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ORIGINAL ARTICLE

Variability in human hepatic MRP4 expression: influence of cholestasis and genotype

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The multidrug resistance protein 4 (MRP4) is an efflux transporter involved in the transport of endogenous substrates and xenobiotics. We measured MRP4 mRNA and protein expression in human livers and found a 38- and 45-fold variability, respectively. We sequenced 2 kb of the 5′-flanking region, all exons and intron/exon boundaries of the *MRP4* gene in 95 patients and identified 74 genetic variants including 10 non-synonymous variations, seven of them being located in highly conserved regions. None of the detected polymorphisms was significantly associated with changes in the MRP4 mRNA or protein expression. Immunofluorescence microscopy indicated that none of the non-synonymous variations affected the cellular localization of MRP4. However, in cholestatic patients the MRP4 mRNA and protein expression both were significantly upregulated compared to non-cholestatic livers (protein: 299 ± 138 vs 100 ± 60a.u., $P < 0.001$). Taken together, human hepatic MRP4 expression is highly variable. Genetic variations were not sufficient to explain this variability. In contrast, cholestasis is one major determinant of human hepatic MRP4 expression.
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Introduction

The multidrug resistance protein 4 (MRP4, gene symbol: *ABCC4*) is one of 12 ATP-binding cassette (ABC) transporters included in the subfamily C of the ABC transporter superfamily. ABC transporters mediate ATP-dependent efflux of a diverse range of endogenous and exogenous substrates across membranes and therefore play a critical role in physiological transport processes, drug transport and detoxification.[1–6]

Human MRP4 is expressed in liver, brain, kidney, colon, lung and placenta. Subcellularly, it has been localized to the basolateral membrane of hepatocytes[7] and to the apical membrane of kidney proximal tubular cells.[8] MRP4 transports endogenous cyclic nucleotides such as cAMP and cGMP[9] out of cells. Other physiological substrates are bile acids,[7,10,11] steroids,[10] prostaglandins[12] and reduced glutathione.[7] Using *Abcc4(-/-)* and wild type mice, it was also demonstrated very recently that Mrp4 is able to transport several sulfate conjugates across the basolateral hepatocyte membrane into blood.[13]

Recently, *in vitro* experiments[14,15] as well as animal models[15–18] of cholestasis have demonstrated that human MRP4 and rodent Mrp4 are induced under cholestatic conditions. Mennone *et al*[19] showed that serum bile acid concentrations are lower in Mrp4 knockout mice than in wild-type CBDL (common bile

duct ligation) mice presumably owing to impaired secretion of bile acids over the basolateral hepatocyte membrane. Moreover, the upregulation of MRP4 mRNA and protein in human liver has been reported for a small number of liver specimens from children diagnosed with progressive familial intrahepatic cholestasis (PFIC)-2 and 3.[20] To the best of our knowledge, there are no systematic data on the influence of cholestasis on human hepatic MRP4 expression.

MRP4 is able to confer resistance to nucleoside analogs used in anticancer and antiviral therapies.[21,22] Recent studies have demonstrated that MRP4 is capable of transporting the antiviral agent ganciclovir and may therefore reduce its antiviral efficacy.[23] It has also been shown that MRP4 is able to transport actively mercaptopurine metabolites, used as anticancer agents, out of cells.[24,25] Furthermore, a polymorphism in the 3′-untranslated region of *MRP4* was associated with higher lamivudine-triphosphate levels in peripheral blood mononuclear cells (PBMCs) of HIV-positive patients indicating a possible role of MRP4 in the resistance to this nucleoside analog.[26] Because MRP4 is expressed in the liver, it may be that inter-individual variations in MRP4 expression affect intrahepatic and systematic drug concentrations. While a wide variation in MRP4 expression has been reported for pediatric leukemia lymphoblasts,[14] little is known on the variability of MRP4 expression in human liver or on factors affecting human MRP4 expression.

We therefore analyzed in a large collection of human livers (1) the variability of human hepatic MRP4 mRNA and protein expression, (2) the influence of cholestasis on human hepatic MRP4 mRNA and protein expression and (3) the impact of *MRP4* polymorphisms/haplotypes on its hepatic expression and localization.

Results

Influence of cholestasis on MRP4 mRNA and protein expression

MRP4 mRNA expression data were not normally distributed and varied 38-fold among the total study population. Fifteen of the 70 patients were cholestatic at the time of tissue resection (Table 1). Comparison of the cholestatic group of liver samples with the non-cholestatic group revealed a significantly higher *MRP4* mRNA expression in liver specimens obtained from patients with cholestasis (204 ± 162 vs 100 ± 76 a.u., $P = 0.03$; Figure 1a).

MRP4 protein expression was determined in those specimens from which nuclear/membrane pellets could be prepared (24 non-cholestatic and 14 cholestatic livers). Representative Western blots for MRP4 of cholestatic and non-cholestatic liver specimens are shown in Figure 1b. MRP4 protein data were not normally distributed and varied 45-fold among the total study population. MRP4 protein expression was 3-fold higher in cholestatic compared to non-cholestatic liver specimens (299 ± 138 vs 100 ± 60 a.u., $P < 0.001$; Figure 1c).

Liver samples of the patients classified as cholestatic were excluded from the following analyses on additional factors

Table 1 Mean blood biochemistry parameters of cholestatic and non-cholestatic patients

Group	*Parameter*	*Total bilirubin*	*Conjugated bilirubin*	*Alkaline phosphatase*	*γ-Glutamyl-transferase*	*Aspartate aminotransferase*	*Alanine aminotransferase*	*Albumin*	*International Normalized Ratio*	*C-Reactive Protein*
	Unit	mg/dl	mg/dl	u/l	u/l	u/l	u/l	g/dl	% of norm	mg/l
	Norm	<1	<0.3	20–180	4–28	4–22	4–17	3.5–5.2	70–125%	<5
Non-cholestatic ($n = 55$)	Mean	0.7	0.2	157	45	15	19	5.1	106	1.2
	s.d.	0.4	0.2	70	38	11	22	6.4	13	1.6
Cholestatic ($n = 15$)	Mean	1.6	1.0	483	233	35	44	3.7	97	4.1
	s.d.	1.3	1.1	279	278	27	52	0.4	22	3.3
Difference between groups	**	***	***	***	***	***	***	***	NS	***

Reference values are according to Pschyrembel, Clinical Dictionary.[38]
***$P \leqslant 0.001$; **$P \leqslant 0.01$; *$P \leqslant 0.05$; NS ($P > 0.05$).

