Korean J Physiol Pharmacol
Vol 17: 525−530, December, 2013
http://dx.doi.org/10.4196/kjpp.2013.17.6.525

Functional Characterization of *ABCB4* Mutations Found in Low Phospholipid-Associated Cholelithiasis (LPAC)

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Multidrug resistance 3 (MDR3) is expressed on the canalicular membrane of the hepatocytes and plays an important role in protecting the liver from bile acids. Altered *ABCB4* gene expression can lead to a rare hepatic disease, low phospholipid-associated cholelithiasis (LPAC). In this study, we characterized 3 *ABCB4* mutations in LPAC patients using various *in vitro* assay systems. We first measured the ability of each mutant to transport paclitaxel and then the mechanisms by which these mutations might change MDR3 transport activity were determined using immunoblotting, cell surface protein biotinylation, and immunofluorescence. Through a membrane vesicular transport assay, we observed that the uptake of paclitaxel was significantly reduced in membrane vesicles expressing 2 *ABCB4* mutations, F165I and S320F. Both mutants showed significantly decreased total and cell surface MDR3 expression. These data suggest two missense mutations of *ABCB4* may alter function of MDR3 and ultimately can be determined as LPAC-causing mutations.

Key Words: *ABCB4*, Functional characterization, LPAC, MDR3, Mutation

INTRODUCTION

Human multidrug resistance 3 (MDR3) coded by ATP-binding cassette, subfamily B, member 4 gene (*ABCB4*) belongs to ABC transporters family [1]. MDR3 is expressed on the canalicular membrane of the hepatocyte and translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane, playing an important role in protection of the liver from bile acids [2]. It was known that dysfunction of MDR3 due to genetic variations can cause several hepatic diseases in human, such as low phospholipid-associated cholelithiasis (LPAC), progressive familial intrahepatic cholestasis 3 (PFIC3), obstetric cholestasis, and drug-induced liver injury [3-7]. LPAC is intrahepatic cholelithiasis characterized by at least two of the following criteria: age onset of symptom less than 40 years, intrahepatic echogenic foci or microlithiasis, and recurrence after cholecystectomy [8,9]. LPAC is occurred higher in female than male, while its exact prevalence rate is not known. LPAC patients often have to undergo cholecystectomy due to biliary pain which could not be relieved by the treatment using ursodeoxycholic acid (UDCA). In addition, a few patients with end-stage liver disease can be candidate for liver transplantation [8].

To date, there were few studies to investigate the expression or function of each *ABCB4* variant in spite of the clinical importance of MDR3 transporter. Most previous functional analysis of *ABCB4* genetic variations have been restricted to missense mutations identified in PFIC3 patients. For example, a *ABCB4* mutation found in PFIC3 patients, I541F, was shown to decrease transport activity through reduction of membrane MDR3 expression [10,11]. Recently, we identified and functionally characterized the *ABCB4* promoter variants through direct sequencing using genomic DNA samples from 126 Koreans and reported 2 common promoter haplotypes of *ABCB4* resulted in significantly decreased promoter activity [12].

Previously, mutation analysis of *ABCB4* was performed using genomic DNA samples from 32 LPAC patients [3]. The authors identified 14 mutations in the coding region including 9 missense and 5 nonsense mutations. They found that all these mutations were not present in other two groups: one was group consisting patients with a classic gallstone disease and the other was group of patients without a history of cholelithiasis. However, a functional characterization of each mutant was not performed in their study.

In this study, we selected 3 novel missense mutations of *ABCB4* that were first reported by Rosmorduc et al. [3] and

Received October 21, 2013, Revised November 25, 2013, Accepted December 3, 2013

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ABBREVIATIONS: MDR3, multidrug resistance 3; LPAC, low phospholipid-associated cholelithiasis; *ABCB4*, ATP-binding cassette, subfamily B, member 4 gene; PFIC3, progressive familial intrahepatic cholestasis 3; UDCA, ursodeoxycholic acid; SNP, single nucleotide polymorphism; TMs, transmembranes; ICDs, intracellular domains; ECs, extracellular loops; TMD-NBD, transmembrane domain-nucleotide binding domain.

investigated the function of each mutant using various *in vitro* assays such as membrane vesicular transport, immunoblotting, and surface protein biotinylation. To our knowledge, this is the first study to characterize functionally *ABCB4* mutations found in LPAC. This study may contribute to the development of diagnostic kits for LPAC in the future.

