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CLINICAL RESEARCH

Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump

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Abstract

AIM: To study the association of three common *ABCB11* and *ABCC2* polymorphisms (*ABCB11*: 1331T>C → V444A; *ABCC2*: 3563T>A → V1188E and 4544G>A → C1515Y) with intrahepatic cholestasis of pregnancy (ICP) and contraceptive-induced cholestasis (CIC).

METHODS: *ABCB11* and *ABCC2* genotyping data were available from four CIC patients and from 42 and 33 ICP patients, respectively. Allele-frequencies of the studied polymorphisms were compared with those in healthy pregnant controls and Caucasian individuals. Furthermore, serum bile acid levels were correlated with the presence or absence of the 1331 C allele.

RESULTS: The *ABCB11* 1331T>C polymorphism was significantly more frequent in cholestatic patients than in pregnant controls: C allele 76.2% (CI, 58.0-94.4) *vs* 51.3% (CI 35.8-66.7), respectively (P = 0.0007); and CC allele 57.1% (CI 36.0-78.3) *vs* 20% (CI 7.6-32.4), respectively (P = 0.0065). All four CIC patients were homozygous carriers of the C allele. In contrast, none of the studied *ABCC2* polymorphism was overrepresented in ICP or CIC patients. Higher serum bile acid levels were found in carriers of the 1331CC genotype compared to carriers of the TT genotype.

CONCLUSION: Our data support a role for the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-glutamyl transferase levels might help to distinguish *ABCB4*- and *ABCB11*-related forms of ICP and CIC.

Key words: Cholestasis of pregnancy; Contraceptive-induced cholestasis; Bile salt export pump; Multidrug resistance associated protein 2; Pharmacogenetics

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INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) and oral contraceptive-induced cholestasis (CIC) are two acquired forms of cholestasis, which are observed in otherwise healthy young women with a normal medical history. Both syndromes are rapidly reversible upon discontinuation of the hormonal challenge, which suggests that female sex hormones play a key pathogenic role in these forms of cholestasis[1,2]. In line with these observations, ICP usually occurs during the third trimester of pregnancy, when serum concentrations of estrogens and progesterone reach their peak[3,4]. Furthermore, women with ICP and female family members of ICP patients have an increased susceptibility to develop intrahepatic cholestasis under oral contraception[5].

A genetic predisposition for both types of hormonal cholestasis has been suspected based upon the strong regional clustering[6], the higher prevalence in female family members of patients with ICP[5,7] and the co-incidence with hereditary cases of progressive familial intrahepatic cholestasis[5,7]. Recently, mutations in the *ABCB4* gene that encodes the canalicular phospholipid flippase multidrug resistance protein

3 (MDR3) have been implicated in the development of ICP and CIC in a subset of affected patients[5,8-12]. MDR3-associated cases of hormonal cholestasis are associated with elevated serum γ-glutamyl transferase (γ-GT) levels in 80% of affected patients, which reflects cholangiocytic damage characteristic of MDR3 dysfunction[11].

In the same study, the majority of ICP women without *ABCB4* mutations exhibited normal γ-GT levels[11], which suggests a different pathogenic mechanism in this subset of patients. Dysfunction of the bile salt export pump (BSEP) or the multidrug resistance associated protein 2 (MRP2) have, therefore, been proposed as alternative candidate proteins involved in the pathogenesis of hormonal cholestasis. BSEP constitutes the predominant bile salt efflux system of hepatocytes, which mediates the cellular excretion of numerous conjugated bile salts into the bile canaliculus[13-16]. In contrast, the bilirubin transporter MRP2 is the main driving force for bile-salt-independent bile flow through canalicular excretion of reduced glutathione[17,18]. Given their important roles in bile formation and bilirubin secretion, inherited and acquired dysfunction of these proteins can lead to severe cholestatic syndromes and conjugated hyperbilirubinemia, respectively[19-21].

In hormonal cholestasis, *in vitro* inhibition of BSEP by estrogen and progesterone metabolites has been proposed as an underlying pathophysiological mechanism[22]. BSEP inhibition by estrogen and progesterone metabolites takes place from the luminal side of the bile canaliculus (so-called trans inhibition), which requires previous MRP2-mediated canalicular secretion of conjugated metabolites[22,23]. Therefore, MRP2 dysfunction might contribute to this form of cholestasis. While sequencing of *ABCB11* in unrelated ICP women has not revealed the presence of disease-causing BSEP mutations[11], only little attention has so far been paid to the possible pathogenic role of functional *ABCB11* and *ABCC2* polymorphisms. Recent observations have suggested that a non-synonymous polymorphism in exon 13 of the *ABCB11* gene (1331T>C) is overrepresented in drug-induced cholestatic liver injury[24]. The same polymorphism has recently been observed more frequently in ICP women compared to healthy controls, pointing towards a possible role of this polymorphism as a susceptibility factor for ICP and CIC[25]. Furthermore, two non-synonymous *ABCC2* polymorphisms (V1188E and C1515Y) showed significant differences in hepatic MRP2 expression levels compared to the wildtype sequence, which could be relevant for the extent of BSEP trans inhibition[25].

The aim of the present study was, therefore, threefold: (1) to compare allele frequencies of the aforementioned *ABCB11* and *ABCC2* polymorphisms in a prospectively recruited group of patients with ICP and CIC; (2) to define the relative risk of the different polymorphisms for the development of ICP; and (3) to determine the extent of the increase in serum bile acid levels as marker of cholestasis in the presence of the different *ABCB11* 1331T>C genotypes.

