The apical conjugate efflux pump ABCC2 (MRP2)

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Abstract ABCC2 is a member of the multidrug resistance protein subfamily localized exclusively to the apical membrane domain of polarized cells, such as hepatocytes, renal proximal tubule epithelia, and intestinal epithelia. This localization supports the function of ABCC2 in the terminal excretion and detoxification of endogenous and xenobiotic organic anions, particularly in the unidirectional efflux of substances conjugated with glutathione, glucuronate, or sulfate, as exemplified by leukotriene C₄, bilirubin glucuronosides, and some steroid sulfates. The hepatic ABCC2 pump contributes to the driving forces of bile flow. Acquired or hereditary deficiency of ABCC2, the latter known as Dubin–Johnson syndrome in humans, causes an increased concentration of bilirubin glucuronosides in blood because of their efflux from hepatocytes via the basolateral ABCC3, which compensates for the deficiency in ABCC2-mediated apical efflux. In this article we provide an overview on the molecular characteristics of ABCC2 and its expression in various tissues and species. We discuss the transcriptional and posttranscriptional regulation of ABCC2 and review approaches to the functional analysis providing information on its substrate specificity. A comprehensive list of sequence variants in the human *ABCC2* gene summarizes predicted and proven functional consequences, including variants leading to Dubin–Johnson syndrome.

Keywords ABCC2 transporter ·

Apical conjugate export pump · Detoxification by ABCC2 ·

Dubin–Johnson syndrome ·

Membrane topology predicted for ABCC2 ·

Multidrug resistance protein 2 ·

Sequence variants of ABCC2 ·

Substrate specificity of ABCC2

Introduction

The multidrug resistance protein 2 (ABCC2) was the second member of the subfamily of MRP efflux pumps to be cloned from rat and human tissues [13, 60, 114, 133, 165]. The function of rat *Abcc2* was recognized long before its cloning by studies on the hepatobiliary elimination of organic anions in normal and transport-deficient mutant rats [54, 55, 64, 131, 163]. The loss of ATP-dependent transport across the hepatocyte canalicular membrane was identified in these mutant rats using inside-out membrane vesicles and various glutathione (GSH) *S*-conjugates as substrates, notably leukotriene C₄ (LTC₄) [57]. Thus, the difference between canalicular membrane vesicles from normal and transport-deficient mutant rats with respect to ATP-dependent transport of various anionic substrates has provided important insight into the substrate specificity of the conjugate export pump *Abcc2*. Moreover, the similar substrate spectrum of *Abcc2* and ABCC1 [89] has supported the identification of substrates for ABCC1, which had been cloned by Cole et al. [21], 2 years before the identification of the first transport substrates of ABCC1 [105, 120]. The link between the ATP-dependent transport of LTC₄ and ABCC1 was made primarily by photoaffinity labeling using LTC₄ as a photolabile high-affinity substrate which served to label mouse *Abcc1* [106] and human

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ABCC1 ([66, 105]; reviewed by Jedlitschky and Keppler [65]). This photoaffinity labeling approach [33, 105, 106] had indicated a 190-kDa integral membrane glycoprotein with properties similar to ABCC1 [106].

The localization of ABCC2 to the apical membrane of various polarized cells involved in the secretion of conjugated endogenous and xenobiotic substances favors the function of this efflux pump in the terminal phase of detoxification [83, 89]. Due to this substrate specificity, ABCC2, rather than ABCB1 or ABCG2, is the optimal pump for the elimination of conjugates of various toxins and carcinogens with GSH, glucuronate, or sulfate from hepatocytes into bile, or from kidney proximal tubules into urine, or from intestinal epithelial cells into the intestinal lumen. Particularly in nonpolarized cell types, additional members of the ABCC subfamily, such as ABCC1, may have this function in terminal detoxification.

ABCC2 plays a decisive role in the elimination of bilirubin glucuronosides from hepatocytes into bile. Accordingly, the absence of functional ABCC2 from the canalicular membrane [72, 82] causes conjugated hyperbilirubinemia, as observed in the hereditary disorder described by Dubin and Johnson [31]. Today, many sequence variants in the *ABCC2* gene have been identified, as described below, but only some of them cause Dubin–Johnson syndrome. The hereditary deficiency of ABCC2 in humans, or of *Abcc2* in the mutant rats mentioned above, and the recently generated *Abcc2* knock-out mouse strains [18, 121, 177] illustrate the key function of this apical efflux pump in the elimination of anionic conjugates from the body. It should be noted, however, that this loss of ABCC2 function is usually well tolerated and compensated by the upregulation of other membrane transporters, particularly of ABCC3 in the basolateral membrane of hepatocytes [30, 82, 91].

