Human Cell (2024) 37:1070–1079
https://doi.org/10.1007/s13577-024-01074-x

RESEARCH ARTICLE

Functional characterization of variants in human ABCC11, an axillary osmidrosis risk factor

Yu Toyoda[1,2] · Hirotaka Matsuo[2] · Tappei Takada[1]

Received: 9 March 2024 / Accepted: 3 May 2024 / Published online: 15 May 2024

Abstract

Human ATP-binding cassette transporter C11 (ABCC11) is a membrane protein exhibiting ATP-dependent transport activity for a variety of lipophilic anions including endogenous substances and xenobiotics such as anti-cancer agents. Accumulating evidence indicates that ABCC11 wild type is responsible for the high-secretion phenotypes in human apocrine glands including wet type of earwax and the risk of axillary osmidrosis. Also, a less-functional variant of ABCC11 was reportedly associated with a risk for drug-induced toxicity in humans. Thus, functional change in ABCC11 may affect individual's constitution and drug toxicity, which led us to reason that functional validation of genetic variations in *ABCC11* should be of importance. Therefore, in addition to p.G180R (a well-characterized non-functional variant of ABCC11), we studied cellular expression and function of 10 variants of ABCC11. In this study, ABCC11 function was evaluated as an ATP-dependent transport of radio labeled-dehydroepiandrosterone sulfate using ABCC11-expressing plasma membrane vesicles. Except for p.G180R, other 10 variants were maturated as an *N*-linked glycoprotein and expressed on the plasma membrane. We found that six variants impaired the net cellular function of ABCC11. Among them, p.R630W was most influential. Including this identification of a significantly-dysfunctional variant, our findings will extend our understanding of genetic variations and biochemical features of ABCC11 protein.

Keywords ABC transporter · Axillary osmidrosis · DHEA-S · MRP8 · Rare variant

Introduction

ATP-binding cassette (ABC) transporters form a large protein family involved in many physiologically important cellular processes [1–3]. Among such proteins, human ABC transporter C11 (ABCC11), a 1382-amino acid *N*-linked glycoprotein, is identified as a membrane protein exhibiting transport activity for a variety of lipophilic anions in an ATP-dependent manner [4, 5]. Especially in early stages of investigation for this transporter protein, considering its cellular function as a drug efflux pump from cytosol into extracellular regions, drug resistance properties of ABCC11 have been studied [6, 7]; in this context, ABCC11 has been called as multidrug resistance-associated protein 8 (MRP8). However, since the identification of *ABCC11* c.538G>A (rs17822931) as the determinant of earwax type—an apocrine gland-related phenotype in humans [8], its physiological impacts have also attracted a lot of interests.

Previous studies on the single nucleotide polymorphism [SNP, minor allele frequency (MAF) ≥ 1%] *ABCC11* c.538G>A revealed that ABCC11 wild type (WT) is responsible for high-secretion phenotypes in human apocrine glands including wet-type earwax [8], apocrine colostrum secretion from the mammary gland [9], and the risk of axillary osmidrosis (AO) [10–12]. This SNP substitutes the glycine at amino acid position 180 to arginine (p.G180R); the p.G180R variant is recognized as an endoplasmic reticulum-associated degradation (ERAD) substrate and thereby losses the cellular function of ABCC11 [11]. In other words, ABCC11 WT is functional; the p.G180R variant is functionally null, which is consistent with the relationship between *ABCC11* genotypes and the apocrine gland-related phenotypes—538G/G and G/A correspond to the wet type of

✉ Yu Toyoda
ytoyoda-tky@umin.ac.jp

1 Department of Pharmacy, The University of Tokyo Hospital, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

2 Department of Integrative Physiology and Bio-Nano Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

earwax and AO risk; 538A/A corresponds to the dry type and little risk of AO, which are apocrine secretion-deficient phenotypes [13, 14].

In *ABCC11*, not only the well-characterized SNP variant p.G180R but also other genetic variations including rare variants (MAF < 1%) have been reported. Among them, in our previous study [11], nine non-synonymous variants (p.R19H, p.A317E, p.T546M, p.R630W, p.V648I, p.V687I, p.K735R, p.M970V, and p.H1344R; details are summarized below) were biochemically characterized regarding their effects on the protein maturation of ABCC11 as an *N*-linked glycoprotein. These variants were expressed as glycoprotein in mammalian cells, contrary to p.G180R. Although a functional study for a several variants of them was conducted using stably ABCC11-expressing cells [15], their effects on protein function of ABCC11 have not been well understood yet. Moreover, while p.T546M was reportedly identified as a risk factor of 5-fluorouracil (FU)-induced leukopenia in a human study [16], little information has been available for phenotypic effects of the other variants due to the lack of investigations focusing on *ABCC11* variants. Given the physiological and clinical impacts of ABCC11, functional validation of such genetic variations in *ABCC11* should be of importance.

To address the possible physiological or pharmacological relevance of genetic variation in *ABCC11*, we herein functionally characterized a total of 10 variants (the nine variants above and p.N1277Y, which is relatively common in some populations) of ABCC11. In addition to cell-based biochemical investigations, using a plasma membrane vesicle system, we quantitatively evaluated the effects of the variants on the ABCC11-mediated transport of [^{3}H]-dehydroepiandrosterone sulfate (DHEA-S), an ABCC11 substrate [6]. Our findings contribute to deepening the understanding of genetic variations in *ABCC11* and the biochemical characteristics of the ABCC11 protein.

Materials and methods

Materials

Critical materials used in this study are summarized in Table 1. All other chemicals used were commercially available and of analytical grade.

Plasmids

The full-length ABCC11 WT (NCBI accession: NM_033151) open reading frame (ORF) in pcDNA3.1/hygro(−) plasmid was constructed in our previous study [11]. Expression vectors for each ABCC11 variant in the plasmid were also from the previous study or generated in this study using a site-directed mutagenesis technique. Introduction of mutations was confirmed by full sequencing using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) with Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) as described previously [17]. Information on *ABCC11* variants studied here is summarized in Table 2. Prior to further experiments described below, all the plasmids were obtained from the same lot using the PureLink HiPure Plasmid Filter Midiprep Kit (Thermo Fisher Scientific, Yokohama, Japan), according to the manufacturer's protocol.

