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Generation of a bile salt export pump
deficiency model using patient-specific
induced pluripotent stem cell-derived
hepatocyte-like cells

(疾患特異的 iPS 細胞由来分化誘導肝細胞を用いた
進行性家族性肝内胆汁うっ滞症 2 型の病態モデル)

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Generation of a bile salt export pump deficiency model using patient-specific induced pluripotent stem cell-derived hepatocyte-like cells

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Bile salt export pump (BSEP) plays an important role in hepatic secretion of bile acids and its deficiency results in severe cholestasis and liver failure. Mutation of the *ABCB11* gene encoding BSEP induces BSEP deficiency and progressive familial intrahepatic cholestasis type 2 (PFIC2). Because liver transplantation remains standard treatment for PFIC2, the development of a novel therapeutic option is desired. However, a well reproducible model, which is essential for the new drug development for PFIC2, has not been established. Therefore, we attempted to establish a PFIC2 model by using iPSC technology. Human iPSCs were generated from patients with BSEP-deficiency (BD-iPSC), and were differentiated into hepatocyte-like cells (HLCs). In the BD-iPSC derived HLCs (BD-HLCs), BSEP was not expressed on the cell surface and the biliary excretion capacity was significantly impaired. We also identified a novel mutation in the 5'-untranslated region of the *ABCB11* gene that led to aberrant RNA splicing in BD-HLCs. Furthermore, to evaluate the drug efficacy, BD-HLCs were treated with 4-phenylbutyrate (4PBA). The membrane BSEP expression level and the biliary excretion capacity in BD-HLCs were rescued by 4PBA treatment. In summary, we succeeded in establishing a PFIC2 model, which may be useful for its pathophysiological analysis and drug development.

Bile salt export pump (BSEP) is a key molecule for the generation of bile flow in humans¹. BSEP deficiency causes severe intrahepatic cholestasis and liver failure. Progressive familial intrahepatic cholestasis (PFIC) is one of the cholestatic diseases in children, and PFIC type 2 (PFIC2) is a form of infantile cholestatic disorder that

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occurs despite normal serum levels of gamma glutamyl transferase (GGT)². PFIC2 is caused by mutations in the *ABCB11* gene that encodes BSEP³. Certain mutations cause deficiency in the membrane expression of BSEP owing to accelerated proteasome-mediated degradation^{4–7}. The main clinical features of BSEP-deficiency are severe jaundice, pruritus, and intrahepatic cholestasis, followed by liver failure and juvenile hepatobiliary carcinoma^{2,8,9}. While orthotopic liver transplantation (OLT) is the major curative approach for BSEP-deficiency^{2,10}, some BSEP-deficient patients treated with OLT experience a relapse of intrahepatic cholestasis due to the presence of autoimmune antibodies against BSEP^{11,12}. Therefore, it is important to elucidate the pathophysiology of PFIC2 in order to develop novel therapies for its treatment.

Knockout mouse models are often used for the elucidation of disease mechanisms. However, *Bsep/Abcb11*-knockout mice are not a valid model for reproducing PFIC2 phenotypes because of species differences in the functions of hepatic transporters¹³. Specifically, multiple drug resistance 1 (Mdr1) compensates for the biliary excretion functions of Bsep in mice, whereas no transporter compensates for these functions in humans¹⁴. To analyze the functions of mutated human *ABCB11*, BSEP-overexpressing cell lines are used widely. BSEP-overexpressing cells are useful for analyzing bile acid kinetics mediated by mutated BSEP, but not for analyzing the function of BSEP having mutations in the non-coding region of the *ABCB11* or other genes. In fact, one-third of patients with PFIC harboring normal GGT do not have mutations in *ABCB11* or *ATP8B1* genes^{15,16}. In addition, some *ABCB11* mutations result in disruption of pre-mRNA splicing¹⁷. To perform pathological analysis of these mutations, primary human hepatocytes should be obtained from patients¹⁸. Therefore, a novel *in vitro* disease model containing the whole genome information of patients with BSEP-deficiency is necessary to further elucidate the disease mechanisms and BSEP regulation.

Human induced pluripotent stem cells (iPSCs) can be obtained by reprogramming somatic cells¹⁹. Patient-specific iPSCs and their derivatives are expected to offer novel disease models²⁰. Moreover, the iPSC technology has already shown great potential for the discovery of new therapies against several diseases²¹. The utilization of patient-specific iPSCs and their derivatives would be advantageous for generating a disease model in humans, because the phenotype can be evaluated without consideration of species differences. In this study, human iPS cell-derived hepatocyte-like cells (HLCs) were generated from patients with BSEP-deficiency. As we have already established a highly efficient hepatocyte differentiation protocol, an almost homogenous hepatocyte population (more than 80%) could be generated from human iPS cells independently of iPS cell lines^{22,23}. In this study, human iPSC lines were established from two patients with BSEP-deficiency (BD-iPSC), and then differentiated into the HLCs. Finally, we examined whether the BD-iPSC derived HLCs (BD-HLCs) recapitulated the pathophysiology of PFIC2, specifically the aberrant splicing of *ABCB11* mRNA, reduction of membrane BSEP expression and impairment of biliary excretion capacity.

Materials and Methods

Ethical statement. This study was approved by the ethics committees of the University of Tsukuba and the National Institutes of Biomedical Innovation, Health and Nutrition. All experiments were performed in accordance with relevant guidelines and regulations, and with the approval of the University of Tsukuba and the National Institutes of Biomedical Innovation, Health and Nutrition. Written informed consent was obtained from the participants or their parent.

Generation of human iPSCs from peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using Ficoll gradient separation, and then cultured with plate-bound anti-CD3 monoclonal antibody (Becton, Dickinson and Company) and X-VIVO10 medium (Lonza) containing 10 ng/mL recombinant IL-2 (Thermo fisher scientific). To generate human iPSCs from PBMCs, Yamanaka four factor-expressing Sendai-virus (SeV) vectors (CytoTune-iPS For Blood Cells; DNASyn) was used²⁴. Twenty-four hours after plating (1.87×10^5 cells/cm²), the PBMCs were transduced with the SeV vector at a multiplicity of infection (MOI) of 20. Twenty-four hours after the transduction, the medium was replaced with fresh X-VIVO 10 medium. The next day, the SeV-transduced PBMCs were plated onto a mitomycin C-treated EmbryoMax Primary Mouse Embryo Fibroblasts (MEF; Merck Millipore) feeder layer. The PBMCs were cultured with ReproStem (ReproCELL) medium containing 5 ng/mL fibroblast growth factor 2 (FGF2; Katayama Chemical Industries) for 2–3 weeks. The SeV genome cDNA synthesized from RNA of human iPSCs was amplified by RT-PCR using Ex Taq DNA polymerase (TaKaRa Bio Inc.). The PCR products were separated by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. The SeV RNA genome was not detected in human iPSCs passaged more than 10 times (Figure S1D). The PCR primer sequences used in this study are described in Table S1.

Human ESCs/iPSCs Culture. Human embryonic stem cell (ESC) line, H9 (WiCell Research Institute), was maintained on MEF feeder layer with ReproStem medium supplemented with 5 ng/mL FGF2. Human ESCs were used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan and furthermore, and the study was approved by Independent Ethics Committee. Human iPSC lines were maintained on MEF feeder layer with ReproStem medium supplemented with 10 ng/mL FGF2.

Primary Human Hepatocyte Culture. Platable cryopreserved human hepatocytes were purchased from VERITAS (lot YOW). The vials of hepatocytes were rapidly thawed in a shaking water bath at 37 °C, and then the contents of the vial were emptied into prewarmed Cryopreserved Hepatocyte Recovery Medium (CHRM, Life Technologies) and the suspension was centrifuged at 750 rpm for 10 min at room temperature. The hepatocytes were seeded at 1.25×10^5 cells/cm² in HCM containing 10% FBS (Life Technologies) onto Cellmatrix Type I-A

acid-soluble type I collagen (Nitta Gelatin)-coated plates. The medium was replaced at 6 hr after seeding. The hepatocytes, which were cultured 48 hr after plating the cells, were used in the experiments.

