Mutations in the canalicular multispecific organic anion transporter (cMOAT) gene, a novel ABC transporter, in patients with hyperbilirubinemia II/Dubin–Johnson syndrome

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Members of the ATP-binding cassette (ABC) transporter superfamily are mutated to cause diseases that include cystic fibrosis, hyperinsulinemia, adrenoleukodystrophy, Stargardt disease and multidrug resistance. We recently isolated a novel human member of ABC transporter superfamily as the candidate transporter for the glucuronide and glutathione-conjugated antitumor agents, and found it highly homologous to the rat cmoat gene. Consistent with recent findings of defects in the homologous cmoat gene in two rat models of hyperbilirubinemia (TR⁻ and Eisai), we report two deletions and a missense mutation in the active transport family signature region in the gene in patients with hyperbilirubinemia II/Dubin–Johnson syndrome (DJS; MIM 237500), respectively. These results strongly implicate the cMOAT gene as responsible for the defects in DJS patients.

INTRODUCTION

Hyperbilirubinemia II/Dubin–Johnson syndrome (DJS; MIM 237500), a hereditary disease transmitted as an autosomal recessive trait, is characterized by conjugated hyperbilirubinemia, an increase in the urinary excretion of co-proporphyrin isomer I, deposition of melanin-like pigment in hepatocytes and prolonged retention of sulfobromophthalein, but otherwise normal liver function (1–4). The genetic basis of DJS is unknown, but early studies suggested defects in excretion rather than in the import or conjugation of bilirubin.

Hepatobiliary excretion of conjugated bilirubin is mediated by an ATP-dependent transport system, the canalicular multispecific organic anion transporter (cMOAT), located in the apical (canalicul) membrane of hepatocytes (5–7). The multidrug resistance-associated protein (MRP) can also transport glutathione conjugates [leucotriene C₄ (LTC₄) and dinitrophenyl glutathione (GS-DNP)] (8,9), which are also putative substrates for transport by cMOAT protein, but the expression of the MRP gene in liver is very low (9). Recently, Paulusma et al. demonstrated that rat cMOAT is a liver-specific homolog of the MRP; they reported that TR⁻ rats, an animal model of DJS, are defective in anion transporter (cMOAT) and found a single nucleotide deletion mutation at nucleotide 1179 in the gene, resulting in reduced mRNA abundance and absence of the protein (10). Ito et al. (11) have reported independently that expression of cmoat is defective in Eisai hyperbilirubinemic rats (EHBR), a second animal model, and found a molecular defect in cmoat in that case as well, a transition mutation (G→A at nucleotide 2564) that creates a premature stop codon.

We recently isolated a novel human member of the ATP-binding cassette (ABC) transporter superfamily as the candidate transporter for the glucuronide and glutathione-conjugated antitumor agents, and found it highly homologous to the rat cmoat gene (12). Recently, >20 genes for the ABC transporter were found in the expressed sequence tag (EST) databases and their genomic map positions determined (13,14). One of these, EST172291 (13), corresponds to the human cMOAT gene (12) by sequence identity and map location. Members of the ABC transporter superfamily (15) are mutated to cause diseases that include cystic fibrosis (16), hyperinsulinemia (17), adrenoleukodystrophy (18), Stargardt disease (19) and multidrug resistance (20).

These results raised the question of whether the human cMOAT gene is responsible for DJS. The mRNA and genomic DNA encoding the cMOAT gene were analyzed in four DJS patients. We detected a deletion caused by a mutation at a splice site in a patient and also a missense mutation and/or a deletion mutation in the active transport family signature region that is characteristic of nucleotide-binding folds of ABC transporters (15) in three

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other patients, strongly suggesting that the mutated cMOAT gene is responsible for the defects in DJS patients.

