

Molecular Pathogenesis of Hepatolithiasis — A Type of Low Phospholipid-Associated Cholelithiasis

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1. ABSTRACT

Hepatolithiasis is prevalent in East Asia, including Japan, but occurs much less frequently in Western countries. Hepatolithiasis appears mostly as brown pigment stones (calcium bilirubinate stones). The disease is characterized by its intractable nature and frequent recurrence, requiring multiple endoscopic or operative interventions, in distinct contrast to gallbladder cholesterol or black pigment stones. In view of the lack of information on the pathogenesis, a multidisciplinary approach has been carried out through the Hepatolithiasis Research Group organized by the Ministry of Health, Labor and Welfare of Japan. In this review, the up-to-date data on the molecular pathogenesis of hepatolithiasis with special reference to a defect in phospholipid metabolism are introduced and discussed. Furthermore, a potential medical treatment targeting hepatic phospholipid transporters is proposed as a future therapeutic option for the disease.

2. PATHOGENESIS OF HEPATOLITHIASIS

Gallstones in hepatolithiasis consist of two groups; i.e., brown pigment stones (calcium bilirubinate stones) and cholesterol stones—the former predominating (1-3). It should be stressed that intrahepatic brown pigment stones contain less bilirubin and bile acid and more cholesterol than those either in the common bile duct or in the gallbladder (2,4,5). The presence of brown pigment with a wider range of cholesterol contents as well as cholesterol stones among hepatolithiasis suggests that the complex nature of the pathogenesis should be considered; e.g., not only the formation and precipitation of calcium bilirubinate but also the solubility of cholesterol in hepatic bile.

In searching for any metabolic defects underlying the formation of cholesterol-rich brown pigment stones, a recent study has suggested hepatic transport and secretion defects of phospholipids (phosphatidylcholines) in the liver of patients with hepatolithiasis (6). This review discusses the role of hepatic transport and secretion defects of phospholipids in the pathogenesis of the disease.

2.1. Role of phospholipids in bile

Phosphatidylcholine (PC), the major class of biliary phospholipids, cholesterol, and bile acids form mixed micelles in bile (Figure 1). This protects the biliary epithelium from detergent action of hydrophobic bile acids (7), which are present at high concentrations in the biliary tree (8,9).

The PC translocating activity of ATP binding cassette protein B4 (Abcb4) formerly known as the multidrug-resistance 2 P-glycoprotein (mdr2), murine homologue of human ABCB4 (MDR3), in the canalicular membranes of hepatocytes is considered the rate-limiting step in biliary phospholipid secretion in vivo (10-12). Evidence in support of Abcb4 as a PC translocator has been provided using membranes of yeast secretory vesicles (13,14).

In mice, the phospholipid-deficiency due to homozygous disruption of Abcb4 results in liver injury associated with chronic nonsuppurative destructive cholangitis (15), as well as spontaneous cholesterol cholecysto- and hepatolithiasis (16). Decreased or even absent expression of ABCB4 has been observed in several clinical forms of cholestasis, such as progressive familial intrahepatic cholestasis type 3 (PFIC-3) (17), intrahepatic cholestasis during pregnancy (18), primary hepatolithiasis (intrahepatic bile duct stones) (6,19) and low phospholipid associated cholelithiasis (20) (Figure 1). These genetic abnormalities provide an important link between defective canalicular transport of phospholipids and the development of hepatochoangiopathies and indicate that ABCB4 may be a target for novel therapeutic strategies.

