, Data not available.
■ Promoter variants, □ intronic variants, ■ synonymous variants, ■ non-synonymous variants.

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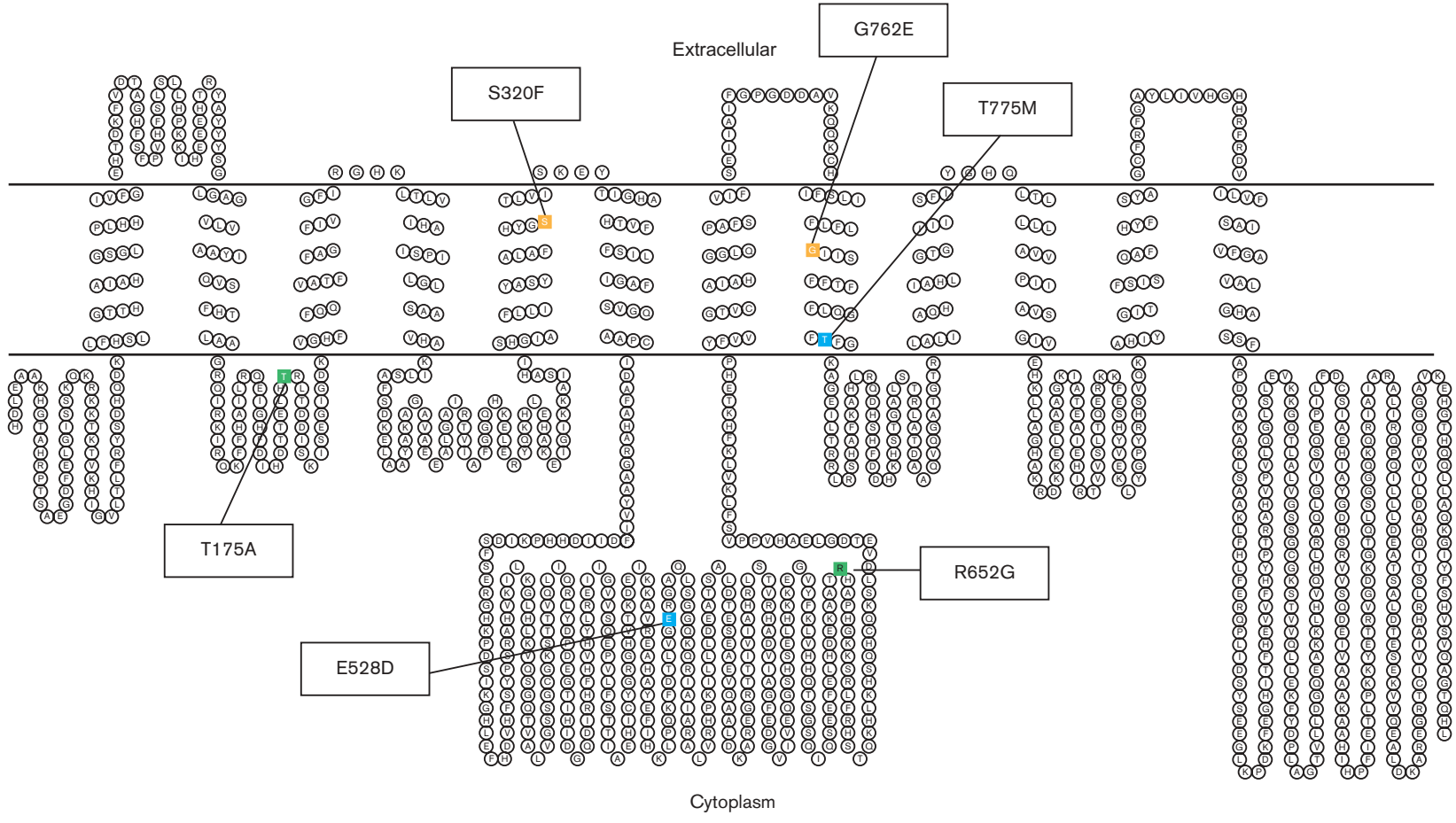
Fig. 2



Secondary structure of multidrug resistance protein 3 with non-synonymous coding region genetic variants. The transmembrane topology scheme was generated using TOPO (S. J. Johns and R. C. Speth, transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO/topo.html). Common variants are shown in green, ICP-specific variants are shown in red, variants specific for the control group are shown in blue.

