Journal of Human Genetics (2018) 63:569–577
https://doi.org/10.1038/s10038-018-0431-1

ARTICLE

Clinical phenotype and molecular analysis of a homozygous *ABCB11* mutation responsible for progressive infantile cholestasis

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Received: 26 July 2017 / Revised: 2 February 2018 / Accepted: 7 February 2018 / Published online: 5 March 2018

Abstract

The bile salt export pump (BSEP) plays an important role in biliary secretion. Mutations in *ABCB11*, the gene encoding BSEP, induce progressive familial intrahepatic cholestasis type 2 (PFIC2), which presents with severe jaundice and liver dysfunction. A less severe phenotype, called benign recurrent intrahepatic cholestasis type 2, is also known. About 200 missense mutations in *ABCB11* have been reported. However, the phenotype–genotype correlation has not been clarified. Furthermore, the frequencies of *ABCB11* mutations differ between Asian and European populations. We report a patient with PFIC2 carrying a homozygous *ABCB11* mutation c.386G>A (p.C129Y) that is most frequently reported in Japan. The pathogenicity of $BSEP^{C129Y}$ has not been investigated. In this study, we performed the molecular analysis of this *ABCB11* mutation using cells expressing $BSEP^{C129Y}$. We found that trafficking of $BSEP^{C129Y}$ to the plasma membrane was impaired and that the expression of $BSEP^{C129Y}$ on the cell surface was significantly lower than that in the control. The amount of bile acids transported via $BSEP^{C129Y}$ was also significantly lower than that via $BSEP^{WT}$. The transport activity of $BSEP^{C129Y}$ may be conserved because the amount of membrane $BSEP^{C129Y}$ corresponded to the uptake of taurocholate into membrane vesicles. In conclusion, we demonstrated that c.386G>A (p.C129Y) in *ABCB11* was a causative mutation correlating with the phenotype of patients with PFIC2, impairment of biliary excretion from hepatocytes, and the absence of canalicular BSEP expression in liver histological assessments. Mutational analysis in *ABCB11* could facilitate the elucidation of the molecular mechanisms underlying the development of intrahepatic cholestasis.

Introduction

Bile salt export pump (BSEP) is a crucial molecule involved in the secretion of bile acids in the human liver [1]. BSEP is encoded by *ABCB11* (NM_003742), which is located on chromosome 2q23.3-2q31.1. Mutations in *ABCB11* cause progressive familial intrahepatic cholestasis type 2 (PFIC2) [2]. An infantile cholestatic disorder characterized by normal serum gamma-glutamyl transpeptidase (GGT) levels occurs due to the impairment of biliary excretion capacity. Normal-GGT PFIC is characterized by mutations in *ATP8B1* (PFIC1) and *ABCB11* (PFIC2, OMIM#:601847) [3], whereas patients carrying a *ABCB4* mutation show high serum GGT levels [4]. The main clinical features of PFIC include

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Electronic supplementary material The online version of this article (https://doi.org/10.1038/s10038-018-0431-1) contains supplementary material, which is available to authorized users.

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jaundice, pruritus, rickets, and cholestasis [3]. PFIC2 is a rare autosomal recessive liver disease, and the population prevalence has been estimated to be approximately 1/100,000 births [5]. In patients with PFIC2, rapid progression to liver failure and juvenile hepatobiliary cancer are also observed [6–8]. Liver transplantation is often required for rescuing patients with PFIC2, and a number of other therapeutic options are currently being developed [8].

Currently, approximately 200 mutations in *ABCB11* have been reported in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php). A less severe form of BSEP disorder, benign recurrent intrahepatic cholestasis type 2 (BRIC2), is also known. Mutations p.E297G and p.D482G in *ABCB11* are more frequently reported in the Western population, which account for 58% of PFIC2 cases [8]. The molecular mechanisms of these frequent *ABCB11* mutations have been extensively studied [9, 10]. In contrast, these common *ABCB11* mutations have not been frequently detected in Asian patients with PFIC2 [11–14], which is consistent with the concept of ethnicity-specific mutations [15]. However, the genotype–phenotype correlations in PFIC2 are not well understood.