Cell culture

Human embryonic kidney 293 (HEK293)-derived 293A cells (Invitrogen, CA, USA) were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), 1% penicillin–streptomycin (Nacalai Tesque), 2 mM L-glutamine (Nacalai Tesque), and 1 × non-essential amino acid (Life Technologies, Tokyo, Japan) at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air as described previously [18]. All experiments were carried out with 293A cells at passages 10–18.

Each vector plasmid for ABCC11 WT or its variants was transiently transfected into 293A cells using polyethyleneimine MAX (PEI-MAX; 1 mg/mL in milliQ water, pH 7.0; Polysciences, Warrington, PA, USA) as described previously [17]. The amount of plasmid DNA used for transfection was adjusted to be the same for ABCC11 WT and its variants. In brief, each plasmid was mixed with PEI-MAX (2 μg of plasmid/10 μL of PEI-MAX for 1×10^6 293A cells) in Opti-MEM (Thermo Fisher Scientific) and incubated for 20 min at room temperature, then subjected to plasmid transfection. The medium was replaced with a fresh medium after the first 24 h of incubation.

RNA extraction and qPCR

Total RNA was extracted from mock or ABCC11-expressing 293A cells using the RNA isoPlus Reagent (Takara Bio, Shiga, Japan), according to the manufacturer's protocol. Reverse transcriptional reaction with ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) and subsequent quantitative PCR (qPCR) using SYBR GreenER qPCR SuperMix Universal (Life Technologies) in an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) were performed as described previously [19]. The expression levels of ABCC11 were normalized by those of β-actin.

Table 1 Key resources

Reagent or resource	Source	Identifier
Antibodies		
Rat monoclonal anti-MRP8 (ABCC11) antibody [M8I-74]	Abcam	Cat# ab91452; RRID: AB_2049125
Rabbit polyclonal anti-α-tubulin	Abcam	Cat# ab15246; RRID: AB_301787
Rabbit polyclonal anti-Na^+/K^+-ATPase α antibody	Santa Cruz Biotechnology	Cat# sc-28800; RRID: AB_2290063
Goat anti-rat IgG-horseradish peroxidase (HRP)-conjugate	GE Healthcare	Cat# NA935V; RRID: AB_772207
Donkey anti-rabbit IgG-horseradish peroxidase (HRP)-conjugate	GE Healthcare	Cat# NA934V; RRID: AB_772206
Chemicals and recombinant proteins		
Dehydroepiandrosterone sulfate, sodium salt, [1,2,6,7-^{3}H(N)] (60.0 Ci/mmol)	PerkinElmer	Cat# NET860
Adenosine 5′-triphosphate disodium salt hydrate, Grade II, ≥ 98.5% (HPLC), crystalline, from microbial	SIGMA	Cat# A3377-5G; CAS: 4369-07-8
Adenosine 5′-monophosphate sodium salt, from yeast, ≥ 99%	SIGMA	Cat# A1752-5G; CAS: 149022-20-8
Creatine phosphokinase from rabbit muscle, Type I, salt-free, lyophilized powder, ≥ 150 units/mg protein	SIGMA	Cat# C3755-17.5KU; CAS: 9001-15-4
Disodium creatine phosphate tetrahydrate	FUJIFILM Wako Pure Chemical Industries	Cat# 030-04584; CAS: 922-32-7
Clear-sol II	Nacalai Tesque	Cat# 09136-83
Polyethelenimine "MAX"	Polysciences	Cat# 24765; CAS: 49553-93-7
Critical commercial assays		
Pierce™ BCA protein assay reagent A & B	Thermo Fisher Scientific	Cat# 23223, Cat# 23224
PureLink™ HiPure plasmid filter Midiprep Kit	Thermo Fisher Scientific	Cat# K210015
Recombinant DNA		
The complete human ABCC11 cDNA	Toyoda et al. [11]	NCBI reference sequence: NM_033151
Experimental models: cell lines		
293A	Invitrogen	R70507
Software and algorithms		
Excel 2019	Microsoft	https://www.microsoft.com/ja-jp/
Statcel4 add-in software	OMS publishing	http://www.oms-publ.co.jp/

Preparation of whole cell lysate

Forty-eight hours after the plasmid transfection, whole cell lysates were prepared in an ice-cold lysis buffer A containing 50 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 1% (w/v) Triton X-100, and a protease inhibitor cocktail for general use (Nacalai Tesque) as described previously [11]. Protein concentration of whole cell lysate was quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard, according to the manufacturer's protocol. For glycosidase treatment, the whole cell lysate samples were incubated with PNGase F (New England Biolabs Japan, Tokyo, Japan) (1.25 U/μg of protein) at 37 °C for 10 min as described previously [20], and then subjected to immunoblotting.

Preparation of ABCC11-expressing plasma membrane vesicles

Plasma membrane vesicles were prepared as the same lot from 293A cells transiently transfected with each ABCC11 variant-expressing or mock plasmid as described previously [17, 18]. Obtained plasma membrane vesicles were rapidly frozen in liquid N_2 and stored at −80 °C until use. Protein concentration of plasma membrane vesicles was quantified using a BCA Protein Assay Kit as described above.

Immunoblotting

Expression of ABCC11 protein in whole cell lysates or plasma membrane vesicles was examined by immunoblotting as described previously [18, 21]. Briefly, the prepared samples were electrophoretically separated on poly-acrylamide gels and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) by electroblotting at 15 V for 70 min. For blocking, the membrane was incubated in Tris-buffered saline containing 0.05% Tween 20 and 5% skim milk (TBST-skim milk). Blots were probed with a rat monoclonal anti-ABCC11 antibody (M8I-74, which had been validated in our previous study [18]; Abcam, Cambridge, MA, USA; diluted 100-fold), a rabbit anti-α-tubulin antibody (ab15246; Abcam; diluted