MATERIALS AND METHODS

Patients and controls

After approval by the Ethics Committee of the University Hospital of Zurich and written informed consent from all participating individuals, blood samples for DNA extraction were obtained from Caucasian patients with ICP or CIC. The total population of analyzed individuals consisted of two different groups: 25 patients (21 ICP_{new} patients and four CIC patients) were prospectively recruited for this study, and a second group of 20 patients (ICP_{old}) had already been described in a previous study by Pauli-Magnus and coworkers[11].

Two hundred and five Caucasian volunteers and patients without cholestasis, as well as Caucasian women with uneventful pregnancies (n = 40), served as a control population for BSEP (*ABCB11*) and MDR3 (*ABCB4*) genetic variants. These controls have already been described in previous studies[11,25,26]. Specifically, pregnant controls were all healthy, as defined by normal serum levels of transaminases, bilirubin, γ-GT, alkaline phosphatase (AP) and bile acids. Caucasian controls from the study of Pauli-Magnus and coworkers[26] (n = 95) were healthy volunteers recruited for participation in phase I studies, with uneventful medical history and normal blood biochemistry. Neither of these two control groups took any regular medication. In the case of the Caucasian control population of Meier and coworkers[25] (n = 110), most patients suffered from extrahepatic malignancies, and cholestatic disease was excluded in all patients. Furthermore, none of these patients used medication known to be associated with the development of cholestasis.

For lack of DNA availability, only 110 out of 205 Caucasian controls could be used for MRP2 sequencing. For the same reason, a new group of Caucasian women with uneventful pregnancies (n = 42) had to be collected for the MRP2 variants. Demographic data and pregnancy course of these women did not differ from the previous control group.

Diagnosis of ICP was based upon: (1) a clinical history of pruritus, which occurred in the third trimester of pregnancy; (2) the presence of laboratory abnormalities suggestive of ICP: fasting serum bile acid $\geqslant$ 1.5 ULN (upper limit of normal) and/or serum AP levels $\geqslant$ 1.5 ULN and/or alanine aminotransferase (ALT) levels $\geqslant$ 1.5 ULN; and (3) spontaneous resolution of clinical symptoms and laboratory findings after delivery. Diagnosis of CIC was based upon laboratory abnormalities as defined for ICP and the exclusion of preexisting liver disease defined by: (1) a negative serology for hepatitis A, B and C; (2) the exclusion of other preexisting medical conditions that could explain liver injury, such as congestive heart failure, systemic infection, or malignancy; (3) normal liver ultrasound; and (4) a clear causal relationship to drug intake. Each case of ICP and CIC was evaluated by at least one obstetrician and one hepatologist, as well as by a clinical pharmacologist.

Full length *ABCB4* and *ABCB11* sequencing data were already available from the control groups, as well as from ICP_{old} patients. To allow detection of additional *ABCB4* and *ABCB11* mutations in the new group, complete sequencing of these two genes was also performed in the 25 newly recruited patients. Genotyping of *ABCC2* included all CIC patients, as well as 17 out of 21 patients from the ICP_{new} group and 16 of 21 patients in the ICP_{old} group, which yielded a total number of 33 patients for *ABCC2*

Table 1 Primers and probes of real-time PCR for allelic discrimination of *ABCC2* SNPs in Caucasians

cDNA position[1]	SNP	Exon	Amino acid change	Eense-/antisense primer	Probes[2]
1249	G>A	10	V417I	5'-CCAACTTGGCCAGGAAGGA-3'/	VIC 5'-CTGTTTCTCCAACGGTGTA-3'
				5'-GGCATCCACAGACATCAGGTT-3'	FAM 5'-ACTGTTTCTCCAATGGTGTA-3'
3563	T>A	25	V1188E	5'-GCACCAGCAGCGATTTCTG-3'/	VIC 5'-ACACAATGAGGTGAGGAT-3'
				5'-AGGTGATCCAGGAAAAGACACATTT-3'	FAM 5'-ACAATGAGGAGAGGAT-3'
4544	G>A	32	C1515Y	5'-GTAATGGTCCTAGACAACGGGAAG-3'/	VIC 5'-AGAGTGCGGCAGCC-3'
				5'-CCAGGGATTTGTAGCAGTTCTTCAG-3'	FAM 5'-ATTATAGAGTACGGCAGCC-3'

[1]cDNA sequence from GenBank accession numbers NM_000392 starting at the ATG; [2]For each SNP two probes were designed and labeled with the fluorescent reporter dyes VIC (allele 1) and FAM (allele 2). SNP: Single nucleotide polymorphism.

genotyping. In nine patients (four ICP_{new} and five ICP_{old}), no *ABCC2* genotyping could be performed for lack of DNA availability.

Sequencing and genotyping

Isolation of DNA and DNA sequencing was done at Epidauros Biotechnology AG, Bernried, Germany. Genomic and cDNA sequences were derived from known sequences (*ABCB4*: AC005068.2 for non-coding exons -3 to 1 and coding exons 2 and 3; AC006154.1 for exons 4 to 12; AC0005045.2 for exons 13 to 28; and NM_000443.2 for cDNA; *ABCB11*: GenBank accession number AC008177.3 for promoter and exons 1 to 21; AC069165.2 for exons 22 to 28 and NM_003742.2 for cDNA).

