Identification of a compound heterozygous mutation of *ABCC2* in a patient with hyperbilirubinemia

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Abstract. Bilirubin is the end product of heme catabolism, which is produced primarily from the breakdown of erythrocyte hemoglobin in the reticuloendothelial system. Hyperbilirubinemia is induced not only by increased bilirubin synthesis, but can also be caused by decreased bilirubin clearance. There are several disorders, which can contribute to hyperbilirubinemia, including Dubin-Johnson syndrome (DJS). DJS is a rare autosomal recessive disorder, which is characterized by predominantly conjugated hyperbilirubinemia without progression to end-stage liver disease. Previous studies have demonstrated that defects in multidrug resistance proteins ATP-binding cassette C2 (ABCC2)/multidrug resistance-associated protein 2 (MRP2) contribute to DJS. In the present study, a case of a patient with hyperbilirubinemia was examined and identified a compound heterozygous mutation in the *ABCC2* gene (p.T435P and W442X). These were predicted to be deleterious by three bioinformatics programs (Polymorphism Phenotyping-2, Sorting Intolerant From Tolerant and MutationTaster). These finding expand on the spectrum of *ABCC2* mutations and provide additional evidence that ABCC2 is key in the development of DJS.

Introduction

Bilirubin is the end product of heme catabolism, which is produced primarily from the breakdown of erythrocyte hemoglobin in the reticuloendothelial system. There are several major steps in the hepatic clearance of bilirubin, including hepatocytes ingesting and storing unconjugated bilirubin, the conjugation of bilirubin to bilirubin glucuronides, the excretion of conjugated bilirubin into bile, and hepatocytes resorbing the conjugated bilirubin (1). Hyperbilirubinemia is not only induced by increased bilirubin synthesis, but it can also be caused by decreased bilirubin clearance (2). There are several inherited disorders, which can contribute to hyperbilirubinemia (3), including Dubin-Johnson syndrome (DJS), Crigler-Najjar syndrome, Gilbert syndrome and Lucey-Driscoll syndrome. As a rare autosomal recessive disorder, DJS is characterized by predominantly conjugated hyperbilirubinemia without progression to end-stage liver disease (4-6).

To date, there are several genes, which have been identified to contribute to hyperbilirubinemia, including *UGT1A1*, *SLCO1B1*/*OATP1B1*, *SLCO1B3*/*OATP1B3*, *MRP2*/*ABCC2* and *ABCG2*/*BCRP* (1,7,8). At present, at least 24 *ABCC2*/*MRP2* point mutations have been reported in DJS, a number of which are predicted to result in truncated proteins. In the present study, the possible causative gene was investigated in a patient with hyperbilirubinemia. The results revealed two novel mutations (c.1303A>C/p.T435P and c.1326G>A/p.W442X) in exon 10 of *ABCC2*. To the best of our knowledge, these mutations have not been reported in previous studies, neither have they been presented in the single nucleotide polymorphism (dbSNP) databases (https://www.ncbi.nlm.nih.gov/projects/SNP/) and Exome Variant Server databases (http://evs.gs.washington.edu/EVS/).

Materials and methods

Patients. In the present study, a family from Hunan province comprising six members across three generations, which were admitted to the Second Xiangya Hospital in October, 2015 (Changsha, China), was included (Fig. 1A; Table I). The proband was diagnosed with hyperbilirubinemia (III2) with a total bilirubin level of 30.6 μmol/l and a direct bilirubin level of 10.5 μmol/l. No hyperbilirubinemia was present in the other family members. Details of the family are listed in Table I. The present study was approved by the Second Xiangya Hospital of Central South University (Changsha, China). All subjects provided consent prior to commencement of the study.

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Table I. Summary of the family with Dubin-Johnson syndrome investigated.

Family member	TBIL/DBIL (μmol/l)	Age (years)	ABCC2 DNA	Protein	Program prediction		
					Polyphen-2	SIFT	MutationTaster
III2 (proband)	30.6/10.5	19	1303A>C	T435P	Possibly damaging	Deleterious	Disease-causing
			1326G>A	W442X	Possibly damaging	Deleterious	Disease-causing
I2	16.0/5.0	70	-	-	-	-	-
I3	13.7/5.5	69	-	-	-	-	-
II1	15.9/4.9	43	1326G>A	W442X	-	-	-
II2	14.9/4.9	44	1303A>C	T435P	-	-	-
III1	13.5/3.8	20	-	-	-	-	-

TBIL, total bilirubin; DBIL, direct bilirubin; ABCC2, ATP-binding cassette C2; Polyphen-2, Polmorphism Phenotyping-2; SIFT, Sorting Intolerant From Tolerant.

