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A Mutation in the Human Canalicular Multispecific Organic Anion Transporter Gene Causes the Dubin-Johnson Syndrome

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The human Dubin-Johnson syndrome (DJS) is a rare autosomal recessive liver disorder characterized by chronic conjugated hyperbilirubinemia. Patients have impaired hepatobiliary transport of non-bile salt organic anions. A highly similar phenotype has been described for a mutant Wistar rat strain, the transport-deficient (TR-0) rat. First, there is chronic conjugated hyperbilirubinemia. Second, the hepatic clearance of intravenously injected bromosulphthalein is delayed, and the gluthathione conjugate, which is normally excreted into bile via the canalicular multispecific organic anion transporter (cMOAT), regurgitates into the plasma. Third, there is an increased urinary excretion of coproporphyrin I, a metabolic byproduct of heme synthesis, which also is a substrate for cMOAT. Finally, liver biopsies from patients with the DJS display a characteristic lysosomal accumulation of black pigment with otherwise normal histology. This pigment is also observed when TR-0 rats are fed a diet supplemented with aromatic amino acids. Studies in the TR-0 rat have greatly contributed to the biochemical characterization of the transport system involved in this defect. These mutant animals lack the hepatobiliary excretion of many organic anions including bilirubin-glucuronide, cysteinyl-leukotrienes, and some divalent bile salt conjugates (among various other glutathione and glucuronide conjugates) (reviewed by Oude Elferink et al.). The transport of these compounds is impaired in DJS as well. The characterization of the human multidrug resistance–associated protein (MRP1) as an organic anion pump with a highly similar substrate specificity as the putative cMOAT has recently led to the cloning of the rat cmoat complementary DNA (cDNA), a liver-specific homologue of MRP1. The identification of a single-nucleotide deletion in this gene in TR-0 rats has unambiguously demonstrated its role in canalicular organic anion transport. Indeed, transfection studies revealed an increased organic anion efflux from cells expressing cMOAT (Paulusma CC, unpublished data, July 1996). In view of the highly similar phenotypes of TR-0 rats and DJS patients, we postulated that a mutation in the human cMOAT gene underlies the transport defect in the DJS.

MATERIALS AND METHODS

Cloning of Human cMOAT cDNA. A human liver 5′-stretch-plus cDNA library (Clontech, Palo Alto, CA) was screened, using a 5-kb fragment of rat cmoat as a probe, as described previously. Three overlapping cDNAs were isolated. The sequence of the 5′ end, encoding the first 11 amino acids of cMOAT, was determined from a human cDNA clone (no. 124379, Soares fetal liver spleen library 1NFLS) obtained from the I.M.A.G.E. consortium. cMOAT was sequenced using the ABI377 automatic sequencer (accession number GenBank U49248).

Patient, Tissues, and Fibroblast Culture. One caucasian, female patient was studied. Liver from this patient was obtained by a needle biopsy. Normal control liver was obtained from surgical pathology specimens. Biopsies were fixed for histology in 4% formaldehyde and embedded in paraffin. Skin fibroblasts from the patient and normal control were obtained by skin biopsy and cultured in Ham F-10 (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin, at 37°C.

Immunohistochemistry. Formaldehyde-fixed, paraffin-embedded liver sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 0.3% (vol/vol) H2O2 in methanol for 30 minutes. Before staining, the sections were pretreated with 0.01 mol/L citric acid (pH 6.0) for 3 × 5 minutes at 100°C. The sections were blocked with normal rabbit serum for 10 minutes and incubated with monoclonal antibody M3,III-6 for 1 hour. This antibody was produced against a bacterial fusion protein containing the 202-amino acid COOH-terminal end of rat cmoat, and characterized as described previously. Immuneactivity was visualized with biotinylated rabbit anti-mouse Fab2 (Dako Copenhagen, Denmark), followed by streptavidin-conjugated horseradish peroxidase (Dako) in phosphate-buffered saline/1% bovine serum albumin, and subsequent staining with 3,3′-diaminobenzidine tetrahydrochloride and 0.02% (vol/vol) H2O2 in phosphate-buffered saline. P-glycoproteins were detected with monoclonal antibody JSB-1. All sections were counterstained with hematoxylin and mounted.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from fibroblasts according to the acid-phenol single-step method. cDNA synthesis was performed with 6 μg of total RNA and random hexamer primers with Moloney murine leukemia virus reverse transcriptase (Life Technologies), at 37°C for 1 hour, followed by 10 minutes at 85°C to inactivate the Moloney murine leukemia virus reverse transcriptase.

Abbreviations: DJS, Dubin-Johnson syndrome; TR-0, transport deficient; cMOAT, canalicular multispecific organic anion transporter; MRP1, multidrug resistance–associated protein; cDNA, complementary DNA; PCR, polymerase chain reaction.

