Rapid Communication

Defect of Multidrug-Resistance 3 Gene Expression in a Subtype of Progressive Familial Intrahepatic Cholestasis

JEAN-FRANÇOIS DELEUZE,[1] EMMANUEL JACQUEMIN,[1, 2] CLAIRE DUBUISSON,[1, 2] DANIÈLE CRESTEIL,[1] MICHELINE DUMONT,[3] SERGE ERLINGER,[3] OLIVIER BERNARD,[1, 2] AND MICHELLE HADCHOUEL[1, 2]

Disruption of the murine *mdr2* (multidrug-resistance) gene, which encodes a phosphatidylcholine flippase, leads to a hepatic disorder because of loss of biliary phospholipid secretion. Among the hereditary human cholestasis, a subtype of progressive familial intrahepatic cholestasis with high γ-glutamyltranspeptidase (GGT) serum activity shares histological, biochemical, and genetic features with mice lacking *mdr2* gene expression (*mdr2* −/− mice). No *MDR3* (human *mdr2* homolog) messenger RNA (mRNA) was detected by Northern blotting in the liver of a patient suffering from this form of PFIC, and the biliary phospholipid level in a second patient was substantially decreased. Thus, the absence of the *MDR3* P-glycoprotein may be responsible for this type of PFIC, which, as in the murine model, may be due to a toxic effect of bile acids on the biliary epithelium in absence of biliary phospholipids. (HEPATOLOGY 1996; 23:904-908.)

The mouse *mdr2* gene (standard gene symbol *Pgy-3*) and its human homolog *MDR3* (also called *MDR2,* standard gene symbol *PGY3*) are members of the multidrug gene family[1-7] that do not confer multidrug resistance[8-11] but encode P-glycoproteins, which act as phosphatidylcholine flippases.[12-17] These P-glycoproteins are, in liver, exclusively found in the canalicular membrane of hepatocytes.[18-21] Disruption of the *mdr2* gene in mice has led to evidence for a physiological role of the *mdr2* P-glycoprotein in biliary excretion.[13] In homozygous *mdr2* −/− mice, phospholipids are absent from bile and the animals suffer from liver disease.[13,22] This disease, characterized by a portal inflammation and a ductular proliferation, is probably the consequence of a toxic effect of bile acids on the biliary epithelium in the absence of biliary phospholipids.[13,22] Because human bile acids are more hydrophobic than those of rodents, it is expected that the absence of *MDR3* P-glycoprotein (the human homolog of *mdr2* P-glycoprotein) would result in severe liver disease manifest in childhood.

Progressive familial intrahepatic cholestasis (PFIC), which is a recessive condition of childhood that results in death of liver failure before adolescence, may correspond to such a liver disease.[23-26] Several studies have recently provided support for the heterogeneity of this clinical entity, suggesting the existence of different causes: clinical, biochemical, and histological features suggest at least three subcategories.[27-30] However, no associated genetic defect has been identified. It has been suggested that one subtype of PFIC may be attributable to a defect in primary bile acid secretion,[29] and it has been established that children affected by another subtype have a defect in primary bile acid synthesis.[30] In both of these subtypes, cholestasis is associated with normal serum γ-glutamyltranspeptidase (GGT) activity, and there is no ductular proliferation as assessed by liver histology.[29-30] By contrast, the pathological mechanism is unknown for the third subtype characterized by high serum GGT activity and ductular proliferation and inflammatory infiltrate in portal areas with patency of intrahepatic and extrahepatic bile ducts. This pattern of nonsuppurative cholangitis is very similar to the hepatic injury observed in *mdr2* −/− mice. Therefore, it can be speculated that an abnormality in the *MDR3* P-glycoprotein gene expression may be the cause of this type of PFIC. To investigate this possibility, we tested for the presence of messenger RNA (mRNA) encoding the *MDR3* P-glycoprotein in the liver of a patient, and we analyzed the bile phospholipid content of another patient, both with high serum GGT activity PFIC.

PATIENTS AND METHODS

Patient 1. The first patient was a North African girl, the third child of first-cousin parents. The two older sisters are

Abbreviations: PFIC, progressive familial intrahepatic cholestasis; GGT, γ-glutamyltranspeptidase; mRNA, messenger RNA; cDNA, complementary DNA.

From the [1]Unité INSERM U 347, le Kremlin Bicêtre, France; [2]Service d'hépatologie, département de pédiatrie, Hôpital de Bicêtre, le Kremlin Bicêtre, France; and [3]Unité INSERM U24, Hôpital Beaujon, Clichy, France.

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Address reprint requests to Michelle Hadchouel, MD, INSERM U347, 80 rue du général Leclerc, 94276, le Kremlin-Bicêtre Cedex, France.

