A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome


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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⁺) rat. First, there is chronic conjugated hyperbilirubinemia. Second, the hepatic clearance of intravenously injected bromosulfophthalein 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⁺ rats are fed a diet supplemented with aromatic amino acids. Studies in the TR⁺ 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⁺ rats has unambiguously demonstrated its role in the 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 Indeed, transfection studies revealed an increased organic anion efflux from cells expressing cMOAT (Paulusma CC, unpublished data, July 1996).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⁺ 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, paraformaldehyde-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 Fab (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 65°C to inactivate the Moloney murine leukemia virus reverse transcriptase.

Abbreviations: DJS, Dubin-Johnson syndrome; TR⁺, 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 DJ5 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 M46-1-6. 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). As
a control, a positive canalicular staining was observed in both
DJS and control liver with JSB-1, 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. 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 syn-
thesis, the normal protein being 1,545 amino acids long (see
also Fig. 1). The mutation results in the loss of a TaqI restric-
tion site, and we have confirmed the absence of this site in
the patient by TaqI digestion of a cMOAT PCR product en-
compassing the site of the mutation (Fig. 4). Digestion of
genomic DNA from patient and control with TaqI, and subse-
quently Southern blot analysis, showed a different hybridiza-
tion pattern in patient and control, indicating that the pa-
tient 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 TR⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓stead 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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REFERENCES


