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ORIGINAL ARTICLE

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Identification of a novel 2026G → C mutation of the MRP2 gene in a Japanese patient with Dubin-Johnson syndrome

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Abstract Dubin-Johnson syndrome is a recessive inherited disorder with conjugated hyperbilirubinemia caused by a dysfunction of multidrug resistance protein 2 (MRP2) on the canalicular membrane of hepatocytes. A mutational analysis of the MRP2 gene was carried out in three Japanese patients and their family members. In two patients, the homozygous mutations c.1901del67 and c,2272del168 were found. In the third patient, a −24C → T polymorphism and the two mutations c.1901del67 and 2026G → C were detected. The 2026G → C mutation was a novel mutation in exon 16 affecting the conversion of Gly^{676} to Arg^{676} (G676R) in the MRP2 protein, and was not detected in fifty healthy volunteers. The G676R mutation was located in the Walker A motif of the first nucleotide binding domain in the MRP2 protein, and it was suggested that the mutation induced the dysfunction of the MRP2 protein. It was concluded that the compound heterozygosity of the two mutations of the MRP2 gene in the third patient contributed to the induction of hyperbilirubinemia in this case.

Keywords Dubin-Johnson syndrome · Multidrug resistance 2 (MRP2) · ATP-binding cassette · Walker A motif · Mutation

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Introduction

Dubin-Johnson syndrome (DJS) is an inherited metabolic disorder characterized by conjugated hyperbilirubinemia and a re-increase of serum bromosulfophtalein (BSP) in the BSP tolerance test, characteristics that are caused by a dysfunction of the canalicular multispecific anion transporter (cMOAT, multidrug resistance protein 2; MRP2).

The human MRP2 gene was identified in cisplatin-resistant tumor cells by Taniguchi et al. (1996) following the identification of the rat cmoat (mrp2) gene in hyperbilirubinemia rats (TR^- rats) by Paulusma et al. (1996). The human MRP2 gene was mapped to chromosome 10 at q24 (Taniguchi et al. 1996) and was found to consist of 32 exons (Toh et al. 1999).

MRP2 protein is a membrane protein with 1545 amino acids; it contains two ATP-binding cassettes (ABCs) and 17 transmembrane sequences (Taniguchi et al. 1996). It is expressed at the canalicular membrane of hepatocytes and apical membrane of the renal uriniferous tuble (Paulusma et al.1996) and is involved in the excretion of glucronide-conjugated bilirubin and glutathione-conjugates from the liver (Taniguchi et al. 1996; Wada et al. 1998).

The first mutation of the MRP2 gene causing hyperbilirubinemia was found by Paulusma et al. (1997) in DJS, and a total of 10 mutations have been found as listed in Table 1.

In the present study, we conducted a mutational analysis in three Japanese DJS patients and found two known deletion mutations (c.1901del67, c.2272del168) and a novel missense mutation of G676R in the first nucleotide-binding domain (NBD1) in the MRP2 protein.

Subjects and methods

Study participants

Three DJS patients and their family members were included in the present study.

Table 1 Mutations of MRP2 in Dubin-Johnson syndrome (DJS)

Nucleotide mutation	Predicted effects	References
Splice site mutation		
1815 + 2T → A	1669del147 (exon13 skipping)	Wada et al. 1998
1967 + 2T → C	1901del67 (exon15 skipping)	Kajihara et al. 1998
2439 + 2T → C	2272del168 (exon18 skipping)	Toh et al. 1999
Deletion mutation		
Del4170–5	Del R^{1392}, M^{1393}	Tsujii et al. 1999
Missense mutation		
2302C → T	R768 W	Wada et al. 1998
3449G → A	R1150H	Mor-Cohen et al. 2001
3517A → T	I1173F	Mor-Cohen et al. 2001
4145A → G	Q1382R	Toh et al. 1999
Nonesense mutation		
3196C → T	R1066X	Paulusma et al. 1997
3928C → T	R1310X	Tate et al. 2002

Patient 1 (DJ1) was a male whose parents were distantly related. He was diagnosed with DJS at the age of 28 years based on hyperbilirunemia, a BSP test, and a histological examination of the liver. Serum bilirubin values of his two daughters and spouse were within the normal range.

Patient 2 (DJ2) was a female whose parents were the first cousins. She was diagnosed with DJS at the age of 23 years based on hyperbilirunemia, a BSP test, and a histological examination of the liver. Serum bilirubin values of her two siblings, daughter, and spouse were within the normal range.