Functional characterization and substrate specificity of ABCC2

Insight into the substrate specificity of human or rodent ABCC2/*Abcc2* may be obtained on several experimental levels. A relatively simple indication whether a compound is transported by rat *Abcc2* is given by the comparative elimination of a compound into the bile of normal and *Abcc2*-deficient mutant rats [55, 64, 131]; however, intracellular metabolism and the presence of alternative efflux pumps in the canalicular membrane may obscure the results. Kinetic constants for rat *Abcc2* may be obtained by comparative studies with inside-out hepatocyte canalicular membrane vesicles isolated from normal and *Abcc2*-deficient liver [57, 126, 127, 187], as detailed in Table 1. Clearly, the most informative data can be obtained with

Table 1 Substrate specificity of human and rat ABCC2

Substrate	K_m value (μ M)	Reference
Human ABCC2 (recombinant in vesicles)		
Bilirubin		
Monoglucuronosyl	0.7	[71]
Bisglucuronosyl	0.9	[71]
Leukotriene C ₄	1	[24]
S-Glutathionyl 2,4-dinitrobenzene	6.5	[32]
17 β -Glucuronosyl estradiol	7.2	[24]
Cholecystokinin peptide (CCK-8)	8.1	[109]
Monoglucuronosyl chlorambucil	10	[156]
Bromosulfophthalein	12	[25]
Ethinylestradiol-3- <i>O</i> -glucuronide	35	[20]
<i>p</i> -Aminohippurate	880	[107]
	2,100	[155]
Methotrexate		[53]
S-Glutathionyl ethacrynic acid		[32]
Ochratoxin A		[107]
Estrone 3-sulfate		[95]
Rat <i>Abcc2</i> (recombinant in vesicles)		
S-Glutathionyl 2,4-dinitrobenzene	0.2	[61]
Bilirubin		
Bisglucuronosyl	0.5	[71]
Monoglucuronosyl	0.8	[71]
Leukotriene C ₄	1.1	[24]
Sulfatolithocholyl taurine	3.9	[1]
17 β -Glucuronosyl estradiol	6.9	[24]
5-(and 6)-Carboxy-2',7'-dichlorofluorescein	140	[185]
<i>p</i> -Aminohippurate	1,900	[173]
Rat <i>Abcc2</i> (normal vs mutant in canalicular membrane vesicles)		
Leukotriene C ₄	0.3	[57]
Leukotriene D ₄	1.5	[57]
Sulfatolithocholyl taurine	1.5	[1]
Fluo-3	3.7	[122]
<i>N</i> -Acetyl leukotriene E ₄	5.2	[57]
Glucuronosyl E3040	5.7	[126]
Glucuronosyl grepafloxacin	7.2	[148]
Sulfatochenodeoxycholyl taurine	8.8	[1]
Bromosulfophthalein	31	[128]
Temocaprilat	93	[58]
Pravastatin	220	[187]
Methotrexate	295	[113]
Leukotriene E ₄		[57]
S-Glutathionyl 2,4-dinitrobenzene		[57]
S-Glutathionyl sulfobromophthalein		[57]
Glutathione disulfide		[35]
Bilirubin		
Monoglucuronosyl		[67]
Bisglucuronosyl		[67, 127]
Glucuronosyl nafenopin		[66]
Glucuronosyl SN38 carboxylate		[19]
Glucuronosyl SN38 lactone		[19]
SN38 carboxylate		[19]
Thromboxane A ₂ receptor antagonist (Z-335)		[77]

inside-out membrane vesicles containing recombinant ABCC2 from human or other species expressed in cell lines such as human embryonic kidney cell line 293 [24], Madin–Darby canine kidney cell line (MDCKII) [24, 32], or Sf9 insect cells [171]. K_m values given for various substrates have been obtained with these inside-out membrane vesicles as summarized in Table 1. ABCC2 purified to homogeneity has also been analyzed with respect to the ATP-dependent transport of LTC₄ and ATPase activity [43, 44]. The ATPase activity of ABCC2 has been studied in detail in membrane vesicles [4]. Although this assay may provide valuable information, it cannot substitute for transport measurements using inside-out membrane vesicles, which allow for the determination of K_m values and for kinetic constants of inhibitors of transport across membranes.

More recently, double- and multiple-transfected polarized cells have been developed, which stably express an uptake transporter of the organic anion transporting polypeptide family in the basolateral membrane and ABCC2 in the apical membrane [25, 81, 95, 149]

(Fig. 1). With this in vitro system, functional analyses and identification of substrates for ABCC2 have become possible in a medium- to high-throughput format. However, K_m values cannot be obtained by the use of such double-transfected cell lines.

The substrate specificity of ABCC2 is broad and comprises many organic anions, with the highest affinity for glucuronate and GSH conjugates of lipophilic substances, as exemplified by LTC₄, which is an arachidonate derivative and a GSH conjugate (Table 1, reviewed by Jedlitschky and Keppler [65] and König et al. [91]). In addition, many anionic substances without anionic conjugate residues have been identified as substrates, such as methotrexate and bromosulphophthalein (Table 1). Reduced and oxidized GSH are additional substrates. Interestingly, Abcc2-deficient mutant rats cannot excrete GSH into their bile [131, 135]. It remains controversial whether this is due to low affinity transport of GSH by Abcc2 or due to the coefflux of GSH with additional compounds in a process well established for ABCC1 [110] and for ABCC4 [140, 141]. The differences in the substrate specificity between ABCC2 and ABCC1 are