ABCB4 and ABCB11: Sequencing of *ABCB4* covered a proximately 8000 bp, including (1) 2000 bp of the upstream promoter region and non-coding exon -3 to 1 and, (2) coding exons 2-28 and 100-350 bp of the intronic sequence around each exon. For *ABCB11*, sequencing covered 10000 bp including (1) non-coding exon 1 and 2400 bp of the upstream promoter region and, (2) coding exons 2-28 and 100-350 bp of the intronic sequence around each exon. Primers for genomic DNA were designed to span all exons and at least 100 bp of the flanking intronic sequence at the 5' and 3' end of each exon. The DNA sequence of purified PCR fragments was analyzed on an ABI3700 capillary sequencer (ABI, Weiterstadt, Germany) and assembled using the phredPhrap, Consed and PolyPhred software (University of Washington). Details regarding the primers, optimized PCR conditions and subsequent purification and sequencing of the fragments are available at info@epidauros.com.

ABCC2: Three non-synonymous polymorphisms with a potential impact on MRP2 function and expression were chosen for genotyping[25]: 1249G>A variant (V417I, rs2273697), 3563T>A (V1188E, rs17222723) and 4544G>A (C1515Y, rs8187710). Genotyping was performed with the Custom TaqMan SNP Genotyping Assays procedure (Applied Biosystems, Foster City, CA, USA) which contained a sense- and an antisense primer and two probes, labeled with fluorescent reporter dyes, either VIC or 6-Fam at the 5' end and a non-fluorescent quencher at the 3' end to distinguish between alleles 1 and 2, respectively. Primer and probe sequences for individual SNPs are given in Table 1. Probe solution (0.625 μL) and 12.5 μL of 2 × Universal PCR Master Mix (Applied Biosystems) were brought to 25 μL with 20 ng of genomic DNA. PCR reaction (2 min at 50℃, followed by 10 min at 95℃ and 40 cycles of 15 s at 92℃ and 1 min at 60℃). Allelic discrimination was processed with an ABI PRISM 7700 Sequence Detector.

Statistical analysis

Genotype distribution, allelic frequencies and odds ratios (ORs) are given with 95% CI. In *ABCB11*, formal statistical analysis was only performed for the 1331T>C polymorphisms (rs2287622), whereas for *ABCC2* analysis, it included two highly linked polymorphisms. No correction according to Bonferroni was, therefore, required. Differences investigated in our study apply to a proportion of diseased *versus* non-diseased individuals within the whole population, using an unmatched case control design. Response (ICP *versus* non-ICP) and predictors (T *versus* C) were both binary variables and were therefore best condensed into a 2 × 2 table. Differences in genotype distribution between patients and controls were calculated with the χ^2 test, and difference in allelic frequencies between two groups was performed using a 2 × 2 Fisher exact test. $P \leqslant 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

A total of 25 unrelated patients with estrogen-associated intrahepatic cholestasis were prospectively enrolled in this study, 21 with ICP and four with oral CIC. Demographic data and laboratory findings in ICP patients are given in Table 2. Only two patients showed elevated γ-GT levels > 1.5 ULN, while total bile acid levels were elevated in all patients in whom it was determined (16 out of 21; range, 1.7-17.3 ULN). Three patients had a previous history of ICP; three pregnancies were twin pregnancies, and one patient experienced cholestasis under previous oral contraception.

Characteristics of patients with CIC are given in Table 3. One patient showed elevated γ-GT levels. Total bile acid levels were elevated in all three patients in whom it was determined (three out of four; range, 1.6-22.3 ULN). Oral contraceptive preparations used in the four patients contained comparable amounts of ethinylestradiol (20-35 μg) while the progesterone-like portion ranged from 50 to 150 μg. All patients had a liver biopsy done for strictly diagnostic reasons, which showed intrahepatic cholestasis in three patients. One patient had a previous history of ICP.

Table 2 New group of patients with ICP (ICP$_{new}$)

Patient ID	Age (yr)	Liver parameters					Comments		Genotypes of SNPs		
		ALT (ULN)	AP (ULN)	γ-GT (ULN)	tBili (ULN)	tBA (ULN)	No of preg/ No ICP	Others	*ABCB11* 1331T>C (V444A)	*ABCC2* 3600T>A (V1188E)	*ABCC2* 4581G>A (C1515Y)
1	36	0.9	2.3	3.3	0.8	10.7	2/1		CC	TA	GA
2	31	1.6	2.5.	0.3	0.8	17.3	3/2		TC	TA	GA
3	35	8.9	2.3	1	0.5	10	1/1		TC	TT	GG
4	29	1.2	3	0.6	0.5	7	2/1		CC	TT	GG
5	42	11.6	1.4	0.4	0.8	6	1/1		TC	TT	GG
6	28	4.8	1.8	0.2	0.8	4.7	2/1	Twins	CC	TA	GA
7	32	11.9	3	1.2	0.9	3.6	nd		CC	TT	GG
8	16	5.2	2	0.4	nd	3.3	nd		TC	TT	GG
9	38	6.2	1.2	1.1	0.4	3	2/1		TC	TT	GG
10	23	1.6	1.3	1.3	0.8	2.4	1/1	Twins	TC	TT	GG
11	30	0.4	0.9	0.2	0.5	2.4	3/1		TC	TT	GG
12	22	0.3	1.8	0.1	0.5	2.1	nd		TC	TA	GA
13	32	0.2	0.9	0.7	0.2	2	2/1		TT	TT	GG
14	30	0.8	1.2	0.3	0.6	1.7	1/1		CC	TT	GG
15	28	7.5	2.3	0.9	0.6	nd	nd		TC	TT	GG
16	31	0.9	2.1	1.2	0.5	8.1	1/1		CC	TT	GG
17	20	3.4	1.1	0.4	0.5	2.6	1/1		TT	TT	GG
18	24	7.2	1.3	0.4	0.8	nd	1/1		CC	nd	nd
19	31	8.9	1.3	1.2	1.5	nd	2/2		CC	nd	nd
20	32	11	2.2	0.4	3.1	nd	3/3	Pruritus with contraceptives	CC	nd	nd
21	41	10.9	2.9	5.7	1.2	nd	1/1	Twins	CC	nd	nd
Summary median (Q1; Q3)	31 (28; 32)	4.8 (0.9; 8.9)	1.8 (1.2; 2.3)	0.6 (0.4; 1.2)	0.6 (0.5; 0.8)	3.5 (2.4; 7.3)					