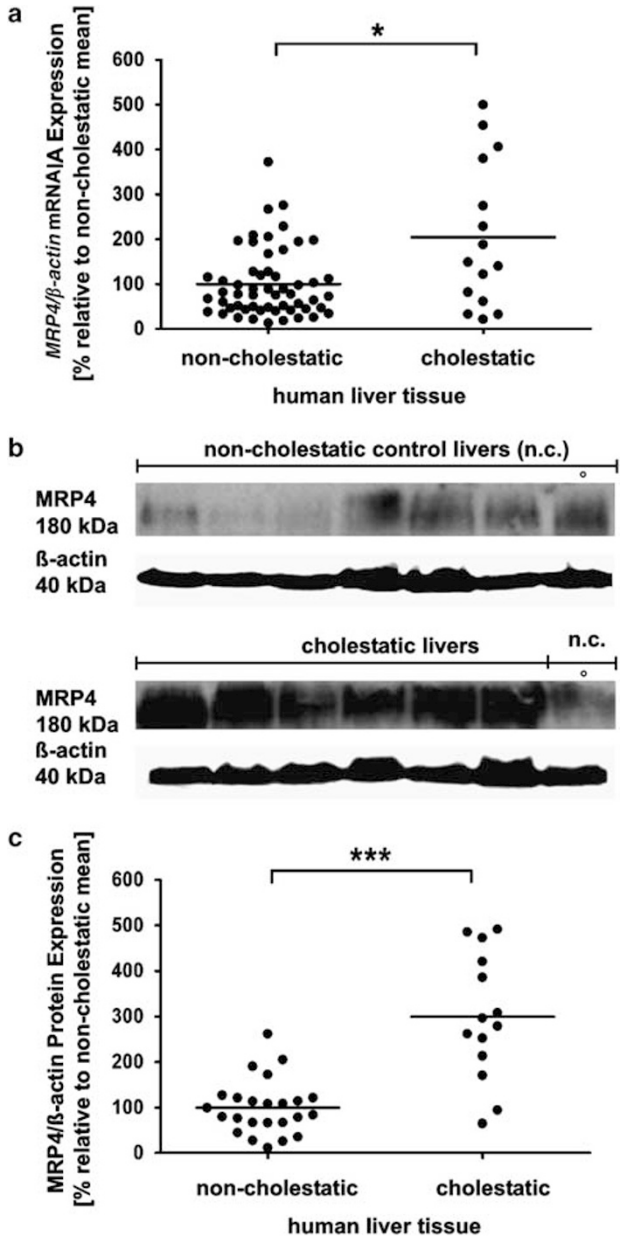


Figure 1 MRP4 expression in non-cholestatic and cholestatic human livers. (**a**) Mean *MRP4* mRNA expression normalized for *β-actin* mRNA expression in 70 livers classified as cholestatic or non-cholestatic. *$P<0.05$. (**b**) Representative Western blots for MRP4 protein in non-cholestatic and cholestatic livers. The circles above the bands on the right side mark the reference sample with the highest MRP4 expression among the non-cholestatic samples (n.c. = non-cholestatic control liver, see Materials and methods). (**c**) Mean MRP4 protein expression normalized for β-actin expression in livers classified as cholestatic or non-cholestatic. *** $P<0.0001$.

potentially influencing MRP4 mRNA or protein expression. The *MRP4* mRNA expression of the remaining non-cholestatic liver samples was not normally distributed (Figure 2a), whereas the protein expression was (Figure 2b). MRP4

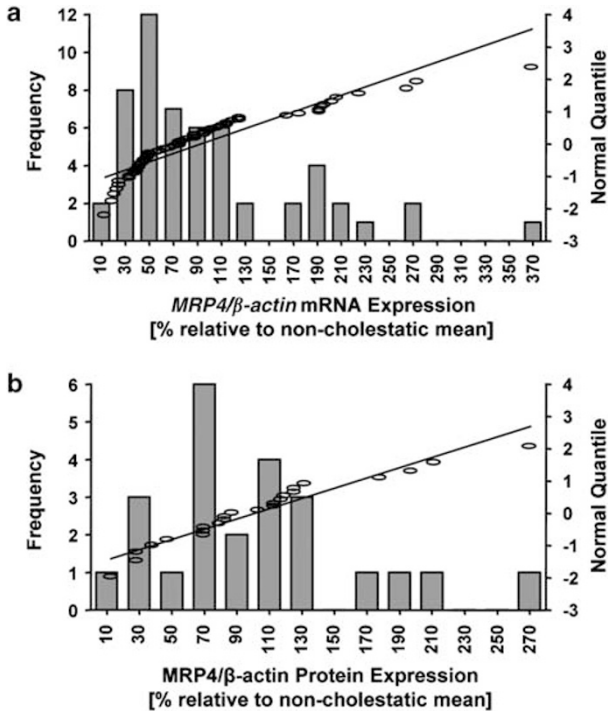


Figure 2 Frequency histogram and normal plot of MRP4 expression in the non-cholestatic study population. (**a**) *MRP4* mRNA expression normalized for *β-actin* mRNA (not normally distributed), (**b**) MRP4 protein expression normalized for β-actin (normally distributed).

mRNA and protein expression were not significantly correlated.

Analysis of hepatic MRP4 mRNA and protein expression of all non-cholestatic patients in relation to age, gender, medication (antihypertensives, analgesics, anti-ulcer drugs, psychopharmaceuticals, estrogens, glucocorticoids, statins, antibiotics, cytostatics), type of hepatobiliary disease (cholangiocellular carcinoma, hepatocellular carcinoma, Klatskin tumor, Caroli syndrome, liver metastasis), smoking or alcohol consumption did not reveal any influence of the analyzed factors. There was no difference in β-actin expression between cholestatic and non-cholestatic livers both for mRNA and protein.

Genetic variants

In total, 74 genetic variants were detected in the *MRP4* gene (Table 2) including 10 exonic variations resulting in amino acid exchanges, four of which have not been described previously (ID 40,41,48,54). In addition, 11 variations within the promoter region, 12 synonymous exonic variations, 36 intronic variations and five variations in the 3′ untranslated region (3′-UTR) could be detected. A summary of all variants, their sequence context and their frequencies is listed in Table 2. Seven of the 10 amino acid exchanges were predicted to be near or within either the transmembrane regions or the ATP-binding domains of MRP4 (Figure 3).

