World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i19.5867

World J Gastroenterol 2014 May 21; 20(19): 5867-5874
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ABCB4 mutations underlie hormonal cholestasis but not pediatric idiopathic gallstones

Milan Jirsa, Jiří Bronský, Lenka Dvořáková, Jan Šperl, Vít Šmajstrla, Jiří Horák, Jiří Nevoral, Martin Hřebíček

Milan Jirsa, Center for Experimental Medicine, Institute for Clinical and Experimental Medicine, 14021 Prague, Czech Republic
Milan Jirsa, Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University in Prague, 12801 Prague, Czech Republic
Jiří Bronský, Jiří Nevoral, Department of Pediatrics, Second Faculty of Medicine, Charles University in Prague and University Hospital Motol, 15006 Prague, Czech Republic
Lenka Dvořáková, Martin Hřebíček, Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, 12808 Prague, Czech Republic
Jan Šperl, Department of Hepatogastroenterology, Institute for Clinical and Experimental Medicine, 14021 Prague, Czech Republic
Vít Šmajstrla, Bormed Private Health Centre, 72200 Ostrava, Czech Republic
Jiří Horák, 1st Department of Internal Medicine, Third Faculty of Medicine, Charles University in Prague, 10034 Prague, Czech Republic
Author contributions: Jirsa M and Hřebíček M designed the study, wrote the draft and performed statistical analysis; Bronský J and Nevoral J selected patients with idiopathic gallstones; Dvořáková L supervised mutation analysis and performed pathogenicity predictions; Šperl J, Šmajstrla V and Horák J provided clinical data and samples of the LPAC families; all contributed to writing the draft.
Supported by The project (Ministry of Health, Czech Republic) for development of research organization 00023001 (IKEM, Prague, Czech Republic) - Institutional support; PRVOUK-P24/LF1/3 and MH CZ - DRO VFN64165 to Dvořáková L and Hřebíček M
Correspondence to: Milan Jirsa, MD, PhD, Center for Experimental Medicine, Institute for Clinical and Experimental Medicine, Vídeňská 1958/9, 14021 Prague, Czech Republic. milan.jirsa@ikem.cz
Telephone: +420-2-61362773 Fax: +420-2-41721666
Received: May 23, 2013 Revised: July 25, 2013
Accepted: August 16, 2013
Published online: May 21, 2014

Abstract

AIM: To investigate the contribution of *ABCB4* mutations to pediatric idiopathic gallstone disease and the potential of hormonal contraceptives to prompt clinical manifestations of multidrug resistance protein 3 deficiency.

METHODS: Mutational analysis of *ABCB4*, screening for copy number variations by multiplex ligation-dependent probe amplification, genotyping for low expression allele c.1331T>C of *ABCB11* and genotyping for variation c.55G>C in *ABCG8* previously associated with cholesterol gallstones in adults was performed in 35 pediatric subjects with idiopathic gallstones who fulfilled the clinical criteria for low phospholipid-associated cholelithiasis syndrome (LPAC, OMIM #600803) and in 5 young females with suspected LPAC and their families (5 probands, 15 additional family members). The probands came to medical attention for contraceptive-associated intrahepatic cholestasis.

RESULTS: A possibly pathogenic variant of *ABCB4* was found only in one of the 35 pediatric subjects with idiopathic cholesterol gallstones whereas 15 members of the studied 5 LPAC kindreds were confirmed and another one was highly suspected to carry predictably pathogenic mutations in *ABCB4*. Among these 16, however, none developed gallstones in childhood. In 5 index patients, all young females carrying at least one pathogenic mutation in one allele of *ABCB4*, manifestation of LPAC as intrahepatic cholestasis with elevated serum activity of gamma-glutamyltransferase was induced by hormonal contraceptives. Variants *ABCB11* c.1331T>C and *ABCG8* c.55G>C were not significantly overrepresented in the 35 examined patients with suspect LPAC.

CONCLUSION: Clinical criteria for LPAC syndrome

caused by mutations in *ABCB4* cannot be applied to pediatric patients with idiopathic gallstones. Sexual immaturity even prevents manifestation of LPAC.

Key words: Idiopathic cholelithiasis; Intrahepatic cholestasis; Oral contraceptives; Low phospholipid-associated cholelithiasis; Gallbladder disease 1

Core tip: Mutations in *ABCB4* are not overrepresented in children with idiopathic gallstones who fulfill the clinical and laboratory criteria for low phospholipid-associated cholelithiasis syndrome (Gallbladder Disease 1, OMIM #600803). Sexual immaturity prevents manifestation of low phospholipid-associated cholelithiasis. In young females, manifestation of low phospholipid-associated cholelithiasis syndrome such as intrahepatic cholestasis with elevated serum activity of gamma-glutamyltransferase may be induced by hormonal contraceptives.

Jirsa M, Bronský J, Dvořáková L, Šperl J, Šmajstrla V, Horák J, Nevoral J, Hřebíček M. *ABCB4* mutations underlie hormonal cholestasis but not pediatric idiopathic gallstones. *World J Gastroenterol* 2014; 20(19): 5867-5874 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5867.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5867

INTRODUCTION

Low phospholipid-associated cholelithiasis syndrome (LPAC, synonym Gallbladder disease 1, OMIM #600803) has been defined as symptomatic and recurring cholelithiasis associated with mutations in *ABCB4* encoding multidrug resistance protein 3 (MDR3), the canalicular phospholipid export pump[1,2]. LPAC should be suspected in patients with symptomatic cholelithiasis in whom at least one minor criterion is present. These minor criteria are proposed as: (a) age below 40 years at the onset of symptoms; (b) recurrence after cholecystectomy; (c) intrahepatic hyperechoic foci with a topography compatible with lipid deposits along the luminal surface of the intrahepatic biliary tree; (d) intrahepatic sludge; (e) microlithiasis; (f) history of gallstones in first-degree relatives; or (g) history of intrahepatic cholestasis of pregnancy[3]. The distribution of associated *ABCB4* mutations in conserved regions of the gene, as well as their type, strongly support the role of partial MDR3 deficiency in LPAC, with decreased MDR3 activity and/or expression altering biliary lipid composition.