2.2. Abnormal lipid composition in hepatic bile of patients with hepatolithiasis

Phospholipid concentrations were significantly decreased in the bile specimens from both the stone-containing hepatic ducts and the unaffected ducts for patients with intrahepatic brown pigment stones, and in the specimens from both the affected ducts and the unaffected ducts for patients with intrahepatic cholesterol stones, compared to the specimens from hepatic ducts of control

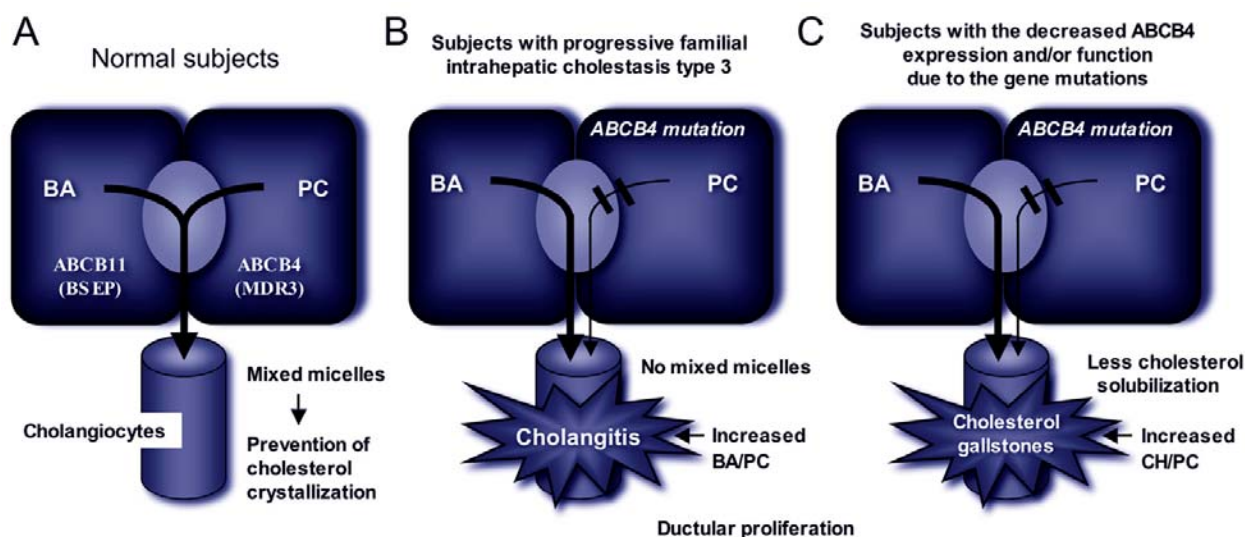


Figure 1. Role of phospholipids in bile. (A) Under normal conditions, phosphatidylcholine (PC) in bile protects cholangiocytes from bile acid toxicity by forming mixed micelles. Also, proper proportions of bile acids to PC in bile are necessary to maintain solubility of cholesterol. (B) ABCB4 deficiency, e.g. progressive familial intrahepatic cholestasis type 3 (PFIC3), results in decreased biliary PC level and high bile acid to PC ratio and causes bile duct injury (cholangitis and ductular proliferation). (C) ABCB4 deficiency results in decreased biliary PC level and high cholesterol to PC ratio, leading to a high biliary cholesterol saturation index (CSI). This will promote lithogenicity of bile with crystallization of cholesterol, which could favor small bile duct obstruction (Adapted with permission from the reference (21)).

subjects and patients with gallbladder cholesterol stones (Table 1) (6).

Cholesterol/phospholipid (Ch/PL) ratios in bile were significantly higher in patients with intrahepatic calculi, in both affected and unaffected sides, than in control subjects and patients with gallbladder cholesterol stones. In contrast, phospholipid/bile acid (PL/BA) ratios in bile were significantly lower in patients with intrahepatic brown pigment stones, in both affected and unaffected sides, than in control subjects and patients with gallbladder cholesterol stones. Cholesterol saturation indices were significantly higher in patients with intrahepatic calculi, for the specimens from both affected and unaffected ducts, than in control subjects and patients with gallbladder cholesterol stones.

Under these conditions of the decreased formation of mixed micelles in the bile, the presence of hydrophobic bile acids in the bile ducts could solubilize the plasma membrane of the biliary epithelia. Hyposecretion of phospholipids, coupled with hydrophobic bile acid (e.g., chenodeoxycholate) secretion, may thus contribute to intractable and long-standing cholangitis in patients with hepatolithiasis (termed chronic proliferative cholangitis) (22,23).