Fig. 1 Immunolocalization of human ABCC2 (green) in different human tissues and in polarized epithelial MDCK cells double-transfected with the cDNAs encoding ABCC2 and the uptake transporter organic anion transporting polypeptide 1B1 (OATP1B1). Pictures were taken with a confocal laser scanning microscope. The panel with the double transfectants shows the normal xy-view and an optical xz-section taken along the dashed line as indicated in the xy-view. ABCC2 is localized in the apical membrane of hepatocytes in liver, of enterocytes in duodenum, and of proximal tubule epithelial cells in kidney. The uptake of organic anions from blood into hepatocytes is mediated by the basolaterally localized OATP1B1, in addition to other organic anion transporters. Double-transfected [25, 81, 108, 109, 149, 157] or quadruple-transfected [95] polarized cells coexpressing organic anion transporters in their basolateral and the conjugate efflux pump ABCC2 in their apical membrane serve as a valuable system to study the vectorial transport of substances that undergo hepatobiliary elimination in humans. Bars, 10 μ m

limited and often detectable only by the determination of kinetic constants [65, 89].

Molecular characterization of ABCC2

Abcc2 cDNA was initially identified and cloned as a fragment from rat liver, using degenerate oligonucleotides complementary to human *ABCC1* [21] and *Leishmania* *ltgpa* [114]. This *Abcc2* cDNA fragment was amplified from normal rat liver, but not from the liver of mutant rats that were deficient in the hepatobiliary excretion of anionic conjugates [114]. The subsequent cloning and analysis of the full-length rat *Abcc2* cDNA identified *Abcc2* [Mrp2; formerly described as cMrp and canalicular multispecific organic anion transporter (cMoat)] as an ABCC1-related protein localized in the canalicular membrane of hepatocytes [13, 133] (Fig. 1). At present, the full-length sequences from six mammalian species, including the orthologs from human, rhesus monkey, rat, rabbit, mouse, dog [13, 22, 37, 133, 165, 172], and from three other vertebrates, are known (Table 2). ABCC2-related sequences

are also present in many other organisms, including *Caenorhabditis elegans* [11] and *Arabidopsis thaliana* [139]. Within the human ABCC subfamily, ABCC2 has the highest degree of amino acid sequence identity with ABCC1 (50%) and the lowest with ABCC11 and cystic fibrosis transmembrane conductance regulator (Table 2). ABCC2 is structurally and functionally very distinct from MDR1 P-glycoprotein (ABCB1), which belongs to a different subfamily of ABC transporters with which ABCC2 shares only about 26% amino acid sequence identity. The human *ABCC2* gene is located on chromosome 10q24 [165], spans about 65 kilobase pairs, and consists of 32 exons with a high proportion of class 0 introns [168, 170].

The predicted topology of the subfamily of ABCC/MRP efflux pumps is related to most of the other human ABC transporters and comprises two polytopic membrane-spanning domains, each followed by a nucleotide-binding domain [45, 96]. However, ABCC2, together with ABCC1, ABCC3, ABCC6, and ABCC10, is unique in having an additional amino-terminal polytopic membrane-spanning domain (Fig. 2) that may be required for proper function

Table 2 Amino acid sequence identity of human ABCC2 with the other members of the human ABCC subfamily and with *Abcc2* orthologs from other species

Species	Symbol	Alternate name	Chromosomal localization	Number of exons ^a	Number of amino acids	Identity (%) ^b	Accession number ^c
The human ABCC subfamily							
Human	ABCC2	MRP2	10q24	32	1,545	100	NP_000383
	ABCC1	MRP1	16p13.1	31	1,531	50	NP_004987
	ABCC3	MRP3	17q22	31	1,527	48	NP_003777
	ABCC4	MRP4	13q32	31	1,325	39	NP_005836
	ABCC5	MRP5	3q27	32 [§]	1,437	38	NP_005679
	ABCC6	MRP6	16p13.1	31	1,503	40	NP_001162
	CFTR	ABCC7	7q31.2	27	1,480	30	NP_000483
	ABCC8	SUR1	11p15.1	41 [§]	1,581	34	NP_000343
	ABCC9	SUR2A	12p12.1	39 [§]	1,549	35	NP_005682
		SUR2B	12p12.1	39 [§]	1,549	35	NP_064693
	ABCC10	MRP7	6p21.1	22	1,464	34	NP_258261
	ABCC11	MRP8	16q12.1	28	1,382	33	NP_149163
	ABCC12	MRP9	16q12.1	29	1,359	35	NP_150229
Human ABCC2 and other <i>Abcc2</i> orthologs							
Human	ABCC2		10q24	32	1,545	100	NP_000383
Rhesus monkey	<i>Abcc2</i>				1,545	96	AAY41167
Dog	<i>Abcc2</i>		28	33 [§]	1,544	84	AAS91646
Rabbit	<i>Abcc2</i>				1,564	82	CAA89004
Mouse	<i>Abcc2</i>		19	32 [§]	1,543	78	AAL36985
Rat	<i>Abcc2</i>		1q51		1,541	78	NP_036965
Chicken	<i>Abcc2</i>		6	31 [§]	1,542	64	XP_421698
Zebrafish	<i>Abcc2</i>		13	26 [§]	1,567	59	NP_956883
Little skate	<i>Abcc2</i>				1,564	57	AAL92112