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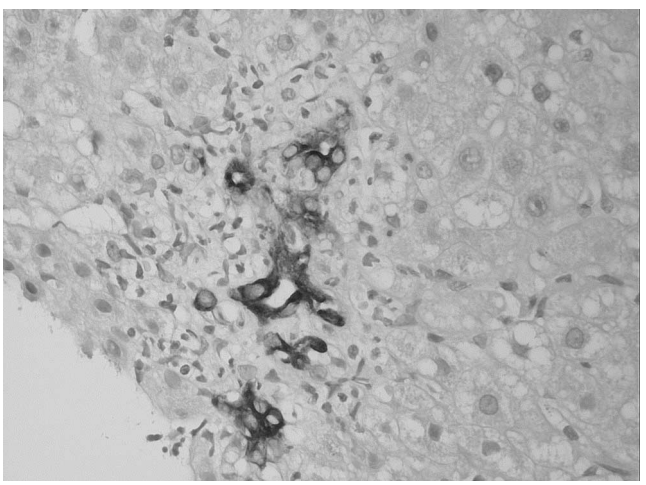
elevated γ-GT levels at the time of pregnancy-associated cholestasis (Table 3). Furthermore, both patients with ICP-specific non-synonymous *MDR3* mutations and three out of four patients with a heterozygous splicing consensus mutation in *MDR3* had a high γ-GT phenotype. Out of these, the woman carrying the newly identified T(+2)A mutation in intron 26 had a family history of pregnancy-associated cholestasis and experienced cholestasis during each of her three pregnancies, one of which was associated with intrauterine fetal death. Furthermore, the same patient experienced recurrent episodes of intrahepatic cholestasis also during non-pregnant periods and during stress or viral infection. A liver biopsy of this patient predominantly showed signs of bile duct destruction and marginal proliferation (Fig. 3), as well as inflammatory infiltrates (Fig. 4).

Of the remaining 10 patients without ICP-specific *MDR3* mutations, six had normal γ-GT values (60%), and four (40%) had elevated γ-GT values (Table 3). Two patients with normal γ-GT values and ICP-specific *MDR3* mutations (patients 1 and 2) carried additional ICP-specific *BSEP* mutations (Table 3).

MDR3 promoter assay

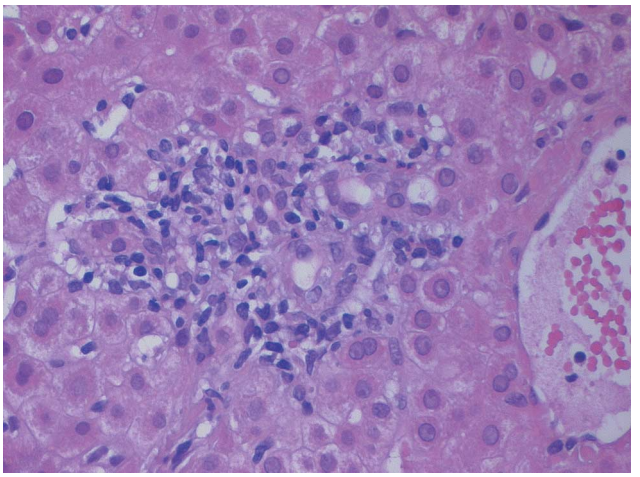
DNA from three different ICP patients was used for the generation of the promoter constructs. *MDR3*-P1 contains the reference sequence. Patients' DNA for the construction of *MDR3*-P2 and *MDR3*-P3 was selected to cover a maximum of common variants (Pro1, Pro4, Pro7, Pro9, Pro10, Pro11, Pro 14 and Pro15) as well as three ICP-specific promoter variants, present in the patient with a family history of pregnancy-associated cholestasis (Pro3, Pro5 and Pro6). Promoter activity was assessed for three different constructs in HepG2 and Huh-7 cells. The activity of the reference promoter (*MDR3*-P1) was set to 100%. In both cell lines, no differences in promoter activity of the mutated constructs were observed with respect to the promoter containing the reference sequence. In HepG2 cells, activity of *MDR3*-P2 and *MDR3*-P3 amounted to 96% and 103% of reference activity, respectively. In Huh-7 cells, the activity of *MDR3*-P2 and *MDR3*-P3 was 90% and 85% of reference activity, respectively (Fig. 5).