Table 2 Genetic variations identified in the *MRP4* gene among 95 Caucasian individuals

Variant ID	*Sequence context 5′*	*Systematic name*	*Sequence context 3′*	*Genetic element*	*Effect*	*Frequency (%)*				
						Heterozygous	*HW*	*Homozygous*	*HW*	*NCBI SNP ID*
1	GAACTCCTGA	g.-2174C>G	GTCAGGTGAT	Promoter		23.7	28.5	5.4	3.0	
2	CACCTGCCTC	g.-2152A>G	GCCTCCCAGA	Promoter		2.2	2.2	0.0	0.0	
3	TGGGCTAAAG	g.-2055C>A	CATCCTCCTG	Promoter		40.9	47.9	39.8	36.3	rs2389235
4	CTAAAGCCAT	g.-2051C>T	CTCCTGTCTC	Promoter		38.7	45.1	46.2	43.0	rs1764419
5	ATTTTTAAAA	g.-1980-1981insA	CTTTTGGTAG	Promoter		39.8	45.5	45.2	42.3	
6	CACTATGCTG	g.-1949G>C	CTAGCCTGTG	Promoter		1.1	1.1	0.0	0.0	
7	CATCTATAAA	g.-1508G>A	TAAGGATAAC	Promoter		12.6	11.8	0.0	0.4	rs868853
8	AAATTATCAG	g.-1235T>C	ATTTGAACTT	Promoter		2.1	2.1	0.0	0.0	
9	TACTTAAACT	g.-1130G>A	AGTTGGGAGG	Promoter		42.4	46.3	15.2	13.3	rs2993579
10	CCATGGCACC	g.-642G>C	TCGTTTGGTC	Promoter		41.9	46.7	41.9	39.6	rs869951,[39]
11	CGCGTCTCCT	g.-289C>G	CCGCCGCGTC	Promoter		2.2	2.2	0.0	0.0	
12	CCGCGGCCAC	g.-49C>T	GCCGCCTGAT	5′ UTR/ Exon 1		1.1	1.1	0.0	0.0	rs3751333,[39]
13	TGCCCGTGTA	g.15C>T	CAGGAGGTGA	Exon 1	synonymous	1.1	1.1	0.0	0.0	
14	**GGACGCGAAC**	**g.52A>C**	**TCTGCTCACG**	**Exon 1**	**I18L**	**2.2**	**2.2**	**97.8**	**97.8**	**rs11568681**
15	GGTGAGTGTC	g.84C>T	CCGCCCGAAA	Intron 1		2.2	2.1	0.0	0.0	rs11568682
16	CACTCTCCCG	g.138G>C	GGCCGCGCCC	Intron 1		1.1	1.0	0.0	0.0	
17	ACCCATGGAG	g.53498G>A	TAGGAGTCTA	Intron 1		46.7	47.4	15.2	14.9	rs9556466
18	TTTTTCCTCAT	g.54215T>C	GTAGGTTCTG	Intron 2		46.7	47.0	14.4	14.3	rs4148437,[39]
19	AGCATGGGTG	g.66387A>G	CAGAGCAAGC	Intron 3		11.9	11.2	0.0	0.4	[39]
20	GGTAAGTGAC	g.66715A>G	TTCAGCATTA	Intron 4		2.2	2.2	0.0	0.0	rs11568637
21	**CATGGCCATG**	**g.90561G>T**	**GGAAGACAAC**	**Exon 5**	**G187W***	**4.3**	**4.2**	**0.0**	**0.0**	**rs11568658**
22	CACCCCTCTT	g.90673T>C	CCCCTTTTAT	Intron 5		25.3	23.7	73.6	74.4	rs899496,[39]
23	TTTCTGGAGG	g.90696C>T	AGGGGCTCAC	Intron 5		38.9	37.5	5.6	6.3	rs4148472,[39]
24	GCAGGGGCTC	g.90705-90708delACTC	TGTTCACACT	Intron 5		44.9	47.7	38.2	36.8	rs3046400
25	TGTGTTCTTA	g.91667C>T	TGGTTTTGCT	Intron 5		1.1	1.1	0.0	0.0	
26	TGCAGGCGAT	g.91765C>T	GCAGTGACTG	Exon 6	synonymous	19.4	19.2	1.1	1.2	rs899494,[39]
27	GTTGTCGGAA	g.93281G>T	CAAAAGCGCT	Intron 6		43.5	41.5	48.9	49.9	rs2274410,[39]
28	GGCTGTGATC	g.93355G>A	CTCAGGCTCA	Intron 6		15.2	17.7	2.2	1.0	rs2274409,[39]
29	CTCATCTCCC	g.93372G>A	TGTCTGGTTC	Intron 6		2.2	2.2	0.0	0.0	rs11568683
30	AAGTTTTACT	g.93595A>G	TGTTTTCACA	Intron 7		43.5	41.5	48.9	49.9	rs2274408,[39]
31	**TCTCTTTCAG**	**g.94534G>T**	**AAGGAGATTT**	**Exon 8**	**K304N**	**17.6**	**17.8**	**1.1**	**1.0**	**rs2274407,**[39]
32	CCTGCCTCAG	g.94573G>A	GGGATGAATT	Exon 8	synonymous	47.2	42.3	6.7	9.2	rs2274406,[39]
33	ATTTGGCTTC	g.94591G>A	TTTTTCAGTG	Exon 8	synonymous	47.2	42.3	6.7	9.2	rs2274405,[39]
34	CAGGTTGGTG	g.94791T>A	CAGATGCCAT	Intron 8		4.4	4.3	0.0	0.0	rs11568702
35	CACTATGTTC	g.94865C>G	AGTGTAATGA	Intron 8		47.2	42.3	46.1	48.5	rs3818494,[39]
36	TGACATTTAA	g.94883C>T	TCTCTCATAA	Intron 8		47.2	42.3	46.1	48.5	rs3818494,[39]
37	TAGAGAATTT	g.106549T>C	GAGGTGTTAC	Intron 9		55.6	49.9	24.4	27.3	rs2274403,[39]
38	GCATTGCAGT	g.114366G>A	GCTTATTCTT	Intron 10		9.8	9.3	0.0	0.2	rs4148487,[39]
39	CTCTGAAAAA	g.114614G>A	GTGAGTGATG	Exon 11	synonymous	1.1	1.1	0.0	0.0	
40	**ATAGGAGATC**	**g.123270G>A**	**GGGAACCACG**	**Exon 12**	**R531Q**	**1.1**	**1.1**	**0.0**	**0.0**	
41	**GCTGACATCT**	**g.123548A>G**	**TCTCCTGGAC**	**Exon 13**	**Y556C***	**1.1**	**1.1**	**0.0**	**0.0**	
42	GAGGTCTCCC	g.130808G>A	AACTTGCAAG	Intron 14		24.4	23.1	1.1	1.8	rs11568663
43	TGCCTGTTTC	g.136709C>T	CCACAGCTTT	Intron 15		18.2	16.5	0.0	0.8	rs4148501,[39]
44	GTTTTCAGGC	g.136862C>T	TATAAGAATT	Exon 16	synonymous	2.1	2.1	0.0	0.0	rs11568666
45	ATGTATGAAA	g.137735T>C	ACTCCAAAAT	Intron 17		16.1	14.8	83.9	84.5	rs11568650
46	GGTTCATTTT	g.138034T>C	AAAAAAATGT	Intron 17		20.7	20.3	1.1	1.3	[39]
47	**AAATGTAACC**	**g.138154G>A**	**AGAAGCTAGA**	**Exon 18**	**E757K**	**1.1**	**1.1**	**0.0**	**0.0**	**rs3765534,**[39]
48	**TGTAGCTACC**	**g.139997G>A**	**TTCTTTTTGG**	**Exon 19**	**V776I**	**1.1**	**1.1**	**0.0**	**0.0**	
49	ACCACTGACA	g.185182T>G	CGGCTTATTT	Intron 19		46.7	49.9	29.3	27.8	rs1678394,[39]
50	TGAATAATAT	g.185207A>G	TTAAATACAT	Intron 19		4.3	4.2	0.0	0.0	rs2296652
51	ATTTGCTGCC	g.185369G>A	CTGACGTTTT	Exon 20	synonymous	1.1	1.1	0.0	0.0	
52	ACAACTCATG	g.217965C>A	AAGTATTTTT	Intron 20		6.7	6.4	93.3	93.4	rs1189437,[39]
53	**GGTTGGTGTG**	**g.218049G>T**	**TCTCTGTGGC**	**Exon 21**	**V854F***	**3.3**	**3.3**	**0.0**	**0.0**	**rs11568694**
54	**TTGGATCGCA**	**g.218085A>G**	**TACCCTTGGT**	**Exon 21**	**I866 V***	**5.6**	**5.4**	**0.0**	**0.1**	
55	CTTGGGAGGC	g.225708C>T	GCAGGTTTTT	Intron 21		28.0	25.8	1.2	2.3	rs11568672

