Original Article

Role of Common Canalicular Transporter Gene Variations in Aetiology of Idiopathic Gallstones in Childhood

(cholelithiasis / polymorphism / *ABCB11* / *ABCG8*)

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Abstract. Variations in genes encoding canalicular transportes, for biliary lipids may affect concentrations of biliary lipids in bile and promote cholesterol crystallization and gallstone formation. In our study we investigated the contribution of heterozygosity for common variations considered either potentially pathogenic or susceptibility alleles for cholesterol cholelithiasis in adults (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*) to the aetiology of paediatric idiopathic gallstone disease. Genotyping was performed in 35 paediatric subjects with idiopathic gallstones with positive family history for gallstones and 150 population controls. The ABCB4 variant p.Thr175Ala was found only in the controls, not in the patients. The frequency of the remaining three variant alleles and the corresponding genotypes did not differ between patients and controls. We conclude that the studied common variations in genes encoding canalicular transporters known to contribute to genetic predisposition to cholesterol gallstones in adulthood do not contribute specifically to the aetiology of paediatric idiopathic gallstones.

Introduction

The prevalence of gallstones in adults of industrialized countries approximates 10 % and shows a tendency to rise. Paediatric gallstones are considered a rare disease; however, prevalence data in this age group are scarce. Adult gallstone disease is predominantly idiopathic and most stones are composed of cholesterol, whereas the same disease in paediatric patients is due to specific causes such as infectious diseases, intestinal, hepatic or haemolytic disorders, and stones are predominantly composed of bilirubin polymers and calcium salts. Idiopathic paediatric gallstones represent less than 50 % cases (Herzog and Bouchard, 2008).

The aetiology of adult cholesterol gallstone disease is multifactorial and beside gender, non-genetic factors such as age, pregnancy, parity, obesity, rapid weight loss, administration of oestrogens or fibrates, preexisting diseases (diabetes mellitus, M. Crohn, short bowel, intestinal diseases, Gaucher disease, Down syndrome) and stasis of bile play a key role. The role of genetic factors is minor; nonetheless, five monogenic diseases associated with defects in *CYP71A* encoding the regulatory enzyme of bile salt synthesis (Pullinger et al., 2002), *ABCB4* encoding the biliary phospholipid export pump (Jacquemin et al., 2001; Rosmorduc et al., 2001), *ABCG8* encoding a protein unit of the heterodimeric canalicular cholesterol and plant sterol exporter (Buch et al., 2007) and two further non-specified loci on chromosome 1 (1.p34 and 1.p36.21) (Puppala et al., 2006) have been reported to date. The list of causes of paediatric idiopathic gallstones may be similar; however, one would expect a more pronounced role for the genetic factors especially in those patients with family history of gallstones in first-degree relatives in whom other known environmental factors are missing.

In our study we focused on the possible role of potentially pathogenic gene variations in *ABCB4* (GeneID: 5244), *ABCB11* (GeneID: 8647), and *ABCG8* (GeneID: 64241). The *ABCB4* variation c.523A>G (p.Thr175Ala) is considered a pathogenic mutation predisposing to low phospholipid-associated cholelithiasis (Rosmorduc and Poupon, 2007). The data on the other common *ABCB4* variation c.1954A>G (p.Arg652Gly) are controversial (Jacquemin et al., 2001; Meier et al., 2006). The *ABCB11* coding variant c.1331T>C (p.Val 444Ala) is associated with the lower expression of BSEP in liver (Meier et al.,

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Abbreviations: ABC – adenosine triphosphate-binding cassette, BSEP – bile salt export pump.

2006) and with lower protein production in an *in vitro* assay (Byrne et al., 2009). With diminished BSEP expression or function, decreased biliary bile-salt concentrations might promote cholesterol crystallization in bile. The *ABCG8* coding variant c.55G>C (Asp19His) has recently been identified as a genetic cause of gallbladder disease 4 in adults (Buch et al., 2007). The aim of our study was to assess the potential role of these variations in the aetiology of idiopathic gallstone disease in childhood.