Fig. 3



Liver biopsy of the patient carrying the T(+2)A splicing mutation in exon 26 of the *MDR3* gene. Cytoceratin 19 staining showing predominant bile duct destructions and marginal bile duct proliferation.

Fig. 4



Liver biopsy of the patient carrying the T(+2)A splicing mutation in exon 26 of the *MDR3* gene. Haemotoxylin and eosin staining showing bile duct infiltration with lymphocytes.

Discussion

Our study is the first analysis of sequence variation in the promoter region as well as the entire exonic and flanking intronic regions of *BSEP* and *MDR3* in patients with intrahepatic cholestasis of pregnancy and a control group of women with uneventful pregnancies.

There is now increasing evidence that mutations in *MDR3*, and possibly also in *BSEP*, are associated with intrahepatic cholestasis of pregnancy [7,10,11,18–20]. A pathogenic involvement of *MDR3* mutations in the development of ICP was first suggested by Jacquemin *et al.* [10], who reported the coexistence of a PFIC3 phenotype and intrahepatic cholestasis of pregnancy associated with a frameshift mutation in *MDR3* in a large consanguineous family. Subsequently, different groups reported additional mutations in *MDR3* associated with the presence of ICP, yielding more than 10 different mutations that have been associated with the pathogenesis of ICP [7,10,11,18–20].

The present study provides further evidence for a pathogenic role of *MDR3* genetic variation in a subset

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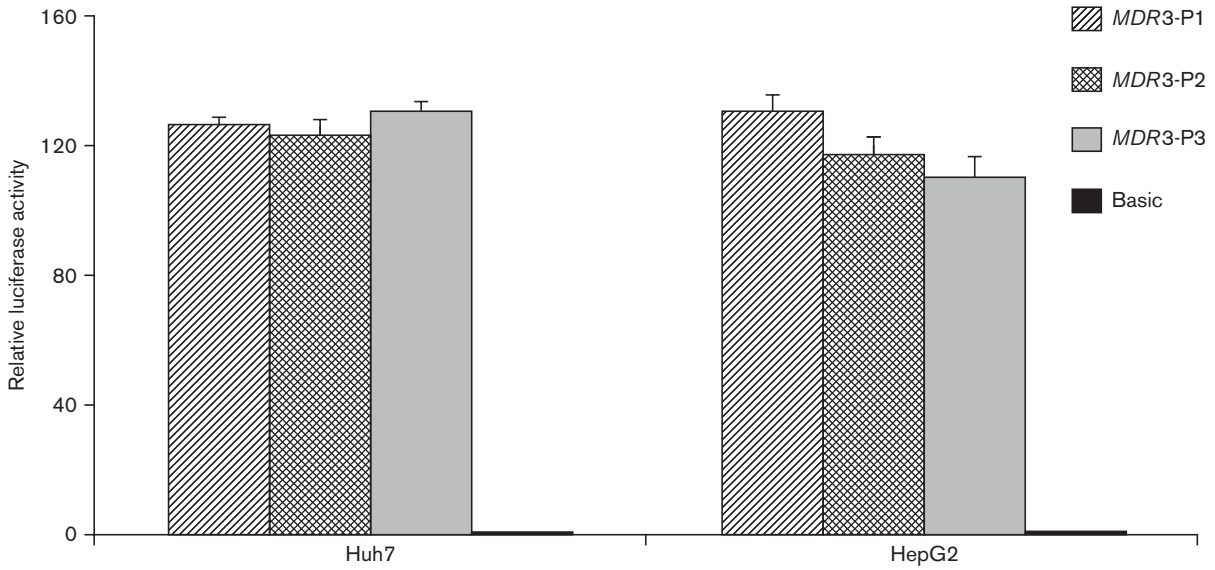
Fig. 5



