発表した成果 （発表題目，口頭・ポスター発表の別）	発表者氏名	発表した場所 （学会等名）	発表した時期	国内・外の別
Hepaticoduodenostomy in hepatectomy for perihilar cholangiocarcinoma: a preliminary report	Yoshida H, Mamada Y, Taniai N, Makino H, Yokoyama T, Maruyama H, Hirakata A, Hotta M, Uchida E	Austin Journal of Surgery	2014年5月	国外
特集：消化器外科手術ピットフォールとリカバリーショット　II．各論　6. 脾臓　a)　巨脾摘出の注意点	吉田　寛，平方　敦史，上田　純志，牧野　浩司，横山　正，丸山　弘，中村　慶春，谷合　信彦，真々田裕宏，内田　英二	外科	2014年10月	国内
難治性腹水の治療	上田　純志，吉田　寛，真々田裕宏，谷合　信彦，吉岡　正人，平方　敦史，川野　陽一，水口　義昭，清水　哲也，神田　知洋，高田　英志，内田　英二	Fluid Management Renaissance	2014年10月	国内
Preoperative Three-dimensional Virtual Simulation for Safe Liver Surgery	Shimizu T, Taniai N, Yoshioka M, Takata H,Kanda T, Mizuguchi Y, Mamada Y, Yoshida H, Uchida E	J Nippon Med Sch	2014年12月	国外
Overall survival in response to sorafenib versus radiotherapy in unresectable hepatocellular carcinoma with major portal vein tumor thrombosis: propensity score analysis	Nakazawa T, Hidaka H, Shibuya A, Okuwaki Y, Tanaka Y, Takada J, Minamino T, Watanabe M, Kokubu S, Koizumi W	BMC Gastroenterology	2014年5月	国外

発表した成果 （発表題目，口頭・ポスター発表の別）	発表者氏名	発表した場所 （学会等名）	発表した時期	国内・外の別
Radiofrequency ablation combined with chemolipiodolization in a porcine liver: Comparison of the pharmacokinetic analysis with cisplatin powder and miriplatin	Ito T, Okubo H, Kokubu S, Miyazaki A, Ando H, Fujimura A, Watanabe S	Hepatol Res	2014年7月	国外
食道・胃静脈瘤治療の内科的アプローチ	林　量司， 國分　茂博， 浅野　朗， 山科　俊平， 川邉　正人， 宮崎　招久， 渡辺　純夫， 松川　正明	日本内科学会雑誌	2014年7月	国外

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。

（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

Ⅳ. 研究成果の刊行物・別刷

Improved Liver Function and Relieved Pruritus after 4-Phenylbutyrate Therapy in a Patient with Progressive Familial Intrahepatic Cholestasis Type 2

Sotaro Naoi, MS[1,*], Hisamitsu Hayashi, PhD[1,*], Takeshi Inoue, MD[2], Ken Tanikawa, MD[3], Koji Igarashi, PhD[4], Hironori Nagasaka, MD, PhD[5], Masayoshi Kage, MD, PhD[3], Hajime Takikawa, MD, PhD[6], Yuichi Sugiyama, PhD[7], Ayano Inui, MD, PhD[8], Toshiro Nagai, MD, PhD[2], and Hiroyuki Kusuhara, PhD[1]

To examine the effects of 4-phenylbutyrate (4PB) therapy in a patient with progressive familial intrahepatic cholestasis type 2. A homozygous c.3692G>A (p.R1231Q) mutation was identified in *ABCB11*. In vitro studies showed that this mutation decreased the cell-surface expression of bile salt export pump (BSEP), but not its transport activity, and that 4PB treatment partially restored the decreased expression of BSEP. Therapy with 4PB had no beneficial effect for 1 month at 200 mg/kg/day and the next month at 350 mg/kg/day but partially restored BSEP expression at the canalicular membrane and significantly improved liver tests and pruritus at a dosage of 500 mg/kg/day. We conclude that 4PB therapy would have a therapeutic effect in patients with progressive familial intrahepatic cholestasis type 2 who retain transport activity of BSEP per se. *(J Pediatr 2014;164:1219-27).*

Progressive familial intrahepatic cholestasis type 2 (PFIC2), an inherited autosomal-recessive liver disease caused by mutations in *ABCB11* encoding the bile salt export pump (BSEP), is characterized by cholestasis and jaundice in the first year of life.[1] This disease progresses to severe cholestasis with sustained intractable itching, jaundice, diarrhea, and failure to thrive, leading to liver failure and death before adulthood. No medical therapy has been established for PFIC2.[1,2] BSEP is an adenosine triphosphate (ATP)-binding cassette transmembrane transporter located on the canalicular membrane of hepatocytes that mediates the biliary excretion of monovalent bile salts.[3-6] Therefore, in patients with PFIC2, biliary bile salt secretion is impaired, bile salts accumulate in hepatocytes, and consequently the hepatocytes are damaged.

We have published experimental evidence that 4-phenylbutyrate (4PB), a drug used to treat ornithine transcarbamylase deficiency (OTCD), has an additional pharmacologic effect to increase the hepatocanalicular expression of BSEP and the biliary excretion capacity of bile salts when given at a clinically relevant concentration in patients with OTCD.[7-9] Greater BSEP expression levels in liver specimens from patients with OTCD after 4PB therapy compared with before 4PB therapy suggest that 4PB treatment increases BSEP expression in humans.[10] These results indicate the possibility that 4PB may be a potential therapeutic compound for patients with PFIC2 who show a reduced BSEP expression at the canalicular membrane but who have retained transport activity of BSEP per se. To test this hypothesis, we investigated the effects of 4PB therapy in a patient with PFIC2 carrying a homozygous c.3692G>A (p.R1231Q) mutation in *ABCB11*.

[^{3}H]-TC	[^{3}H]-Taurocholate
4PB	4-Phenylbutyrate
ALT	Alanine aminotransaminase
AST	Aspartate aminotransaminase
ATP	Adenosine triphosphate
BSEP	Bile salt export pump
cDNA	Complementary DNA
D-Bil	Direct bilirubin
EV	Empty vector
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGT	Gamma-glutamyl transferase
GPCR	G protein-coupled receptor
HA	Hemagglutinin
mRNA	Messenger RNA
OTCD	Ornithine transcarbamylase deficiency
PCR	Polymerase chain reaction
PFIC2	Progressive familial intrahepatic cholestasis type 2
P-gp	P-glycoprotein
qPCR	Quantitative polymerase chain reaction
T-Bil	Total bilirubin
TfR	Transferrin receptor
UDCA	Ursodeoxycholic acid
WT	Wild type

From the [1]Laboratory of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan; [2]Department of Pediatrics, Dokkyo Medical University, Koshigaya Hospital, Saitama, Japan; [3]Department of Diagnostic Pathology, Kurume University Hospital, Fukuoka, Japan; [4]Bioscience Division, Reagent Development Department, TOSOH Corporation, Kanagawa, Japan; [5]Department of Pediatrics, Takarazuka City Hospital, Takarazuka-shi, Japan; [6]Department of Medicine, Teikyo University School of Medicine, Tokyo, Japan; [7]Sugiyama Laboratory, RIKEN Innovation Center, Research Cluster for Innovation, RIKEN, Yokohama, Japan; and [8]Department of Pediatric Hepatology and Gastroenterology, Saiseikai Yokohamashi Tobu Hospital, Yokohama, Japan

*Contributed equally.

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Methods

We obtained approval for the study from the institutional ethics review boards. Informed consent was provided by the patient's parents before assessment because the patient was younger than 18 years of age. A detailed description of the materials and methods is presented in the Appendix (available at www.jpeds.com). All materials and methods used standard techniques and commercially available reagents.

The patient, a girl who is now 1 year of age, was born to parents of Pakistani descent. She developed hepatocellular cholestasis and jaundice with normal serum gamma-glutamyl transferase (GGT) activity at the age of 2 months and was diagnosed with PFIC2 by the presence of the c.3692G>A (p.R1231Q) mutation in both alleles of *ABCB11* and no detectable immunosignal for BSEP at the canalicular membrane of a liver section sample (Figure 1, A and B). Despite treatment with ursodeoxycholic acid (UDCA; 120 mg/day), she continued to have severe cholestasis with sustained intractable itch, jaundice, diarrhea, and failure to thrive. 4PB therapy was started at the age of 4 months. The administration of UDCA was maintained during and after the course of 4PB treatment.

Sequence Analysis of *ATP8B1* and *ABCB11*

Genomic DNA was isolated from peripheral blood leukocytes using a Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin), and all exons of *ATP8B1* and *ABCB11* and flanking intron–exon boundaries were amplified by polymerase chain reaction (PCR). Primer sequences and PCR conditions were designed according to previous reports.[11,12] Both strands were analyzed using BigDye Direct Cycle Sequencing Kit (Applied Biosystems, Foster City, California).

In Vitro Studies

pShuttle (Clontech, Palo Alto, California) containing complementary DNA (cDNA) of human BSEP with a hemagglutinin (HA) tag at the N-terminus (HA-BSEP$^{\text{wild type [WT]}}$) and that of HA-BSEP^{WT} with the c.3692G>A (p.R1231Q) or p.T1210P mutation (HA-BSEP^{R1231Q} and HA-BSEP^{T1210P}) were used for this study.[8] The c.3692G>A (p.R1231Q) and p.T1210P mutations were introduced into pShuttle containing HA-BSEP^{WT} cDNA by site-directed mutagenesis as described previously.[9] HEK293T cells and McA-RH7777 cells transfected with pShuttle containing HA-BSEP^{WT}, HA-BSEP^{R1231Q}, or HA-BSEP^{T1210P} cDNA, or empty vector (EV) (HA-BSEP^{WT}, HA-BSEP^{R1231Q}, HA-BSEP^{T1210P}, or EV HEK293T cells and HA-BSEP^{WT}, HA-BSEP^{R1231Q}, HA-BSEP^{T1210P}, or EV McA-RH7777 cells) were subjected to analysis of quantitative PCR (qPCR), cell surface biotinylation, immunofluorescence, and transport. All in vitro experiments were performed as described previously,[7,9] and a detailed description of the experiments is presented in the Appendix. The cells were treated with 1 mM 4PB for 24 hours before the in vitro experiments, which were designed to examine the effects of 4PB on HA-BSEP^{WT}, HA-BSEP^{R1231Q}, and HA-BSEP^{T1210P}.