Here, we report a patient with PFIC2 harboring a homozygous *ABCB11* mutation (c.386G>A (p.C129Y)), which has only been previously identified in Japan [11]. Detailed clinical descriptions of patients carrying this *ABCB11* mutation have not been reported, and the effects of this mutation remain unknown. To determine the prognoses and develop personalized treatments in individual patients with PFIC2, further elucidation of the relationships between genotype and molecular mechanisms is required [16]. Therefore, in this study, we examined the pathogenicity of the homozygous *ABCB11* mutation, c.386G>A (p.C129Y), in a Japanese patient with PFIC2 and performed molecular functional analyses.

Materials and methods

Ethics

This study was approved by the ethics committee of the University of Tsukuba.

Plasmids

The pShuttle vector containing human BSEP cDNA N-terminally tagged with a hemagglutinin antigen (HA) (pShuttle–HA–BSEP^{WT}) was constructed as described previously [17]. Site-directed mutagenesis was performed as described previously [9] to introduce the c.386G>A (p.C129Y) mutation into HA–BSEP^{WT} (HA–BSEP^{C129Y}).

Cell culture

HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA) (ATCC Number: CRL-11268). HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 and 95% humidity. To examine thermal influence on the expression of HA–BSEP^{WT} and HA–BSEP^{C129Y}, the cells were cultured at 27 °C for the last 24 h.

In vitro studies to characterize the c.386G>A (p.C129Y) mutation in *ABCB11*

HEK293T cells were transfected with the indicated vector using XtremeGene HP DNA transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. The cells were analyzed by quantitative PCR (qPCR), cell surface biotinylation, and immunocytochemistry as described previously 48 h after the transfection [17, 18], or were used for the preparation of membrane vesicles. The isolated membrane vesicles were subjected to immunoblotting and transport assays [17, 19]. Transport assays were performed using the rapid filtration method reported previously [19].

qPCR

Total RNA was isolated using Isogen II (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription was performed using ReverTra Ace® qPCR reverse transcription master mix with genomic DNA remover (Toyobo, Osaka, Japan). BSEP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were determined by qPCR using a LightCycler 480 system II (Roche Diagnostics, Mannheim, Germany), the appropriate software (v. 3.53; Roche Diagnostics), and Thunderbird SYBR qPCR mix (Toyobo) as described previously [17]. qPCR was performed using the following primers: 5′-TGCCCAGTGCATCATGTTTA-3′ and 5′-CCCTGGAAGTTGTCCCATTT-3′ (BSEP), and 5′-GGGGAGCCAAAAGGGTCATCATCT-3′ and 5′-GACGCCTGCTTCACCACCTTCTTG-3′ (GAPDH). Gene expression for each reaction was normalized to the expression of human GAPDH.

Cell surface biotinylation

Cell surface biotinylation for preparing cell surface fractions was performed as previously reported [17, 18]. The isolated biotinylated protein was analyzed by immunoblotting.