Table 2 *Continued*

Variant ID	*Sequence context 5′*	*Systematic name*	*Sequence context 3′*	*Genetic element*	*Effect*	*Frequency (%)*				*NCBI SNP ID*
						Heterozygous	*HW*	*Homozygous*	*HW*	
56	TTTCCCACTT	g.225789G>A	TCATCTTCTC	Exon 22	synonymous	7.9	7.6	0.0	0.2	rs1678339,[39]
57	CCCGCTGGTT	g.227028C>T	GCCGTCCGTC	Exon 23	synonymous	7.4	7.1	0.0	0.1	rs1189466,[39]
58	TCAGAAGATT	g.229696T>G	AGTGCTATGT	Intron 25		4.3	4.2	0.0	0.0	rs11568667
59	TAAGGAAGAA	g.238593A>G	ATGTCAATCA	Exon 26	synonymous	34.1	33.9	4.5	4.7	rs1751034,[39]
60	CAAGTCATAG	g.238627C>A	GTCCTTACCT	Intron 26		28.4	29.0	3.4	3.1	rs11568656
61	GACCGTGACT	g.248116A>G	TTTTGGGCAC	Intron 26		6.5	8.2	1.1	0.2	rs11568643
62	**AATGAGCACA**	**g.248189C>T**	**GGATGAGGAA**	**Exon 27**	**T1142M****	**3.2**	**3.2**	**0.0**	**0.0**	**rs11568644**
63	GTTAGCCTCA	g.248262G>A	TATGTGTATA	Intron 27		3.3	3.3	0.0	0.0	rs7324065
64	TACCTTTACA	g.248285G>A	CATTGCAGGA	Intron 27		5.6	5.4	0.0	0.1	
65	TAAGTGTGTA	g.257099A>C	ATGGGAAACA	Intron 28		31.2	29.2	2.2	3.1	rs12875235
66	TAGAAACCCC	g.257724T>C	AGTAAGTTAA	Intron 29		8.8	8.4	0.0	0.2	
67	CCTAGTAAGT	g.257732T>A	AATTTTCTGT	Intron 29		20.4	20.0	1.1	1.3	rs9302041
68	GGCTCCCTCC	g.266763G>A	CACAATTTGG	Intron 30		1.1	1.1	0.0	0.0	
69	GAAGGCATTT	g.279778T>G	CCACTAGTTT	Exon 31/ 3′ UTR		42.6	44.9	12.8	11.6	rs3742106,[39]
70	TCATTTGAAT	g.279870A>G	TTTCTCCCAA	3′ UTR		1.1	1.1	0.0	0.0	
71	TCCAAATCAG	g.279962A>G	GGTGCAGGCC	3′ UTR		1.1	1.1	0.0	0.0	
72	GTTAAATGCC	g.279987G>A	TCTATCAGGT	3′ UTR		1.1	1.1	0.0	0.0	
73	GAGTAGGACA	g.280051A>G	AGTTGTCACA	3′ UTR		43.6	45.2	12.8	12.0	rs4148551,[39]
74	AAAAAAAAAA	g.280318delA	TGGATACATG	3′ UTR		44.7	45.6	42.6	42.1	rs4148552,[39]

Abbreviation: SNP, single nucleotide polymorphism.
Genotype frequencies are given as observed frequencies and expected frequencies are calculated according to the law of Hardy–Weinberg (HW).
Non-synonymous SNPs are given in bold letters and those predicted to be possibly or probably damaging for transporter function by *in silico* analysis are marked with one or two asterisks, respectively.

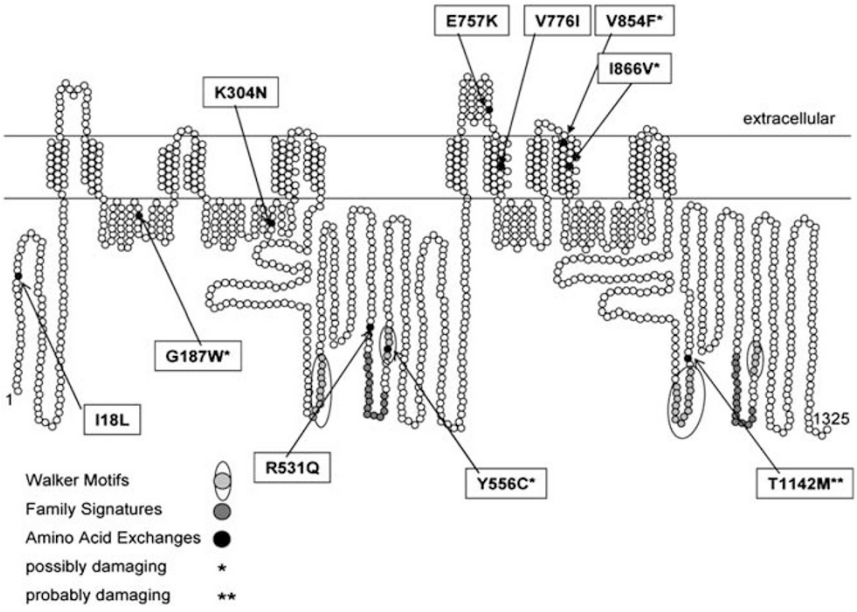


Figure 3 Predicted two-dimensional protein structure of the MRP4 protein. Prediction was made by using the CBS Prediction Server of the Technical University of Denmark DTU and the DAS-Transmembrane Prediction Server of the Stockholm Bioinformatics Center. *MRP4* polymorphisms resulting in amino acid exchanges are indicated as filled circles and those predicted to alter transporter expression and/or function are marked by asterisks.