n/a: Not available; ALT: Alanine aminotransferase; tBili: Total bilirubin; tBA: Total bile acids.

Table 3 Characteristics of patients with oral CIC

Patient ID	Oral contraceptive	Age (yr)	Exposure time	Liver parameters					Comments		Genotypes of SNPs		
				ALT (ULN)	AP (ULN)	γGT (ULN)	tBili (ULN)	tBA (ULN)	Clinical features	Histology	*ABCB11* 1331T>C (V444A)	*ABCC2* 3600T>A (V1188E)	*ABCC2* 4581G>A (C1515Y)
1[1]	30 μg ethinylestradiol/ 75 μg gestodene	32	nd	4.9	1.7	1	10.9	22.3	Jaundice	Intrahepatic cholestasis	CC	TT	GG
2	30 μg ethinylestradiol/ 150 μg levonorgestrel	15	21 d	1	3	1	4.2	nd	Jaundice, nausea, pruritus	Extensive intrahepatic cholestasis	CC	TT	GG
3	35 μg ethinylestradiol/ 50 μg levonorgestrel	40	2 yr	3.9	2.8	3.6	0.5	1.6	Pruritus	Bland	CC	TT	GG
4	35 μg ethinylestradio/ 2 mg cyproteron	34	nd	1	1.3	nd	2.8	1.6	Jaundice	Extensive canalicular cholestasis, mild portal inflamation	CC	TA	GA

[1]Patient exhibited previous episodes of ICP.

Sequence analysis

ABCB4 and ABCB11: Sequence analysis in the 25 newly recruited patients with estrogen-associated cholestasis revealed no disease-associated non-synonymous mutations in *ABCB4* or *ABCB11*. Furthermore, in line with previous findings[11], no *ABCB4* polymorphism was found to be overrepresented in the ICP and CIC groups compared to pregnant women without cholestasis and healthy Caucasian individuals. All of the detected genetic variants in *ABCB11* and *ABCB4* were in Hardy Weinberg equilibrium.

In contrast, the *ABCB11* 1331T>C → V444A polymorphism was significantly more frequent in ICP and CIC patients compared to the two control groups. Specifically, the CC genotype was encountered in 57.1% of all ICP patients (ICP$_{new}$, 47.6% and ICP$_{old}$, 67.7%) and 100% of CIC patients compared to 20 and 32.2% in pregnant

Table 4 Genotype distribution of non-synonymous *ABCB11* variant site 1331T>C in patients and controls

Genotype SNP	ICP_{old}		ICP_{new}		ICP_{total}		Pregnant controls		Caucasian controls	
	n (%)	95 % CI	*n* (%)	95 % CI	*n* (%)	95 % CI	*n* (%)	95 % CI	*n* (%)	95 % CI
ABCB11 1331T>C (V444A)	21 (100)		21 (100)		42 (100)		40 (100)		205 (100)	
TT (VV)	-	-	2 (9.5)	0.0-22.1	2 (4.8)	0.0-13.9	7 (17.5)	5.7-29.3	38(18.5)	13.2-23.9
CC (AA)	14 (67.7)	46.5 -86.8	10 (47.6)	26.3-69.0	24 (57.1)	36.0-78.3	8 (20)	7.6-32.4	66 (32.2)	25.8-38.6
TC (VA)	7 (33.3)	13.2-53.5	9 (42.9)	21.7-64.0	16 (38.1)	17.3-58.9	25 (62.5)	47.5-77.5	101 (49.3)	42.4-56.1
Frequency C allele	35 (83.3)	67.4-99.3	29 (69.0)	49.3-88.8	64 (76.2)	58.0-94.4	41 (51.3)	35.8-66.7	233 (56.8)	50.1-63.6
Frequency T allele	7 (16.7)	0.7-32.6	13 (31.0)	11.2-50.7	20 (23.8)	5.6-42.0	39 (48.8)	33.3-64.2	177 (43.2)	36.4-50.0
ABCC2 3563T>A (V1188E)	16 (100)		17 (100)		33 (100)		42 (100)		110 (100)	
TT (VV)	15 (93.8)	71.3-98.6	13 (76.5)	52.3-90.4	28 (84.8)	68.9-93.3	37 (88.1)	74.3-96.1	95 (86.4)	68.9-93.3
AA (EE)	-	-	-	-	-	-	-	-	1 (0.9)	0.0-5.0
TA (VE)	1 (3.1)	0.0-15.8	4 (23.5)	9.6-47.7	5 (15.2)	6.7-31.1	5 (11.9)	3.9-25.7	14 (12.7)	7.1-20.5
ABCC2 4544G>A (C1515Y)	16 (100)		17 (100)		33 (100)		42 (100)		110 (100)	
GG (CC)	15 (93.8)	71.3-98.6	13 (76.5)	52.3-90.4	28 (84.8)	68.9-93.3	36 (85.7)	71.4-94.6	95 (86.4)	68.9-93.3
AA (YY)	-	-	-	-	-	-	-	-	1 (0.9)	0.0-5.0
GA (CY)	1 (3.1)	0.0-15.8	4 (23.5)	9.6-47.7	5 (15.2)	6.7-31.1	6 (14.3)	5.4-28.6	14 (12.7)	7.1-20.5