Table II. Primer sequences used for mutation sequencing of *UGT1A1*, *ABCC2* and *OATP1B1* genes.

Gene	Primer sequences 5'→3'
UGT1A1 1-1	F: ACATTAACTTGGTGTATCGA
	R: AGCCAGACAAAAGCATAG
UGT1A1 1-2	F: TTGTTAGTCTCGGGCATA
	R: GTCCTGGACAGTCACCTCT
UGT1A1 1-3	F: GCAGCGGGTGAAGAACAT
	R: ATGCCAAAGACAGACTCAAACC
UGT1A1 2	F: TAATTCTGTAAGCAGGAAC
	R: TAATAGTTGGGAAGTGGC
UGT1A1 3	F: GAAGTTGCCAGTCCTCAG
	R: AATTTGACCCTGGTTTGA
UGT1A1 4	F: TGCTGACATCCTCCCTAT
	R: AACGCTATTAAATGCTACG
UGT1A1 5	F: CCAGGCATAACGAAACTG
	R: CCTTATTTCCCACCCACT
ABCC2 1	F: AACTGGTGAGTCTCCCTG
	R: AATTGCACATCTAACATTTCTG
ABCC2 2	F: TGTGAAAGCAGTGGGATG
	R: CTGGCTCTACCTGAGACAAT
ABCC2 3	F: ATCACCGGAAACCATTCT
	R: AAAGGTAAACAGGGCAGA
ABCC2 4	F: CCCTCAGCCCTCCTTTCT
	R: TACCTCCTCATGTCATCCACTC
ABCC2 5	F: TGTATTAGAGGGATTTGATC
	R: TACCTTATTCTGGGCTTG
ABCC2 6	F: TTAGAGTCCCATGAAGTT
	R: AGTAAGGATACAGCCAAT
ABCC2 7	F: TTCTGATAGAAGTGGTGGAG
	R: TACCCTTGCCTGAAACAT
ABCC2 8	F: GGCAGCTAGAAGGGCAGAA
	R: AAGGAGGGTGGCAGAGGA
ABCC2 9	F: TGAACTATGATCCTGCCACT
	R: CCTGCCGTATTCTGCTTA
ABCC2 10	F: GAGGCAAGAAGTCACAGT
	R: CTCCCATTAAGAATTAGAGT

Table II. Continued.

Gene	Primer sequences 5'→3'
ABCC2 11	F: GAGCAGAGTGGGCAAAGA
	R: CAGGAGGACATGAAACAAAA
ABCC2 12	F: AAACATGGGTGGATCAGA
	R: TGCCAGCTAGTCTATCAAAA
ABCC2 13	F: GCTCTGGTCCTAGTAATCC
	R: GATGTGATAGCCAGTCATT
ABCC2 14	F: CATCTGTCTATGGTGGGA
	R: GAATAAGTTTGGGAAGCA
ABCC2 15	F: AGCCAGCACTTAGCAGAA
	R: TGGAAAGAAGGCAACTCA
ABCC2 16	F: AACTACTCTTCAATACCCAACC
	R: CTAGCCCTCAGTGCCTTC
ABCC2 17	F: GCTCCATTTGTTTCTTCC
	R: TTCACCACCATCCTCACT
ABCC2 18	F: CTCCCTATTAGATTCTGTG
	R: CTTCCCTGTCTTACTTGC
ABCC2 19	F: TTGAAAGGCAAGGTGAGA
	R: ACAGAACCCAGAAAGCAG
ABCC2 20	F:TGTTCATAGGACTGACAGGGAT
	R: GCGCATTTCAGGGCAGAT
ABCC2 21	F: GGTCATCTGCCCTGAAAT
	R: AGCCCACAGCCTCTGCTA
ABCC2 22	F: GTTGGCATTCTAGGTGAT
	R: GTACAGGGTCCAGACAGA
ABCC2 23	F: AATCTGTCTGGACCCTGTA
	R: ATGTTCATCCCTCAATCT
ABCC2 24	F: TTGGTTATTGGGGCAAGC
	R: GGGCTCCTGGGTATGTCA
ABCC2 25	F: GGAGGAAGATGGTGGATG
	R: CTTGGTAAACGGCAGAGC
ABCC2 26	F: TGTAGGATTCCCTTAGTTC
	R: TCAGTCTTCTTTAGTCCCT
ABCC2 27	F: TCCCTTGTAGAGTCCAGC
	R: ATTAGGTCCTTTGAGTTAGA
ABCC2 28	F: GACTGTTCGGCTGAGTTG

Table II. Continued.