Influence of MRP4 variations on mRNA and protein expression in human liver

None of the 74 detected polymorphisms was significantly associated with changes in the *MRP4* mRNA expression. MRP4 protein expression in samples carrying non-synonymous polymorphisms relative to the control group ($n=8$) was as follows: G187W 58% ($n=2$), K304N 54% ($n=3$), R531Q 20% ($n=1$), Y556C 60% ($n=1$), E757K 86% ($n=1$), V776I 161% ($n=1$), V854F 96% ($n=1$), I866V 78% ($n=4$) and T1142M 40% ($n=2$). None of these changes was statistically significant owing to the small sample size (Figure 4).

MRP4 protein expression in samples carrying the homozygous or heterozygous single nucleotide polymorphism (SNP) g.279778T>G (internal variant ID #69, $n=12$), which was previously shown to affect the lamivudine-triphosphate concentration in PBMC of HIV-positive patients,[26] was about 72% of samples with the homozygous wild type at this position ($n=13$). This difference was not quite significant ($P=0.07$).

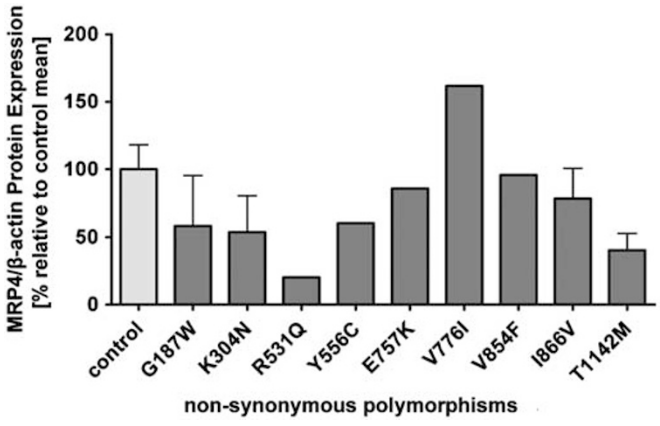


Figure 4 MRP4 protein expression normalized for β-actin in non-cholestatic livers of patients carrying non-synonymous *MRP4* polymorphisms relative to control livers.

Influence of MRP4 haplotypes on mRNA and protein expression in human liver

The use of SNPs may not have sufficient power to track unobserved, but evolutionarily linked genetic markers. Therefore, gene-based haplotypes have been established. Two hundred and fifty-one theoretical haplotypes were constructed from our genotype data by the Phase program.[27] Seven haplotypes (termed A-G) were observed with a frequency of at least 2.0% each and represent in total 38.8% of all alleles (Table 3). Haplotype D is associated with significantly lower *MRP4* mRNA expression (32 ± 16a.u., $n=6$) compared to individuals without this haplotype (107 ± 77a.u., $n=63$; $P=0.03$). Haplotype D carries single nucleotide polymorphisms with the ID27,30 (100% linked), 32,33,35,36 (all four 100% linked).

Haplotype F does not carry any of the above-mentioned variations and is associated with a significantly higher *MRP4* mRNA expression level (203 ± 8 a.u., $n=3$) compared to individuals without this haplotype (97 ± 75 a.u., $n=85$; $P=0.05$). The remaining haplotypes named A, B, C, E and G did not show any significant influence on *MRP4* mRNA expression.

No significant association between MRP4 haplotypes and protein expression was detected in those cases where protein expression could be determined (A–E).

Influence of non-synonymous variations on the localization of MRP4 in human hepatocytes

As previously described[7], immunofluorescence microscopy of MRP4 indicates that it is localized to both the basolateral membrane and to intracellular structures of human hepatocytes. None of the investigated non-synonymous polymorphisms appeared to affect MRP4 hepatic tissue distribution (Figure 5). A representative staining for MRP4 and the basolateral membrane marker desmoplakin in control liver tissue from a patient with no amino acid exchange and one with the amino acid exchange V854F is shown in Figure 5.