Apart from LPAC, mutations in *ABCB4* that reduce but do not abrogate the activity of MDR3 can cause a variety of milder forms of familial intrahepatic cholestasis type 3 (OMIM #602347), with slowly progressive or non-progressive hepatobiliary disease or anicteric cholestasis with varying liver fibrosis in adulthood[4]. Several reports[5-7] have shown that intrahepatic cholestasis of pregnancy is associated with *ABCB4* mutations in some women. Finally, the idea that contraceptive-induced cholestasis (CIC) may be associated with mutations in *ABCB4* has also been proposed. Asymptomatic gallstones and clinically silent cirrhosis, diagnosed later as progressive familial intrahepatic cholestasis type 3, became manifest in a 17-year-old girl when cholestasis developed on ingestion of contraceptive pills containing ethinylestradiol 30 μg, and levonorgestrol 150 μg[8], and isolated gallstone disease unmasked by oral contraception and associated with *ABCB4* mutation has been reported[1]. In contrast, no mutations in *ABCB4* were found in 5 subjects with CIC studied by Lang *et al*[9].

In our previous study[10] we focused on the role of the common variants c.523A>G (p.Thr175Ala) and c.1954A>G (p.Arg652Gly) in *ABCB4*, c.1331T>C (p.Val444Ala) in *ABCB11* and c.55 G>C (p.Asp19His) in *ABCG8* in pediatric gallstone disease. These variants are considered either as potentially pathogenic or as susceptibility alleles for cholesterol cholelithiasis in adults; however, they were not observed to contribute to genetic predisposition to gallstones in childhood[10].

In this study we investigated: (1) the role of *ABCB4* mutations in the etiology of pediatric idiopathic gallstones; and (2) the capability of hormonal contraceptives to unmask hitherto clinically silent MDR3 deficiency.

MATERIALS AND METHODS

Pediatric patients with gallstones

Pediatric patients with gallstones were selected as described[10] (see Figure 1 for the selection algorithm). Briefly, 109 children (53 males and 56 females) with gallbladder gallstones who had been hospitalized at the Department of Pediatrics, Faculty Hospital Motol, Prague, between 1995-2004, were considered. In 22 patients, gallstones were clearly associated with another disease such as Down syndrome, Gaucher disease, cystic fibrosis, hemolytic anemia, inflammatory bowel disease, immune deficiency and Gilbert syndrome. Thirty-three of the 87 invited patients did not respond. In 13 of 54 patients, the etiology of gallstones was uncertain. However, as these 13 patients had at least one of the following: long-term parenteral nutrition, treatment with cephalosporins or furosemide, dyslipidemia, hepatobiliary infectious disease or obesity (BMI > 27), *e.g.* conditions that could promote gallstone formation, they were not enrolled. In 41 patients, gallstones were most likely idiopathic. For *ABCB4* mutation testing, only 35 of these 41 patients (including only one of the monozygous twins) with idiopathic gallstones were selected who had at least one parent or grandparent with gallstones. These subjects (15 males and 20 females with positive family history), all unrelated Caucasians of Czech origin, met the major criterion and minor criteria (a) and (f) of Rosmorduc and Poupon[3]. The mean age at diagnosis of cholelithiasis was 10.7 ± 5.0 years (range 1-17). Nineteen of these 35

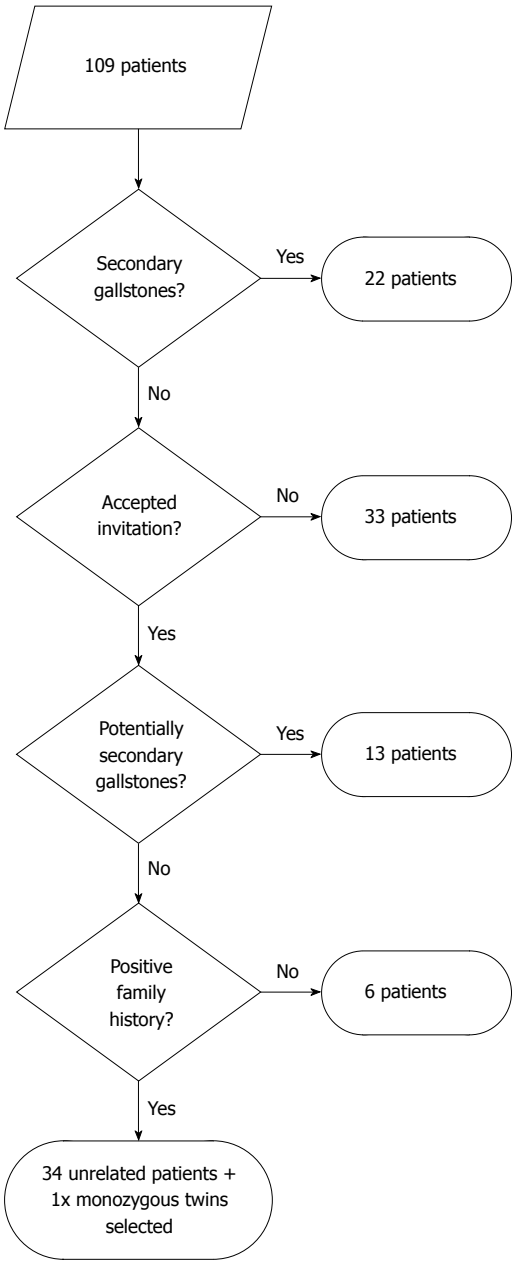

Figure 1 Flowchart describing the algorithm for selection of pediatric patients with low phospholipid-associated cholelithiasis syndrome-like idiopathic gallbladder gallstones.