^aCompiled from Haimeur et al. [45] and König et al. [91] and “Gene” database at <http://www.ncbi.nlm.nih.gov/entrez> (symbol §)

^bAnalyzed by the GAP tool of the Heidelberg Unix Sequence Analysis Resources program package at <http://genome.dkfz-heidelberg.de>

^cProtein accession number from “Protein” database at <http://www.ncbi.nlm.nih.gov/entrez>

and localization [34, 39, 183]. The extracellular location of the amino terminus was first predicted for rat *Abcc2* by computational analysis [13] and subsequently proven experimentally [24]. Different algorithms, which have been used to calculate the probability of an amino acid sequence to be a transmembrane-spanning helix in a polytopic membrane protein, predicted only four transmembrane-spanning helices between the first and second nucleotide-binding domain of ABCC2 [91] (Fig. 2). This is in contrast to the established six transmembrane-spanning helices in the corresponding part of ABCC1 [21].

Localization of ABCC2 in polarized cells and tissues

The ABCC2 protein was, for the first time, localized in the apical (canalicular) membrane of rat [13] and human [82, 134] hepatocytes and, therefore, initially termed canalicular MRP (cMRP) [13] or cMOAT [133, 134, 165]. The subsequent identification of *Abcc2* in the apical membrane of proximal tubule epithelial cells of rat kidney was the first demonstration of an extrahepatic localization of this conjugate efflux pump [150]. Besides human kidney [151], other polarized human tissues with ABCC2 being present in the apical membrane of the polarized cells include the small intestine [38, 147], colon [91], gallbladder [144], bronchi [91, 147], and placenta [159] (Fig. 1). Many of these polarized cell types express additional ABCC/MRP efflux pumps that are basolaterally localized, as exemplified in human hepatocytes where ABCC3 [90, 94], ABCC4 [140], and ABCC6 [6, 91] were identified in the sinusoidal membrane. In contrast to these polarized tissues, the

ABCC2 protein is apparently not present in the human blood–testis barrier [5, 147] and in the blood–brain barrier in the human cortex [125, 147, 188]. However, ABCC2 was identified in brain capillaries of hippocampus specimens from patients with temporal lobe epilepsy [2] and in the rat blood–brain barrier after pilocarpine-induced epileptic seizures [52]. Moreover, some studies described *Abcc2* in brain capillaries of normal rats [115, 176]. Various human tissues do not express the ABCC2 protein in detectable amounts, such as the skin, exocrine pancreas, female reproductive system, lymphatic system, cardiovascular system, and connective tissue [147].

In addition to normal human tissues, the ABCC2 protein is also present in a number of human malignant tumors, as evidenced by immunostaining of clinical specimens from renal clear-cell [147, 151], hepatocellular [123, 190], ovarian [3, 147], and colorectal carcinomas [49, 147]. Using tissue microarrays, ABCC2 was detected in lung, breast, and gastric carcinomas [147]. In contrast, ABCC2 protein expression was negligible or absent in primary testicular tumors [5], pancreatic adenocarcinomas [92], and gliomas [12]. Because of its ability to confer resistance to a wide variety of anticancer drugs [17, 24, 53, 78], ABCC2 may be of clinical relevance by contributing to the multidrug resistance phenotype of several solid malignant human tumors.

Several cell lines endogenously expressing ABCC2 have been used to study ABCC2 function in intact cells. Some rat and human hepatoma cells acquire a hepatocyte-like polarity after several days in culture and form apical vacuoles or bile canaliculus-like structures between adjacent cells [14, 122]. Secretion of fluorescent ABCC2

Fig. 2 Predicted membrane topology of human ABCC2. Amino acids within the nucleotide-binding domains constituting the Walker A and B motifs and the family signature are in *black*. Tree-like structures indicate the location of putative *N*-glycosylation sites. This topology prediction with four transmembrane segments between the first and second nucleotide-binding domain is based on different algorithms, including TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) and TopPred2 (<http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>). Sequence variants causing Dubin–Johnson syndrome are shown as detailed in Table 3. MSD, membrane-spanning domain

substrates into these apical vacuoles can be followed by live cell video microscopy [14, 91, 122]. Caco-2 cells, which are derived from a human colon carcinoma and also endogenously express ABCC2, gain an epithelial polarity with ABCC2 being present in the apical membrane when grown on certain filter membranes [9, 182]. Polarized MDCK (or rather MDCKII) cells expressing recombinant human ABCC2 in their apical membrane are better defined as cell systems than Caco-2 cells to study the ABCC2-mediated efflux of substances from cells [24, 32]. A major advancement came with the development of MDCK cells coexpressing one [25, 149] or more [95] human organic anion uptake transporters, localized in the basolateral membrane, together with the human apical efflux pump ABCC2 (Fig. 1). These double or quadruple transfectants are useful to study the vectorial transport of substances that undergo hepatobiliary elimination in humans without the need for studies with inside-out membrane vesicles containing the ABCC2 protein [25, 81, 95, 108, 109, 149, 157].