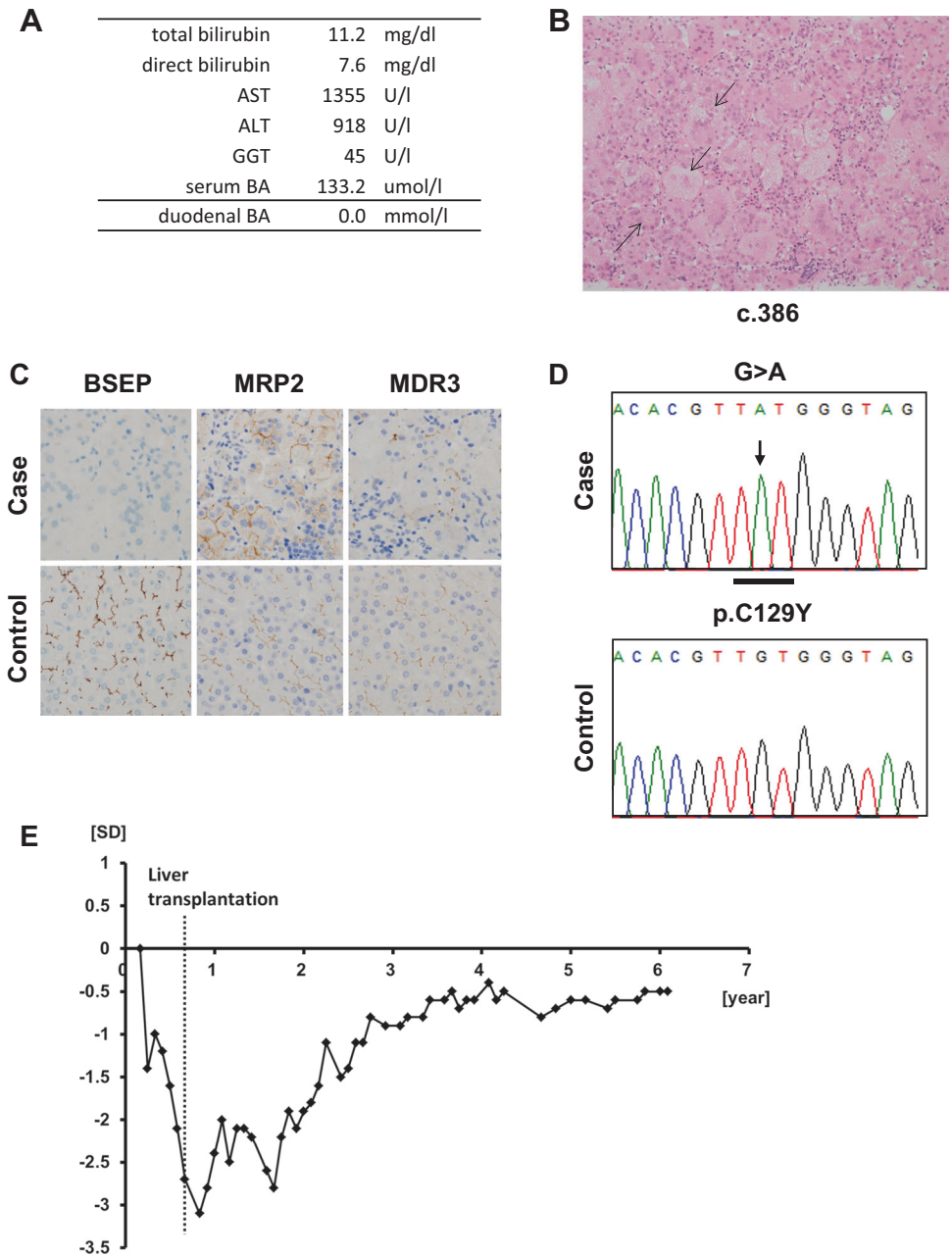

Fig. 1 Pathological examination and genetic sequencing. **a** Profile of biochemical analyses in serum and duodenal drainage. **b** Staining of liver sections with hematoxylin and eosin. Black arrows indicate giant cell transformation, hepatocellular swelling, and bile plug. **c** Immunostaining analyses of liver sections for BSEP, MRP2, and MDR3 (original magnification: 400×). **d** Sequence analysis of *ABCB11* using DNA extracted from the blood cells. The homozygous c.386G>A (p.C129Y) mutation is shown by a black bar. **e** The schematic diagram of a height gain curve

Immunoblotting

Specimens, except the non-reducing ones, were boiled with 3% (v/v) 2-mercaptoethanol at 60° C for 5 min, loaded into the wells of a 7% sodium dodecyl sulfate (SDS)-polyacrylamide gel with a 3.75% stacking gel, electrophoresed, and subjected to immunoblotting as described previously [17, 18]. The intensity of the band indicating each protein was quantified using the Multi Gauge software (v. 2.0; Fujifilm, Tokyo, Japan).

Immunocytochemistry

The transfected HEK293T cells were cultured on glass coverslips (Matsunami Glass Ind. Ltd., Osaka, Japan), fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) for 10 min, permeabilized in 0.1% saponin/PBS for 10 min, blocked with 3% bovine serum albumin (BSA)/PBS for 30 min, and stained with anti-HA and anti-Na+/K+-ATPase α1-subunit (plasma membrane marker) for 2 h. The cells were then stained with Alexa Fluor 488 donkey anti-rat immunoglobulin G and Alexa Fluor 546 donkey anti-mouse immunoglobulin G for 1 h. These staining procedures were performed at room temperature. The cells were mounted

onto glass slides with VECTASHIELD mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) and then visualized using a Leica TCS SP5 II laser scanning confocal microscope (Leica, Solms, Germany).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). The differences between two variables and multiple variables were assessed at the 95% confidence level using Student's *t*-tests and analysis of variance with a post-hoc Dunnett's test, respectively. Data were analyzed using Prism software (v. 6; GraphPad Software, La Jolla, CA).