Hepatocyte differentiation *in vitro*. For hepatocyte differentiation from human ESC and iPSC lines, the differentiated cells were constantly removed by manually picking them up. Before the initiation of hepatocyte differentiation, human ESCs/iPSCs were dissociated into clumps by using dispase (Roche Diagnostics) and plated onto BD Matrigel Basement Membrane Matrix Growth Factor Reduced (Becton, Dickinson and Company). These cells were cultured in the mouse embryo fibroblasts-conditioned medium for 3–4 days. The differentiation protocol for the induction of definitive endoderm cells, hepatoblast-like cells, and hepatocyte-like cells was based on our previous reports with some modifications^{22,23}. Briefly, in the definitive endoderm differentiation, human ESCs/iPSCs were cultured with the L-Wnt3A-expressing cell (CRL2647; ATCC)-conditioned RPMI1640 medium (Sigma) containing 100 ng/mL Activin A (R&D Systems), 1% GlutaMAX (Thermo fisher scientific), 0.2% fetal bovine serum (FBS; Thermo fisher scientific), and 1 × B27 Supplement Minus Vitamin A (Thermo fisher scientific) for 4 days. For the induction of hepatoblasts, the definitive endoderm cells were cultured with RPMI1640 medium containing 30 ng/mL bone morphogenetic protein 4 (BMP4) (R&D Systems) and 20 ng/mL fibroblast growth factor 4 (R&D Systems), 1% GlutaMAX, and 1 × B27 Supplement Minus Vitamin A for 5 days. To perform the hepatocyte differentiation, the hepatoblasts were cultured with RPMI1640 medium containing 20 ng/mL hepatocyte growth factor (HGF) (R&D Systems), 1% GlutaMAX, and 1 × B27 Supplement Minus Vitamin A for 5 days. Finally, the cells were cultured with hepatic maturation medium (hepatic maturation medium consists of Hepatocyte Culture Medium (HCM; Lonza, without epidermal growth factor (EGF)) containing 20 ng/mL oncostatin M (OsM) and 3% GlutaMAX) for 6 days. On day 20, the Matrigel solution (diluted by the hepatic maturation medium; the final Matrigel concentration was 0.25 mg/mL) was overlaid on the cells. The next day, the medium was changed with the hepatic maturation medium, and then the cells were cultured until day 25.

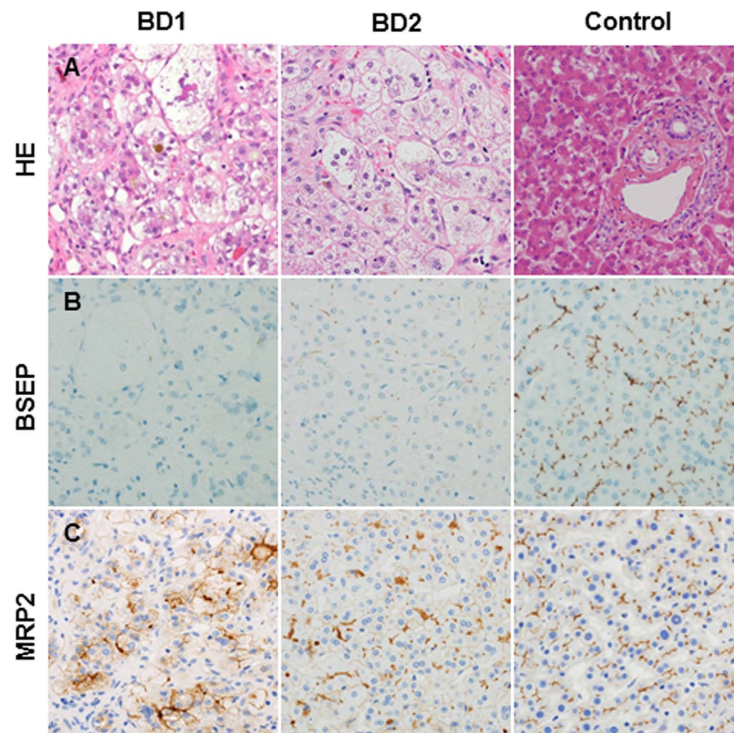
DNA sequence analysis. The exonic sequences of the *ABCB11* gene were analyzed in the PBMCs/iPSCs of a control and patients with BSEP deficiency by direct sequencing of PCR products. The DNA was extracted from the PBMCs/iPSCs of a control and a patient with BD using a DNeasy Blood & Tissue Kit (Qiagen). The cDNA sequence of the *ABCB11* gene was also analyzed by the direct sequencing of PCR products. The cDNA was synthesized from RNA of the Control, BD1 and BD2-HLCs. The PCR products were gel-purified, extracted with the GENECLAN kit (MP Biomedical) according to the manufacturer's instructions, and used as templates for the sequencing reaction with the Big Dye Terminator kit v3.1 (Applied Biosystems). Sequence analysis was performed by a Genetic Analyzer 3130 (Applied Biosystems). The sequences were compared to the reference sequence (GenBank accession number NM_003742.2). The nucleotide numbering was relative to the first adenine (+1) of initiation ATG codon. The PCR primer sequences used in this study are described in Table S2.

Biliary excretion index assay. To evaluate the biliary excretion capacity *in vitro*, the biliary excretion index (BEI) was calculated^{25,26}. The Control, BD1, and BD2-HLCs on day 25 of culture were prepared, and then the cells were washed with HBSS buffer three times. Ten minutes after the cells were incubated with HBSS buffer or calcium-free HBSS buffer, the buffer was exchanged for HBSS buffer containing 5 μM cholesteryl-l-lysyl-fluorescein (CLF), and the cells were incubated for 10 minutes. Uptake of CLF was stopped by adding cold HBSS buffer. The cells were washed with HBSS buffer, and lysed by 1% Triton X-100. The amount of CLF in the lysate was estimated by measuring the fluorescence at 492 nm and 536 nm by using a microplate reader (Genios, Tecan). BEI was calculated as follows: $BEI = 100 \times (HBSS - HBSS(Calcium\ free)) / HBSS\%$

Results

Patients with BSEP-deficiency. A BSEP-deficient patient, named as BD1, was a 6-year-old girl. At the age of 3 months, she experienced severe jaundice and cholestasis with normal serum GGT levels. In addition, liver histological findings showed cholestasis, giant cell transformation, hepatocellular swelling (Fig. 1A, left), and negative BSEP immunostaining (Fig. 1B, left). At 8 months of age, due to liver failure, a successful OLT was performed. The clinical course since OLT has been good, and currently she does not have any hepatic or extrahepatic symptoms. She was diagnosed as having progressive cholestasis with normal-GGT and BSEP deficiency but there were no mutations in the exons of the *ABCB11* gene. The other BSEP-deficient patient, named as BD2, was a 13-year-old boy. He suffered from severe jaundice and cholestasis with normal serum GGT at the age of 2 months. Gene analysis of peripheral blood cells showed novel compound heterozygous mutations in the *ABCB11* gene, c.-24C > A (5'-UTR; five prime untranslated region) and c.2417G > A (p.G806D). Trio analysis identified that these *ABCB11* mutations were transmitted from the patient's parents. Liver histological findings showed evidence for PFIC2; giant cell transformation, hepatocellular swelling, and cholestasis (Fig. 1A, middle). Consistently, liver immunohistochemistry indicated extremely low canalicular BSEP expression compared to control liver (Fig. 1B, middle). The other canalicular transporter, MRP2 was expressed in the BD2 patient's liver to a degree similar to the control liver (Fig. 1C, middle). These findings were compatible with the typical symptoms of PFIC2. At 2 years of age, he underwent a successful liver transplantation from his mother. Clinical course since the procedure has been good, and the patient currently has no symptoms. Detailed information about these patients with BSEP-deficiency is shown in Fig. 1D.

Generation of patient-specific iPSCs. An overview of human iPSC generation is shown in Figure S1A. Two patients with BSEP-deficiency (BD1 and BD2) were included in this study. To generate human iPSCs from these subjects, blood cells were collected, and the separated peripheral blood mononuclear cells (PBMCs) were transduced with Yamanaka four factor-expressing Sendai viral (SeV) vectors²⁴. Twenty days after the SeV transduction, BD1 and BD2-iPSC colonies were obtained. Control-iPSCs had been generated previously from the peripheral blood cells of a healthy female²⁷.



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Patient	Age at presentation	GGT (U/L)	peak ALT (U/L)	BA ($\mu\text{mol/L}$)	BSEP IHC	ABCB11 exonic mutation	Age at OLT	Extrahepatic symptoms
BD1	3 m	26	1340	155.8	not detected	not detected	8 m	not found
BD2	2 m	32	655	350	extremely low	c.-24 C>A c.2417 G>A	2 y 8 m	not found

Figure 1. Pathological examination in liver sections. (A) Staining of liver sections with hematoxylin and eosin. Immunostaining analyses of liver sections with BSEP (B) and MRP2 (C) (original magnification: 400 \times). (D) Characteristics of both the BSEP-deficient patients.

DNA sequence analyses showed that there were no differences in *ABCB11* exons between the iPSCs and their parent PBMCs. This meant that the *ABCB11* exonic mutations in BD2-iPSCs were consistently inherited from the parental PBMCs. Next, to characterize the human iPSC lines, immunostaining and real-time RT-PCR analyses of pluripotent markers were performed. Human iPSCs as well as ESCs (H9) were positive for OCT4, a pluripotency marker (Fig. S1B). The gene expression levels of the pluripotent markers (*OCT4* and *NANOG*) in the iPSCs were higher than those in the PBMCs, and similar to those in human ESCs (Fig. S1C). We confirmed that the SeV RNA genome was not detected in human iPSCs (Fig. S1D). To examine whether the iPSCs have the ability to differentiate into cells of the three germ layers, embryoid bodies (EBs) were generated. Human iPSC-derived EBs were positive for the endodermal marker (*AFP*), mesodermal marker (*cTnT*), and ectodermal marker (*Nestin*) (Fig. S1E). The gene expression levels of the endodermal markers (*AFP* and *TTR*), mesodermal markers (*NKX2.5* and *BRACHYURY*), and ectodermal markers (*NEUROD1* and *PAX6*) were higher in iPSC-derived EBs than in undifferentiated cells (Fig. S1F). Taken together, these results indicated that human iPSCs were successfully generated from patients with BSEP-deficiency.