Gene	Primer sequences 5'→3'
	R: AATGATGAAGGCTTAGGG
ABCC2 29	F: CCTCTTACCTCCTGTGAC
	R: GTAGACCGTGGAATTGAC
ABCC2 30	F: ATAAACCGAGGACTTCTAACC
	R: GCCAGGCATCACCTAACA
ABCC2 31	F: TGCGTCTTTCCTTGGTCT
	R: CTGCCATCAGGTGTTTCC
ABCC2 32	F: CACAACTTAGTCCTGGTT
	R: ATGGGTGTTCACTTATCC
OATP1B1 1	F: TAAGAGGAATAAAGGGTG
	R: CTCAGAATGTAAGCGTGT
OATP1B1 2	F: GACATAGTAGACCCTGAG
	R: CTATACATTAAAGTTCCCTA
OATP1B1 3	F: CCCCTTTCCTTCTGATTT
	R: ACCCCTGACCTCTACCTT
OATP1B1 4	F: CATTGTCTTTGAGGGAAGG
	R: ATAGTGGCACAGAGGTTT
OATP1B1 5	F: AATGGTGCAAATAAAGGG
	R: GTTGTTAATGGGCGAACT
OATP1B1 6	F: TTAGCAGCATAAGAATGG
	R: AGTAGACAAAGGGAAAGT
OATP1B1 7	F: TCACTTTCCCTTTGTCTA
	R: GGTACCTTGTTCTGGTTG
OATP1B1 8	F: TTCCCTGAACCTATTGTA
	R: GAGTTGGGTATGCTTTATT
OATP1B1 9	F: GGCTATTCTCACTCTTTG
	R: CAGAGCAATAGTGACATC
OATP1B1 10	F: AAAACCTAGATGACAGTT
	R: TGGTTAACATATTATGCA
OATP1B1 11	F: CCATTTCGTCATCATCAA
	R: CACCCATCACAATAACAG
OATP1B1 12	F: TATTTGCAGCACTGTTAG
	R: AATGGAAGAATTAGAGGC
OATP1B1 13	F: TATTGCTCAAGTGTTTGC
	R: CACAGAAATAGAAAGGATA
OATP1B1 14	F: ATGATTTGGGTCTTTGAG
	R: AGATACGAGATTGCTTGA
OATP1B1 15	F: TCTATCGTTATGCCCCAAT
	R: GGACCAGGAACTCCTCAA

F, forward; R, reverse.

DNA extraction. Genomic DNA was extracted from the peripheral blood of the proband and other family members using a DNeasy Blood & Tissue kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol, on the QIAcube automated DNA extraction robot (Qiagen, Inc.), as previously described (9).

Mutation sequencing. Through the use of polymerase chain reaction (PCR), several genes were amplified, including *UGT1A1* (Refseq: NM_000463), *ABCC2* (Refseq: NM_000392) and *OATP1B1* (Refseq: NM_0,06446). PCR was performed using 25 μl reaction volumes, containing 0.3 mM dNTPs, 1X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100 and 0.01% w/v gelatin), 2.0 mM $MgCl_2$, 0.5 μM of each primer (forward and reverse), 1.5 U of Taq polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 50 ng of genomic DNA. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 95˚C for 30 sec, annealing at 55-61˚C for 30 sec and extension at 72˚C for 1 min. A final extension step was performed at 72˚C for 7 min. The sequences of the PCR products were obtained using the ABI 3100 genetic analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Results were compared with normal control samples, as defined in our previous study (9).

Bioinformatics sequence analysis and mutation prediction. In several species, the multiple ABCC2 protein sequences were aligned (version 3.6; http://www.ncbi.nlm.nih.gov). Polymorphism Phenotyping-2 (Polyphen 2; http://genetics.bwh.harvard.edu/pph2/) (10), Sorting Intolerant From Tolerant (SIFT; http://sift.bii.astar.edu.sg/) (11) and MutationTaster (www.mutationtaster.org) (12) were used to predict the effects of these sequence variants on the function of the protein.