Table 2 Summary of genetic variations in *ABCC11* gene studied in this study and their effects on the cellular function of ABCC11 protein

rs number	Nucleotide change	Amino acid change	Alternative allele frequency in each study population[†]					Protein maturation[#]	Effect on net cellular function of ABCC11[$]
			African	Europe	East Asian	South Asian	American		
rs16945988	c.56G>A	p.Arg19His	0.246	0.101	0.019	0.044	0.089	Yes	N.S
rs17822931	c.538G>A	p.Gly180Arg	0.012	0.136	0.780	0.482	0.140	No	Functionally null*
rs11863236	c.950C>A	p.Ala317Glu	0.298	0.101	0.045	0.044	0.089	Yes	N.S
rs17822471	c.1637C>T	p.Thr546Met	0.005	0.066	0.000	0.015	0.052	Yes	Strongly decreased
rs41282045	c.1888C>T	p.Arg630Trp	0.003	0.004	0.000	0.002	0.004	Yes	Almost all disrupted
rs16945930	c.1942G>A	p.Val648Ile	0.002	0.016	0.300	0.005	0.049	Yes	Moderately decreased
rs16945928	c.2059G>A	p.Val687Ile	0.023	0.000	0.001	0.000	0.004	Yes	N.S
rs16945926	c.2204A>G	p.Lys735Arg	0.014	0.000	0.000	0.000	0.003	Yes	Moderately decreased
rs41280943	c.2908A>G	p.Met970Val	0.000	0.006	0.000	0.011	0.011	Yes	Moderately decreased
rs61739606	c.3829A>T	p.Asn1277Tyr	0.078	0.092	0.000	0.016	0.063	Yes	Moderately decreased
rs16945916	c.4031A>G	p.His1344Arg	0.387	0.168	0.045	0.033	0.137	Yes	N.S

N.S. not significant

[†]Information on allele frequency of each genetic variation in *ABCC11* gene was from NCBI dbSNP (https://www.ncbi.nlm.nih.gov/snp/; last accessed 2022/12/13) in which referenced study name was 1000Genomes; [#]referred to Fig. 3a; [$]referred to Fig. 4b; *p.G180R was not subjected to functional assay in this study; however, this variant had been identified as functionally null [8, 11]

1000-fold), or a rabbit polyclonal anti-Na^+/K^+-ATPase α antibody (sc-28800; Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1000-fold) followed by incubation with a goat anti-rat immunoglobulin G (IgG)-horseradish peroxidase (HRP)-conjugated antibody (NA935V; GE Healthcare; diluted 2000-fold) or a donkey anti-rabbit IgG-HRP-conjugated antibody (NA934V; GE Healthcare; diluted 3000-fold). All antibodies were used in TBST-skim milk. HRP-dependent luminescence was developed using the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and detected using a multi-imaging Analyzer Fusion Solo 4 system (Vilber Lourmat, Eberhardzell, Germany).

Vesicle transport assay

Experiments to examine the in vitro transport of [1,2,6,7-^{3}H(N)]-DHEA-S (PerkinElmer, Waltham, MA, USA), an ABCC11 substrate [6], into ABCC11-expressing and control (derived from 293A cells transfected with mock plasmid) plasma membrane vesicles were performed using a rapid filtration technique [21, 22], with a minor modification. Briefly, the plasma membrane vesicles were incubated with 100 nM of [1,2,6,7-^{3}H(N)]-DHEA-S in the reaction mixture (10 mM Tris/HCl, 250 mM sucrose, 10 mM $MgCl_2$, 10 mM creatine phosphate, 1 mg/mL creatine phosphokinase, and 50 mM ATP or AMP as a substitute of ATP, at pH 7.4) for 5 min at 37 °C. Then the radioactivity derived from the incorporated DHEA-S was measured.

In this vesicle transport assay, the transport activity in each group was calculated as incorporated clearance, [μL/mg protein/min = incorporated level of DHEA-S (disintegrations per minute (DPM)/mg protein/min)/DHEA-S level in the incubation mixture (DPM/μL)]. ATP-dependent DHEA-S transport was calculated by subtracting the DHEA-S transport activity in the absence of ATP from that in the presence of ATP; ABCC11-mediated DHEA-S transport activity was calculated by subtracting the ATP-dependent DHEA-S transport activity for control plasma membrane vesicles from that for ABCC11-expressing plasma membrane vesicles.

Schematic illustration of ABCC11 protein

Based on the amino acid sequence of ABCC11 protein (NCBI accession: NP_149163; ABCC11 isoform a that is encoded by NM_033151), its topology model was predicted by the open-source tool Protter [23] and SOSUI system [24–26], as an updated 2D structure of previously reported topologies [27–29]. The obtained topology data were plotted and modified using the T(E)Xtopo package [30]. Information of highly conserved peptide motifs among ABC transporters, such as Walker A, Walker B, and signature C, in ABCC11 protein were obtained from previous reports [28, 31].

Multiple sequence alignment

To compare amino acid sequences of ABCC11 in several mammalians, multiple sequence alignments and homology calculations were conducted using the GENETYX software (GENETYX, Tokyo, Japan) with the ClustalW2.1 Windows program, as described previously [19].

Statistical analyses

All statistical analyses were performed using Excel 2019 with Statcel4 add-in software (OMS publishing, Saitama, Japan). Different statistical tests were used for different experiments as described in the figure legends. Briefly, when analyzing multiple groups, the similarity of variance between groups was compared using Bartlett's test. When passing the test for homogeneity of variance, a Dunnett's test for comparisons with a control group was used. In the case of a single pair of quantitative data, one-sample *t* test was performed. Statistical significance was defined in terms of *P* values less than 0.05 or 0.01.