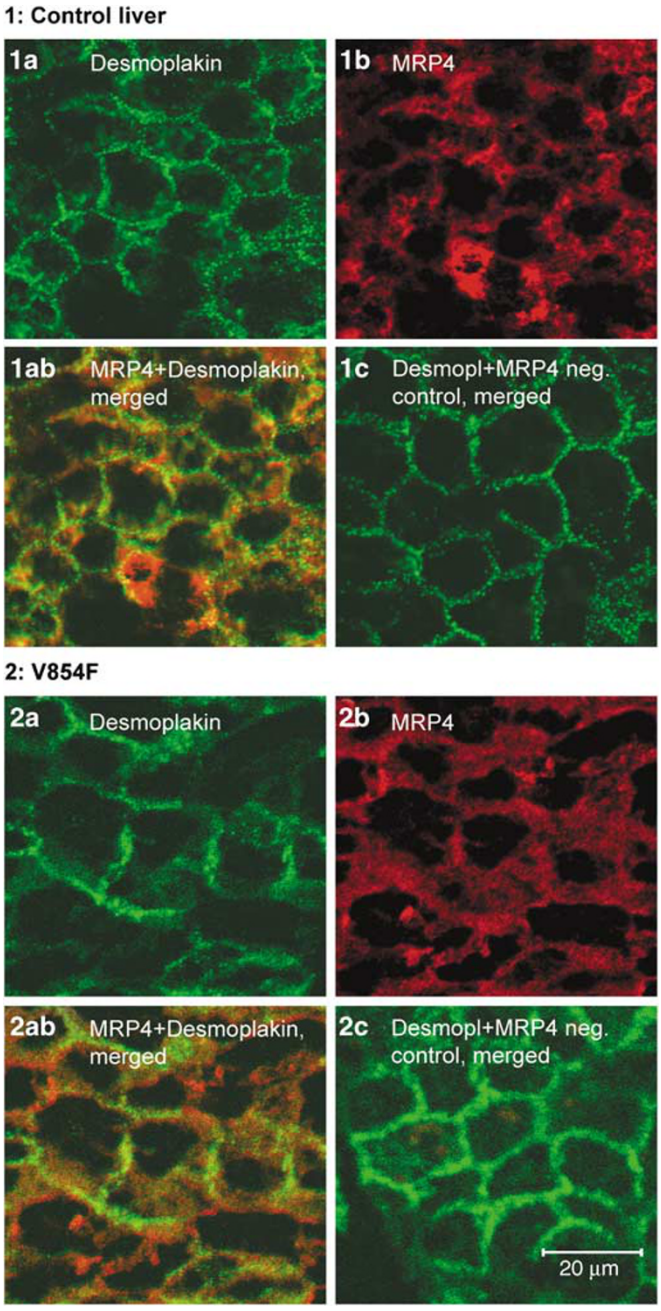
Table 3 ***MRP4*** **haplotypes**

Haplotype ID	*SNP*																*Frequency*
	Promoter					*Intron 1*	*Intron 2*	*Intron 5*		*Intron 6*	*Intron 7*	*Exon 8*				*Intron 8*	*Intron 9*	*Intron 19*	*Exon 26*	*Exon 31*	*3′UTR*	*%*
SNP ID	3	4	5	9	10	17	18	23	24	27	30	32	33	35	36	37	49	59	69	73		
										100% linkage		100% linkage										
A	**A**	**T**	**i**	**A**	**C**	G	T	C	**d**	**T**	**G**	**G**	**G**	**G**	**T**	T	**G**	**A**	T	A		15.2
B	**A**	**T**	**i**	**A**	**C**	**A**	**C**	C	**d**	G	A	A	A	C	C	**C**	T	G	T	A		5.6
C	**A**	**T**	**i**	**A**	**C**	**A**	**C**	**T**	r	**T**	**G**	**G**	**G**	**G**	**T**	**C**	**G**	**A**	T	A		4.5
D	C	C	r	G	G	G	T	C	**d**	**T**	**G**	**G**	**G**	**G**	**T**	T	**G**	**A**	T	A		4.5
E	**A**	**T**	**i**	**A**	**C**	**A**	**C**	**T**	r	**T**	**G**	**G**	**G**	**G**	**T**	**C**	T	G	T	A		3.4
F	**A**	**T**	**i**	**A**	**C**	**A**	**C**	C	**d**	G	A	A	A	C	C	**C**	T	**A**	T	A		2.8
G	**A**	**T**	**i**	**A**	**C**	**A**	**C**	C	**d**	G	A	A	A	C	C	**C**	**G**	**A**	T	A		2.8

Abbreviations: MRP4, multidrug resistance protein 4; SNP, single nucleotide polymorphism; d, deletion; i, insertion; r, no deletion/insertion.
The most common *MRP4* haplotypes (A–G) deduced from 20 *MRP4* variations. SNP IDs are according to Table 2. Nucleotides that are different from the reference sequence are in bold letters.

Prediction of functional effects of non-synonymous variations

In silico analysis of all 10 detected amino acid exchanges revealed that five of them (I18L, K304N, R531Q, E757K, V776I) can be considered benign, whereas others especially near or within transmembrane regions or ATP-binding domains are possibly (G187W, Y556C, V854F, I866V) or in one case (T1142M) even very likely damaging for protein localization and/or function (Figure 3).



Discussion

Variations in genes encoding transport proteins influence drug action, toxicity and endogenous transport processes.[1–5] In this study, we investigated the impact of genetic polymorphisms and other factors on the MRP4 mRNA and protein expression in human liver as well as on its hepatocellular localization.

One of the major findings of this study was that MRP4 mRNA and protein expression, determined in a large collection of human liver samples, were two- to threefold higher in patients classified as cholestatic vs those being non-cholestatic. Studies in cell culture[14,15] and in animal models[15–18] have shown increased hepatic MRP4/Mrp4 mRNA and protein expression under cholestatic conditions. Moreover, Mennone *et al*[19] showed that serum bile acid levels are lower in Mrp4 knockout mice than in wild-type CBDL (common bile duct ligation) mice, indicating a role of murine Mrp4 in the adaptive response to obstructive cholestatic liver injury. Keitel *et al*[20] reported a significant increase in MRP4 mRNA and protein expression in a small number of liver samples from children diagnosed with PFIC-2 and -3. These children were treated with tauroursodeoxycholate making it difficult to judge whether this upregulation of MRP4 was owing to their medication with tauroursodeoxycholate,[20] the cholestatic condition itself or a combination of both. Marschall *et al.*[28] recently studied the influence of UDCA (ursodeoxycholic acid) treatment on MRP4 mRNA and protein expression in non-cholestatic gallstone patients and found an upregulation of MRP4 protein and mRNA, the latter data being not significant. Taken together, these data and our findings indicate that upregulation of MRP4 in human liver may be an adaptive mechanism to increased bile salt levels in cholestasis and can also be accomplished by UDCA treatment. An underlying mechanism via farnesoid X receptor (FXR) inhibition was proposed very recently by Stedman *et al.*[29] but seems to be indirect, that is FXR does not directly regulate Mrp4.[15,30]

Figure 5 Immunolocalization of MRP4 in cryosections of human liver. (1) Control liver tissue from a patient with no amino acid exchanges in MRP4. (2) Liver tissue from a patient with the amino acid exchange V854F in MRP4. Of each liver tissue sample two cryosections (5 μm) were incubated with either a purified rabbit polyclonal MRP4 antiserum and desmoplakin (basolateral marker protein[36]) antibody (**a**,**b**,**ab**) or only with desmoplakin antibody (**c**, negative control for MRP4). This was in both sections followed by incubation with a secondary antibody conjugated with Cy2 (green) and a secondary antibody conjugated with Cy3 (red). Panels 1a and 2a show the green fluorescence of stained desmoplakin, panels 1b and 2b show the red fluorescence of stained MRP4, panels 1ab and 2ab show the merged confocal laser scanning picture of desmoplakin (green) and MRP4 (red), which were mostly colocalized indicated by the resulting yellow color. Panels 1c and 2c show the merged confocal laser scanning pictures of sections incubated with desmoplakin primary antibody and both secondary antibodies and therefore served as negative control for the MRP4 staining. No difference in the localization of MRP4 was observed between control liver and the V854F variant and livers from patients with other non-synonymous variations (staining not shown).