Hepatocyte differentiation from human iPSCs. The protocol for hepatocyte differentiation was based on our previous report with some modifications (Fig. 2A)^{22,23}. HLCs were overlaid with Matrigel on day 20 of hepatic cell differentiation, to promote the formation of bile canaliculi; the morphology was similar to that of human hepatocytes²⁸. Moreover, transmission electron microscopy showed biliary canaliculi and tight junction structures in the HLCs (Fig. 2B). To investigate whether the biliary canaliculi were constructed between the HLCs, a bile secretion assay was performed using cholesteryl-l-lysyl-fluorescein (CLF), a fluorescent bile acid analog. CLF accumulation was detected in bile canaliculi of the Control-iPSC derived HLCs (Control-HLCs) overlaid with Matrigel (Fig. S2). To estimate the hepatocyte differentiation capacity of the iPSCs, albumin (*ALB*) expression levels were examined by real-time RT-PCR, immunostaining, flow cytometry and enzyme-linked immuno-sorbent assay (ELISA). The *ALB* gene expression levels in the iPSC-HLCs were similar to those in primary human hepatocytes (PHH) plated for 48 h (Fig. 2C). The HLCs stained positively for ALB (Fig. 2D), and

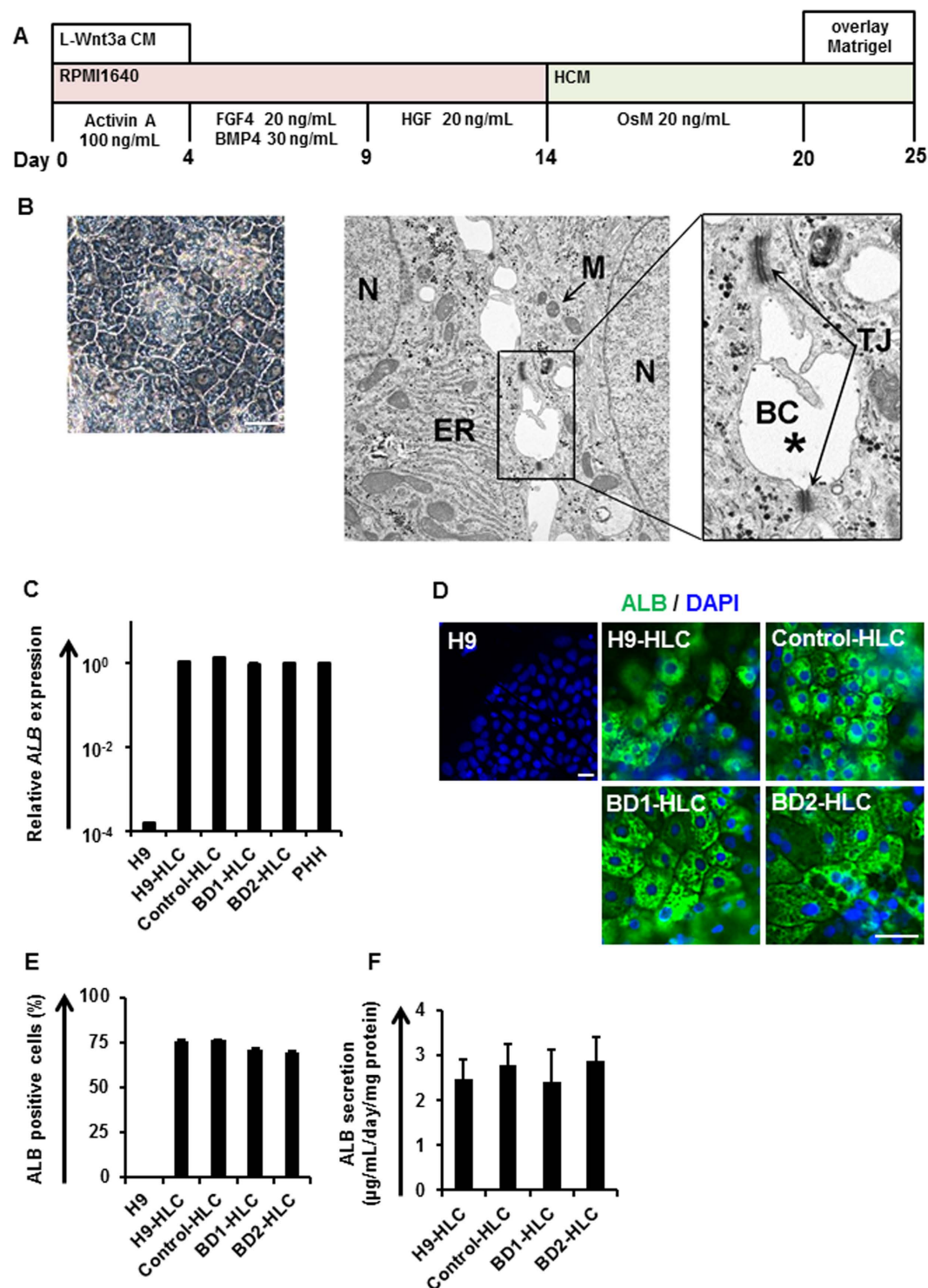


Figure 2. Differentiation of human iPSCs into hepatocyte-like cells. (A) The protocol for hepatocyte differentiation from human ESCs (H9) and human iPSCs. (B) Left panel is a phase contrast image of Control-HLCs at day 25. Middle panel is a transmission electron microscopy image of Control-HLCs (original magnification: 9300 \times). Right panel shows a further magnified image. Black asterisk indicates the canalicular space. BC, bile canaliculus; TJ, tight junction; ER, endoplasmic reticulum; M, mitochondria; N, nucleus. (C) Gene expression levels of *ALB* in HLCs derived from H9 cells or human iPSCs as examined by real-time RT-PCR. The gene expression level in PHH was taken as 1.0. Data represent mean \pm SD ($n = 3$). (D) Immunostaining analyses of ALB in the HLCs derived from H9 cells or human iPSCs. Nuclei were counterstained with DAPI. (E) Percentage of ALB-positive cells in HLCs derived from H9 cells or iPSCs, as measured by flow cytometry. Data represent mean \pm SD ($n = 3$). (F) ALB secretion levels of HLCs derived from H9 cells or human iPSCs as examined by ELISA. Data represent mean \pm SD ($n = 8$). Scale bars represent 20 μ m.