Material and Methods

Patients

One hundred and nine children (53 males and 56 females) with gallbladder gallstones who had been hospitalized at the Department of Paediatrics, Faculty Hospital Motol, Prague, in the years 1995-2004 were considered. In 22 patients, gallstones were clearly associated with another disease such as Down syndrome, Gaucher disease, cystic fibrosis, haemolytic anaemia, inflammatory bowel disease, immune deficiency and Gilbert syndrome. Eighty-seven patients were invited to participate in the study. Thirty-three patients did not respond. In 13 of 54 patients, the aetiology of gallstones was uncertain; however, as long-term parenteral nutrition, treatment with cephalosporins or furosemide, dyslipidaemia, hepatobiliary infectious disease or obesity could promote gallstone formation, these patients were not enrolled. In 41 patients, gallstones were most likely idiopathic, with none of the risk factors, either mentioned above or known to contribute to cholelithiasis in adulthood (gender, pregnancy, parity, obesity, or rapid weight loss), playing any role. For *ABCB4* mutation testing, only those 35 patients of 41 with idiopathic gallstones were selected whose at least one parent or grandparent had gallstones. All were unrelated Caucasians of Czech origin. The mean age at the diagnosis of cholelithiasis was 10.7 ± 5.0 years (range 1–17). Nineteen patients (13 girls and 6 boys) underwent cholecystectomy. In none did gallstones recur after surgery. At the time of writing, all patients were well, without reported subjective problems such as abdominal pain, jaundice, or loss of appetite. Blood samples from 150 anonymous controls from the Czech Republic were used for survey of allele frequencies.

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

Method

Genomic DNA was isolated from 5 ml of peripheral blood collected into EDTA tubes. A standard method reported by Miller et al. (1988) was used for DNA isolation. The variations c.523A>G (p.Thr175Ala) and c.1954A>G (p.Arg652Gly) of *ABCB4*, the low-expression allele c.1331T>C of *ABCB11* and the variation c.55G>C of *ABCG8* were detected as restriction fragment length polymorphism after digestion of PCR products with allele-specific restriction endonucleases. The digested PCR products were analysed by gel electrophoresis in agarose or polyacrylamide gels. Primer sequences and further details of the individual procedures are presented in Table 1. The presence of the studied variations in *ABCB4* and *ABCB11* in patients was confirmed by DNA sequencing in automated DNA sequencers (AlfExpress, Pharmacia, Uppsala, Sweden; ABI-PRISM 3100-Avant, Applied Biosystems, Foster City, CA).

Results and Discussion

The *ABCB4* variation c.523A>G was not present in any of our patients with paediatric gallstones. By con-

Table 1. Primer sequences, restriction enzymes and size of the restriction fragments used for detection of polymorphisms in ABCB4, ABCB11 and ABCG8. Overhangs used for DNA sequencing are written in uppercase, gene-specific sequences are in lowercase, mismatched nucleotides are in bold and underlined.

Variation	Primer sequence	PCR product	Enzyme	Size (bp)	Allele
ABCB4 c.523A>G Thr175Ala	*5′*-AATACGACTCACTATAGtcctgaaagaga tgaataaaggatg *5′*-CAGGAAACAGCTATGACTTccttgacat attttcacacag	669 bp	*Hha*I	669 573+96	A (Thr) G (Ala)
ABCB4 c.1954A>G Arg652Gly	*5′*-AATACGACTCACTATAGtccttgattgag aagcagttagg *5′*-CAGGAAACAGCTATGACcaaagagtatgg ctcatagtagc	487 bp	*Tsp*RI	487 247+240	A (Arg) G (Gly)
ABCB11 c.1331T>C Val444Ala	*5′*-TAATACGACTCACTATAGgatttcagtgg acgttgctttg *5′*-TGAAACAGCTATGACCATGctatgcatgc caggacagtctc	420 bp	*Bsu*RI	420 235+185	T (Val) C (Ala)
ABCG8 c.55G>C Asp19His	*5′*-atggccgggaaggcggcagaggagag *5′*-acttcccattgctcactcaccgagg**g**at	83 bp	*Bam*HI	83 56+27	C (His) G (Asp)