Huh7 or HepG2 cells were transfected with the indicated constructs. Multidrug resistance protein 3 (MDR3) reporter constructs (*MDR3*-P1) showed a 100-fold higher luciferase activity compared to the empty pGL3 basic vector (Basic) in both cell lines. Genetic variants in the MDR3 promoter sequence (*MDR3*-P2, *MDR3*-P3) did not affect basal luciferase activity. Promoter activity is shown as the ratio of luciferase to β-galactosidase. Results are expressed as the mean ± SE of four to six transfections.

of patients with pregnancy-associated cholestasis and identifies new, possibly disease-causing, *MDR3* mutations. The percentage of ICP-specific variants in *MDR3* was more than two-fold higher than in *BSEP* (37.5% versus 17.6%) and the percentage of *MDR3* specific ICP-variants is higher than in the control group (37.5% versus 31.4%) even though the control collective was twice the size of the ICP collective. Out of the 15 ICP-specific *MDR3*-variants, six were promoter sites. None of the tested promoter variants found in both study collectives had a functional impact on baseline promoter activity, supporting the notion that they probably do not play a pathogenic role in the development of cholestasis. Furthermore, the three ICP-specific promoter variants found in the patient with a family history of cholestasis did not show any difference in promoter activity compared to the promoter containing the reference sequence. Because this patient carries one of the newly detected splicing consensus mutations [intron 26, T(+2)A] the absence of a functional involvement of the promoter mutations indicates a pathogenic role of the splicing mutation in intron 26.

Out of the coding region changes, two were newly identified non-synonymous mutations located in highly conserved regions of the protein (S320F and G762E). Interestingly, these non-synonymous mutations detected in the ICP collective were located in transmembrane domains of MDR3, whereas none of the non-synonymous variants encountered in the healthy Caucasian population were located in this region of the protein [17]. It has recently been suggested that the amino acid diversity of membrane proteins is significantly lower in transmembrane domains than in loop domains. This difference is especially pronounced for members of the ATP-binding cassette superfamily of transporters, in which very little variation has been observed in transmembrane domains even for unconserved residues [21]. Furthermore, the two ICP-specific mutations lead to marked changes with respect to amino acid size and electric charge. The presence of such important changes in highly conserved regions in the transmembrane domain of the MDR3 protein might therefore indicate a possible functional consequence of these mutations. Additionally, four new heterozygous *MDR3* splicing variants [intron 21, G(+1)A; intron 25, G(+5)C and C(−3)G; and intron 26, T(+2)A] were detected in our ICP-collective, all of which were absent in the control patients.

From a clinical perspective, two different forms of intrahepatic cholestasis of pregnancy can be distinguished according to γ-GT values and clinical evolution. High γ-GT values are often found in ICP patients with MDR3-deficiency, suggesting that this form would be MDR3-related [10,11]. This is in parallel to the observation in patients with an MDR3-related form of progressive familial intrahepatic cholestasis (PFIC3), who also show a high γ-GT phenotype [7]. In a recent study in a collective of 81 women with obstetric cholestasis, 30% exhibited such a high γ-GT form of ICP [22]. In our collective, nine out of 19 patients for

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whom γ-GT values were available (47%) had elevated γ-GT levels and seven of these patients (77%) carried ICP-specific *MDR3* mutations. Of the newly detected *MDR3* splicing mutations, three were present in patients with high γ-GT cholestasis. On the other hand, only two of the eight patients with normal γ-GT levels (25%) harbored ICP specific *MDR3* variants. In both patients (patients 1 and 2), these *MDR3* variants were combined with ICP-specific *BSEP* mutations. The predominance of a high γ-GT phenotype in patients with ICP-specific *MDR3* genetic variation might support a pathogenic role of MDR3 deficiency in high γ-GT obstetric cholestasis.

In contrast to *MDR3*, evidence for a pathogenic involvement of hereditary *BSEP* mutations is controversial. A possible role of BSEP in the development of pregnancy-associated cholestasis was first reported in a mother with an affected PFIC2 child [7]. Another study investigated the association of two intragenic *BSEP* marker single nucleotide polymorphisms in Finnish patients with ICP and unaffected controls and suggested that *BSEP* might also be a susceptibility gene for pregnancy-associated intrahepatic cholestasis [12]. However, genetic analysis of flanking markers for *BSEP* and *MDR3* in 16 individuals from two Finnish ICP families could not find such an association [23].