2.3. ABCB4 expression in livers of patients with hepatolithiasis

Phospholipid concentrations are significantly decreased in the hepatic bile issuing from both affected and unaffected bile ducts of hepatolithiasis patients in this study, irrespective of stone category (6). This is supported

by the decrease in both affected and unaffected hepatic segments of mRNA and protein levels of ABCB4 (Figures 2 and 3), which is rate-limiting for phospholipid secretion into bile (10-12).

To elucidate whether the decreased ABCB4 mRNA and protein expression in hepatolithiasis patients is associated with the gene mutations, a mutation analysis on the cDNA of the ABCB4 gene with special reference to the coding region was performed using the liver specimens of hepatolithiasis patients (24).

By sequence analysis of the gene (Figure 4), a 77-bp deletion at nucleotides 537–613 in exon 7 in transmembrane domain (TMD) 3, which results in a frameshift at codon 179 and an early stop codon predicting a truncated protein, was found as a heterozygous mutation in 2 of the 16 patients. A 1-bp deletion at nucleotide 1015 in exon 10 in TMD 6 was found as a heterozygous mutation in one of those 2 patients, and a 242-bp deletion at nucleotides 2683–2924 in exons 22–23 in TMD 11 was found as a heterozygous mutation in the same patient. No other mutations were found in the other 14 patients. In real-time PCR, no significant difference was found between the mRNA levels of ABCB4 in the 2 HL patients with mutations nor in the other 14 patients without mutations (Figure 4).

The immunostaining of ABCB4 outlined the canalicular membrane domain but was less defined and focally absent in the liver of patients with hepatolithiasis, especially in affected segments (6). In the hepatolithiasis we studied here, histology of biopsied liver specimens showed minor changes associated with cholestasis in the

Table 1. Biliary Lipid Composition

Subjects	n	Cholesterol		Phospholipid		Bile acid		Ch / PL ratio	PL / BA ratio	Cholesterol Saturation index
		mM	molar%	mM	molar%	mM	molar%			
Controls	13	1.5 ± 0.1 ¹	3.6 ± 0.2	10.4 ± 0.4	23.7 ± 0.6	30.7 ± 1.0	72.7 ± 0.7	0.08 ± 0.01	0.33 ± 0.01	64 ± 3
Patients with gallbladder cholesterol stones	20	2.3 ± 0.12 ^{b2}	5.4 ± 0.5	10.8 ± 0.8	24.1 ± 1.1	32.3 ± 2.5	70.7 ± 1.3	0.12 ± 0.01	0.35 ± 0.03	99 ± 10
Intrahepatic brown pigment stones	(+)	16	1.5 ± 0.3 ^d	11.9 ± 0.5 ^{b,d}	2.2 ± 0.2 ^{b,d}	17.5 ± 1.0 ^{b,d}	8.6 ± 0.9 ^{b,d}	0.36 ± 0.06 ^{b,d}	0.25 ± 0.02 ^{a,d}	383 ± 43 ^{b,d}
Intrahepatic brown pigment stones	(-)	26	1.9 ± 0.1 ^c	8.1 ± 0.4 ^{b,d}	4.7 ± 0.2 ^{b,d}	19.6 ± 0.5 ^{b,d}	17.2 ± 0.9 ^{b,d}	0.21 ± 0.01 ^{b,c}	0.27 ± 0.01 ^{a,d}	196 ± 11 ^{b,d}

Abbreviations: Ch, cholesterol; PL, phospholipid; BA, bile acid; (+), bile specimens from the affected bile ducts; (-), bile specimens from the unaffected bile ducts, ¹Values represent means ± SEM; ^{2a}*P* < 0.05, ^b*P* < 0.01, significantly different from controls; ^c*P* < 0.05, ^d*P* < 0.01, significantly different from patients with gallbladder cholesterol stones