ABCC2 is exclusively localized in the apical membrane of polarized cells. A cell type-dependent localization, as detected for ABCC4, which is present in the basolateral membrane of human hepatocytes [140] and in the apical membrane of kidney proximal tubule epithelial cells [174], has not been observed for ABCC2. Under certain experimental conditions, ABCC2 may be redistributed to the basolateral membrane, e.g., in rat hepatocyte couplets shortly after their isolation [142] and in human HepG2 cells after protein kinase C activation [100].

The molecular mechanisms by which ABCC2 is targeted to the apical membrane are incompletely understood. Some proteins have been reported to interact with ABCC2 in vitro. Radixin, a member of the ezrin/radixin/moesin family cross-linking actin with several integral membrane proteins [10], is apparently required for the proper apical localization of Abcc2 because radixin knock-out mice exhibit conjugated hyperbilirubinemia and a selective loss of the Abcc2 protein from the hepatocyte canalicular membrane [84]. Canalicular localization of human ABCC2 may similarly be dependent on radixin interaction [88]. Other proteins interacting with ABCC2 in vitro belong to the PSD95/Dlg/ZO-1 (PDZ) family and bind to C termini with the conserved sequence T/S-X-Φ, with X being any amino acid and Φ a hydrophobic amino acid. Interaction of ABCC2 with PDZK1 and other PDZ proteins has been described [48, 86] and most of the known ABCC2 orthologs (Table 2) have a consensus PDZ-binding motif at their C termini. Interestingly, PDZK1 knock-out mice have a proper apical localization of Abcc2 in kidney proximal tubule epithelial cells, indicating that the interaction of Abcc2 with PDZK1 is either not required in vivo or that other PDZ proteins are able to compensate [87].

Whereas one report proposed that the PDZ-binding motif is the apical targeting signal of ABCC2 [46], this was not confirmed by all other studies [34, 93, 124, 183]. These latter studies indicate that the apical targeting motif of ABCC2 is not a distinct, narrowly defined amino acid sequence, but rather appears to be composed of several motifs within different parts of ABCC2 that come together only in the intact protein.

Transcriptional and posttranscriptional regulation of ABCC2

ABCC2 is not only constitutively expressed, but its expression is also regulated on the transcriptional and posttranscriptional level in response to many endogenous and xenobiotic substances and to different disease states. Transcriptional regulation may result from changes in the intracellular concentrations of bile acids and of a number of lipophilic compounds that are ligands for nuclear hormone receptors. Additional posttranscriptional mechanisms may allow for a short-term regulation of ABCC2. ABCC2 regulation is often accompanied by a coordinate regulation of other bile salt transporters and of conjugating enzymes. These processes are described in detail by Gerk and Vore [40], Kullak-Ublick et al. [102], and van de Water et al. [175].

Cloning and characterization of the rat *Abcc2* and the human *ABCC2* promoters identified several consensus binding sites for both liver-specific and ubiquitous transcription factors [74, 160, 164]. The region between nucleotides –517 and –197 in front of the translation initiation codon is important for human *ABCC2* expression [160], with the transcriptional start-site being at nucleotide –247. In addition, a hormone response element in the rat *Abcc2* promoter (ER-8) was identified, which is bound by heterodimers of the retinoid X receptor α (RXRα, NR2B1 [129]) with the ligand-activated transcription factors farnesoid X receptor (FXR, NR1H4), pregnane X receptor (PXR, NR1I2), or constitutive androstane receptor (CAR, NR1I3) [73]. These nuclear receptors are, for example, activated by bile acids via FXR [132] by various xenobiotics such as the antibiotic rifampicin, the synthetic glucocorticoid dexamethasone, and pregnenolone 16α-carbonitrile via PXR [8, 41, 85], or by phenobarbital via CAR [161]. Knowledge of the presence of the hormone response element ER-8 in the rat *Abcc2* promoter [73] may now explain studies which describe the induction of *Abcc2* mRNA and Abcc2 protein in primary cultures of rat hepatocytes by a number of xenobiotics, including the carcinogen 2-acetylaminofluorene, the anticancer drug cisplatin, the antifungal agent clotrimazole, the antibiotic cycloheximide, dexamethasone, the chemopreventive agents oltipraz and sulforaphane, phenobarbital, and pregnenolone 16α-carbonitrile [23, 73, 75, 98,

[137]. Additional factors are probably involved in ABCC2/*Abcc2* regulation in vivo because phenobarbital or oltipraz treatment of rats did not increase *Abcc2* mRNA expression [23, 40, 42, 68]. In contrast, the feeding of mice with several compounds, e.g., the bile acids ursodeoxycholic acid and cholic acid [36], or the herbicide 2,4,5-trichlorophenoxyacetic acid [184], induced *Abcc2* mRNA and *Abcc2* protein expression, which may prevent the accumulation of potentially toxic bile acid conjugates or xenobiotics. Similarly, ABCC2 induction was detected in the liver of nonhuman primates [76] and in the intestines of humans [38] after treatment with the PXR ligand rifampicin.