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**RESULTS**

The human cMOAT cDNA was obtained after screening a human liver cDNA library using a 5-kb fragment of rat *cmoat* as a probe. Three overlapping cDNAs were isolated, which lacked the 5' 30 coding nucleotides, when compared with the rat *cmoat* sequence. The missing 5' end was present in a cDNA clone (#124379), which we obtained from the I.M.A.G.E. consortium. The full-length cDNA encoding cMOAT contains a single open reading frame of 1,545 amino acids (Fig. 1), with a predicted molecular weight of 174 kD, which shares 77.7% and 88.7% sequence identity and similarity, respectively, with the rat cmoat protein. The cMOAT cDNA described in this study is identical to the *cMRP/cMOAT* cDNA recently reported by others. The tissue distribution of cMOAT in humans and rats is highly similar, with high expression in liver, and low expression in kidney and duodenum (Kool M, et al., submitted).

We have studied a patient (age 54) who was diagnosed for DJS at the age of 20. She frequently complained of pains in the upper abdomen. General liver function was normal except for chronic elevated conjugated (38 to 70 μmol/L) and unconjugated (12 to 25 μmol/L) serum bilirubin levels. It was not possible to visualize the gallbladder after administration of oral contrast reagent, a characteristic feature of DJS. The patient showed a delayed plasma clearance of intravenously injected bromosulphophthalein, followed by a secondary rise in plasma bromosulphophthalein levels. At the age of 32, the patient underwent cholecystectomy. A characteristic black liver was observed, and microscopic analysis of a liver section revealed mild fibrosis and the pigment accumulation indicative of DJS (Fig. 2B).

Paraffin-embedded liver sections of DJS and control liver were examined for the presence and localization of the cMOAT protein, using monoclonal antibody M4III-6. In human control liver (Fig. 2A), the antibody stained the canalicular membrane of the hepatocyte. In DJS liver (Fig. 2B), no canalicular staining was observed, indicating that this patient lacked the cMOAT protein. The same results were obtained in liver slices of Wistar and TR rats (not shown).
a control, a positive canalicular staining was observed in both DJS and control liver with JSB-1, 20 an antibody against P-glycoprotein (not shown).

To investigate the nature of the genetic defect, total RNA was isolated from cultured fibroblasts obtained from a skin biopsy of both the patient and a normal control. cDNA was prepared and the total cMOAT cDNA was amplified by the “touch down” PCR protocol. 22 Sequence analysis of multiple independent clones revealed a mutation in the patient at codon 1066 (CGA to TGA; arginine to stop-codon) (Fig. 3), which leads to premature termination of cMOAT protein synthesis, the normal protein being 1,545 amino acids long (see also Fig. 1). The mutation results in the loss of a TaqI restriction site, and we have confirmed the absence of this site in the patient by TaqI digestion of a cMOAT PCR product encompassing the site of the mutation (Fig. 4). Digestion of genomic DNA from patient and control with TaqI, and subsequent Southern blot analysis, showed a different hybridization pattern in patient and control, indicating that the patient is homozygous for the mutation in codon 1066 (not shown).

**DISCUSSION**

This article describes the identification of the genetic defect that underlies the phenotype observed in patients with DJS, and that corresponds to the genetic defect identified in the animal model for DJS, the TR0 rat. 18 Kartenbeck et al. 26 previously described the absence of the canalicular immunostaining in a liver section of a DJS patient, using an antibody directed to MRP1. This antibody gave both lateral and canalicular staining in control liver, but only lateral staining in DJS liver. Because MRP1 is only present in the basolateral membrane of epithelial cells, 27 we conclude that the antibody crossreacts with the apically localized, and homologous, cMOAT protein, a conclusion supported by more recent work of this group. 19 The antibody used in our work does not crossreact with MRP1 and only stains the canaliculus. Indeed, staining with this antibody was completely negative in the DJS patient.

The mutated cMOAT gene in the patient under study results in a truncated protein. Our antibody is raised against the C-terminal 202 amino acids (of rat cmoa1), which lie behind the mutation in this patient. We do not know, therefore, whether the truncated protein is present in the canaliculus, whether it is mistargeted, or broken down. Because the mutation leads to the absence of four membrane-spanning domains and the complete second adenosine triphosphate–binding cassette, it can be assumed that the protein is not functional. In relation to this, it was recently shown that expression of the NH2- or COOH-proximal halves of the MRP1 protein separately (each containing one adenosine triphosphate–binding cassette) did not result in adenosine triphosphate–dependent leukotriene C4 transport, while coexpression of both half-molecules restored this transport, 28 a phenomenon that also might apply to related adenosine triphosphate–binding cassette transporter proteins, including cMOAT.

An association has been observed between DJS and Factor-VII deficiency in a patient group from Jewish communities in Iran and Iraq. 29 In these communities, a high incidence of several genetic disorders, including DJS, is caused by a high degree of consanguinity. 30 The genes encoding human Factor-VII and cMOAT have been localized to chromosomes 13q34 and 10q23-q24, 25,32 respectively. Thus, a primary linkage between DJS and clotting Factor-VII deficiency can be ruled out. Our demonstration of a low but detectable expression of the cMOAT gene in fibroblasts allows a simple identification of this inherited disorder, without the need for liver biopsy.
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