Genetic analysis

Sequencing of *ABCB11* was performed at Kazusa DNA Research Institute (Kisarazu, Japan).

Homology model of BSEP

The structure of the BSEP protein was constructed by homology modeling using the program SWISS-MODEL [20–22] using the crystal structure of mouse multidrug resistance protein 1A (MDR1A) (gene Abcb1a) (RSCB Protein Data Bank entry 4KSD) as a template. The structure was illustrated using PyMOL (Schrödinger, Inc. http://www.pymol.org/).

Results

A patient with a homozygous BSEP mutation (c.386G>A (p.C129Y))

A Japanese girl was delivered normally at term to non-consanguineous parents and did not have a specific family history of disease. At the age of 45 days, she was referred to our hospital because of the presence of jaundice and acholic stool. Blood tests gave the following results: 1355 U/L aspartate aminotransferase (AST), 918 U/L alanine aminotransferase (ALT), 7.6 mg/dL direct bilirubin (D-Bil), and 133.2 µmol/L total bile acids (TBA). Severe liver dysfunction and cholestasis were observed with normal GGT activity. Analysis of duodenal drainage fluid indicated low bile acid levels, and lipase levels were elevated. The results of these biochemical assessments are shown in Fig. 1a. These data suggested that biliary excretion from hepatocytes was impaired in this patient.

Percutaneous liver biopsy was performed at 2 months of age. Liver histological examination showed cholestasis, giant cell transformation, and hepatocellular swelling (Fig. 1b). Immunostaining for BSEP was negative, whereas the canalicular multidrug-resistance protein 2 (MRP2, *ABCC2*) and multidrug-resistance 3 (MDR3, *ABCB4*) protein were observed (Fig. 1c). These pathological findings were similar to the PFIC2 phenotype [23]. Furthermore, genomic sequencing analysis of peripheral blood cells showed a homozygous mutation in *ABCB11*, c.386G>A (p.C129Y) (Fig. 1d). The minor allele frequency of C129Y in 1200 entries in the Japanese Human Genetic Variation Database (http://www.hgvd.genome.med.kyoto-u.ac.jp/index.html) and in the Exome Aggregation Consortium dataset (http://exac.broadinstitute.org/) had not been reported, indicating C129Y to be a very rare variant. The effect of this missense mutation was predicted to be tolerated by Sorting Intolerant From Tolerant (http://sift.jcvi.org/; J. Craig Venter Institute, Rockville, MD, USA), although it was predicted to be probably damaging by Polymorphism Phenotyping-2 (http://genetics.bwh.harvard.edu/pph2/; Harvard Medical School, Boston, MA, USA; Table S1). PFIC2 cases harboring a heterozygous mutation in ABCB11, c.386 G > A (p.C129Y), had been recently reported in Japanese individuals [11], and these findings led to the diagnosis of PFIC2 in this patient. These findings provided a diagnosis of PFIC2 for this patient.

The patient was administered ursodeoxycholic acid (5 mg/kg/day) and fat-soluble vitamins for 6 months. However, cholestasis did not improve, and the patient suffered from a failure to thrive and dysfunction of the liver. A living related-donor liver transplantation was performed at 8 months of age. The clinical course since the surgical procedure has been good and cholestasis has not been observed for more than 5 years, although a recurrence triggered by antibodies against BSEP was reported [24–26]. In addition, the patient exhibited catch-up growth after liver transplantation (Fig. 1e).

Effects of the c.386G>A (p.C129Y) mutation in *ABCB11*

We constructed a homology model of BSEP (Fig. 2a). To investigate whether a disulfide bond was formed between residues C107 and C129, we used HEK293T cells transfected with the cDNA of HA-BSEP^{WT} and HA-BSEP^{C129Y}. A disulfide bond might not be formed because HA-BSEP^{WT} was detected to have identical molecular weight as HA-BSEP^{C129Y}, under both reducing and non-reducing conditions (Fig. 2b).