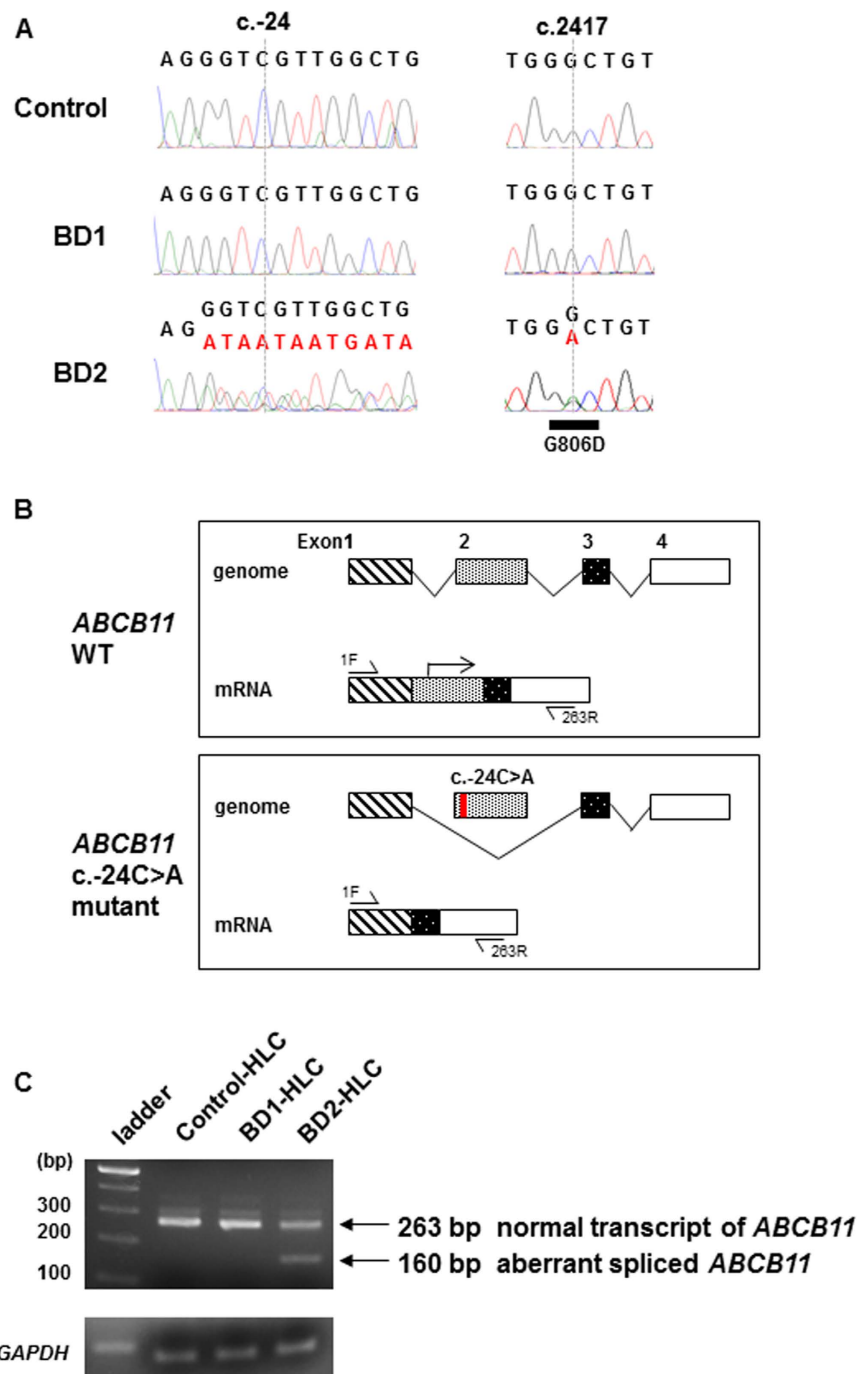


Figure 3. Sequence analysis of *ABCB11* mRNA using RNA extracted from the HLCs. (A) Sequence analysis of *ABCB11* mRNA using RNA extracted from the Control, BD1, and BD2-HLCs. (B) A schematic diagram of wild type (WT) and mutant form (BD2 patient) of *ABCB11* mRNA. (C) RT-PCR of normal and aberrantly spliced mRNA products of *ABCB11* in the different HLCs.

the percentage of ALB-positive cells was more than 70% (Fig. 2E). In contrast, undifferentiated H9 cells were negative for ALB (Fig. 2D). The HLCs could abundantly secrete ALB into the culture medium (Fig. 2F). These results demonstrate that both efficient hepatocyte differentiation and comparative analysis of the HLCs could be achieved using our differentiation protocol.

Sequencing analysis of *ABCB11* mRNA in the HLCs. Up to now, a mutation in the 5'-UTR of the *ABCB11* gene has not been reported in BSEP-deficiency^{2,10}. To examine whether the *ABCB11* mutation in the 5'-UTR mutation (c.-24C>A) would cause aberrant splicing in the HLCs derived from BD2-iPSCs (BD2-HLCs), direct sequence analysis of *ABCB11* cDNA was performed. Exon 2 sequence of *ABCB11* cDNA was heterozygously eliminated in BD2-HLCs (Fig. 3A). This suggested aberrant splicing of *ABCB11* in BD2-HLCs. A schematic

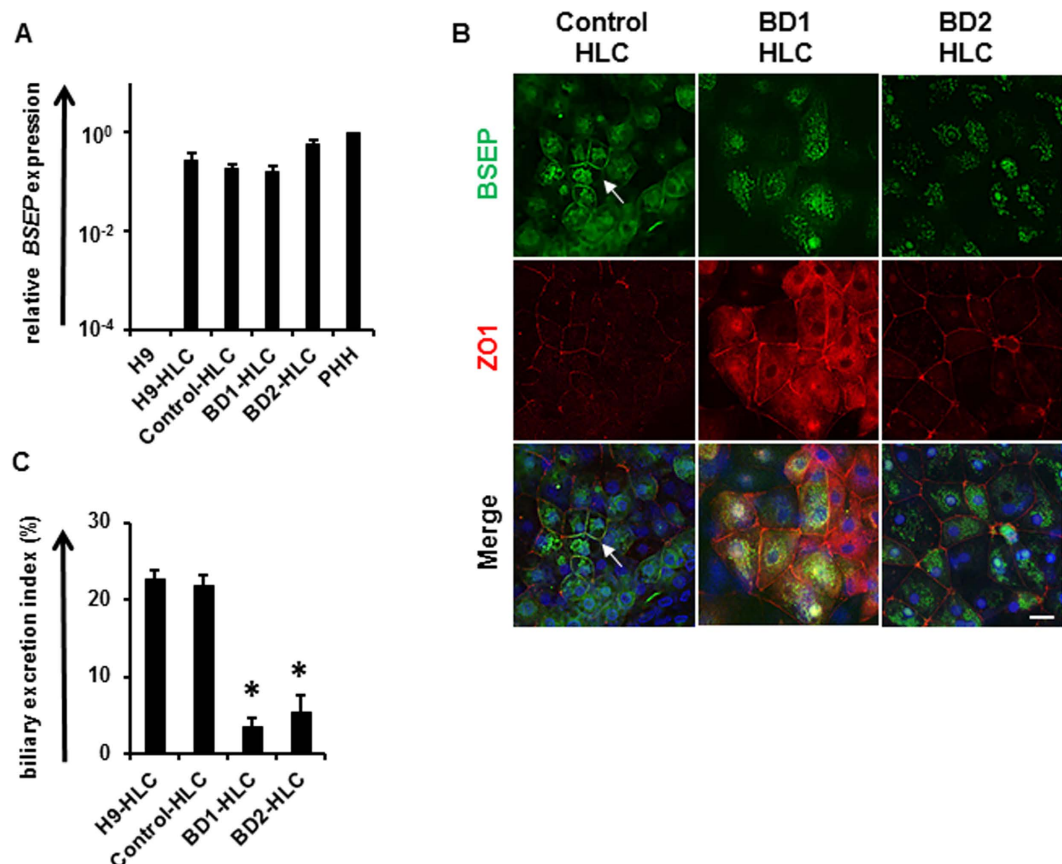


Figure 4. Analysis of BSEP expression and biliary excretion in the BD-HLCs. (A) Gene expression levels of *BSEP* in the HLCs as examined by real-time RT-PCR. The gene expression levels in PHH were taken as 1.0. Data represent mean \pm SD ($n = 3$). (B) Immunofluorescence double staining of BSEP and ZO-1 in the different HLCs. White arrow indicates the co-localization of BSEP and ZO-1. (C) BEI of CLF-treated HLCs. Data represent mean \pm SE (three independent differentiation experiments). Statistical significance was evaluated by one-way ANOVA in Tukey's test. * $P < 0.05$. The scale bar represents 20 μ m.

diagram of the predicted *ABCB11* mRNA is presented in Fig. 3B (upper panel). In BD2-HLCs, aberrant splicing resulted in elimination of the proper initiation codon in *ABCB11* (Fig. 3B, lower panel, the red mark indicates the 5'-UTR mutation). These results suggested that the mutation in the 5'-UTR of the *ABCB11* gene (c.-24C > A) prevented normal BSEP translation. To investigate the aberrant splicing forms, RT-PCR analysis for *ABCB11* exons was performed, using primers (1F and 263R, Table S1) amplifying the region indicated in Fig. 3B. Agarose gel electrophoresis of the PCR product identified a smaller band of 160 bp in the BD2-HLC lane (Fig. 3C). The size of this PCR product was consistent with the size of aberrantly spliced form of *ABCB11* containing exons 1, 3, and 4. A heterozygous missense *ABCB11* gene mutation (c.2417G > A, p.G806D) was also observed in the transcripts of BD2-HLCs (Fig. 3A). The pathogenicity of this novel missense mutation was predicted by Polyphen-2 (Polymorphism Phenotyping V2, <http://genetics.bwh.harvard.edu/pph2/>) and SIFT (Sorting Intolerance From Tolerant, <http://sift.jcvi.org/>) tools, and its effect is anticipated to be damaging (Table S3).

Recapitulation of the PFIC2 phenotype using BD-HLCs. Next, we investigated whether the BD-HLCs could recapitulate the PFIC2 phenotype. The *BSEP* expression levels in the BD-HLCs were similar to those of Control-HLCs (Fig. 4A). In addition, *farnesoid X receptor (FXR)*, which regulates the transcription of *BSEP*, was expressed in BD-HLCs (Fig. S3A). These results suggested that the expression level of *BSEP* transcripts in BD-HLCs was similar to that in Control-HLCs. To analyze the localization of BSEP in the HLCs, immunofluorescent double staining of BSEP and ZO-1 was performed, because ZO-1 is located on the canalicular membrane²⁹. BSEP and ZO-1 were not co-localized in BD-HLCs, but were co-localized in the Control-HLCs (Fig. 4B, white arrow). This finding suggested that membrane BSEP was not expressed on BD-HLCs, which was also observed in the liver of the patients with BSEP deficiency (Fig. 1B, left and middle). It is also suggested that there might be a problem in BSEP trafficking in these two patients. Taken together, this data showed that BD-HLCs reproduced a pathological feature of PFIC2.