Table 2. Genotypes in potentially pathogenic loci of ABCB4, ABCB11 and ABCG8 found in 35 paediatric subjects with idiopathic gallstones

Patient ID	*ABCB4* variations		*ABCB11* low-expression allele	*ABCG8* variation
	c.523A>G Thr175Ala rs58238559	c.1954A>G Arg652Gly rs2230028	c.1331T>C Val444Ala rs2287622	c.55G>C Asp19His rs11887534
1	AA	AA	CC	GG
2	AA	AA	TC	GG
3	AA	AA	TC	GG
4	AA	AG	TC	GG
5	AA	AA	CC	GG
6	AA	AA	TC	GG
7	AA	AA	TC	GG
8	AA	AG	TC	GG
9	AA	AA	TT	GG
10	AA	AA	CC	GG
11	AA	AA	TC	GG
12	AA	AA	CC	GG
13	AA	AA	TC	GG
14	AA	AA	CC	GG
15	AA	AA	TC	GC
16	AA	AA	TC	GC
17	AA	AA	TC	GG
18	AA	AA	TC	GC
19	AA	AA	TC	GC
20	AA	AA	TT	GG
21	AA	AA	TC	GG
22	AA	AA	TT	GG
23	AA	AA	TT	GG
24	AA	AA	CC	GG
25	AA	AA	TC	GG
26	AA	AG	TC	GG
27	AA	AA	TC	GG
28	AA	AA	TT	GG
29	AA	AA	TT	GG
30	AA	AA	CC	GC
31	AA	AA	TT	GC
32	AA	AA	TT	GG
33	AA	AA	TC	GG
34	AA	AA	TT	GG
35	AA	AA	CC	GG
Allelic frequency of variant alleles in patients with gallstones, HapMap populations and Czech population controls				
Allele	**G**	**G**	**C**	**C**
Gallstone patients	0	0.043	0.471	0.086
HapMap CEU	n.a.	0.075	0.408	0.085
HapMap HCB	n.a.	0.023	0.333	0.022
HapMap JPT	n.a.	0.023	0.261	0.011
HapMap YRI	n.a.	0.392	0.425	0.042
150 Czech controls	0.027	0.090	0.400*)	0.067**)

n.a. – data not available, *) N = 149, **) frequency in 285 Czech controls reported by Hubacek et al., 2001

trast, we found that 8 of 150 heterozygotes carried guanine at position 523 (8/300, representing 2.7 % of control alleles from the Czech population, see Table 2). The *ABCB4* variation c.1954A>G (p.Arg652Gly) was found in heterozygous state in subjects 4, 8 and 26. However, the c.1954G allele was not overrepresented (3/70, allelic frequency 0.043) in our patients as compared with a healthy Czech Caucasian population (allelic frequency 0.090, 27 heterozygotes in 150 controls, see Tables 2 and 3).

The frequency, 0.743, of carriers of the *ABCB11* low-expression variant c.1331T>C was not significantly different from the frequency of 0.631 and 0.650 found in, respectively, 149 Czech population controls and 120 Utah residents of northern and western European ancestry (named CEU population) included in the HapMap Project (www.hapmap.org). The differences between the frequencies of homozygotes for the same variant were non-significant as well (0.229 gallstone patients, 0.161 Czech population controls, 0.167 HapMap CEU controls, see Table 2). Finally, the frequency, 0.171, found for the carriers of the *ABCG8* variant c.55G>C, was not substantially different from the frequency of 0.126 found in 285 Czech population controls and 0.153 found in the HapMap CEU population sample (N = 118, see Tables 2 and 3 for details).

Table 3. Genotype frequencies of the possibly pathogenic variations in ABCB4, ABCB11 and ABCG8 identified patients with paediatric gallstones and in the Czech population controls. Odds ratio (OR), confidence interval (CI), and the P value were calculated for carriers of at least one variant allele (genotypes 12 + 22) vs. homozygous state for the wild-type allele (genotypes 11).