Treatment of the Patient with PFIC2 with 4PB

Oral administration of 4PB (AMMONAPS; Swedish Orphan Inter AB, Stockholm, Sweden) was started at a daily dosage of 200 mg/kg/day divided into 4 doses a day. After 1 month, the dosage was increased to 350 mg/kg/day, which was maintained for an additional month. Because neither a therapeutic effect nor any side effects were observed, the dosage was increased up to 500 mg/kg/day, which is a clinically relevant dosage for OTCD, and this dosage was maintained for the next 4 months.

A liver biopsy sample was collected before and after the course of 4PB treatment. A part of the sample was preserved in RNAlater (QIAGEN, Hilden, Germany) for RNA preparation and stored at −20°C. Another portion was fixed in 4% formaldehyde at room temperature for histological analysis, and the remaining portion was snap-frozen in liquid nitrogen for preparation of membrane fractions and stored at −70°C in a deep freezer. Serum was collected before, during, and after the course of 4PB treatment. Liver tests were performed using standard methods immediately after collection, and the remaining specimens were preserved at −70°C for further analysis.

The severity of pruritus was scored according to a previous report[13]: 0, none; 1, mild scratching when undistracted; 2, active scratching without abrasion; 3, abrasions; or 4, cutaneous mutilation, with bleeding and scarring. Serum concentrations of total bile acids, histamine, and tryptase were measured by an enzyme cycling method (Wako Pure Chemicals, Osaka, Japan), a competitive enzyme immunoassay (Bertin Pharma, Montigny le Bretonneux, France), and a fluoroenzyme immunoassay (USCN Life Science Inc, Hubei, People's Republic of China), according to the manufacturer's instruction. The concentration and activity of autotaxin in serum were assessed using a specific two-site enzyme immunoassay and the measurement of choline liberation from the substrate lysophosphatidylcholine.[14]

Histologic Analysis of Human Liver Specimens

Liver biopsies were fixed in 10% formalin and embedded in paraffin. Then, 4-μm thick sections from the liver specimens were prepared by a Microm HM340E (Microm International GmbH, Walldorf, Germany), adhered to the glass coverslips, and subjected to hematoxylin and eosin staining and immunohistochemistry followed by microscopic analysis with an Olympus CX41 (Olympus, Tokyo, Japan) to evaluate the degree of cholestasis, giant cell transformation, inflammation in the liver tissues, and BSEP expression at the canalicular membrane.

Statistical Analyses

The data in the Figures are presented as the mean ± SE. The significance of differences between 2 variables and multiple variables was calculated at the 95% confidence level by Student *t* test and by one-way analysis of variance with Tukey test, respectively, using Prism software (GraphPad Software, Inc, La Jolla, California).

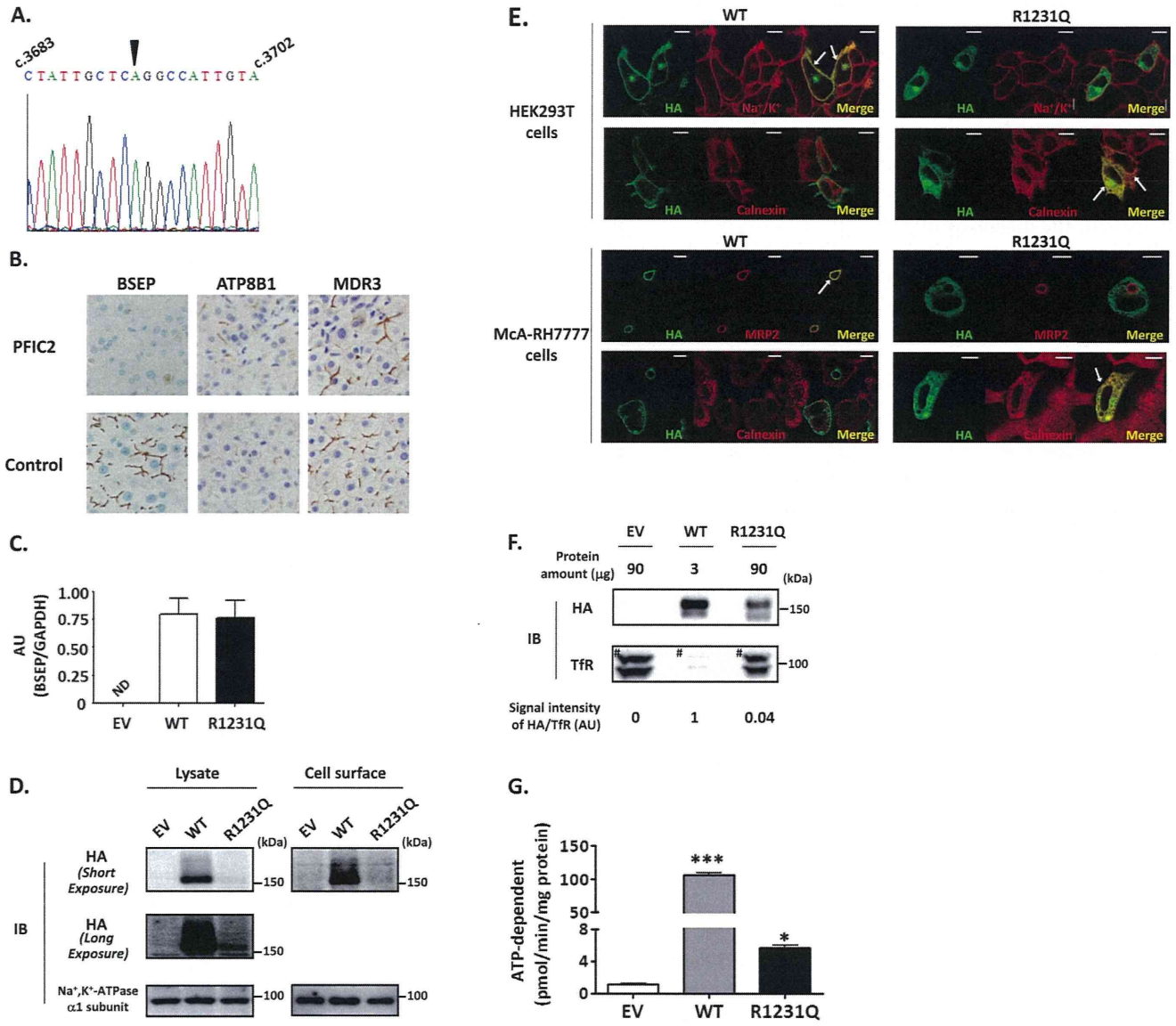

Figure 1. **A, B,** Diagnosis of PFIC2 in a patient who developed intrahepatic cholestasis with normal GGT activity. **A,** Genetic sequencing analysis. The homozygous c.3692G>A (p.R1231Q) mutation in *ABCB11* identified is shown by the *arrowhead*. **B,** Immunohistochemistry. Liver sections prepared from the liver biopsy specimens of the patient with PFIC2 and of control subjects were subjected to immunohistochemistry as described in the Methods. A typical image under each condition is shown. Original magnification, ×400. **C, D,** Determination of mRNA and protein expression levels of BSEP^{R1231Q}. EV, HA-BSEP^{WT}, or HA-BSEP^{R1231Q} HEK293T cells were subjected to **C,** qPCR and **D,** cell surface biotinylation and then analyzed as described in the Methods. **E,** Cellular localization of BSEP^{R1231Q}. HEK293T cells (*top*) and McA-RH7777 cells (*bottom*) expressing HA-BSEP^{WT} (WT; *left*) or HA-BSEP^{R1231Q} (R1231Q, *right*) were analyzed by confocal immunofluorescence microscopy as described in the Methods. *Arrows* in the merged images indicate colocalization. *Scale bar*: 10 μm. **F, G,** BSEP^{R1231Q}-mediated uptake of [^{3}H]-TC. Membrane vesicles (**F,** 3 or 90 μg in and **G,** 10 μg in prepared from EV, HA-BSEP^{WT}, and HA-BSEP^{R1231Q} HEK293T cells were subjected to **F,** immunoblotting and **G,** an uptake assay. In **C-G,** a representative result of 2-3 independent experiments is shown. Bars represent the mean ± SE of each experiment in triplicate. $^{*}P < .05$, $^{***}P < .001$, *AU*, arbitrary units; *IB*, immunoblotting; *ND*, not detected because of low expression levels.

Results

Progressive familial intrahepatic cholestasis type 1 is caused by mutations in *ATP8B1* encoding a membrane protein that is expressed on the canalicular membrane of hepatocytes and that translocates phosphatidylserine from the outer leaflet to the inner leaflet. Patients with progressive familial intrahepatic cholestasis type 1 present with similar clinical

symptoms and biologic variables as patients with PFIC2. Therefore, all encoding exons and flanking areas of both *ATP8B1* and *ABCB11* were sequenced, and a homozygous c.3692G>A (p.R1231Q) mutation in *ABCB11* was identified, which has been reported previously in European white patients with PFIC2 (Figure 1, A).[12] This result, combined with the immunosignal of ATP8B1, but not of BSEP at the canalicular membrane of liver sections (Figure 1, B), was the basis of the diagnosis of PFIC2.

