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## WHAT THE AMISH CAN TELL US ABOUT. . . CHOLESTASIS

*Bull LN, van Eijk MJT, Pawlikowska L, DeYoung JA, Juijn JA, Liao M, Klomp LWJ, Lomri N, Berger R, Scharschmidt BF, Knisely AS, Houwen RHJ, Freimer NB.* A gene encoding a P-type ATPase is mutated in two forms of hereditary cholestasis. *Nat Genet* 1998;18:219-224. (Reprinted with permission.)

## ABSTRACT

Cholestasis, or impaired bile flow, is an important but poorly understood manifestation of liver disease. Two clinically distinct forms of inherited cholestasis, benign recurrent intrahepatic cholestasis (BRIC) and progressive familial intrahepatic cholestasis type 1 (PFIC1), were previously mapped to 18q21. Haplotype analysis narrowed the candidate region for both diseases to the same interval of less than 1 cM, in which we identified a gene mutated in BRIC and PFIC1 patients. This gene (called FIC1) is the first identified human member of a recently described subfamily of P-type ATPases; ATP-dependent aminophospholipid transport is the previously described function of members of this subfamily. FIC1 is expressed in several epithelial tissues and, surprisingly, more strongly in small intestine than in liver. Its protein product is likely to play an essential role in enterohepatic circulation of bile acids; further characterization of FIC1 will facilitate understanding of normal bile formation and cholestasis.

*de Vree JML, Jacquemin E, Sturm E, Cresteil D, Bosma PJ, Aten J, Deleuze J-F, Desrochers M, Burdelski M, Bernard O, Oude Elferink RPI, Hadchouel M.* Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis. *Proc Natl Acad Sci U S A* 1998;95:282-287. (Reprinted with permission.)

## ABSTRACT

Class III multidrug resistance (MDR) P-glycoproteins (P-gp), *mdr2* in mice and MDR3 in man, mediate the translocation of phosphatidylcholine across the canalicular membrane of the hepatocyte. Mice with a disrupted *mdr2* gene completely lack biliary phospholipid excretion and develop progressive liver disease, characterized histologically by portal inflammation, proliferation of the bile duct epithelium, and fibrosis. This disease phenotype is very similar to a subtype of progressive familial intrahepatic cholestasis, hallmarked by a high serum  $\gamma$ -glutamyltransferase ( $\gamma$ -GT) activity. We report immunohistochemistry for MDR3 P-gp, reverse transcription-coupled PCR sequence analysis, and genomic DNA analysis of MDR3 from two progressive familial intrahepatic cholestasis patients with high serum  $\gamma$ -GT. Canalicular staining for MDR3 P-gp was negative in liver tissue of both patients. Reverse transcrip-

tion-coupled PCR sequencing of the first patient's sequence demonstrated a homozygous 7-bp deletion, starting at codon 132, which results in a frameshift and introduces a stop codon 29 codons downstream. The second patient is homozygous for a nonsense mutation in codon 957 (C - T) that introduces a stop codon (TGA). Our results demonstrate that mutations in the human MDR3 gene lead to progressive familial intrahepatic cholestasis with high serum  $\gamma$ -GT. The histopathological picture in these patients is very similar to that in the corresponding *mdr2* (-/-) mouse, in which *mdr2* P-gp deficiency induces complete absence of phospholipid in bile.

## COMMENTS

Progressive familial intrahepatic cholestasis (PFIC) is the name given to a group of autosomal recessive disorders in which cholestasis develops in early infancy and progresses to end-stage liver disease before adulthood.<sup>1,2</sup> Patients with PFIC type 1 (PFIC1), or Byler disease, named for the extended Amish kindred in which it was first described,<sup>3</sup> exhibit disproportionately low serum  $\gamma$ -glutamyl transpeptidase (GGTP) and cholesterol levels for the degree of cholestasis, along with a bland intracanalicular cholestasis by light microscopy and coarsely granular bile on transmission electron microscopy.<sup>4</sup> By using material from the original Byler kindred, the gene responsible for PFIC1 was mapped to chromosome 18q21-22.<sup>5</sup> This same region had been linked earlier to benign recurrent intrahepatic cholestasis (BRIC), a disease characterized by episodic attacks of jaundice and pruritus but without progressive liver disease<sup>6</sup>; this suggested that the two phenotypes are allelic variants of the same disease. In contrast, there is a second group of patients with PFIC who exhibit high serum GGTP levels and bile duct proliferation along with portal inflammatory infiltrates on liver histology.<sup>7</sup> These changes resemble those that have been observed following disruption of the *mdr2* gene, the mouse homolog of human MDR3, which encodes a canalicular P-glycoprotein required for biliary phospholipid secretion.<sup>8</sup> In one patient with this second type of PFIC, MDR3 messenger RNA was undetectable and in another phospholipid in bile was markedly reduced.<sup>9</sup> The biochemical features of PFIC had been previously linked to defects in either bile salt synthesis<sup>10</sup> or bile salt secretion.<sup>11</sup> Total biliary bile acids were markedly reduced ( $1.1 \pm 1.4$  mmol/L) in seven patients with PFIC compared with values in eight control patients with other cholestatic diseases ( $88.9 \pm 83.2$  mmol/L).<sup>11</sup> Strikingly low percentages of chenodeoxycholic acid were observed in gallbladder bile from the PFIC patients.<sup>11</sup>