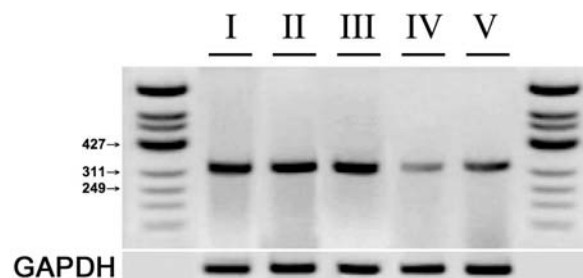


Figure 2. RT-PCR-assisted amplification of ABCB4 mRNA in the liver of a control subject, a patient with gallbladder stones, a patient with obstructive cholestasis, and a patient with intrahepatic brown pigment stones. Lane I, a control subject; Lane II, a patient with gallbladder stones; Lane III, a patient with obstructive cholestasis; Lane IV, the affected hepatic segment from a patient with intrahepatic stones; Lane V, the unaffected segment from a patient with intrahepatic stones. The abundance of GAPDH mRNA was determined as an internal standard. The PCR products were 319 bp in size for ABCB4 and 311 bp for GAPDH.

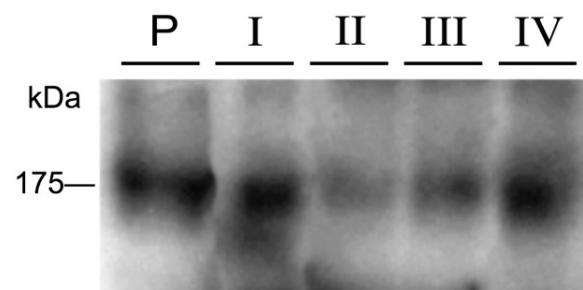


Figure 3. Immunoblot analysis of P-glycoproteins in the crude plasma membrane fractions isolated from the liver of a control subject, a patient with intrahepatic brown pigment stones, and a patient with obstructive cholestasis. Lane P, positive control; Lane I, a control subject; Lane II, the affected hepatic segment from a patient with intrahepatic stones; Lane III, the unaffected segment from a patient with intrahepatic stones; Lane IV, a patient with obstructive cholestasis. The membrane vesicles from LLC-PK1 cells transfected with human ABCB1 (MDR1) cDNA were used as the positive control for P-glycoproteins.

affected segments but almost no changes in the unaffected segments. It can therefore be assumed that decreased

expression of ABCB4 is primary rather than secondary to stone-associated biliary obstruction.

It is quite important to determine whether the amount of canalicular ABCB4 protein is altered in hepatolithiasis patients with the ABCB4 gene mutations. Immunostaining of ABCB4 protein was found in the bile canaliculi of liver sections from the two patients with mutations (Figure 4). The results suggest that in primary hepatolithiasis the decreased transcription levels of ABCB4 in the liver are not due to the mutations detected in the coding region of the gene, and that either unknown mutations in the promotor region of the ABCB4 gene or a possible involvement of unknown genes possibly regulating ABCB4 transcription and/or trafficking in the liver.

In hepatolithiasis, the observed biochemical/metabolic defects in the liver may be interpreted as the consequence of primary events, since these changes are specific to patients with hepatolithiasis and observed in both affected and unaffected segments of the liver, irrespective of whether the stones are brown pigment or cholesterol. However, specified factors initiating the defects in the liver, e.g., food and environmental issues stated before, or even gene mutations, have not been well elucidated yet.

2.4. Intracellular transporter expressions in livers of patients with hepatolithiasis

The intracellular transporters SCP2, PCTP, and HBAB for cholesterol, phospholipid, and bile acid, respectively, in the liver were determined by RT-PCR (Figure 5) (6). Levels of PCTP mRNA were significantly decreased, in both hepatic segments affected by stones and unaffected segments of patients with intrahepatic brown pigment stones, and in both affected segments and unaffected segments of patients with intrahepatic pure cholesterol stones, irrespective of stone category, compared to the levels of control subjects, gallbladder stone patients, and patients with obstructive cholestasis. However, levels of SCP2 and HBAB mRNAs in the liver of intrahepatic stone patients, in both affected and unaffected segments, were not significantly different from the levels of control subjects, gallbladder stone patients, and patients with obstructive cholestasis.