METHODS

Construction of ABCB4 plasmids

To construct the plasmid containing a reference *ABCB4* gene, vector (BC_042531) was purchased (Thermo Fisher Scientific Inc., Waltham, MA, USA) and subcloned into the pcDNA3.1(+) vector. Plasmids containing the mutant *ABCB4* sequences were produced using QuikChange® II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with the primers listed in Table 1 from the pcDNA3.1-*ABCB4* plasmid. Nucleotide location numbers were assigned from the translational start site according to the *ABCB4* mRNA sequence (GenBank accession number; NM_018849.2).

Membrane vesicle preparation

Preparation of membrane vesicles was performed according to a previously described method [13]. Briefly, *ABCB4* reference or mutant-bearing plasmids were transfected into HEK-293T (Human embryonic kidney) cells using the Calcium Phosphate Transfection Kit (Life Technologies Corporation, Carlsbad, CA, USA). Cells were harvested 48 h later in a homogenization buffer supplemented with a protease inhibitor cocktail. Harvested cells were then subjected to nitrogen cavitation at 350 pounds per square inch for 15 min and transferred to a tube containing 0.5 M EDTA. The membrane vesicle fractions were collected by sucrose density gradient centrifugation at 1,000,000×g for 90 min. Immediately after preparation, the vesicles were suspended in buffer containing 250 mM sucrose and 50 mM Tris (pH 7.4) at a protein concentration of 4∼8 mg/ml.

Adenosine triphosphatase (ATPase) assay for paclitaxel transport

ATPase assays for paclitaxel transport were performed according to a previously described method with some modifications [14]. The vanadate-sensitive ATPase activity was measured using the SensoLyte® MG Phosphate Assay Kit (71103, AnaSpec, Fremont, CA, USA), to assess the paclitaxel transporting capacity of MDR3. Briefly, membrane vesicles (4 μg/well) were incubated in 4 mM $MgCl_2$, 5 mM 3-(Nmorpholino)-propanesulfonic acid-Tris (pH 7.0), 4 mM ATP, and various concentrations of paclitaxel with or without 1 mM sodium orthovanadate at 37°C for 15 min. Finally, absorbance was measured at 620 nm using a microplate reader. ATPase activities were determined as the difference in the inorganic phosphate liberation in the presence or absence of sodium orthovanadate.

Table 1. Oligonucleotide primers used in the construction of *ABCB4* mutants

F165I	5'-AGG AAA TAG GAT GG**A** TTG ACA TCA ATG ACA-3'
M301T	5'-GCA AAC ATT TCC A**C**G GGT ATT GCC TTS CTG-3'
S320F	5'-AGG ACA CAA A**T**C AGA CAG CAT CAA AGG GAA-3'

The SNP sites were marked by bold-faced letters with underlines.

Immunoblotting

The *ABCB4* reference or mutant-bearing plasmids were transfected into HEK-293T cells using the Lipofectamine LTX and Plus reagents (Life Technologies). 48 h after transfection, immunoblotting was performed using the following primary antibodies: a mouse anti-MDR3 antibody (P3II26, Abcam, Cambridge, UK), rabbit anti-neomycin phosphotransferase II antibody (06-747, Millipore, Billerica, MA, USA), or goat anti-β-actin antibody (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The intensity of each band was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Biotinylation of cell surface proteins

Biotinylation experiments were conducted using a Cell Surface Protein Isolation Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol using the HEK-293T cells obtaining from transfection of the *ABCB4* reference or mutant-bearing plasmids. A rabbit polyclonal anti-Na^+/K^+ ATPase α-1 antibody (06-520, Millipore) was used as an internal standard.

Immunofluorescence

For immunofluorescence, HEK-293T cells were grown on coverslips in a 24-well plate and the reference or mutant *ABCB4*-bearing plasmids were transfected into HEK-293T cells. 48 h after transfection, cells were incubated with CellMask Plasma Membrane stain solution (Life Technologies) and washed 3 times. After fixation with 4% PFA, cells were permeabilized with acetone and blocking was performed. For detection of MDR3, the anti-MDR3 antibody and Alexa Fluor® 488 rabbit anti-mouse IgG (11059, Life Technology) was used. Confocal images were captured using confocal laser scanning microscope (LSM5 PASCAL; Carl Zeiss, Oberkochen, Germany) and the digital images were analyzed using a LSM Image Examiner (Carl Zeiss).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 software package (GraphPad Software, Inc., San Diego, CA, USA). All p values were calculated using one-way analysis of variance followed by Dunnett's two-tailed test, and p values less than 0.05 were considered to be statistically significant.