Results are given with 95 percent confidence interval (95% CI).

Table 5 1331T>C (V444A): Fisher's exact test and ORs for the presence of homozygous CC variant and the C allele in the different groups

	CC *vs* TT			C *vs* T		
	Fisher	Odds ratio	95% CI	Fisher	Odds ratio	95% CI
IC_{Pold} *vs* Pregnant controls	0.0041	nd[1]	-	0.0004	4.8	2.2-15.0
IC_{Pold} *vs* Caucasian controls	0.0029	nd[1]	-	0.0005	3.8	1.9-11.1
IC_{Pnew} *vs* Pregnant controls	0.1082	4.4	0.7-27.2	0.0441	2.1	1.0-4.7
IC_{new} *vs* Caucasian controls	0.1461	2.9	0.6-13.8	0.0850	1.7	0.9-3.4
IC_{Ptotal} *vs* Pregnant controls	0.0065	10.5	1.9-63.7	0.0007	3.0	1.7-6.4
IC_{Ptotal} *vs* Caucasian controls	0.0025	6.9	1.6-32.1	0.0006	2.4	1.5-4.5

[1]Could not be determined, as TT = 0.

women without cholestasis and healthy Caucasian controls, respectively (Table 4). In line with these findings, the ORs of C *versus* T were 3.0 (1.7-6.4) for all ICP patients (ICP_{new} + ICP_{old}) *versus* healthy pregnant control women (ICP_{new}, 2.1; 1.0-4.7 and ICP_{old} 4.8; 2.2-15.0) (Table 5 and Figure 1). With the exception of this polymorphism and two intronic variants that were found to be closely linked to the 1331T>C polymorphism in previous studies [intron 13: (+70) C>T and intron 14 (+32) T>C][26], the allele frequency of the remaining common variants in the patients with ICP and CIC was comparable to that observed in healthy pregnant and Caucasian controls. Due to the small sample size, no significance levels could be calculated for the CIC group. However, all patients in this group were homozygous for the C at position 1331, which is highly suggestive of an overrepresentation of this allele compared to the control groups.

ABCC2: The 1249G>A polymorphism was not found in our patients. In contrast, 3563T>A and 4544G>A were strongly linked and distributed similarly in all groups (Table 4). No significant difference in the frequency of these two polymorphisms was observed between affected ICP and CIC patients and healthy controls. Heterozygous carriers of the 3563A and the 4544A alleles were found in 15.2% of ICP patients (ICP_{new}, 23.5% and ICP_{old}, 3.1%) compared to 11.9 and 12.7% in pregnant women without cholestasis and healthy Caucasian controls, respectively (Table 4). Furthermore, one CIC patient was a heterozygous carrier for the two variant alleles at positions 3563 and 4544.

Relation of serum bile acid levels and the ABCB11 1331T>C genotype

For correlation of bile acid levels with the corresponding genotype at position 1331 of *ABCB11*, ICP and CIC samples were analyzed together. Bile acid levels were available for 16 out of 21 ICP_{new} patients, seven out of 20 ICP_{old} patients, and three out of four CIC patients, which yielded a total of 26 samples (CC, 14 patients; CT, 10 patients; and TT, two patients). Interindividual variability in serum bile acid levels was high and ranged from 1.7 to 22.3 ULN and 1.7 to 17.3 ULN in CC and CT patients, respectively. Serum bile acid levels gradually increased from carriers of the TT genotype to carriers of the CC genotype, with medians of 2.3 ULN (Q1, 2.2; Q3, 2.5), 3.2 ULN (Q1, 2.4; Q3, 6.2) and 4.4 ULN (Q1, 2.2; Q3, 7.8) for TT, TC and CC, respectively (Figure 2).

DISCUSSION

We investigated the risk association between different *ABCB11* and *ABCC2* polymorphisms in ICP and CIC, and correlated different genotypes with serum bile acid levels