To characterize the effect of the c.3692G>A (p.R1231Q) mutation on BSEP, HA-BSEP^{WT} and HA-BSEP^{R1231Q} were expressed ectopically in HEK293T cells and McA-RH7777 cells, a rat hepatoma cell line that develops canalicular membranes through the formation of couplets as hepatocytes. qPCR analysis showed that the BSEP messenger RNA (mRNA) expression level was similar between HA-BSEP^{WT} and HA-BSEP^{R1231Q} in HEK293T cells (Figure 1, C). In contrast, a cell-surface biotinylation study showed much lower cell surface expression of HA-BSEP^{R1231Q} than HA-BSEP^{WT} in HEK293T cells (Figure 1, D). Immunofluorescence analysis showed no colocalization of HA-BSEP^{R1231Q} with the plasma membrane marker, Na^+, K^+-ATPase α1 subunit, in HEK293T cells or with the canalicular membrane marker, MRP2, in McA-RH7777 cells, which was consistent with the results of immunohistochemistry (Figure 1, B and E). HA-BSEP^{R1231Q} was localized predominantly in the endoplasmic reticulum (Figure 1, E), suggesting that this mutation induces incomplete folding of BSEP molecules retained in the endoplasmic reticulum followed by proteasomal degradation, as has been reported for CFTRΔF508,[15] resulting in a decrease in HA-BSEP^{R1231Q} expression at the cell surface. The ATP-dependent uptake of [^{3}H]-taurocholate ([^{3}H]-TC), into membrane vesicles isolated from HA-BSEP^{WT} and HA-BSEP^{R1231Q} HEK293T cells was almost linear up to 5 minutes (data not shown). Its uptake value per minute was about 97- and 4.8-fold greater in vesicles from HA-BSEP^{WT} and HA-BSEP^{R1231Q} HEK293T cells, respectively, than in vesicles from EV-HEK293T cells (Figure 1, G). Normalizing the BSEP expression levels in the membrane vesicles based on the results of the immunoblotting, in which the expression of HA-BSEP^{WT} was 25-fold greater than that of HA-BSEP^{R1231Q} (Figure 1, F), showed that the transport activity of [^{3}H]-TC mediated per unit mass of HA-BSEP^{R1231Q} molecules did not differ significantly from that mediated by HA-BSEP^{WT}.

Effect of 4PB Treatment on the Cell-Surface Expression and Transport Capacity of HA-BSEP^{R1231Q}

qPCR analysis and the cell-surface biotinylation study demonstrated that 4PB treatment for 24 hours at 1 mM, a clinically relevant concentration in patients with OTCD,[7,16] increased the cell surface expression of HA-BSEP^{WT} and HA-BSEP^{R1231Q} by 2.2- and 2.0-fold, respectively, in HEK293T cells without significantly changing the expression level of each mRNA (Figure 2, A and B). The expression level of HA-BSEP^{WT} and HA-BSEP^{R1231Q} and of their mRNAs was evaluated after normalization by that of the transferrin receptor (TfR) and of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, respectively, which were not affected by 4PB treatment (data not shown). Consistent with the cell surface biotinylation study, immunofluorescence analysis showed that HA-BSEP^{R1231Q} colocalized partially with the canalicular membrane marker, MRP2, in McA-RH7777 cells in the 4PB-treated condition (Figure 2, C). Treatment with 4PB also increased the expression of HA-BSEP^{WT} and HA-BSEP^{R1231Q} by 1.7 and 2.2 times, respectively, in membrane vesicles prepared from HEK293T cells expressing each form of BSEP (Figure 2, D). This was accompanied by an increase in the ATP-dependent uptake of [^{3}H]-TC. The HA-BSEP^{WT}- and HA-BSEP^{R1231Q}–mediated uptake of [^{3}H]-TC, which was calculated by subtracting the ATP-dependent uptake of [^{3}H]-TC into membrane vesicles of EV HEK293T cells from that of HA-BSEP^{WT} and HA-BSEP^{R1231Q} HEK293T cells, was increased by 1.6- and 2.0-fold, respectively (Figure 2, E). These results suggest that 4PB treatment at a clinically relevant dosage for humans could increase BSEP expression at the canalicular membrane in patients with PFIC2 with the c.3692G>A (p.R1231Q) mutation in *ABCB11* and, consequently, expand the capacity to secrete bile salt into bile.

Treatment with 4PB increased the cell surface expression of HA-BSEP^{R1231Q} in HEK293T cells cultured at 30°C to the same extent as in HEK293T cells cultured at 37°C (Figure 2, F). Considering that a low culture temperature around 30°C is capable of correcting the trafficking to the plasma membrane of the mutated misfolded protein,[7,17,18] this finding indicates that the process that modulates the trafficking of BSEP to the plasma membrane is unlikely to contribute to the mechanism underlying the increase in the cell surface expression of HA-BSEP^{R1231Q} with 4PB treatment.

Therapeutic Effect of 4PB in the Patient with PFIC2 with the c.3692G>A (p.R1231Q) Mutation in *ABCB11*

Serum liver tests and the itching score did not improve during the period of 4PB treatment at the dosages of 200 and 350 mg/kg/day. However, the serum level of aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) started to decrease when the dosage was increased to 500 mg/kg/day. Their values finally reached the range of 70-85 U/L, which is close to the reference range (AST, <55 U/L; ALT, <40 U/L; Figure 3, A). Consistent with the decrease in AST and ALT levels, the concentrations of total bilirubin (T-Bil) and direct bilirubin (D-Bil) decreased after the start of 4PB treatment at 500 mg/kg/day. One month after this dosage was started, both measurements were normalized to within the reference range (T-Bil, <18 μM; D-Bil, <5 μM; Figure 3, B). In contrast, the concentration of serum bile acids remained above the reference range (<10 μM) during the period of 4PB treatment, probably as the result of the coadministration of UDCA. The itching score was reduced concomitantly with

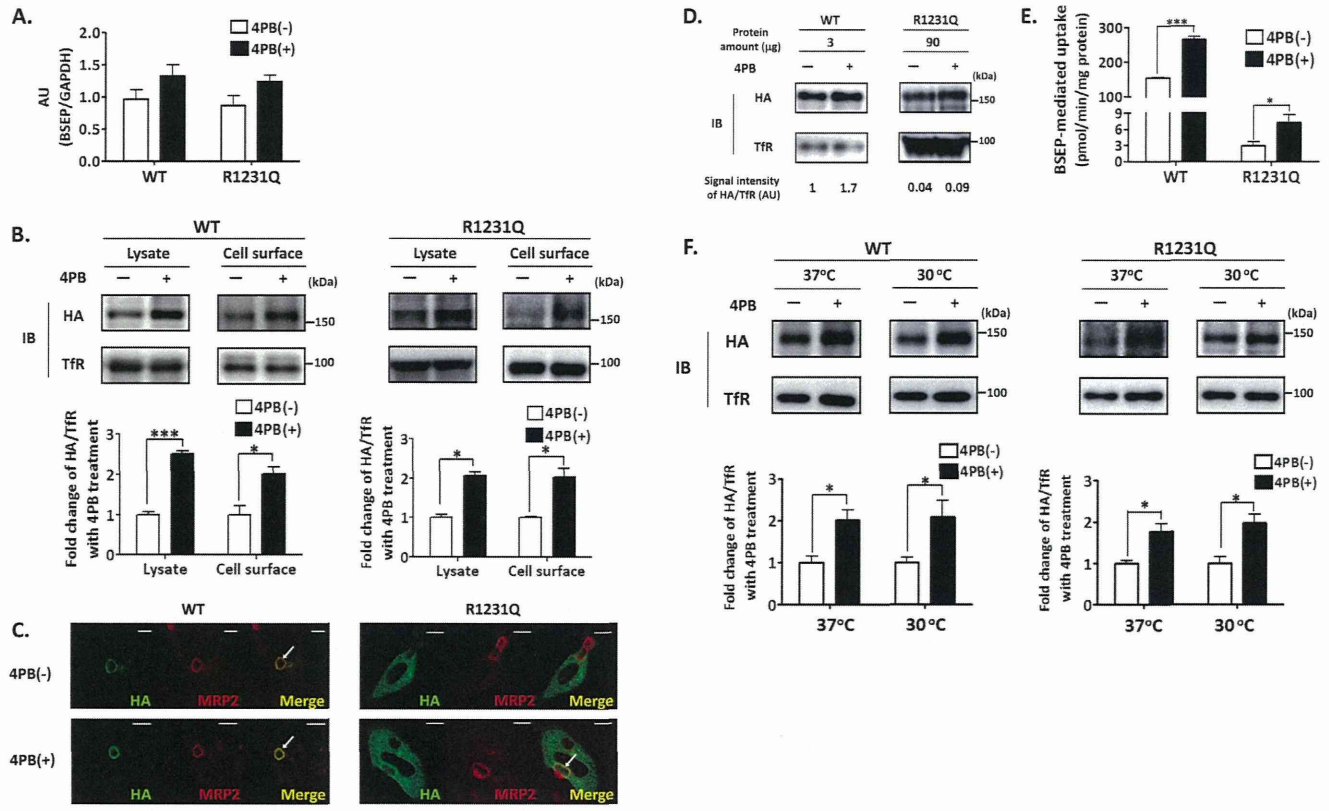

Figure 2. **A, B,** Effects of 4PB treatment on mRNA and protein expression levels of BSEP^{R1231Q}. HA-BSEP^{WT} (WT) and HA-BSEP^{R1231Q} (R1231Q) HEK293T cells were treated with or without 1 mM 4PB for 24 hour and subjected to **A,** qPCR and **B,** cell surface biotinylation and analyzed as described in Methods. **C,** Effect of 4PB on cellular localization of BSEP^{R1231Q}. McA-RH7777 cells expressing HA-BSEP^{WT} (WT, *left*) or HA-BSEP^{R1231Q} (R1231Q, *right*) were treated with or without 1 mM 4PB for 24 hours and then subjected to confocal immunofluorescence microscopy as described in **Figure 1**, **E.** *Arrows* in the merged images indicate co-localization. *Scale bar*: 10 μm. **D, E,** Effect of 4PB on transport of [^{3}H]-TC by BSEP^{R1231Q}. Membrane vesicles (**E,** 3 or 90 μg in and **D,** 10 μg in prepared from HA-BSEP^{WT} and HA-BSEP^{R1231Q} HEK293T cells after treatment with or without 1 mM 4PB were subjected to immunoblotting **D,** and an **E,** uptake assay as described in Effect of 4PB on BSEP^{R1231Q} expression at a low temperature. *Top*, HA-BSEP^{R1231Q} HEK293T cells were cultured for 24 hours at 37°C or 30°C in the presence or absence of 1 mM 4PB, biotinylated, and analyzed as described in the Methods. In **A-F,** a representative result from 2 or 3 independent experiments is shown. *Bars* represent the mean ± SE of each experiment in triplicate. $^{*}P < .05$, $^{***}P < .001$.

the improved results of liver tests (**Figure 3**, C-E). Almost complete and persistent relief of pruritus helped improve the child's ability to sleep and thus increased her quality of life. However, the itching score did not correlate significantly with the serum levels of autotaxin, bile acids, or autotaxin activity (**Figure 3**, C-E), all of which have been proposed as potential pruritogens in cholestasis.[19] No decrease in other possible pruritogens, the serum levels of histamine and tryptase, was observed as well (data not shown).