In this context, two independent groups have now defined the molecular defects underlying various forms of PFIC. The gene mutated in PFIC1 and BRIC patients, termed FIC1 (for

familial intrahepatic cholestasis 1), exhibits significant similarity to a subfamily of P-type ATPase genes that encode putative aminophospholipid transporters.<sup>12</sup> Five mutations in FIC1 were identified in patients with PFIC1, including two missense mutations that replace amino acids that are highly conserved in P-type ATPases, suggesting that these are critical to normal protein structure and/or function. In patients with BRIC, two different mutations were identified, including a missense mutation and a small deletion, that are in less highly conserved regions of the gene. This finding provides a potential explanation for the differences among the clinical manifestations of BRIC and PFIC1, because the BRIC mutations are expected to have less of an effect on FIC1 structure and function. A surprising finding was preferential expression of FIC1 in the small intestine, consistent with a role for this gene product in the intestinal handling of bile acids. Although malabsorption and diarrhea have been described in patients with PFIC1,<sup>3,13</sup> the clinical manifestations of PFIC1 and BRIC led many to suspect that the defect underlying these disorders would be localized to the liver. Furthermore, FIC1 was identified in a wide variety of tissues, including sites not associated with bile acid handling, such as pancreas and stomach. This finding suggests that FIC1 may have a general role in absorptive and secretory processes. Other members of the subfamily to which FIC1 belongs are believed to maintain an asymmetric distribution of phospholipids in membranes by the transport of aminophospholipids from outer to inner leaflets.<sup>12</sup> Clearly, additional studies are required to determine how FIC1 functions and how mutations cause disease. Nevertheless, the identification of FIC1 as the defect in PFIC1 and BRIC represents a significant shift in our understanding of the pathophysiology of cholestatic liver disease.

In the second study, two children with PFIC and high serum GGTP levels were found to have mutations in MDR3. Canalicular staining for MDR3 P-glycoprotein was absent in liver biopsy specimens in both children. As a control, staining of the canalicular membranes with antibodies directed against MDR1 P-glycoprotein and MRP2/cmoat, the canalicular transporter involved in the secretion of nonbile acid organic anions, was normal. Two mutations were identified using reverse transcription-polymerase chain reaction; a seven-nucleotide deletion and a single nonsense mutation, both resulting in the introduction of stop codons and truncated messenger RNA. This finding is another milestone in our understanding of cholestatic liver disease. By starting with a gene, in this case *mdr2* in the mouse, and determining its function to define a phenotype, it represents a fundamentally different but equally successful approach to that pursued in the first study in which a defined phenotype was used to identify a defective gene.

Another locus for PFIC has been recently identified on chromosome 2q24 and designated PFIC2.<sup>14</sup> This locus corresponds to that of the human sister P-glycoprotein (SPGP) gene.<sup>15</sup> The protein product of this liver-specific gene is abundantly expressed on the canalicular membrane.<sup>16</sup> Rat *spgp* expressed in *Xenopus* oocytes and in membrane vesicles isolated from transfected Sf9 insect cells was recently found to mediate adenosine triphosphate-dependent bile acid transport.<sup>17</sup> Furthermore, taurochenodeoxycholate, the concentration of which is strikingly low in gallbladder bile from PFIC patients,<sup>11</sup> was the preferred substrate for *spgp*.<sup>17</sup> These findings suggest that *spgp* is a canalicular bile acid trans-

porter and that mutations of the human liver SPGP gene are responsible for PFIC type 2. In three patients with PFIC and normal serum GGTP levels, SPGP messenger RNA was normal but SPGP was undetectable on the canalicular membrane by immunohistochemistry,<sup>18</sup> consistent with a trafficking defect. To date, 15 mutations in SPGP have been identified in patients with PFIC2 (Richard Thompson, Department of Pediatrics, University College London Medical School, London, UK, personal communication).

In summary, the molecular basis for all forms of PFIC may now have been identified. However, the implications of these findings may extend far beyond a mere understanding of a group of relatively rare inherited cholestatic disorders. *Lith1*, a gene that has been identified as a determinant of cholesterol gallstone susceptibility in inbred mice, co-localizes with *spgp*.<sup>19</sup> Thus, a potential link has been established between canalicular bile salt secretion and cholesterol gallstone formation. Further characterization of the function of the gene products of FIC1, MDR3, and SPGP holds the promise of even more advances in bridging the gap between the bench and the bedside.