RESULTS

Mutations of ABCB4 examined in this study

To perform a molecular characterization of *ABCB4* muta-

tions found in LPAC patients, we selected some novel mutations that were reported by Rosmorduc et al. [3]. They identified 14 *ABCB4* mutations including 9 missense mutations through direct sequencing of genomic DNA samples from 32 LPAC patients. Among these mutations, we excluded mutations that resulted in protein truncation or whose effect on other ABC transporters had already been investigated. Table 2 shows the 3 missense *ABCB4* mutations included in this study. It has known that MDR3 consists of 12 transmembranes (TMs), 6 intracellular domains (ICDs), 6 extracellular loops (ECs), and a linker connecting the N-terminal to the C-terminal transmembrane domain-nucleotide binding domain (TMD-NBD) [15]. The *ABCB4* mutant, F165I might be located in the ICD1, while other two mutants, M301T and S320F are located in the TM5 [3].

The effect of mutations on the transport activity of MDR3

It has been previously shown that MDR3 transports paclitaxel [16]. To characterize the functional effects of the *ABCB4* mutations, we first measured the ability of each mutant to transport paclitaxel. Fig. 1 shows the results of the ATP-dependent paclitaxel uptake in inside-out membrane vesicles expressing *ABCB4* reference or mutations at various concentrations (0.01, 1, 10, and 1,000 μM) of paclitaxel. To exclude ATPase activity by other endogenous ABC transporters including MDR1, values for transport activity were obtained by subtracting the uptake in empty vector-transfected cells from that in cells transfected with *ABCB4* reference or mutant-bearing vectors, at each corresponding paclitaxel concentration. The uptake of paclitaxel

Table 2. *ABCB4* mutations included in this study

cDNA position	Amino acid substitution	Predicted domain[a]
c.495T>A	F165I	ICD1
c.902T>C	M301T	TM5
c.959C>T	S320F	TM5

Position of each mutant was based upon the translational start site. [a]Predicted domain was determined by previous study [3]. ICD, intracellular domain; TM, transmembrane.

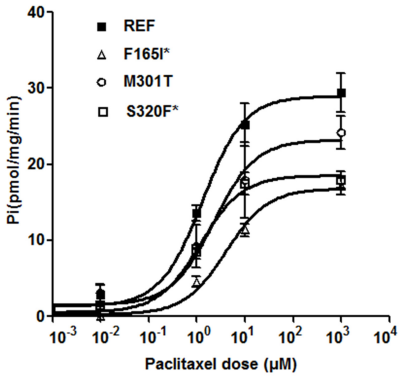

Fig. 1. Effect of *ABCB4* mutants on the transport activity. Inside-out membrane vesicles were prepared after transfection of *ABCB4* reference (REF) or mutant-bearing plasmids into HEK-293T cells and ATP-dependent transport of paclitaxel was measured using various concentrations of paclitaxel. Values for transport activity were obtained by subtracting the uptake in empty vector (EV, pcDNA3.1)-transfected cells from that in cells transfected with *ABCB4* reference or mutant-bearing vectors, at each corresponding paclitaxel concentration. The X-axis represents paclitaxel concentration. The Y-axis represents the amount of inorganic phosphate which was produced by the ATPase activity of MDR3 during the transport of paclitaxel. The data shown represent mean±SD from 5 separate experiments, with each experiment performed in triplicate wells. *$p<0.05$ vs. reference.