patients (13 girls and 6 boys) underwent cholecystectomy with no recurrence after surgery. As of this writing, all cholecystectomized patients are well, without abdominal pain or jaundice.

Young adults with suspect LPAC

Five young adult female patients with symptomatic gallstones (age below 40 years at the onset of symptoms), a history of intrahepatic cholestasis, and a family history of gallstones in first-degree relatives were referred for *ABCB4* analysis. Their clinical characteristics are summarized in Table 1. None of the patients had hyperechoic foci in the liver parenchyma or proven intrahepatic sludge; duodenal bile was not investigated for microlithiasis. Nonethless, all met the proposed criteria for LPAC[3]. In patient I , intrahepatic cholestasis associated with exposure to an oral contraceptive containing ethinylestradiol 0.030 mg, and levonorgestrel 0.125 mg (Minisiston; Jenapharm, Jena, Germany) was the first clinical symptom of LPAC. Cholestasis resolved rapidly after withdrawal of the contraceptive. However, the patient developed cholecystolithiasis within one year despite ursodeoxycholic acid administration (15 mg/kg) and underwent cholecystectomy. One year later, ursodeoxycholic acid was withdrawn because the patient was completely asymptomatic with normal clinical/laboratory test results. Rechallenge with another oral contraceptive containing ethinylestradiol 0.020 mg and desogestrel 0.150 mg (Mercilon; Organon, Oss, The Netherlands) two years after cholecystectomy was followed within several weeks by a second attack of cholestasis. Clinical and laboratory findings improved again rapidly when the medication was withdrawn. None of the other 4 index patients mentioned any problems associated with the use of contraceptives; the data on contraceptives presented in Table 1 were obtained in part from clinical records and in part by specific questioning.

The patient studies were approved by the Institutional Review Board of the Faculty Hospital Motol. Either both parents or the examined subjects, when aged over 15 years, gave written informed consent before blood sampling.

Mutational analysis

Twenty-seven fragments covering all exonic (protein-coding) regions of *ABCB4* and including portions of adjacent intronic sequences were amplified from genomic DNA by PCR (primer sequences are available from the corresponding author). The DNA sequence of purified PCR products was analyzed on an ABI-PRISM 3100-Avant automated DNA sequencer (Applied Biosystems, Foster City, CA). Ensembl Acc. No. ENSG00000005471 and GenBank Acc. No. NM_018849.2 served as genomic and cDNA reference sequences. Mutations found by DNA sequencing were independently confirmed by restriction fragment length polymorphism analysis after digestion of the corresponding PCR product with restriction enzymes. In addition, *ABCB4* was scanned for deletions/duplications by multiplex ligation-dependent probe amplification, using SALSA MLPA KIT P109 *ABCB4* (MRC-Holland, Amsterdam, The Netherlands) according to manufacturer's instructions.

The low expression allele c.1331T>C of *ABCB11*[11] was detected as the presence of a PCR-*Bsu*RI restriction fragment length polymorphism. The variation c.55G>C in *ABCG8* associated with cholesterol gallstones in adults[12] was detected as described by Hubáček *et al*[13]. Pathogenicity of missense variations was predicted *in silico* by SIFT[14], PMut[15], PolyPhen-2[16] and MutationTaster[17].

Statistical analysis

The data are presented as mean and standard deviation,

Table 1 Characteristics of young women with low phospholipid-associated cholelithiasis syndrome and proven *ABCB4* mutations

Patient ID	Year of birth	Age at cholecystectomy (yr)	Age at liver biopsy (yr)	Age at pregnancies (yr)	Age (yr), contraceptive, complication
I	1980	20	19, periportal fibrosis	Nulliparous	18 Minisiston, withdrawn for CIC 22 Mercilon, withdrawn for CIC
II	1978	17	21, periportal fibrosis	Nulliparous	18 Tri-regol, withdrawn for CIC
III	1973	22	22, periportal fibrosis	30, with ICP	20-22 Cilest, withdrawn for CIC
IV	1967	28	31, normal histology	19, without ICP	28 Tri-regol, withdrawn for CIC 39-now Lunafem, tolerated
V	1973	20	20, periportal fibrosis	23, without ICP 32, without ICP	19-?, withdrawn for CIC 24-31 Marvelon, tolerated 32-now Mirena, permanent pruritus, GGT twice normal