Furthermore, the nuclear receptors pregnane X receptor and constitutive androstane receptor have been discussed as possible regulators at least for mouse Mrp4.[15] It remains to be investigated to what extent MRP4 can compensate for impaired bile salt export and to what extent it shares this function with the recently identified organic solute carrier OSTα/β.[31]

A wide variation in *MRP4* mRNA expression has been described earlier for leukemia lymphoblasts[14] in children. Here, we report that a high variability of MRP4 mRNA and protein expression can as well be demonstrated in human livers. Age, medication, smoking, type of hepatobiliary disease or alcohol consumption of the respective individuals had no impact on this highly variable MRP4 mRNA and protein expression. Interestingly, also the gender of the patients did not influence MRP4 mRNA or protein expression in neither the non-cholestatic nor the cholestatic group of patients although differences in Mrp4 expression have been reported in mice.[32] To evaluate whether sequence variations in the *MRP4* gene influence the MRP4 expression, we sequenced 2 kb of the 5′-flanking region and all exons including the intron/exon boundaries of the *MRP4* gene of all patients. In total, we identified 74 genetic variants in the *MRP4* gene including 10 exonic SNPs resulting in amino acid exchanges. Of special interest are seven SNPs, four of them being novel, predicted near or within either the transmembrane regions or the ATP-binding domains of MRP4 (Figure 3). Amino acid exchanges in those regions are likely to influence transporter localization and/or function. Computational prediction of functional effects of all detected amino acid exchanges confirmed these speculations for at least five of them. According to our analysis, none of the 74 analyzed non-synonymous SNPs leads to a significant change in *MRP4* mRNA expression. Five of the analyzed non-synonymous polymorphisms (Figure 4) were associated with lower MRP4 protein expression levels of at least 40% decrease compared to livers from patients without any non-synonymous variations. One patient showed an increase of more than 60%. Owing to the small numbers of patients carrying one specific polymorphism, we cannot conclude that these findings are statistically significant.

Because Anderson *et al*[26] had reported a significant association of the polymorphism g.279778T>G (internal variant ID #69, Table 2) with lamivudine-triphosphate concentration in PBMCs of HIV-positive patients, we also analyzed whether this polymorphism influenced MRP4 protein expression. We found a non-significant trend towards lower MRP4 protein expression in livers from patients with the GT or GG variant compared with those carrying TT at this position. This would be in line with the data from Anderson *et al*[26] who found significantly elevated lamivudine-triphosphate concentrations in PBMC of individuals with the GT or GG genotypes and could suggest that these effects have been accomplished by a lower MRP4 expression. The fact that the difference in protein expression between the two genotypes did not quite reach significance ($P=0.07$) in our study, could be owing to the relatively small sample size of only 12 and 13 specimens, respectively. Finally, we also investigated the impact of non-synonymous *MRP4* polymorphisms on the localization of MRP4 in human hepatocytes. Especially those amino acid exchanges located within the transmembrane domains of MRP4 (Figure 3) could possibly influence MRP4 localization. According to our findings, none of these polymorphisms affected the localization of MRP4 in the hepatocyte membrane.

In conclusion, our findings demonstrate the upregulation of MRP4 mRNA and protein in human cholestatic livers, which is likely to represent a major escape route for bile salts during cholestasis. We report a wide variability of human hepatic MRP4 mRNA and protein expression and the identification of 74 genetic variations in the *MRP4* gene, among them 10 non-synonymous variations, four of which have not been reported previously.

Some of the detected non-synonymous single nucleotide polymorphisms might influence MRP4 protein expression but these changes were not significant and less profound than the impact of cholestasis on the MRP4 expression and they did not change the localization of MRP4 in the basolateral membrane and intracellular structures of hepatocytes. Furthermore, certain haplotypes may be associated with hepatic MRP4 expression.

Further studies are required to test the functional consequences of the polymorphisms described in this study.

Materials and methods

DNA and liver samples

Blood samples were obtained from a total of 95 Caucasian patients. Genomic DNA was isolated by standard methods using the Qiagen blood isolation kit (QiaAmp DNA Blood BioRobot 9604 Kit, Qiagen, Hilden, Germany) on a Qiagen 9604 biorobot.

Human liver tissue was available from 91 of these 95 Caucasian patients and was obtained as non-tumorous tissue adjacent to surgically removed liver tumors or metastases or the material that was surgically resected for other reasons as described previously.[33,34] Resected tissue samples were morphologically examined by a pathologist, and only histologically normal liver tissue was used for further studies. The tissue samples were stored at -80°C before subsequent processing. Extensive documentation was obtained for each of the samples, including demographic data of the patient, diagnosis, smoking status, medication and blood biochemistry. Since ursodeoxycholic acid has been shown to induce hepatic MRP4 protein expression,[20,28] livers from the patients treated with ursodeoxycholic acid were excluded from expression analysis. The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the *a priori* approval by the ethics committee of the Medical Faculty of the Charité, Humboldt-University Berlin (Germany). Written informed consent was obtained from each patient.

PCR and sequencing of genomic DNA

PCR for generating *MRP4* fragments were generally performed in a reaction volume of 50 μl with 20–30 ng of

genomic DNA, 10 × PCR buffer, 1 mM $MgCl_2$ in addition to $MgCl_2$ in the PCR buffer, 200 μM dNTPs, 0.2 μM (promoter 5, exons 3–8, 12, 13, 15, 16, 18, 23–27, 29), 0.4 μM (promoter 3, 4, exons 1 (additionally 4% dimethylsulfoxide) 17, 19–22, 30, 31, 3′-UTR) and 0.5 μM (exon 9, exon 10) of each primer and 1 unit Taq polymerase (all reagents from Qiagen, Hilden, Germany). Exons 2, 14, 28 (all 0.2 μM primer), exons 11 (0.3 μM primer) and promoter 2 (all 0.4 μM primer) were amplified without additional 1 mM $MgCl_2$.

PCR fragments were generated in a GeneAmp PCR System 9700 (ABI, Weiterstadt, Germany) with an initial denaturation step of 2 min at 94°C, followed by 34 cycles of denaturation at 94°C for 45 s, annealing for 45 s at 62°C and extending for 1 min at 72°C. The initial denaturation temperature for fragment exon 1 was 96°C for 5 min followed by 34 cycles of denaturation 96°C for 45 s, annealing for 45 s at 62°C and extending for 1 min at 72°C. Information about oligonucleotide primers used for PCR is available on request. Subsequently purified amplicons were directly sequenced for genetic polymorphisms on PE ABI 3700 DNA Analyzers by using BigDye Terminator cycle sequencing reactions (ABI, Weiterstadt, Germany). Sequences were analyzed and polymorphisms identified using the PHRED/PHRAP/CONSED/POLYPHRED software package (University of Washington, Seattle, WA, USA).

Blood biochemistry

Complete blood biochemistry was available from 70 of the 91 patients from whom liver samples had been obtained. Available presurgery liver serum parameters included total bilirubin, conjugated bilirubin, alkaline phosphatase, γ-glutamyltransferase, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), albumin, international normalized ratio and c-reactive protein. An internist (blinded for MRP4 expression) classified the livers as cholestatic or non-cholestatic according to diagnosis and blood biochemistry values. In general, patients with highly elevated levels of the cholestasis indicating enzymes γ-glutamyltransferase and alkaline phosphatase together with increased levels of bilirubin and the aminotransferases ASAT and ALAT were classified as cholestatic (Table 1).