Results

The present study reported on a patient with hyperbilirubinemia with a total bilirubin level of 30.6 μmol/l and direct bilirubin of 10.5 μmol/l, whereas the reference standard values are 5.1-17.1 and 0-6.0 μmol/l, respectively. The possibility of the induction of hyperbilirubinemia by known genes was investigated. Using Sanger sequencing, a missense mutation (c.1303A>C/p.T435P) and a nonsense mutation (c.1326G>A/p.W442X) in ABCC2 were identified and co-segregated with the affected members. (Fig. 1B and C). The allelic segregation analysis revealed that the missense mutation was carried by the mother, whereas the nonsense mutation was inherited from the father. These newly identified missense mutations c.1303A>C and c.1326G>A were not found in a cohort of 200 controls, as described in our previous study (9). In addition, these two mutations were not present in the dbSNP and Exome Variant Server databases. In humans, macaques, cats, mice and zebrafish, the amino acid sequences of ABCC2 were found to be aligned, which revealed that the affected amino acids were evolutionarily conserved (Fig. 2A and B). Three programs were used for analyzing the protein functions of ABCC2; polyphen2, SIFT and Mutation Taster, predicted that the two variants were likely to be damaging, deleterious and disease-causing, respectively. The consistent findings of the detrimental effects of the variants by all these bioinformatics programs suggested that these mutations are important in the function of ABCC2.

Discussion

The present study presented a case of hyperbilirubinemia associated with a compound heterozygous mutation (c.1303A>C/p.T435P and c.1326G>A/p.W442X) in exon 10 of *ABCC2*. A previous study found that mutations in *ABCC2* may cause DJS, and this syndrome was characterized by

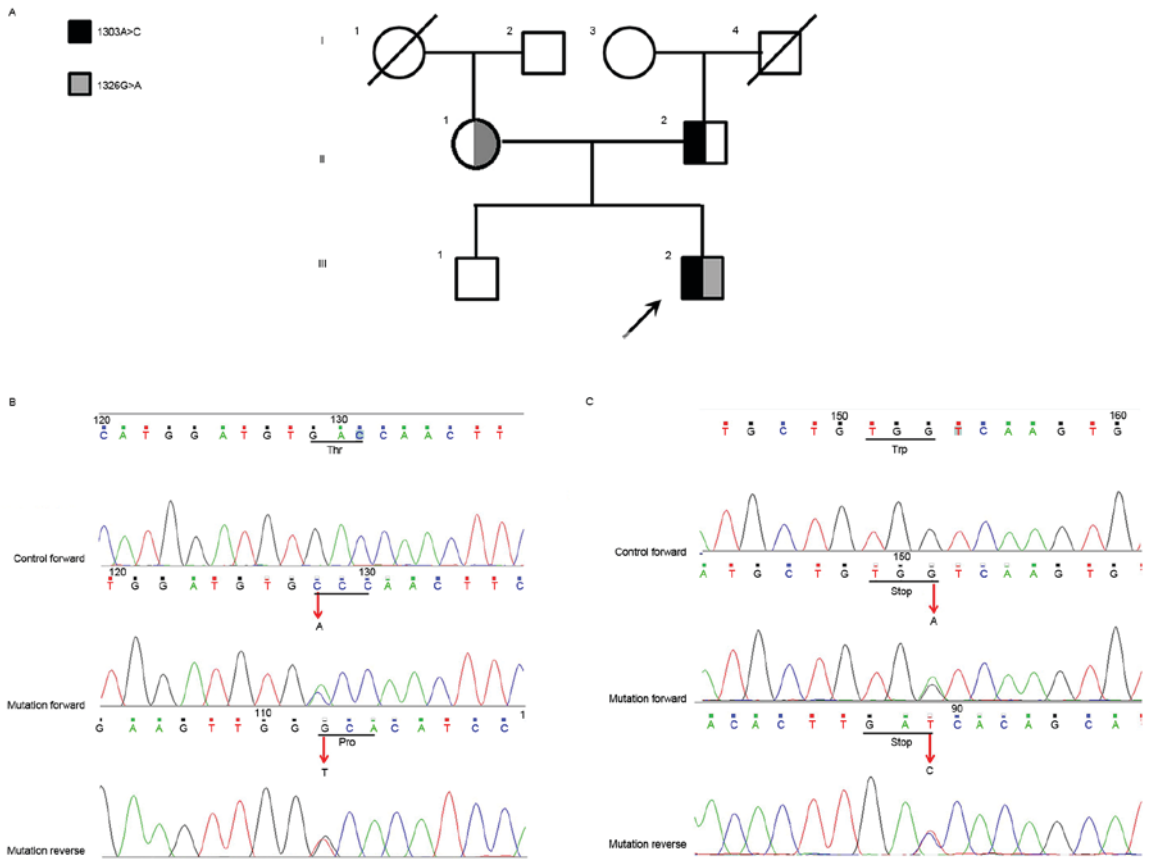

Figure 1. (A) Pedigree of the family examined in the present study. Squares represent males; circles represent females; horizontal lines indicate that the individual is deceased; the white/grey circle represents the affected member with c.1326G>A/p.W442X; the black/white square represents the affected member with c.1303A>C/p.T435P; the black/grey square represents the proband with c.1326G>A/p.W442X and c.1303A>C/p.T435P. (B) Analysis of *ABCC2*. The chromatograms show the partial sequence of *ABCC2*, c.1326G>A. (C) Analysis of *ABCC2*. The chromatograms show the partial sequence of *ABCC2*, c.1303A>C. ABCC2, ATP-binding cassette C2.