Chemical composition of contraceptives: (1) Minisiston (Jenapharm, Jena, Germany): ethinylestradiol 0.030 mg; levonorgestrel 0.125 mg; (2) Mercilon (Organon, Oss, The Netherlands): ethinylestradiol 0.020 mg; desogestrel 0.150 mg; (3) Tri-regol (Chemical Works of Gedeon Richter, Budapest, Hungary): ethinylestradiol 0.030 - 0.040 - 0.030 mg; levonorgestrel 0.050 - 0.075 - 0.125 mg; (4) Cilest (Janssen Pharmaceutica, Beerse, Belgium): ethinylestradiol 0.035 - 0.035 - 0 mg; norgestimate 0.250 - 0.250 - 0 mg; (5) Lunafem (Bayer Schering Pharma, Berlin, Germany): ethinylestradiol 0.020 mg; gestodene 0.075 mg; (6) Marvelon (Organon, Oss, The Netherlands): ethinylestradiol 0.030 mg; desogestrel 0.150 mg; (7) Mirena (Bayer Schering Pharma, Berlin, Germany): levonorgestrel 0.020 mg. Estrogen free intrauterine application. CIC: Contraceptive-induced cholestasis.

or as frequencies when appropriate. We used chi-square testing to check whether genotype frequencies were consistent with Hardy-Weinberg equilibrium. Differences between genotype frequencies were analyzed by two-sided Fisher exact testing, using the approximation of Katz, with the InStat3 program (GraphPad Software, La Jolla, CA).

RESULTS

Pediatric patients with gallstones

In the group of pediatric patients with idiopathic gallstones selected for genetic examination, analysis of protein-coding exons and intron/exon junctions of *ABCB4* identified no obvious pathogenic mutations. In patient 31, a novel heterozygous variation was found (c.2222C>T, leading to predicted conservative amino acid substitution p.Pro741Leu in the extracellular loop between transmembrane domains 7 and 8). The substitution was rated as neutral by all four pathogenicity prediction programs. Another predicted amino acid substitution (p.Gly773Val, localized in transmembrane domain 8 and caused by the novel mutation c.2318G>T) was found in a heterozygous state in patient 32. This conservative substitution was rated as disease-causing by MutationTaster, possibly pathogenic by PolyPhen-2, and neutral by SIFT and PMut. In addition, 6 known coding (5 synonymous) and 6 known non-coding variations were found (Table 2). None of these changes is reportedly associated with hepatobiliary disease, with the possible exception of c.1954A>G (p.Arg652Gly), found previously in a heterozygous state in subjects 4, 8 and 26[10]. However, the c.1954A>G variant was not overrepresented (3/70, allelic frequency 0.043) in our patients as compared with a healthy adult Czech Caucasian population (allelic frequency 0.090, 27 heterozygotes in 150 controls, OR = 0.48, 95%CI: 0.16-1.48, $P = 0.17$).

Families with suspect LPAC

Two of the five probands carried a single heterozygous nonsense mutation, two were heterozygotes for the missense mutation c.523A>G (p.Thr175Ala, rs58238559), and one was a compound heterozygote for the same missense mutation (c.523A>G) and for the frameshift mutation c.1371delG (p.Gln458Argfs*7) (Figure 2). The variation c.523A>G was found on 30% (3/10) of alleles in patients with LPAC, whereas only 2.7% of control alleles from the Czech population carried guanine at the position 523 (8/300, 8 heterozygotes in 150 control individuals, OR = 8.00, CI: 2.20-29.24, $P = 0.012$). While the number of patients was too low to make the result fully convincing, this observation suggests that p.Thr175Ala at least confers susceptibility to hepatobiliary disease. All three null mutations were novel to our best knowledge.

No deletions/duplications in *ABCB4* were detected in index patients by multiplex ligation-dependent probe amplification.

Two probands were homozygous and the other three probands were heterozygous for the low-expression *ABCB11* variant c.1331T>C (p.Val444Ala) (Figure 2). One proband had a c.55G|C genotype while four other probands were homozygous for the wildtype allele c.55G in *ABCG8*. To assess the segregation of the genotype and phenotype in the families of all index patients, first degree relatives were examined. As can be seen from the family trees depicted in Figure 2, the parents in families I, II, IV, and V who carried the same mutation as the probands in a heterozygous state were symptomatic. This indicates that the null mutations in families I - III and even the missense mutation leading to p.Thr175Ala in families IV and V all are likely sufficient in a heterozygous state to promote the LPAC phenotype. In contrast, variations *ABCB11* c.1331T>C and *ABCG8* c.55G>C, found in probands and 11 family members carrying mutations in *ABCB4*, do not seem to affect the penetrance of LPAC (Figure 2).

DISCUSSION

The only possible pathogenic mutation in *ABCB4* found

Table 2 Known variations in *ABCB4*, *ABCB11* and *ABCG8* found in 35 pediatric subjects with idiopathic gallstones