A liver biopsy was performed 4 months after the onset of 4PB therapy at the dosage of 500 mg/kg/day and studied with the specimens isolated for the diagnosis of PFIC2 before the enrollment in this intervention study. Histologic analysis showed that in the specimens obtained after 4PB therapy, giant cell transformation in the hepatic lobuli and the portal and lobular inflammation were relieved, compared with those before the therapy, and cholestasis almost disappeared (**Figure 4**, A). qPCR and immunoblot analysis demonstrated that in the patients with PFIC2, the liver *BSEP* mRNA expression before 4PB therapy in the patient with PFIC2 was almost the same as that in age-matched and adult control subjects, whereas the protein expression in the membrane fraction was approximately 2.5% of that in age-matched control subjects. 4PB therapy partly restored the reduced BSEP expression without increase in the mRNA expression, although the expression level of BSEP was still 12%-13% of that in age-matched control subjects (**Figure 4**, B and C). The specimens of the age-matched and adult control subjects were obtained from the pediatric patients without cholestasis when they underwent liver transplantation and the healthy organ donors. The expression level of BSEP mRNA and BSEP was evaluated after normalization by that of P-glycoprotein (P-gp)

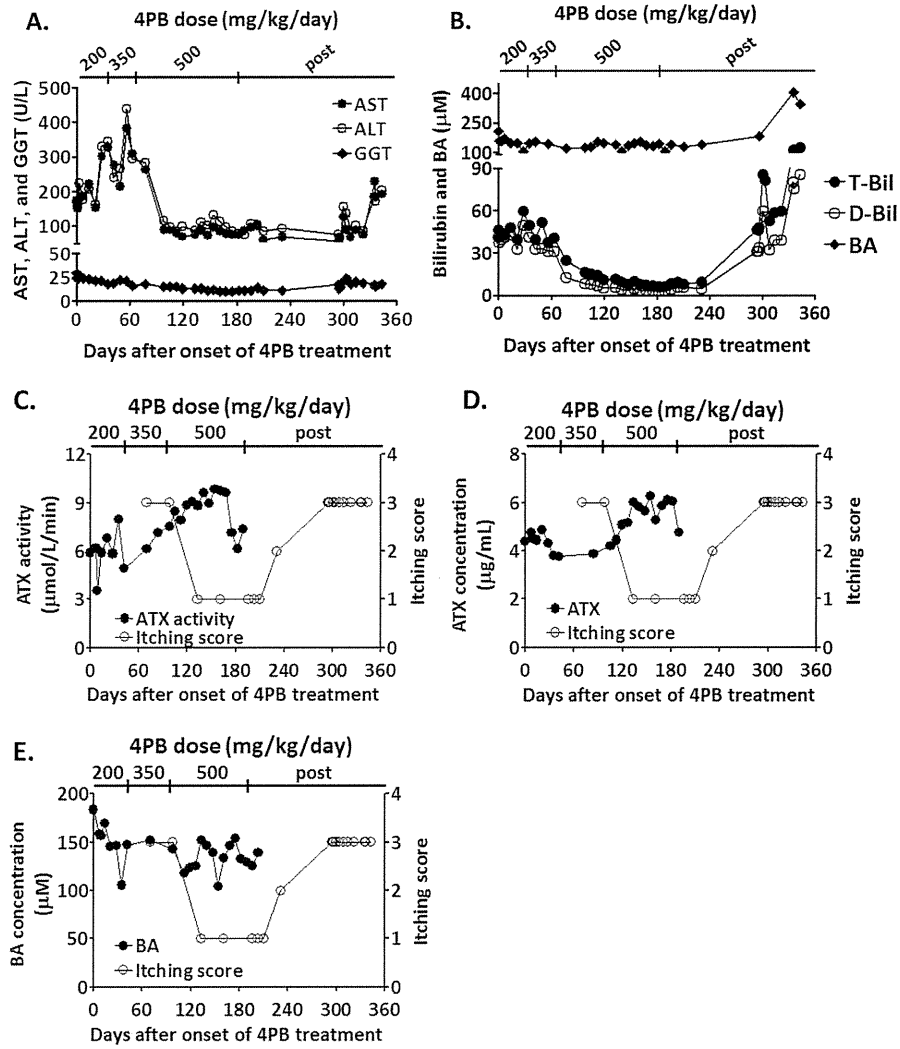

Figure 3. **A, B,** Levels of **A,** serum AST, ALT, GGT and **B,** T-Bil, D-Bil, and bile acid were monitored during and after the period of 4PB therapy. **C-E,** Correlation diagram of itching scores for the patient with PFIC2 with **C,** autotaxin activity and **D,** serum concentrations of autotaxin and **E,** bile acid in the patient during and after 4PB therapy. Pruritus severity was scored ranging from 0 (no pruritus) to 4 (cutaneous mutilation, with bleeding and scarring). *ATX*, autotoxin; *BA*, bile acid.

mRNA and of TfR, respectively, which were not affected by the treatment with 4PB.[7,10,16]

The liver tests and itching score remained unchanged 1.5 months after the end of 4PB therapy but gradually returned to values almost equal to those before her enrollment in this intervention study (Figure 3). During and up to 1.5 months after the end of 4PB therapy, the patient's height and weight continued to increase. No severe side effects were observed during or after 4PB therapy.

Discussion

PFIC2 is an autosomal-recessive, inherited liver disease resulting from mutations in *ABCB11*. Currently, partial external biliary diversion and liver transplantation are the most commonly used therapeutic procedures. Herein, we provide clinical evidence that 4PB therapy at a dosage of 500 mg/kg/day, a clinically relevant dosage used in patients with OTCD, biochemically and histologically improved liver functions and persistently relieved pruritus in a patient with PFIC2 who had decreased BSEP expression at the canalicular membrane but retained transport activity of BSEP per se. These findings suggest that 4PB therapy could help improve the quality of life of patients with this type of mutation in *ABCB11* and their parents. This beneficial effect of 4PB is supported further by our experimental evidence of the effect of 4PB on expression and transport activity of BSEP (Figure 5; available at www.jpeds.com). Gonzales et al[20] demonstrated that 4PB therapy improved liver tests in a patient with PFIC2 with a homozygous p.T1210P mutation in BSEP ($BSEP^{T1210P}$),[20] which markedly reduced BSEP

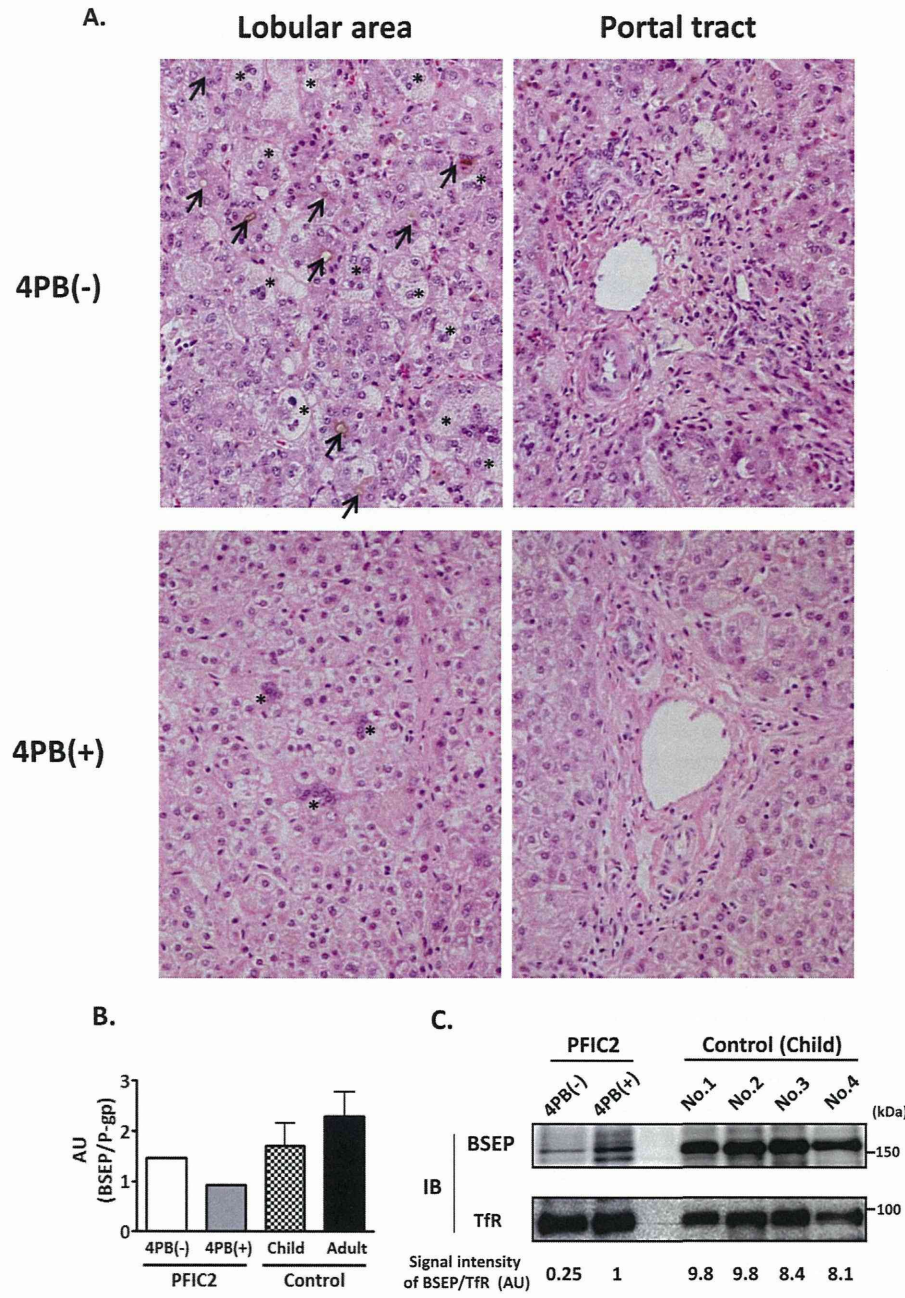

Figure 4. **A,** Histologic characteristics of liver sections. Liver sections prepared from the liver biopsy specimens of the patient with PFIC2 before and after the 4PB therapy were subjected to hematoxylin and eosin staining as described in the Methods. A typical image under each condition is shown. *Arrows* and *asterisks* indicate cholestasis and giant hepatocytes, respectively. Original magnification, ×200. **B, C,** mRNA and protein expression levels of BSEP. Liver biopsy specimens from the patient with PFIC2 before and after the 4PB therapy and from the control subjects were subjected to **B,** qPCR and **C,** immunoblotting and analyzed as described in the Methods. In **B,** the data were obtained from triplicate determination. Each *bar* represents the mean ± SE of individual specimens. *4PB(–)*, before 4 PB treatment; *4PB(+)*, after 4PB treatment.

expression at the canalicular membrane but did not abolish its transport activity (Figure 5).