To investigate the capacity for bile acid uptake and synthesis in the BD-HLCs, the gene expression levels of the bile acid uptake transporter (Na^+ -taurocholate cotransporting polypeptide, *NTCP*) and bile acid synthesis enzyme (cytochrome P4507A1, *CYP7A1*) were examined by real-time RT-PCR. The gene expression levels of *NTCP* and *CYP7A1* were higher in the BD-HLCs than in PHHs (Fig. S3B,C). These results indicated that the

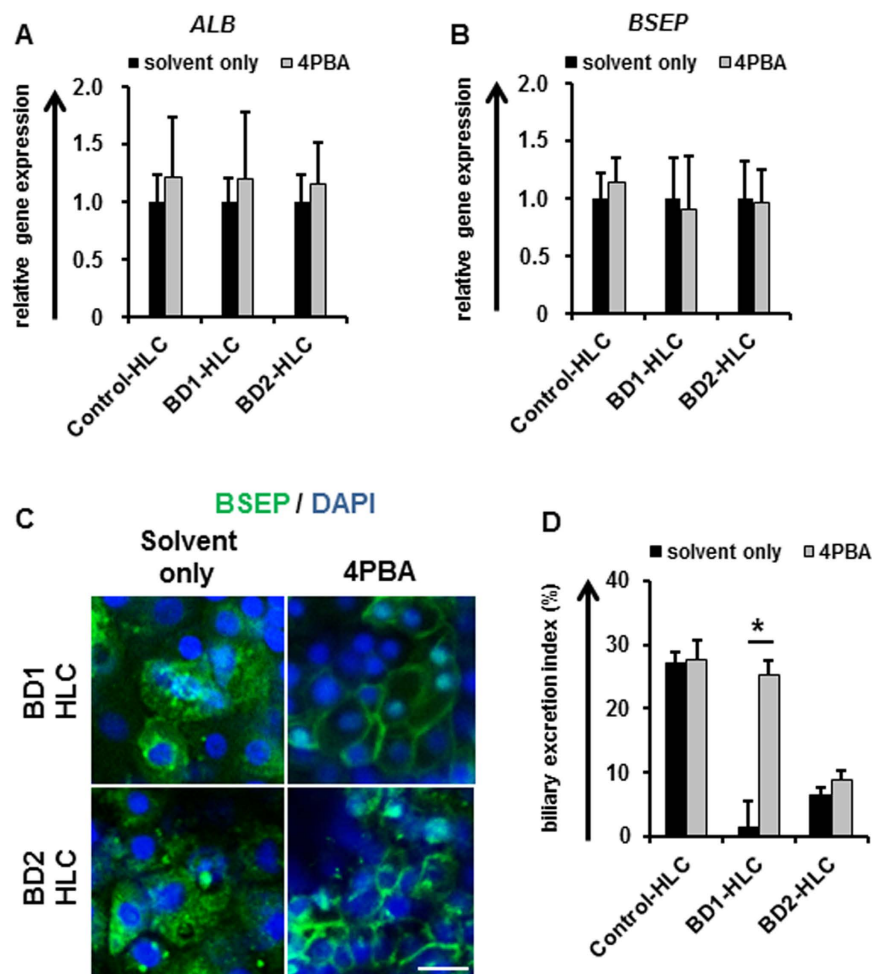


Figure 5. Analysis of the BD-HLCs treated with 4-phenylbutyrate. The BD1, BD2, and Control HLCs were treated with 1 mM 4PBA for 24 hours. (A,B) Gene expression levels of *ALB* and *BSEP* in the HLCs as examined by real-time RT-PCR. The gene expression levels in solvent-treated HLCs were taken as 1.0. Data represent mean \pm SD ($n = 3$). (C) Immunofluorescence staining of BSEP in the HLCs. The scale bar represents 20 μ m. (D) BEI of 4PBA-treated HLCs. Data represent mean \pm SE (three independent differentiation experiments). Statistical significance was evaluated by Student's t-test ($n = 3$). * $P < 0.05$.

BD-HLCs had the capacity to take up and synthesize bile acid. Next, to evaluate the biliary excretion capacity of HLCs, the biliary excretion index (BEI) was calculated using CLF^{25,26}. The BEI values of the BD-HLCs were significantly lower than those of the Control-HLCs (Fig. 4C). Taken together, these results showed that the BD-HLCs could reproduce the pathophysiology of PFIC2, specifically the impairment of biliary excretion.

Drug efficacy evaluation of 4PBA in the BD-HLCs. Finally, to investigate whether BD-HLCs could demonstrate measurable responses to therapeutic agents, we examined the drug efficacy of 4PBA, a promising drug for treating patients with PFIC2^{30–33}. It is known that 4PBA can improve membrane BSEP expression and biliary excretion capacity³⁰. To investigate whether 4PBA treatment affected the BD-HLCs, the gene expression level of *ALB* was examined by real-time RT-PCR (Fig. 5A). No significant alteration of *ALB* gene expression levels was observed by 4PBA treatment, suggesting that 4PBA did not affect the hepatic function of the HLCs. Next, the *BSEP* gene expression level and membrane BSEP expression level in the 4PBA-treated HLCs were examined by real-time RT-PCR (Fig. 5B) and immunostaining (Fig. 5C), respectively. Although there was no significant alteration of *BSEP* expression level by this treatment, the membrane BSEP expression was increased by 4PBA treatment. These results suggested that 4PBA treatment did not promote transcriptional activation of BSEP, but altered other pathways, such as the pathway for membrane-protein trafficking and degradation^{4,34}. In addition, the BEI was calculated to examine whether the biliary excretion capacity of the BD-HLCs could be rescued by 4PBA treatment. The BEI in BD1-HLCs treated with 4PBA was significantly higher than that in these cells treated with solvent only (Fig. 5D). On the other hand, 4PBA treatment did not increase the biliary excretion capacity of BD2-HLCs. This might be explained by assuming that the biliary excretion capacity of the mutated BSEP was very low that is in agreement with the previous bioinformatics analyses of the mutated BSEP function (Table S3), although the membrane BSEP expression levels were increased by 4PBA treatment. These results suggested that the drug efficacy could be evaluated by using BD-HLCs.

Discussion

The aim of this study was to examine whether HLCs derived from patients with BSEP-deficiency could reproduce the disease characteristics of PFIC2. We show here that the pathophysiologic features of PFIC2, i.e., abnormality of BSEP expression and impairment of biliary excretion, were reproduced in BD-HLCs. To the best of our knowledge, this is the first case generating a cholestatic disease model, using iPSC technology²⁰.

One-third of patients with PFIC are not diagnosed genetically¹⁶. Recently, mutations in tight junction protein 2 (*TJP2*) and *FXR* were found in patients with BSEP-deficiency, who harbored normal serum GGT and had no mutations in the *ABCB11* genes^{15,35}. However, the gene or mutation responsible for PFIC has not been identified in the BD1 patient or other BSEP-deficient patients with normal serum GGT¹⁰. The combination of iPSC technology and next-generation sequencing technology is expected to provide some clues^{15,35–37}. Future studies may reveal the function of novel PFIC-related genes identified using next-generation sequencing analysis with patient-specific iPSC-derived HLCs.

In this study, we identified two novel compound heterozygous *ABCB11* mutations in c.-24C > A (5'-UTR) and c.2417G > A (p.G806D) and demonstrated that the 5'-UTR mutation resulted in aberrant splicing of mRNA followed by elimination of the proper initiation codon (Fig. 3A,B). The aberrantly spliced mRNA from the allele with this 5'-UTR mutation (c.-24C > A) does not appear to be translated into a functional BSEP protein. Some studies have shown that the *ABCB11* mRNA is aberrantly spliced in PFIC2³⁸; however, the effects of mutations in the 5'-UTR of the *ABCB11* gene have not been reported previously. As the expression of the *ABCB11* gene is observed only in hepatocytes, the spliced form of *ABCB11* mRNA cannot be analyzed using other types of cells. It is difficult to obtain biopsy samples of the liver, although blood cells and dermal fibroblasts are often used to analyze genomic DNA or mRNA. Thus, we were able to analyze aberrant splicing of *ABCB11* gene using the HLCs instead of liver biopsy samples. The amino acid mutation (p.G806D) may also cause severe dysfunction of the BSEP protein as predicted by the Polyphen-2 and SIFT tools (Table S3). Accordingly, our data showed that the biliary excretion capacity of the BD2-HLCs was lower compared to Control-HLCs (Fig. 4C).

4PBA treatment promoted the membrane expression of BSEP (Fig. 5C). However, 4PBA treatment did not improve the capacity of bile secretion in BD2-HLCs (Fig. 5D). This discrepancy may be explained by the existence of mutation which causes defect in the transport activity. Consistently, Naoi *et al.* suggested that 4PBA may be an effective compound for patients with PFIC2 who retain transport activity of BSEP³¹. Because it is reported that CLF is also transported into bile canaliculi by not only BSEP but also other transporters³⁹, it might be important to perform further analysis for evaluating BSEP function using the HLCs.

Our findings suggest a potential for dissecting PFIC2 pathogenesis at the cellular and molecular level in patient-specific HLCs. Our research also provides hope for discovering novel therapeutic options for treating BSEP deficiency and cholestasis in the future. In conclusion, this is the first report establishing a PFIC2 model in human hepatocytes, using iPSC technology. We believe that this model will promote further innovations in BSEP research.

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Author Contributions

K.I., K. Takayama, R.S., and H.M. designed research; K.I., M.S., and R.S. collected clinical data; K.I., K. Takayama, S.I., K. Tanikawa, K. Harada performed research; K.I., K. Takayama, S.I. contributed new reagents/analytic tools; K.I., K. Takayama, K. Tanikawa, M.S., K. Harada, M.T., F.S., E.N., K. Hirata, M.K., K.K., R.S., and H.M. analyzed data; and K.I., K. Takayama, R.S. and H.M. wrote the paper.