ABCC2/*Abcc2* regulation in disease states can also, at least in part, result from mediation via transcription factors. Down-regulation of *Abcc2* mRNA and *Abcc2* protein in rat liver after experimental cholestasis induced either by bile duct ligation or endotoxin treatment [99, 169, 178] may be explained by an upregulation of the inflammatory cytokine interleukin-1 β , which in turn down-regulates the heterodimeric retinoic acid receptor α /RXR α , leading to down-regulation of *Abcc2* promoter activity [27]. Interestingly, *Abcc2* expression in the kidney is preserved or up-regulated during cholestasis [27, 103]. In humans, ABCC2 mRNA levels were reduced in livers from patients with primary sclerosing cholangitis [130] or with obstructive cholestasis [153]. Obstructive cholestasis leads to reduced ABCC2 protein levels in the intestine without an effect on ABCC2 mRNA expression [28].

Besides this transcriptional regulation, several studies describe the posttranscriptional regulation of ABCC2/*Abcc2*. One mechanism is the modulation of the amount of the conjugate efflux pump in the apical membrane by recruitment of transporter molecules from intracellular pools or their retrieval into these pools. Insertion of *Abcc2* into the canalicular membrane of rat hepatocytes may depend on the lipid kinase phosphoinositide 3-kinase [116], on protein kinase C [7], or on cyclic AMP [142]. Retrieval of rat *Abcc2* transporter molecules from the canalicular membrane into the hepatocyte has been detected under different cholestatic conditions [119, 136, 169], after phalloidin treatment [143], after cytokine stimulation [29, 99], and under hyperosmolar conditions [29, 97]. Under these conditions, immunostaining of *Abcc2* is no longer confined to the canalicular membrane, but appears “fuzzy,” which is interpreted as an accumulation of transporter molecules within the hepatocyte. A comparable fuzzy immunostaining, indicative of endocytic retrieval of transporter molecules, has been described in human liver diseases, e.g., inflammation-induced icteric cholestasis [189], obstructive cholestasis [153, 186], and advanced stages of primary biliary cirrhosis [88, 101].

The amount of transporter molecules in the hepatocyte canalicular membrane can also be regulated by a change in

their synthesis or degradation rate. The physiological half-life of rat *Abcc2* in the hepatocyte canalicular membrane is about 27 h [69]. In normal rat liver, the synthesis and degradation rates result in *Abcc2* protein levels that appear to be similar within a lobule [136]. During cholestasis, however, *Abcc2* in the periportal hepatocytes is more rapidly degraded than under normal conditions, leading to the observation that *Abcc2* is concentrated near the central (perivenous) area of the liver lobule [136]. Decreased *Abcc2* protein levels, but unaltered *Abcc2* mRNA levels, were also detected in rats treated with ethinylestradiol [169] and in pregnant rats in comparison with control rats [15]. Clofibrate, which is a ligand for the peroxisome proliferator-activated receptor α (PPAR α , NR1C1 [59]), also decreased rat *Abcc2* protein levels by posttranscriptional mechanisms [68].

Sequence variants of ABCC2 and the hereditary deficiency of ABCC2 in human Dubin–Johnson syndrome and in animal models

More than 200 naturally occurring sequence variants have been identified in the exons, introns, and the 5'- and 3'-flanking regions of the human ABCC2 gene. Many of these sequence variants are single nucleotide changes in the introns or in the exons without leading to amino acid changes, and are, therefore, probably without functional consequences [62, 63, 117, 146, 162, 179] (single nucleotide polymorphism database, <http://www.ncbi.nlm.nih.gov/SNP>). Sequence variants that result in the absence of a functionally active ABCC2 protein from the canalicular membrane of hepatocytes are the molecular basis of Dubin–Johnson syndrome in humans [72, 82, 134, 170]. This syndrome is an autosomal, recessively inherited disorder characterized by conjugated hyperbilirubinemia (i.e., increased concentration of bilirubin glucuronosides in blood) and deposition of a dark pigment in hepatocytes, so that the liver of an affected individual appears dark blue or black [31, 145, 158]. The incidence of Dubin–Johnson syndrome ranges from 1:1,300 among Iranian Jews [152] to 1:300,000 in a Japanese population [70].

Sequence variants of the human ABCC2 gene identified in patients with Dubin–Johnson syndrome include splice-site mutations resulting in exon loss and subsequent premature stop codons [70, 118, 168, 180], missense mutations [47, 111, 112, 117, 166, 168, 180, 181], a deletion mutation leading to the loss of two amino acids from the second nucleotide-binding domain [170], a deletion/in-frame insertion mutation [16], and nonsense mutations leading to premature stop codons [134, 154, 166] (Table 3, Fig. 2). Although all sequence variants associated with Dubin–Johnson syndrome result in the absence of a

Table 3 Nucleotide sequence variants in the human *ABCC2* gene (NM_000392) leading to amino acid changes in the ABCC2/MRP2 protein (NP_000383)