3. Potential therapies for hepatolithiasis

Our studies (6,24) and studies from other groups (19,20) suggest that the decreased hepatic transport and biliary secretion of phospholipids is involved in the etiological process of hepatolithiasis. Therefore, future

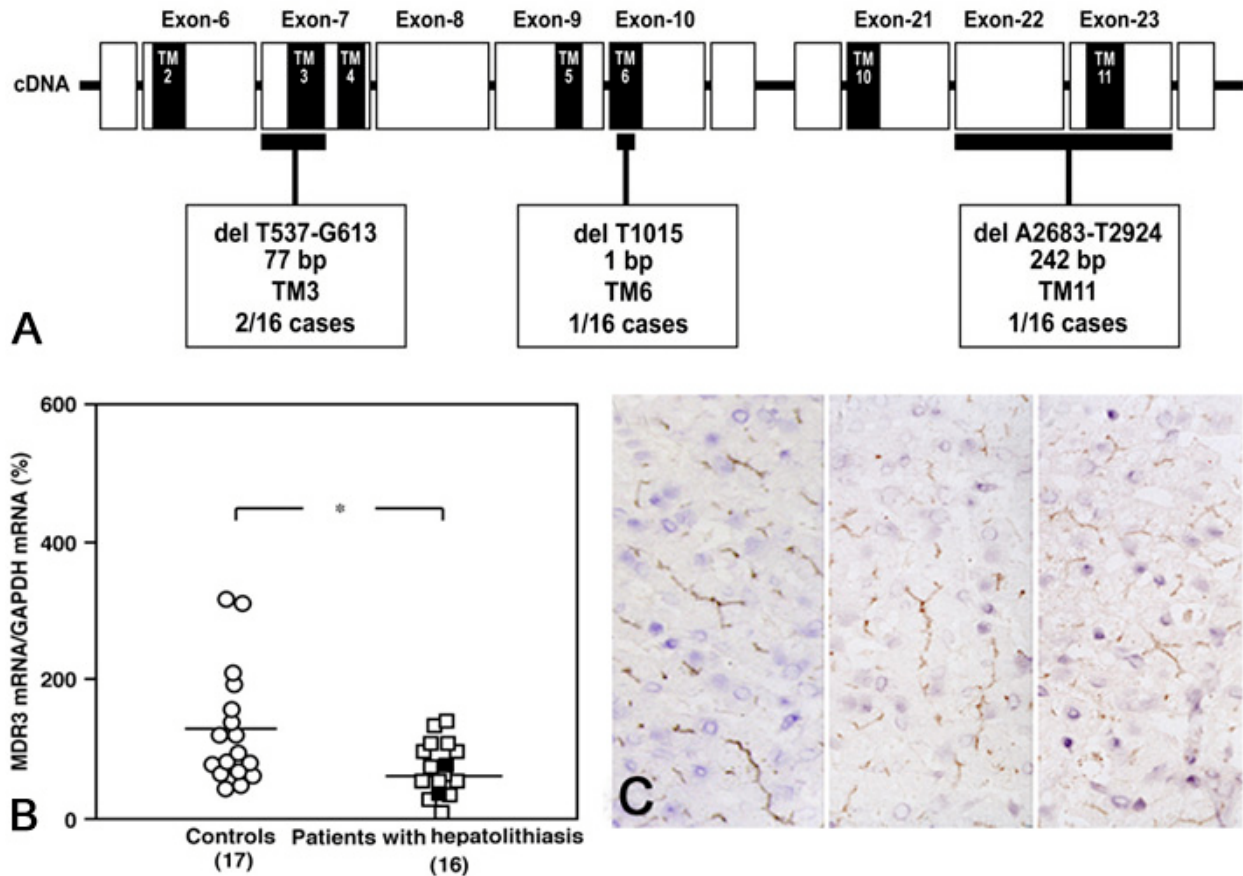


Figure 4. (A) Localization of mutations on ABCB4 cDNA. Exons 6–12 and 25–28 are represented by boxes. Black boxes represent the transmembrane domains (TM 6–11). The different ABCB4 mutations are localized on the cDNA by bars. (B) Real-time PCR of ABCB4 mRNAs in liver specimens from control subjects (O) and patients with primary HL (■, with the gene mutations; □, without the gene mutations). The abundance of GAPDH mRNA was determined as an internal standard. PCR was performed in triplicate. The data are expressed as averaged percentages of the amounts of GAPDH mRNA present in specimens. ** $P < 0.05$, significantly different from control subjects. (C) Immunohistochemical localizations of ABCB4 in the livers. Immunostaining of ABCB4 shows a diffuse and linear pattern outlining the canalicular membrane domain of the liver section from a control subject (A: original magnification $\times 100$). However, in sections of the livers from a hepatolithiasis patient with ABCB4 gene mutations (B: original magnification $\times 100$) and a hepatolithiasis patient without mutations (C: original magnification $\times 100$), the staining pattern of ABCB4 outlines the canalicular membrane domain but is less defined and focally absent. No significant difference was found between hepatic localization of ABCB4 in the hepatolithiasis patients with and those without ABCB4 gene mutations.

medical treatments with some drugs and agents which target the molecules important for the pathogenesis of the disease should be proposed.