Our study in a collective of unrelated Caucasian ICP women does not support a strong role of *BSEP* mutations in the pathogenesis of pregnancy-associated cholestasis. In contrast to *MDR3*, the total number of *BSEP* variants in the ICP collective was only half of that observed for *MDR3* (17 versus 40). Furthermore, the higher number of *BSEP* sequence variants in the control group can be explained by the higher number of control subjects compared to ICP subjects. Additionally, only three (17.6%) of the *BSEP* mutations were specific for the ICP collective. Of these ICP-specific *BSEP* mutations, one was a new non-synonymous variation located in a highly conserved region of the intracellular domain of the BSEP protein (N519S) that had previously not been observed in healthy Caucasian controls [17]. Whether this mutation has any functional significance remains subject to further investigation.

Several conclusions can be drawn from our study. First, direct comparison of the genetic variation in *BSEP* and *MDR3* in patients with pregnancy-associated cholestasis and healthy control women further supports a possible involvement of *MDR3* genetic variation in the pathogenesis of ICP, whereas analysis of *BSEP* sequence variation indicates that this gene is probably less important for the pathogenesis of pregnancy-associated cholestasis. The high percentage of high γ-GT obstetric cholestasis in patients carrying ICP-specific *MDR3* mutations further indicates a pathogenic role of *MDR3* deficiency in this form of cholestasis. Second, genetic variation in *BSEP* and *MDR3* has so far only been described in patients with pregnancy-associated dysfunction of these transporters, while the extent of genetic variability in the normal population was unknown. However, in the absence of functional analysis *in vitro* or expression data obtained *in vivo*, the interpretation of mutation data is more meaningful if it can be compared to the pattern of genetic variation present in healthy controls, because disease-causing mutations should be absent in the control group. Third, all of the possibly disease-causing mutations identified through analysis of sequence variation in our patient collective have not been previously described. This genetic heterogeneity of ICP patients does not allow the easy genotyping of affected women for marker mutations in defined positions of the gene but requires a full sequencing of the exonic and flanking intronic regions to identify mutations. Finally, the absence of any possibly disease-causing genetic variation in *BSEP* or *MDR3* in more than half of the affected patients allows the conclusion that the aetiology of ICP is heterogeneous and that a genetic predisposition only plays a role in a subset of patients. This finding is supported by recent work by Savander *et al.* [23] who were able to exclude a pathogenic involvement of *BSEP* and *MDR3* in two Finnish ICP families through genotyping for flanking markers of these two genes [23]. In the future, the better characterization of hormonal and environmental factors associated with the development of ICP might help to more accurately identify those patients with a genetic predisposition.

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References

1 Kenyon AP, Piercy CN, Girling J, Williamson C, Tribe RM, Shennan AH. Obstetric cholestasis, outcome with active management: a series of 70 cases. *Br J Obstet Gynaecol* 2002; **109**:282–288.
2 Heikkinen J, Maentausta O, Ylostalo P, Janne O. Changes in serum bile acid concentrations during normal pregnancy, in patients with intrahepatic cholestasis of pregnancy and in pregnant women with itching. *Br J Obstet Gynaecol* 1981; **88**:240–245.
3 Lunzer M, Barnes P, Byth K, O'Halloran M. Serum bile acid concentrations during pregnancy and their relationship to obstetric cholestasis. *Gastroenterology* 1986; **91**:825–829.
4 Bacq Y, Sapey T, Brechot MC, Pierre F, Fignon A, Dubois F. Intrahepatic cholestasis of pregnancy: a French prospective study. *Hepatology* 1997; **26**:358–364.
5 Reyes H, Simon FR. Intrahepatic cholestasis of pregnancy: an estrogen-related disease. *Semin Liver Dis* 1993; **13**:289–301.
6 Gonzalez MC, Reyes H, Arrese M, Figueroa D, Lorca B, Andresen M, *et al.* Intrahepatic cholestasis of pregnancy in twin pregnancies. *J Hepatol* 1989; **9**:84–90.
7 Jacquemin E, De Vree JM, Cresteil D, Sokal EM, Sturm E, Dumont M, *et al.* The wide spectrum of multidrug resistance 3 deficiency: from