Variation	Patients	Genotype 11	12	22	Controls	Genotype 11	12	22	OR	CI	P
ABCB4 c.523A>G p.Thr175Ala	Gallstone patients (N = 35)	35	0	0	Czech controls (N = 150)	142	8	0	0.24	0.01-4.19*	0.29
ABCB4 c.1954A>G p.Arg652Gly	Gallstone patients (N = 35)	32	3	0	Czech controls (N = 150)	123	27	0	0.43	0.12-1.50	0.17
					HapMap CEU (N = 120)	102	18	0	0.53	0.15-1.92	0.33
ABCB11 c.1331T>C p.Val444Ala	Gallstone patients (N = 35)	9	18	8	Czech controls (N = 149)	55	70	24	1.69	0.74-3.87	0.21
									1.48**	0.59-3.73*	0.40**
					HapMap CEU (N = 120)	42	58	20	1.56	0.67-3.62	0.30
									1.54**	0.63-3.80*	0.34**
ABCG8 c.55G>C p.Asp19His	Gallstone patients (N = 35)	29	6	0	Czech controls (N = 285)	249	34	2	1.60	0.61-4.14	0.33
					HapMap CEU (N = 118)	100	16	2	1.28	0.46-3.55	0.63

Genotype symbols: 1 – wild-type allele, 2 – variant allele
* As one of the cells of the 2 × 2 contingency table used for determination of OR contained zero, OR, CI and P were calculated with modified maximum likelihood estimator c = 0.5 as reviewed by Agresti (1999). Briefly, for the calculations 0.5 was added to numbers in each cell of the 2 × 2 contingency table.
** calculated for homozygotes for the allele C vs. carriers of genotypes TC and TT

The frequency (0.027) of the *ABCB4* variation c.523A>G in Czech controls is compatible with the allelic frequency of 0.025–0.032 in other Central European Caucasian population samples (Pauli-Magnus et al., 2004 a,b; Lang et al., 2007). The threonine residue at position 175 is highly conserved, lying in a Thr-Arg-Leu-Thr cluster required for MDR3 adenosine triphosphatase activity. While the functional consequences of substitution 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 (Kwan and Gros, 1998), in which the substitution p.Thr169Ile resulted in a complete loss of substrate-induced P-glycoprotein ATPase activity. The substitution p.Thr175Ala is thus considered a possible disease-associated mutation or at least a susceptibility allele (Rosmorduc and Poupon, 2007). Our finding in paediatric gallstone patients strongly indicates that this likely pathogenic variant does not significantly contribute to the genetic aetiology of idiopathic gallstones in childhood.

The *ABCB4* variation c.1954A>G (p.Arg652Gly), which occurred in paediatric patiens with idiopathic gallstones, is common in the European Caucasian and African general population (see Table 2), but it has also been found in a patient with low phospholipid-associated cholelithiasis and low biliary phospholipids in whom p.Arg652Gly was hypothesized to be a conditional mutation leading to clinical symptoms only under certain circumstances, such as pregnancy, or when combined with another mutation (Jacquemin et al., 2001). By contrast, no correlation of the *ABCB4* genotype c.1954A>G with MDR3 expression level in the liver measured by Western blot was observed in a study by Meier et al. (2006). This, however, does not exclude functional deficiency. Our finding that the genotype c.1954A>G was neither overrepresented nor significantly underrepresented in patients with gallstones may indicate little role for this variation in the aetiology of paediatric idiopathic gallstones. Similar conclusions could be drawn for both carriers and homozygotes for the low-expression variant p. Val444Ala of BSEP and for the carriers of the *ABCG8* variation c.55G>C (Tables 2 and 3).

The results obtained in paediatric patients with idiopathic gallstones still do not rule out the possibility that other variations in genes encoding canalicular transporters of biliary lipids contribute to genetic predisposition of the disease. To answer this question, complete mutational analysis of the corresponding genes would be necessary. Alternatively, other pathogeneses of idiopathic gallstones in childhood should be considered. Since we did not assay biliary lipid concentrations in bile from our paediatric subjects, we cannot state what kind of changes in biliary lipid concentration was responsible for gallstone formation. Such examination was, however, not possible.

We conclude that the studied common variations in genes encoding canalicular transporters that contribute to genetic predisposition to cholesterol gallstones in adulthood do not contribute more significantly to the aetiology of idiopathic gallstones in childhood.

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