To elucidate the effects of the mutation c.386G>A (p.C129Y) in *ABCB11*, mRNA and protein expression, cellular localization, and transport activity of HA-BSEP^{C129Y} were evaluated. qPCR analysis showed no significant impact of the mutation c.386G>A (p.C129Y) on the expression of *ABCB11* gene (Fig. 3a). Next, the expression of the mutated BSEP protein was investigated by cell

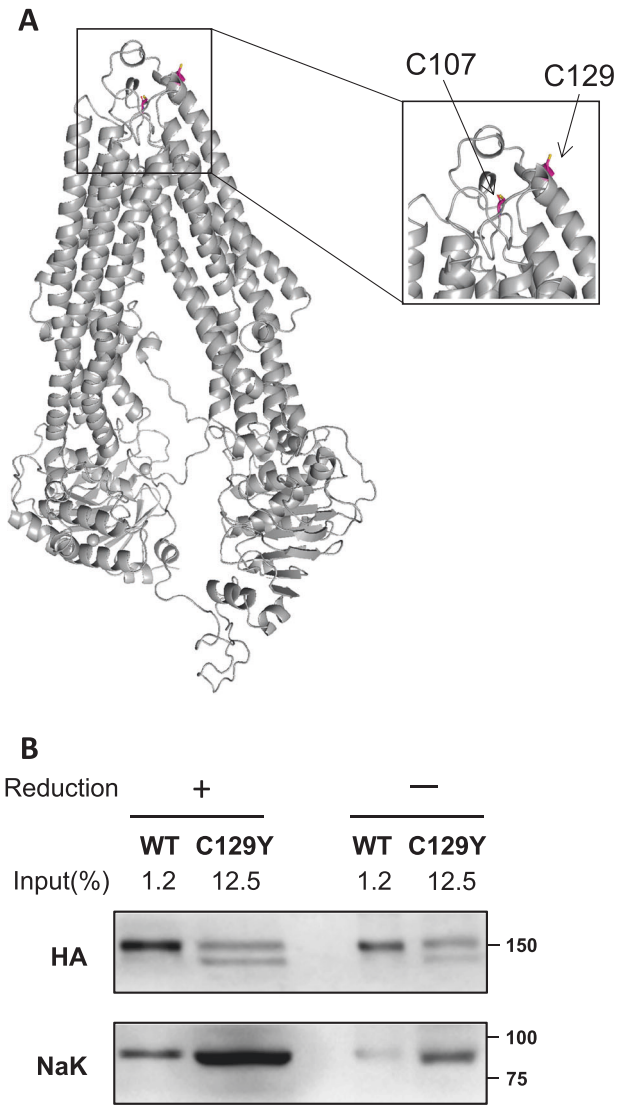

Fig. 2 A disulfide bond between residues C107 and C129. **a** The whole protein is shown with cysteine (Cys) 107 and Cys 129 in magenta. Atoms are shown as sticks, with yellow sticks indicating sulfur. The model was created using PyMOL. **b** HEK293T cells were transfected with pcDNA3.1(+)-HA-BSEP^{WT} or HA-BSEP^{C129Y} and lysed. The prepared specimens were either treated with 3% (v/v) 2-mercaptoethanol at 60 °C for 5 min, or left untreated, and then analyzed by immunoblotting

surface biotinylation and subsequent immunoblotting. These data showed that the expression of HA-BSEP^{C129Y} on the cell surface was significantly lower than that seen with the control (Fig. 3b). Immunocytochemical assessment showed that the expression of HA-BSEP^{C129Y} was distributed to endoplasmic reticulum (ER)-like structures, although HA-BSEP^{WT} was correctly localized to the plasma membrane (Fig. 3c). These results suggested that the trafficking of BSEP^{C129Y} to the plasma membrane is impaired compared to BSEP^{WT}. To explore the transport activity of the mutated BSEP, the uptake of [^{3}H]-taurocholate ([^{3}H]-TC) into membrane vesicles isolated from HEK293T cells expressing HA-BSEP^{WT} and HA-BSEP^{C129Y} was investigated. Immunoblotting analysis indicated that the expression of HA-BSEP^{WT} was 12.5-times higher than that of HA-BSEP^{C129Y} (Fig. 3d). The amount of [^{3}H]-TC transported via HA-BSEP^{C129Y} was significantly lower than that seen with HA-BSEP^{WT} (Fig. 3e). The transport activity of BSEP^{C129Y} would be conserved; indeed, these results suggested that the amount of membrane BSEP^{C129Y} corresponded to the uptake of [^{3}H]-TC into membrane vesicles.