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neonatal cholestasis to cirrhosis of adulthood. *Gastroenterology* 2001; **120**:1448–1458.
8 Meng LJ, Reyes H, Palma J, Hernandez I, Ribalta J, Sjovall J. Effects of ursodeoxycholic acid on conjugated bile acids and progesterone metabolites in serum and urine of patients with intrahepatic cholestasis of pregnancy. *J Hepatol* 1997; **27**:1029–1040.
9 Meier PJ, Stieger B. Bile salt transporters. *Annu Rev Physiol* 2002; **64**:635–661.
10 Jacquemin E, Cresteil D, Manouvrier S, Boute O, Hadchouel M. Heterozygous non-sense mutation of the MDR3 gene in familial intrahepatic cholestasis of pregnancy. *Lancet* 1999; **353**:210–211.
11 Dixon PH, Weerasekera N, Linton KJ, Donaldson O, Chambers J, Egginton E, *et al*. Heterozygous MDR3 missense mutation associated with intrahepatic cholestasis of pregnancy: evidence for a defect in protein trafficking. *Hum Mol Genet* 2000; **9**:1209–1217.
12 Eloranta ML, Häkli T, Hiltunen MJ, Helisalmi S, Punnonen KR, Heinonen ST. Association of single nucleotide polymorphisms of the bile salt export pump with intrahepatic cholestasis of pregnancy. *Scand J Gastroenterol* 2003; **38**:648–652.
13 Stieger B, Fattinger K, Madon J, Kullak-Ublick GA, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* 2000; **118**: 422–430.
14 Huang L, Smit JW, Meijer DK, Vore M. Mrp2 is essential for estradiol-17beta (beta-D-glucuronide)-induced cholestasis in rats. *Hepatology* 2000; **32**:66–72.
15 Strautnieks SS, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, *et al*. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nature Genet* 1998; **20**:233–238.
16 van der Bliek AM, Kooiman PM, Schneider C, Borst P. Sequence of mdr3 cDNA encoding a human P-glycoprotein. *Gene* 1988; **71**: 401–411.
17 Pauli-Magnus C, Kerb R, Lang T, Fattinger K, Kullak Ublick GA, Beuers U, *et al*. BSEP and MDR3 sequence diversity and haplotype structure in primary sclerosing cholangitis and primary biliary cirrhosis [Abstract]. *American Association for the Study of Liver Diseases*. Boston: AASLD; 2003.
18 Eloranta ML, Heiskanen JT, Hiltunen MJ, Mannermaa AJ, Punnonen KR, Heinonen ST. Multidrug resistance 3 gene mutation 1712delT and estrogen receptor alpha gene polymorphisms in Finnish women with obstetric cholestasis. *Eur J Obstet Gynecol Reprod Biol* 2002; **104**:109–112.
19 Gendrot C, Bacq Y, Brechot MC, Lansac J, Andres C. A second heterozygous MDR3 nonsense mutation associated with intrahepatic cholestasis of pregnancy. *J Med Genet* 2003; **40**:E32.
20 Lucena JF, Herrero JI, Quiroga J, Sangro B, Garcia Foncillas J, Zabalegui N, *et al*. A multidrug resistance 3 gene mutation causing cholelithiasis, cholestasis of pregnancy, and adulthood biliary cirrhosis. *Gastroenterology* 2003; **124**:1037–1042.
21 Leabman MK, Huang CC, DeYoung J, Carlson EJ, Taylor TR, de la Cruz M, *et al*. Natural variation in human membrane transporter genes reveals evolutionary and functional constraints. *Proc Natl Acad Sci USA* 2003; **100**:5896–5901.
22 Milkiewicz P, Gallagher R, Chambers J, Eggington E, Weaver J, Elias E. Obstetric cholestasis with elevated gamma glutamyl transpeptidase: incidence, presentation and treatment. *J Gastroenterol Hepatol* 2003; **18**:1283–1286.
23 Savander M, Ropponen A, Avela K, Weerasekera N, Cormand B, Hirvioja M-L, *et al*. Genetic evidence of heterogeneity in intrahepatic cholestasis of pregnancy. *Gut* 2003; **52**:1025–1029.

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