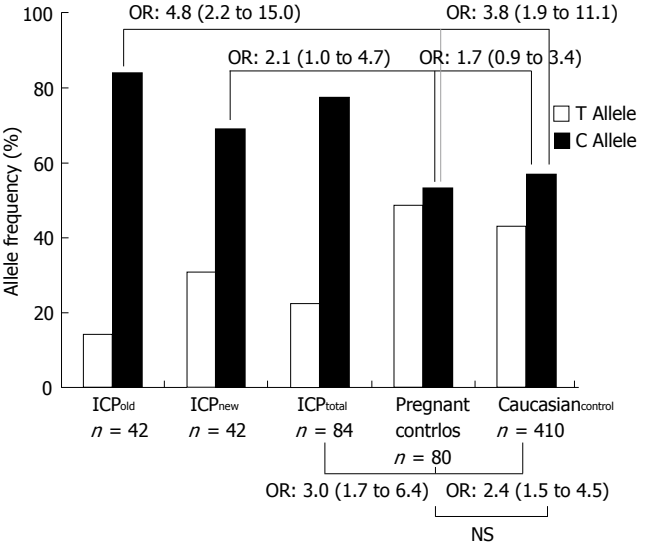

Figure 1 Allelic frequency of the T allele (white panel) and C allele (black panel) of the ABCB11 1331T>C (1331T>C) polymorphism. 21 ICP_{new} patients (42 alleles); 21 ICP_{old} patients (42 alleles); 42 ICP_{total} patients (84 alleles); 20 $ICP_{control}$ patients (40 alleles); 205 $Caucasian_{control}$ patients (410 alleles). Allelic frequencies and ORs are given with 95% CI. Groups were compared with Fisher's exact test.

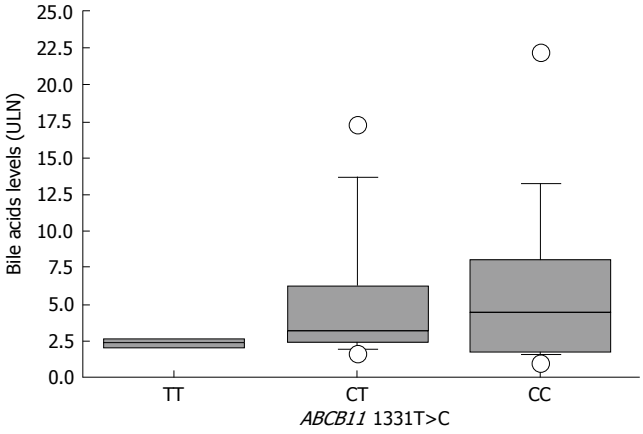

Figure 2 Bile acid levels in patients harboring different 1331C>T genotypes. TT: two patients; CT: 10 patients; CC: 14 patients.

as a marker of cholestasis. In our group of 25 patients with estrogen-associated cholestasis (21 with ICP and four with CIC), there was a highly significant association between the presence of the C allele at position 1331 of *ABCB11* and the presence of cholestasis, which confirms preliminary results from another collective of ICP women, in whom such an overrepresentation was first observed[11]. While *in vitro* function of both BSEP variants, as measured by taurocholate transport activity, is comparable[24], BSEP expression in healthy liver tissue of Caucasian individuals has recently been found to be lower in carriers of the 1331C allele[25]. Such differences in hepatic BSEP expression levels might offer one valuable explanation for the increased susceptibility to the development of cholestasis under specific circumstances, such as hormonal challenges. Furthermore, serum bile acids as a marker of *in vivo* BSEP function was influenced by the underlying genotype. Lowest bile acid levels were observed in patients with the TT genotype and highest levels in carriers of the CC genotype. Although the accepted level of statistical significance was not reached due to high interindividual variability, this observation is in line with the hypothesis that the underlying genotype at position 1331 is a determinant of BSEP function, and hence contributes to the individual risk of developing cholestasis.

The homozygous state for the 1331T>C polymorphism has only recently been observed in a very severe case of pregnancy-associated cholestasis with serum bile acid levels > 40-fold above the ULN. Interestingly, decreased BSEP expression levels were found in a liver biopsy obtained from this patient[27]. Although this patient carried an additional *ABCB4* mutation, the presence of decreased hepatic BSEP expression and highly elevated bile acid levels strongly support a BSEP-related mechanism as a predominant pathogenetic factor. The same patient also developed severe cholestasis under previous use of oral contraceptives, which supports the notion that the same polymorphism also predisposes to oral CIC.

In our group, seven patients (from ICP_{old}) carried additional *ABCB4* mutations, while no such mutations were detected in the remaining 35 out of 42 ICP patients (ICP_{old}, 14 and ICP_{new}, 21). This finding suggests that the *ABCB11* 1331T>C polymorphism independently contributes to an individual's risk for developing cholestasis under certain conditions. On the other hand, it can be speculated whether the combination of the 1331T>C polymorphism with *ABCB4* mutations might be a risk constellation for a severe disease course, as observed by Keitel and coworkers[27].

In contrast, no association was found between the presence of the non-synonymous polymorphisms at positions 1188 and 1515 of MRP2 and the presence of ICP or CIC. A possible pathogenic role of these two polymorphisms in ICP and CIC was suspected based upon the genotype-dependent alteration in hepatic MRP2 expression levels in healthy human liver tissue[25]. Specifically, heterozygous carriers of the glutaminic acid at position 1188 and tyrosine at position 1551 showed significantly higher levels of MRP2 in their liver than homozygous carriers of valine and cysteine, respectively[25]. As BSEP inhibition by estrogen and progesterone metabolites requires prior MRP2-mediated secretion into the bile canaliculus, high MRP2 expression was suspected as a risk factor for the development of estrogen-dependent cholestasis[22].