Quantification of MRP4 *mRNA in human liver samples*

Total RNA was isolated as described previously.[34] One or 2 μg of the isolated RNA were reverse-transcribed using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time quantitative PCR was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) by utilizing the 5′-nuclease assay with TaqMan probes. PCR reactions of cDNA synthesized from 10 or 20 ng of total RNA were carried out in triplicates as described previously.[34] The forward primer (5′-CGAGTAGCCATGTGCCATATGA-3′) was positioned in exon 5 and the reverse primer (5′-CTTCTGTTGGTGTCCGGTCTATC-3′; MWG Biotech, Ebersberg, Germany) in exon 6 of the *MRP4* mRNA sequence (GenBank accession: NM_005845). The TaqMan probe (5′-TATCGGAAGGCACTTCG-3′; Applied Biosystems, Warrington, UK) was labeled with the reporter FAM (6-carboxyfluorescein) at the 5′-end and with MGB (Minor Groove Binder) at the 3′-end and contained sequences of both exons 5 and 6. Standard curves for this assay were calculated by using serial dilutions of known amounts of linearized *MRP4* plasmid cDNA (kindly provided by Professor D Keppler, Division of Tumor Biochemistry, DKFZ, Heidelberg, Germany). The results for *MRP4* mRNA were normalized to β-actin as housekeeping gene as described previously[34] with the modification that 1:20 dilutions of the cDNAs were used.

Quantification of MRP4 protein in human liver samples

Nuclear/membrane pellets were prepared as described previously[34], sonicated for 30 s (50%, MS72, Bandelin US200) and stored at −80°C. Protein concentration was measured in duplicates by use of the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's standard protocol.

Sixty micrograms of total protein from nuclear/membrane pellet fractions were incubated in sample buffer at 95°C for 5 min before separation on 10% SDS-polyacrylamide gels. Transfer of protein onto nitrocellulose membranes was performed using the Mini-PROTEAN 3 cell blotting system (Bio-Rad, Munich, Germany). Nonspecific binding sites were saturated by incubation with blocking buffer for 1 h. The primary antibody anti-MRP4 (monoclonal antibody M_4I-10; Alexis, Grünberg, Germany) was diluted 1:1000. Membranes were incubated overnight, washed and incubated in peroxidase-conjugated secondary antibody (dilution 1:1000; goat anti-rat IgG; Jackson ImmunoResearch laboratories, West Grove, PA) for 1 h. After the final wash immunoreactive proteins were visualized by chemiluminescence detection solution and development on films for chemiluminescence (ECL Western Blotting detection reagents and hyperfilm ECL; Amersham, Buckinghamshire, UK). Densitometry was performed using Version 4.5 of the Gel-Pro Analyzer Software (Media Cybernetics, Silver Spring, MD, USA). For β-actin detection membranes were stripped (Restore Western Blot Stripping Buffer; Pierce, Rockford, IL, USA) and reprobed deploying the same method as for MRP4 except that the incubation with anti-β-actin antibody (dilution 1:10000, clone AC-15; Sigma-Aldrich, St Louis, MO, USA) lasted for 1 h. The peroxidase-conjugated secondary antibody was goat anti-mouse IgG (dilution 1:10000; Sigma-Aldrich, St Louis, MO, USA). Each probe was analyzed in duplicates, normalized to β-actin and expression was analyzed in percent of the same reference liver sample (#192) that was loaded on each gel (° in Figure 1b).

Nomenclature

Genetic polymorphisms are described according to den Dunnen and Antonarakis.[35] Accession numbers AL356257.14, AL157818.12 and AL139381.24 served as reference for the genomic *MRP4* sequence. The reference sequence used for the cDNA is NM_005845.2. The indicated positions are given with respect to the *MRP4* translational

start site with the A of ATG depicted as +1 and the immediately following 5′-base as –1.

Haplotype analysis
MRP4 haplotypes and frequencies were calculated by the phase program.[27] Haplotype calculation was restricted to the 20 most frequent *MRP4* polymorphisms each polymorphism with a frequency greater than 20%.

Prediction of protein secondary structure and tolerability of amino acid variations
The two-dimensional structure of the MRP4 protein was predicted using the CBS Prediction Server of the Technical University of Denmark DTU and the DAS-Transmembrane Prediction Server of the Stockholm Bioinformatics Center.

The prediction of functional effects of amino acid variations at all positions was calculated using the PolyPhen software of the European Molecular Biology Laboratory EMBL, Heidelberg, Germany.

Immunofluorescence microscopy
Human liver tissue samples were cut in 5 μm sections and fixed on microscope slides with ice-cold acetone. Sections were permeabilized with 1% triton in phosphate buffered saline (PBS) for 30 min, unspecific binding was blocked with 1.5% goat serum in PBS for 1 h, followed by 1 h of incubation with primary and then 45 min of secondary antibodies at the following concentrations. Primary antibodies: anti-desmoplakin for staining of the basolateral hepatocyte membrane[36] (Desmoplakin 1&2 mouse monoclonals, dilution: 1:2; Progen Biotechnik, Heidelberg, Germany) and purified anti-MRP4 SNG (raised in rabbits against the 15 C-terminal amino acids 1311–1325 SNGQPSTLTIFETAL of human MRP4 as described previously,[37] dilution: 1:100), secondary antibodies: Cy3-conjugated AffiniPure goat anti-rabbit IgG (Dianova, Hamburg, Gemany) 1:300 and Cy2-conjugated AffiniPure goat anti-mouse IgG (Dianova, Hamburg, Germany) 1:200. The purified anti-MRP4 SNG antibody described above was used for immunofluorescence microscopy, since the monoclonal antibody M_4I-10 used for the Western blots did not show specific staining with immunofluorescence microscopy experiments. Green and red fluorescence was detected by confocal laser scanning microscopy with an Axiovert 100M (Karl Zeiss GmbH, Jena, Germany).

Statistics
Data are presented as mean $\pm$ s.d. Expression data were tested for significance by Mann–Whitney *U*-test, Student's *t*-test or Kruskal–Wallis-Test as appropriate. A *P*-value $\leqslant 0.05$ was considered statistically significant. Calculations were carried out using GraphPad Prism Version 4.01 (GraphPad Software Inc, San Diego, CA, USA) and Analyze-It for Microsoft Excel Software Version 1.71 (Analyze-It Software, Ltd, Leeds, UK).

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Duality of Interest

There are no dualities of interest.

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