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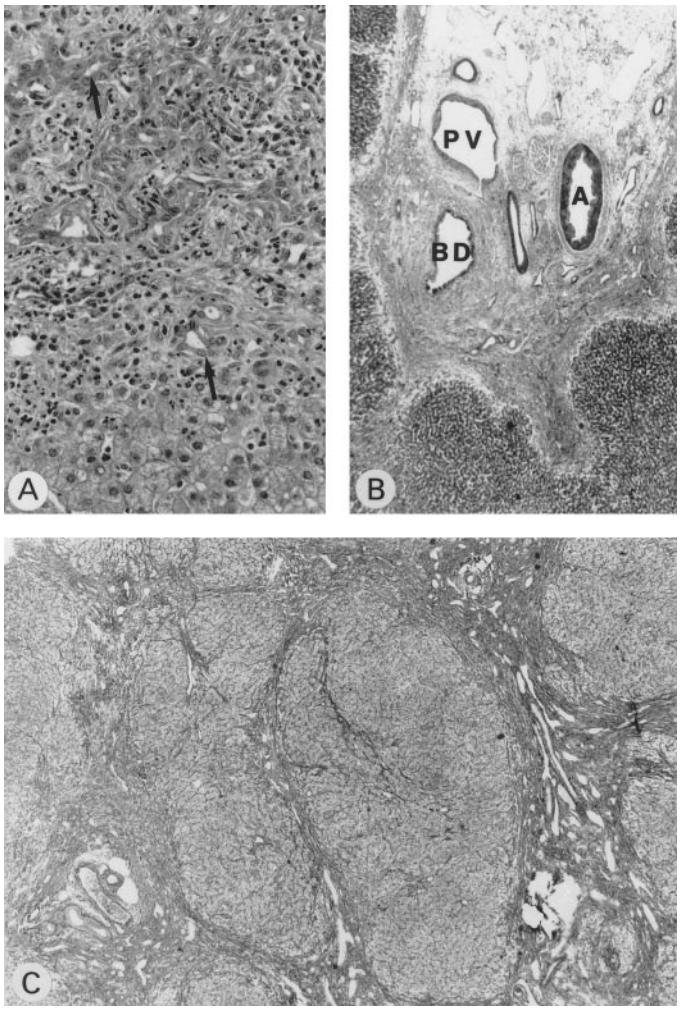

FIG. 1. Histology of the liver from patient 1 suffering from progressive familial intrahepatic cholestasis with high serum GGT activity. (A) First biopsy at 2 years; there is slight portal fibrosis with ductular proliferation (arrow) and mixed inflammatory infiltrate. (Original magnification ×200.) (B) Resected liver, a large portal area with portal vein (PV), hepatic artery (A), normal bile duct (BD). (Original magnification ×25.) (C) Resected liver, extensive portal fibrosis. (Original magnification ×25.)

unaffected, but two cousins died at 7 and 8 years of age of similar intrahepatic cholestasis with patent extrahepatic bile ducts. At 1 year of age, she presented with pruritus and pale stools without jaundice. At 2 years, she was admitted to another hospital; firm hepatomegaly and splenomegaly were present, and there were signs of biochemical cholestasis with high alkaline phosphatase and GGT activities but normal serum bilirubin concentration and prothrombin time. Liver histology showed slight portal fibrosis and ductular proliferation with mixed inflammatory infiltrate (Fig. 1A).

When 5 years old, she was admitted to Bicêtre Hospital for gastrointestinal bleeding; she presented with jaundice, pruritus, and hepatosplenomegaly. Weight and height were −1 and −3 standard deviations below normal, respectively. Liver function tests showed high serum alanine transaminase activity (8 × N), elevated conjugated bilirubin concentration (210 μmol/L), high serum GGT (17 × N) and alkaline phosphatase (7 × N) activities. Liver transplantation was performed at age 6, and at 10 years she is in good condition with normal liver function tests, receiving alternate-day prednisone therapy and cyclosporin. Patency of extrahepatic biliary tree was confirmed during the transplantation procedure. Histological examination of the resected liver showed patency of main intrahepatic ducts. There was extensive portal fibrosis with ductular proliferation, interlobular bile ducts were present in most of the portal tracts, and there was neither periductal fibrosis nor biliary epithelium injury (Fig. 1B and 1C). Cholestasis was present both in the lobule and in some ductules containing bile plugs.