Several conclusions can be drawn from this study. First, our data point toward a pathogenic relevance of the *ABCB11* 1331T>C polymorphism in ICP and CIC. While these types of cholestasis are so far mainly attributed to different disease-causing mutations in *ABCB4*[5,11,12], our data support a clear association between the presence of a frequent *ABCB11* polymorphism and ICP. Interestingly, all of the patients with CIC were homozygous carriers of the C allele at position 1331. It can be speculated that lower estrogen levels in CIC compared to second or third trimester pregnancy require two low-function alleles to result in cholestasis. Furthermore, the 1331T>C variant was also found to be associated with other inherited and acquired forms of cholestasis, such as benign recurrent intrahepatic cholestasis and drug-induced cholestasis[24,28,29]. This suggests a role for this polymorphism as a risk factor for different cholestatic conditions, which have so far been regarded as different disease entities[20,30].

Second, while γ-GT levels are elevated in ICP patients who carried a disease-causing *ABCB4* mutation[11], serum bile acid levels are influenced by the BSEP genotype at position 444 of *ABCB11*. It can, therefore, be speculated that these two parameters allow us to clinically distinguish between MDR3- and BSEP-related forms of estrogen-related cholestasis, as it is already done for progressive forms of inherited familial intrahepatic cholestasis[5,21]. From a prognostic point of view, this might help to distinguish patients that carry a common susceptibility factor from those who carry a disease-causing *ABCB4* mutation, which in some cases, has been associated with disease progression[7,11,12,31]. Third, although a pathogenic involvement of MRP2 in estrogen-induced cholestasis has longly been suspected, common *ABCC2* polymorphisms have not been associated with the development of cholestasis. We did not exclude the presence of disease-associated *ABCC2* mutations in our group, but normal bilirubin levels in all but one patient suggests no major MRP2 dysfunction, which should result in a Dubin Johnson phenotype[32].

In summary, our data support a role for the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-GT levels might help to distinguish *ABCB4* and *ABCB11*-related forms of ICP and CIC.

COMMENTS

Background

Intrahepatic cholestasis of pregnancy (ICP) and oral contraceptive-induced cholestasis (CIC) are two acquired forms of cholestasis, which are observed in otherwise healthy young women with a normal medical history. The bile salt export pump (BSEP, *ABCB11*) and the multidrug resistance protein 2 (MRP2, *ABCC2*) might be of pathogenetic importance in both conditions.

Research frontiers

A genetic predisposition for both types of hormonal cholestasis has been suspected based upon the strong regional clustering, the higher prevalence in female family members of patients with ICP, and the co-incidence with hereditary cases of progressive familial intrahepatic cholestasis. While mutations in the *ABCB4* gene that encodes the canalicular phospholipid flippase multidrug resistance protein 3 (MDR3) have been implicated in the development of ICP and CIC in a subset of affected patients, the role of genetic variants in *ABCB11* and *ABCC2* remains unclear.

Innovations and breakthroughs

Our data support a role of the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-GT levels might help to distinguish *ABCB4*- and *ABCB11*-related forms of ICP and CIC.

Applications

While the clinical consequences of such findings are still uncertain at this time, they provide important new insights in the role of genetically determined differences in canalicular transporter expression and function for the development of estrogen-induced cholestasis. In the future, the integration of different factors that predict cholestasis might be used to counsel pregnant patients or to avoid certain medications in susceptible patients.

Terminology

ICP: Intrahepatic cholestasis of pregnancy; CIC: contraceptive-induced cholestasis; BSEP: Bile Salt Export Pump (*ABCB11*); MRP2: Multidrug Resistance Protein 2 (*ABCC2*); MDR3: Multidrug Resistance Protein 3 (*ABCB4*).

Peer review

The study characterized a potential underlying defect in the subgroup of normal γ-GT ICP patients and contributes to a clinical risk assessment for the future. This study from a group with longstanding experience in transporter genomics is well designed and presented in a clearly written manuscript.