The observed increase in BSEP expression after 4PB therapy in patients with PFIC2 with $BSEP^{R1231Q}$ or $BSEP^{T1210P230}$ suggests that the 4PB treatment restored biliary excretion of bile salts, relieved bile salt accumulation in hepatocytes, and thereby improved liver tests in these patients. The BSEP expression in the membrane fractions from liver specimens from the patient with PFIC2 with $BSEP^{R1231Q}$ and in the canalicular membrane of liver sections from the patient with PFIC2 with $BSEP^{T1210P}$ was still lower than that found in specimens of age-matched control patients even after treatment

with 4PB. It is conceivable that partial restoration of BSEP expression at the canalicular membrane is sufficient to fulfill the physiologic role of BSEP, leading to improvement in liver functions in the patients with PFIC2 with $BSEP^{R1231Q}$ or $BSEP^{T1210P}$. This finding is consistent with experimental evidence showing that liver pathology in mice with *mdr2* deficiency, which completely diminishes the biliary excretion of phosphatidylcholine, is markedly corrected by the relatively little expression of *MDR3*, a human orthologue of *mdr2*.[21] Alternatively, 4PB may have other as-yet-identified roles in combating intrahepatic cholestasis in addition to the increase in BSEP expression at the canalicular membrane.

We have provided experimental evidence suggesting that treatment with 4PB retards degradation of the canalicular membrane-resident forms of $BSEP^{WT}$, $BSEP^{E297G}$, and $BSEP^{D482G}$, the mutation found most frequently in patients with PFIC2, by interrupting the internalization process, which increases their expression at the canalicular membrane.[7,8,10] In this study, although we could not measure the degradation rate of cell surface-resident HA-$BSEP^{R1231Q}$ because of its low expression levels, treatment with 4PB had no significant effect on the level of mRNA expression of $BSEP^{R1231Q}$ and $BSEP^{T1210P}$ and was less likely to affect the trafficking to the plasma membrane of $BSEP^{R1231Q}$ (Figure 2, A, F; Figure 4, B; and Figure 5, A, E). Given that modulation of mRNA expression and trafficking to the plasma membrane is a mechanism that increases cell surface expression of both types of mutated BSEP, these results strongly suggest that degradation of $BSEP^{R1231Q}$ and $BSEP^{T1210P}$ trafficked to the canalicular membrane was inhibited with 4PB treatment, as is the case for $BSEP^{WT}$, $BSEP^{E297G}$, and $BSEP^{D482G}$, resulting in an increase in BSEP expression at the canalicular membrane in patients with PFIC2 with the respective mutations. Analysis using an in vitro minigene system has suggested that c.3692G>A (p.R1231Q) in *ABCB11* causes aberrant *ABCB11* splicing.[22] However, because BSEP was detected around 160 kDa, which is identical to the value in the control patients (Figure 4, C) and to the reported molecular weight,[9] it is likely that the correct splicing occurred to some degree in our patient despite having c.3692G>A (p.R1231Q) in *ABCB11* and its resultant protein product on the canalicular membrane was prevented from degradation by 4PB therapy. At present, we do not know the reason for the presence of some WT splicing in our patient, but this might be explained by the balance in the expression levels of splicing factors; for example, because the aberrant splice product associated with some missense mutation in *ABCB11* is abolished completely by transduction of SC35, but not of the other splicing factors assessed.[22]

In our patient with PFIC2, the pruritus disappeared concomitantly with the improvement in liver tests after the onset of 4PB therapy at 500 mg/kg/day. However, we observed no decrease in the factors suspected to be implicated in causing the itch (Figure 3, C-E). 4PB and/or its metabolites may modulate the local concentrations of these pruritogens, which may not have been detected by systemic measurements. The physiologic function of autotaxin, an enzyme secreted extracellularly that generates lysophosphatidic acid, is thought to be mediated predominantly by activation of G protein-coupled receptors (GPCRs).[23] TGR5, a GPCR activated by bile salts, on sensory nerves could contribute to bile salt-induced itching[24]; if so, 4PB and/or its metabolites might antagonize the GPCRs responsible for itch signaling and therefore attenuate activation of sensory neurons. Alternatively, 4PB therapy may affect unidentified pruritogens or antipruritogens.

In conclusion, our study demonstrated that 4PB therapy provides a favorable outcome—improvement in liver function and relief of refractory itching—in a patient with PFIC2 with decreased BSEP expression at the canalicular membrane but with retained BSEP transport activity. This therapeutic effect of 4PB and its safety should be validated by future clinical studies with more patients and for longer periods than was possible in this study. ■

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Reprint requests: Hisamitsu Hayashi, PhD, Laboratory of Molecular Pharmacokinetics, Department of Medical Pharmaceutics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: hayapi@mol.f.u-tokyo.ac.jp or Hiroyuki Kusuhara, PhD, Laboratory of Molecular Pharmacokinetics, Department of Medical Pharmaceutics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: kusuhara@mol.f.u-tokyo.ac.jp

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Appendix

4PB for the in vitro studies was purchased from Sigma-Aldrich (St Louis, Missouri). AMMONAPS, a pharmaceutical grade of 4PB for the treatment of a PFIC2 patient, was purchased from Swedish Orphan Inter AB. [^{3}H]-TC (2 Ci/mmol) was obtained from American Radiolabeled Chemicals (St Louis, Missouri). Anti-HA antibody was obtained from Roche Diagnostics (Mannheim, Germany). Antibodies against BSEP for immunoblotting, Na^+, K^+-ATPase α1 subunit, and calnexin were purchased from Abcam (Cambridge, United Kingdom). Antibodies against TfR and Alexa Fluor secondary antibodies were obtained from Invitrogen (Carlsbad, California). Anti-BSEP, ATP8B1, and MDR3 antibodies for immunohistochemistry were purchased from Santa Cruz Biotech (Dallas, Texas), Sigma-Aldrich, and Enzo Life Sciences (Plymouth Meeting, Pennsylvania), respectively. All other chemicals were of analytical grade.

Cell Culture. HEK293 T cells and McA-RH7777 cells were purchased from the American Type Culture Collection (ATCC number: CRL-11268 [HEK293 T] and CRL-1601 [McA-RH7777], Manassas, Virginia). HEK293 T cells and McA-RH7777 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 and 95% humidity.

Measurement of BSEP mRNA Level. HEK293 T cells were seeded in 24-well plates at a density of 2.5×10^5 cells per well; transfected with pShuttle containing HA-BSEP^{WT}, HA-BSEP^{R1231Q}, or HA-BSEP^{T1210P} cDNA or EV using FuGENE HD (Promega); and treated with or without 1 mM 4PB for 24 hours. Total RNA was isolated using Isogen II (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. Total RNA from the liver specimens of humans was isolated using an RNeasy Mini Kit (QIAGEN).

Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. BSEP, P-gp, and GAPDH mRNA levels were measured by qPCR using a LightCycler instrument and the appropriate software (Ver. 3.53; Roche Diagnostics) as described previously.[1] qPCR was performed using the following primers: 5′-TGCCCAGTGC ATCATGTTTA-3′ and 5′-CCCTGGAAGTTGTCCCATTT-3′ (BSEP), 5′-GGCCAACATACATGCCTTCATCGAG-3′ and 5′-TGTCCAGGGCTTCTTGGACAACC-3′ (P-gp), and 5′-GGGGAGCCAAAAGGGTCATCATCT-3′ and 5′-GACGCC TGCTTCACCACCTTCTTG-3′ (GAPDH). *BSEP* gene expression in each reaction was normalized by the expression of *GAPDH* in HEK293T cells or *P-gp* in human liver specimens as appropriate.

Cell Surface Biotinylation. HEK293T cells were seeded at a density of 5.0×10^5 cells per well in 6-well plates coated with poly-L-lysine and poly-L-ornithine; transfected with pShuttle containing HA-BSEP^{WT}, HA-BSEP^{R1231Q}, or HA-BSEP^{T1210P} cDNA or EV using FuGENE HD; and treated with or without 1 mM 4PB for 24 hours. Forty-eight hours after the transfection, cell surface biotinylation was performed to investigate the expression on the plasma membrane as described previously.[2] The isolated biotinylated proteins were subjected to immunoblotting.

Preparation of and Transport Assays with Membrane Vesicles. HEK293T cells were seeded in a 15-cm dish at a density of 6.0×10^6 cells; transfected with pShuttle containing BSEP^{WT}, BSEP^{R1231Q}, or BSEP^{T1210P} cDNA or EV using FuGENE HD; and treated with or without 1 mM 4PB for 24 hours. The membrane vesicles were prepared 48 hours after transfection as described previously.[3] The isolated membrane vesicles were subjected to immunoblotting and transport assays. Transport assays were performed using the rapid filtration method reported previously.[3] Membrane vesicles were incubated at 37°C with [^{3}H]-TC (1 mM) for 5 min in the presence of 5 mmol/L ATP or AMP. ATP-dependent uptake of ligand was obtained by subtracting the value in the absence of ATP from that in its presence. To calculate BSEP-mediated uptake of [^{3}H]-TC, the value of its ATP-dependent uptake by the membrane vesicles of EV HEK293T cells was subtracted from that of HA-BSEP^{WT}, HA-BSEP^{R1231Q}, or HA-BSEP^{T1210P} HEK293T cells.