RESULTS AND DISCUSSION

Using RT-PCR and sequence analysis, we analyzed the entire cMOAT cDNA sequence from four patients that had high concentrations of T (total)- and D (direct)-bilirubin [5.0, 5.2, 1.3 and 1.3 mg/dl for T- and ND (not determined), 3.8, 0.8 and 0.8 mg/ml for D-bilirubin in patients DJ1, DJ7, DJ4 and DJ5, respectively]. Patients DJ4 and DJ5 were brothers, and the others are unrelated. We identified a missense mutation 2302 (C→T) in the active transport family signature (15) present in cDNA of three patients, DJ1, DJ4 and DJ5 (Figs 1 and 2). This mutation was homozgygous in genomic DNA of the severely affected patient DJ1 and heterozygous in patients DJ4 and DJ5 and their father DJ2 (Fig. 2). The second alteration, 2272del168, a deletion of 168 nucleotides from 2272 to 2439 in PCR product G, was also detected in cDNA from peripheral blood leucocyte of DJ4 and DJ5 (Figs 1 and 3a). Sequence, RT-PCR and restriction analyses (loss of the AcI site) of DJ4, DJ5 and their family members showed perfect co-segregation of the mutations with the DJS trait (Figs 2 and 3a). Patients DJ4 and DJ5 are compound heterozygote for both mutations, whereas parents DJ2 and DJ3, who have both been diagnosed as carriers by urinary excretion of co-proporphyrin I (21), are heterozygotes for one of each mutation, respectively (Figs 2 and 3a). Their sister DJ6 does not have both mutations (Figs 2 and 3a). Restriction analysis of RT-PCR product F was also consistent with these results. The product F of an allele corresponding to the deletion mutation 2272del168 is not expected to be detected in DJ3, DJ4 and DJ5, because one of the primers used for amplification of the product F locates in the deleted region described above. As a result, the product with mutation 2302 (C→T) was detected in DJ4 and DJ5, the product with no mutation 2302 (C→T) was detected in DJ3 and DJ6, and the product of both types was detected in DJ2 (Fig. 3b). The base substitution 2302 (C→T) and the deletion 2272del168 were not detected in any of 50 unrelated controls. RT-PCR analysis of peripheral blood leukocytes also revealed a shorter than expected band with primer pair H in subjects DJ4 and DJ5. However, this deletion, 2748del136, was also detected in some control samples, and proved on sequencing to represent an apparent product of alternative splicing, with one exon skipped. Genomic sequencing of DJ4 and DJ5 revealed no alteration of the apparent product of alternative splicing, with one exon skipped.

A transition two bases after the 3′ boundary of the corresponding exon (the splice donor site) was identified in genomic DNA isolated from patient DJ7 (Fig. 4b). No deletion was detected in 50 unrelated controls, and no other change was observed in the cMOAT of patient DJ7 (data not shown).

In contrast to the two animal models for DJS, we observed similar levels of cMOAT mRNA in the liver of patient DJ7 (the only one for whom liver tissue was available) compared with normal liver. The deletion mutation in patient DJ7, and presumably the missense mutation as well, thus most likely affects protein formation or function rather than an absence of transcription. This interpretation of the mutation analysis is supported further by the demonstration of the absence of the cancular isoform of the MRP in DJS (22) and by the segregation profile of the mutation (Figs 2 and 3a), which is consistent with the autosomal recessive inheritance of the syndrome (2,4) and the chromosomal location, 10q21, of the human cMOAT gene (12). During the preparation of this report, Paulusma et al. have reported a nonsense mutation at codon 1066 in another DJS patient (23). Although this report was not accompanied by genomic analysis data, it further supports the conclusion reached here.