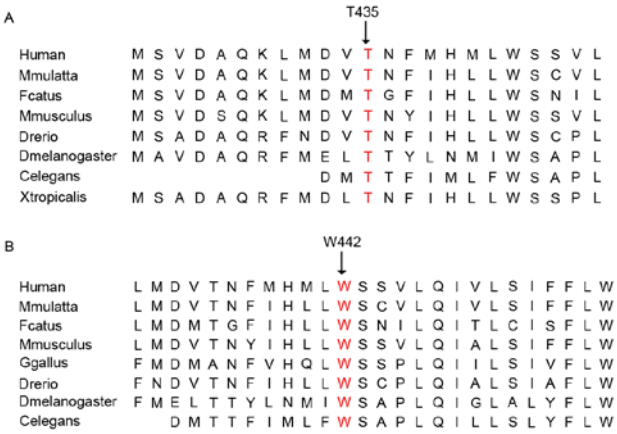

Figure 2. Conservation analysis of ATP-binding cassette C2. (A) T435 sites are highlighted in red. This locus was found to be highly conserved at the protein level across different species. (B) W442 sites are highlighted in blue. This locus was found to be highly conserved at the protein level across different species.

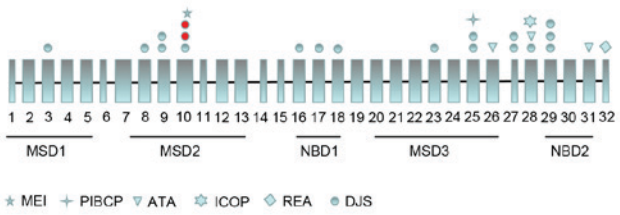

Figure 3. Summary of missense or nonsense mutations in ATP-binding cassette C2 and the associated diseases. Mutations identified in the present study are indicated in red. MSD, membrane spanning domain; NBD, nucleotide-donmain; MEI, methotrexate elimination, impaired; PIBCP, pruritis in biliary cirrhosis patients; ATA, altered transport activity; ICOP, intrahepatic cholestasis of pregnancy; REA, reduced efflux activity; DJS, Dubin-Johnson syndrome.

biphasic, predominantly conjugated, hyperbilirubinemia. At present, ~17 point mutations of *ABCC2* have been reported in patients with DJS (Fig. 3). The outcome of the molecular genetic investigations performed in the present study was consistent with and confirmed the clinical diagnosis of DJS.

Under normal conditions, hepatocytes take up unconjugated bilirubin by transporters of the organic anion-transporting polypeptide family, followed by conjugation with glucuronic acid and ATP-dependent transport into bile. This efflux across the canalicular membrane is mediated by ABCC2/MRP2, which has a high affinity and efficiency for monoglucuronosyl and bisglucuronosyl bilirubin into bile. Therefore, mutations in ABCC2 may lead to DJS (13,14).

In the present study, the missense and nonsense mutations were located in a conserved membrane-spanning domain (MSD), namely MSD2, of the ABCC2 protein (15). The

nonsense mutation can also lead to the absence of the complete nucleotide-binding domain (NBD)-1, MSD3 and NBD2. Mutations in MSD2 may affect the subcellular localization of ABCC2, and the truncated mutation may cause the functional defect of ABCC2. These two point mutations were present in patients, and this compound heterozygous mutation was associated with DJS recessive hereditary mode.

Among the compound heterozygous mutations in DJS, 13 cases have been reported, including that identified in the present study (5,16). At present, a total of 24 *MRP2/ABCC2* point mutations have been reported, 17 of which are associated with DJS (17-19) (Fig. 3). In the last 25 years, different types of viral vectors have been used in clinical trials for the treatment of a variety of monogenetic disorders. It has been suggested that this technique may be used to treat hereditary hyperbilirubinemia (20,21), however, further investigation and improvements are required (22).

In conclusion, the present study identified an *ABCC2* compound heterozygous mutation (c.1303A>C/p.T435P and c.1326G>A/p.W442X) in a patient with DJS. To the best of our knowledge, this may be the first report of these two mutations worldwide. The results of the present study offer further support for the significant involvement of *ABCC2* in DJS. The results also expand on the spectrum of *ABCC2* mutations, and contribute to the genetic diagnosis and counseling of families with DJS.

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