Patient. ID	Variations in the coding sequence of *ABCB4*						*ABCB11* low expression allele	*ABCG8* variation
	c.147C>T Ser49Ser rs8187789	c.175C>T Leu59Leu rs2302387	c.459T>C Phe153Phe rs2230027	c.504C>T Asn168Asn rs1202283	c.711A>T Ile237Ile rs2109505	c.1954A>G Arg652Gly rs2230028	c.1331T>C Val444Ala rs2287622	c.55G>C Asp19His rs11887534
1	CC	CC	TT	TT	AA	AA	CC	GG
2	CC	CC	TT	CT	AA	AA	TC	GG
3	CC	CC	TT	CT	AA	AA	TC	GG
4	CC	CC	TT	CT	AT	AG	TC	GG
5	CC	CC	TT	TT	AA	AA	CC	GG
6	CC	CC	TT	CC	AA	AA	TC	GG
7	CC	CC	TT	CT	AA	AA	TC	GG
8	CC	CC	TT	CC	AA	AG	TC	GG
9	CC	CC	TT	TT	AA	AA	TT	GG
10	CC	CC	TT	TT	AA	AA	CC	GG
11	CC	CC	TT	TT	AA	AA	TC	GG
12	CC	CC	TT	TT	AA	AA	CC	GG
13	CC	CC	TT	CT	AA	AA	TC	GG
14	CC	CC	TT	CC	AA	AA	CC	GG
15	CC	CC	TT	TT	AA	AA	TC	GC
16	CC	CC	TT	CC	AA	AA	TC	GC
17	CC	CC	TT	CC	AA	AA	TC	GG
18	CC	CC	TT	TT	AA	AA	TC	GC
19	CC	CC	TT	CT	AA	AA	TC	GC
20	CC	CC	TT	CT	AA	AA	TT	GG
21	CC	CC	TT	TT	AA	AA	TC	GG
22	CC	CC	TT	CT	AA	AA	TT	GG
23	CC	CC	TT	CT	AA	AA	TT	GG
24	CC	CT	TT	CC	AT	AA	CC	GG
25	CC	CC	TT	CC	AA	AA	TC	GG
26	CT	CT	TC	CC	AA	AG	TC	GG
27	CC	CC	TT	TT	AA	AA	TC	GG
28	CC	CC	TT	TT	AA	AA	TT	GG
29	CC	CC	TT	CT	AA	AA	TT	GG
30	CC	CC	TT	TT	AA	AA	CC	GC
31	CC	CC	TT	CT	AA	AA	TT	GC
32	CC	CC	TT	CT	AA	AA	TT	GG
33	CC	CC	TT	TT	AA	AA	TC	GG
34	CC	CC	TT	CC	AA	AA	TT	GG
35	CC	CC	TT	TT	AA	AA	CC	GG
Allelic frequency of variant alleles in patients with gallstones, HapMap populations and Czech population controls								
Allele	T	T	C	T	T	G	C	G
Gallstone patients	0.014	0.029	0.014	0.571	0.029	0.043	0.471	0.086
HapMap CEU	0	0.112	0[1]	0.664	0.175	0.075	0.408	0.085
HapMap HCB	0	0.167	0[1]	0.344	0.222	0.023	0.333	0.022
HapMap JPT	0	0.273	0[1]	0.442	0.300	0.023	0.261	0.011
HapMap YRI	0.042	0.525	0.11	0	0.362	0.392	0.425	0.042
Czech controls (n = 150)	n.d.	n.d.	n.d.	n.d.	n.d.	0.090	0.400	0.067[2]

[1]Results from corresponding populations studied in Environmental Genome Project (NIEHS ES15478 project). HapMap population data were not available for this variation; [2]Frequency in 285 Czech controls[15]. n.d.: Not done.

in pediatric patients with idiopathic gallstones who met clinical criteria for the diagnosis of LPAC was the variation c.2318G>T (p.Gly773Val) found in a heterozygous state in only one affected subject. The nucleotide change c.1954A>G found in 3 other pediatric gallstone subjects is common in the European, Caucasian, and African general population, but it has also been found in a patient with LPAC and low biliary phospholipid in whom its predicted consequence p.Arg652Gly was hypothesized to be conditionally penetrant, leading to clinical symptoms only under certain circumstances, such as pregnancy, or when combined with another mutation[18]. In contrast, no correlation of the *ABCB4* genotype c.1954A|G with the MDR3 expression level in the liver, as measured by Western blot, was observed in a study by Meier *et al*[11] and the substitution was rated as neutral by all software tools used. Our finding that the genotype c.1954A|G was neither overrepresented nor significantly underrepresented in patients with gallstones may indicate the negligible role of this variation in etiology of pediatric idiopathic gallstones. Similar conclusions could be drawn for both carriers and homozygotes for the low expression variant of the bile salt export pump and for the carriers of the *ABCG8* variation c.55G>C.

Interestingly, the *ABCB4* variation c.523A>G (p.Thr175Ala), found in three index patients with LPAC,