Location	Nucleotide change ^a	Deduced effect on protein ^a	Causing Dubin–Johnson syndrome ^b	Predicted effect by PolyPhen database ^c	Experimentally proven functional consequence	Average frequency of indicated nucleotide exchange in population	NCBI SNP ID ^d and/or references
Exon 2	c.56 C>T ^e	p.P19L	DJS	Probably damaging		T: 0.007	[63]
Exon 2	c.116 T>A	p.F39Y		Benign		A: 0.010 A: 0.008	rs927344 rs17222603
Exon 3	c.298 C>T	p.R100X ^f		Possibly damaging		A: 0.007	[154]
Exon 3	c.299 G>A ^e	p.R100Q					[63]
Exon 7	c.736 A>C	p.M246L		Benign		C: 0.002 C: 0.002	rs8187667 rs17222744
Exon 7	c.842 G>A	p.S281N	DJS	Benign		A: 0.006–0.056	[117]
Exon 8	c.998 A>G	p.D333G		Possibly damaging		G: 0.002 G: 0.004	rs8187668 rs17222674
Exon 9	c.1058 G>A	p.R353H		Benign		A: 0.009 A: 0.014	rs7080681 rs17216205
Exon 9	c.1177 C>T	p.R393W		Probably damaging			[104, 112]
Exon 10	c.1234 A>G	p.R412G		Probably damaging	Deficient methotrexate transport function		[56]
Exon 10	c.1249 G>A	p.V417I		Benign	None apparent [50]	A: 0.163 A: 0.158 A: 0.125 A: 0.183–0.312 [117]	rs2273697, [146] rs17216184
Exon 10	c.1457 C>T	p.T486I		Benign		T: 0.002 T: 0.002	rs8187670 rs17222589
Exon 11	c.1483 A>G	p.K495E		Possibly damaging		G: 0.002 G: 0.002	rs8187672 rs17222561
Exon 13	c.1686 T>G	p.F562L		Benign		G: 0.002 G: 0.002	rs8187673 rs17216233
Exon 16	c.2009 T>C	p.I670T		Benign			rs8187676 rs17222632
Exon 16	c.2026 G>C	p.G676R	DJS	Probably damaging		C: 0.006	[181]
Exon 17	c.2125 T>C	p.W709R	DJS	Probably damaging			[111]
Exon 17	c.2153 A>G	p.N718S		Possibly damaging			rs3740072
Exon 17	c.2215 C>T	p.L739F		Probably damaging		T: 0.006	[51]
Exon 18	c.2302 C>T	p.R768W	DJS	Probably damaging	Deficient maturation and impaired sorting [47]	T: 0.010 [62]	[168, 180]

Table 3 (continued)

Location	Nucleotide change ^a	Deduced effect on protein ^a	Causing Dubin–Johnson syndrome ^b	Predicted effect by PolyPhen database ^c	Experimentally proven functional consequence	Average frequency of indicated nucleotide exchange in population	NCBI SNP ID ^d and/or references
Exon 18	c.2366 C>T	p.S789F		Probably damaging	Reduced protein levels [50]	T: 0.010 [62]	
Exon 19	c.2546 T>G	p.L849R		Benign		G: 0.002 G: 0.006 A: 0.007	rs8187689 rs17222617 [63]
Exon 20	c.2647 G>A ^e	p.D883N		Benign			rs3740071
Exon 20	c.2677 G>C	p.E893Q		Benign			
Exon 21	c.2882 A>G ^e	p.K961R		Benign		G: 0.007	[63]
Exon 22	c.2901 C>A	p.Y967X ^f				A: 0.002 A: 0.002 G: 0.002 G: 0.002	rs8187683 rs17222547 rs8187684 rs17222554
Exon 22	c.2944 A>G	p.I982V		Benign		T: 0.007 C: 0.002 C: 0.004	[63] rs8187685 rs17216149
Exon 22	c.3057 G>T ^e	p.Q1019H		Benign			
Exon 23	c.3107 T>C	p.I1036T		Possibly damaging		G: 0.002 G: 0.002	rs8187686 rs17222540
Exon 23	c.3188 A>G	p.N1063S		Benign			[134]
Exon 23	c.3196 C>T	p.R1066X ^f	DJS		No ABCC2 protein in liver		
Exon 25	c.3449 G>A	p.R1150H	DJS	Probably damaging	Deficient transport function	A: 0–0.009	[117]
Exon 25	c.3517 A>T	p.I1173F	DJS	Probably damaging	Deficient maturation and impaired sorting, deficient transport function	T: 0–0.029 [117]	[80, 117]
Exon 25	c.3521 G>A ^e	p.R1174H		Probably damaging		A: 0.007	[63]
Exon 25	c.3542 G>T	p.R1181L		Possibly damaging		T: 0.039 T: 0.034	rs8187692 rs17222702
Exon 25	c.3563 T>A	p.V1188E		Benign		A: 0.059 A: 0.059	rs8187694 rs17222723
Exon 26	c.3732 T>G ^e	p.N1244K		Possibly damaging		G: 0.014	[63]
Exon 27	c.3817 A>G	p.T1273A		Benign		G: 0.002 G: 0.004	rs8187699 rs17222582
Exon 27	c.3825 C>G	p.Y1275X ^f	DJS		No ABCC2 protein in liver		[104]
Exon 28	c.3872 C>T	p.P1291L		Possibly damaging		T: 0.012 T: 0.010	rs8187700 rs17216317
Exon 28	c.3895 A>C	p.K1299Q		Benign			rs4148400, [146]
Exon 28	c.3928 C>T	p.R1310X ^f	DJS				[166]
Exon 29	c.4100 C>G ^e	p.S1367C		Possibly damaging		G: 0.007	[63]
Exon 29	c.4145 A>G	p.Q1382R	DJS	Probably	Deficient		[47, 168]