Low-temperature treatment is reported to be capable of correcting the trafficking of other PFIC2-type mutated BSEP [10]. However, 24 h-culture at 27 °C increased the immature ER-resident form (<150 kDa), but not mature cell surface-resident form (>150 kDa) of HA-BSEP^{C129Y} (Fig. 3f), suggesting that the low-temperature treatment might have stabilized the immature ER-resident form of HA-BSEP^{C129Y}, but was unable to restore its impaired trafficking. Increased expression of the mature cell surface-resident forms (>150 kDa) of HA-BSEP^{WT} and HA-BSEP^{C129Y}, accounting for 1.6- and 2.3-fold change, respectively, was observed following a treatment with sodium 4-phenylbutyrate (4PB), a potential therapeutic drug for patients with PFIC2, who maintain BSEP transport activity [10, 17, 18] (Fig. 3g).

These data demonstrated that c.386G>A (p.C129Y) in *ABCB11* was a causative mutation that correlated with the phenotype of patients with PFIC2, the impairment of biliary excretion into bile canaliculi, and the absence of canalicular BSEP expression in liver histological assessments.

Discussion

Mutations in *ABCB11* confer various phenotypes, including PFIC2 and BRIC2, which is a less severe form of BSEP disorder. Moreover, mutations in *ABCB11* resulting in a phenotypic continuum between BRIC2 and PFIC2 have also been reported [27, 28]. The phenotype–genotype correlation has not been elucidated as of yet because hotspot mutations have not been found in the *ABCB11* gene. The mutations p.E297G and p.D482G in *ABCB11* are those most frequently observed in Western patients with PFIC2 and have been extensively studied with regard to in vitro pathogenicity (Table S2). However, these common missense mutations have not been identified in Japanese patients with PFIC2. In Japan, the *ABCB11* mutation c.386G>A (p.C129Y), which was also identified in our case here, has been found most frequently (Table 1). The missense mutations in *ABCB11* reported previously in Japan are shown in Table 1 [39, 40]. The characteristics of *ABCB11* gene mutations in Japan may be different from those in other areas [3, 8]. The existence of ethnicity-