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#### HEREDITARY HEMOCHROMATOSIS—SOMETIMES HAVING A REAL COMPLEX CAN BE A GOOD THING

Feder JN, Penny DM, Irrinki A, Lee VK, Lebron JA, Watson N, Tsuchihashi Z, Sigal E, Bjorkman PJ, Schatzman RC. The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc Natl Acad Sci U S A* 1998;95:1472-1477. (Reprinted with permission.)

#### ABSTRACT

We recently reported the positional cloning of a candidate gene for hereditary hemochromatosis called HFE. The gene product, a member of the major histocompatibility complex class I-like family, was found to have a mutation, Cys-282 Tyr (C282Y), in 85% of patient chromosomes. This mutation eliminates the ability of HFE to associate with  $\beta_2$ -microglobulin ( $\beta_2m$ ) and prevents cell-surface expression. A second mutation that has no effect on  $\beta_2m$  association, H63D, was found in eight out of nine patients heterozygous for the C282Y mutant. In this report, we demonstrate in cultured 293 cells overexpressing wild-type or mutant HFE proteins that both the wild-type and H63D HFE proteins form stable complexes with the transferrin receptor (TfR). The C282Y mutation nearly completely prevents the association of the mutant HFE protein with the TfR. Studies on cell-associated transferrin at 37°C suggest that the overexpressed wild-type HFE protein decreases the affinity of the TfR for transferrin. The overexpressed H63D protein does not have this effect, providing the first direct evidence for a functional consequence of the H63D mutation. Addition of soluble wild-type HFE/ $\beta_2m$  heterodimers to cultured cells also decreased the apparent affinity of the TfR for its ligand under steady-state conditions, both in 293 cells and in HeLa cells. Furthermore, at 4°C, the added soluble complex of HFE/ $\beta_2m$  inhibited binding of transferrin to HeLa cell TfR in a concentration-dependent manner. Scatchard plots of these data indicate that the added heterodimer substantially reduced the affinity of TfR for transferrin. These results establish a molecular link between HFE and a key protein involved in iron transport, the TfR, and raise the possibility that alterations in this

regulatory mechanism may play a role in the pathogenesis of hereditary hemochromatosis.

#### COMMENTS

In recent years, many disease genes have been successfully cloned by establishing a linkage between the gene and markers from known locations in the genome. One of the virtues of a positional cloning approach to identify a disease-related gene is that information about the function of the gene is not required for success. As long as patients with the disease can be unequivocally identified, the discovery of mutations within a candidate gene in affected individuals but not in healthy individuals should be sufficient to prove that the gene is correct. Positional cloning has identified many disease genes; however, there are several situations in which a disease gene bore no resemblance to genes of known function and, thus, provided little insight into pathogenesis. In other instances, a disease gene resembled known genes but the relationship between the gene and the disease process was initially obscure. Such was the case for hereditary hemochromatosis, whose gene named HFE was cloned in 1996.<sup>1</sup>

HFE is similar in sequence to major histocompatibility complex class I genes, a family of polymorphic integral membrane proteins that associate noncovalently with a 12-kd protein  $\beta_2$  microglobulin ( $\beta_2m$ ). Within the endoplasmic reticulum, class I molecules capture and bind peptides derived from proteolytic digestion of intracellular proteins. Once peptide is bound to a class I molecule, the complex exits the endoplasmic reticulum and moves to the cell surface where peptides from viral proteins that are expressed in an infected cell can be recognized as foreign and can target the cell for destruction by T-lymphocytes. There is another group of major histocompatibility complex class I proteins that are atypical in that they have maintained the basic structure of class I molecules, but have other biological roles, as in the case of CD1 and the neonatal Fc receptor.<sup>2</sup>

Although the mechanism by which an abnormality in a class I-like gene would lead to iron overload was initially quite obscure, the observation fit well with previous data that mice homozygous for a targeted disruption in the  $\beta_2m$  gene develop iron overload (presumably as a consequence of loss of  $\beta_2m$  and, therefore, loss of the functional HFE- $\beta_2m$  complex).<sup>3</sup> Subsequently, it has been shown that mice homozygous for a targeted disruption of HFE also develop iron overload similar to that in hereditary hemochromatosis. Together these findings confirm that functional absence of HFE leads to hemochromatosis.<sup>4</sup>

Because loss of HFE results in iron overload, it is reasonable to expect that HFE interacts in some fashion with proteins of iron metabolism. To attempt to identify potential HFE-interacting proteins, Feder et al. analyzed immunoprecipitates from cells in which HFE was overexpressed. Initially, cell-surface proteins were labeled with N-hydroxysuccinamide, and HFE was immunoprecipitated with antibodies to the native HFE C-terminus or to an epitope tag inserted in the overexpressed HFE. In these experiments, HFE and  $\beta_2m$  were coimmunoprecipitated as expected, but in addition, other proteins were identified at approximately 100 and 200 kd. When known proteins of iron metabolism were considered, it became apparent that the 100- and 200-kd bands were quite similar to those seen in immunoprecipitations of the transferrin receptor (TfR), which can migrate as a dimer as well as in a monomeric form of approximately 100 kd. The identity of