The Walker A, B motif and the active transport family signature, also called the C motif at the nucleotide-binding site, are highly conserved among members of the ABC transporter superfamily, and are important for both ATP binding and hydrolysis. Missense mutation of the active transport family signature, as we found in patients DJ1, DJ4 and DJ5, is also reported in other ABC transporter disorders, including cystic fibrosis and Stargardt disease (19,24). As in DJS, the loss of the transporter function is severe but not lethal to the affected cells. In DJS, this combination of features permits the survival of liver
MATERIALS AND METHODS

Subjects

Four patients were analyzed in this study. Patients DJ4 and DJ5 are brothers and were diagnosed as having DJS by their serum bilirubin concentration (1.3 and 1.3 mg/dl for T-bilirubin and 0.8 and 0.8 mg/ml for D-bilirubin, respectively) and co-proporphyrin retention (94.5 and 93.6% for urinary co-proporphyrin I, respectively) (21). Other laboratory findings in DJ4 and DJ5 were retention (0.8 mg/ml for D-bilirubin, respectively) and co-proporphyrin bilirubin concentration (1.3 and 1.3 mg/dl for T-bilirubin and 0.8 mg/ml for D-bilirubin, respectively) and co-proporphyrin bilirubin concentration (1.3 and 1.3 mg/dl for T-bilirubin and 0.8 mg/ml for D-bilirubin, respectively). The mother, DJ3, and sister, DJ6, of DJ4 and DJ5 do not have this mutation. Sequencing profiles of the sense strand of only DJ3 (wild-type), DJ4 (heterozygous mutant) and DJ1 (homozygous mutant) are presented. (b) Restriction digestion analysis of the genomic region around the mutation 2302(C→T) in DJ1, DJ4, DJ5 and their family members. The mutation 2302(C→T) destroys a naturally occurring AciI restriction site. PCR-amplified genomic DNA (170 bp) was digested with AciI and the products were electrophoresed on a 3% agarose gel. Wild-type products are digested into 88 and 82 bp (DJ2, DJ3, DJ4, DJ5, DJ6 and control). The 2302(C→T) mutant products are not cleaved (DJ1, DJ2 DJ4 and DJ5). This shows that DJ1 is homozygous and DJ2, DJ4 and DJ5 are heterozygous for the mutation. The 100 bp ladder marker was run in lane M.

cells that have developed the other phenotype associated with cMOAT loss, failure to transport certain conjugated drugs.
The sequences of the 12 pairs of primer used for RT-PCR sequencing analysis were as follows, with the nucleotide numbers in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′ in parentheses: A(–28 to 513), 5′ CAGTC3′.

DJS patients was isolated using the ISOGEN (Nippongene Co., Tokyo, Japan). First-strand synthesis from total RNA was performed using random hexanucleotide primers and MMLV reverse transcriptase (Gibco BRL, Gaithersburg, MD). The single-stranded cDNA was PCR amplified with 2 pmol of the forward and reverse primers described above using AmpliTaq-Gold™ DNA polymerase (Perkin Elmer, Tokyo, Japan). For PCR, 40 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s) and extension (72°C for 45 s) were performed. The PCR products were sequenced directly or after subcloning into pMOSBlue vector (Amersham, Buckinghamshire, UK).

**Sequencing and identification of mutations**

In order to avoid PCR artifacts, we sequenced at least 10 subclones for each sample or sequenced PCR products directly, using a DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan) and a DNA sequencing system (model 373S, Applied Biosystems). Both sense and antisense strands were sequenced for confirmation.

**Amplification of genomic DNA**

Genomic DNAs were prepared from either peripheral blood leukocyte cells or liver tissue (DJ7) following standard methods. The nucleotide sequences of the primers used to amplify the genomic fragments containing each mutation were as follows (the region to which these primers correspond are described parentheses: gF5′, 5′TTAGGAGTGGGCAAGTACC3′ (intron at the beginning of the cDNA G fragment, see above); gF3′, 5′CATGAGCCTCCAGTGCAC3′ (F and G fragments of cDNA); gE5′, 5′AAGGAGTGCCATTAGGGCC3′ (intron at the beginning of the cDNA E fragment); gE3′, 5′AGTCTAGTCGAACTCCAG3′ (intron at the end of the cDNA E fragment).

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