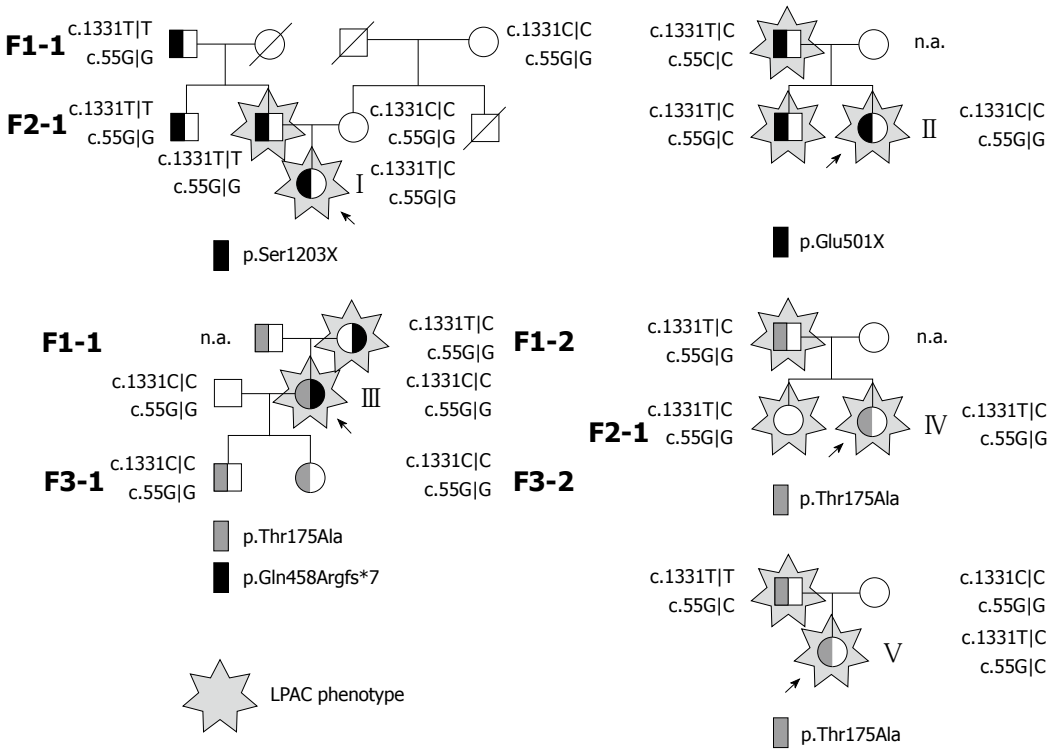

Figure 2 Family trees of the five unrelated female probands with low phospholipid-associated cholelithiasis syndrome and *ABCB4* mutations. Phenotype of low phospholipid-associated cholelithiasis syndrome (LPAC) is indicated with gray asterisks, index patients are marked with arrows. Genotypes for *ABCB11* c.1331T>C and *ABCG8* c.55G>C are shown at the genealogical symbols. n. a. - DNA not available. The father´s brother of the proband Ⅰ (F2-1) aged 53 years had no signs of LPAC. The proband´s grandfather (F1-1) developed gallstones after 40 years of age. Similarly, the father of proband Ⅲ (F1-1) experienced his first attack of biliary pain at the age of 60 years. His DNA sample was not available for analysis; however, he is most likely a heterozygous carrier of c.523A>G (p.Thr175Ala) as depicted. The children (F3-1 and F3-2) of proband Ⅲ are nine year old twins with no signs of LPAC. The DNA of the sister of proband Ⅳ (F2-1), who met the clinical criteria of LPAC, was analyzed for *ABCB4* mutations with negative results.

was not present in any of our patients with pediatric gallstones. The allele c.523G is linked to cholestatic disease[1] although it is also found in healthy Caucasian populations at an allelic frequency of 0.025-0.032[9,19,20]. The threonine residue at position 175 is highly conserved, lying in a Thr-Arg-Leu-Thr cluster required for MDR3 adenosine triphosphatase (ATPase) activity. While the functional consequences of replacement of threonine at position 175 by a neutral amino-acid residue having a hydrophobic side chain were not evaluated in MDR3, they were studied in yeast in the close homologue P-glycoprotein[21], in which the substitution p.Thr169Ile resulted in a complete loss of substrate-induced P-glycoprotein ATPase activity. The substitution p.Thr175Ala, predicted uniformly to impair protein function by SIFT, PMut, PolyPhen-2 and MutationTaster is thus considered a disease-associated mutation[3] with incomplete penetrance.

Neither the 14 confirmed and 1 suspected heterozygous carriers of *ABCB4* mutations investigated in the second part of our study nor the heterozygotes reported previously by others[1,2,4,22-24] developed symptomatic gallstones without progressive familial intrahepatic cholestasis in childhood. This suggests that other pathogeneses of idiopathic gallstones in childhood should be sought. Since we did not assay phospholipid and cholesterol concentrations in bile from our 35 pediatric subjects, we cannot definitively claim that they did not have LPAC; only that, if they had LPAC, it was associated neither with demonstrable *ABCB4* mutation (this study) nor with the studied variations in *ABCB11* and *ABCG8*[10]. We suggest that to carry out *ABCB4* sequencing in pediatric patients with idiopathic cholesterol gallstones who meet only some of the present criteria for assigning the diagnosis of LPAC may be unproductive. We believe that the validity of these criteria for LPAC associated with *ABCB4* mutation should be re-assessed, in pediatric patients at least, and propose that the present criteria at this juncture be considered to apply only to adults aged less than 40 years.

The observations that LPAC syndrome becomes manifest after middle adolescence and that young females heterozygous for pathogenic mutations in *ABCB4* developed CIC and/or manifested previously asymptomatic gallstones during administration of combined oral contraceptives are most likely explained by known changes in biliary lipid composition during the second decade of life. Gallstones hardly ever occur in children, but are frequent in adults; this difference seems to be due to the low concentrations of cholesterol in the bile of children[25]. Children have reduced biliary cholesterol:bile salt excretion ratios[26]. Therefore, even at low rates of phospholipid secretion caused by incomplete MDR3 deficiency, bile is not saturated with cholesterol. The known increase in the biliary cholesterol saturation index in

young adults[26], together with the decreased biliary secretion rate of phosphatidylcholine in carriers of mutations in *ABCB4*, shifts the cholesterol-solubility equilibrium to the borderline. Even the low load of exogenous hormones contained in contraceptives or other hormonally active drugs, which inhibit bile salt secretion[27] and further decrease secretion of phospholipids into bile proportionally to bile salt flow[28], can precipitate cholestasis and promote cholesterol crystallization from supersaturated bile, with formation of intrahepatic sludge and of gallstones.

A practical question can be raised on the safety of contraceptives in women with MDR3 deficiency. Our patients heterozygous for null mutations developed CIC rapidly and consequently contraceptives had to be withdrawn. In contrast, two patients heterozygous for the missense MDR3 variant p.Thr175Ala tolerated long-term administration of oral contraceptives after cholecystectomy without apparent worsening in hepatobiliary disease. Interestingly, patient V reported pruritus and her serum levels of GGT were repeatedly increased twofold when she used estrogen-free intrauterine contraception. We therefore believe that the heterozygous state for missense mutations in *ABCB4* is not an *a priori* contraindication to oral contraception. However, monitoring of clinical status and clinical-laboratory indices of hepatobiliary injury is essential in such cases.