Fibrates are antihyperlipidemic drugs, which up-regulate the expression of Abcb4 through gene transcription and promote biliary phospholipid secretion (25). Biliary PC secretion is limited by the amount of ABCB4 present in the canalicular membrane of the hepatocytes (11,17,26,27). Clinically, treatment with bezafibrate (BF) improves the elevated serum levels of biliary enzymes in patients with primary biliary cirrhosis (28,29), a chronic cholestatic liver disease characterized by progressive inflammatory destruction of the intrahepatic bile ducts. The beneficial therapeutic effects of BF in patients with primary biliary cirrhosis suggest the possibility that the drug promotes the PC translocating function of ABCB4 through increasing

the protein amount in the bile canaliculi and improves the bile duct injury through protecting the cholangiocytes from hydrophobic bile acid toxicity by forming mixed micelles with the increased biliary PC.

Importantly, the expression and function of Abcb4 is regulated by peroxisome proliferator-activated receptor alpha (PPARalpha) in mice (30) and BF, acting as a ligand for PPARalpha (31), up-regulates the transcriptional level of PPARalpha (31). When we explored whether BF-induced changes in ABCB4 expression and function are mediated by PPARalpha, BF may enhance the capacity of human hepatocytes to transport PC into bile canaliculi by promoting insertion of ABCB4 molecules into the canalicular membrane via PPARalpha-mediated mechanism (Figure 6) (32). These observations provide a rationale for the use of BF to restore canalicular ABCB4 expression and

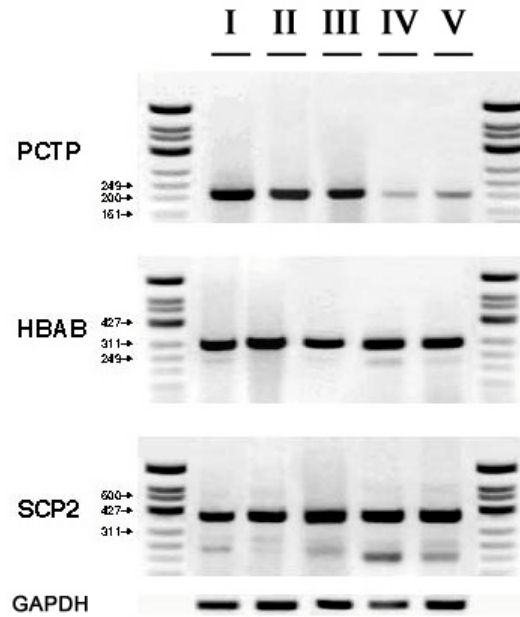


Figure 5. RT-PCR-assisted amplifications of SCP2, PCTP, and HBAB mRNAs in the liver of a control subject, a patient with gallbladder stones, a patient with obstructive cholestasis, and a patient with intrahepatic brown pigment stones. Lane I, a control subject; Lane II, a patient with gallbladder stones; Lane III, a patient with obstructive cholestasis; Lane IV, the affected hepatic segment from a patient with intrahepatic stones; Lane V, the unaffected segment from a patient with intrahepatic stones. The abundance of GAPDH mRNA was determined as an internal standard. The PCR products were 388 bp in size for SCP2, 216 bp for PCTP, 303 bp for HBAB, and 311 bp for GAPDH.