Patient 3 (DJ3) was a male whose parents were distantly related. He was diagnosed with chronic hepatitis and DJS at the age of 25 years based on serum aminotransferase levels, conjugated hyperbilirunemia, a BSP test, and a histological examination of the liver. Thereafter, he was diagnosed as having DJS with chronic hepatitis C from positive results for anti-HCV antibody and HCV RNA. Among his two daughters, spouse, and six siblings, there was only a moderate hyperbilirubinemia in his older sister.

Mutational analyses

mRNA analysis

Total RNA was extracted from peripheral white blood cells with an Isogen WB kit (Wako Pure Chemicals, Japan), and cDNA was obtained by reverse transcription of 1 μg RNA with M-MLV transcriptase, RNase inhibitor (RNaseOUT, Invitrogen, Carlsbad, Calif.), and random hexamer primers (Invitrogen). Polymerase chain reaction (PCR) was performed with *Taq* DNA polymerase (Promega, Tokyo, Japan) and twelve primer sets originally reported by Wada et al. (1998). PCR products were separated on a 1.5% agarose gel by electrophoresis and directly sequenced by a Sequencing High detection kit (Toyobo, Tokyo, Japan).

Genomic DNA analysis

Genomic DNA was isolated from peripheral white blood cells with a DNA isolation kit (Puregene Blood Kit, Gentra Systems, Minneapolis, Minn.) and used for PCR amplification of 32 exons with flanking intronic regions by the primer sets reported by Toh et al. (1999). Direct sequencing analysis was performed by ABI PRISM Dye Terminators in an ABI 3700 Genetic Analyzer (Applied Biosystems, Foster City, Calif.).

Segregation of a novel mutation within the family

A mismatch primer set was used for the novel mutation site. The primers used to detect the mutation were the forward primer 5'-gataggccctgtcggctctC-3' (the underlined sequence indicates the mismatched site) and the reverse primer 5'-gggaaggagaatcacagtccaca-3'. PCRs were conducted with 30 cycles of 30 s denaturation at 95°C, 30 s anneraling at 66°C, and 30 s extension at 74°C. PCR products of 234 bp were detected by electrophoresis on a 1.5% agarose gel.

Restriction analysis of polymorphisms

Following the identification of the −24C → T polymorphism in exon 1 by sequence analysis, restriction analysis with *Bbs*I (New England Biolabs, Beverly, Mass.) was performed to detect this polymorphism according to the method of Mor-Cohen et al. (2001). Exon 1 was amplified by using a reported primer set (Toh et al. 1999); the product was digested by 10 U *Bbs*I for 12 h at 37°C and separated on a 1.5% agarose gel.

Results

Mutational analysis of the MRP2 mRNA from three DJS patients revealed two types of deletion mutation, c.2272del168 and c.1901del67, which were previously known as shown in Table 1.

In the study of DJ1, the homozygous mutation c.2272del168, which causes skipping of exon 18, was detected by PCR of the MRP2 cDNA by using primer set G (forward: 5'-ggagatttggctgagattggagaga-3', reverse: 5'-cttcagatgcctgccattggaccta-3') designed by Wada et al. (1998), and which covers c.2245–2608, and by direct sequencing of the PCR product (data not shown). The two daughters (DJ1/C1 and DJ1/C2) of DJ1 were heterozygous for this mutation (Table 2).

In the case of DJ2, a homozygous mutation of c.1901del67, which causes skipping of exon 15, was revealed by PCR amplification by using primer set E (forward: 5'-accttcattcagagaccaag-3', reverse: 5'-ggctgatatcaaggaggatt-3') as shown in Fig. 1A. One daughter and one of two siblings of DJ2 were heterozygous for this mutation (Table 2).

In the case of DJ3, as shown in Fig. 1A, the patient was heterozygous for c.1901del67; this result was supported by sequence analysis of exon 15 with the flanking intronic region, which revealed the 1967 + 2T → C mutation of the splicing site (Fig. 1B). In addition, sequencing analysis of exons 16 and 1 detected a novel missense mutation of 2026G → C (G676R) in the first Walker A motif (Fig. 2) and a single nucleotide polymorphism (SNP) of −24C → T in the non-coding region of exon 1 (Fig. 3). The c.1901del67 mutation was detected in two other siblings and two daughters of DJ3. Mismatch PCR revealed that the 2026G → C mutation was not distributed in the six siblings and two children (Table 2, Fig. 4). With respect to the polymorphism −24C → T, we also employed a restriction enzyme assay with *Bbs*I; the results showed that his spouse and one daughter were heterozygous for this SNP, but other

Table 2 Mutations of the MRP2 gene in DJS patients and family members (*wild* wild-type, *nd* not determined)