Results

To grasp the positional information of all the variants addressed in this study in ABCC11 protein, we schematically illustrated them in a topology model of ABCC11 protein (Fig. 1). In silico analyses using Protter and SOSUI system independently predicted 11 transmembrane domains (TMDs) including 10 common TMDs and one different TMD in each other, respectively (Supplementary Table S1). Given previously predicted topology models [27–29], together with the fact that a typical structure of ABC proteins consists of 12 TMDs and two intracellular ATP-binding cassettes, we herein integrated all the predicted results and constructed the topology model harboring 12 TMDs (Fig. 1), which allowed an ATP-binding cassette located on the C-terminus of ABCC11 protein to be intracellular. Also, this topology model is consistent with a predicted 3D structure of ABCC11 protein put on the AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/entry/Q96J66, accessed 2022/8/13) [32, 33]. Based on these pieces of information, three variants (p.G180R, p.A317E,

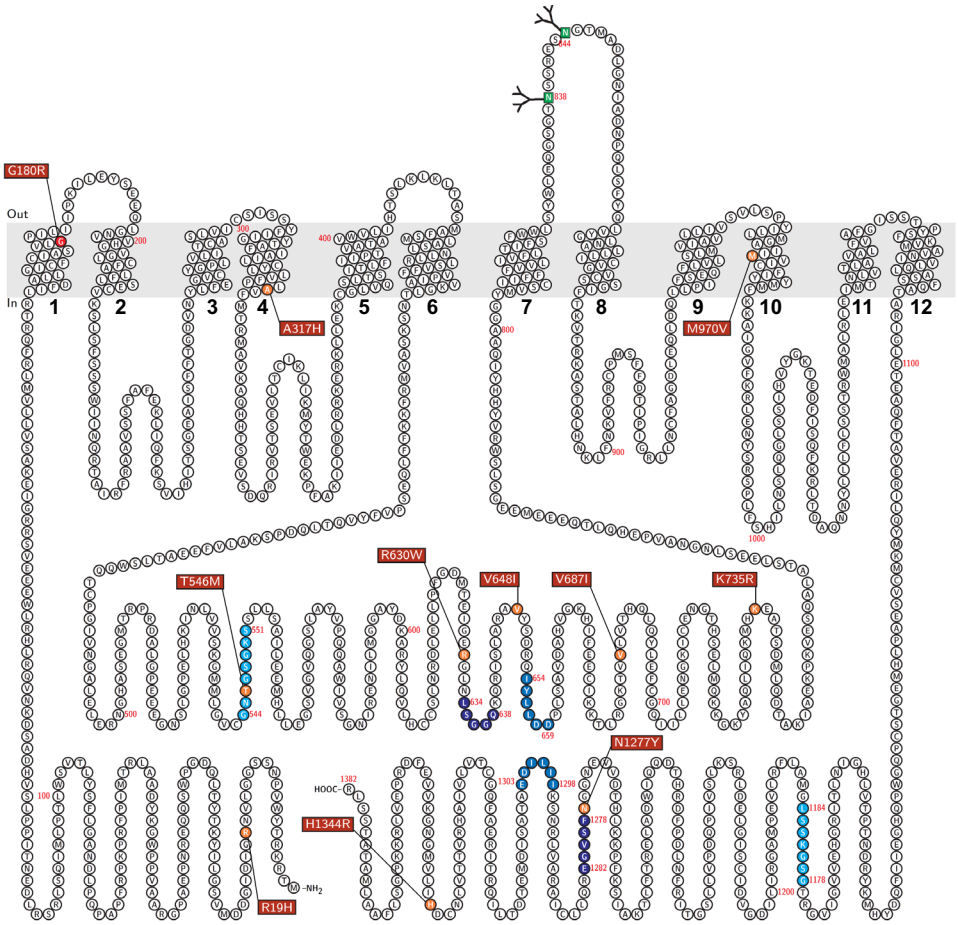

Fig. 1 Schematic illustration of a putative topological model of human ABCC11 protein. ABCC11 variants analyzed in this study are indicated with their amino acid substitution. Helices in the transmembrane domain are numbered (1 to 12); details of their prediction are shown in the main text. Asn838 and Asn844 are *N*-linked glycosylation sites. Within ATP-binding cassettes, there are several highly conserved motifs, which characterize ABC transporter proteins at the amino acid sequence level, including Walker A, Walker B, and signature C: Walker A (amino acids 544–551 and 1178–1184), Walker B (amino acids 654–659 and 1298–1303), and signature C (amino acids 634–638 and 1278–1282) in ABCC11 protein are indicated by colors

and p.M970V) are in transmembrane helices; the others are in intracellular regions.

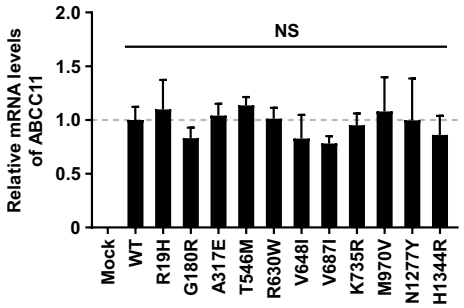

Fig. 2 Effects of each mutation on the mRNA levels of ABCC11 in transiently transfected 293A cells. Quantitative PCR was conducted using total cDNA samples that were prepared 48 h after plasmid transfection; as an internal control, β-actin mRNA was used. ABCC11 mRNA levels were normalized to the wild-type (WT) level. Data are expressed as mean ± SD; $n = 3$. *NS* not significantly different compared with WT (Dunnett's test)

To examine the effects of each ABCC11 mutation on the cellular processing and protein function of ABCC11, we conducted a series of in vitro experiments as follows. When transiently expressed in 293A cells, mRNA levels of each ABCC11 variant were not significantly different from those of WT (Fig. 2), suggesting minimal effects of each mutation on mRNA stability. Regarding protein expression, among 11 variants tested in this study, only the p.G180R variant was not maturated as a glycoprotein (Fig. 3a), which was consistent with previous studies demonstrating that this variant is recognized as an ERAD substrate and degraded in cells [11, 15, 18]. The other variants were expressed as matured glycoproteins (Fig. 3a) and their protein levels in the 293A cells were not significantly different from that of ABCC11 WT (Fig. 3b).