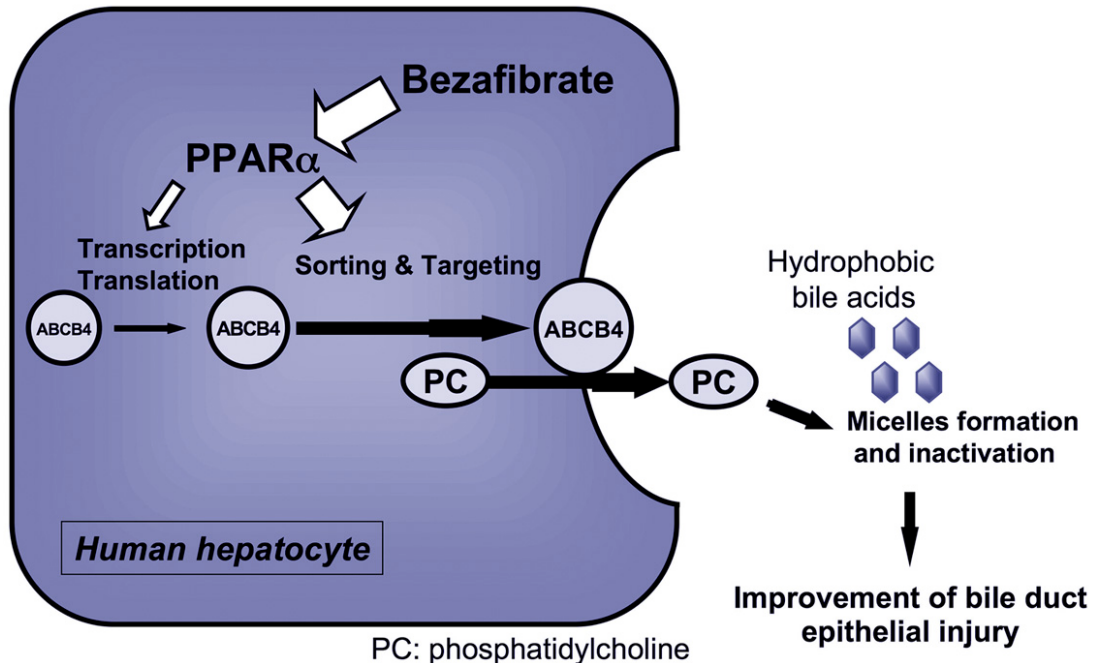


Figure 6. Bezafibrate (BF) may enhance the capacity of human hepatocytes to transport and secrete phosphatidylcholine (PC) by a novel mechanism through the stimulation of exocytosis and insertion of ABCB4 into the bile canaliculi. By treatment with BF, the vesicular targeting of ABCB4 into the bile canaliculi may be enhanced through the PPAR α -mediated mechanism. These provide a rational for the beneficial role of BF in stimulation and restoration of defective ABCB4 expression and function in various types of cholestatic hepatobiliary diseases.

function in various types of cholestatic hepatobiliary diseases, including hepatolithiasis.

In therapeutic aspects for hepatolithiasis, adjuvant treatment with BF after surgical or endoscopic stone removals, which target the ABCB4 gene product, should be introduced in future clinical practice, especially for the intractable cases.

4. ACKNOWLEDGEMENT

This work was supported in part by Grants-in-Aid for Research on Hepatolithiasis from the Ministry of Health, Labor, and Welfare, Japan.

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Key Words: Hepatolithiasis, Pathogenesis, Phospholipid, Transporter defect, ABCB4, Potential therapies, Review

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