Patients and family members[a]		Bilirubin (mg/dl)		Alteration in MRP2 gene	Predicted effects
		Total	Conjugated		
1	DJ1	2.7	1.8	2439 + 2T → C/2439 + 2T → C	2272del168/2272del168
	DJ1/C1	0.6	0.2	2439 + 2T → C/wild	2272del168/wild
	DJ1/C2	0.6	0.2	2439 + 2T → C/wild	2272del168/wild
2	DJ2	3.0	2.4	1967 + 2T → C/1967 + 2T → C	1901del67/1901del67
	DJ2/S1	0.4	0.1	1967 + 2T → C/wild	1901del67/wild
	DJ2/S2	0.5	0.2	Wild/wild	–
	DJ2/C1	0.8	0.2	1967 + 2T → C/wild	1901del67/wild
3	DJ3	3.4	2.8	1967 + 2T → C/2026G → C	1901del67/G676R
	DJ3/S1	0.8	0.3	Wild/wild	–
	DJ3/S2	0.9	0.3	Wild/wild	–
	DJ3/S3	0.9	0.3	Wild/wild	–
	DJ3/S4	0.9	0.3	1967 + 2T → C/wild	1901del67/wild
	DJ3/S5	0.3	0.1	Wild/wild	–
	DJ3/S6	1.3	0.6	1967 + 2T → C/wild	1901del67/wild
	DJ3/C1	nd	nd	1967 + 2T → C/wild	1901del67/wild
	DJ3/C2	0.7	0.3	1967 + 2T → C/wild	1901del67/wild

[a] *C* child, *S* siblings

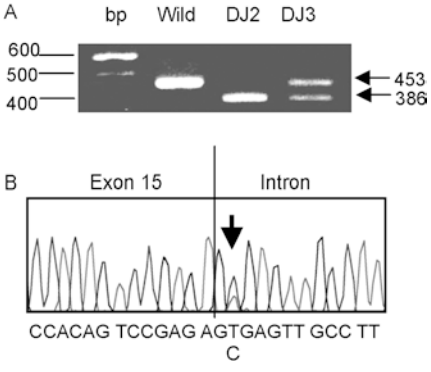

Fig 1A, B The c.1901del67 mutation found in DJ2 and DJ3. **A** The c.1901del67 mutation in the polymerase chain reaction (PCR) products of cDNA from DJ2 and DJ3. PCR of the cDNA from position 1597–2049 in DJ3 amplified a normal product of 453 bp (cf. *Wild*) and a smaller product of 386 bp that was confirmed to be a deletion of 67 bp from 1901 to 1967 by sequencing analysis (data not shown). **B** Sequencing analysis of exon 15 in the MRP2 gene from DJ3 revealed a heterozygous point mutation at the splice site, 1967 + 2T → C (*arrow* site of the mutation)

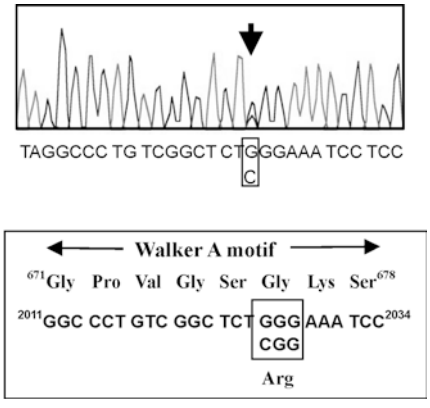

Fig. 2 Sequencing analysis of exon 16 in the MRP2 gene from DJ3 (*arrow* site of the heterozygous missense mutation 2026G → C). The mutation was predicted to change the amino acid sequence of the MRP2 protein from glycine[676] to arginine[676]

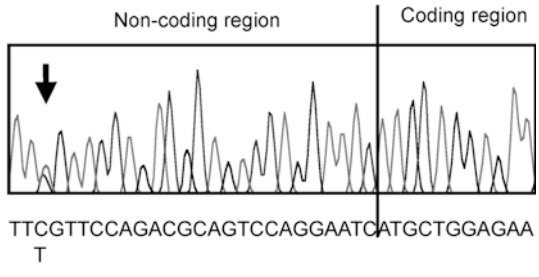

Fig. 3 The −24C → T polymorphism in the non-coding region in exon 1 of the MRP2 gene from DJ3 (*arrow* site of the polymorphism)

family members did not possess it (Fig. 4). There were no data for the parents and one sibling who were deceased. The new 2026G → C mutation was not found in 50 healthy individuals (100 alleles). These results indicated that the mutations c.1901del67 and 2026G → C were located on different alleles, and that 2026G → C was a disease-associated mutation. DJ3 was a compound heterozygote for these mutations.

Discussion

In the present study, we have found a novel missense mutation that is predicted to affect the substitution of glycine[676] with arginine[676] (G676R), together with two known deletion mutations. Glycine[676] is one of the conserved amino acids (underlined) in the Walker A motif ([671]GPVGSGKS[678]) in the ABC of MRP2 protein (Taniguchi et al. 1996).