In conclusion, our findings indicate that clinical criteria for LPAC caused by mutations in *ABCB4* cannot be applied to pediatric patients with idiopathic gallstones. Sexual immaturity prevents manifestation of LPAC even in carriers of pathogenic mutations in *ABCB4*. In young females, manifestation of LPAC as intrahepatic cholestasis with elevated serum activity of gamma-glutamyltransferase may be triggered by hormonal contraceptives.

ACKNOWLEDGMENTS

We thank A S Knisely, Institute of Liver Studies, King's College Hospital, London, United Kingdom, for comments on the manuscript, and Lucie Budišová, IKEM, and Michaela Boučková, Institute of Inherited Metabolic Diseases, both Prague, Czech Republic, for technical assistance.

COMMENTS

Background

Mutations in *ABCB4*, the variation c.55G>C in *ABCG8* and the low expression allele c.1331T>C of *ABCB11* may affect biliary lipid composition and increase saturation of bile with cholesterol. Mutations in *ABCB4* are known to cause low phospholipid-associated cholelithiasis (LPAC) in young adults. The variation c.55G>C in *ABCG8* has been linked with gallstones in adults.

Research frontiers

Since saturation of bile with cholesterol in children is lower than in adults, the authors anticipated a strong contribution of the above listed genetic variations to pediatric idiopathic gallstones and conducted a genetic study in pediatric LPAC-like gallstone patients and in young adults with suspect LPAC who came to medical attention due to contraceptive-induced cholestasis. Whereas young adult females with clinically defined LPAC carried mutations in *ABCB4*, no association with the studied variants was found in pediatric LPAC-like subjects.

Innovations and breakthroughs

Sexual immaturity prevents manifestation of LPAC even in carriers of pathogenic mutations in *ABCB4*.

Applications

Clinical criteria for LPAC caused by mutations in *ABCB4* cannot be applied to pediatric patients with idiopathic gallstones and to carry out *ABCB4* sequencing in pediatric patients with idiopathic cholesterol gallstones may be unproductive. Heterozygous state for some missense mutations in *ABCB4* is not an *a priori* contraindication to oral contraception; however, monitoring of clinical status and clinical/laboratory indices of hepatobiliary injury is essential in such cases.

Terminology

Biliary lipid secretion is mediated by three ABC transporters: *ABCB11* encodes the bile salt export pump, *ABCB4* encodes the canalicular lecithin pump MDR3 (multidrug resistance protein 3) and the genes *ABCG5* and *ABCBG8* encode two proteins named sterolins which form a heterodimeric ABC transporter responsible for biliary secretion of cholesterol and plant sterols.

Peer review

It is an excellent manuscript submitted to reevaluate the criteria for LAPC associated with *ABCB4* mutation and provide the data of both pediatric idiopathic gallstone and young women with LAPC hormonal cholestasis by oral contraceptives. The mechanism of cholelithiasis formation with *ABCB11* and *ABCG8* mutation was also considered and 5 probands with detailed pedigrees were presented. Ethics of the research was given by written informed consent.

REFERENCES

1 **Rosmorduc O**, Hermelin B, Poupon R. MDR3 gene defect in adults with symptomatic intrahepatic and gallbladder cholesterol cholelithiasis. *Gastroenterology* 2001; **120**: 1459-1467 [PMID: 11313316 DOI: 10.1053/gast.2001.23947]

2 **Rosmorduc O**, Hermelin B, Boelle PY, Parc R, Taboury J, Poupon R. ABCB4 gene mutation-associated cholelithiasis in adults. *Gastroenterology* 2003; **125**: 452-459 [PMID: 12891548 DOI: 10.1016/S0016-5085(03)00898-9]

3 **Rosmorduc O**, Poupon R. Low phospholipid associated cholelithiasis: association with mutation in the MDR3/ABCB4 gene. *Orphanet J Rare Dis* 2007; **2**: 29 [PMID: 17562004 DOI: 10.1186/1750-1172-2-29]

4 **Ziol M**, Barbu V, Rosmorduc O, Frassati-Biaggi A, Barget N, Hermelin B, Scheffer GL, Bennouna S, Trinchet JC, Beaugrand M, Ganne-Carrié N. ABCB4 heterozygous gene mutations associated with fibrosing cholestatic liver disease in adults. *Gastroenterology* 2008; **135**: 131-141 [PMID: 18482588 DOI: 10.1053/j.gastro.2008.03.044]

5 **Floreani A**, Carderi I, Paternoster D, Soardo G, Azzaroli F, Esposito W, Variola A, Tommasi AM, Marchesoni D, Braghin C, Mazzella G. Intrahepatic cholestasis of pregnancy: three novel MDR3 gene mutations. *Aliment Pharmacol Ther* 2006; **23**: 1649-1653 [PMID: 16696816 DOI: 10.1111/j.1365-2036.2006.02869.x]

6 **Schneider G**, Paus TC, Kullak-Ublick GA, Meier PJ, Wienker TF, Lang T, van de Vondel P, Sauerbruch T, Reichel C. Linkage between a new splicing site mutation in the MDR3 alias ABCB4 gene and intrahepatic cholestasis of pregnancy. *Hepatology* 2007; **45**: 150-158 [PMID: 17187437 DOI: 10.1002/hep.21500]