Next, we addressed the effects of each mutation except for p.G180R, which was already identified as functionally null, on the transport activities of ABCC11. For this purpose, we first prepared ABCC11-expressing plasma membrane vesicles. Immunoblotting analyses confirmed that like ABCC11 WT, each variant protein was expressed in the plasma

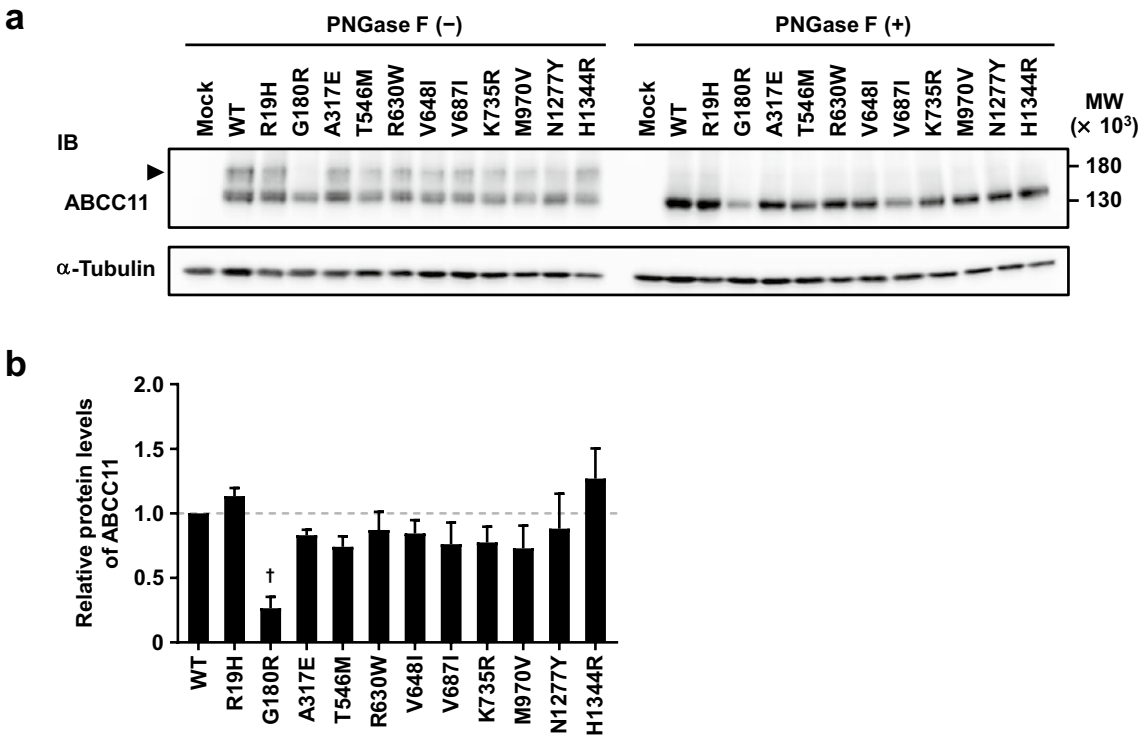

Fig. 3 Effects of each mutation on the maturation status and protein levels of ABCC11 in transiently transfected 293A cells. **a** Immunoblot detection of ABCC11 wild-type (WT) and its variants in the whole cell lysate samples that were prepared 48 h after the transfection. Immuno-reactive bands corresponding to the matured form of ABCC11 as a glycoprotein (*arrowhead*) disappeared after peptide *N*-glycosidase F (PNGase F) treatment. α-Tubulin, a loading control. **b** Relative protein levels of ABCC11 WT and its variants. After PNGase F treatment, whole cell lysate samples were subjected to immunoblotting for densitometoric analysis of the protein levels of ABCC11. The signal intensity ratios (ABCC11/α-tubulin) of the immunoreactive bands were determined and normalized to that in ABCC11 WT-expressing cells. Data are expressed as mean ± SEM; $n = 5$. $^{\dagger}P < 0.05$ ($P < 0.004$, after Bonferroni correction) vs. WT (one-sample *t* test, two-sided: there were no significant differences except for p.G180R)

membrane vesicles (Fig. 4a); however, in some variants, signal intensities of immunoreactive band were lower compared with WT. These results might reflect the deficiency in plasma membrane localization of such variants, at least in 293A cells. Then using the ABCC11-expressing plasma membrane vesicles, functional assays were conducted where ABCC11 function was evaluated as an ATP-dependent transport of [^{3}H]-DHEA-S into the vesicles (Fig. 4b). The results show that two mutations—p.T546M and p.R630W—strongly decreased (< 25%) the function of the ABCC11 protein; four mutations—p.V648I, p.K735R, p.M970V, and p.N1277Y—moderately affected the ABCC11-mediated transport activities. Except for p.R630W, such differences in the ABCC11 function between WT and these variants disappeared after the normalization of DHEA-S transport activity by ABCC11 protein level on the plasma membrane vesicles (Fig. 4c). These results suggest that unlike p.R630W, the remaining five mutations—p.T546M, p.V648I, p.K735R, p.M970V, and p.N1277Y—could minimally affect ABCC11 function qualitatively (via alteration of its intrinsic transporter activity), but rather could do so quantitatively (via decreasing protein levels on the plasma membrane). Also, under this normalization, p.H1344R variant showed higher transport activity than WT (Fig. 4c). Given that the net function of p.H1344R variant was almost comparable to that of WT (Fig. 4b), the increasing effect on the transport activity could be canceled out by the effect on protein level of this variant.

Finally, we integrated the results of biochemical analyses and classified the 11 variants according to their effects on the intracellular processing and protein function of ABCC11. As summarized in Table 2, in addition to p.G180R (a null variant), six variants impaired the net function of ABCC11. Among the seven variants, five original amino acids (G180, T546, R630, V648, and N1277) were conserved among seven mammalian species examined in this study (Fig. 5).