Preparation of Crude Membrane Fractions from Human Liver. Human liver specimens were homogenized in hypotonic buffer (1 mM EDTA, 5 mM sodium phosphate, pH 7.0) supplemented with protease inhibitor cocktail using QIAshredder (QIAGEN), and then centrifuged at $800 \times g$ for 10 minutes at 4°C. The supernatant was ultracentrifuged at $100\,000 \times g$ for 1 hour at 4°C, and the pellet was dissolved in lysis buffer comprising hypotonic buffer with 1% Triton-X.

Immunoblotting. Specimens were loaded into each well of a 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis plate with a 3.75% stacking gel and subjected to immunoblotting as described previously.[2] Immunoreactivity was detected with an ECL Advance Western Blotting Detection Kit (Amersham Biosciences, Piscataway, New Jersey). The intensity of the band was quantified using Multi Gauge software (ver. 2.0; Fuji Film). Expression of BSEP and each form of HA-BSEP was normalized by the expression of TfR or Na^+, K^+-ATPase a1 subunit as appropriate.

Immunocytochemistry. HEK293T cells and McA-RH7777 cells were transfected with pShuttle containing HA-BSEP^{WT}, HA-BSEP^{R1231Q}, or HA-BSEP^{T1210P}; seeded on glass coverslips (Matsunami Glass Ind Ltd, Osaka, Japan) in 12-well plates at a density of 2.5×10^5 cells per well; and treated with or without 1 mM 4PB for 24 hours. The cells were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 10 minutes, permeabilized in 0.1% saponin/PBS for 10 minutes, blocked with 3% bovine serum

albumin/PBS for 30 minutes, and stained with anti-HA and anticalnexin (endoplasmic reticulum marker) or anti-Na^+, K^+-ATPase α1-subunit (HEK293T cells)/anti-MRP2 (McA-RH7777 cells; plasma membrane marker) for 2 hours. The cells were then stained with Alexa Fluor 488 donkey anti-rat immunoglobulin G and Alexa Fluor 546 donkey anti-rabbit immunoglobulin G or Alexa Fluor 546 donkey antimouse immunoglobulin G for 1 hour. These staining procedures were performed at room temperature. The cells were mounted onto glass slides with VECTASHIELD mounting medium (Vector Laboratories Inc, Burlingame, California) and then visualized by confocal microscopy using a Leica TCS SP5 II laser scanning confocal microscope (Leica, Solms, Germany).

Immunohistochemistry. Four-micrometer-thick sections embedded in paraffin were adhered to coated glass slides and stained with VENTANA BenchMark ULTRA fully automated staining instrument (Ventata Medical Systems, Inc, Tucson, Arizona) with anti-BSEP antibody and Leica BOND-III (Leica), the fully automated immunohistochemistry and in situ hybridization staining system, with antibody against MDR3 or ATP8B1 according to the manufacturer's instructions.

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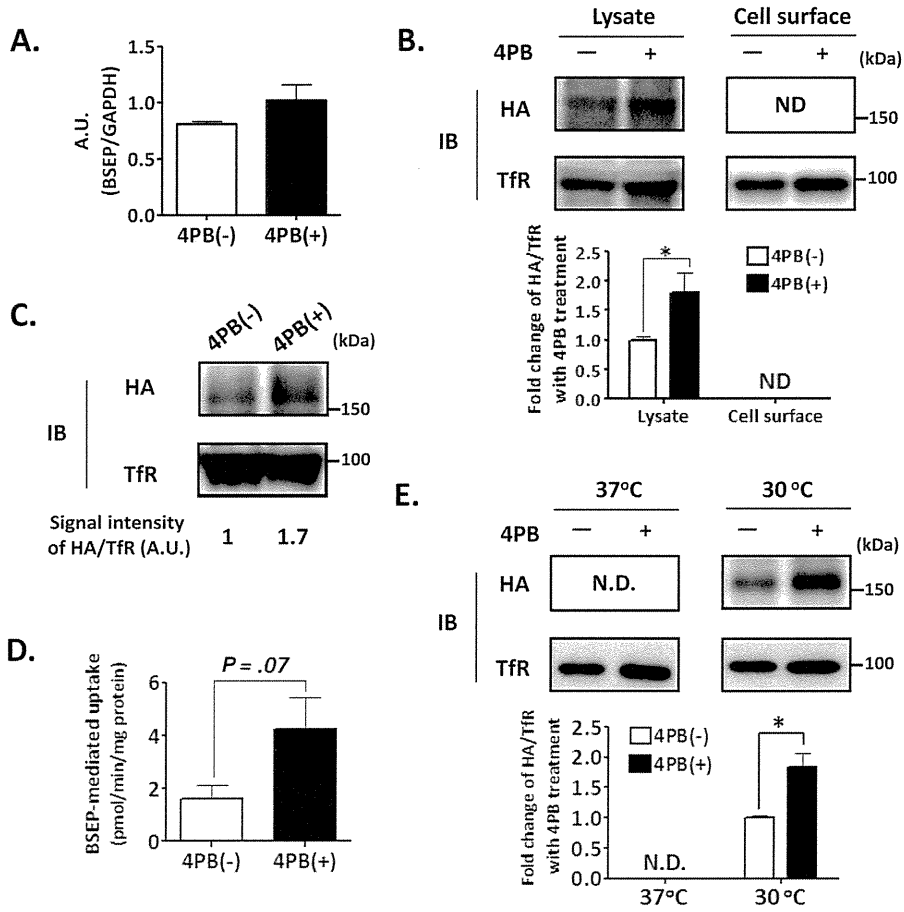

Figure 5. **A, B,** Effects of 4PB treatment on mRNA and protein expression of BSEP^{T1210P}. HA-BSEP^{T1210P} HEK293T cells were treated with or without 1 mM 4PB for 24 hours and subjected to **A,** qPCR and **B,** cell-surface biotinylation and then analyzed as described in Figure 2, A and B. **C, D,** Effect of 4PB on transport of [^{3}H]-TC by BSEP^{T1210P}. Membrane vesicles (**C,** 90 μg in and **D,** 10 μg in were prepared from HA-BSEP^{T1210P} HEK293T cells after treatment with or without 1 mM 4PB and were subjected to **C,** immunoblotting and **D,** the uptake assay as described in Figure 2, D and E. **E,** Effect of 4PB on BSEP^{T1210P} expression at a low temperature. Top, HA-BSEP^{T1210P} HEK293T cells were cultured for 24 hours at 37°C or 30°C, with or without 1 mM 4PB, biotinylated, and analyzed as described in Figure 2, F. In **A-E,** a representative result from 2 independent experiments is shown. *Bars* represent the mean ± SE of each experiment performed in triplicate. $^*P < .05$.

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特集II 門脈圧亢進症の治療法の選択とその成績

肝臓への血小板集積は肝硬変における脾摘の効果に関与するか*

近藤礼一郎**,***,****
矢野博久**
鹿毛政義***,****

Key Words : thrombocytopenia, platelet, transforming growth factor-beta (TGF-β), megakaryocyte

はじめに

末梢血血小板数減少は，慢性肝炎・肝硬変の重要な合併症である．慢性肝炎・肝硬変に伴う末梢血血小板減少の発生機序としては，脾臓への血小板のpooling[1]や脾臓での血球破壊の亢進[2]，骨髄での産生低下[3]など複数の病態が報告されている．最近，われわれは，慢性肝炎・肝硬変において，肝臓内にみられる血小板は肝線維化に伴い漸増し，肝臓への血小板集積も慢性肝炎・肝硬変に伴う末梢血血小板減少の重要な病態であることを報告した[4]．慢性肝炎・肝硬変に伴う末梢血血小板減少の治療として，脾機能亢進症を有する症例には脾臓摘出手術(脾摘)[5]や部分的脾動脈塞栓術(PSE)[6]が用いられているが，その治療成績と肝臓への血小板集積の関係は検討されていない．

また，近年，脾摘やPSEは，末梢血血小板減少の改善だけでなく，肝機能や肝線維化の改善にも寄与することが報告されている[7)~10)]．最近のわれわれの検討では，脾機能亢進症を有する肝硬変症例7例に対して脾摘時と脾摘後に肝生検を行い，4例で脾摘後に肝線維化の改善が病理組織学的に確認された[10]．慢性肝炎・肝硬変の病態における脾機能亢進症は，末梢血血小板減少だけに止まらず，重要な意味を持つことが示唆されている．

脾機能亢進症と肝線維化については，脾臓でのtransforming growth factor-β1(TGF-β1)の産生が関与していると報告されている[7)~9)]．肝硬変での脾機能亢進症では，脾臓でのTGF-β1産生が増加し，産生されたTGF-β1は門脈血を介して肝線維化に寄与しているとされている[7)]．脾臓でのTGF-β1産生にはマクロファージの関与が報告されているが，十分には明らかにされていない[7)8)]．

本研究では，肝臓への血小板集積が，肝硬変による末梢血血小板減少に対する脾摘の効果に関与するかを検討した．さらに，脾臓におけるTGF-β1発現について病理学的な検討を行った．

方　法

肝細胞癌のため一期的に肝切除と脾摘を行った脾機能亢進症合併肝硬変症例12例(C型肝炎：9例，B型肝炎：1例，自己免疫性肝炎：1例，非アルコール性脂肪性肝炎：1例，平均年齢：61±9歳)を対象として用いた．なお，コントロールとして慢性肝炎，脾腫，末梢血血小板減少を伴わない外

* Accumulation of platelets in the liver may be an important contributing factor to the therapeutic efficacy of splenectomy in patients with liver cirrhosis.