7 **Floreani A**, Carderi I, Paternoster D, Soardo G, Azzaroli F, Esposito W, Montagnani M, Marchesoni D, Variola A, Rosa Rizzotto E, Braghin C, Mazzella G. Hepatobiliary phospholipid transporter ABCB4, MDR3 gene variants in a large cohort of Italian women with intrahepatic cholestasis of pregnancy. *Dig Liver Dis* 2008; **40**: 366-370 [PMID: 18083082 DOI: 10.1016/j.dld.2007.10.016]

8 **Ganne-Carrié N**, Baussan C, Grando V, Gaudelus J, Cresteil D, Jacquemin E. Progressive familial intrahepatic cholestasis type 3 revealed by oral contraceptive pills. *J Hepatol* 2003; **38**: 693-694 [PMID: 12713886 DOI: 10.1016/S0168-8278(03)00049-7]

9 **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 [PMID: 17264802 DOI: 10.1097/01.fpc.0000230418.28091.76]

10 **Bronský J**, Jirsa M, Nevoral J, Hrebícek M. Role of common canalicular transporter gene variations in aetiology of idiopathic gallstones in childhood. *Folia Biol* (Praha) 2010; **56**: 9-13 [PMID: 20163776]

11 **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 [PMID: 16799996 DOI: 10.1002/hep.21214]

12 **Buch S**, Schafmayer C, Völzke H, Becker C, Franke A, von Eller-Eberstein H, Kluck C, Bässmann I, Brosch M, Lammert F, Miquel JF, Nervi F, Wittig M, Rosskopf D, Timm B, Höll C, Seeger M, ElSharawy A, Lu T, Egberts J, Fändrich F, Fölsch UR, Krawczak M, Schreiber S, Nürnberg P, Tepel J, Hampe J. A genome-wide association scan identifies the hepatic cholesterol transporter ABCG8 as a susceptibility factor for human gallstone disease. *Nat Genet* 2007; **39**: 995-999 [PMID: 17632509 DOI: 10.1038/ng2101]

13 **Hubácek JA**, Berge KE, Stefková J, Pitha J, Skodová Z, Lánská V, Poledne R. Polymorphisms in ABCG5 and ABCG8 transporters and plasma cholesterol levels. *Physiol Res* 2004; **53**: 395-401 [PMID: 15311998]

14 **Ng PC**, Henikoff S. Predicting deleterious amino acid substitutions. *Genome Res* 2001; **11**: 863-874 [PMID: 11337480 DOI: 10.1101/gr.176601]

15 **Ferrer-Costa C**, Gelpí JL, Zamakola L, Parraga I, de la Cruz X, Orozco M. PMUT: a web-based tool for the annotation of pathological mutations on proteins. *Bioinformatics* 2005; **21**: 3176-3178 [PMID: 15879453 DOI: 10.1093/bioinformatics/bti486]

16 **Adzhubei IA**, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. *Nat Methods* 2010; **7**: 248-249 [PMID: 20354512 DOI: 10.1038/nmeth0410-248]

17 **Schwarz JM**, Rödelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nat Methods* 2010; **7**: 575-576 [PMID: 20676075 DOI: 10.1038/nmeth0810-575]

18 **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 [PMID: 11313315 DOI: 10.1053/gast.2001.23984]

19 **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 [PMID: 14999697 DOI: 10.1002/hep.20159]

20 **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 [PMID: 15077010]

21 **Kwan T**, Gros P. Mutational analysis of the P-glycoprotein first intracellular loop and flanking transmembrane domains. *Biochemistry* 1998; **37**: 3337-3350 [PMID: 9521654 DOI: 10.1021/bi972680x]

22 **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 [PMID: 12624161 DOI: 10.1136/jmg.40.3.e32]

23 **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 [PMID: 12671900 DOI: 10.1053/gast.2003.50144]

24 **Gotthardt D**, Runz H, Keitel V, Fischer C, Flechtenmacher C, Wirtenberger M, Weiss KH, Imparato S, Braun A, Hemminki K, Stremmel W, Rüschendorf F, Stiehl A, Kubitz R, Burwinkel B, Schirmacher P, Knisely AS, Zschocke J, Sauer P. A mutation in the canalicular phospholipid transporter gene, ABCB4, is associated with cholestasis, ductopenia, and cirrhosis in adults. *Hepatology* 2008; **48**: 1157-1166 [PMID: 18781607 DOI: 10.1002/hep.22485]

25 **Niessen KH**, Theisen M. [Why do children rarely have gallstones? Examinations of the lithoindices and the bile acid pattern in infants and children in health and disease (author's transl)]. *Monatsschr Kinderheilkd* 1980; **128**: 551-557 [PMID: 6107835]

26 **Heubi JE**, Soloway RD, Balistreri WF. Biliary lipid composition in healthy and diseased infants, children, and young adults. *Gastroenterology* 1982; **82**: 1295-1299 [PMID: 7067953]

27 **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 [PMID: 10648470 DOI: 10.1016/S0016-5085(00)70224-1]

28 **Oude Elferink RP**, Ottenhoff R, van Wijland M, Smit JJ, Schinkel AH, Groen AK. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. *J Clin Invest* 1995; **95**: 31-38 [PMID: 7814632 DOI: 10.1172/JCI117658]

P- Reviewer: Han TQ **S- Editor**: Wen LL
L- Editor: O'Neill M **E- Editor**: Liu XM

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 1007-9327