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背景・目的

胆汁酸トランスポーター (BSEP) はヒトにおいて胆汁流量を規定する重要な分子で、その機能欠損は重度の胆汁うっ滞を惹起し、肝不全へと至る[1]。進行性家族性肝内胆汁うっ滞症 (PFIC) は小児期発症の胆汁うっ滞性疾患の一つで、PFIC2 型 (PFIC2) は BSEP 異常によって起きる (図 1) [2-4]。一部の BSEP 変異では、プロテアソーム系で分解を受けやすくなっているために、細胞膜上での BSEP 発現が低下していることが示されている[5-9]。PFIC2 の主要病像として、重度の黄疸、掻痒、肝内胆汁うっ滞があり、それらに引き続いて肝不全や若年性肝細胞癌に至ることが報告されている[3, 4, 10]。現在のところ、PFIC2 に対する治療として肝移植が主な治療法になっているが、移植後の遠隔期に BSEP タンパクに対する自己抗体が体内で産生され、PFIC2 病態の再燃が近年報告されている[11-14]。そのため、PFIC2 に対する新しい治療法の開発が求められており、その病態の解明が重要である。

疾患解析研究においてノックアウトマウスはしばしば用いられるが、PFIC2 の解析で Bsep ノックアウトマウスはあまり用いられてこなかった。ヒトに比べて胆汁酸毒性が低いことや、Mdr1 などの他のトランスポーターが胆汁酸輸送を補うことなどで PFIC2 のような重度の胆汁うっ滞性肝障害を呈さないと考えられている[15]。ヒト BSEP の機能解析研究では、変異 BSEP cDNA をプラスミドやウイルスベクターで各種細胞株に発現させ、輸送活性やタンパク発現を観察する手法が広く用いられている[6, 7]。しかし、BSEP 遺伝子の非コード領域に変異がある場合や、BSEP 遺伝子以外に変異があつて PFIC の病態を示す場合の解析が難しい。また、スプライシング異常の有無を観察することも難しい。PFIC の病態を示す患者のうち 30%程度で BSEP などのこれまでに報告されて

いる遺伝子の異常を認めず、この疾患領域の重要な課題の一つとされている[16]。

PFIC 患者から得た肝細胞で疾患解析を行うことは、疾患メカニズムや BSEP 分子の制御機構の解明に役立つと考えられる。

ヒト iPS 細胞は体細胞のリプログラミングによって得られる[17]。患者由来 iPS 細胞とその派生細胞は新たな疾患モデルになることが期待され、いくつかの疾患においてはすでに新しい治療法の可能性が報告されている (図 2) [18-21]。患者由来 iPS 細胞は種差による表現型の違いを減じることができるため、ヒト疾患モデルとして有用であると考えられる。そこで、本研究では PFIC2 患者から iPS 細胞を作製し、さらに分化誘導した肝細胞を用いて BSEP スプライシング異常、肝細胞膜上における BSEP 発現の異常、胆汁輸送能の低下などの PFIC2 病態を再現できるかどうかを検討した。

図1 BSEP 異常による胆汁うっ滞

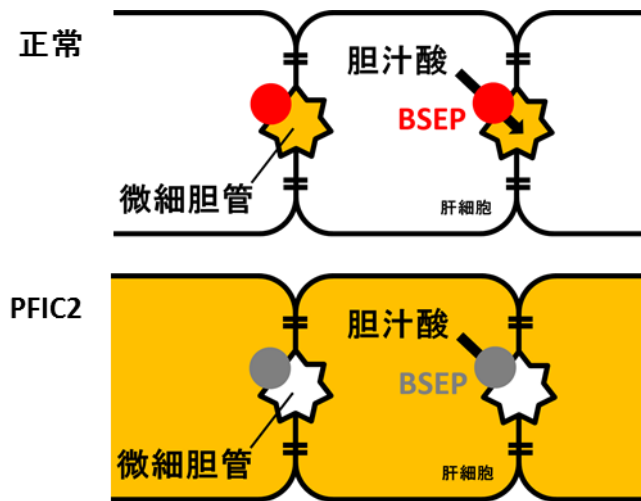
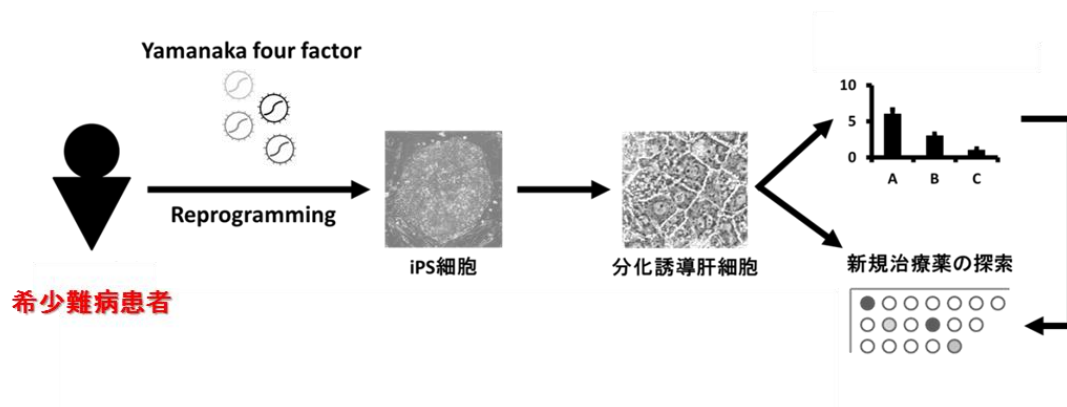


図2 患者由来 iPS 細胞と分化誘導細胞を用いた研究



対象と方法

遺伝子変異あるいは肝病理 BSEP 免疫染色により診断された PFIC2 患者 2 症例を対象とした。本研究は筑波大学附属病院倫理委員会の承認を受け、各患者の両親からの同意を書面で得た上で行われた。

患者由来 iPS 細胞は末梢血から作製した。全血からフィコールによる比重遠心で単核球を分離し、抗 CD3 抗体を固相化したプレート上で培養した。IL-2 添加培地で 5~7 日培養し、山中 4 因子発現センダイウイルスベクターを MOI20 で導入し、FGF2 添加幹細胞培地とフィーダー細胞（マイトマイシン C 処理マウス線維芽細胞）上で iPS 細胞へと誘導した。14~20 日後にヒト ES 細胞様コロニーをピックアップし、安定的に継代できたものを以後の実験に用いた。10 回以上継代した iPS 細胞からはセンダイウイルスゲノムは検出されなかった (Figure S1D)。

ヒト ES 細胞およびヒト iPS 細胞は FGF2 添加幹細胞培地とフィーダー細胞（マイトマイシン C 処理マウス線維芽細胞）上で培養した。ヒト ES 細胞を用いた解析は医薬基盤研究所で行った。幹細胞から下記の方法で肝細胞へ分化誘導した (Figure 2A)。フィーダー細胞を除いた iPS 細胞をマトリゲル上に播種し、L-Wnt3A コンディショナル RPMI 培地とアクチビン A 100ng/mL を含む RPMI 培地で 4 日間培養し、内胚葉系細胞に分化誘導した。内胚葉系細胞に BMP4 30ng/mL と FGF4 20ng/mL を 4 日間作用させ、肝芽細胞へと分化誘導し、次の 5 日間は HGF 20ng/mL を添加して肝細胞様細胞に誘導した。さらに 12 日間オンコスタチン M 20ng/mL を添加して肝細胞の成熟化を図った。また、毛細胆管形成を促すため、分化開始後 20 日目からマトリゲル重層培養を行い、分化開始後 25 日目に解析を行った。また、三胚葉系への分化能確認のため胚様

体形成を行った。10%FBS 含有 DMEM 培地内で 4 日間低接着培養し、次の 10 日間接着培養した。

末梢血単核球と iPS 細胞からゲノムを抽出し、それぞれの BSEP のダイレクトシーケンスを行った。また、分化誘導肝細胞から RNA を抽出し、逆転写反応で cDNA を合成し、BSEP のダイレクトシーケンスを行った。PCR 産物をアガロースゲルから抽出し、サンガー法でシーケンス解析した。

培養細胞から抽出した RNA から cDNA を合成し、SYBR Green 法を用いて定量的 RT-PCR 解析を行った。内在コントロールには GAPDH 遺伝子を用いた。また、培養細胞の培養液上清を用いて ELISA 法により肝細胞分泌のアルブミンを定量した。

細胞蛍光免疫染色では、4%パラホルムアルデヒドで固定した細胞を解析に用いた。室温 1 時間でブロッキング作業を行い、1 次抗体を 4℃で 16 時間インキュベートし、2 次抗体は室温 1 時間で反応させた。肝臓病理標本はホルマリン固定後パラフィン包埋されたものを使用し、BSEP・MRP2 の DAB 染色で評価した。分化誘導肝細胞の電子顕微鏡像は 2%パラホルムアルデヒド・2%グルタルアルデヒドで固定後に超薄切切片を作製し、観察した。