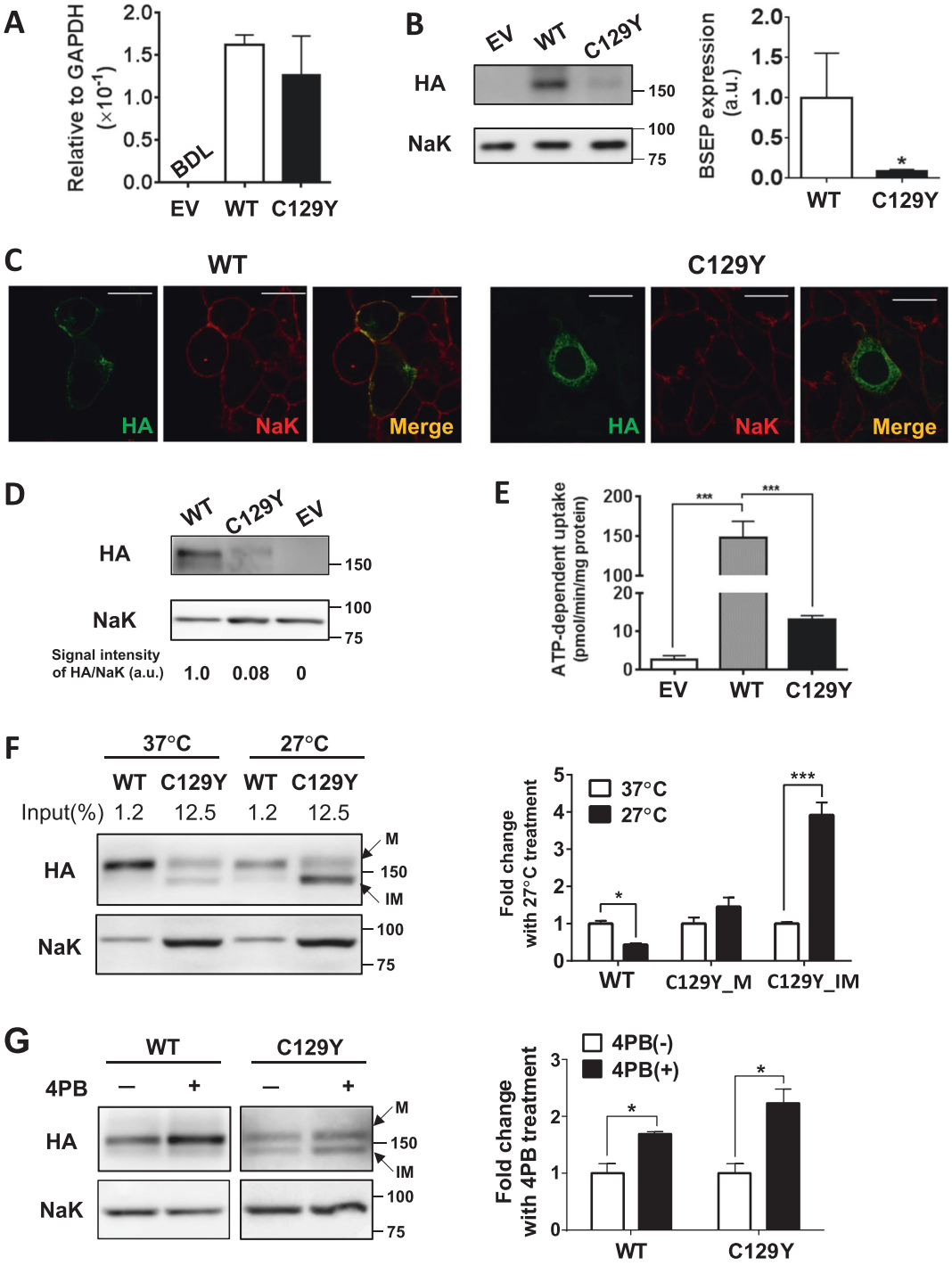

Fig. 3 Characterization of c.386G>A (p.C129Y) mutation in *ABCB11*. HEK293T cells were transfected with pcDNA3.1(+)-HA-BSEP^{WT}, HA-BSEP^{C129Y}, or corresponding empty vector. Determination of mRNA and protein levels of HA-BSEP^{C129Y}. The cells were subjected to RNA preparation (**a**) and cell surface biotinylation (**b**) and then analyzed by qPCR (**a**) and immunoblotting (**b**). **c** Cellular localization of HA-BSEP^{C129Y}. The cells were stained with anti-HA and anti-Na/K antibodies and analyzed by confocal immunofluorescence microscopy. Yellow in the merged images indicates colocalization. Scale bar: 10 µm. **d**, **e** HA-BSEP^{C129Y}-mediated uptake of [^{3}H]-taurocholate (TC) (**e**). Membrane vesicles were prepared from the cells and subjected to immunoblotting (**d**) and an uptake assay using [^{3}H]-TC (**e**). In **a**–**e**, a representative result of more than two independent experiments is shown. Bars represent the mean ± SEM of each experiment in triplicate (**a**, **b**) or quadruplicate (**e**). Influence of low temperature (**f**) and 4PB (**g**) on the expression of HA-BSEP^{C129Y}. The transfected cells were cultured either at 27 °C, or left untreated with the low-temperature stimulus (**f**) or treated with 1 mM 4PB (**g**) for the last 24 h, lysed, and analyzed by immunoblotting. In **a**–**g**, a representative result of more than two independent experiments is shown. Bars represent the mean ± SEM of each experiment in triplicate (**a**, **b**, **f**, **g**) or quadruplicate (**e**). $^*P < 0.05$, $^{***}P < 0.0001$; a.u. arbitrary units, BDL below detection limits because of low expression levels, EV empty vector, IM immature ER-resident form, M mature cell surface-resident form, NaK Na^+/K^+-ATPase subunit α1, WT wild type