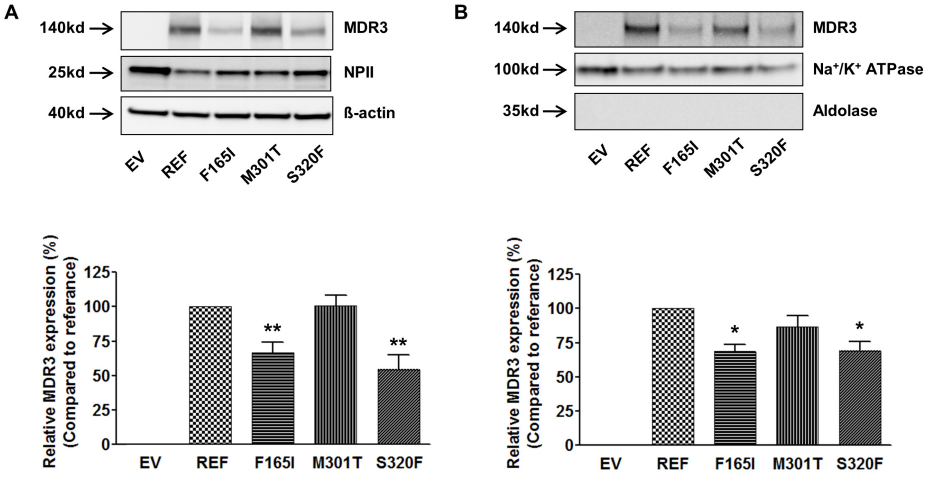

Fig. 2. Effect of *ABCB4* mutants on the MDR3 expression. (A) HEK-293T cells expressing reference or mutant *ABCB4* genes were lysed and proteins were immunoblotted with antibodies against MDR3, neomycin phosphotransferase II (NPII), and β-actin. (B) HEK-293T cells expressing reference or mutant *ABCB4* genes were surface biotinylated and proteins were immunoblotted with antibodies against MDR3, Na^+/K^+ ATPase α-1, and aldolase. Immunoblotting with the anti-aldolase antibody confirmed that the samples were not contaminated with the cytosolic fraction of MDR3 protein. The intensity of each band was normalized by neomycin phosphotransferase II and β-actin (A) or Na^+/K^+ ATPase α-1 (B). Data (mean±SD) are from 3 separate experiments. *$p<0.05$, **$p<0.01$ vs. reference.

was significantly reduced in membrane vesicles expressing 2 *ABCB4* mutations, F165I and S320F. M301T mutant also showed a reduction in the transport activity compared to that of the reference, although statistical significance was not observed. The paclitaxel V_{max} and K_m values for the *ABCB4* reference or mutant genes are shown in Table 3. We observed that the average values of V_{max}/K_m for F165I and S320F were significantly reduced compared to that of the *ABCB4* reference. This was attributable to a dramatically reduced V_{max}. In the case of K_m, these mutants showed similar K_m values compared to that of the reference.

The effect of mutations on the expression level of MDR3

Cell surface biotinylation and immunoblotting were performed to investigate the mechanisms through which *ABCB4* mutations alter the transport activity. Fig. 2A shows the results from immunoblotting of the total cell lysates after transfection of *ABCB4* reference or mutant-bearing plasmids into HEK-293T cells. The 2 mutations, F165I and S320F that showed decreased transport activities as compared to the reference, had significantly decreased MDR3 expression as compared to reference. The MDR3 expression level of M301T was comparable with that of the reference. Then, we investigated MDR3 expression levels of these mutants on the plasma membrane by cell surface biotinylation and observed that those of F165I and S320F were significantly decreased by 31%, compared to that of the reference (Fig. 2B). The results from immunoblotting or cell surface biotinylation experiments could explain the reason of altered transport activities of *ABCB4* mutants, F165I and S320F; the decreased transport activities of these mutants were due to the reduced expression of functional MDR3 on the plasma membrane.

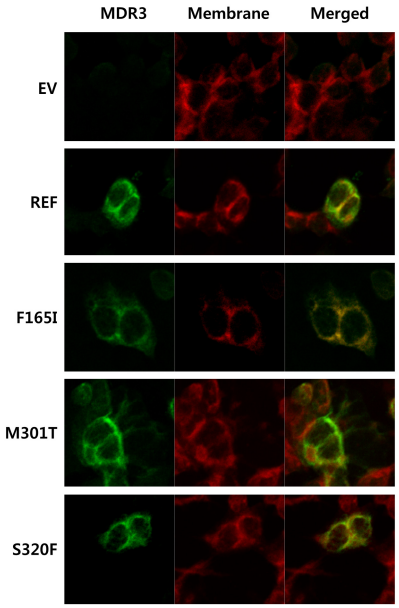

Fig. 3. Subcellular localization of the *ABCB4* mutants. Immunofluorescence was performed after transfection of *ABCB4* reference or mutant-bearing plasmids into HEK-293T cells. MDR3 protein was detected using the P3II26 antibody and Alexa Fluor® 488 rabbit anti-mouse IgG (green). Plasma membrane was stained by CellMask Plasma Membrane stain solution (red).

The effect of mutations on the subcellular localization of MDR3

To investigate the subcellular localization of MDR3 and its mutants, immunofluorescence was performed after transfection of *ABCB4* reference or mutant-bearing plasmids into HEK-293T cells. Fluorescence microscope showed that the majority of the reference MDR3 was restricted to the plasma membrane and was co-localized with the plasma membrane staining (Fig. 3). The subcellular expression of M301T was comparable with that of the reference while the co-localization of F165I and S320F with plasma membrane was decreased. These findings were consistent with the results obtained in immunoblotting and cell surface biotinylation experiments.

DISCUSSION

MDR3 is highly expressed on the canalicular membrane of hepatocytes and mediates the excretion of bile acids [1]. Functional changes in the activity or expression of MDR3 caused by genetic variants have been implicated as the primary genetic risk factor of LPAC.