REFERENCES

1 **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
2 **Kenyon AP**, Piercy CN, Girling J, Williamson C, Tribe RM, Shennan AH. Obstetric cholestasis, outcome with active management: a series of 70 cases. *BJOG* 2002; **109**: 282-288
3 **Gonzalez MC**, Reyes H, Arrese M, Figueroa D, Lorca B, Andresen M, Segovia N, Molina C, Arce S. Intrahepatic cholestasis of pregnancy in twin pregnancies. *J Hepatol* 1989; **9**: 84-90
4 **Reyes H**, Sjovall J. Bile acids and progesterone metabolites in intrahepatic cholestasis of pregnancy. *Ann Med* 2000; **32**: 94-106
5 **Jacquemin E**, De Vree JM, Cresteil D, Sokal EM, Sturm E, Dumont M, Scheffer GL, Paul M, Burdelski M, Bosma PJ, Bernard O, Hadchouel M, Elferink RP. The wide spectrum of multidrug resistance 3 deficiency: from neonatal cholestasis to cirrhosis of adulthood. *Gastroenterology* 2001; **120**: 1448-1458
6 **Reyes H**, Gonzalez MC, Ribalta J, Aburto H, Matus C, Schramm G, Katz R, Medina E. Prevalence of intrahepatic cholestasis of pregnancy in Chile. *Ann Intern Med* 1978; **88**: 487-493
7 **Leevy CB**, Koneru B, Klein KM. Recurrent familial prolonged intrahepatic cholestasis of pregnancy associated with chronic liver disease. *Gastroenterology* 1997; **113**: 966-972
8 **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
9 **Lucena JF**, Herrero JI, Quiroga J, Sangro B, Garcia-Foncillas J, Zabalegui N, Sola J, Herraiz M, Medina JF, Prieto J. A multidrug resistance 3 gene mutation causing cholelithiasis, cholestasis of pregnancy, and adulthood biliary cirrhosis. *Gastroenterology* 2003; **124**: 1037-1042
10 **Mullenbach R**, Linton KJ, Wiltshire S, Weerasekera N, Chambers J, Elias E, Higgins CF, Johnston DG, McCarthy MI, Williamson C. ABCB4 gene sequence variation in women with intrahepatic cholestasis of pregnancy. *J Med Genet* 2003; **40**: e70
11 **Pauli-Magnus C**, Lang T, Meier Y, Zodan-Marin T, Jung D, Breymann C, Zimmermann R, Kenngott S, Beuers U, Reichel C, Kerb R, Penger A, Meier PJ, Kullak-Ublick GA. Sequence analysis of bile salt export pump (ABCB11) and multidrug resistance p-glycoprotein 3 (ABCB4, MDR3) in patients with intrahepatic cholestasis of pregnancy. *Pharmacogenetics* 2004; **14**: 91-102
12 **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
13 **Byrne JA**, Strautnieks SS, Mieli-Vergani G, Higgins CF, Linton KJ, Thompson RJ. The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* 2002; **123**: 1649-1658
14 **Noe J**, Stieger B, Meier PJ. Functional expression of the canalicular bile salt export pump of human liver. *Gastroenterology* 2002; **123**: 1659-1666
15 **Kullak-Ublick GA**, Stieger B, Meier PJ. Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* 2004; **126**: 322-342
16 **Meier PJ**, Stieger B. Molecular Mechanisms in Bile Formation. *News Physiol Sci* 2000; **15**: 89-93
17 **Keppler D**, Konig J. Hepatic secretion of conjugated drugs and endogenous substances. *Semin Liver Dis* 2000; **20**: 265-272
18 **Nies AT**, Keppler D. The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch* 2007; **453**: 643-659
19 **Jansen PL**, Strautnieks SS, Jacquemin E, Hadchouel M, Sokal

EM, Hooiveld GJ, Koning JH, De Jager-Krikken A, Kuipers F, Stellaard F, Bijleveld CM, Gouw A, Van Goor H, Thompson RJ, Muller M. Hepatocanalicular bile salt export pump deficiency in patients with progressive familial intrahepatic cholestasis. *Gastroenterology* 1999; **117**: 1370-1379

20 **Noe J**, Kullak-Ublick GA, Jochum W, Stieger B, Kerb R, Haberl M, Mullhaupt B, Meier PJ, Pauli-Magnus C. Impaired expression and function of the bile salt export pump due to three novel ABCB11 mutations in intrahepatic cholestasis. *J Hepatol* 2005; **43**: 536-543

21 **Strautnieks SS**, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM, Thompson RJ. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat Genet* 1998; **20**: 233-238

22 **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

23 **Vallejo M**, Briz O, Serrano MA, Monte MJ, Marin JJ. Potential role of trans-inhibition of the bile salt export pump by progesterone metabolites in the etiopathogenesis of intrahepatic cholestasis of pregnancy. *J Hepatol* 2006; **44**: 1150-1157

24 **Lang C**, Meier Y, Stieger B, Beuers U, Lang T, Kerb R, Kullak-Ublick GA, Meier PJ, Pauli-Magnus C. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet Genomics* 2007; **17**: 47-60

25 **Meier Y**, Pauli-Magnus C, Zanger UM, Klein K, Schaeffeler E, Nussler AK, Nussler N, Eichelbaum M, Meier PJ, Stieger B. Interindividual variability of canalicular ATP-binding-cassette (ABC)-transporter expression in human liver. *Hepatology* 2006; **44**: 62-74

26 **Pauli-Magnus C**, Kerb R, Fattinger K, Lang T, Anwald B, Kullak-Ublick GA, Beuers U, Meier PJ. BSEP and MDR3 haplotype structure in healthy Caucasians, primary biliary cirrhosis and primary sclerosing cholangitis. *Hepatology* 2004; **39**: 779-791

27 **Keitel V**, Vogt C, Haussinger D, Kubitz R. Combined mutations of canalicular transporter proteins cause severe intrahepatic cholestasis of pregnancy. *Gastroenterology* 2006; **131**: 624-629

28 **Kubitz R**, Keitel V, Scheuring S, Kohrer K, Haussinger D. Benign recurrent intrahepatic cholestasis associated with mutations of the bile salt export pump. *J Clin Gastroenterol* 2006; **40**: 171-175

29 **Lang C**, Meier Y, Stieger B, Beuers U, Lang T, Kerb R, Kullak-Ublick GA, Meier PJ, Pauli-Magnus C. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet Genomics* 2007; **17**: 47-60

30 **Pauli-Magnus C**, Stieger B, Meier Y, Kullak-Ublick GA, Meier PJ. Enterohepatic transport of bile salts and genetics of cholestasis. *J Hepatol* 2005; **43**: 342-357

31 **de Vree JM**, Jacquemin E, Sturm E, Cresteil D, Bosma PJ, Aten J, Deleuze JF, Desrochers M, Burdelski M, Bernard O, Oude Elferink RP, Hadchouel M. Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis. *Proc Natl Acad Sci USA* 1998; **95**: 282-287

32 **Borst P**, Zelcer N, van de Wetering K. MRP2 and 3 in health and disease. *Cancer Lett* 2006; **234**: 51-61

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