胆汁排泄能を調べるため、分化誘導 25 日目の肝細胞で biliary excretion index (BEI) アッセイを行った。カルシウムを含む HBSS プラスバッファーとカルシウムを含まない HBSS マイナスバッファーでそれぞれ 10 分間インキュベートし、胆汁排泄蛍光試薬 CLF を含むバッファーでさらに 10 分間インキュベートした。それぞれの条件の細胞ライセートを回収し、マイクロプレートリーダーで蛍光を測定し、計算によって CLF の毛細胆管内量を求めた (図 3)。

図 3 Biliary excretion index assay



$$\text{BEI}(\%) = \frac{\text{Ca}(+) - \text{Ca}(-)}{\text{Ca}(+)} \times 100$$

$$= \frac{\text{微細胆管}}{\text{細胞内} + \text{微細胆管}} \times 100$$

結果

症例 1 は生後 3 か月時に γ GTP 正常の重度黄疸と胆汁うっ滞を示し、肝生検で巨細胞肝炎、胆汁うっ滞、BSEP 免疫染色陰性を示し、PFIC2 と診断された。8 か月時に肝不全を適応に肝移植が施行され、以後胆汁うっ滞の再燃や肝外症状の出現はなかった。移植後の遺伝子解析では BSEP 遺伝子に変異は同定されなかった (Figure 1A-D)。症例 2 は生後 2 か月時に γ GTP 正常の重度黄疸と胆汁うっ滞を示し、肝生検で巨細胞肝炎、胆汁うっ滞が認められた。BSEP 遺伝子検査によりコンパウンドヘテロ変異が認められ、PFIC2 と診断された。2 歳時に肝移植が施行され、以後胆汁うっ滞の再燃や肝外症状の出現はなかった (Figure 1A-D)。

2 名の患者の末梢血から iPS 細胞を作製した (Figure S1A)。患者 2 の BSEP 変異は iPS 細胞にも継承されていた。作製された iPS 細胞は OCT4 や NANOG といった未分化マーカーをヒト ES 細胞と同様に発現していた (Figure S1B,C)。また、iPS 細胞にセンダイウイルスベクターのゲノムが残存していないことを PCR で確認した (Figure S1D)。胚様体形成により、三胚葉への分化能を確認した (Figure S1E,F)。

続いて肝細胞への分化誘導を行った。内胚葉系細胞、肝芽細胞を経て肝細胞様細胞へと分化誘導した (Figure 2A)。肝細胞様細胞は多角の肝細胞様形態を示し、細胞間に毛細胆管構造を形成した (Figure 2B,S2)。また、肝細胞で特異的に発現するアルブミンを定量的 RT-PCR や免疫染色、ELISA で認め、肝細胞としての特性を有することが示された (Figure 2C-F)。

肝細胞様細胞から RNA を抽出し、合成した cDNA の BSEP 遺伝子ダイレクトシーケンスを行い、スプライシング異常有無を解析した (Figure 3A)。症

例 2 ではエクソン 2 がスキップしており、5'非翻訳領域の一塩基置換がスプライシング異常を惹起していることが明らかになった (Figure 3B)。電気泳動でもエクソン 2 欠失のサイズと一致するバンドが検出された (Figure 3C)。また、アミノ酸置換 G806D はこれまでに報告のない箇所の BSEP 変異で、in silico 解析を行ったところ damaging であると推測された (Table S3)。

患者 iPS 細胞由来分化肝細胞では、BSEP やそれを調節する転写因子 FXR は発現していた (Figure 4A,S3)。免疫染色で患者群はコントロールと比較して細胞膜上の BSEP 発現が認められず、PFIC2 の病態を反映していることが示唆された (Figure 4B)。また、胆汁排泄能 BEI は患者群で低値であった (Figure 4C)。PFIC2 の治療薬候補として報告されているフェニル酪酸を患者 iPS 細胞由来分化肝細胞に作用させたところ、BSEP の遺伝子発現量は変化しなかったが、細胞膜上の BSEP 発現パターンが改善し、胆汁排泄能も改善した (Figure 5A-D)。

考察

本研究では進行性家族性肝内胆汁うっ滞症 2 型 (PFIC2) と診断された 2 名の患者の血液細胞から iPS 細胞を作製し、分化誘導して得られた肝細胞を解析した。ヒト iPS 細胞は 2007 年に初めて報告され[17]、多分化能を有して肝臓や心臓、神経などの生検で得難い組織の細胞を分化誘導できることから、再生医療や疾患解析、創薬研究への活用が報告されている[22, 23]。肝疾患患者由来 iPS 細胞を用いた研究では、 $\alpha 1$ アンチトリプシン欠損症や嚢胞線維症などの疾患で報告がある[24-27]。一部の疾患では、患者由来 iPS 細胞と分化誘導肝細胞を用いて既存薬ライブラリーによる治療薬候補のスクリーニングやゲノム編集技術による表現型改善などが示されており、疾患解析だけでなく新規治療法の開発が進んでいる[18-21]。しかし、PFIC2 患者由来 iPS 細胞を用いた研究はこれまでに報告がないことから新たな疾患モデルの作出を目的に本研究を実施するに至った。

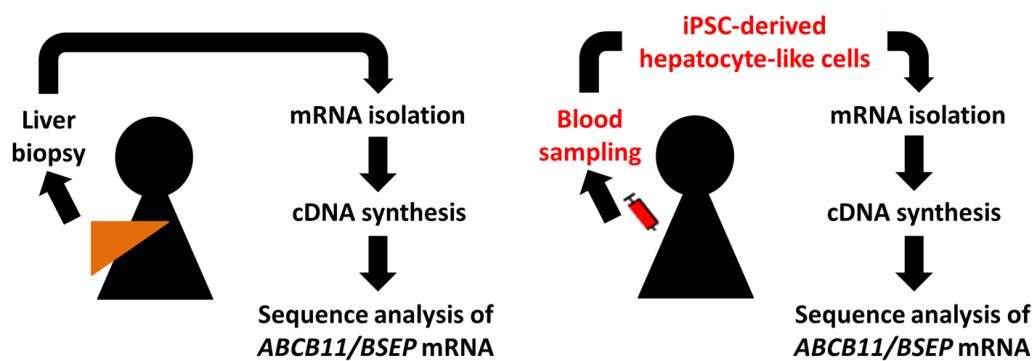
PFIC2 患者数は約十万人に一人と推定されている希少疾患である[28]。PFIC2 患者における病気の進行度は多様であり、肝移植を要さない比較的軽症の良性反復性肝内胆汁うっ滞 (BRIC) があることも知られている[29]。PFIC2 患者でみられる BSEP 遺伝子変異にはホットスポットは無く、100 か所以上の変異箇所が知られている[3, 4]。また、BSEP 遺伝子に変異が特定されないにも関わらず、PFIC2 と同じような臨床像を呈する患者が 3 割程度存在することが報告されており、この疾患領域における重要な課題とされている[16]。近年、これら PFIC の表現型を示す診断未確定患者に対する網羅的遺伝子解析が行われ、新たな疾患遺伝子が報告されている[30-33]。

本研究で対象にした患者 BD2 では BSEP エクソンに 2 か所 (5'非翻訳領域と

第 8 膜貫通領域) に変異を認めた。これらの変異は過去に報告がなく、その変異意義は不明であった。これまで、BSEP の機能解析は肝細胞を用いた解析や、モデル動物を用いた解析はほとんどなく、mutagenesis 法で変異を導入した BSEP cDNA をプラスミド等でトランスフェクションされた HEK293 細胞等の細胞株で発現パターンや胆汁酸輸送活性を評価されている[6, 7]。本研究における症例のように BSEP エクソンに変異の無い症例や 5'非翻訳領域に変異のある症例では、この従来の解析手法を用いて評価することが困難であった。BSEP 遺伝子は肝細胞のみで発現するため、通常は肝生検検体から mRNA を抽出して cDNA のシーケンスを行うが、患者 BD2 のような自己肝を失っている肝移植後患者ではそれが不可能である。本研究では、iPS 細胞由来肝細胞を用いることでスプライシング異常の存在を見出すことができた。病状が進行中の症例においては、iPS 細胞樹立・肝細胞分化誘導に要する時間を考慮すると上記手法の活用は限定的であるが、肝移植後遠隔期の患者における解析では本研究の手法が利用できることが示唆された。症例 BD1 では BSEP エクソンの変異が認められないが、患者の肝生検検体で BSEP 発現が消失しており、また分化誘導肝細胞で細胞膜上 BSEP 発現低下や胆汁排泄能の低下が認められた。全エクソン解析を行い、胆汁酸代謝だけでなく細胞内移送、細胞極性などに関わる分子を含めて遺伝子解析を進めていきたい。

近年普及してきた高速シーケンサーを用いた遺伝子解析は広く活用されるようになり、小児期胆汁うっ滞性疾患の診断にも役立てられている[34, 35]。特に、既知の疾患遺伝子に変異が無い PFIC の表現型を呈する症例を集積して網羅的にエクソーム解析した研究では、新しい疾患遺伝子がいくつか同定されている[30-33]。新しい疾患遺伝子の発見は、当該分野における病態や分子機構の解明に寄与するだけでなく、希少疾患の原因遺伝子や原因分子から糖尿病や高