** Reiichiro KONDO, M.D., Ph.D. & Hirohisa YANO, M.D., Ph.D.: 久留米大学医学部病理学講座〔〒830-0011 福岡県久留米市旭町67〕; Department of Pathology, Kurume University School of Medicine, Kurume, Fukuoka 830-0011, JAPAN
*** Masayoshi KAGE, M.D., Ph.D.: 久留米大学病院病理部
**** 久留米大学病院腫瘍センター

表1 血液検査所見まとめ

	ALT<38 IU/l(n=5)	ALT≧38 IU/l(n=7)
末梢血白血球数(/μl)		
脾摘前	2,960±829(2,100～4,300)	2,886±570(2,100～4,000)
脾摘3か月後	5,520±3,014(3,300～10,400)	5,028±1,490(2,400～6,700)
末梢血赤血球数(/μl)		
脾摘前	365±56(273～417)	403±45(343～476)
脾摘3か月後	333±56(255～377)	384±60(324～503)
末梢血血小板数(×10^4/mm^3)		
脾摘前	5.6±1.5(4.4～8)	6±1.6(3.1～7.7)
脾摘3か月後	26±7.5(17.4～35.8)	19.4±7.9(9.2～29.7)
ALT(IU/l)		
脾摘前	13±7(6～24)	59±19(38～89)
脾摘3か月後	18±10(8～32)	48±17(25～72)
T. bil(mg/dl)		
脾摘前	1.1±0.5(0.5～1.7)	1.1±0.3(0.7～1.5)
脾摘3か月後	0.7±0.2(0.4～0.9)	0.9±0.5(0.5～1.9)
PT(%)		
脾摘前	76±8(68～89)	82±11(69～99)
脾摘3か月後	81±15(65～100)	74±10(61～89)
Alb(g/dl)		
脾摘前	3.6±0.2(3.3～3.8)	3.8±0.5(3.1～4.5)
脾摘3か月後	3.6±0.4(3～4)	3.7±0.5(3.2～4.6)

科切除症例(肝臓5例，脾臓9例)を用いた．

1．臨床的検討

対象において，脾摘前と脾摘3か月後の血液検査所見を検討した．

2．病理組織学的検討

全症例の肝組織に対して，ヘマトキシリン・エオジン(HE)染色を行い，新犬山分類を用いて肝線維化と炎症の評価を行った．

次に，すべての肝組織と脾組織にCD41(αIIb integrin)とTGF-β1の免疫組織化学を行い，組織内の血小板，巨核球とTGF-β1発現を病理学的に検討した．CD41は血小板，巨核球に対する特異的な表面マーカーである[11]．

また，鏡検下で肝組織から門脈域周囲5か所，脾組織から赤脾髄5か所を無作為に選択し，血小板面積とTGF-β1陽性面積を画像解析ソフト(Win ROOF software package version 6.1, Mitani Corporation, Fukui, Japan)を用いて測定した．

結　果

1．臨床的検討

対象の血液検査所見のまとめを表1に示す．術前の血中ALT値が基準値である38 IU/l未満であった症例(ALT基準値内症例)は5例で，術前の血中ALT値が38 IU/l以上であった症例(高ALT症例)は7例であった．ALT基準値内症例，高ALT症例のいずれにおいても脾摘後に末梢血血小板数が改善したが，ALT基準値内症例と高ALT症例を比較すると，高ALT症例はALT基準値内症例より脾摘3か月後の末梢血血小板数改善が乏しかった(図1)(末梢血血小板数，脾摘前 → 脾摘3か月後，6±1.6×10^4/mm^3 → 19.4±7.9×10^4/mm^3 vs. 5.6±1.5×10^4/mm^3 → 26±7.5×10^4/mm^3，P=0.14)．

2．病理組織学的検討

(1)肝臓の線維化と炎症

対象の全症例で，肝組織にはびまん性に，小葉のひずみを伴う線維性架橋形成がみられた(新犬山分類F4)．肝組織にみられる炎症は，高ALT症例の多くに門脈域の限界板を破壊した炎症がみられ，高ALT症例にみられる炎症はALT基準値内症例より有意に高度であった(図2)(新犬山分類A，2±1 vs. 1±1，P=0.01)．

コントロールの肝組織には，肝線維化や壊死炎症反応はみられなかった(新犬山分類F0 A0)．

(2)肝組織内の血小板と巨核球

コントロールを含む全症例で肝組織の類洞に血小板が観察された．コントロールでは，肝小葉の類洞に散在性に血小板がみられたが，対象では炎症が強い門脈域周囲の類洞に血小板が集積していた．高ALT症例では，炎症によって破壊された門脈域の限界板に沿って，またはその周囲の類洞に血小板の集積がみられた(図3)．肝組織内の血小板面積は，高ALT症例がALT基準値内症例より大きかった(図3)(15,217±8,058 μm² vs. 8,745±5,970 μm²，P=0.16)．対象とコントロールを含めて，肝組織内に巨核球はまったく認めなかった．

(3)脾組織内の血小板と巨核球

コントロールを含む全症例の脾組織で，血小板は赤脾髄の脾洞および脾索に，びまん性にみられた．また，対象12例のうち，9例(75%)で赤脾髄の脾索に巨核球を認めた．コントロールの脾組織に巨核球は認めなかった．脾組織内の血小板面積は，高ALT症例がALT基準値内症例より小さかった(図4)(50,652±22,250 μm² vs. 69,203±16,033 μm²，P=0.1)．

(4)肝脾組織でのTGF-β1発現

対象の全症例で，肝および脾組織にTGF-β1の発現がみられた．対象の肝組織におけるTGF-β1発現は，線維性に拡大した門脈域および線維性隔壁に沿ってみられた(図5)．対象の脾組織におけるTGF-β1発現は，主に赤脾髄の脾洞および脾索を構成する細胞に，びまん性にみられた(図5)．

(5)脾臓内の巨核球とTGF-β1発現

CD41とTGF-β1の二重免疫組織化学では，対象

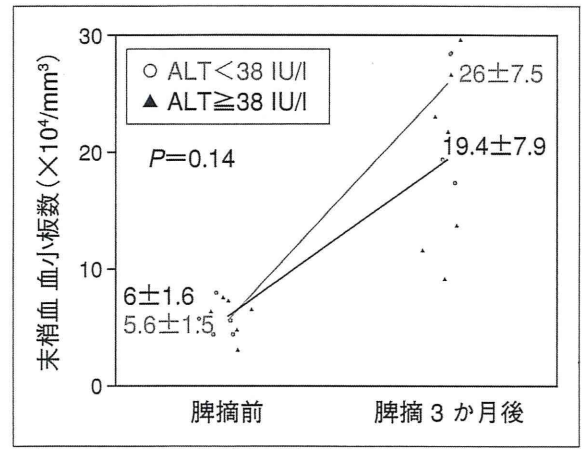

図1　脾摘前後での末梢血血小板数の推移
術前の血中ALT値が基準値(38 IU/l)以上であった7例(高ALT症例)は，ALT基準値内症例より脾摘3か月後の末梢血血小板数の改善が乏しい傾向にあった(末梢血血小板数，脾摘前→脾摘3か月後，6±2 mm³ → 19±8 mm³ vs. 6±1 mm³→26±8 mm³，P=0.14)．

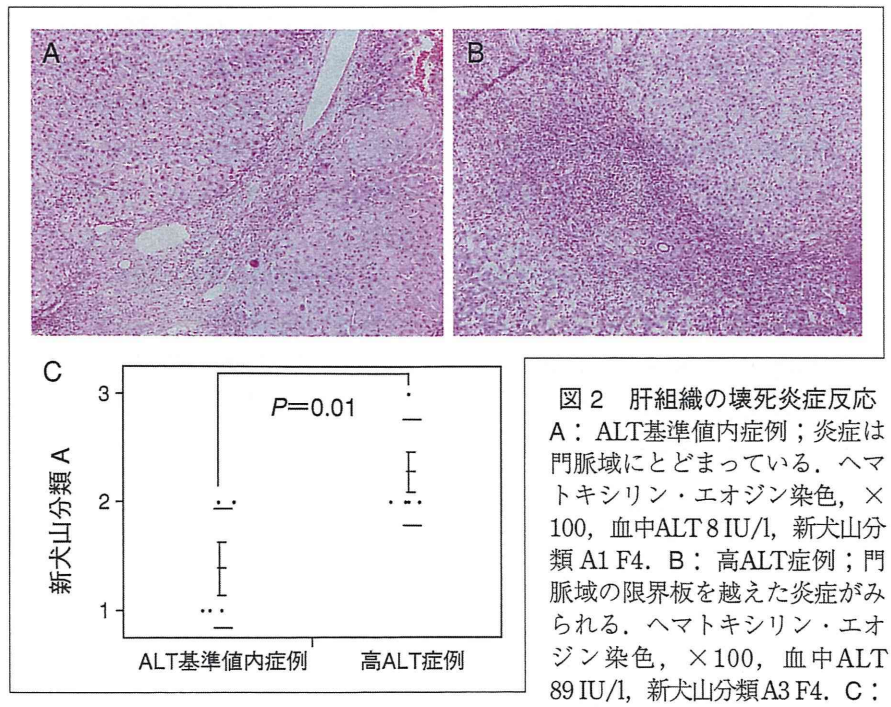

図2　肝組織の壊死炎症反応
A：ALT基準値内症例；炎症は門脈域にとどまっている．ヘマトキシリン・エオジン染色，×100，血中ALT 8 IU/l，新犬山分類 A1 F4．B：高ALT症例；門脈域の限界板を越えた炎症がみられる．ヘマトキシリン・エオジン染色，×100，血中ALT 89 IU/l，新犬山分類A3 F4．C：高ALT症例にみられる炎症はALT基準値内症例より有意に高度であった(新犬山分類 A，2±1 vs. 1±1，P=0.01)．

の脾組織において，赤脾髄の脾索にTGF-β1を発現した巨核球を認めた(図6)．脾組織内のTGF-β1陽性面積は，脾臓内に巨核球を認める症例が巨核球を認めない症例より有意に大きかった(図6)($P<0.01$)．