Table 3 (continued)

Location	Nucleotide change ^a	Deduced effect on protein ^a	Causing Dubin–Johnson syndrome ^b	Predicted effect by PolyPhen database ^c	Experimentally proven functional consequence	Average frequency of indicated nucleotide exchange in population	NCBI SNP ID ^d and/or references
Exon 30	c.4175_4180del6	p.R1392_M1393del2	DJS	damaging	transport function [47] No ABCC2 protein in liver [170] Impaired sorting and trafficking [79]		[79, 170]
Exon 31	c.4348 G>A	p.A1450T		Possibly damaging	Reduced protein levels [50]	T: 0.010 [62]	[62]
Exon 31	c.4430 C>T	p.T1477M		Benign		T: 0.006	[51]
Exon 32	c.4544 G>A	p.C1515Y		Benign		A: 0.047 A: 0.116	rs8187710 rs17222568

NCBI National Center for Biotechnology Information, SNP single nucleotide polymorphism, DJS Dubin–Johnson syndrome

^aAs recommended by the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>) and by ref [26], nucleotide position +1 is the A of the ATG of the translation initiation codon in the *ABCC2* cDNA sequence, “c.” describes a nucleotide change in relation to the *ABCC2* cDNA sequence, “p.” describes a change in relation to the deduced *ABCC2* protein sequence, and “X” denotes a premature stop codon

^bIf not indicated otherwise, the diagnosis of Dubin–Johnson syndrome was based on the histopathology of the liver and on elevated serum levels of conjugated bilirubin

^cPolyPhen online tool for assessing potential effects of amino acid substitutions, <http://genetics.bwh.harvard.edu/pph>

^dSingle nucleotide polymorphism database, <http://www.ncbi.nlm.nih.gov/SNP>; data based on NCBI dbSNP build 125, regular updates available

^eIdentified in cell lines derived from Japanese individuals

^fProbably reduced *ABCC2* mRNA levels due to nonsense-mediated mRNA decay [167]

functionally active *ABCC2* protein from the canalicular membrane, their effects on the synthesis and function of the *ABCC2* protein differ. Premature stop codons may cause rapid degradation of the mutated *ABCC2* mRNA by nonsense-mediated decay, a mechanism which cotranslationally recognizes when a stop codon precedes the last splice-site [167], thus leading to the absence of the *ABCC2* protein in some cases of Dubin–Johnson syndrome. In fact, a truncated *ABCC2* protein has not been detected so far [134, 170]. Other Dubin–Johnson syndrome-associated sequence variants lead to deficient maturation and impaired sorting of the *ABCC2* protein or to an apically localized, but functionally inactive, *ABCC2* protein [47, 79, 80, 117] (Table 3).

Many naturally occurring sequence variants have been identified whose consequences on *ABCC2* function are not yet known (Table 3). The potential effects of these single amino acid substitutions can be tentatively assessed by the online tool Polymorphism Phenotyping (PolyPhen, <http://genetics.bwh.harvard.edu/pph> [138]). Interestingly, all amino acid substitutions causing Dubin–Johnson syndrome are classified in this database as “probably

damaging” (Table 3). However, the PolyPhen tool cannot replace the experimental analysis of each individual amino acid substitution to proof changes of *ABCC2* function, as exemplified by some published transfection studies [79, 80, 117].

Two different mutant hyperbilirubinemic rat strains, the GY/TR[−] rats [64] and the Eisai hyperbilirubinemic rats (EHBR) [54, 163], have a hereditary defect in the secretion of anionic conjugates into bile. In consequence of the molecular identification and cloning of rat *Abcc2* as the apical conjugate efflux pump [13, 60, 133], which is defective in these rat strains [13, 60, 133], both can now be considered as animal models of human Dubin–Johnson syndrome. Two distinct naturally occurring sequence variants in the rat *Abcc2* gene, either at codon 401 (GY/TR[−]) [133] or at codon 855 (EHBR) [60], lead to premature stop codons and to a lack of the *Abcc2* protein from the hepatocyte canalicular membrane [13, 114, 133]. Recently, *Abcc2*-knock-out mice have been generated and characterized by several groups [18, 121, 177]. These *Abcc2*-deficient mice are apparently healthy and fertile, as are

the Abcc2-deficient rats. It will be of interest to crossbreed these mutants with mice lacking other Abc transporters, such as knock-out mice lacking Abcc3, Abcc4, Abcb1a and Abcb1b (Mdr1a and Mdr1b P-glycoprotein), and Abcg2 (Bcrp1).

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