図 4 分化誘導肝細胞を用いた BSEP スプライシング解析



脂血症などの対象者の多い疾病治療薬が開発されるなど、分子創薬への応用も期待される[36, 37]。実際の全エクソン解析では、数万か所のバリエントが検出され、多段階のフィルタリングを受けて数百か所にまで絞り込まれる[38]。続いて患者の表現型などを参照しながら病的遺伝子変異の候補となるものを絞り込む[39]。これまでに報告のない遺伝子変異であれば、同様の表現型を呈する他家系でも同じ遺伝子に変異を持つことが証明されると新規疾患遺伝子と決定される。しかし、希少疾患であることから、症例数が少なく、他家系とのマッチングが難しいことが多い。患者由来 iPS 細胞とゲノム編集技術を用い、全エクソン解析で検出されたバリエントが疾患遺伝子であるかどうかを示す研究が報告され、iPS 技術が遺伝子解析研究にも応用できることが示唆されている[40]。本研究の成果を基に、未解明の胆汁うっ滞性疾患の解析に取り組みたい（図 5）。

フェニル酪酸は変異 BSEP の細胞膜上における発現改善と胆汁酸輸送能改善の作用を有することが報告されている[41]。同薬剤は先天性代謝異常症に対する治療薬として既に上市されており、肝臓の BSEP 発現を改善させる作用が報告され[42]、PFIC2 あるいはその他の胆汁うっ滞症において胆汁うっ滞を改善させる作用があることが報告されている[43-46]。また、胆汁うっ滞症患者でみられる強い痒みに対する改善効果も報告されている（表 1）[47, 48]。フェニル酪酸が胆汁うっ滞を改善させる機序としては、ユビキチン化を受けた変異 BSEP の肝細胞の頂端膜側における内在化を減じる作用により、BSEP の細胞膜上での発現改善に寄与することが報告されている（図 6）[5, 9, 41]。本研究における 2 症例の iPS 細胞由来肝細胞では、フェニル酪酸の薬効を一部評価することができた。症例 BD1、BD2 とともに細胞膜側の BSEP 発現が改善し、BD1 では胆汁排泄能 BEI 値も有意に改善した。一方で BD2 では BEI 値はやや上昇したが有意な改善は認めなかった。変異 BSEP の輸送活性が失われている場合、タンパ

図 5 高速シーケンサーと iPS 細胞を用いた新規疾患遺伝子の探索

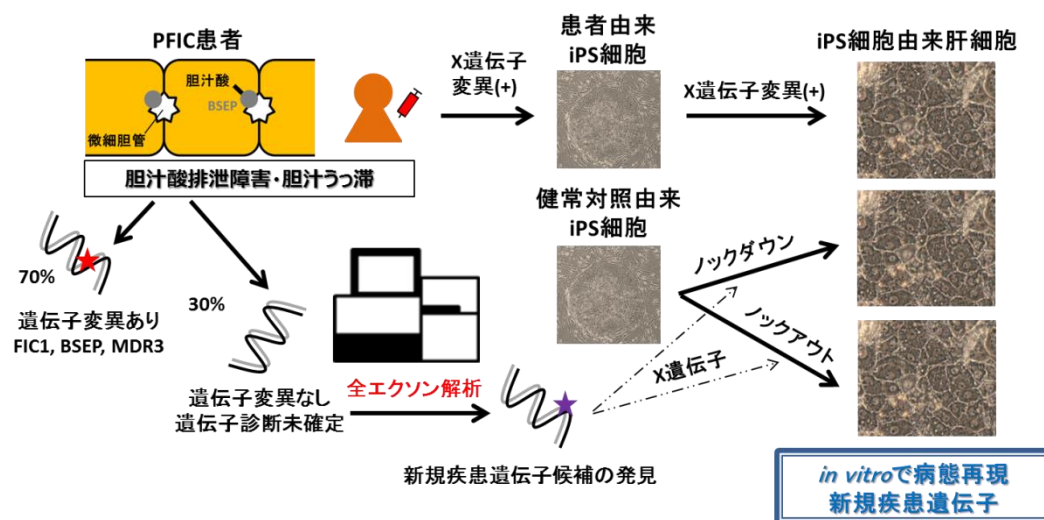
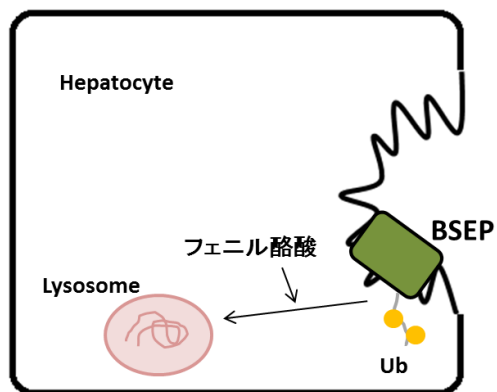


表 1 胆汁うっ滞症に対するフェニル酪酸の使用例

年	国名	疾患	肝BSEP発現	ALT	血清胆汁酸	直接ビリルビン	掻痒	文献
2016	日本	超早産児の胆汁うっ滞症	改善	正常	正常	正常	NA	43
2015	日本	良性反復性肝内胆汁うっ滞症2型(BRIC2)	NA	正常	低下	正常	改善	44
2015	フランス	進行性家族性肝内胆汁うっ滞症2型(PFIC2)	改善	正常	不変	正常	改善	45
2015	フランス	進行性家族性肝内胆汁うっ滞症2型(PFIC2)	改善	正常	低下	正常	改善	45
2015	フランス	進行性家族性肝内胆汁うっ滞症2型(PFIC2)	NA	不変	低下	不変	改善	45
2015	フランス	進行性家族性肝内胆汁うっ滞症2型(PFIC2)	改善	低下	低下	不変	改善	45
2014	日本	進行性家族性肝内胆汁うっ滞症2型(PFIC2)	改善	低下	不変	正常	改善	47
2014	日本	進行性家族性肝内胆汁うっ滞症1型(PFIC1)	改善	不変	不変	不変	改善	48
2014	日本	進行性家族性肝内胆汁うっ滞症1型(PFIC1)	改善	不変	不変	不変	改善	48
2014	日本	進行性家族性肝内胆汁うっ滞症1型(PFIC1)	不変	不変	不変	不変	改善	48

図 6 フェニル酪酸はユビキチン化を受けた BSEP の分解を減じる



Soroka et al., Mol Aspects Med 2014 より改変 [49]

ク発現パターンが改善しても胆汁酸の輸送量が改善しなかった可能性が示唆された。

現在、PFIC2 は主に肝移植により治療される。肝移植により PFIC2 は根治できると考えられていたが、体内で産生された抗 BSEP 抗体による移植肝の BSEP 機能不全が報告され、移植後に症状の再燃が起きることが知られるようになった[12-14]。したがって、肝移植以外の治療法開発が求められている。フェニル酪酸は PFIC2 の治療薬候補の一つとして、本邦で 2016 年 11 月から医師主導臨床試験が開始された[50]。一方で、フェニル酪酸投与によっても胆汁酸が十分低下しない PFIC2 症例も報告されている[45]。本研究の成果を基に、PFIC2 患者由来肝細胞を用いた BSEP 発現や胆汁排泄改善に寄与する薬剤のハイスループット探索系の開発に取り組みたい。胆汁排泄を改善させる作用のある薬剤が発見できれば、PFIC2 の治療薬としてだけでなく、利胆薬として肝疾患領域全体に応用できる可能性がある。

iPS 細胞の利用は新規治療法の開発において、薬剤スクリーニングだけでなく、再生治療分野でも期待される。本邦における肝移植は年間 400～500 件で、脳死ドナーの不足により 9 割以上で生体肝移植が行われている[51]。また、先天性代謝異常症の患者に移植時余剰肝から分離した肝細胞を門脈経由で移植し、治療効果を得た症例が報告され、臓器移植だけでなく細胞治療による肝疾患の治療法が開発されている[52]。肝移植医療における慢性的なドナー不足を背景に、ヒト ES 細胞やヒト iPS 細胞から分化誘導した肝細胞を用いた肝再生医療に期待が寄せられている[53-55]。PFIC2 は肝移植を要する疾患の一つであり、肝移植を代替する治療法として再生医療も選択肢の一つとして考えられる。ゲノム編集技術を用いて変異箇所を修復した分化誘導細胞を移植する戦略がこれまでに報告されている[56]。この方法では患者本人の細胞を利用できる点で移植時の免

疫反応を回避できる利点があるが、移植細胞を準備するまでの工程に多大な時間や労力を要する。そのため、再生医療の実用化に向け、HLA ホモドナーから作製した iPS 細胞をストックし、レシピエントと拒絶反応の起きにくい HLA 型の iPS 細胞から分化誘導した細胞を治療用に用いることが計画されている[57, 58]。PFIC2 患者などの肝移植を要する患者に対する治療戦略として、iPS 細胞由来分化誘導肝細胞による細胞治療が将来実現するかもしれない。

本研究で得た成果を基に PFIC2 に対する新しい治療法の開発や、疾患遺伝子の新規同定など広く応用していきたい。

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