Discussion

The present study deepens the functional understanding of non-synonymous variants of ABCC11. Consistent with previous studies [11, 18], p.G180R disrupted the maturation of ABCC11 as an *N*-linked glycoprotein. The others examined in this study were maturated and reached to the plasma membrane; some variants exhibited lower protein levels on the plasma membrane compared with WT, suggesting their influences on intracellular trafficking and/or stability on

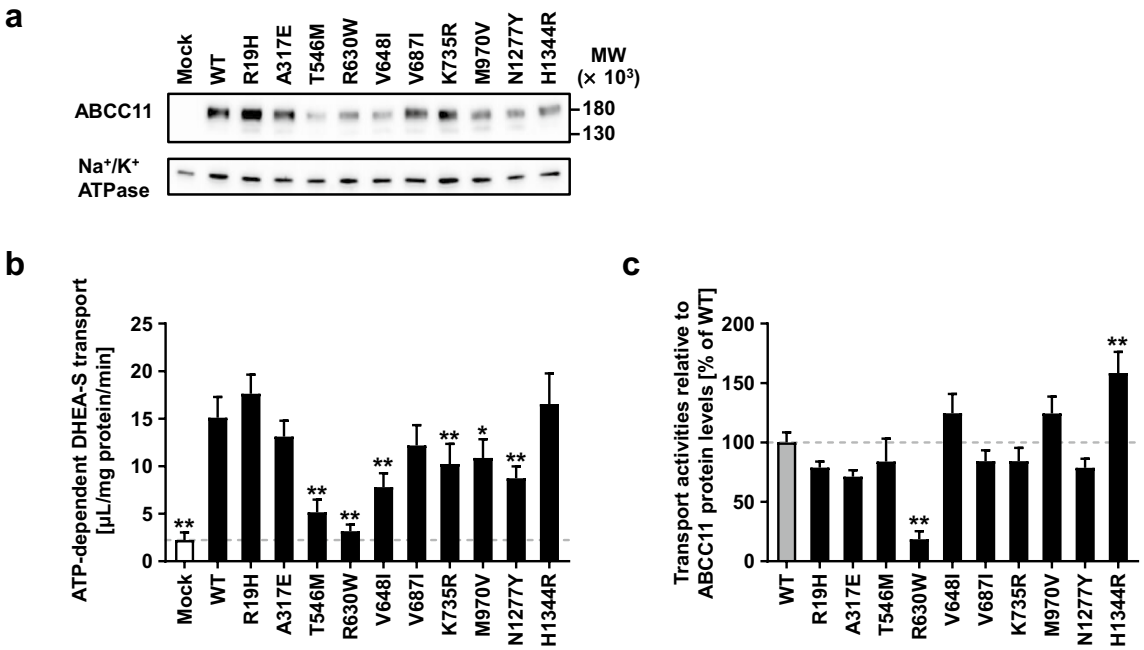

Fig. 4 Effects of each mutation of ABCC11 function determined by vesicle transport assay. **a** Immunoblot detection of ABCC11 wild type (WT) and its variants expressed in plasma membrane vesicles prepared from 293A cells. Na^+/K^+ ATPase, a loading control. **b** ATP-dependent transport of [1,2,6,7-^{3}H(N)]-dehydroepiandrosterone sulfate (DHEA-S) by ABCC11 WT and its variants. ATP-dependent DHEA-S transport into membrane vesicles was measured for 5 min. **c** DHEA-S transport activities normalized by ABCC11 protein levels. ABCC11 protein levels were determined semi-quantitatively by immunoblotting. For this purpose, the signal intensity ratio (ABCC11/Na^+/K^+ ATPase) of the immunoreactive bands shown in **a** was determined and normalized to that in ABCC11 WT-expressing plasma membrane vesicles. The data are shown as % of WT. Data are expressed as the mean ± SEM; $n = 4$. Statistical analyses for significant differences was performed using Bartlett's test, followed by a Dunnett's test (*$P < 0.05$; **$P < 0.01$ vs. WT)