Table 1 Missense mutations in BSEP reported in Japan

Protein domain	Amino acidic change	*n*	Reference
Extracellular	C129Y	3	Our case, [11]
Cytoplasm	R487H	2	[11] [39]
Cytoplasm	E636G	1	[39]
Cytoplasm	A570V	1	[11]
TM10	S901R	1	[40]
Cytoplasm	Y1041H	1	[11]
Cytoplasm	C1083Y	1	[40]
Cytoplasm	R1231Q	1	[17]
Cytoplasm	R1268W	1	[11]
Cytoplasm	I1280N	1	[11]

TM transmembrane

specific mutations in *ABCB11* has been described previously [15]. As mutational analyses in the laboratory are required in order to understand the molecular functions of the mutated BSEP, its effects are still unknown. Therefore, in the present study we investigated the function of $BSEP^{C129Y}$, located on the first extracellular loop (ECL1) of BSEP. ECL1 is the largest extracellular domain of BSEP, with 61 amino acid residues. This domain is exclusively found in BSEP, but not in any other of its close relatives such as MDR1 and MDR3 and is likely to possess some unique functional role yet to be uncovered [24]. The data here show that the gene expression of *BSEP* was not altered; however, membrane BSEP expression was significantly reduced, and bile acid transport was also significantly impaired. These results are similar to those of previous analyses of mutated BSEP [9].

One-third of patients with PFIC are not diagnosed genetically [16]. Recently, causative mutations in *TJP2* and *FXR* were found in patients with normal-GGT PFIC, who demonstrated normal serum GGT and who had no mutations in the *ABCB11* gene [29, 30]. However, patients with normal GGT PFIC who do not harbor mutations in *TJP2* and *FXR* have also been reported. Molecular genetic analyses of Japanese patients with infantile cholestasis revealed that seven of 12 patients (58%) with PFIC did not bear mutations in the genes responsible for PFIC, i.e., *ATP8B1*, *ABCB11*, *ABCB4*, and *TJP2* [11]. Thus, the percentage of Japanese patients with the PFIC phenotype who have not been genetically diagnosed may be higher than that in other populations. This discrepancy suggests the possible existence of ethnicity-specific genes that are responsible for the PFIC phenotype. Accordingly, a comprehensive genomic analysis of undiagnosed patients may elucidate novel causative genes for PFIC.

4PB is a drug with a potential application in the treatment of PFIC2 [10, 18]. It can increase the expression of BSEP on the canalicular membrane by reducing ubiquitination and subsequent internalization from the canalicular membrane [9, 31, 32]. Intrahepatic cholestasis may be ameliorated by increasing the canalicular expression of mutated BSEP protein that retains the capacity to transport bile acids [17, 33]. Several reports have demonstrated the drug's therapeutic efficacy in patients with PFIC2 [34, 35]. In addition, the efficacy of 4PB therapy in preterm infants with cholestasis has also been reported [36]. Interestingly, patients with PFIC1 often exhibit intractable pruritus, and 4PB therapy successfully reduces pruritus in patients with this disease [37]. 4PB therapy may have been effective in our patient with PFIC2 harboring the ABCB11 mutation, c.386G>A (p.C129Y), who retained BSEP transport activity and showed partial restoration of BSEP expression following treatment with 4PB at a clinically relevant concentration (Fig. 3d–f). Moreover, mutational analyses may be effective in estimating the efficacy of therapeutic options. Cell-based models, such as those employed in the present study, may be useful for performing functional analyses as BSEP-knockout mice do not reproduce the PFIC2 phenotype [38]. Thus, further cases and mutational analyses are necessary to fully elucidate the pathogenesis of PFIC2 and to develop novel therapies for this disease.

Acknowledgements We thank Dr. Hiroko Fukushima, Dr. Aiko Sakai, and Dr. Hisato Suzuki for critical discussions. We also thank all the members of our laboratory for constructive comments, suggestions, and discussions. We would like to thank Enago (www.enago.jp) for the English language review. We would like to thank Editage (www.editage.jp) for English language editing.

Funding KI is supported by JSPS KAKENHI Grant Number 17K16240. This work is supported by Japan Agency for Medical Research and Development, AMED, under Grant Number JP17ak0101036 to HH. This research is also funded by Japan Agency for Medical Research and development, AMED, to RS.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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