Patient 2. The second patient was an Italian girl, born to unrelated parents. At 1 year of age, she presented with hepatosplenomegaly. At age 5, she was admitted to Bicêtre Hospital; weight and height were normal for age; she complained of pruritus; there was no jaundice. Firm hepatomegaly and major splenomegaly were observed, and there were signs of biochemical cholestasis with high alkaline phosphatase activity but normal serum bilirubin concentration. Liver histology showed portal fibrosis with ductular proliferation and lobular cholestasis. Percutaneous cholecystography showed patency of extrahepatic bile ducts. When 10 years old, she presented with gastrointestinal bleeding, greatly enlarged liver and spleen, biochemical cholestasis with high GGT activity (12 × N) with normal serum bilirubin concentration and normal prothrombin time. A surgical mesocaval shunt was performed and proved to be patent 6 months later. Jaundice occurred 1 year later and increased over the following 2 years. Orthotopic liver transplantation was performed at age 13. Liver function tests at the time of transplantation showed elevated serum bilirubin concentration 205 μmol/L, slightly elevated serum alanine transaminase activity (2.5 × N), and high serum GGT activity (13 × N), and prolonged prothrombin time (60%) (N >70%). At 20 years she is in good condition with normal liver function tests, receiving alternate-day prednisone therapy and cyclosporin. Histological examination of the resected liver showed extensive portal fibrosis with ductular proliferation, interlobular bile ducts were present, and there was no biliary epithelial injury. Cholestasis was present both in the lobule and in some ductules containing bile plugs.

Liver Samples. Expression of the *MDR3* gene was studied in six liver samples obtained at time of transplantation. One was from patient 1. The others were used as controls and were obtained from patients with other cholestatic diseases: two from patients affected with PFIC with normal GGT serum activity, one from a patient suffering from Alagille syndrome, and one from a patient with extrahepatic biliary atresia. One normal liver sample was obtained from a healthy donor after reduction of the size of the graft.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from the frozen liver samples using the acid guanidium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi.[31] Poly A^+ RNA was isolated by oligodT column purification.[32] The poly A^+ RNA (7 μg) was separated by electrophoresis through a 1% agarose gel containing 2.2 mol/L formaldehyde. The RNA was transferred to a nylon membrane (Hybond N; Amersham International, Oakville, Canada) by capillary action and hybridized with complementary DNA (cDNA) probes radiolabeled by random primer extension or nick translation with [α-^{32}P]dATP. The membranes were washed for 5 minutes at room temperature in 2× sodium saline citrate/0.1% sodium dodecyl sulfate then for 30 minutes at 42°C in 2× sodium saline citrate/0,1% sodium dodecyl sulfate and finally 30 minutes at 65°C in 1× sodium saline citrate/0.1% sodium dodecyl sulfate. The blots were

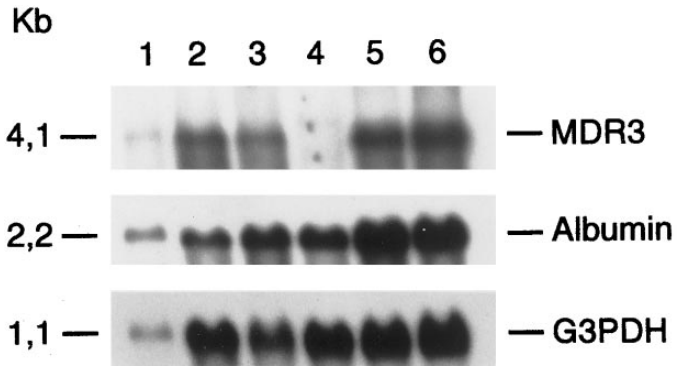

FIG. 2. Northern blot analysis of human liver mRNA. Poly A^+ RNA from: healthy donor (lane 1), 2 patients with PFIC with normal serum GGT activity (lanes 2 and 3), 1 patient with PFIC with high serum GGT activity (lane 4), 1 patient with Alagille syndrome (lane 5), 1 patient with extrahepatic biliary atresia (lane 6); all were hybridized with a ^{32}P-labeled *MDR3* cDNA. As a control for RNA loading, blots were rehybridized with ^{32}P-labeled human G3PDH cDNA and mouse albumin cDNA as probes.

exposed for 2 to 10 days at −70°C, with intensifying screens. As a control for RNA loading, the blots were successively probed with cDNAs corresponding to human glyceraldehyde-3-phosphodehydrogenase (G3PDH) and mouse albumin that cross-hybridized with the human albumin.