Previously, mutation analysis of *ABCB4* was performed using genomic DNA samples from 32 patients with LPAC [3]. The authors identified 14 mutations in the coding region including 9 missense and 5 nonsense mutations that can be considered as potential disease-causing mutations. However, the functional characterization of each mutant was not performed in their study.

The current study was conducted to characterize functionally the *ABCB4* mutations found previously by Rosmorduc et al. [3]. Among these 14 *ABCB4* mutations, we selected 3 mutations after excluding mutations that were nonsense mutations or had already been investigated in relation to other ABC transporters. Through a membrane vesicular transport assay using paclitaxel, we found that 2 mutants, F165I and S320F, showed significantly decreased transport activity compared to the reference (Fig. 1). F165I and S320F are located in the ICD1 and TM5 of MDR3, respectively, that might be involved in coupling the energy from ATP hydrolysis to substrate transport and the conformational change involved in substrate extrusion, respectively [3,15]. M301T, showing a comparable transport activity with that of the reference is also located in TM5.

Table 3. Kinetic values of paclitaxel uptake in membrane vesicles expressing *ABCB4* reference or mutations

	V_{max} (nmol mg^{-1} per min)	K_m (mM)	V_{max}/K_m ratio (nmol mg^{-1} min^{-1} per mM)
Reference	28.98±1.565	1.114±0.1391	27.13±5.232
F165I	16.82±1.565*	1.028±0.1189	15.56±4.658*
M301T	23.23±0.8641	1.206±0.2875	20.60±5.628
S320F	18.55±2.726*	1.185±0.1064	15.99±3.736*

Data (mean±SD) are from 5 separate experiments. *$p < 0.05$ vs. reference.

The different effect of genetic variations which are located closely on the transport activity was also reported previously [17]. These data suggest that the function of each variation could not be determined only by its location on the transporter. The reduced transport function of F165I and S320F mutants also might be due to the decreased expression of functional MDR3 on the plasma membrane (Fig. 2). In the kinetic studies using paclitaxel, we observed that all V_{max} values of the mutants showing decreased transport activity were significantly decreased compared to the reference, whereas the changes in the K_m values were not statistically significant (Table 3). These data support that the reduced transport activity of these mutants is primarily attributable to the reduced expression of functional MDR3 on the plasma membrane.

One of the *ABCB4* mutations, I541F, was found in PFIC3 patients and was shown to be a trafficking-defective mutation [10,11]. This mutant demonstrated decreased transport activity due to the marked reduction of membrane MDR3 expression. 2 mutants, F165I and S320F, examined in this study also showed decreased transport activity and protein expression, although the extent of reduction was less than that of I541F. Therefore, PFIC3 patients are usually candidates for liver transplantation before adulthood due to complications such as hepatic failure, liver cirrhosis, and hepatocellular carcinoma, while the majority of LPAC patients can be controlled by UDCA if this treatment is started early [8,18].

According to the previous study by Rosmorduc et al. [3], only 18 patients had *ABCB4* mutations among the 32 patients clinically diagnosed as LPAC. As mentioned above, UDCA treatment should be started early to control LPAC effectively. Therefore, it would be valuable to develop the diagnostic kits for the rapid detection of *ABCB4* mutations.

In this study, we could not assess the correlation between the severity of clinical symptoms and the molecular function of mutants because the previous study that was referred in this study [3] did not provide the clinical symptoms and mutant type of each patient. Recently, screening of genomic DNA samples from 156 LPAC patients identified 46 *ABCB4* mutations, consisting of 13 nonsense and 33 missense mutations. Among the 33 missense mutations, 15 were novel [9]. By investigating the genotype-phenotype relationships in LPAC patients, the authors found that the nonsense mutations were associated with an earlier onset of symptoms, while biliary complications or the frequency and severity of intrahepatic cholestasis of pregnancy were not related with the mutant type. However they didn't perform *in vitro* assay of each mutant. To clarify these relationships further, the functional characterization of each mutant would be necessary.

In conclusion, we characterized the *ABCB4* mutations found in LPAC patients and revealed that 2 mutants showed significantly decreased transport activity, mainly due to decreased functional MDR3 expression on the cell membrane. To our knowledge, this is the first study to characterize functionally *ABCB4* mutations that cause LPAC. This study may contribute to the development of diagnostic kits for LPAC in the future.

ACKNOWLEDGEMENTS

This study was supported by the National Research Foundation of Korea (NRF) grants funded by the Korean government (MEST, 2010-0003262 and MSIP, 2010-0029353).

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