考　察

慢性肝炎・肝硬変の一般診療には，インター

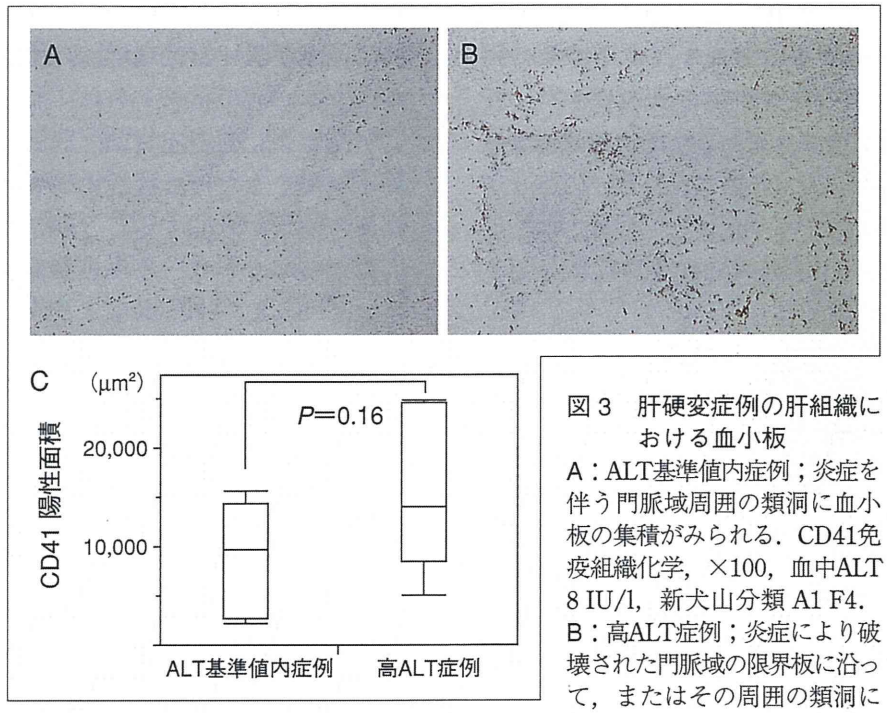

図3　肝硬変症例の肝組織における血小板
A：ALT基準値内症例；炎症を伴う門脈域周囲の類洞に血小板の集積がみられる．CD41免疫組織化学，×100，血中ALT 8 IU/l，新犬山分類 A1 F4．B：高ALT症例；炎症により破壊された門脈域の限界板に沿って，またはその周囲の類洞に血小板の集積がみられる．CD41免疫組織化学，×100，血中ALT 89 IU/l，新犬山分類 A3 F4．C：肝組織内の血小板面積は，高ALT症例がALT基準値内症例より大きい傾向にあった(15,217±8,058 μm² vs. 8,745±5,970 μm²，$P=0.16$)．

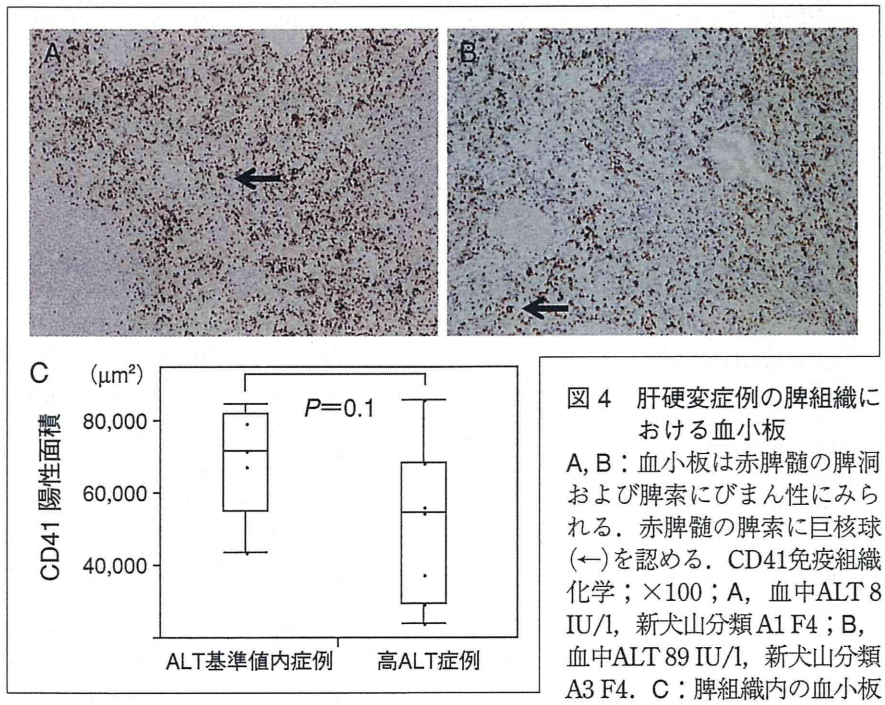

図4　肝硬変症例の脾組織における血小板
A, B：血小板は赤脾髄の脾洞および脾索にびまん性にみられる．赤脾髄の脾索に巨核球(←)を認める．CD41免疫組織化学；×100；A，血中ALT 8 IU/l，新犬山分類A1 F4；B，血中ALT 89 IU/l，新犬山分類A3 F4．C：脾組織内の血小板面積は，高ALT症例がALT基準値内症例より低い傾向にあった(50,652±22,250 μm² vs. 69,203±16,033 μm²，$P=0.1$)．

フェロン治療などの抗ウイルス治療あるいは胃食道静脈瘤や肝細胞癌に対する観血的治療が含まれ，慢性肝炎・肝硬変に伴う末梢血血小板減少は治療方針を左右する大きな要因となる．

本研究では，肝硬変における肝臓，脾臓への血小板の分布を病理組織学的に明らかにし，肝硬変に伴う末梢血血小板減少に対する脾摘の効果と肝臓への血小板集積について検討した．本研究で，対象の高ALT症例は，ALT基準値内症例より脾摘後の末梢血血小板数の改善が乏しかった．高ALT症例は，ALT基準値内症例より肝組織に高度の壊死炎症反応がみられ，肝組織内により多くの血小板を認めた．肝組織における血小板は炎症が強い門脈域周囲の類洞に集積がみられた．本研究の結果からは，肝臓の壊死炎症反応が高度な肝硬変症例は，肝臓に血小板がより多く集積するため，脾摘による末梢血血小板数改善の効果が減弱することが示唆された．

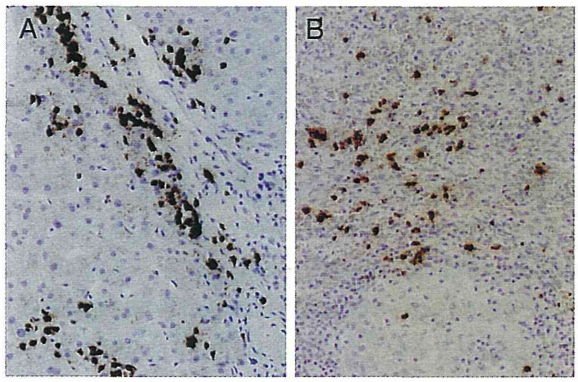

図5　肝硬変症例の肝脾組織におけるTGF-β1発現
A：肝組織；線維性に拡大した門脈域および線維性隔壁に沿ってTGF-β1の発現がみられる．TGF-β1免疫組織化学，×200．B：脾組織；主に赤脾髄の脾洞および脾索を構成する細胞に，TGF-β1の発現がびまん性にみられる．TGF-β1免疫組織化学，×200．

血管内において，血小板は血管内皮細胞が傷害を受けると，フォンヴィレブランド因子(von Willebrand factor；vWF)を介してコラーゲンなどの細胞外基質に接着し，偽足を伸展させ扁平に形態変化する．さらに，強固に粘着するため血小板の膜糖蛋白であるGP IIb/IIIa(integrin αIIbβ3)が活性化する[12]．肝組織においても血管内と同様に，類洞内皮細胞が傷害されると血小板が内皮細胞に粘着することが報告されている[13]．

血小板シンチグラフを用いた検討では，Kinuyaら[14]が，脾臓と肝臓に分布する血小板の比(spleen/

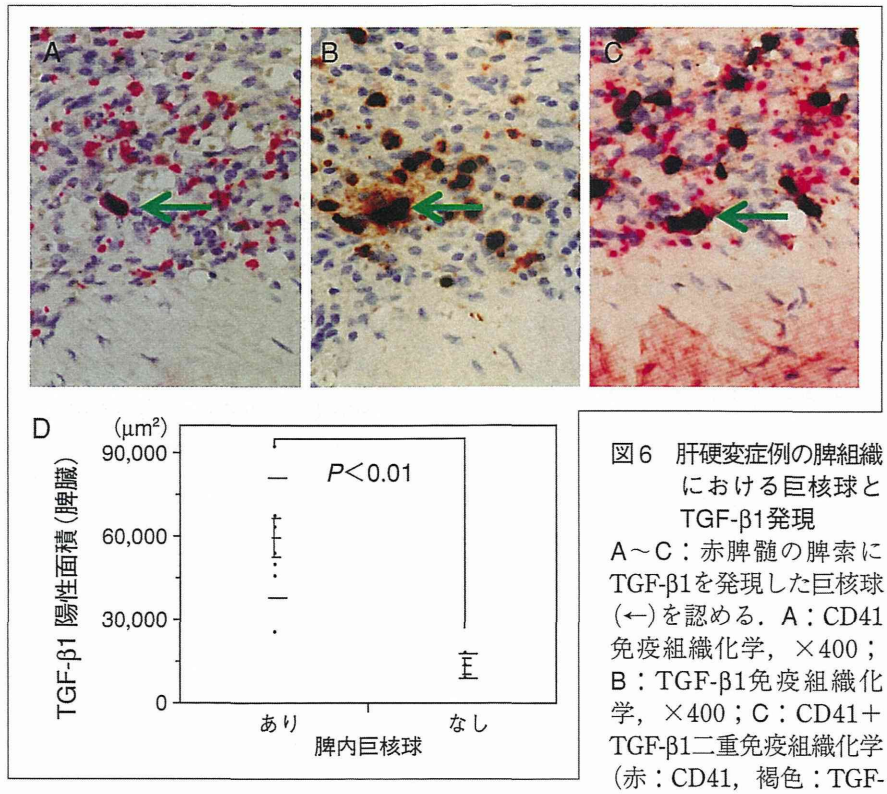

図6　肝硬変症例の脾組織における巨核球とTGF-β1発現
A～C：赤脾髄の脾索にTGF-β1を発現した巨核球(←)を認める．A：CD41免疫組織化学，×400；B：TGF-β1免疫組織化学，×400；C：CD41＋TGF-β1二重免疫組織化学(赤：CD41，褐色：TGF-β1)，×400．D：脾組織内のTGF-β1陽性面積は，脾臓内に巨核球を認める症例が巨核球を認めない症例より有意に大きかった($P<0.01$)．