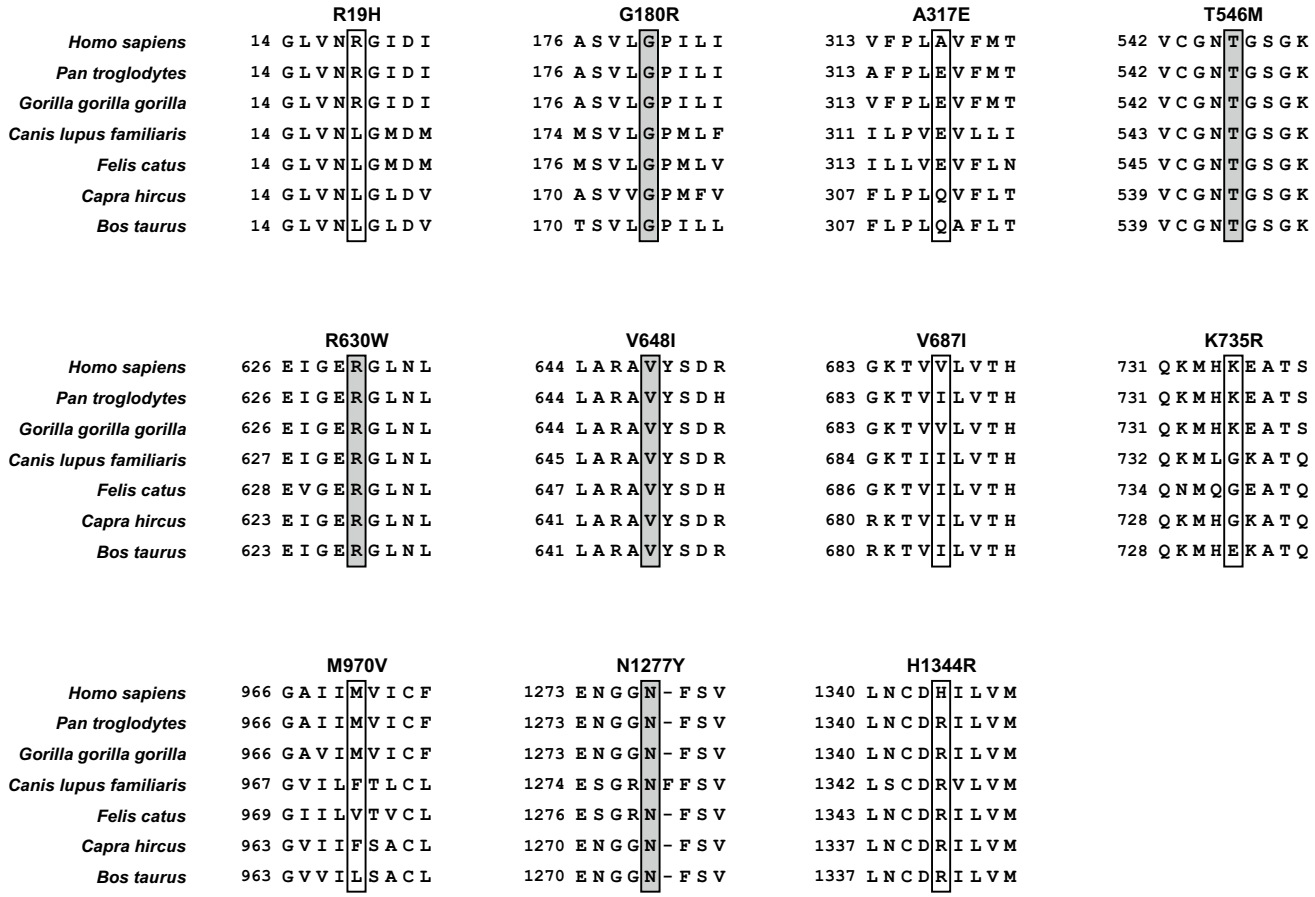

Fig. 5 ABCC11 amino acids evolutionary conserved among seven mammalian species. The positions of non-synonymous substitutions conserved among seven species examined in the present study are grey labeled. Regarding Abcc11 protein in each species, NCBI Reference Sequence ID and amino acid sequence identity (vs. human ABCC11, NP_115972) are summarized as below: *Pan troglodytes* (Chimpanzee, XP_009429028), 98%; *Gorilla gorilla gorilla* (Gorilla, XP_004057661, 98%; *Canis lupus familiaris* (Dog, XP_003638982), 75%; *Felis catus* (Cat, XP_003998059), 75%; *Capra hircus* (Goat, XP_005692011), 72%; *Bos taurus* (Bovine, XP_024834705), 71%

the plasma membrane. Our results showed that due to such quantitative effects than qualitative effects, five mutations—p.T546M, p.V648I, p.K735R, p.M970V, and p.N1277Y—would affect the net cellular function of ABCC11. Moreover, we revealed that p.R630W variant was in both quantity and quality inferior to WT, resulting in the extensive disruption of ABCC11 function.

Our findings provide insights into amino acid positions influencing ABCC11 function. In addition to G180 [8, 11] and T546 [15, 16] of which significance has been identified, R630 could be a biochemically important residue for ABCC11 considering that p.R630W affected the intrinsic transport function of ABCC11 (Fig. 4c). Based on a 3D structure of ABCC11 protein predicted by the AlphaFold (Supplementary Fig. S1), the side chain of R630 can be oriented to an intracellular cavity surrounded by two intracellular domains of ABCC11 protein. When focusing on the physical property of amino acid, the Arg to Trp substitution at the residue means the loss of positive charge of arginine side chain with the uncharged tryptophan side chain. Given that ABCC11 prefers anionic compounds as its substrates [5], the R630 may be involved in the substrate recognition. Also, as another possibility, its potential role for the regulation of ATP binding can be assumed, since the R630 is close to an ATP-binding cassette. To address this biochemical matter raised by the present study, further studies are required.

In a previous study, since p.R19H and p.A317E were in almost complete genetic linkage in Caucasians ($r^2 = 0.95$), their double variant (p.R19H/p.A317E) was constructed and analyzed [15]. Thus, the effects of each mutation on ABCC11 function remain unclear. However, given the difference in their MAF in other populations [e.g. 0.019 (p.R19H) and 0.045 (p.A317E) in East Asian (Table 2)], these two variants cannot be always in genetic linkage. In this context, functional characterization of each single variant should be a matter. With this point, the present study provides a progress (Fig. 4). Our results revealed

that both of them could hardly affect the net function of ABCC11, which is consistent with the previous study showing the minimal effect of p.R19H/p.A317E on the ABCC11 function [15].

Some limitations warrant mention. First, although we found six mutations affecting ABCC11 function in addition to p.G180R (a functionally null variant), their clinical relevance remains unclear except for p.T546M. To reveal the effects of such less-functional or dysfunctional mutations on *ABCC11*-related phenotypes, further clinicogenetic analyses should be required in future. ABCC11-related phenotypes such as AO risk in individuals who are compound heterozygous for p.G180R and p.R630W will be of interest. Second, there is an inconsistency regarding the effects of p.T546M on ABCC11 between the present study and a previous study [15], although both studies showed that this mutation would impair the net cellular function of ABCC11. In detail, as the molecular basis of functional impairment, the present results showed that p.T546M did affect ABCC11 protein levels on the plasma membrane with minimal effect on its intrinsic transport activity (Fig. 4), whereas the previous study suggested the change in the intrinsic activity. In any case, what is important is that both studies have provided valuable insights into the potential impact of p.T546M on ABCC11-related phenotypes.

In summary, we studied 11 non-synonymous variants of ABCC11 in this study. Among them, we identified p.R630W as a novel significantly dysfunctional variant. Our findings will deepen our understanding of genetic variations and biochemical characteristics of ABCC11 protein. Given the recent increasing of interests for rare variants associated with human health, further identification of those with functional impacts in ABCC11 will be important.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13577-024-01074-x.

Acknowledgements The authors thank Dr. Toshihisa Ishikawa for his continuous encouragement for this study and for kindly providing the expression vectors for some ABCC11 variants as well as Dr. Hiroshi Suzuki for his support. YT is an Excellent Young Researcher in MEXT Leading Initiative for Excellent Young Researchers.

Author contributions Conceptualization: Yu Toyoda; methodology: Yu Toyoda; resources: Yu Toyoda; validation: Yu Toyoda; formal analysis: Yu Toyoda; investigation: Yu Toyoda; writing—original draft preparation: Yu Toyoda; writing—review and editing: Yu Toyoda, Hirotaka Matsuo, and Takada Tappei; supervision: Takada Tappei; project administration: Yu Toyoda; funding acquisition: Yu Toyoda; all authors read and approved the final manuscript.

Funding This study was supported by JSPS KAKENHI Grant Numbers 15H05610, 19K16441, and 21H03350 (to YT).

Data availability Data are provided within the manuscript or supplementary information files.

Declarations

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

Ethics approval Not applicable.

Informed consent Not applicable.