Two mutations within the two ABCs in the MRP2 protein have been described previously in DJS patients in Japan as shown in Table 1. One of these mutations (R768 W) is located within the first Walker C motif, and the other (Q1382R) lies within the second ABC (Toh

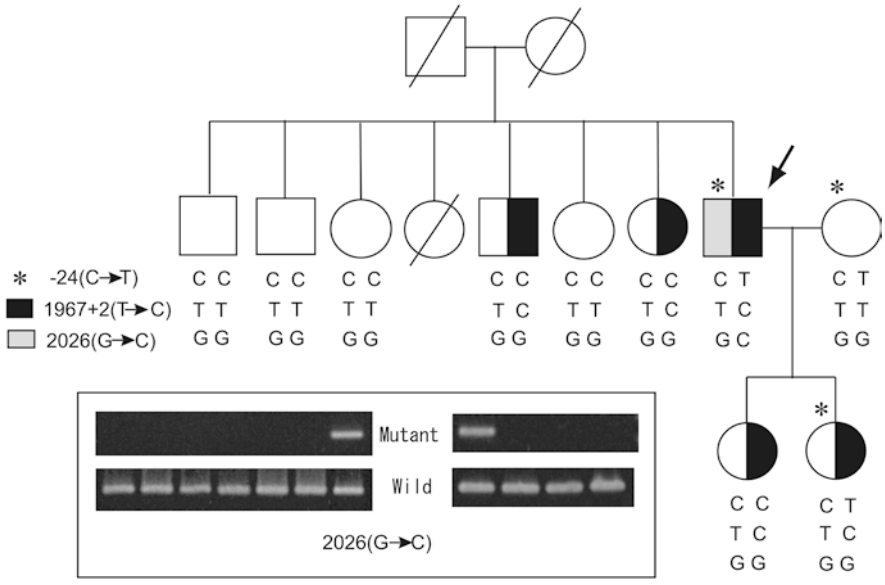

Fig. 4 Distribution of the −24C → T polymorphism and the mutations c.1901del67 and 2026G → C in the family members of DJ3 (*arrow* DJ3). *Insert* Electrophoresis showing results of the PCR with mismatch primers for the detection of the 2026G → C mutation (*upper* mutant, *lower* wild-type). *Left* Results for the siblings of DJ3. The order is identical with the pedigree. *Right* DJ3 (*lane 1*), spouse (*lane 2*), and children (*lanes 3, 4*)

et al. 1999). The G676R conversion is the first reported mutation of the Walker A motif in MRP2 protein.

The Walker A motif consists of a sequence of eight amino acids (GPVGSGKS), and those underlined are consensus amino acids as described above. The G676R mutation is located at the 5'-flanking end of lysine, an amino acid shown to bind the γ-phosphate of ATP (Konig et al. 2003) in NBD1. In the MRP1 protein, which is a member of the same family as MRP2, the substitution of lysine with methionine in NBD1 decreases ATP-binding to NBD2 and diminishes the transport activity of the protein (Gao et al. 2000; Hou et al. 2002). In the transporters associated with antigen processing (TAP1 and TAP2), the conversion of lysine to methionine in the Walker A motif has been shown to cause a loss of transport activity (Lapinski et al. 2001).

Hashimoto et al. (2002) have reported that the artificial mutation K677R of the MRP2 gene and the missense mutation R768 W cause deficient maturation and impaired sorting.

The G676R mutation is associated with a change of hydrophobicity and electronic charge. It has been speculated that G676R causes two basic amino acids, arginine and lysine, to interact and/or a change in the conformation of NBD1. The local changes in NBD1 attributable to this mutation may cause impaired expression of the MRP2 variant or may decrease the function of the two ABCs and the transport activity of the protein. From the segregation analysis of the two mutations and the −24C → T polymorphism shown in Fig. 4, the segregation of c.1901del67 and −24C → T in DJS3 and his family members demonstrates that these two variations were transferred independently. Thus, these two variants were on different alleles in DJS3, and −24C → T was on the 2026G → C variant allele. This was supported by the absence of −24C → T in siblings without the 2026G → C mutation.

Because the parents and one sibling were deceased, the genetic route and correct segregation of the G676R mutation remain unclear. Further investigation of the siblings of the parents and their family members may indicate whether this mutation is genetically transferred.

In conclusion, a novel disease-associated mutation, 2026G → C, which is predicted to cause a G676R conversion in the MRP2 protein, has been detected in a DJS patient. This mutation is located within the first Walker A motif in the MRP2 protein.

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