DNA Probes. The human *MDR3* probe was a 4-Kb full-length cDNA cloned into plasmid PJ3omega obtained from the American Tissue Culture Collection (Ref. 65706, Rockville, MD). Human G3PDH probe was a 1.1-Kb cDNA obtained from Clontech laboratories (Palo Alto, CA). The albumin probe was a 450-bp mouse albumin cDNA sequence excised from plasmid pmalb2.[33]

Analysis of Biliary Lipids. Bile samples from patient 2 and from three control children with sclerosing cholangitis were obtained after percutaneous transhepatic cholecystography and were kept frozen at −20°C. Total bile acid concentration was measured by an enzymatic technique using the modified 3α-hydroxysteroid dehydrogenase method.[34] Cholesterol concentration was determined by an enzymatic and colorimetric method (kit from Boehringer Mannheim, Germany). Phospholipids were assayed by an enzymatic method using phospholipase D and choline oxidase[35] (kit Biotrol, Chennevieres les Louvres, France). This method gives a measure of phosphatidylcholine, which is the major phospholipid in bile.[36]

RESULTS AND DISCUSSION

No *MDR3* mRNA was detected by Northern blot analysis in the liver of patient 1 with PFIC and high serum GGT activity (lane 4), whereas *MDR3* mRNA was found in all of the other livers tested (lanes 1, 2, 3, 5, 6, Fig. 2). The absence of hybridization was not due to a smaller amount of loaded RNA, as shown by reprobing the same membrane successively with a glyceraldehyde-3-phosphodehydrogenase (G3PDH) and an albumin probe (Fig. 2). Thus, in this patient, the association of high GGT serum activity PFIC with the absence of the *MDR3* mRNA mimics the situation encountered in the *mdr2*−/− mice.

So, the homozygous disruption of the *mdr2* gene leading to a well-defined mouse hepatic disease[13] led to the identification of a corresponding human disease: a subtype of PFIC characterized by high serum GGT activity.[28,30] There are substantial similarities between the mouse and human diseases: both are intrahepatic cholestasis of early onset characterized by ductular proliferation without extrahepatic obstruction;[13,22,28,30] they share a common genetic basis, because PFIC is a recessive disease and only homozygous *mdr2* −/− mice are affected.[13,25] In *mdr2* −/− mice, the loss of biliary phospholipid secretion is the primary consequence of the *mdr2* gene disruption, showing that the *mdr2* P-glycoprotein is required for the secretion of phospholipids into bile.[13] It acts by promoting the flip of the phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane.[15-17] The loss of phospholipid secretion appears to be the main cause of the liver anomalies observed.[13] Indeed, phosphatidylcholine emulsifies bile acids secreted by the hepatocytes to protect the biliary epithelium from their detergent action.[37-40] Thus, phospholipid-free bile would be expected to damage the bile canaliculi (bile salts micelles will tend to elute phospholipid from membranes), resulting in liver injury. It would be of interest to analyze the phospholipid content in the bile of the patient lacking liver *MDR3* mRNA expression to confirm the Northern blot result, but unfortunately, no bile sample from this patient is available. However, we have studied the bile of a second patient with high serum GGT activity PFIC (patient 2) for whom liver RNA isolation was not possible. The phospholipid concentration was 2.2 mmol/L, which was only 7.2% of biliary lipids, whereas bile acid and cholesterol concentrations were 26.8 mmol/L and 1.5 mmol/L, respectively, corresponding to 87.9% and 4.9% of biliary lipids. In contrast, in control cholestatic children, the mean phospholipid concentration was 36.6 mmol/L (19.8% of biliary lipids), and mean bile acid and cholesterol concentrations were 143.4 mmol/L (77.7%) and 4.6 mmol/L (2.5%). These percentage values for control patients are similar to those previously reported in normal human gallbladder bile.[41] Thus, in patient 2, the biliary phospholipid level is dramatically decreased despite the presence of bile acids, suggesting that, as in the *mdr2*−/− mice, high GGT serum activity PFIC was caused by the toxic effect of bile acids on biliary epithelium in the absence or presence of very low concentration of phospholipids.

These observations are consistent with PFIC with high serum GGT activity being the human equivalent of mouse *mdr2* −/− liver injury. However, we cannot conclude that there is a mutation in the *MDR3* gene. The molecular defect may affect transcription or *MDR3* mRNA stability or a gene regulating its expression. Additional molecular studies are required to investigate these alternatives. Several reports on the human *MDR* gene family locus provide good background data for such studies.[42-47] The *MDR3* gene has been mapped to 7q21, and this may allow genetic linkage studies.[42,43] Such a linkage analysis for the first class of PFIC has been recently reported and the locus mapped to chromosome 18.[48] Despite the availability of biological tools,

further analysis is hampered by the small number of patients affected with this disease. The involvement of *MDR3* gene in patients with high-GGT-activity PFIC can be confirmed, at the RNA and phospholipid levels, by the study of liver and biliary samples in a prospective manner as part of our transplantation program. At the DNA level, screening for mutations and linkage genetic studies could include previous transplantation patients for whom liver and bile samples are no longer available.

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