References

1. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE, Gottesman MM. Revisiting the role of ABC transporters in multidrug-resistant cancer. Nat Rev Cancer. 2018;18:452–64.
2. Linton KJ. Structure and function of ABC transporters. Physiology (Bethesda). 2007;22:122–30.
3. Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. J Bioenerg Biomembr. 2001;33:453–8.
4. Toyoda Y, Hagiya Y, Adachi T, Hoshijima K, Kuo MT, Ishikawa T. MRP class of human ATP binding cassette (ABC) transporters: historical background and new research directions. Xenobiotica. 2008;38:833–62.
5. Kruh GD, Guo Y, Hopper-Borge E, Belinsky MG, Chen ZS. ABCC10, ABCC11, and ABCC12. Pflug Arch. 2007;453:675–84.
6. Chen ZS, Guo Y, Belinsky MG, Kotova E, Kruh GD. Transport of bile acids, sulfated steroids, estradiol 17-beta-D-glucuronide, and leukotriene C4 by human multidrug resistance protein 8 (ABCC11). Mol Pharmacol. 2005;67:545–57.
7. Guo Y, Kotova E, Chen ZS, et al. MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. J Biol Chem. 2003;278:29509–14.
8. Yoshiura K, Kinoshita A, Ishida T, et al. A SNP in the ABCC11 gene is the determinant of human earwax type. Nat Genet. 2006;38:324–30.
9. Miura K, Yoshiura K, Miura S, et al. A strong association between human earwax-type and apocrine colostrum secretion from the mammary gland. Hum Genet. 2007;121:631–3.
10. Inoue Y, Mori T, Toyoda Y, et al. Correlation of axillary osmidrosis to a SNP in the ABCC11 gene determined by the smart amplification process (SmartAmp) method. J Plast Reconstr Aesthet Surg. 2010;63:1369–74.
11. Toyoda Y, Sakurai A, Mitani Y, et al. Earwax, osmidrosis, and breast cancer: why does one SNP (538G>A) in the human ABC transporter ABCC11 gene determine earwax type? FASEB J. 2009;23:2001–13.
12. Nakano M, Miwa N, Hirano A, Yoshiura K, Niikawa N. A strong association of axillary osmidrosis with the wet earwax type determined by genotyping of the ABCC11 gene. BMC Genet. 2009;10:42.
13. Toyoda Y, Gomi T, Nakagawa H, Nagakura M, Ishikawa T. Diagnosis of human axillary osmidrosis by genotyping of the human ABCC11 gene: clinical practice and basic scientific evidence. Biomed Res Int. 2016;2016:7670483.
14. Ishikawa T, Toyoda Y, Yoshiura K, Niikawa N. Pharmacogenetics of human ABC transporter ABCC11: new insights into apocrine gland growth and metabolite secretion. Front Genet. 2013;3:306.
15. Arlanov R, Lang T, Jedlitschky G, et al. Functional characterization of common protein variants in the efflux transporter ABCC11 and identification of T546M as functionally damaging variant. Pharmacogenom J. 2016;16:193–201.

16. Magdy T, Arlanov R, Winter S, et al. ABCC11/MRP8 polymorphisms affect 5-fluorouracil-induced severe toxicity and hepatic expression. Pharmacogenomics. 2013;14:1433–48.
17. Toyoda Y, Mancikova A, Krylov V, et al. Functional characterization of clinically-relevant rare variants in ABCG2 identified in a gout and hyperuricemia cohort. Cells. 2019;8:363.
18. Toyoda Y, Takada T, Gomi T, Nakagawa H, Ishikawa T, Suzuki H. Clinical and molecular evidence of ABCC11 protein expression in axillary apocrine glands of patients with axillary osmidrosis. Int J Mol Sci. 2017;18:417.
19. Toyoda Y, Takada T, Miyata H, Ishikawa T, Suzuki H. Regulation of the axillary osmidrosis-associated ABCC11 protein stability by N-linked glycosylation: effect of glucose condition. PLoS One. 2016;11: e0157172.
20. Nakagawa H, Wakabayashi-Nakao K, Tamura A, Toyoda Y, Koshiba S, Ishikawa T. Disruption of N-linked glycosylation enhances ubiquitin-mediated proteasomal degradation of the human ATP-binding cassette transporter ABCG2. FEBS J. 2009;276:7237–52.
21. Saito H, Toyoda Y, Hirata H, et al. Soy isoflavone genistein inhibits an axillary osmidrosis risk factor ABCC11: in vitro screening and fractional approach for ABCC11-inhibitory activities in plant extracts and dietary flavonoids. Nutrients. 2020;12:2452.
22. Toyoda Y, Takada T, Suzuki H. Febuxostat inhibited axillary osmidrosis risk factor ATP-binding cassette transporter C11 in vitro. J Dermatol. 2020;47:1198–9.
23. Omasits U, Ahrens CH, Muller S, Wollscheid B. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics. 2014;30:884–6.
24. Hirokawa T, Boon-Chieng S, Mitaku S. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics. 1998;14:378–9.
25. Mitaku S, Hirokawa T. Physicochemical factors for discriminating between soluble and membrane proteins: hydrophobicity of helical segments and protein length. Protein Eng. 1999;12:953–7.
26. Mitaku S, Hirokawa T, Tsuji T. Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. Bioinformatics. 2002;18:608–16.
27. Yabuuchi H, Shimizu H, Takayanagi S, Ishikawa T. Multiple splicing variants of two new human ATP-binding cassette transporters, ABCC11 and ABCC12. Biochem Biophys Res Commun. 2001;288:933–9.
28. Tammur J, Prades C, Arnould I, et al. Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. Gene. 2001;273:89–96.
29. Bera TK, Lee S, Salvatore G, Lee B, Pastan I. MRP8, a new member of ABC transporter superfamily, identified by EST database mining and gene prediction program, is highly expressed in breast cancer. Mol Med. 2001;7:509–16.
30. Beitz E. T(E)Xtopo: shaded membrane protein topology plots in LAT(E)X2epsilon. Bioinformatics. 2000;16:1050–1.
31. Jones PM, George AM. The ABC transporter structure and mechanism: perspectives on recent research. Cell Mol Life Sci. 2004;61:682–99.
32. Varadi M, Anyango S, Deshpande M, et al. AlphaFold protein structure database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. Nucleic Acids Res. 2022;50:D439–44.
33. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596:583–9.